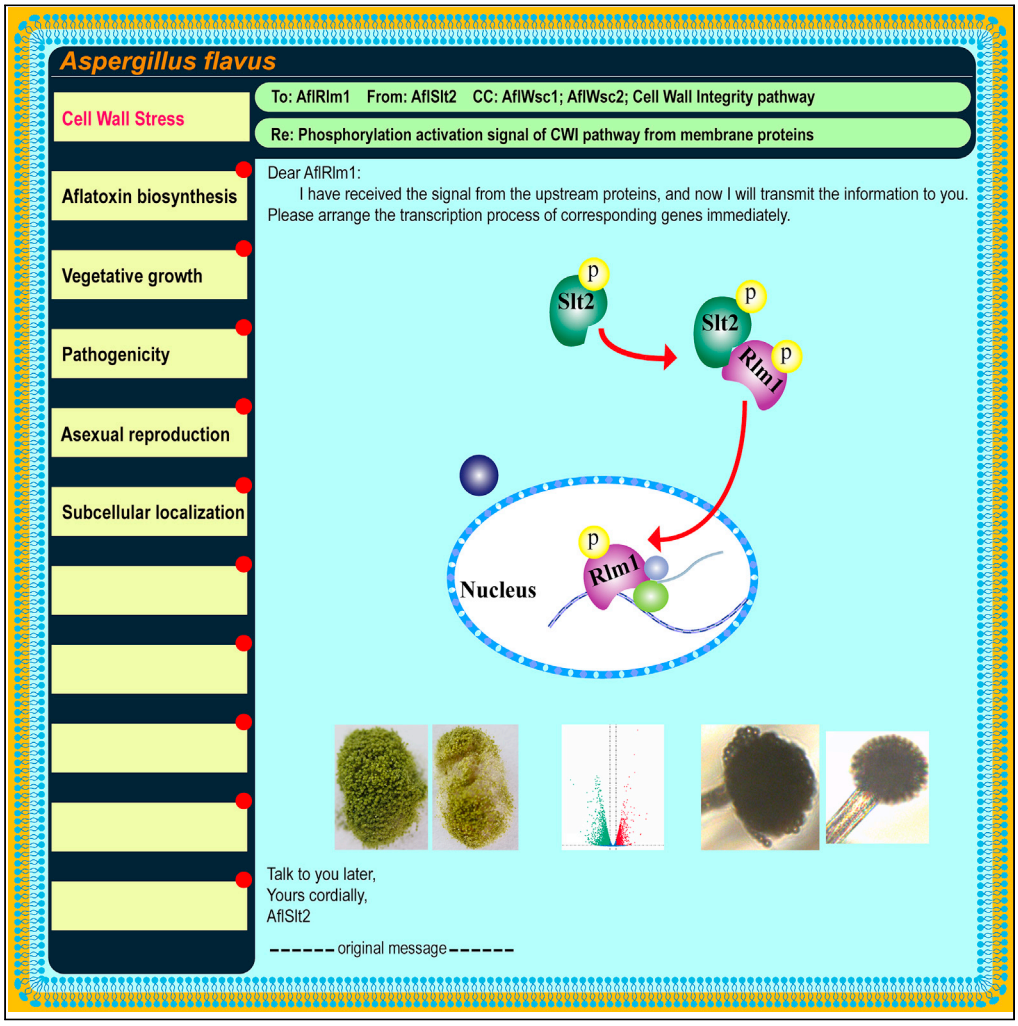
iScience

CellPress
OPEN ACCESS

Article

CWI pathway participated in vegetative growth and pathogenicity through a downstream effector AflRlm1 in *Aspergillus flavus*



Can Tan, Ji-Li Deng, Feng Zhang, ..., Meng-Juan Zhang, Jun Yuan, Shi-Hua Wang

wshyyl@sina.com

Highlights

Linked the CWI pathway from membrane receptors to transcription factors in *A. flavus*

Found the phosphorylate activation and subcellular metastasis of AflRlm1 in stress

Discovered the important role of AflRlm1 in aflatoxin biosynthesis

Tan et al., iScience *24*, 103159
October 22, 2021 © 2021 The Author(s).
https://doi.org/10.1016/j.isci.2021.103159

iScience

CellPress
OPEN ACCESS

Article

CWI pathway participated in vegetative growth and pathogenicity through a downstream effector AflRlm1 in *Aspergillus flavus*

Can Tan,[1] Ji-Li Deng,[1] Feng Zhang,[1] Zhuo Zhu,[1] Li-Juan Yan,[1] Meng-Juan Zhang,[1] Jun Yuan,[1] and Shi-Hua Wang[1,2,*]

SUMMARY

The cell wall is an essential dynamic structure for shielding fungus from environmental stress, and its synthesizing and remodeling are regulated by the cell wall integrity (CWI) pathway. Here, we explored the roles of a putative downstream effector AflRlm1 of CWI pathway in *Aspergillus flavus*. The results showed that AflRlm1 played a positive role in conidia production, sclerotium formation, aflatoxin biosynthesis, and pathogenicity. Furthermore, we provided evidence for the physical connection between AflRlm1 and AflSlt2 and determined the role of AflSlt2 in the phosphorylation of AflRlm1. Then, we discovered the importance of WSCs (cell wall integrity and stress response component) to the CWI signal and the process of AflRlm1 transferring to the nucleus after receiving the signal. Overall, this study clarified the transmission process of CWI signals and proves that the CWI pathway plays a key role in the development of *A. flavus* and the production of aflatoxin combined with transcriptome data analysis.

INTRODUCTION

The life activities of all known living organisms are based on complex and subtle metabolic processes. A prominent molecular pathway for regulating metabolism is the cascade of protein kinases known as the mitogen-activated protein kinase (MAPK) module (Chavel et al., 2014; Widmann et al., 1999). There are different MAPKs on diverse functionally distinct cascades in *Saccharomyces cerevisiae*, such as the pheromone response pathway, the filamentous growth pathway, the cell wall integrity (CWI) pathway, and the high-osmolarity glycerol (HOG) pathway (Chen and Thorner, 2007; Gustin et al., 1998; Mao et al., 2011; Mizuno et al., 2015). The CWI pathway controls the processes of the remodeled and polarized manner of the cell wall, responding to environmental stimuli, growth, and morphogenesis (Fuchs and Mylonakis, 2009; Nishida et al., 2014; Sanz et al., 2017). In yeast, WSC (cell wall integrity and stress response component) sensors transmit signals to the inside of the cell (Ohsawa et al., 2017), and then, the signals are transmitted to small GTPase Rho1, which activates protein kinase C (Pkc1) through the GDP/GTP exchange factor Rom2 (Philip and Levin, 2001). Pkc1 phosphorylates the downstream key kinase Bck1, while Mkk1/2 and Slt2 will be phosphorylated later in other bio-processes (Cruz et al., 2013); then, the two transcription factors Rlm1 and SBF complexes are activated by Slt2 (Kock et al., 2016). The components of the CWI pathway usually play important roles in the growth and development of organisms. It was reported that the deletion of *pkc1* triggers cell death in *Aspergillus nidulans* and *Magnaporthe grisea* (Sugahara et al., 2019). The loss of Bck1 homolog posed a serious threat to the growth of the strains, such as *Cryphonectria parasitica*, *M. oryzae*, and *A. flavus* (Jeon et al., 2008; Kim et al., 2016; Zhang et al., 2020b). In *S. cerevisiae*, the loss of Mkk1 and Mkk2 together caused the phenomenon of temperature-sensitive cell autolysis, whereas overexpression inhibited the autolysis of temperature-sensitive *pkc1* mutant (Irie et al., 1993). The deletion of Slt2 encoding gene also caused serious growth defects in *C. parasitica* and *A. flavus* (So et al., 2017; Zhang et al., 2020a).

As a direct control element of metabolism activities, the function and mechanism of the CWI transcription factor have always been the focus of attention. In *S. cerevisiae*, Rlm1 has been proven to be a downstream transcription factor in the CWI pathway, and transcriptional activation by Rlm1 requires its C-terminal sequences (Dodou and Treisman, 1997). The Δ*rlm1* satellite-cell phenotype was suppressed by deletion of either Slt2 or Swi4 (Piccirillo et al., 2017). Under cell wall stress, Rlm1 is recruited to the promoters of Rlm1 and Slt2, exerting positive feedback mechanism on the expression of both genes (Garcia et al., 2016). It

[1]Key Laboratory of Pathogenic Fungi and Mycotoxins of Fujian Province, Key Laboratory of Biopesticide and Chemical Biology of Education Ministry, and School of Life Sciences, Fujian Agriculture and Forestry University, Fuzhou 350002, China

[2]Lead contact

*Correspondence: wshyyl@sina.com

https://doi.org/10.1016/j.isci.2021.103159

This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

was found that Rlm1 activation in response to catecholamines is a result of oxidative stress in yeast, and the oxidant hydrogen peroxide was found to activate transcription of an Rlm1 reporter (Staleva et al., 2004). In *C. glabrata*, the Rlm1 deletion strains are sensitive to cell wall stress, while overexpression of Rlm1 increases the resistance to micafungin, whether Slt2 was deleted or not (Miyazaki et al., 2010). In *C. albicans*, Rlm1 mediates cell wall remodeling during carbon adaptation (Oliveira-Pacheco et al., 2018), and Rlm1 indirectly controls caspofungin-induced Sko1 transcription (Heredia et al., 2020). It is also found that Rlm1 mutant was hypersensitive to cell wall stress, and genes involved in carbohydrate catabolism were significantly downregulated in Rlm1 mutant (Delgado-Silva et al., 2014).

Besides transcription factors, cell membrane sensors of the CWI pathway are also important. WSC family proteins are reported as cell membrane sensors of the CWI pathway from yeast to fungi (Ketela et al., 1999; Maddi et al., 2012). In *S. cerevisiae*, five putative WSC sensors of the CWI pathway were distributed in the cell membrane (Tong et al., 2016). After knockout of Wsc1 and/or Wsc3, yeast could not grow on YPD medium without sorbitol (Verna et al., 1997; Wilk et al., 2010). Moreover, incomplete Wsc1 prevents yeast from growing normally on the medium (Lodder et al., 1999). Deletion of Wsc1 incurred growth damage under cell wall stress (Bermejo et al., 2010; Straede and Heinisch, 2007). It was also found that there was a high frequency of swollen hyphae under hypo-osmotic conditions in *wscA* knockout strain of *A. nidulans* (Futagami et al., 2011).

