Microbiology and Infectious Disease

The TOR signaling pathway regulates vegetative development, aflatoxin biosynthesis, and pathogenicity in *Aspergillus flavus*

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Abstract

The target of rapamycin (TOR) signaling pathway is highly conserved and plays a crucial role in diverse biological processes in eukaryotes. However, the underlying mechanism of the TOR pathway in *A. flavus* remains elusive. In this study, we identified and characterized seven genes encoding various components of the TOR pathway in *A. flavus,* and investigated their biological function. The FK506-binding protein Fkbp3 and its lysine succinylation are important for aflatoxin production and rapamycin resistance. As a significant downstream effector molecule of the TorA kinase, the Sch9 kinase regulates aflatoxin B_1 (AFB_1) synthesis, osmotic and calcium stress response in *A. flavus,* and this regulation is mediated through its S_TKc, S_TK_X domains, and the ATP binding site at K340. We also showed that the Sch9 kinase may have a regulatory impact on the high-osmolarity glycerol (HOG) signaling pathway. TipA, the other downstream component of the TorA kinase, plays a significant role in regulating sclerotia formation and cell wall stress response in *A. flavus*. The members of the TapA-phosphatase complexes, SitA and Ppg1, are crucial for various biological processes in *A. flavus*, including vegetative growth, sclerotia formation, AFB_1 biosynthesis, and pathogenicity. Furthermore, we showed that SitA and Ppg1 are involved in regulating lipid droplets (LDs) biogenesis and cell wall integrity (CWI) signaling pathways. In addition, another phosphatase complex, Nem1/Spo7, plays critical roles in hyphal development, conidiation, aflatoxin, and lipid droplets biogenesis. This study provides an important insight into the regulatory network of the TOR signaling pathway and the molecular mechanism of aflatoxin biosynthesis in *A. flavus*.

eLife assessment

This **important** study presents relevant information about the involvement of TOR pathway in aflatoxin production by Aspergillus flavus. However, some of the presentation is confusing, leaving the study in its current form is **incomplete**. The strength of the evidence could be augmented with additional experiments and reorganization of the manuscript aiming to fully understand and characterize the involvement of TOR pathway in A. flavus aflatoxin production.

Introduction

Aspergillus flavus is one of the most important phytopathogenic fungi that frequently contaminate various plants, causing economic losses in agricultural commodities and posing serious health concerns in developing countries [1]. *A. flavus* is also an opportunistic human pathogenic fungus that can cause aspergillosis in immune-compromised patients [2]. Aflatoxins (AFs), the toxic and carcinogenic secondary metabolites produced by *A. flavus*, are considered the most potent carcinogen present in nature, causing liver cancer in both humans and animals [3]. The aflatoxin biosynthesis is a complex process involving a series of reactions. Although the majority of the enzyme reactions and genes involved in aflatoxin biosynthesis have been elucidated [4], the related signaling pathway network and specific regulatory mechanisms governing aflatoxin biosynthesis remain elusive. Therefore, exploring the regulatory mechanism of the aflatoxin biosynthesis signaling pathway would provide new insights for preventing *A. flavus* and aflatoxin contamination.

In eukaryotes, the target of rapamycin (TOR) signaling pathway is highly conserved and plays essential roles in various significant biological processes, including ribosome biosynthesis, cell growth, and autophagy [5]. The serine/threonine-protein kinase Tor serves as a crucial protein component in the TOR signaling pathway, which interacts with other proteins to form multi-protein complexes [6]. In *Saccharomyces cerevisiae*, TOR exists in two distinct multiprotein functional complexes, namely TORC1 and TORC2 [7]. TORC1-mediated signaling governs cellular growth by modulating various growth-related mechanisms and exhibits sensitivity to rapamycin. Conversely, TORC2-mediated signaling primarily regulates cytoskeletal remodeling but remains unaffected by rapamycin [7]. In yeast, there are two *tor* genes, *tor1* and *tor2*, whereas in higher eukaryotes such as plants, animals, and filamentous fungi, there is only one *tor* gene [8–10].

In the fungal kingdom, the TOR signaling pathway has been extensively investigated in three prominent species: the budding yeast *S. cerevisiae* [11–12], the fission yeast *Schizosaccharomyces pombe* [13–15], and the human pathogen *Candida albicans* [16–17]. In *S. cerevisiae*, macrolide antibiotic rapamycin interacts with the cytosolic protein Fkbp12 to form a complex, and the Fkbp12-rapamycin complex effectively inhibits the activity of the Tor kinase function by binding to the FRB domain [18]. The Tap42 phosphatase complex serves as the primary target of the Tor kinase. Additionally, the Tap42 protein interacts with the Tip41 protein to collaboratively regulate phosphatases, including PP2A and Sit4 [19–20]. Phosphatase Sit4 can dephosphorylate the transcription factor Gln3, regulating the nitrogen source metabolism pathway [21]. In addition, another immediate effector of TORC1 is the AGC kinase Sch9, which participates in various cellular functions processes, such as ribosome biosynthesis, translation initiation, and entry into the G_0 phase [22]. The TOR signaling pathway has been documented in various filamentous fungi, including *Fusarium graminearum* [23–24], *Magnaporthe oryzae* [25], *Phanerochaete chrysosporium* [26], *Podospora anserine* [27], *A. nidulans* [28], *A. fumigatus* [29–30], *F. fujikuroi* [31], *Botrytis cinerea* [32], *F. oxysporum* [33], and *Mucor circinelloides* [34]. However, the TOR signaling pathway has not yet been reported in *A. flavus*.

In eukaryotic cells, the TOR signaling pathway and the mitogen-activated protein kinase (MAPK) signaling pathways play an important role in regulating adaptive responses to extra- and intracellular conditions [35]. Several studies have demonstrated that the TOR signaling pathway interacts with various other signaling pathways, such as MAPK and cell wall integrity (CWI) pathway. These pathways engage in crosstalk, forming a complex metabolic network that regulates cellular processes [36–40]. However, the underlying mechanisms of multiple crosstalks between the TOR and other signaling pathways in *A. flavus* remain unclear. Therefore, we intended to identify the genes associated with the TOR signaling pathway and elucidate their roles and contributions in regulating vegetative development and aflatoxin biosynthesis in *A.*

flavus. Our results demonstrated that the TOR signaling pathway is involved in multiple cellular processes in *A. flavus*. Our findings revealed a complex interplay between the TOR pathway and other signaling pathways (such as HOG and CWI) in *A. flavus*, which play a crucial role in regulating cellular growth and survival under environmental stress conditions.

Results

Rapamycin exhibits inhibitory effects on the growth, sporulation, sclerotia formation, and aflatoxin production in *A. flavus*

Rapamycin, a secondary metabolite synthesized by *Streptomyces hygroscopicus*, exhibits efficacy in suppressing specific filamentous fungi. In *F. graminearum*, rapamycin has a significant inhibitory impact on growth and asexual reproduction [23]. To investigate the effects of rapamycin on vegetative development, sclerotia formation, and secondary metabolism in *A. flavus*, we conducted an assay to determine the sensitivity of the wild-type strain to rapamycin. We observed that the wild-type strain exhibited a high sensitivity to rapamycin. We found that 100 ng/mL of rapamycin significantly inhibited mycelial growth and conidia formation (**Fig. 1A, 1D, 1E**). More importantly, the wild-type strain exhibited a significant reduction in sclerotia formation and aflatoxin synthesis (**Fig. 1B, 1C, 1F, 1G**). Additionally, rapamycin resulted in a significant decrease in spore germination rate and sparser conidiophores (Fig. S1A, S1B). These results illustrated that the TOR signaling pathway may be involved in regulating the growth, development, and secondary metabolism of *A. flavus*. Calcofluor white is a chemifluorescent blue dye commonly employed for its nonspecific binding affinity to fungal chitin. Fluorescence microscopy analysis revealed a notable reduction in the blue fluorescence on the cell wall following treatment with rapamycin (Fig. S1C). The intracellular lipid droplets within the hyphae were labeled with BODIPY. We observed that the hyphae treated with rapamycin contained more lipid droplets compared to the control group (Fig. S1D). These findings suggest that the TOR pathway may play important roles in maintaining cell wall integrity and facilitating the biogenesis of lipid droplets.

To investigate the potential regulatory role of the TOR pathway in modulating the sporulation, sclerotia formation, and aflatoxin biosynthesis in *A. flavus*, we examined the expression levels of conidia-related, sclerotia formation-related, and aflatoxin-related genes after 3, 6, and 9 hours of treatment with rapamycin. To determine the effects of rapamycin on conidiation, qRT-PCR was conducted to assess the transcript levels of two conidia-related genes, *abaA* and *brlA*. We also conducted qRT-PCR to evaluate the expression levels of three regulators (*nsdC*, *nsdD*, and *sclR*) involved in sclerotia development, and genes involved in AFB_1 biosynthesis, such as the regulatory gene *aflS*, as well as several structural genes (*aflC* and *aflQ*). The strains treated with rapamycin exhibited a significant reduction in the expression levels of genes related to sporulation, sclerotia formation, and aflatoxin synthesis (**Fig. 1H, 1I, 1J**). Overall, these results indicated that the TOR pathway plays a crucial role in regulating the growth, sporulation, sclerotia formation, and aflatoxin biosynthesis in *A. flavus* by modulating the expression of these genes.

The impact of Fkbp3 and its lysine succinylation on AFB_1 biosynthesis and rapamycin resistance in *A. flavus*

FK506-binding proteins (Fkbps) belong to a highly conserved immunophilin family, which are involved in various cellular functions, such as protein folding, immunosuppression, signaling transduction, and transcription [41]. Fkbps function as molecular switches by binding to target proteins and inducing conformational changes [41]. In *S. cerevisiae*, Fkbp12 and rapamycin interact to form a complex that exerts a negative regulatory effect on the activity of the Tor kinase [42]. The *fkbp12* gene homologue *fprA* in *A. nidulans* shares high levels of homology with that

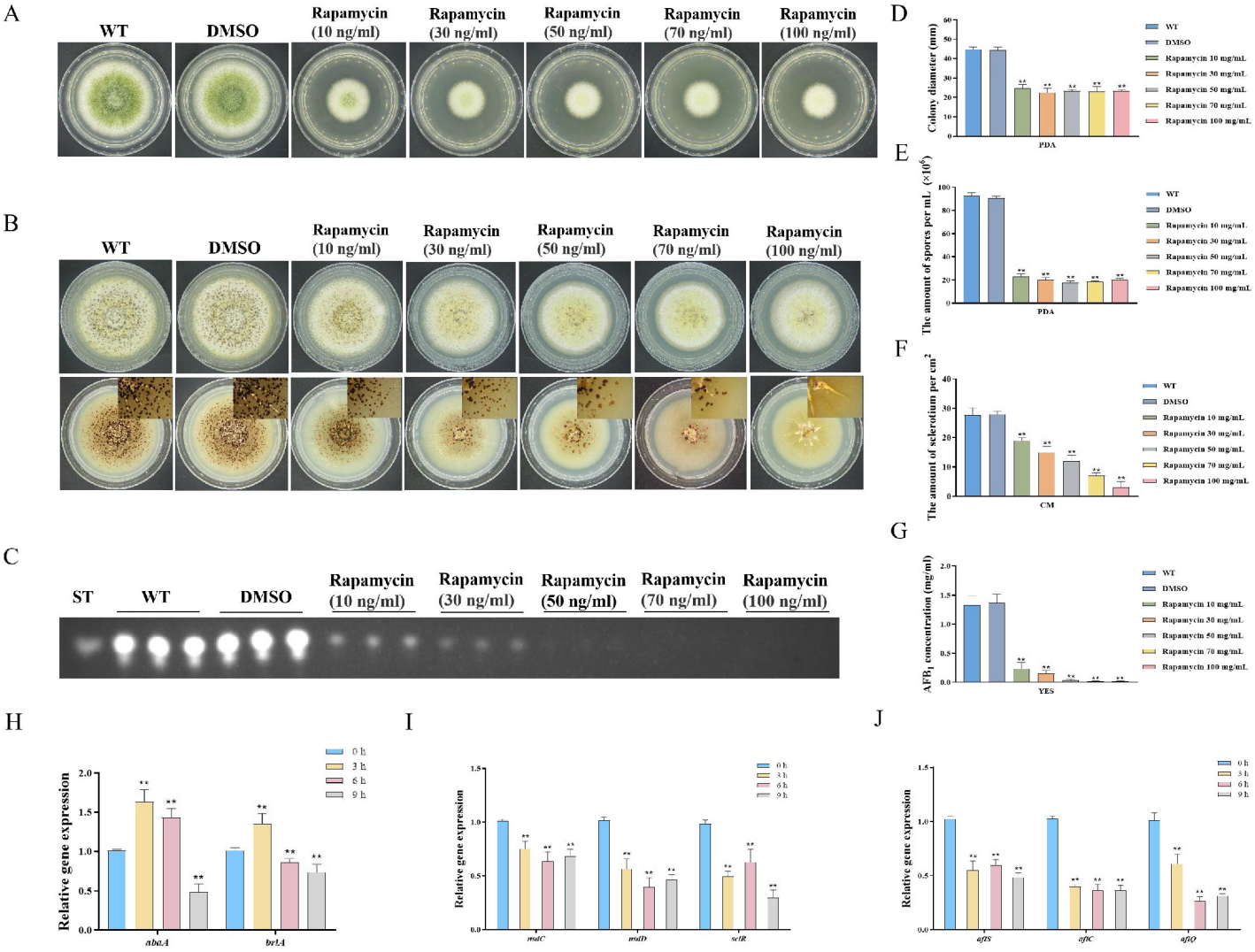

Fig. 1

Impacts of rapamycin on the growth, sporulation, sclerotia formation,and aflatoxin production of *Aspergillus flavus*.

(A) Colony morphology of the WT strain cultured on PDA medium amended with different concentrations of rapamycin at 37°C for 5 days.
(B) Colony phenotype of the WT strain grown on CM medium amended with different concentrations of rapamycin at 37°C for 7 days.
(C) TLC assay of AFB_1 production by the WT strain cultured in YES liquid media amended with different concentrations of rapamycin at 29°C for 6 days.
(D) Statistical analysis of the colony diameter by the WT strain treated with different concentrations of rapamycin described in (A).
(E) Conidial quantification of the WT strain treated with different concentrations of rapamycin as mentioned in (A).
(F) Quantitative analysis of sclerotium formation by the WT strain treated with different concentrations of rapamycin described in (B).
(G) Relative quantification of AFB_1 production by the WT strain treated with different concentrations of rapamycin as mentioned in (C)
(H) Relative expression levels of conidia-related genes in WT strain treated with 100 ng/mL rapamycin after 3, 6, 9 h.
(I) Relative expression levels of sclerotia-related genes in WT strain treated with 100 ng/mL rapamycin after 3, 6, 9 h.
(J) Relative expression levels of aflatoxin biosynthesis regulatory and structural genes in WT strain treated with 100 ng/mL rapamycin after 3, 6, 9 h.

from *S. cerevisiae*[28]. According to the protein sequence of FprA from *A. nidulans*, four genes encoding putative proteins with FK506 binding domain, named *fkbp1* (AFLA_126880), *fkbp2* (AFLA_087480), *fkbp3* (AFLA_010910), and *fkbp4* (AFLA_128200), have been identified from the *A. flavus* genome. We generated four *fkbp* knockout strains using homologous recombination, and all *fkbp* disruption strains were confirmed by PCR and sequencing analysis (Fig. S2). Phenotypic analyses showed that the mycelial growth and conidia formation of all the *fkbp* mutants were similar to the wild-type strain on the PDA medium (**Fig. 2A**), suggesting that Fkbps have minimal influence on the process of vegetative development. We evaluated AFB_1 synthesis utilizing thin layer chromatography (TLC), and the findings demonstrated a noteworthy reduction in AFB_1 synthesis upon deletion of Fkbp3, in comparison to the wild-type strain (**Fig. 2B, 2D**). Additionally, the quantity of sclerotia exhibited a significant decrease in all *fkbp* mutants compared to the wild-type strain (Fig. S3A, S3B). The aforementioned data indicated that Fkbp3 plays a crucial role in sclerotia formation and aflatoxin biosynthesis in *A. flavus*.

To determine the specific Fkbp responsible for rapamycin sensitivity, all mutant strains were cultured on PDA solid plates amended with 100 ng/mL rapamycin. The strain with disrupted *fkbp3* showed significant resistance to rapamycin, while the deletion mutants of *fkbp1*, *fkbp2*, and *fkbp4* did not exhibit any alteration in resistance to rapamycin (**Fig. 2A, 2C**). These results demonstrated that Fkbp3 serves as the principal target for rapamycin in *A. flavu*s. In the FK506-sensitivity analysis, we observed a significant suppression of mycelial growth and conidia formation in wild-type strain with the addition of 10 ng/mL FK506 to the PDA medium. Additionally, the deletion of *fkbp3* led to the heightened resistance to FK506 compared to the wild-type strain (**Fig. 2A, 2C**). These findings suggested that Fkbp3 is mainly involved in the modulation of FK506 resistance in *A. flavus*.

Previous studies have shown that lysine succinylation plays a significant role in aflatoxin production and pathogenicity in *A. flavus* [43]. Based on the analysis of our succinylome data, we have identified six reliable succinylation sites (K5, K19, K40, K42, K55, and K65) on the Fkbp3 protein. To identify the function of Fkbp3 succinylation sites, site-directed mutations were constructed according to the homologous recombination strategy. Compared to the wild-type strain, all point mutants exhibited a similar phenotype in vegetative growth and conidiation. Interestingly, we observed that the K19A mutation resulted in increased resistance to rapamycin (**Fig. 2E, 2F**). Additionally, the K19A strain exhibited a significant reduction in the production of AFB_1 compared to the wild-type strain (**Fig. 2G, 2H**). These findings suggested that the succinylation of Fkbp3 at K19 plays a crucial role in rapamycin resistance and aflatoxin biosynthesis. Furthermore, we found that various point mutants, including K40A, K42A, and K55A, exhibited reduced levels of AFB_1 production in comparison to the wild-type strain (**Fig. 2G, 2H**), suggesting that the succinylation of Fkbp3 at other sites also contributes to AFB_1 biosynthesis in *A. flavus*. The above results indicated that the K19 site likely plays a more prominent role in Fkbp3 succinylation compared to other sites.

The Sch9 kinase is involved in aflatoxin biosynthesis and the HOG pathway

Sch9, a Ser/Thr kinase belonging to the AGC family, is directly phosphorylated by TORC1. The phosphorylation of Sch9 is inhibited by rapamycin, as well as by carbon or nitrogen starvation [22]. The putative *sch9* gene (AFLA_127440) in *A. flavus* encodes a protein consisting of 706 amino acids, exhibiting 80.31% identity to the Sch9 protein in *A. nidulans*. To investigate the role of Sch9 in *A. flavus*, we attempted to create a *sch9* deletion mutant using a homologous recombination strategy. The Δ*sch9* strain exhibited typical vegetative growth and conidiation similar to the wild-type strain (**Fig. 3B**). TLC assay and quantitative analysis showed a significantly decreased aflatoxin production in the Δ*sch9* strain compared to the wild-type strain (**Fig. 3C, 3G**). These results indicated that Sch9 regulates aflatoxin biosynthesis in *A. flavus*.

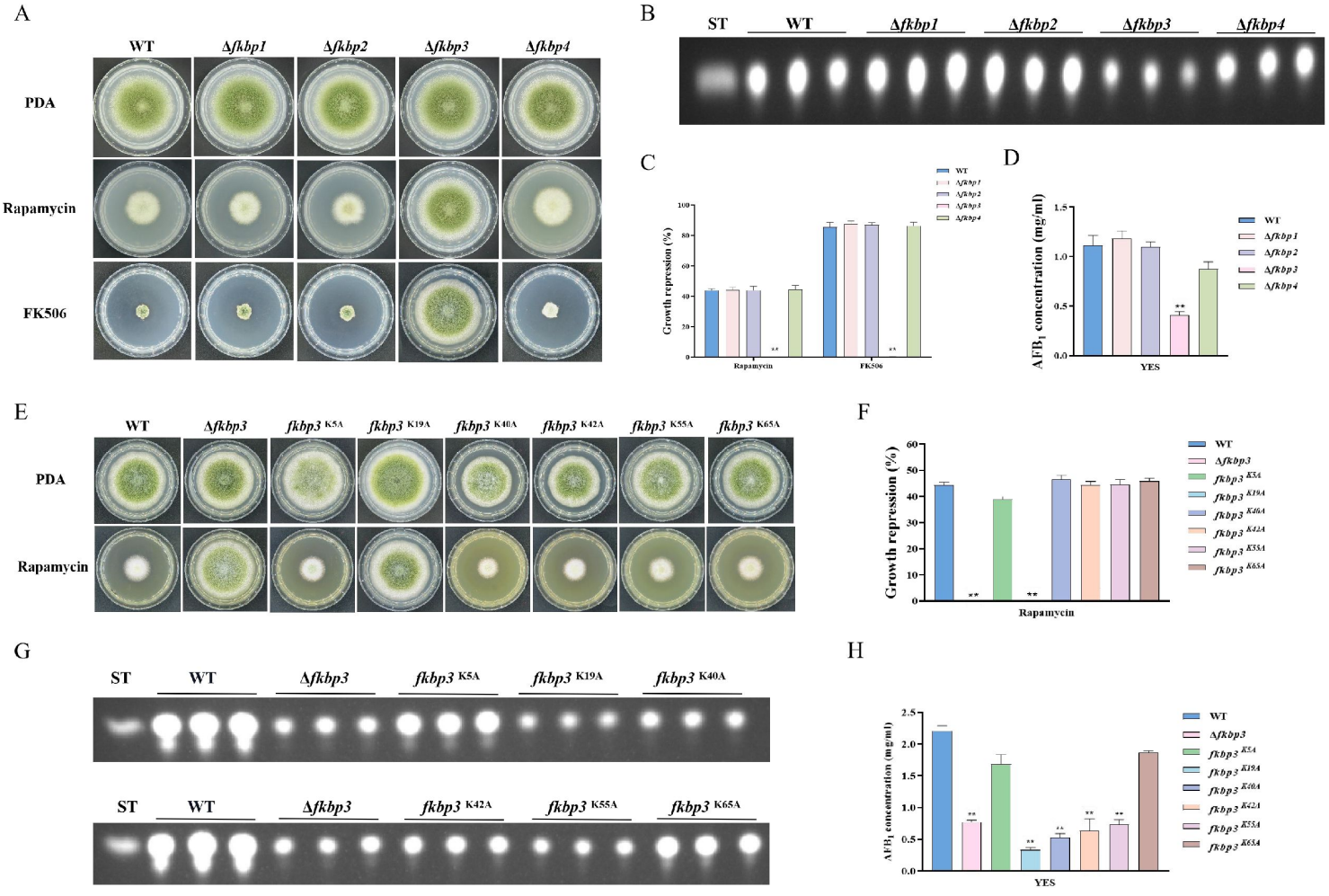

Fig. 2

Disruption of *fkbp3* significantly increases the resistance of *A. flavus* to rapamycin and FK506.

(A) Phenotype of the WT and all mutant strains (Δ*fkbp1*, Δ*fkbp2*, Δ*fkbp3*, and Δ*fkbp4*) grown on PDA amended with rapamycin and FK506 at 37°C for 5 days.
(B) TLC analysis of AFB_1 production by the WT and all mutant strains (Δ*fkbp1*, Δ*fkbp2*, Δ*fkbp3*, and Δ*fkbp4*) cultured in YES liquid medium at 29°C for 6 days.
(C) The growth inhibition rate of the WT and all mutant strains (Δ*fkbp1*, Δ*fkbp2*, Δ*fkbp3*, and Δ*fkbp4*) under rapamycin and FK506 stress.
(D) AFB_1 quantitative analysis of the WT and all mutant strains (Δ*fkbp1*, Δ*fkbp2*, Δ*fkbp3*, and Δ*fkbp4*) as described in (B).
(E) Phenotype of the WT and all mutant strains (Δ*fkbp3*, K5A, K19A, K40A, K42A, K55A, and K65A) grown on PDA amended with 100 ng/mL rapamycin at 37°C for 5 days.
(F) The growth inhibition rate of the WT and all mutant strains (Δ*fkbp3*, K5A, K19A, K40A, K42A, K55A, and K65A) under rapamycin stress.
(G) TLC assay of AFB_1 production by the WT and all mutant strains (Δ*fkbp3*, K5A, K19A, K40A, K42A, K55A, and K65A) cultured in YES liquid medium at 29°C for 6 days.
(H) Relative quantification of AFB_1 production in the WT and all mutant strains (Δ*fkbp3*, K5A, K19A, K40A, K42A, K55A, and K65A) as mentioned in (G).