Aspergillus flavus is a widely distributed filamentous fungus, which can contaminate many crops causing enormous economic losses (Amaike and Keller, 2011). In addition, *A. flavus* is the major airborne opportunistic pathogen resulting in diseases like aspergillosis and liver cancer in animals and humans (Krishnan-Natesan et al., 2008; Krishnan et al., 2009; Lan et al., 2018). Its secondary metabolites aflatoxin, a level 1 carcinogen, can directly or indirectly lead to serious diseases and death in organisms (Hedayati et al., 2007; Heinemann et al., 2004; Yang et al., 2018). Although some results have been achieved in the sequencing of the *A. flavus* genome and the identification of aflatoxin-producing gene clusters (Weaver et al., 2019; Yu et al., 2004), there is still no effective method to prevent and control *A. flavus* and aflatoxin contamination so far. It has been reported that the MAPK pathway is related to aflatoxin syntheses (Tumukunde et al., 2019; Zhang et al., 2020a). We have previously found that the absence of some CWI elements (AflBck1, AflMkk2, and AflSlt2) can change the amount of aflatoxin biosynthesis, but the role of other components, especially cell membrane sensors and downstream factors in aflatoxin production, is still unclear in *A. flavus*. In this study, we aimed to explore the involvement of downstream effector AflRlm1 and WSC family proteins in the CWI pathway of *A. flavus*. We proved that AflRlm1 plays a positive role in the process of hyphal growth and aflatoxin biosynthesis. AflRlm1 physically interacted with AflSlt2 *in vivo*, which played an important role in the phosphorylation of AflRlm1. The absence of both WSC membrane proteins blocks the transmission of cell wall stress signal to AflSlt2. Our work provides new possibilities and potential targets for the development of methods for controlling *A. flavus* and aflatoxin contamination.

RESULTS

aflrlm1 encodes a putative MADS-box transcription factor of the AflSlt2-MAPK pathway

Aspergillus flavus AflRlm1 protein was identified using the National Center for Biotechnology Information (NCBI) database with the reference sequence ScRlm1 (NCBI: NP_015236.1) from *S. cerevisiae*. We also obtained the AflRlm1 homologous proteins from *A. nidulans* (NCBI: XP_660588.1), *A. niger* (NCBI: XP_001400349.1), *A. fumigatus* (NCBI: XP_754763.1), *A. oryzae* (GenBank: EIT79711.1), *C. albicans* (GenBank: KHC63039.1), *M. robertsii* (NCBI: XP_007824324.1), *F. oxysporum* (NCBI: XP_031044163.1), *B. bassiana* (NCBI: XP_008598657.1), and *P. digitatum* (NCBI: XP_014538286.1). According to the phylogenetic analysis, the AflRlm1 was highly conserved with other fungi homologs, especially *A. oryzae* (Figure S1A). Domain analysis showed that it contained one MADS-box domain predicted in these protein sequences (Figure S1B). The above information indicated that putative transcription factor, Rlm1, was relatively conservative in fungi and has a conserved MADS-box domain.

For studying the biofunctions of *aflrlm1* gene in *A. flavus*, the knockout mutant strain (Δ*aflrlm1*) and complementary strain (Δ*aflrlm1*^{C}) were constructed according to the homologous recombination strategy (Figure S2A). Δ*aflrlm1* and Δ*aflrlm1*^{C} were verified by PCR, reverse transcription PCR (RT-PCR), and Southern blot. As shown in Figure S2B, the transcriptional expression of *aflrlm1* could not be detected in Δ*aflrlm1* by RT-PCR. PCR analysis (Figure S2C) showed that open reading frame fragment could not be amplified from Δ*aflrlm1*, whereas AP and BP fragments (from upstream or downstream homologous fragments to

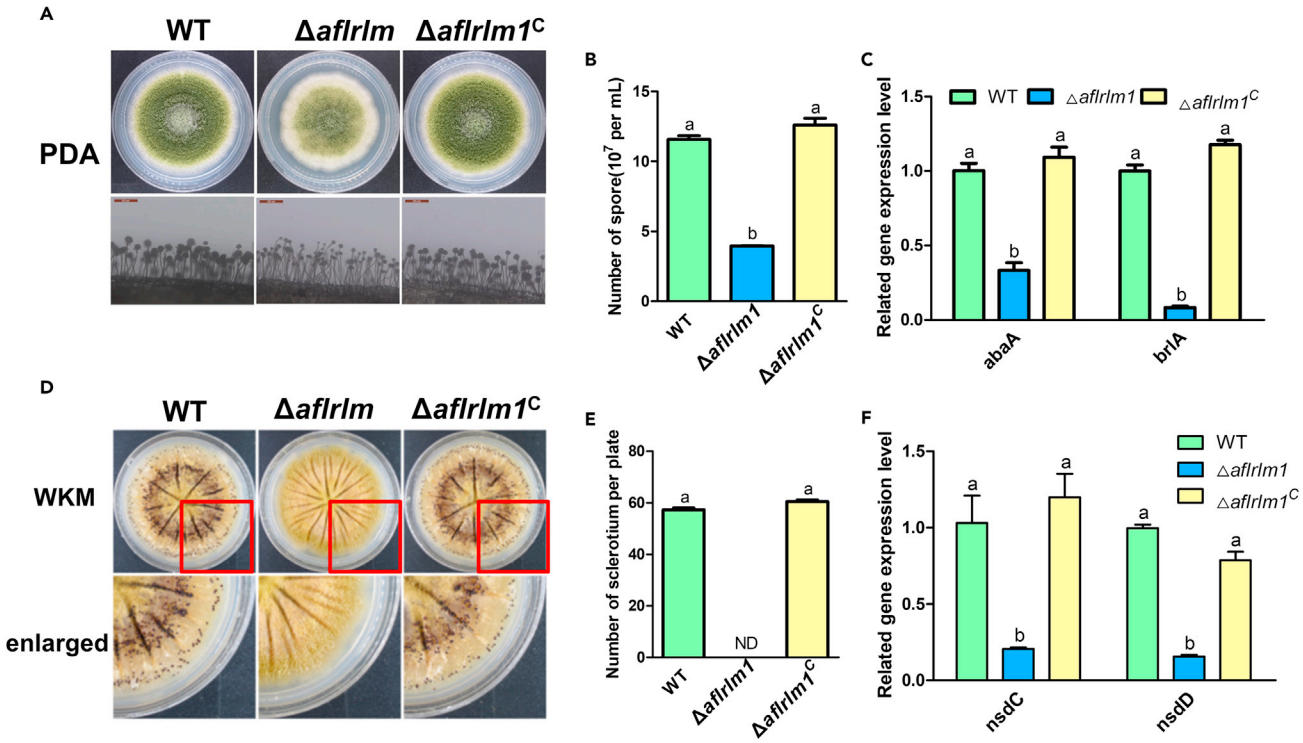


Figure 1. Deletion of *aflRlm1* leads to severe damage in sporulation and sclerotia production

(A) Deletion of *aflRlm1* leads to a significantly reduced yield in both conidia and the formation of conidiophores.

(B) Spore count statistics chart.

(C) The qPCR results of relative expression of sporulation-related genes.

(D) Knockout of *aflRlm1* makes the strain lose the ability to produce sclerotia (WKM: sclerotia-inducing Wickerham medium).

(E) Sclerotia count statistics chart.

(F) The qPCR results of relative expression of sclerotia formation-related genes. (Eerror bars in the figure: data are represented as mean ± SEM. Lowercase letters indicated significant differences at $p < 0.01$)

afupyrG partial fragments) were amplified from Δ*aflRlm1* and Δ*aflRlm1*^{C}. At the same time, we also verified Δ*aflRlm1* and Δ*aflRlm1*^{C} by Southern blot, and the result gave the right detection bands (Figure S2D). All these results confirmed that Δ*aflRlm1* and Δ*aflRlm1*^{C} had been successfully constructed.

AflRlm1 was important for growth and morphogenesis in *A. flavus*

For revealing the roles of AflRlm1 in the growth and morphogenesis of *A. flavus*, the conidia of above three *A. flavus* strains (wild type [WT], Δ*aflRlm1*, and Δ*aflRlm1*^{C}) were cultured on Potato Dextrose Agar medium (Figure 1A). Statistical analysis showed that the absence of AflRlm1 significantly inhibited conidia production (Figure 1B). Furthermore, the transcription levels of conidia formation-related genes, *aflabaA* and *aflbrlA*, in Δ*aflRlm1* were notably lower than those in WT and Δ*aflRlm1*^{C} (Figure 1C), suggesting that AflRlm1 was beneficial to hyphal growth and conidia formation. The result also showed that the Δ*aflRlm1* strain could not form sclerotia at all (Figures 1D and 1E). In order to find out the cause of this phenomenon, the expression level of sclerotia-formation-related genes (*nsdC* and *nsdD*) was further detected by qPCR, and the result showed that the expression levels of *nsdC* and *nsdD* in Δ*aflRlm1* were significantly decreased when compared to WT and Δ*aflRlm1*^{C} (Figure 1F), indicating that AflRlm1 is essential for sclerotia formation. All above results showed that AflRlm1 played important roles in the growth and morphogenesis in *A. flavus*.