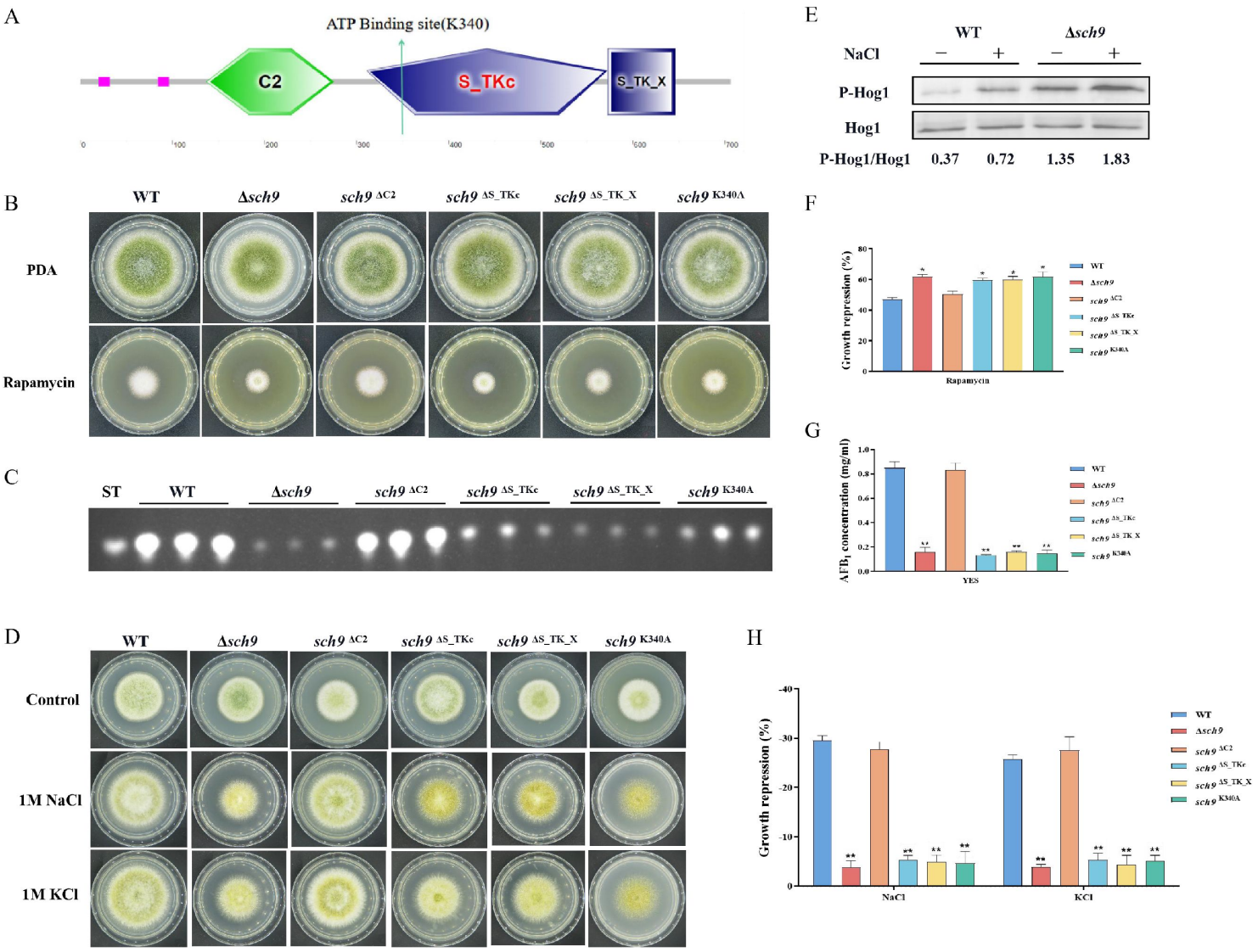

Fig. 3

The Sch9 kinase participates in aflatoxin biosynthesis and the HOG pathway.

(A) The structure diagram of the Sch9 kinase.
(B) Phenotype of the WT, Δ*sch9*, *sch9*$^{\Delta C2}$, *sch9*$^{\Delta S\_TKc}$, *sch9*$^{\Delta S\_TK\_X}$, and *sch9*^{K340A} strains grown on PDA medium amended with 100 ng/mL rapamycin at 37°C for 5 days.
(C) TLC assay of AFB_1 production from the WT, Δ*sch9*, *sch9*$^{\Delta C2}$, *sch9*$^{\Delta S\_TKc}$, *sch9*$^{\Delta S\_TK\_X}$, and *sch9*^{K340A} strains cultured in YES liquid medium at 29°C for 6 days.
(D) The phenotypes of the WT, Δ*sch9*, *sch9*$^{\Delta C2}$, *sch9*$^{\Delta S\_TKc}$, *sch9*$^{\Delta S\_TK\_X}$, and *sch9*^{K340A}strains on YGT media amended with 1 M NaCl and 1 M KCl for 3 days.
(E) The phosphorylation levels of Hog1 in the WT and Δ*sch9* strains were determined with or without osmotic stress.
(F) The growth inhibition rate of the WT, Δ*sch9*, *sch9*$^{\Delta C2}$, *sch9*$^{\Delta S\_TKc}$, *sch9*$^{\Delta S\_TK\_X}$, and *sch9*^{K340A} strains under rapamycin stress.
(G) Quantitative analysis of AFB_1 as shown in (C).
(H) The growth inhibition rate of the WT, Δ*sch9*, *sch9*$^{\Delta C2}$, *sch9*$^{\Delta S\_TKc}$, *sch9*$^{\Delta S\_TK\_X}$, and *sch9*^{K340A} strains under osmotic stress.

In yeast, Sch9 plays an important role in the transcriptional activation of osmostress inducible genes [44]. To characterize the roles of Sch9 in stress response, we examined the sensitivity of the *sch9* deletion strain to various stresses including osmotic stress and calcium stress. Compared with the wild-type strain, the Δ*sch9* mutant significantly increased sensitivity to calcium stress (Fig. S4A, S4B), and decreased sensitivity to osmotic stress induced by NaCl and KCl (**Fig. 3D, 3H**). In *A. flavus*, the Sch9 kinase consists of several domains, including the protein kinase C conserved region 2 (C2), protein kinase (S_TKc), AGC-kinase C-terminal (S_TK_X) domain, and an ATP binding site (**Fig. 3A**). To explore the role of these domains and the ATP binding site in Sch9 kinase, we constructed deletion strains for both domains and a $sch9^{K340A}$ mutant. The results revealed that the $sch9^{\Delta S\_TKc}$ strain, $sch9^{\Delta S\_TK\_X}$ strain, and $sch9^{K340A}$ mutant exhibited similar phenotypes in osmotic stress and AFB_1 production as the Δ*sch9* mutant (**Fig. 3B, 3C, 3D**). Therefore, we speculate that the protein kinase domain, kinase C-terminal domain, and the ATP binding site at K340 play a crucial role in modulating the impact of Sch9 on aflatoxin biosynthesis and stress response in *A. flavus*.

To investigate the potential function of Sch9 in the HOG and TOR pathways in *A. flavus*, we conducted a sensitivity analysis of the Δ*sch9* strain to rapamycin. Compared to the wild-type strain, the *sch9* deletion strain exhibited a slightly higher sensitivity to rapamycin (**Fig. 3B, 3F**). The MAKP Hog1 is an element of the high osmolarity glycerol (HOG) pathway. To verify the connection between Sch9 and the HOG-MAPK pathway, the phosphorylation levels of Hog1 kinase were measured under osmotic stress (1M NaCl). Western blot analysis showed that the phosphorylation level of Hog1 increased significantly in the Δ*sch9* strain (**Fig. 3E**). This finding suggested that Sch9 plays a pivotal role in modulating the response of Hog1 to osmotic stress. Taken together, these results reflected that Sch9 regulates osmotic stress response via the HOG pathway in *A. flavus*.

TipA participate in regulation of sclerotia development and cell wall stress response

In *S. cerevisiae*, Tip41 was identified as a Tap42-interacting protein and has been measured to function as a negative regulator of Tap42, which downregulates TORC1 signaling via activation of PP2A phosphatase [45]. The putative orthologue of *tip41* in *A. flavus* is named *tipA* (AFLA_047310). The amino acid sequence of TipA exhibits a similarity of 40.49% to Tip41 in *S. cerevisiae*. To elucidate the function of the TOR signaling pathway protein TipA in *A. flavus*, we generated the *tipA* deletion mutants. The Δ*tipA* strain exhibited a slightly reduced number of conidia produced on PDA compared to the wild-type strain (**Fig. 4A, 4C**). Meanwhile, the Δ*tipA* strain exhibited decreased sensitivity to rapamycin (**Fig. 4A, 4E**). The Δ*tipA* strain was unable to produce any sclerotia (**Fig. 4B, 4F**). Whereas Δ*tipA* strain do not affect aflatoxin production(**Fig. 4D, 4G**). In addition, the Δ*tipA* strain was more sensitive to the cell wall and cell membrane stress, including CR and SDS (**Fig. 4H, 4I**). Additionally, the expression levels of *nsdC* and *nsdD* exhibited a decrease in Δ*tipA* strain compared to the wild-type strain (**Fig. 4J**). These results indicated that TipA is crucial for the sclerotial formation and cell wall stress in *A. flavus*.

Phosphatase SitA and Ppg1 are involved in growth, conidiation, and sclerotial formation

In *S. cerevisiae*, Tap42 has been identified to interact with the catalytic subunit of type 2A protein phosphatases, including Pph3, Pph21, and Pph22, as well as the type 2A-like phosphatases Sit4 and Ppg1[19]. Phosphatase *sitA* (AFLA_108450) and *ppg1* (AFLA_132610) were identified in *A. flavus*, encoding putative orthologs of *sit4* and *ppg1* in *S. cerevisiae.* To determine the biological functions of phosphatase SitA and Ppg1 in *A. flavus*, we disrupted the *sitA* and *ppg1* by homologous recombination strategy. To further investigate the relationship between phosphatase SitA and Ppg1, we additionally generated complemented and double deletion mutants. The vegetative

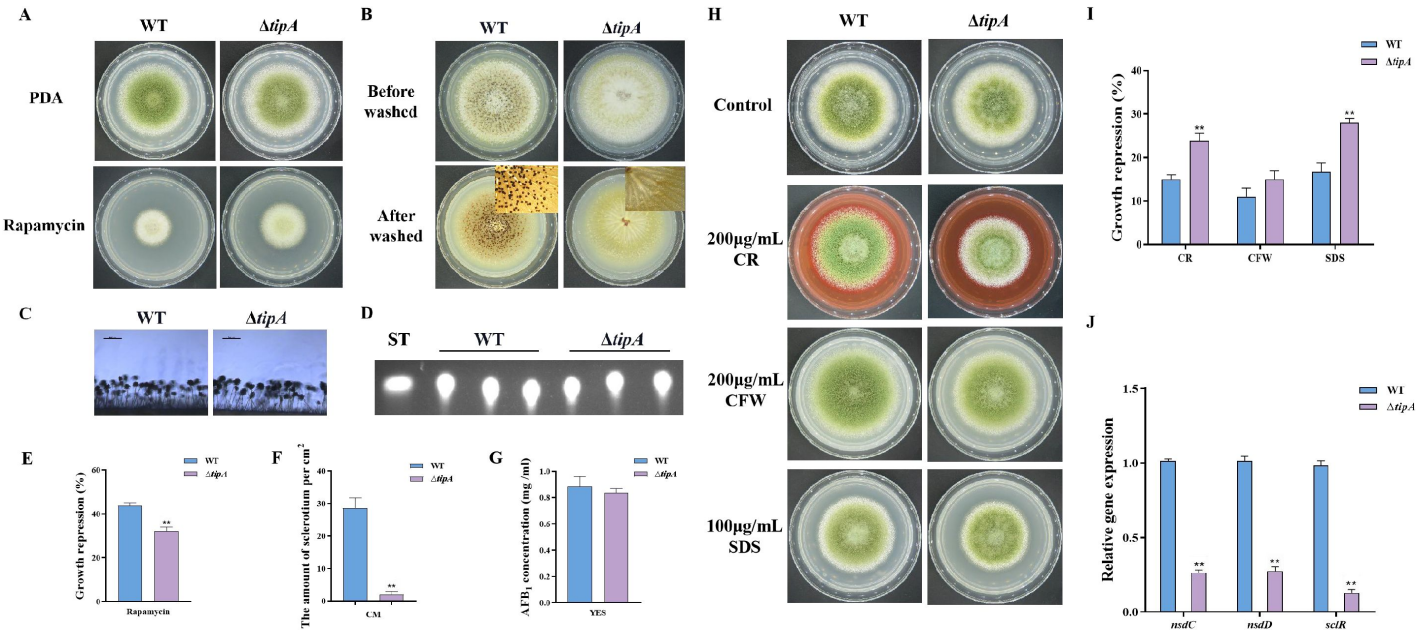

Fig. 4

TipA regulates clerotia development and cell wall stress in *A. flavus*.