AflRlm1 positively regulates AFB1 biosynthesis

In order to confirm that AflRlm1 is involved in the regulation of secondary metabolites synthesis, we cultured these strains in the medium for the detection of aflatoxin synthesis. Thin-layer chromatography (TLC) was used to detect aflatoxin production, and the result of quantitative analysis showed that the

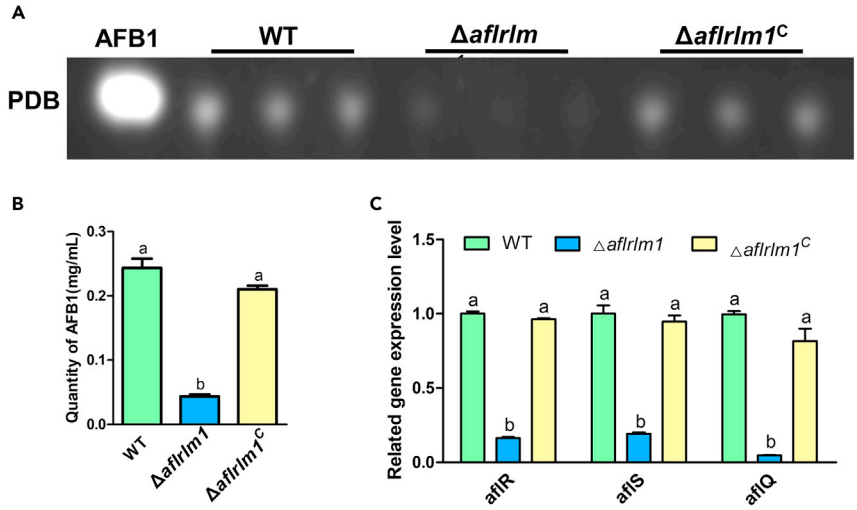


Figure 2. Deletion of *aflRlm1* greatly reduced aflatoxin production

(A) TLC result showed that the absence of *aflRlm1* caused a significant decrease in aflatoxin production.

(B) Optical density quantification of AFB1 production.

(C) The qPCR results of gene expression related to aflatoxin product. (Error bars in the figure: data are represented as mean ± SEM. Lowercase letters indicated significant differences at $p < 0.01$)

amount of aflatoxin AFB1 was decreased significantly in Δ*aflRlm1*, when compared to WT and Δ*aflRlm1*^{C} (Figures 2A and 2B). To explore the regulation of AflRlm1 at the transcription level in aflatoxin biosynthesis, qPCR was used to test the aflatoxin biosynthesis-related genes in these three strains. Statistical analysis showed that the expression levels of the three aflatoxin biosynthesis-related genes *aflR*, *aflS*, and *aflQ* in Δ*aflRlm1* were significantly lower than those in WT and Δ*aflRlm1*^{C} (Figure 2C). All these results indicated that AflRlm1 positively regulates AFB1 biosynthesis by regulating aflatoxin biosynthesis-related genes in *A. flavus*.

AflRlm1 was vital for *A. flavus* pathogenicity

A. flavus is a pathogenic fungus for plants. In order to study the influence of AflRlm1 on the pathogenicity of *A. flavus*, we carried out pathogenicity tests on peanut and maize. The result showed that the surface of the peanut or maize infected by WT and Δ*aflRlm1*^{C} was covered with dense conidia, but seeds infected by Δ*aflRlm1* were not completely covered with conidia (Figure 3A). According to statistical analysis, the conidia amount on the surface of Δ*aflRlm1* was sharply decreased when compared to WT and Δ*aflRlm1*^{C} (Figures 3B and 3C). For further detecting the aflatoxin biosynthesis of these three strains in the host, the aflatoxin AFB1 was extracted from the infected crops and detected by TLC. As shown in Figures 3D, 3E, and 3F, the AFB1 produced by Δ*aflRlm1* was far less than that by WT and Δ*aflRlm1*^{C}. Thus, AflRlm1 plays an irreplaceable role in the pathogenicity of *A. flavus*.

AflRlm1 was involved in maintaining cell wall integrity

Slt2-MAPK is a pathway related to cell wall integrity. To study whether AflRlm1 is involved in regulating cell wall integrity of *A. flavus*, stress experiments were carried out in this study. As shown in Figure 4, the growth of the three strains was inhibited due to cell wall damage reagents. It was worth noting that the growth inhibition rate caused by three kinds of cell wall damage reagents was the most obvious for Δ*aflRlm1* (Figures 4A and 4B). Under Calcofluor white (CFW) stress, the expression levels of chitin synthase genes *chsA*, *chsB*, *chsC*, and *chsD* were examined, and the results showed that the expression levels of those genes were all sharply diminished in Δ*aflRlm1* when compared to those in WT and Δ*aflRlm1*^{C} (Figure 4C). All the above results showed that AflRlm1 was involved in maintaining cell wall integrity in *A. flavus*.

AflSlt2 played an important role in AflRlm1 phosphorylation and physically associated in *vivo*

To identify whether AflRlm1 was phosphorylated or not, AflRlm1-HA strain was constructed. In addition, we constructed Δ*aflslt2* mutant based on AflRlm1-HA strain for revealing the role of AflSlt2 in AflRlm1 phosphorylation. Comparing the SDS-PAGE result with Phos-tag SDS-PAGE of AflRlm1-HA strain, we found

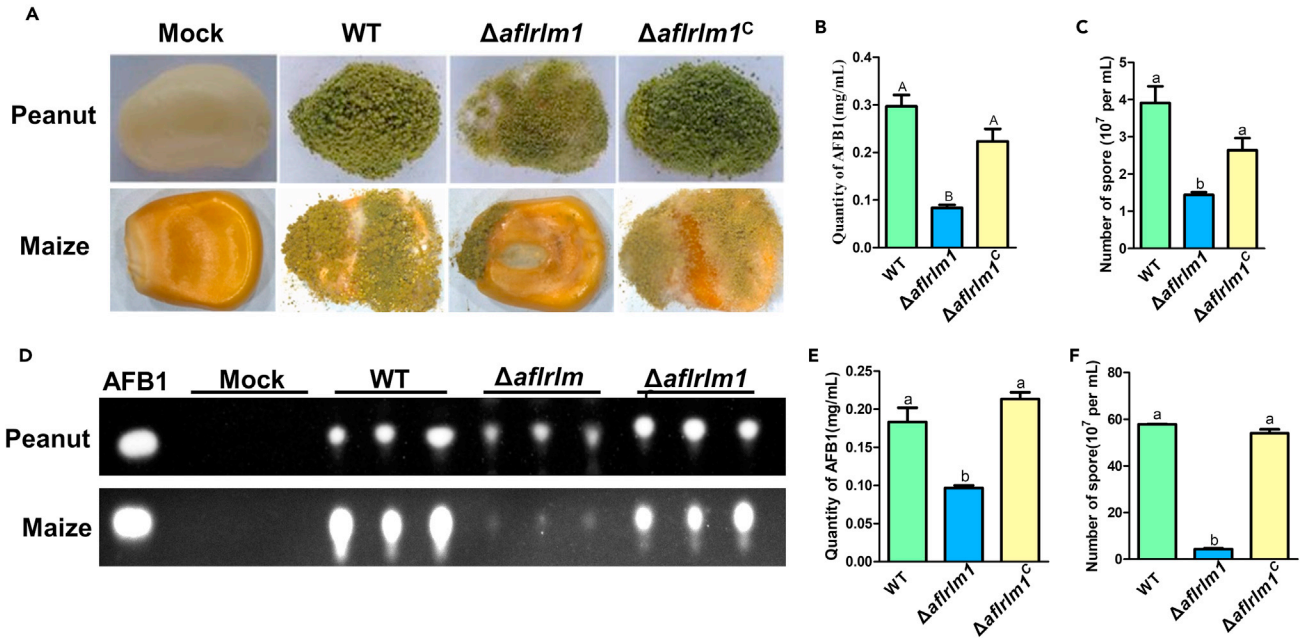


Figure 3. *A. flavus* AflRlm1 plays an important role in the pathogenicity of crop infections

(A) Knockout of *aflrlm1* reduces the pathogenicity of *A. flavus* in peanut and maize.

(B) Statistics of spore production from peanut infection.

(C) Statistics of spore production from corn infection.

(D) TLC result showed that the *aflrlm1* knockout strain reduces the amount of aflatoxin produced in infecting crops.

(E) Optical density quantification of AFB1 production from peanut infection.

(F) Quantification of AFB1 production from maize infection. (Error bars in the figure: data are represented as mean ± SEM. The capital letters represented significant differences at $p < 0.05$, and lowercase letters indicated significant differences at $p < 0.01$.)

that AflRlm1 was in a phosphorylation state in a normal condition (Figure 5A). Interestingly, the phosphorylation level of AflRlm1 was decreased in Δ*aflslt2* strain. These results showed that AflSlt2 was important for the phosphorylation of AflRlm1. To test whether AflSlt2 was associated with AflRlm1 *in vivo*, the tagged strain containing 3×HA fusion with AflRlm1 was employed. After incubation with magnetic beads, AflRlm1-HA was specifically captured from the whole-cell lysate of AflRlm1-HA strains (Figure 5B). Subsequently, co-immunoprecipitation (Co-IP) experiment was performed on two tag strains, respectively, and the results showed that AflSlt2 can be co-immunoprecipitated by AflRlm1 (Figure 5C), meaning that AflRlm1 was physically associated with AflSlt2 *in vivo*. All the results indicated that AflSlt2 played an important role in phosphorylation of AflRlm1 and was physically associated with AflRlm1 in *A. flavus*.

AflRlm1-GFP fusion protein aggregates in the nucleus under stress

We constructed AflRlm1-GFP fusion under the control of the native promoter to investigate the subcellular localization of AflRlm1 during natural and Congo red (CR) stress. A laser scanning confocal microscope observed that AflRlm1-GFP localized in the cytoplasm and nucleus (Figure 6), and the quantitative analysis of fluorescence intensity showed that there is significant aggregation in the nucleus compared to the cytoplasm (Figures S3A and S3B). To further reveal the subcellular localization of AflRlm1 under cell wall stress, CR was added to the hypha in phosphate-buffered saline suspension. The quantification result showed that AflRlm1 had a significantly higher degree of fluorescence intensity at the nucleus under cell wall stress, in comparison to the natural conditions. On the contrary, the fluorescence intensity of the cytoplasm under cell wall stress conditions was much lower than that under non-stress conditions (Figures S3C and S3D). All these subcellular localization results showed that the AflRlm1 transfers from the cytoplasm to the nucleus under cell wall stress in *A. flavus*.