(A) Phenotype of the WT and Δ*tipA* strains grown on PDA amended with 100 ng/mL rapamycin at 37°C for 5 days.
(B) Phenotypic characterization of the WT and Δ*tipA* strains grown on CM medium at 37°C for 7 days.
(C) Morphology of conidiophores in the WT and Δ*tipA* strains observed by microscope.
(D) TLC assay of AFB_1 production by the WT and Δ*tipA* strains cultured in YES liquid medium at 29°C for 6 days.
(E) The growth inhibition rate of the WT and Δ*tipA* strains under rapamycin stress.
(F) Amount of sclerotia produced by the WT and Δ*tipA* strains.
(G) AFB_1 quantitative analysis of the WT and Δ*tipA* strains described in (D).
(H) Colony morphology of the WT and Δ*tipA* strains grown on PDA media supplemented with 200 μg/mL CR, 200μg/mL CFW, and 100 μg/mLSDS at 37°C for 5 days.
(I) The growth inhibition rate of the WT and Δ*tipA* strains under cell wall and cell membrane stress.
(J) Relative expression levels of sclerotia formation genes in the WT and Δ*tipA* strains.

development phenotype analysis showed that the Δ*sitA*, Δ*ppg1*, and Δ*sitA*/Δ*ppg1* strains grew significantly more slowly than the wild-type strain on PDA (**Fig. 5A, 5D**). Additionally, the Δ*sitA*, Δ*ppg1*, and Δ*sitA*/Δ*ppg1* strains were more sensitive to rapamycin (**Fig. 5A**, 5E). In addition, the Δ*sitA*, Δ*ppg1*, and Δ*sitA*/Δ*ppg1* strains formed lower number of conidiophores (**Fig. 5A**). To gain further insight into the role of SitA and Ppg1 in the process of conidiation, qRT-PCR was employed to determine the transcript levels of conidiation-related genes (*abaA* and *brlA*). The expression levels of the *abaA and brlA* genes were significantly downregulated in the Δ*sitA*, Δ*ppg1*, and Δ*sitA*/Δ*ppg1* mutants when compared to the wild-type and complemented strains (**Fig. 5G**). The analysis of sclerotial formation revealed that the Δ*sitA*, Δ*ppg1*, and Δ*sitA*/Δ*ppg1* mutants exhibited a complete inability to produce any sclerotia (**Fig. 5B, 5E**). To further prove these findings, we conducted qRT-PCR to determine the expression levels of three regulators (*nsdC*, *nsdD*, and *sclR*) of sclerotia development. Our findings indicated that the expression levels of *nsdC*, *nsdD*, and *sclR* were all significantly reduced in the Δ*sitA*, Δ*ppg1*, and Δ*sitA*/Δ*ppg1* mutants (**Fig. 5H**), which is consistent with the observed decrease in sclerotia production in the Δ*sitA*, Δ*ppg1*, and Δ*sitA*/Δ*ppg1* mutants. These results showed that SitA and Ppg1 are crucial for hyphal development, conidiation, and sclerotia formation in *A. flavus*.

SitA and Ppg1 play crucial roles in aflatoxin biosynthesis and pathogenicity

We assayed AFB_1 biosynthesis in the Δ*sitA*, Δ*ppg1*, and Δ*sitA*/Δ*ppg1* mutants. TLC analyses revealed that the Δ*sitA* and Δ*sitA*/Δ*ppg1* strains exhibited a decrease in AFB_1 production, whereas the Δ*ppg1* strain showed a significant increase in AFB_1 production compared to the wild-type and complemented strains (**Fig. 5C, 5F**). The expression levels of genes related to aflatoxin synthesis, including structural genes (*aflO* and *aflP*) and regulatory genes (*aflR* and *aflS*), were determined by qRT-PCR in the Δ*sitA*, Δ*ppg1*, and Δ*sitA*/Δ*ppg1* mutants. The results revealed a significant downregulation of these genes in the Δ*sitA* and Δ*sitA*/Δ*ppg1* mutants, while upregulated in the Δ*ppg1* mutants, compared to the wild-type and complemented strains (**Fig. 5I**). These results indicated that SitA and Ppg1 play different roles in regulating aflatoxin biosynthesis in *A. flavus*.

We conducted pathogenicity tests on peanuts and maize to examine the pathogenicity of the phosphatases SitA and Ppg1 on crops. The results showed that the wild-type and complemented strains demonstrated complete virulence on all peanut and maize seeds. In contrast, the Δ*sitA*, Δ*ppg1*, and Δ*sitA*/Δ*ppg1* mutants displayed compromised colonization on peanut and maize seeds (**Fig. 6A**). In addition, the number of conidial productions in these mutants on the infected peanut seeds also dramatically decreased compared to those of the wild-type and complemented strains (**Fig. 6C**). The thin-layer chromatography analyses revealed that the Δ*ppg1* mutant exhibited increased production of AFB_1 in peanuts and maize. Conversely, the levels of aflatoxin produced by the Δ*sitA* and Δ*sitA*/Δ*ppg1* mutants on peanut and maize were significantly reduced compared to the wild-type and complemented strains (**Fig. 6B, 6D**). This observation aligns with the above results of aflatoxin biosynthesis from all mutant strains in the YES medium. All these data showed that SitA and Ppg1 are crucial for crop seeds' pathogenicity.

SitA and Ppg1 are involved in regulating cell wall integrity

To explore the role of phosphatases SitA and Ppg1 in response to cell wall stress, all strains were cultivated on PDA amended with CFW, CR, and SDS. We found that the relative growth inhibition of Δ*sitA*, Δ*ppg1*, and Δ*sitA*/Δ*ppg1* mutants induced by cell wall stress was significantly increased compared to the wild-type and complemented strains (**Fig. 7A, 7B**). Further investigations using qRT-PCR revealed a notable reduction in the expression levels of genes related to the synthesis of cell walls, specifically chitin synthase genes *chsA*, *chsD*, and *chsE* in the Δ*sitA*, Δ*ppg1*, and Δ*sitA*/Δ*ppg1* strains (**Fig. 7C**). Western blot analysis also revealed that the phosphorylation level of Slt2

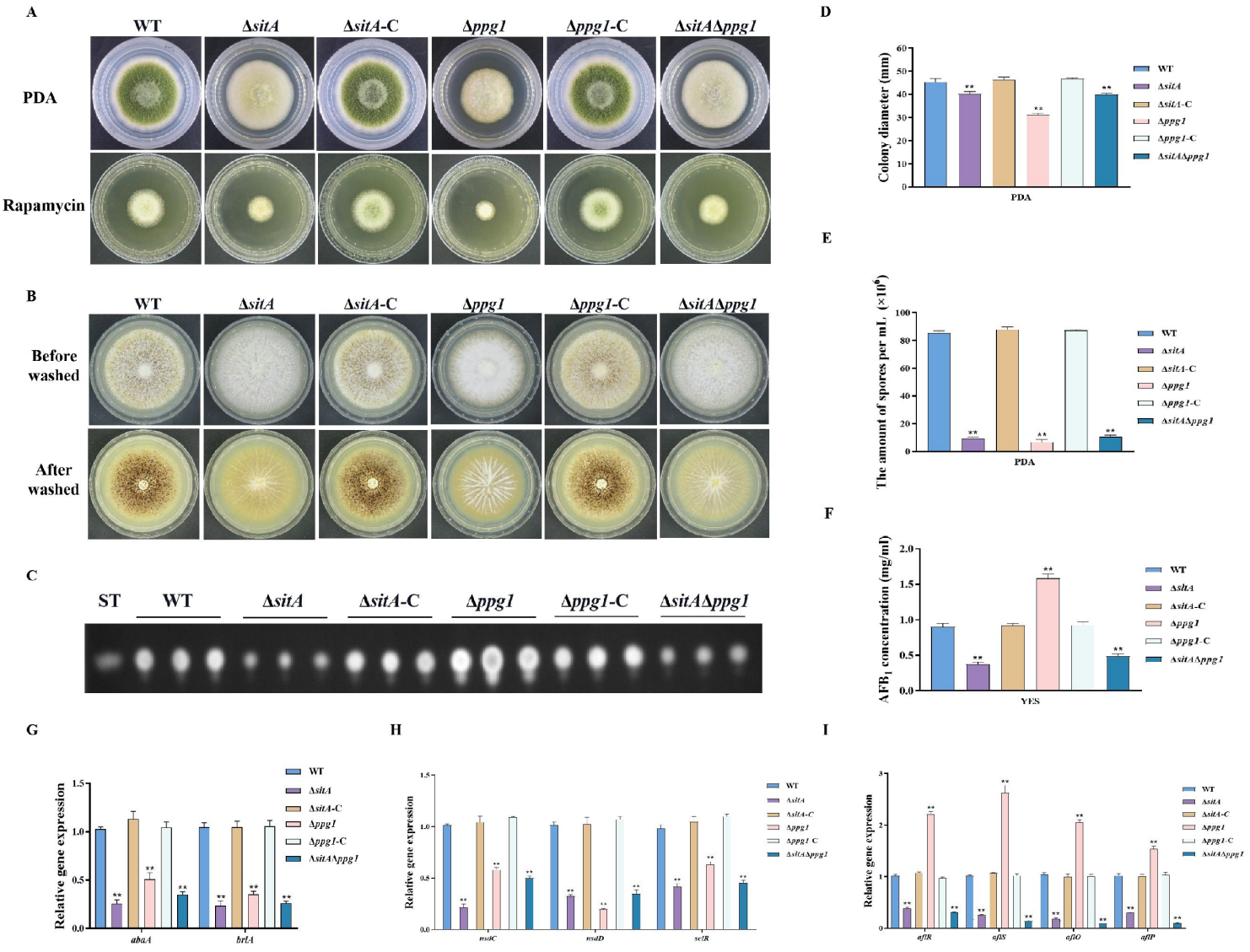

Fig. 5

The impact of the phosphatases SitA and Ppg1 on the growth, conidiation, and aflatoxin biosynthesis in *A. flavus*.

(A) Colony morphology of the WT, single knockout, and double knockout strains cultured on PDA medium amended with 100 ng/mL rapamycin at 37°C for 5 days.
(B) Colony morphology of the WT, single knockout, and double knockout strains cultured on CM medium at 37°C for 7 days.
(C) TLC analysis of AFB_1 production from the WT, single knockout, and double knockout strains cultured in YES liquid medium at 29°C for 6 days.
(D) Growth diameter of the WT, single knockout, and double knockout strains on PDA media.
(E) The growth inhibition rate of the WT, single knockout, and double knockout strains under rapamycin stress.
(F) AFB_1 quantitative analysis of the WT, single knockout, and double knockout strains.
(G) Relative expression levels of conidia synthesis genes in the WT, single knockout, and double knockout strains.
(H) Relative expression levels of sclerotia synthesis genes in the WT, single knockout, and double knockout strains.
(I) Relative expression levels of aflatoxin biosynthesis genes in the WT, single knockout, and double knockout strains.

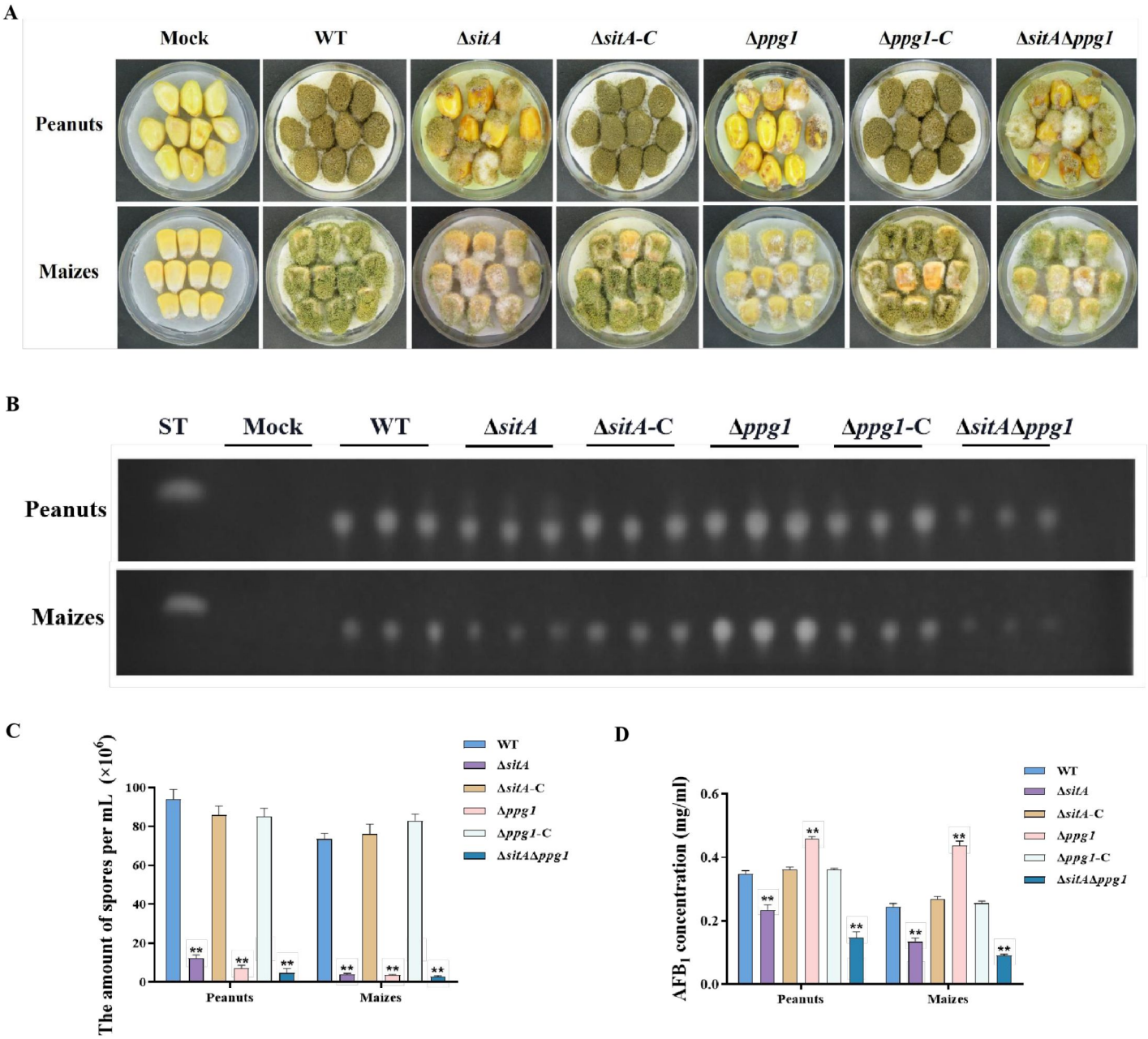

Fig. 6

Pathogenicity analysis of the phosphatase SitA and Ppg1 in *A. flavus*.

(A) Phenotypes of peanut and maize infected by the WT, single knockout, and double knockout strains.
(B) TLC was used to detect the AFB_1 from peanut and maize infected by the WT, single knockout, and double knockout strains.
(C) Quantitative analysis of conidia production from peanut and maize infected by the WT, single knockout, and double knockout strains.
(D) Quantitative analysis of AFB_1 from peanut and maize infected by the WT, single knockout, and double knockout strains.

was significantly elevated in the Δ*sitA*, Δ*ppg1*, and Δ*sitA*/Δ*ppg1* strains under cell wall stress (200 μg/ml CFW) (**Fig. 7D**). In conclusion, the phosphatases SitA and Ppg1 play a crucial role in regulating cell wall integrity.

The phosphatase complex Nem1/Spo7 is important for growth, aflatoxin biosynthesis, and LD biogenesis

Lipid droplets (LDs) are highly dynamic spherical organelles, which appear in the cytosol of most eukaryotic cell types[46]. Rapamycin treatment led to the accumulation of lipid droplets in various filamentous fungi [24]. The FgTOR pathway regulates the phosphorylation status of FgNem1 mainly via the FgPpg1/Sit4-FgCak1/other kinase cascade in *F. graminearum* [24]. In this study, we observed that the treatment of hyphae with rapamycin resulted in the accumulation of LD biogenesis (**Fig. 8A**). This finding suggested that the TOR pathway may regulate the LD biogenesis. However, the underlying regulatory mechanism of LD biosynthesis remain largely unknown in *A. flavus*. To investigate the regulatory mechanism of the TOR pathway in *A. flavus* that controls LD biogenesis, we examined LD accumulation in Δ*sch9*, Δ*sitA*, and Δ*ppg1* mutants. BODIPY staining assays revealed that LD biogenesis was not observed in the Δ*sitA* and Δ*ppg1* strains upon treatment with rapamycin, in contrast to the wild-type and Δ*sch9* strains (**Fig. 8A, 8B, 8C, 8D**). These results indicated that LD biogenesis induced by rapamycin is predominantly reliant on the phosphatases SitA and Ppg1 in *A. flavus*. In *F. graminearum*, the phosphatase FgNem1/FgSpo7 complex was involved in the induction of LD biogenesis by rapamycin[24]. Phosphatase *nem1* (AFLA_002649) and *spo7* (AFLA_028590) were identified in *A. flavus*, which encode putative orthologs of *nem1* and *spo7* in *F. graminearum.* To examine the role of the *nem1* and *spo7* genes in *A. flavus*, we generated Δ*nem1* and Δ*spo7* mutants using homologous recombination.

The Δ*nem1* and Δ*spo7* mutants displayed a notable reduction in both vegetative growth and conidiation compared to the wild-type strain (**Fig. 9A, 9C, 9D**). TLC assay revealed a significantly decreased aflatoxin production in the Δ*nem1* and Δ*spo7* compared to the wild-type strain (**Fig. 9B**). In addition, BODIPY staining showed no visible lipid droplets in the hyphae of Δ*nem1* and Δ*spo7* after rapamycin treatment (**Fig. 9E**). Taken together, these results indicated that Nem1/Spo7 are involved in vegetative growth, conidiation, aflatoxin production, and LD biogenesis.

Discussion

Aflatoxins (AFs) are a class of toxic secondary metabolites synthesized by *A. flavus*, including aflatoxin B_1, B_2, G_1, and G_2, among which AFB_1 has been regarded as the most pathogenic and toxic aflatoxin[47]. Therefore, understanding the regulatory mechanisms involved in fungal secondary metabolites biosynthesis would be instrumental for effectively addressing aflatoxin control in *A. flavus*. In this study, we demonstrated that the target of rapamycin (TOR) signaling pathway plays a pivotal role in regulating growth, sporulation, sclerotia formation, aflatoxin production, and various stress responses in *A. flavus*. Through conducting functional studies on genes within the TOR signaling pathway, we explored the conservation and complexity exhibited by the TOR signaling pathway in *A. flavus*. In addition, we constructed a crosstalk network between the TOR and other signaling pathways (HOG, CWI) and analyzed the specific regulatory process. We proposed that the TOR signaling pathway interacts with multiple signaling pathways, including HOG and CWI pathways, to modulate the cellular responses to diverse environmental stresses (**Fig. 10**).

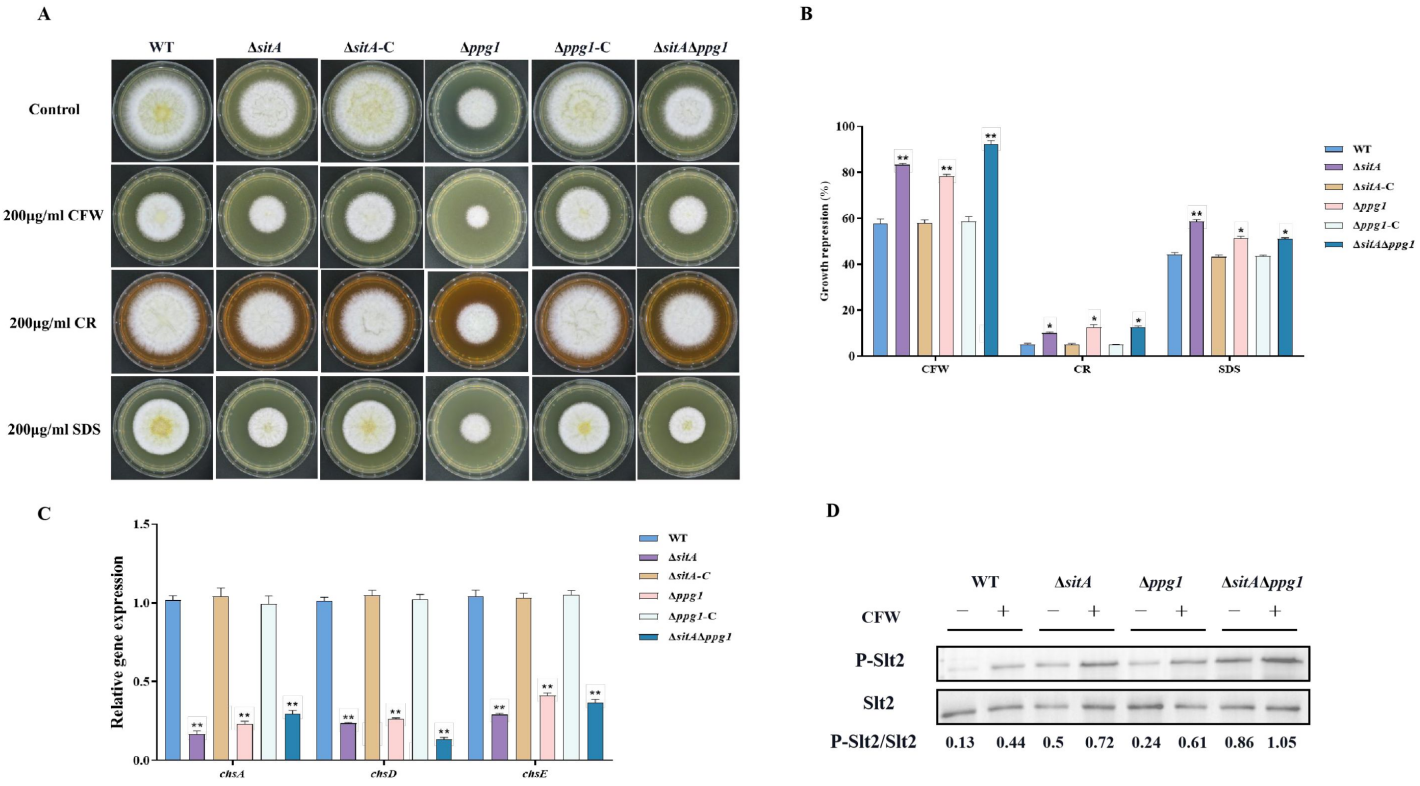

Fig. 7

Sensitivity of phosphatase SitA and Ppg1 to cell wall damaging agents in *A. flavus*.

(A) Morphology of the WT, single knockout, and double knockout strains grown on YES media supplemented with 200 μg/mL CR, 200 μg/mL CFW, or 100 μg/mL SDS at 37°C for 5 days.
(B) The growth inhibition rate of the WT, single knockout, and double knockout strains under cell wall and cell membrane stress.
(C) Relative expression levels of cell wall synthesis genes (*chsA*, *chsD* and *chsE*) in the WT, single knockout, and double knockout strains.
(D) The phosphorylation level of Slt2 in the WT, single knockout, and double knockout strains was detected with or without cell wall stress.

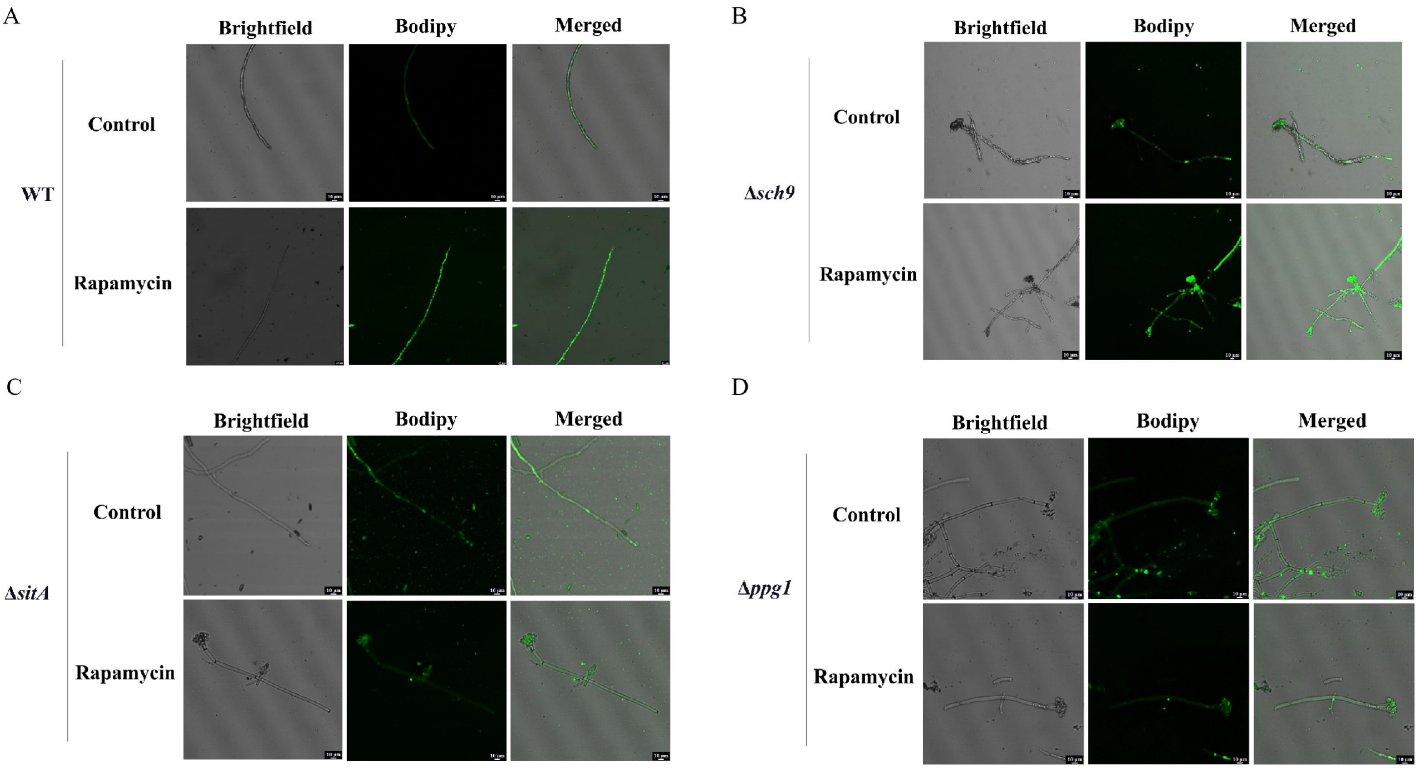

Fig. 8

Phosphatase SitA and Ppg1 are involved in regulating lipid droplet biogenesis.