Transcriptome analysis of Δ*aflrlm1* mutant

Here, RNA-seq was used to further explore the function of *aflrlm1* in *A. flavus*. The total RNA of WT strains and Δ*aflrlm1* mutants was extracted for transcriptome experiments. The quality control documents showed that

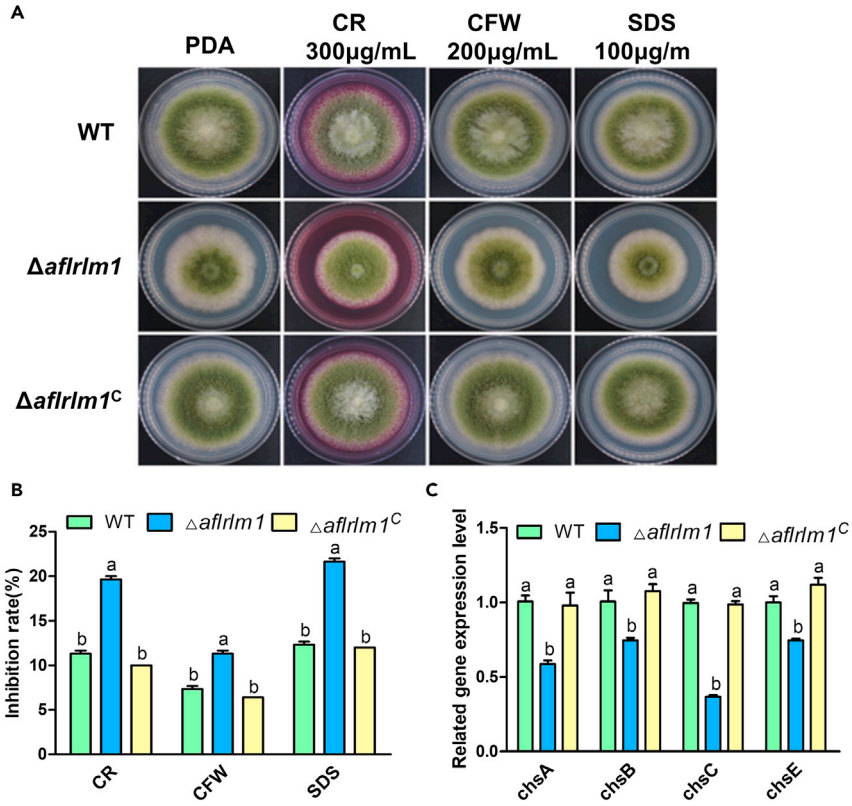


Figure 4. Deletion of *aflRlm1* reduced tolerance to cell wall stress agents

(A) Knockout of *aflRlm1* increased the sensitivity of cell wall stimulation.

(B) Statistics of growth inhibition rate.

(C) The qPCR results of gene expression related to cell wall synthesis. (Error bars in the figure: data are represented as mean ± SEM. Lowercase letters indicated significant differences at $p < 0.01$.)

these data have convincing parallelism (Figure S4). The result showed that 4,413 differential expressed genes (DEGs) (1,751 upregulated genes and 2,662 downregulated genes) were identified in Δ*aflRlm1* strain (Figure 7A). The Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment showed that biosynthesis metabolism-relevant pathways present fluctuation in the transcription level, and the translation-related pathways were upregulated remarkably in Δ*aflRlm1* strains, including ribosome and aminoacyl-tRNA. Partial genes of some metabolic pathways were downregulated significantly in Δ*aflRlm1* strains, especially biosynthesis of secondary metabolites, tyrosine metabolism, and degradation of aromatic compounds (Figure 7B). Gene ontology (GO) enrichment showed that in biological process, cellular component, or molecular function, the upregulated items were almost all related to polypeptide translation and processing, rRNA and various components in the translation complex, translation initiation and regulation, ATP purveyance, and peptide folding (Figure 7C). According to the annotation file, we found that those genes involved in transmembrane transport, integral component of membrane, and monooxygenase activity were significantly downregulated in Δ*aflRlm1* strains (Figure 7D). At the same time, we detected the transcription level of glucan-related genes, an important component of the cell wall, and results indicated that 1,3-alpha-D-glucan synthase and 1,6-alpha-glucosidase homologous genes showed a downward trend in almost all mutant strains, but 1,4-alpha-D-glucan-4- glucanohydrolase homologous genes showed upward trends in mutants (Figure S5A). This further illustrates the importance of the CWI pathway to the normal structure of the cell wall in *A. flavus*.

aflRlm1 deletion affected genes in aflatoxin biosynthesis pathway

Our experiments on culture medium and crop infection have shown that AflRlm1 plays an important role in the production of aflatoxin, so we further analyzed this mechanism through transcriptome data in *A. flavus* WT and Δ*aflRlm1*. Excitingly, we identified that 9 genes (*AFLA_006310*, *AFLA_006309*, *AFLA_006296*, *AFLA_006304*, *AFLA_006289*, *AFLA_006302*, *AFLA_006292*, *AFLA_006291*, and *AFLA_00290*) were mapped in the aflatoxin

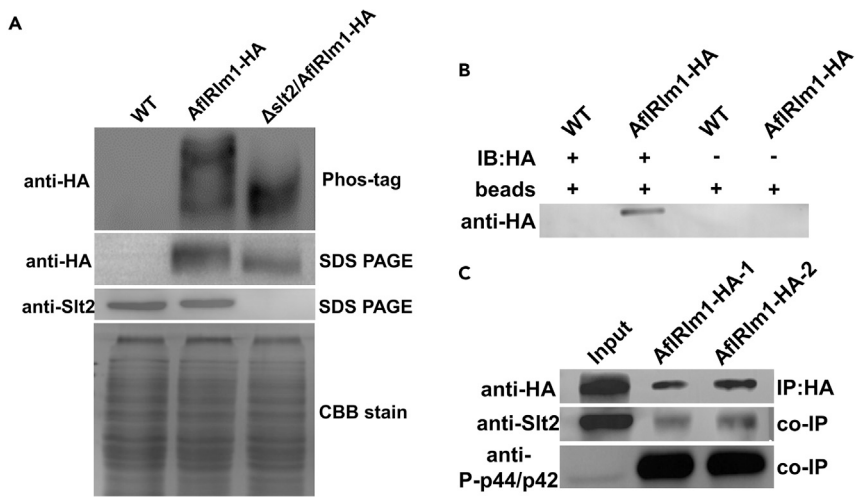


Figure 5. AflSlt2 was important for maintaining phosphorylation of AflRlm1 and physically connected to AlfRlm1

(A) AflSlt2 plays an important role in phosphorylation of aflRlm1.

(B) Immuno-specific quality control.

(C) Co-IP result indicated that AflRlm1 was physically associated with AflSlt2 *in vivo* (AflRlm1-HA-1 and AflRlm1-HA-2 indicate two independent experiments.).

synthesis pathway, and all these 9 genes were downregulated in Δ*aflrlm1* (Figure 8A). This result has also been verified by qPCR of aflatoxin-related genes (Figure 8B). Correspondingly, the first and second significant differences found in the downregulation of KEGG enrichment are metabolic pathways and secondary metabolic biosynthesis, and aflatoxin biosynthesis was also found to be significantly enriched in downregulation (Figure 8C). Moreover, GO biology process analysis also found that the aflatoxin biosynthetic process was significantly downregulated, second only to transmembrane transport. The raw material for the synthesis of aflatoxin precursor is coenzyme A, which is also the raw material for lipid metabolism (Fanelli and Fabbri, 1989; Watanabe et al., 1996). Our analysis of coenzyme A-related genes in the transcriptome found that the effect of *aflrlm1* on its metabolic regulation is not clear, with partial upregulation and partial downregulation (Figure 8D). Interestingly, transcriptome data analysis of lipid metabolism found that most genes related to lipid metabolism were upregulated in Δ*aflrlm1*(Figure S5B). Consequently, it was easy to associate that the *aflrlm1* knockout may affect the aflatoxin biosynthesis by downregulating the related genes of aflatoxin biosynthesis pathway, while upregulating the related genes of lipid metabolism in *A. flavus*.

Putative CWI sensors WSCs were located at the periphery of the cell and required for the activation of AflSlt2

WSC is the CWI pathway membrane sensor that has been reported (Philip and Levin, 2001). To further study WSC homologs in *A. flavus*, five WSC homologous genes were identified in *A. flavus* in this study. Conserved domain analysis revealed that only two WSC proteins contained both WSC domain and transmembrane domain, which were named AflWsc1 and AflWsc2, accordingly. The fluorescently expressing strains were obtained in WT background, and the fluorescence results indicated that both WSCs were located on the periphery of the cell (Figure 9A). After *aflwsc1* and *aflwsc2* were single and double knocked out successfully (Figure S6); cell wall stress experiments showed that WSC double knockout significantly increases the sensitivity to CR (Figures 9B and 9C). To verify whether WSC participates in the process of the CWI pathway to respond to cell wall stimulation, we performed the same CR stress on the WSC knockout strains and WT and tested the phosphorylation of AflSlt2, the core kinase of CWI pathway. Western blotting result showed that only double knockout strain had an inadequate phosphorylation of Slt2 with CR stimulation (Figure 9D). These indicate that AflWsc1 and AflWsc2 were indispensable in the perception of cell wall stimulus but functional redundancy.

WSC knockout caused growth and pathogenic defects

Conidia statistics and observation showed that WSC knockout resulted in a decrease of spore production in *A. flavus*, of which Δ*aflwsc1* and Δ*aflwsc2* were more significant (Figures 10A and 10B). At the same time, the

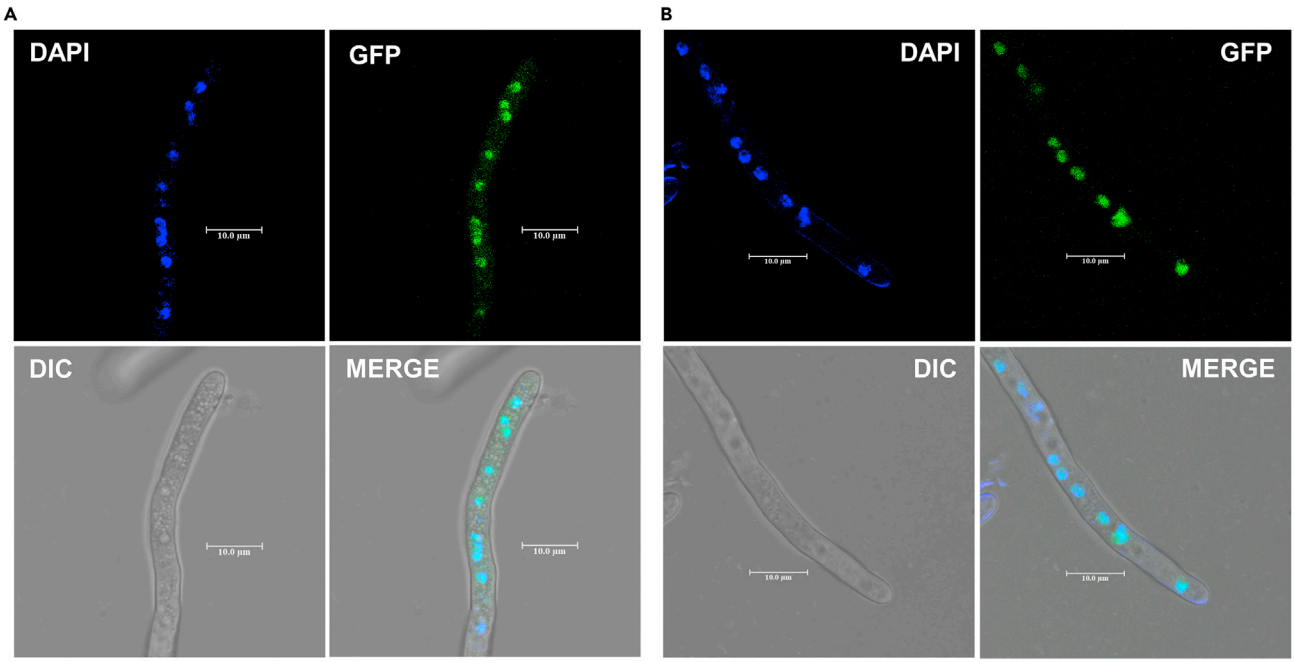


Figure 6. CR stimulation promotes the accumulation of AflRlm1-GFP from the cytoplasm to the nucleus

(A) AflRlm1-GFP subcellular localization in the cytoplasm and nucleus in nature.

(B) AflRlm1-GFP subcellular localization gathers in the nucleus under CR stress. (White scale indicated 10 μm)

formation of conidiophores in double-knockout strains is also significantly reduced (Figure 10A). While observing the mycelium tip, it was found that the *aflwsc2* knockout appeared as apical autolysis, and the same phenomenon also existed in the double-knockout strain (Figure S7A). The sclerotia formation experiments showed that WSC1 had a stronger effect than AflWsc2 during the sclerotia formation process (Figures S7B and S7C). Crop infection experiments found that the yield of spores decreased after WSC was knocked out (Figures 10C and 10D), and the production of aflatoxins also decreased significantly in Δ*aflwsc1* and Δ*aflwsc2* (Figures 10E and 10F), showing that WSCs play an important role in the pathogenicity of *A. flavus*. Overall, WSCs play important roles in the growth and pathogenicity of *A. flavus*.

DISCUSSION

As a monitor and regulator system for cell wall status, the CWI pathway is involved in multiple processes such as cell wall formation, degradation, and damage repair (Jung et al., 2002; Valiante et al., 2015). Our team has reported some of the kinases in this pathway (Zhang et al., 2020a, 2020b), but the roles of other important components remain unelucidated in pathogenic *A. flavus*, especially cell membrane sensors and downstream effectors. Here, we confirmed two CWI sensors and a downstream effector of AflRlm1. The cell wall stimulating signals were monitored by WSCs and then transmitted to MAPK kinases. The MAPK kinase AflSlt2 is activated by phosphorylation; then, the downstream effector AflRlm1 will be phosphorylated for activation. Finally, the transcriptions of relevant genes will be regulated. The functional model of the CWI pathway in *A. flavus* is given in Figure S8.

Our previous work found that the absence of protein kinase AflBck1, AflMkk2, or AflSlt2 seriously inhibited the growth and the formation of conidia (Zhang et al., 2020a, 2020b). In this study, AflRlm1 plays important roles in conidia formation and sclerotia production and also played positive roles in the toxin production and pathogenicity of *A. flavus*. In *S. cerevisiae*, the mechanism of the Pkc1 pathway promoting bud emergence and morphogenesis did not involve the AflRlm1-dependent gene expression regulation (Gray et al., 1997). In *C. albicans*, the growth rate of the AflRlm1 homozygous knockout strain was significantly lower than that of WT and complementary strains, and the biosynthesis rate of some secondary metabolites including glycerol was also declined (Oliveira-Pacheco et al., 2018). The CWI pathway was usually reported to be involved in stress response. In *C. albicans*, Δ*rlm1* mutant was very sensitive to CR, CFW, and caspofungin, and the mannan content of the mutant was decreased significantly, but the chitin content was

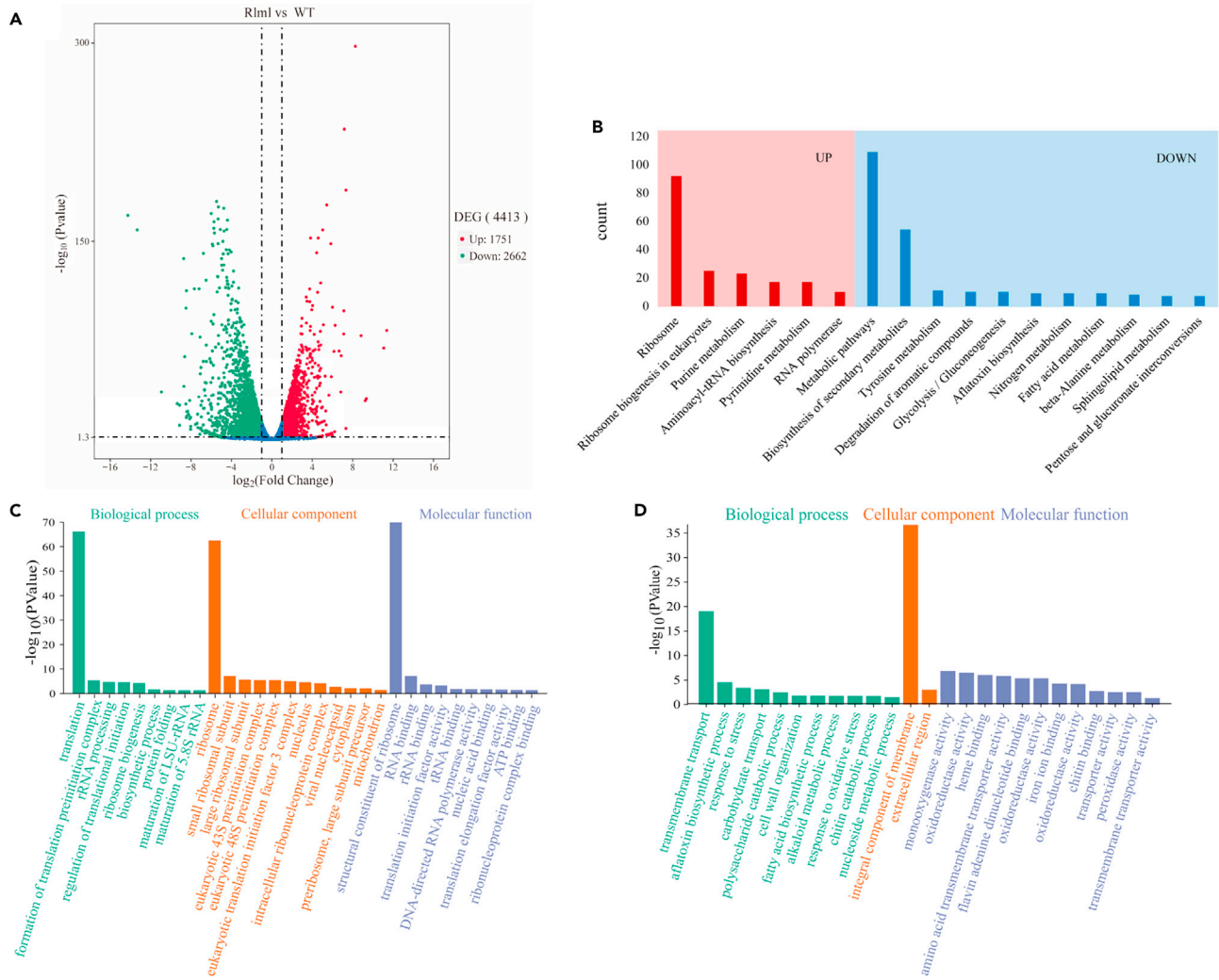


Figure 7. Transcriptome analysis shows that AflRm1 was widely involved in transcription, translation, and material metabolism

(A) Transcriptome DEG volcano plot.

(B) KEGG pathway enrichment (red bar means upregulated items and blue bar means downregulated items).

(C) The upregulated items of GO chart.

(D) The downregulated items of GO chart.

opposite (Delgado-Silva et al., 2014). Under the condition of cell wall stress, Δ*aflrlm1* also showed the same trend in *A. flavus* (Figure 4). It was reported that *A. nidulans* Δ*rlmA* showed sensitivity to cell wall stress (CR and CFW) and oxidative stress (H_2O_2) (Kovacs et al., 2013). *A. niger* Δ*rlm1* was sensitive to cell wall stress reagents, and the mRNA of α-1,3-glucan synthase AgsA cannot be detected under CFW stress when *rlm* was knocked out (Damveld et al., 2005). Besides, Mpk1 MAP kinase and Rlm1 transcription factor mediated endoplasmic reticulum stress through the increased expression of Ptp2 tyrosine phosphatase (Mizuno et al., 2018). In *C. glabrata*, overexpression of Rlm1 increased the tolerance to micafungin (Nagayoshi et al., 2014). The transcription level of the cell wall synthesis-related gene (*chsB*) in Δ*rlmA* of *A. nidulans* was decreased after micafungin treatment (Futagami et al., 2014). It was consistent with the decreasing trend of the transcription level of cell wall synthesis-related genes when treated with cell wall damage reagent in *A. flavus* in this study.