(A) The intracellular lipid droplets were stained by boron dipyrromethene difluoride (BODIPY) in hyphae of the WT strain and observed under fluorescence microscopy. The phenotype of lipid droplets accumulation in the mycelia of the WT strain treated with or without 100 ng/mL rapamycin for 6 h (Bar, 10 μm).
(B) Phenotype of lipid droplets accumulation in the mycelia of the Δ*sch9* strain treated with or without 100 ng/mL rapamycin for 6 h (Bar, 10 μm).
(C) Phenotype of lipid droplets accumulation in the mycelia of the Δ*sitA* strain treated with or without 100 ng/mL rapamycin for 6 h (Bar, 10 μm).
(D) Phenotype of lipid droplets accumulation in the mycelia of the Δ*ppg1* strain treated with or without 100 ng/mL rapamycin for 6 h (Bar, 10 μm).

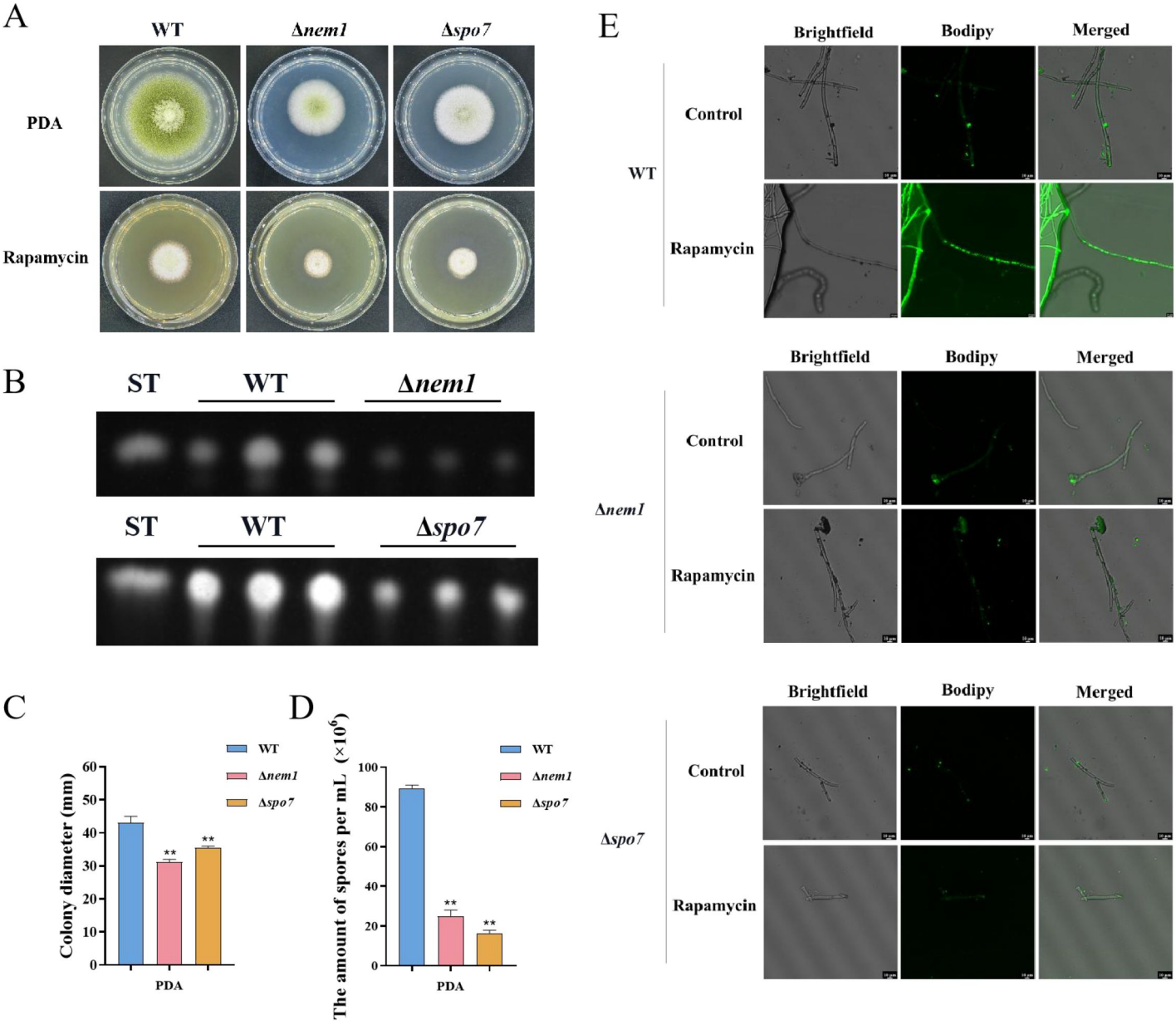

Fig. 9

Phosphatase complex Nem1/Spo7 play significant role in growth, conidiation, aflatoxin, and lipid droplet biogenesis.

(A) Phenotype of the WT, Δ*nem1*, and Δ*spo7* strains grown on PDA amended with 100 ng/mL rapamycin at 37°C for 5 days.
(B) TLC assay of AFB_1 production from the WT, Δ*nem1*, and Δ*spo7* strains cultured in YES liquid medium at 29°C for 6 days.
(C) Growth diameter of the WT, Δ*nem1*, and Δ*spo7* strains on PDA media.
(D) Statistical analysis of the sporulation by the WT, Δ*nem1*, and Δ*spo7* strains.
(E) Phenotype of lipid droplets accumulation in the mycelia of the WT, Δ*nem1*, and Δ*spo7* strains treated with or without 100 ng/mL rapamycin for 6 h (Bar, 10 μm).

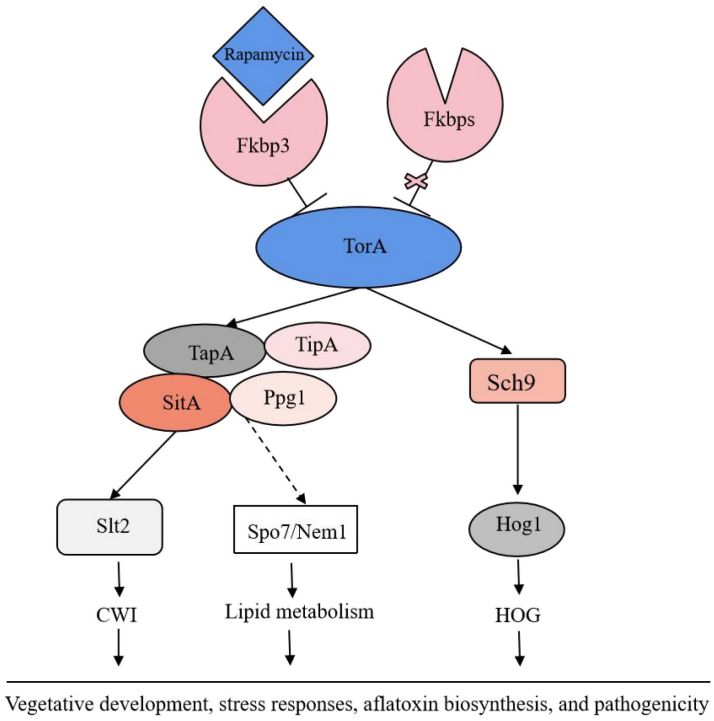

Fig. 10

The proposed model of the target of rapamycin (TOR) pathway in *A. flavus*.

Based on the above results, we propose a hypothesis regarding the TOR signaling pathway model in *A. flavus.* Rapamycin forms a complex with Fkbp3, and this particular complex can bind to the TorA kinase, thereby impeding its regular functionality. Sch9, functioning as a downstream element of the TorA kinase, regulates aflatoxin biosynthesis and HOG signaling pathway. As another important target of the TorA kinase, TapA-phosphatase complex is involved in the regulation of growth, sporulation, sclerotia, aflatoxin production, CWI signaling pathway, lipid droplet synthesis, and other processes. In conclusion, the TOR signaling pathway plays a crucial role in various aspects of *A. flavus*, including vegetative development, stress response, aflatoxin biosynthesis, and pathogenicity.

We have observed that rapamycin has a significant impact on the growth and sporulation of *A. flavus* (**Fig. 1A**). Additionally, it exhibits a strong inhibitory effect on sclerotia production and aflatoxin biosynthesis (**Fig. 1B, 1C**). The inhibitory effect has been documented in various fungal species, including *Candida albicans* [48], *Cryptococcus neoformans* [48], *Podospora anserine* [27], *A. nidulans* [28], *Magnaporthe oryzae* [39], and *F. graminearum* [23]. This suggested that the TOR signaling pathway may have a conserved function in filamentous fungi. The number of FK506-binding proteins varies among different species: there are four Fkbps proteins in *S. cerevisiae* and three Fkbps proteins in *S. pombe* [49]. In *S. cerevisiae*, rapamycin can bind to Fkbp protein, resulting in the irreversible inhibition of the G_1 phase of the cell cycle and the regulation of cell growth [50]. In *A. nidulans*, the *fkbp12* gene homologue *fprA* displays considerable sequence similarity with homologues identified in the *S. cerevisiae* [28]. In this study, based on the homologous alignment of the protein sequence of FprA, it was observed that four *fkbp* genes are present in *A. flavus*. Among these, deletion of *fkbp3* exhibited enhanced tolerance to rapamycin (**Fig. 2A**). So we speculated that rapamycin forms a complex with Fkbp3 in *A. flavus*, subsequently targeting downstream target genes to exert its biological function. We found that Fkbp3 is involved in sclerotia formation and aflatoxin biosynthesis in *A. flavus* (**Fig. 2A**, S3), and subsequent analysis revealed that the underlying mechanism may be associated with succinylation modification. The succinylation sites of Fkbp3 were identified through the utilization of succinylation proteomics data. By constructing point mutant strains and conducting phenotype experiments, we found that the succinylation of Fkbp3 at residue K19 is involved in rapamycin resistance and aflatoxin biosynthesis (**Fig. 2E, 2G**). Previous studies have demonstrated that Fkbp12 has been identified as the receptor for FK506 and rapamycin, which inhibits calcineurin and target rapamycin complex 1 (TORC1), respectively [50]. The deletion of Fkbp12 confers the resistance to rapamycin in *C. albicans* [48] and *C. neoformans* [48]. The entomopathogenic fungus *Beauveria bassiana* contains three putative *fkbp* genes, and disruption of *Bbfkbp12* significantly increases the resistance to rapamycin and FK506 [51]. The plant pathogenic fungus *F. graminearum* harbors three Fkbps proteins, among which deletion of *Fgfkbp12* leads to resistance to rapamycin [23]. Thus, we propose that these Fkbps may exhibit analogous functionalities in conferring resistance to rapamycin. In *Botrytis cinerea*, deletion of *Bcfkbp12* caused a reduction of the virulence of strain T4, while knockout of the *fkbp12* gene did not affect the pathogenic development of strain B05.10 [32]. These findings demonstrated that the *fkbp12* homologous genes exhibited conservation in terms of rapamycin resistance. However, there is significant variation in other biological functions among fungal species, which may be attributed to species or strain evolutionary divergence.

The Tor kinase functions as a fundamental component of the TOR signaling pathway, which exhibits evolutionary conservation across fungi, plants, and mammals [52]. In *S. cerevisiae*, the Tor kinase can interact with a diverse range of proteins, resulting in the formation of complex structures known as TORC1 and TORC2 [53]. TORC1 can detect various signals, including nutrients, growth factors, and environmental stress. It plays a crucial role in regulating cellular processes such as gene transcription, protein translation, ribosome synthesis, and autophagy [54]. A characteristic that distinguishes TORC1 is its interaction with KOG1 (Raptor in mammals) and its sensitivity to the rapamycin-FKBP12 complex [55]. Almost all other eukaryotes, including plants, worms, flies, and mammals, have a single *tor* gene, whereas *S. cerevisiae* has two *tor* genes [54]. The Tor kinase is widely conserved both in terms of its structural characteristics and its role as the target of Fkbp-rapamycin. Typically, the Tor kinase exhibits similar structural characteristics, including the HEAT-like, FAT, FRB, kinase, and FATC domains. The FRB domain is the binding region of the Fkbp-rapamycin complex, and rapamycin resistance is usually caused by the disruption of the FRB domain [54]. The comparison of homology and the analysis of phylogenetics revealed that there is only one predicted *torA* gene in *A. flavus*. To ascertain its functionality, several ectopic transformants were obtained through multiple protoplast transformations, however, no successful knock-out transformants were obtained. We speculated that the deletion of the *torA* gene may be lethal in *A. flavus*. This observation aligns with the situation observed in *F. graminearum* [23]. Previous findings demonstrated that the Tor kinase

could function as a molecular switch connecting an iron cue to defend against pathogen infection in *C. elegans*[56]. In *A. fumigatus*, the Tor kinase represents a central regulatory node controlling genes and proteins involved in nutrient sensing, stress response, cell cycle progression, protein biosynthesis, and degradation, as well as adaptation to low iron conditions[57]. In summary, the Tor kinase acts as a central regulator in the TOR signaling pathway, playing a crucial role in the regulation of the cell cycle, protein synthesis, and cellular energy metabolism.