We further performed transcriptome to analyze the underlying mechanism of AflRlm1 in *A. flavus*. Our transcriptome data set identified more than 4,400 differentially expressed genes, of which 2,662 genes were significantly downregulated (Figure 7A) in *aflrlm1* knockout strain. Further mining data discovered that

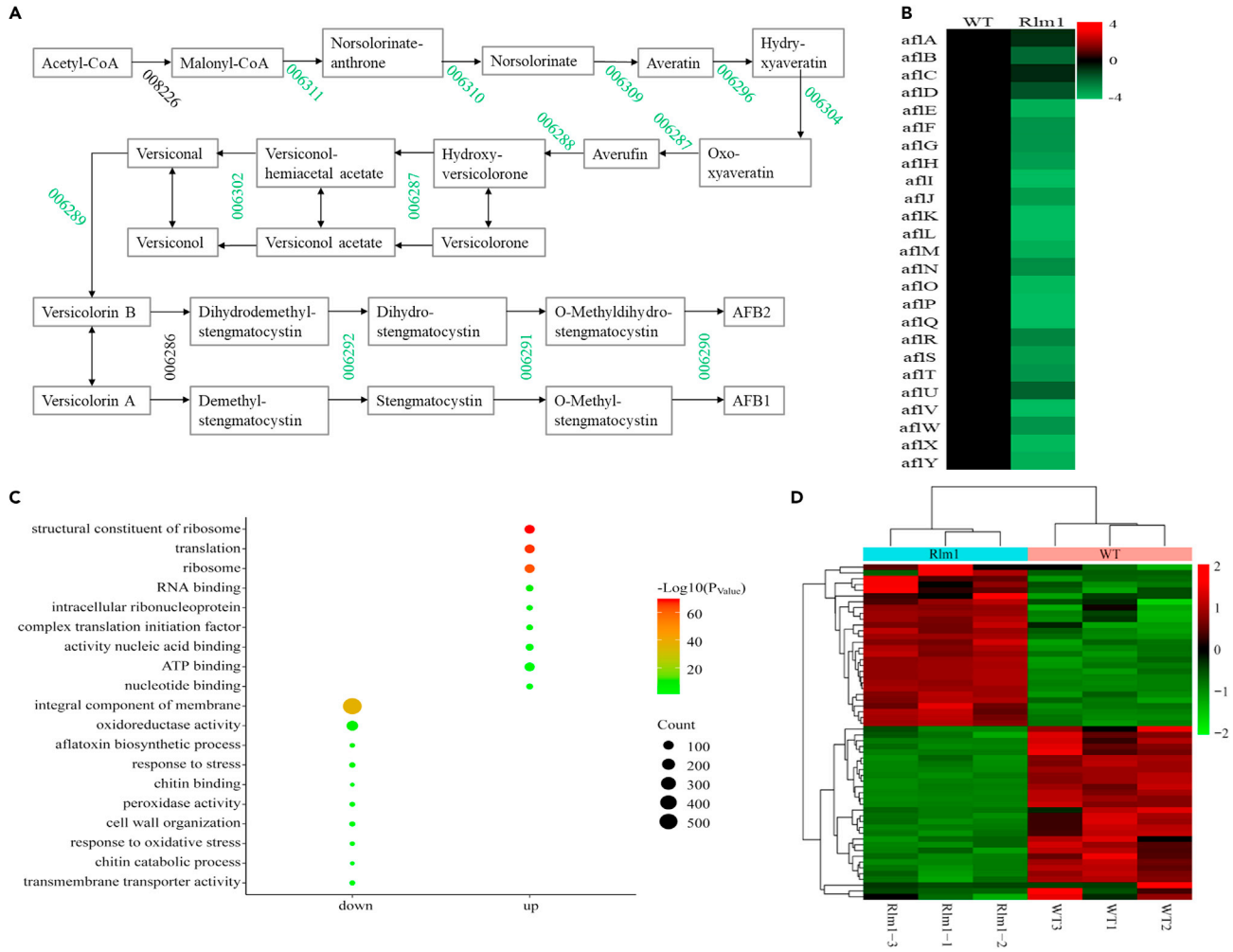


Figure 8. Transcriptome analysis shows that *afrlm1* knockout downregulates aflatoxin synthesis

(A) Nine genes in aflatoxin biosynthesis pathway were downregulated (green mark).

(B) The qPCR verification of aflatoxin biosynthesis-related genes in transcription level.

(C) The GO enrichment of DEGs between Δ*afrlm1* and WT strains.

(D) Transcriptome analysis of Co-A metabolism.

the cell wall-related (chitin and glucan) genes were downregulated significantly (Figure S5A). GO clustering showed that ribosomes and translation processes are significantly enriched. The sensitivity increased to cell wall damage was observed in a large number of mutants, in which genes were related to transcription, translation, amino acid metabolism, and protein modifications, implying that cellular adaptation to cell wall damage mainly relies on transcriptional regulation in *S. cerevisiae* (Garcia et al., 2015). We speculated that this is due to cellular stress caused by downregulation of the overall transcription level. Regarding the metabolism of the main secondary metabolite aflatoxin, KEGG pathway enrichment of transcriptome data showed that almost all the genes in aflatoxin biosynthesis pathway were downregulated in Δ*aflrlm1* strain, which is very consistent with the phenotypic results, and qPCR results of aflatoxin cluster genes also confirmed this. There are special steps for polyketide synthesis in the synthesis of aflatoxin precursors, and this process consumes a lot of coenzyme A, which is also the raw material for lipid metabolism (Hitchman et al., 2001; Watanabe et al., 1996; Yu et al., 2004). We analyzed genes related to polyketide precursor synthesis in the metabolism of aflatoxin in AflRlm1 knockout strains and found that the transcriptional level of aflatoxin precursor synthesis genes including *aflA*, *aflB*, and *aflC* was all significantly downregulated. This result may cause a large amount of coenzyme A to remain. As we speculated, the lipid metabolism-related genes were upregulated (Figure S5B). These results indicated that when the lipid metabolism pathway

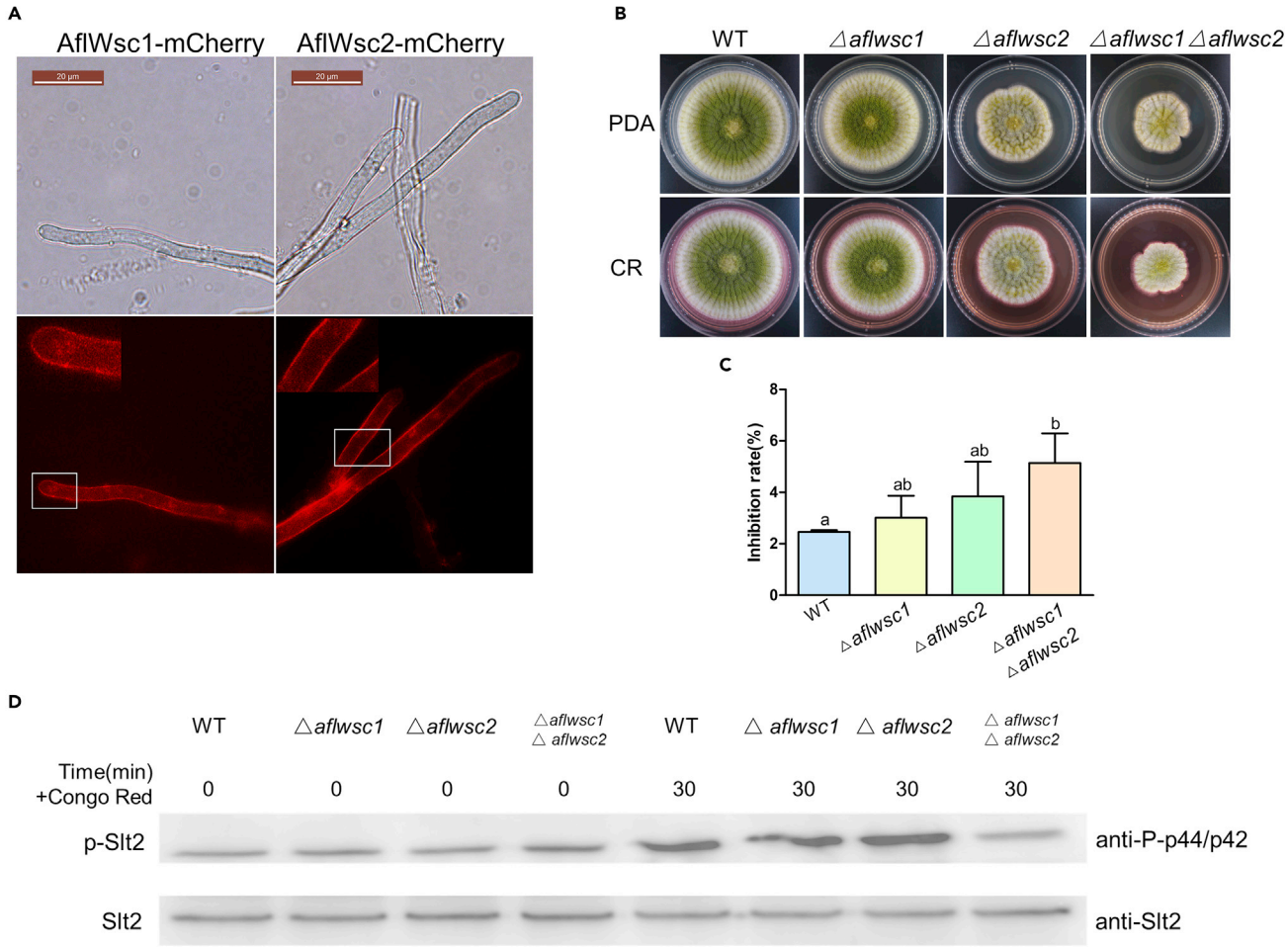


Figure 9. Slt2 phosphorylation requires the participation of membrane proteins AflWSC1 and AflWSC2 in response to cell wall stress stimulation

(A) AflWsc1 and AflWsc2 fusion protein subcellular localization were mainly in the cell periphery (white scale indicated 20 μm).

(B) Knockout of WSC increased the sensitivity to cell wall stress.

(C) Inhibition rate statistics chart.

(D) AflSlt2 cannot be phosphorylated normally during CR stimulation in WSC double knockout strains. (Error bars in the figure: data are represented as mean ± SEM. Lowercase letters indicated significant differences at $p < 0.01$)

operates efficiently in *A. flavus*, aflatoxin metabolism will be reduced due to the reduction of precursor synthesis. Moreover, transcriptome analysis based on GO and KEGG enrichment showed that the AflRlm1 might have an unclear important function for post-transcriptional translation.