In *S. cerevisiae*, the protein kinase Sch9 is a major downstream effector of TORC1, and this kinase is known to have crucial functions in stress resistance, longevity, and nutrient sensing [44]. In addition, the TORC1-Sch9 pathway acts as a crucial mediator of chronological lifespan [44]. In filamentous fungi, an intricate network of interactions exists between the TOR and HOG pathways. In *F. graminearum* [38], FgSch9 and FgHog1 mutants displayed heightened susceptibility to osmotic and oxidative stresses. The phosphorylation level of FgHog1 increased significantly in FgSch9 mutant. Additionally, the affinity capture-MS assay showed that the Tor kinase was among the proteins co-purifying with FgSch9. These results suggest that FgSch9 serves as an intermediary for the TOR and HOG signaling pathways [38]. Furthermore, FgSch9 plays a crucial role in the regulation of various biological processes such as hyphal differentiation, asexual development, virulence, and DON biosynthesis[38]. In *A. fumigatus*, the SchA mutant was sensitive to rapamycin, high concentrations of calcium and hyperosmotic stress, and SchA was involved in iron metabolism. The SchA mutant exhibited enhanced phosphorylation of SakA in response to osmotic stress [29]. In this study, we discovered that Sch9 is involved in regulating aflatoxin biosynthesis (**Fig. 3C**), and responses to osmotic stress and rapamycin stress (**Fig. 3B**, 3D). The S_TKc domain deletion strain (*sch9*$^{\Delta S\_TKc}$), S_TK_X domain deletion strain (*sch9*$^{\Delta S\_TK\_X}$), and *sch9*^{K340A} mutant displayed a similar phenotype to the Δ*sch9* strain (**Fig. 3B, 3C, 3D**). These findings revealed that Sch9 plays a significant role in the biological synthesis of aflatoxin and in responding to various stresses. This role is primarily mediated through its kinase domain, kinase-C domain, and ATP binding site K340. We also found that the phosphorylation level of Hog1 increased significantly in the Δ*sch9* strain under osmotic stress (**Fig. 3E**). Based on the above results, we hypothesized that Sch9 was involved in the osmotic stress response by modulating the HOG pathway.

Tap42, a protein that associates with phosphatase 2A, serves as the direct target of the Tor kinase in yeast. Tap42 plays a crucial role in various Tor functions, particularly in the regulation of transcriptional processes [58]. The genome of *A. flavus* contains a homolog of *tap42* named *tapA* (AFLA_092770), which is predicted to encode a 355 amino-acid protein (sharing 31.9% identity with *S. cerevisiae* Tap42). To further determine the function of the TapA regulator in the TOR signaling pathway of *A. flavus*, we conducted targeted gene deletion experiments on *tapA*. We failed to obtain a null mutant, we hypothesised that *tapA* knockout was lethal in *A. flavus*. In *S. pombe*, the protein Tip41, which interacts with Tap42, plays a significant role in the cellular responses to nitrogen sources by regulating type 2A phosphatases [59]. In *S. cerevisiae*, Tap42-phosphatase complexes associate with TORC1, whereas Tip41 functions to attenuate TORC1 signaling by stimulating the PP2A phosphatase [45]. Unlike its yeast counterpart Tip41, TIPRL, a mammalian Tip41-like protein, exerts a positive influence on mTORC1 signaling by interacting with PP2Ac [60]. While the interaction between Tip41 and Tap42 was not observed in the filamentous fungi. The yeast two-hybrid (Y2H) assay showed that FgTip41 interacts with the phosphatase FgPpg1 rather than FgTap42, while the FgTap42 bind with phosphatases FgPp2A, implying that FgTap42, FgPpg1, and FgTip41 form a heterotrimer in *F. graminearum* [23]. In the rice blast fungus *M. oryzae*, MoTip41 does not interact with MoTap42. However, MoTip41 can bind with the phosphatase MoPpe1, thereby facilitating crosstalk between the TOR pathway and the cell wall integrity pathway [40]. In this study, we found that TipA played a crucial role in sclerotia production and cell wall stress in *A. flavus* (**Fig. 4B**, 4H). However, the interaction between TapA and TipA is not yet clear in *A. flavus*.

In *S. cerevisiae*, Tap42 can bind and regulate various PP2A phosphatases, including Pph3, Pph21, Pph22, Sit4, and Ppg1. This interaction plays a crucial role in the TOR signaling pathway, particularly in response to rapamycin treatment or nutrient deprivation [45]. The phosphatase Sit4 plays a crucial role in regulating cell growth, morphogenesis, and virulence in *C. albicans* [61]. FgSit4 and FgPpg1 are associated with various cellular processes, including mycelial growth, conidiation, DON biosynthesis, and virulence in *F. graminearum* [23]. In addition, FgSit4 and FgPpg1 are involved in the regulation of various signaling pathways, such as the CWI pathway and lipid droplets biogenesis regulation pathway [23]. In this study, we found that the phosphatase SitA and Ppg1 play critical role in vegetative growth, conidiation, sclerotia formation, and aflatoxin biosynthesis in *A. flavus* (**Fig. 5A, 5B, 5C**). Additionally, the phosphatase SitA and Ppg1 also affect pathogenicity and LD biogenesis (**Fig. 6A**, **8C, 8D**). In addition, SitA, Ppg1, and TipA exhibits similar functions in sclerotia production and cell wall stress response. Based on these observations, we hypothesized that TapA-phosphatase complexes play a collaborative role in the regulation of the sclerotia formation and cell wall stress response in *A. flavus*.

After the deletion of the *sitA* and *ppg1* genes, the expression level of genes related to cell wall synthesis has decreased significantly, resulting in damage to the cell wall and showing a higher sensitivity to cell wall integrity stress (**Fig. 7C**). Western blotting results showed that the phosphorylation level of MAPK kinase Slt2 has increased significantly in the Δ*sitA* and Δ*ppg1* strains under cell wall stress, indicating that SitA and Ppg1 exert a negative regulatory effect on the CWI pathway (**Fig. 7D**). In *M. oryzae*, MoPpe1 and MoSap1 serve as an adaptor complex that connects the CWI and TOR signaling pathways. Activation of the TOR pathway results in the inhibition of the CWI pathway [39]. In addition, the phosphatase MoPpe1 interacts with the phosphatase-associated protein MoTip41, facilitating the exchange of information between the TOR and CWI signaling pathways [39]. These results suggest that the phosphatase Ppe1 engages in interactions with various proteins to effectively coordinate the TOR and CWI signaling pathways, thereby regulating the growth and pathogenicity of the rice blast fungus *M. oryzae* [39]. Taken together, we speculated that the phosphatase SitA and Ppg1 might exhibit differential regulation in the CWI and TOR pathways across species in order to fulfill their respective functions.

In *F. graminearum,* the phosphatase complex FgNem1/Spo7-FgPah1 cascade plays significant roles in fungal development, DON production, and lipid droplet (LD) biogenesis [24]. In this study, our results indicated that Nem1 and Spo7 are important for vegetative growth and secondary metabolism of *A. flavus* (**Fig. 9A, 9B**). We have also discovered that LD biogenesis is facilitated by the Nem1/Spo7 and SitA/Ppg1 pathways (**Fig. 8C, 8D**, **9E**). These observations suggest that the PP2A phosphatases play a central role as core phosphatases in the LD biogenesis signaling network. However, there are likely other kinases involved in the regulation of LD biogenesis. To gain a comprehensive understanding of the precise molecular mechanism, further research should focus on investigating the interplay between phosphatases and other kinases.

In conclusion, we identified the complex regulatory network that provides insight into the interplay between the TOR, HOG, and CWI signaling pathways, and how they collectively regulate the development, aflatoxin biosynthesis, and pathogenicity of *A. flavus*. By elucidating the functions of genes associated with the TOR signaling pathway, our research could establish a solid foundation for comprehending the molecular mechanism underlying the aflatoxin biosynthesis of *A. flavus*. This, in turn, can provide novel insights into the management of aflatoxin contamination and the mitigation of *A. flavus* infection.

Materials and methods

Strains and cultural conditions

The *A. flavus* strains in this study were shown in Table S1. All strains were cultivated on three different types of agar media: potato dextrose agar (PDA), yeast extract-sucrose agar (YES), and yeast extract-glucose agar (YGT). The growth and conidiation assays were conducted at 37°C. Additionally, aflatoxin production was assessed using YES liquid medium at 29°C.

Constructions of gene deletion and complemented mutants

The construction of gene deletion and complemented strains was carried out using the SOE-PCR strategy, following previously established protocols [62]. The primers utilized for the amplification of the flanking sequences of each gene can be found in Table S2. The putative gene deletion mutants were validated through PCR assays using the appropriate primers (Fig. S2), and their confirmation was subsequently obtained through sequencing analysis.

Growth, conidiation, and sclerotia analysis

To evaluate mycelial growth and conidia formation, 1 μL of 10^6 spores/mL conidia of all strains was point inoculated onto the center of the PDA medium, and then cultured at 37°C for 5 days in the dark. The conidia were washed with 0.07% Tween-20 and counted using a hemocytometer and microscope [43]. For sclerotia formation analysis, each strain was inoculated and cultivated on CM agar medium at 37°C in the dark for 7 days, and then 75% ethanol was used to wash away mycelia and conidia on the surface of the medium [63]. These assays were repeated at least three times.

Extraction and quantitative analysis of aflatoxin

To extract and quantify aflatoxins (AFs) production, 1 μL of 10^6 spores/mL conidia of all strains was point inoculated onto liquid YES medium, and cultures were then incubated at 29°C in the dark for 6 days. AFs were extracted from the media and detected using thin layer chromatography (TLC) [63]. In addition, the standard AFB_1 was employed for visual comparison, and AFs were quantitatively analyzed using the Image J tool. The assays were repeated at least three times.

Stress assay

To determine the roles of the gene in *A. flavus* responsing to various environmental stresses, 1 μL of 10^6 spores/mL conidia of all strains were point inoculated onto these PDA solid plates supplemented with various agents. These agents included 1M NaCl, 1M KCl, and 1M $CaCl_2$ for inducing osmotic stress, as well as 100 μg/mL SDS, 200 μg/mL Calcofluor white (CFW), and 200 μg/mL Congo red (CR) for inducing cell wall stress. After 5 days of incubation at 37°C in the dark, the relative rates of inhibition were determined [43]. The assays were repeated at least three times.

Seeds infection assays

To measure the pathogenicity of *A. flavus*, all peanut and maize seeds were washed in 0.05% sodium hypochlorite, 75% ethanol, and sterile water, respectively. Subsequently, all peanut and maize seeds were immersed in sterile water containing 10^6 spores/mL of all strains at 29°C in the dark for 60 min. After the conidia adhesion, the seeds were cultured at 29°C for 6 days in the dark [64].

Quantitative RT-PCR

For the real-time quantitative PCR (qPCR) assays, the *A. flavus* strains were cultivated in the PDA medium at 29°C for 3 days in the dark. Total RNA was extracted from the mycelia of all strains, and cDNA was synthesized with the First Strand cDNA Synthesis Kit, and then the cDNA was used as the template for qRT-PCR analysis with SYBR Green qPCR mix. The relative transcript level of each gene was calculated by the $2^{-\Delta\Delta CT}$method [65].

Western blot analysis

To extract the total protein, the mycelia of each strain were cultivated in YES liquid medium for 48 h, then the sample was grinded under liquid nitrogen to a fine powder and transferred to a 1.5 mL microcentrifuge tube added with RIPA lysis buffer and 1 mM PMSF protease inhibitor. Proteins were separated by 12% SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane. Phosphorylation levels of Hog1 and Slt2 were detected by Phospho-p38 MAPK antibody (Cell Signaling Technology, Boston, MA, USA) and Phospho-p44/42 MAPK (Erk 1/2) antibody (Cell signaling Technology, MA, USA), respectively. The experiment was conducted three times independently.

Statistical analysis

All data were measured with means ± standard deviation (SD) of three biological replicates. Graph Pad Prism 7 software was used for conducting statistical and significance analysis, and p-values < 0.05 represented statistical significance. Student's *t*-test was used to compare the two means for differences, whereas Tukey's multiple comparisons were used for testing multiple comparisons.

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Author contributions

Guoqi Li and Xiaohong Cao designed the experiments. Guoqi Li, Xiaohong Cao, and Qianhua Zeng performed the experiments. Guoqi Li, Xiaohong Cao, and Shihua Wang analyzed the data. Guoqi Li, Elisabeth Tumukunde, and Shihua Wang wrote the manuscript. The manuscript was discussed and revised by all the authors.

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Reviewer #1 (Public Review):

While I acknowledge the authors' effort in conducting Southern blot analysis to address my prior concern regarding the presence of dual copies of torA and tapA, I find their current resolution inadequate. Specifically, the simple deletion of the respective result sections for torA and tapA significantly impacts the overall significance of this study. The repeated unsuccessful attempts to generate correct mutants only offer circumstantial evidence, as technical issues may have been a contributing factor. Therefore, instead of merely removing these sections, it is essential for the authors to present more compelling experimental data demonstrating that torA and tapA are indeed vital for the viability of A. flavus. Such data would enhance the overall significance of this study.

https://doi.org/10.7554/eLife.89478.3.sa1

Reviewer #2 (Public Review):

In this study, authors identified TOR, HOG and CWI signaling network genes as modulators of the development, aflatoxin biosynthesis and pathogenicity of A. flavus by gene deletions combined with phenotypic observation. They also analyzed the specific regulatory process and proposed that the TOR signaling pathway interacts with other signaling pathways (MAPK, CWI, calcineurin-CrzA pathway) to regulate the responses to various environmental stresses. Notably, they found that FKBP3 is involved in sclerotia and aflatoxin biosynthesis and rapamycin resistance in A. flavus, especially that the conserved site K19 of FKBP3 plays a key role in regulating aflatoxin biosynthesis. In general, the study involved a heavy workload and the findings are potentially interesting and important for understanding or controlling the aflatoxin biosynthesis. However, the findings have not been deeply explored and the conclusions mostly are based on parallel phenotypic observations.

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Author Response

The following is the authors' response to the original reviews.

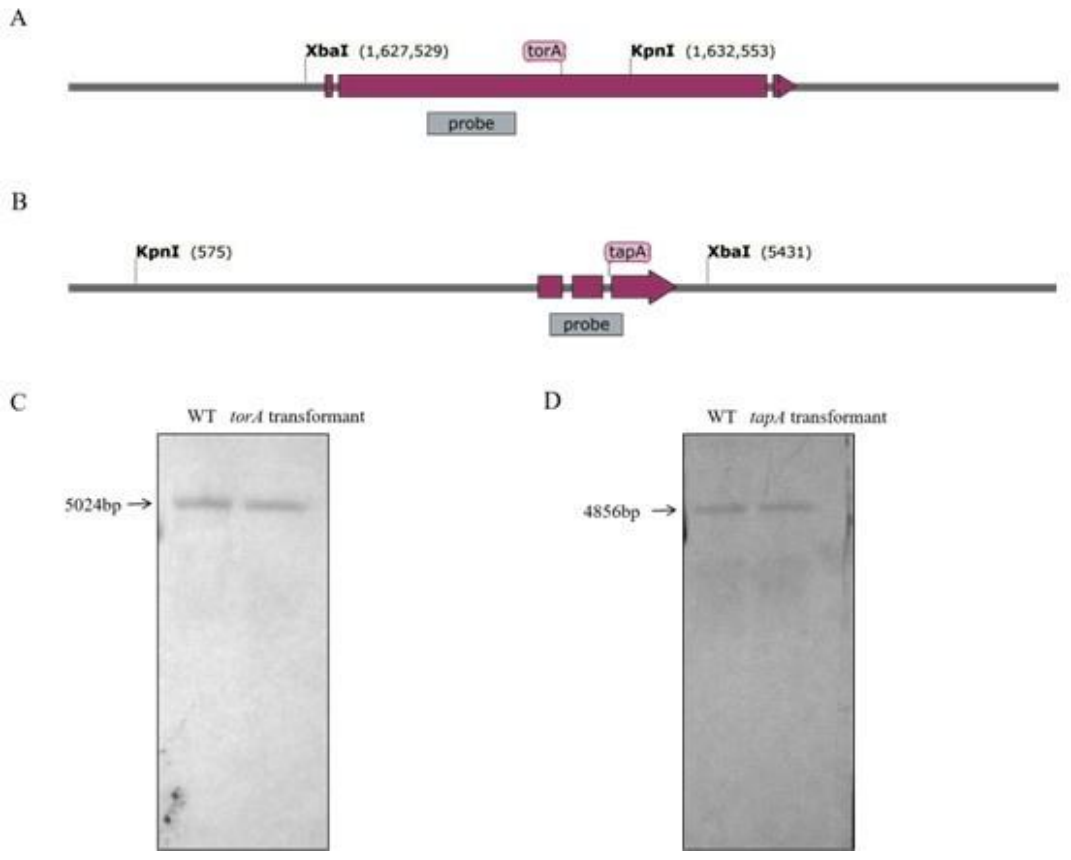
> ***Response to Reviewer 1 Comments (PublicReview)***
>
> *Point 1: First, the authors should provide more convincing data showing that tor and tapA genes are indeed duplicated genes in A. flavus. The authors appeared to use the A. flavus PTS strain as a parental strain for constructing the tor and tapA mutants. If so, the A. flavus CA14 strain (Hua et al., 2007) should be the parental wild-type strain for the A. flavus PTS strain. I did a BLAST search in NCBI for the torA (AFLA_044350) and tapA (AFLA_092770) genes using the most recent CA14 genome assembly sequence (GCA_014784225.2) and only found one allele for each gene: torA on chromosome 7 and tapA on chromosome 3. I could not find any other parts with similar sequences. Even in*

> *another popular A. flavus wild-type strain, NRRL3357, both torA and tapA exist as a single allele. Based on the published genome assembly data for A. flavus, there is no evidence to support the idea that tor and tapA exist as copies of each other. Therefore, the authors could perform a Southern blot analysis to further verify their claim. If torA and tapA indeed exist as duplicate copies in different chromosomal locations, Southern blot data could provide supporting results.*

Response 1: We thank the reviewer for their insightful observation. Based on the southern blot analysis results presented in Figure 1, we have determined that torA and tapA are single-copy genes. Additionally, we conducted protoplast transformation experiments repeated several times. which revealed that both torA and tapA transformants exhibited ectopic mutations. It is plausible that the deletion of torA and tapA genes may lead to the demise of A. flavus, this phenomenon is consistent with previous studies conducted on the fungus Fusarium graminearum[1].To ensure the rigor of the study, we have retracted the previously incorrect conclusion. We once again express our heartfelt appreciation to the experts for their valuable suggestions.

Author response image 1.

Fig.1 Southern blot hybridization analyses of WT, torA, and tapA transformants. (A) The structure diagram of the torA gene. (B) The structure diagram of the tapA gene. (C) Southern blot hybridization analyses of torA gene. (D) Southern blot hybridization analyses of tapA gene.



> *Point 2: Second, the authors should consider the possibility of aneuploidy for their constructed mutants. When an essential gene is targeted for deletion, aneuploidy often occurs even in a fungal strain without the "ku" mutation, which results in seemingly dual*

> *copies of the gene. As the authors appear to use the A. flavus PTS strain having the "ku" mutation, the parental strain has increased genome instability, which may result in enhanced chromosomal rearrangements. So, it will be necessary to Illumina-sequence their tor and tapA mutants to make sure that they are not aneuploidy.*

Response 2: Thank you for your comment. Based on the sequencing results of the torA and tapA mutants, it was determined that the torA and tapA genes were still present in both mutants. In this case, it suggests that the torA and tapA genes may have undergone a genetic rearrangement or insertion at a different site in the mutant strains.

> *Point 3: Furthermore, the genetic nomenclature +/- and -/- should be reserved for heterozygous and homozygous mutants in a diploid strain. As A. flavus is not a diploid strain, this type of description could cause confusion for the readers.*

Response 3: Thank you for your suggestion. We acknowledge your concerns about potential confusion caused by using this type of description, and we agree that it is best to avoid any misunderstandings for readers. Therefore, we have decided to remove this part of the content from the manuscript.

> ***Response to Reviewer 2 Comments (PublicReview)***
>
> *Point 1: However, findings have not been deeply explored and conclusions are mostly are based on parallel phenotypic observations. In addition, there are some concerns for the conclusions.*

Response 1: We are grateful for the suggestion. We conduct additional experiments and analyses to provide a more comprehensive understanding and address concerns raised.

> ***Response to Reviewer 3 Comments (PublicReview)***
>
> *Point 1: The paper by Li et al. describes the role of the TOR pathway in Aspergillus flavus. The authors tested the effect of rapamycin in WT and different deletion strains. This paper is based on a lot of experiments and work but remains rather descriptive and confirms the results obtained in other fungi. It shows that the TOR pathway is involved in conidiation, aflatoxin production, pathogenicity, and hyphal growth. This is inferred from rapamycin treatment and TOR1/2 deletions. Rapamycin treatment also causes lipid accumulation in hyphae. The phenotypes are not surprising as they have been shown already for several fungi. In addition, one caveat is in my opinion that the strains grow very slowly and this could cause many downstream effects. Several kinases and phosphatases are involved in the TOR pathway. They were known from S. cerevisiae or filamentous fungi. The authors characterized them as well with knock-out approaches.*

Response 1: Thank you for your comment. The role of the target of rapamycin (TOR) signaling pathway is of fundamental importance in the physiological processes of diverse eukaryotic organisms. Nevertheless, its precise involvement in regulating the developmental and virulent characteristics of opportunistic pathogenic fungi, such as A. flavus, has yet to be fully elucidated. Furthermore, the mechanistic underpinnings of TOR pathway activity specifically in A. flavus remain largely unresolved. Consequently, our study represents a significant contribution as the first comprehensive exploration of the conserved TOR signaling pathway encompassing a majority of its constituent genes in A. flavus.

> ***Response to Reviewer 1 Comments (Recommendations For The Authors)***
>
> *Point 1: In Table S3, the authors indicated that the Δku70 ΔniaD ΔpyrG::pyrG strain is A. flavus wild-type strain. However, this strain is not a wild-type strain because it seems like*

> *a control strain after introducing the pyrG gene into the A. flavus PTS strain (Δku70 ΔniaD ΔpyrG). So please indicate the real wild-type A. flavus strain name to help readers find out its original genome sequence data. Also, the reference for this Δku70 ΔniaD ΔpyrG::pyrG strain is "saved in our lab". This is not an eligible reference. If you use this control strain for the first time in this study, it should be described as "In this study". Otherwise, please indicate the proper reference for which the strain was first used.*

Response 1: Thank you for your valuable feedback on our manuscript. We appreciate your attention to detail and the opportunity to clarify the information regarding the strain in Table S3. The A. flavus CA14 strain which produces aflatoxins and large sclerotia was isolated from a pistachio bud in the Wolfskill Grant Experimental Farm (University of Davis, Winters, California, USA)[2]. The A. flavus CA14 strain is the parental wild-type strain for the A. flavus CA14 PTs (Δku70, ΔniaD, ΔpyrG) strain. The recipient strain CA14 PTs has been used satisfactorily in gene knockout and subsequent genetic complementation experiments[3]. In this study, the A. flavus CA14 PTs strain was used as the transformation recipient strain, and the control strain (Δku70, ΔniaD, ΔpyrG::pyrG) created by introducing the pyrG gene into the A. flavus CA14 PTs strain. Refer to previously published literature[4]，this control strain (Δku70, ΔniaD, ΔpyrG::pyrG) was named wild-type strain. Therefore, this control strain was also named wild-type strain in this study. As this control strain is indeed used in this study, we will revise the reference to "In this study" Once again, we appreciate your keen attention to detail and thank you for bringing these issues to our attention.

> ***Response to Reviewer 2 Comments (Recommendations For The Authors)***
>
> *Point 1: As in response: However, the tor gene in A. flavus exhibited varying copy numbers, as was confirmed by absolute quantification PCR at the genome level (Table S1). However, it is hard to understand Table S1: Estimation of copy number of tor gene in A. flavus toro and sumoo stand for the initial copy number, and the data are figured as the mean {plus minus} 95% confidence limit. CN is copy number. As indicated in the section of Method, using sumo gene as reference, the tor and tapA gene copy number was calculated by standard curve. In Table S1 of WT, for tor gene, CN value is 1412537 compared to 1698243 in tor+/-, for the reference gene sumo,794328 compared to1584893, how these data could support copy gene numbers of tor?*

Response 1: Thank you for your suggestion. We understand the confusion with the data presented in Table S1 regarding the copy number estimation of the tor gene in A. flavus. We apologize for not providing a clear explanation for the data in the table. Quantitative real-time PCR (qPCR) is widely used to determine the copy number of a specific gene. It involves amplifying the gene of interest and a reference gene simultaneously using specific primers and probes. By comparing the amplification curves of the gene of interest and the reference gene, you can estimate the relative copy number of the gene.

To address your concern and provide more accurate information, we have re-performed the copy number analysis using southern blot. Southern blot analysis allows for the direct estimation of gene copy number by hybridizing genomic DNA with a specific probe for the gene. This method provides more reliable and accurate results in determining gene copy numbers. The southern blot analysis results are presented in Figure 1.

We appreciate your input and apologize for any confusion caused by the earlier presentation of the data.

> *Point 2: In response: For the knockout of the FRB domain, we used the homologous recombination method, but because tor genes are double-copy genes, there are also double copies in the FRB domain. Despite our efforts, we encountered challenges in*

> *precisely determining the location of the other copy of the tor gene. I could not understand these consistent data, why not for using sequencing?*

Response 2: Thank you for your comment. We observed that the torA gene is a single copy. We removed this part of the results to avoid any ambiguity or potential misinterpretation.

> *Point 3: Response in Due to the large number of genes involved, we did not perform a complementation experiment. If there were no complementation data, how to demonstrate data are solid?*

Response 3: Thank you for your important suggestion. We understand that complementation experiments are commonly used to validate gene deletions. Therefore, to ensure the reliability of our data, we have conducted supplementary experiments on specific gene deletions, such as ΔsitA-C and Δppg1-C. Thank you again for your positive comments and valuable suggestions to improve the quality of our manuscript.

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