The weak CWI regulation often incurred damage to growth and development. In yeast, the growth defect caused by WSC knockout has been repeatedly reported (Lodder et al., 1999; Tong et al., 2016; Verna et al., 1997; Wilk et al., 2010). In this study, the deletion of *aflwsc1* or *aflwsc2* impaired the growth and conidia production of *A. flavus*, and the double-knockout mutant was more significant. This growth impairment has also been observed in CWI component knockout strains in other species. The deletion of *Bck1* or *Slt2* genes in *C. minitans* formed a similar colony shape, which showed that both strains lost the ability of conidia formation and the hypha appeared to undergo autolysis (Zeng et al., 2012). The mycelium tip rupture was observed in *aflwsc2* knockout mutant and WSC double-knockout mutants in *A. flavus*. A similar phenomenon was also found in the *A. nidulans wscA* knockout strains, which had a high frequency of swollen hyphae under hypo-osmotic conditions (Futagami et al., 2011). The lysis defect of Δ*wsc* mutant has related to the defect in transcriptional regulation by Rlm1 due to the expression of a reporter gene controlled by Rlm1 was significantly reduced in Δ*wsc* mutants (Zu et al., 2001). This phenomenon was also shown in *aflbck1* knockout strains (Zhang et al., 2020b), and the mycelial tip of Δ*aflslt2* also appears to be abnormal (Zhang

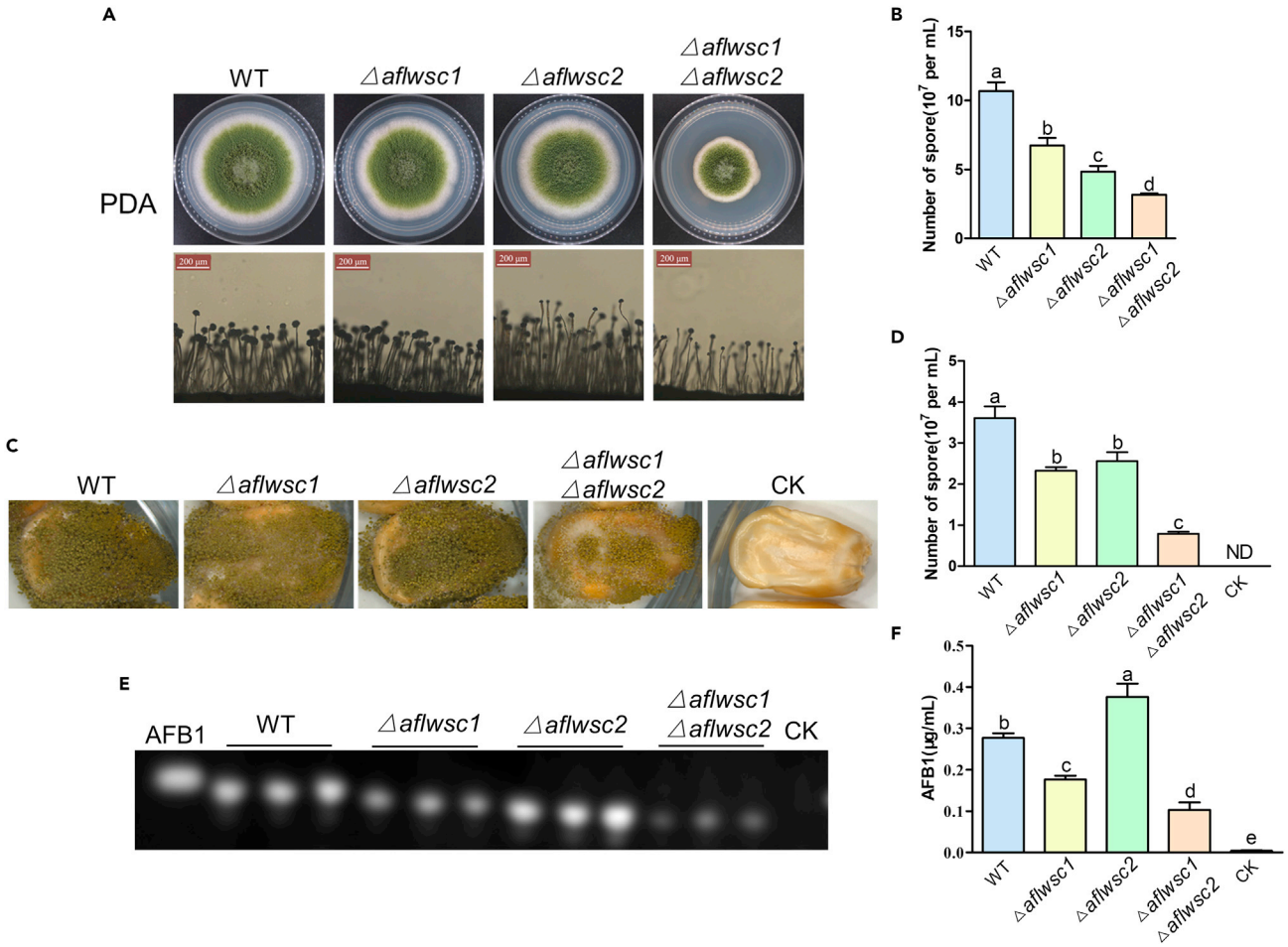


Figure 10. WSC double knockout reduced sporulation capacity and infection pathogenicity

(A) Deletion of WSC impaired the spores and conidiophores formation.

(B) Spore count statistics.

(C) WSC knockout reduces the pathogenicity of maize.

(D) Spore count statistics from maize infection.

(E) TLC result showed that WSC knockout decreases aflatoxin production when maize is infected.

(F) Quantitative optical density of aflatoxin B1 production from maize infection. (Error bars in the figure: data are represented as mean ± SEM. Lowercase letters indicated significant differences at $p < 0.01$)

et al., 2020a). WSC was also reported to be involved in the process of the cell's perception of negative environmental stimuli, such as temperature (Verna et al., 1997), osmotic pressure (Futagami et al., 2011), cell wall damage agent (Tong et al., 2016), multidrug resistance (Dichtl et al., 2012; Nishida et al., 2014), and alkali sensitivity (Raquel et al., 2006). Our data showed that WSC in *A. flavus* was involved in response to cell wall-interfering agents. Our previous works found that Δ*aflbck1* and Δ*aflslt2* strains were sensitive to cell wall stress, and the expression of cell wall-related genes was decreased (Zhang et al., 2020a, 2020b). The Δ*slt2* of *P. digitatum* was also sensitive to cell wall stress reagents (Gandía et al., 2019). In *C. glabrata*, Δ*slt2* decreased the resistance to high temperature and cell wall stress, while overexpression of *slt2* was the opposite (Miyazaki et al., 2010). Therefore, the function of CWI pathway in eukaryote cells is conservative, and this pathway can maintain the stability of multi-stress conditions.

The response of the CWI pathway to the cell wall damage agents was based on the raised phosphorylation level of Slt2, and a much higher level of dually phosphorylated Slt2 rescued the glucan synthesis-related genes (Nobel et al., 2000). Our previous work also observed an increased phosphorylation level of AflSlt2 when *A. flavus* was under cell wall stress (Zhang et al., 2020a). The global *A. fumigatus* phosphoproteome

under CR stress revealed that 485 proteins are potentially involved in the cell wall damage response, and the phosphorylation site mutants of several proteins showed an increase in the sensitivity to cell wall damage agents while there was a reduction in MpkA phosphorylation during the CR stress (Mattos et al., 2020). In *A. flavus*, phosphorylated AflSlt2 cannot be detected in *aflbck1* knockout and key-site mutant strains, but the phosphorylated AflSlt2 re-appeared in AflBck1/Mkk2^{DD} constitutive activation mutant (Zhang et al., 2020b). In this study, Phos-tag western blot results showed that AflSlt2 was important for AflRlm1 phosphorylation (Figure 5A). At the same time, the physical interaction between AflRlm1 and AflSlt2 was verified by Co-IP *in vivo* (Figure 5C). For this, we proposed that AflSlt2 may be a direct upstream activator for AflRlm1. In yeast, the cell surface protein senses compressive stress and then activates the Pkc1/Mpk1MAPK pathway (Mishra et al., 2017). The western blot results of WSC knockout strains showed that, whether knockout *aflwsc1* or *aflwsc2*, the AflSlt2 phosphorylation status of both mutants was similar to that of WT under the CR stimulation, which means that AflSlt2 can receive stress signals and get activated by phosphorylation through one of the WCS. After double knockout *aflwsc1* and *aflwsc2*, AflSlt2 could not be activated under stress conditions (Figure 9D). This result showed that WSC double knockout blocks the stress signal transduction, which means that WSCs are the indispensable factor for the CWI pathway to obtain external stress.

For *A. flavus*, pathogenicity is a focal point that deserved concern. In this study, the crop infection experiments indicated that the absence of AflRlm1 seriously affected pathogenicity, and productions of conidia and aflatoxin were both reduced in Δ*aflrlm1* during crop infection. The same situation also occurred in other pathogenic fungi, such as *B. bassiana* (He et al., 2020), *M. grisea* (Mehrabi et al., 2008), *C. glabrata* (Miyazaki et al., 2010), and *A. fumigatus* (Rocha et al., 2016). The pathogenicity was weakened or lost due to the lack of Rlm1 homolog. The mortality of worms infected by CWI component AfuRho1-induced expression strains was far lower than that of its parent strain, whereas overexpression of AfRho1 did not alter the virulence of *A. fumigatus* in *G. mellonella* (Zhang et al., 2018). It was also found that Δ*aflbck1* and Δ*aflslt2* strains could hardly grow and form conidia on the surface of crops (Zhang et al., 2020a, 2020b). Infection experiment of WSC mutants suggested that WSCs play an important role in pathogenicity in *A. flavus*. Consequently, the WSCs, core kinases (AflBck1, AflMkk2, and AflSlt2) of the CWI pathway, and the downstream transcription factor AflRlm1 play important roles in the virulence or pathogenicity of pathogenic fungi.

In summary, this study revealed that the CWI pathway involved in hyphal morphogenesis, aflatoxin production, and is pathogenic in *A. flavus*. Under the extracellular stimulus, WSC transmits the signal into the cellular component, and protein kinases were activated sequentially by phosphorylation. Then, the downstream transcript factors AflRlm1 were activated and transferred into the nucleus. As a result, the transcription level of many relevant genes was increased, and some associated proteins were expressed. Eventually, the fungus showed signs responding to external stimuli. At the same time, we found that the subcellular localization of *A. flavus* Rlm1 was changed in response to stress. We further used RNA-seq to explore the potential mechanism of the CWI pathway involved in pathogenicity and aflatoxin production. All these results may provide an important reference and potential targets for the prevention and control of *A. flavus* and aflatoxins.

Limitations of the study

This study revealed the regulatory mechanism of the CWI pathway in *A. flavus* and proved that the membrane proteins, AflWsc1 and AflWsc2, mediated the phosphorylation of AflSlt2. The phosphorylated AflSlt2 was physically associated with AflRlm1, and AflSlt2 was necessary for the phosphorylation activation of AflRlm1. The activated AflRlm1 was transferred to the nucleus to perform biological functions. However, this study still lacks in-depth understanding of specific regulatory sites and precise mechanisms in time and space.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.103159.

ACKNOWLEDGMENTS

We especially thank Professor Perng Kuang Chang (Southern Regional Research Center, United States Department of Agriculture, New Orleans, LA) for his kindness to provide the *A. flavus* CA14 strains. This work was supported byNational Natural Science Foundation of China (Grant No. 31972214).

AUTHOR CONTRIBUTIONS

C.T., J.-L.D., F.Z., J.Y., and S.-H.W. conceived and designed the experiments, C.T. and J.-L.D. performed most of the experiments and analyzed the data; Z.Z., L.-J.Y., and M.-J.Z. participated in part experiments; C.T., J.-L.D., and S.-H.W. wrote the paper, and S.-H.W. projected administration and supervision.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: April 7, 2021
Revised: August 1, 2021
Accepted: September 17, 2021
Published: October 22, 2021

REFERENCES

Amaike, S., and Keller, N.P. (2011). Aspergillus flavus. Annu. Rev. Phytopathol *49*, 107–133. 10.1146/annurev-phyto-072910-095221.

Bermejo, C., Garcia, R., Straede, A., Rodriguez-Pena, J.M., Nombela, C., Heinisch, J.J., and Arroyo, J. (2010). Characterization of sensor-specific stress response by transcriptional profiling of Wsc1 and mid2 deletion strains and chimeric sensors in *Saccharomyces cerevisiae*. Omics *14*, 679–688. 10.1089/omi.2010.0060.

Chang, P.K., Ehrlich, K.C., and Hua, S.S. (2006). Cladal relatedness among *Aspergillus oryzae* isolates and *Aspergillus flavus* S and L morphotype isolates. Int. J. Food Microbiol. *108*, 172–177. 10.1016/j.ijfoodmicro.2005.11.008.

Chavel, C.A., Caccamise, L.M., Li, B., and Cullen, P.J. (2014). Global regulation of a differentiation MAPK pathway in yeast. Genetics *198*, 1309–1328. 10.1534/genetics.114.168252.

Chen, R.E., and Thorner, J. (2007). Function and regulation in MAPK signaling pathways: lessons learned from the yeast *Saccharomyces cerevisiae*. Biochim.Biophys. Acta *1773*, 1311–1340. 10.1016/j.bbamcr.2007.05.003.

Cruz, S., Munoz, S., Manjon, E., Garcia, P., and Sanchez, Y. (2013). The fission yeast cell wall stress sensor-like proteins Mtl2 and Wsc1 act by turning on the GTPase Rho1p but act independently of the cell wall integrity pathway. Microbiologyopen *2*, 778–794. 10.1002/mbo3.113.

Damveld, R.A., Arentshorst, M., Franken, A., vanKuyk, P.A., Klis, F.M., van den Hondel, C.A.M.J.J., and Ram, A.F.J. (2005). The *Aspergillus niger* MADS-box transcription factor RlmA is required for cell wall reinforcement in response to cell wall stress. Mol. Microbiol. *58*, 305–319. 10.1111/j.1365-2958.2005.04827.x.

Delgado-Silva, Y., Vaz, C., Carvalho-Pereira, J., Carneiro, C., Nogueira, E., Correia, A., Carreto, L., Silva, S., Faustino, A., Pais, C., et al. (2014). Participation of *Candida albicans* transcription factor RLM1 in cell wall biogenesis and virulence. PLoS ONE *9*, e86270. 10.1371/journal.pone.0086270.

Dichtl, K., Helmschrott, C., Dirr, F., and Wagener, J. (2012). Deciphering cell wall integrity signalling in *Aspergillus fumigatus*: identification and functional characterization of cell wall stress sensors and relevant Rho GTPases. Mol. Microbiol. *83*, 506–519. 10.1111/j.1365-2958.2011.07946.x.

Dodou, E., and Treisman, R. (1997). The *Saccharomyces cerevisiae* MADS-box transcription factor Rlm1 is a target for the Mpk1 mitogen-activated protein kinase pathway. Mol.Cell Biol. *17*, 1848–1859. 10.1128/mcb.17.4.1848.

Fanelli, C., and Fabbri, A.A. (1989). Relationship between lipids and aflatoxin biosynthesis. Mycopathologia *107*, 115–120.

Fuchs, B.B., and Mylonakis, E. (2009). Our paths might cross: the role of the fungal cell wall integrity pathway in stress response and cross talk with other stress response pathways. Eukaryot.Cell *8*, 1616–1625. 10.1128/EC.00193-09.

Futagami, T., Nakao, S., Kido, Y., Oka, T., Kajiwara, Y., Takashita, H., Omori, T., Furukawa, K., and Goto, M. (2011). Putative stress sensors WscA and WscB are involved in hypo-osmotic and acidic pH stress tolerance in *Aspergillus nidulans*. Eukaryot.Cell *10*, 1504–1515. 10.1128/EC.05080-11.

Futagami, T., Seto, K., Kajiwara, Y., Takashita, H., Omori, T., Takegawa, K., and Goto, M. (2014). The putative stress sensor protein MtlA is required for conidia formation, cell wall stress tolerance, and cell wall integrity in *Aspergillus nidulans*. Biosci.Biotechnol.Biochem. *78*, 326–335. 10.1080/09168451.2014.878218.

Gandía, M., Garrigues, S., Hernanz-Koers, M., and al, e. (2019). Differential roles, crosstalk and response to the Antifungal Protein AfpB in the three Mitogen-Activated Protein Kinases (MAPK) pathways of the citrus postharvest pathogen *Penicillium digitatum*. Fungal Genet. Biol. *124*, 17–28.

Garcia, R., Botet, J., Rodriguez-Pena, J.M., Bermejo, C., Ribas, J.C., Revuelta, J.L., Nombela, C., and Arroyo, J. (2015). Genomic profiling of fungal cell wall-interfering compounds: identification of a common gene signature. BMC Genomics *16*, 683. 10.1186/s12864-015-1879-4.

Garcia, R., Sanz, A.B., Rodriguez-Pena, J.M., Nombela, C., and Arroyo, J. (2016). Rlm1 mediates positive autoregulatory transcriptional feedback that is essential for Slt2-dependent gene expression. J.Cell Sci. *129*, 1649–1660. 10.1242/jcs.180190.

Gray, J.V., Ogas, J.P., Kamada, Y., Stone, M., Levin, D.E., and Herskowitz, I. (1997). A role for the Pkc1 MAP kinase pathway of *Saccharomyces cerevisiae* in bud emergence and identification of a putative upstream regulator. EMBO J. *16*, 4924–4937.

Gustin, M.C., Albertyn, J., Alexander, M., and Davenport, K. (1998). MAP kinase pathways in the yeast *Saccharomyces cerevisiae*. Microbiol. Mol. Biol. Rev. *62*, 1264–1300.

He, Z., Song, Y., Deng, J., Zhao, X., Qin, X., Luo, Z., and Zhang, Y. (2020). Participation of a MADS-box transcription factor, Mb1, in regulation of the biocontrol potential in an insect fungal pathogen. J. Invertebr Pathol. *170*, 107335. 10.1016/j.jip.2020.107335.

Hedayati, M.T., Pasqualotto, A.C., Warn, P.A., Bowyer, P., and Denning, D.W. (2007). *Aspergillus flavus*: human pathogen, allergen, and mycotoxin producer. Microbiology *153*, 1677–1692. 10.1099/mic.0.2007/007641-0.

Heinemann, S., Symoens, F., Gordts, B., Jannes, H., and Nolard, N. (2004). Environmental investigations and molecular typing of *Aspergillus flavus* during an outbreak of postoperative infections. J. Hosp.Infect. *57*, 149–155. 10.1016/j.jhin.2004.02.007.

Heredia, M.Y., Ikeh, M.A.C., Gunasekaran, D., Conrad, K.A., Filimonava, S., Marotta, D.H., Nobile, C.J., and Rauceo, J.M. (2020). An expanded cell wall damage signaling network is comprised of the transcription factors Rlm1 and Sko1 in *Candida albicans*. PLoS Genet. *16*, e1008908. 10.1371/journal.pgen.1008908.

Hitchman, T.S., Schmidt, E.W., Trail, F., Rarick, M.D., Linz, J.E., and Townsend, C.A. (2001). Hexanoate synthase, a specialized type I fatty acid synthase in aflatoxin B1 biosynthesis. Bioorg. Chem. *29*, 293–307. 10.1006/bioo.2001.1216.

Irie, K., Takase, M., Lee, K.S., Levin, D.E., Araki, H., Matsumoto, K., and Oshima, Y. (1993). MKK1 and MKK2, which encode *Saccharomyces cerevisiae* mitogen-activated protein kinase-kinase homologs, function in the pathway mediated by protein kinase C. Mol.Cell Biol. *13*, 3076–3083. 10.1128/MCB.13.5.3076.

Jeon, J., Goh, J., Yoo, S., Chi, M.H., Choi, J., Rho, H.S., Park, J., Han, S.S., Kim, B.R., Park, S.Y., et al. (2008). A putative MAP kinase kinase kinase, MCK1, is required for cell wall integrity and pathogenicity of the rice blast fungus, *Magnaporthe oryzae*. Mol. Plant Microbe Interact. *21*, 525–534. 10.1094/MPMI-21-5-0525.

Jung, U.S., Sobering, A.K., Romeo, M.J., and Levin, D.E. (2002). Regulation of the yeast Rlm1 transcription factor by the Mpk1 cell wall integrity MAP kinase. Mol. Microbiol. *46*, 781–789.

Ketela, T., GREEN, R., and BUSSEY, H. (1999). *Saccharomyces cerevisiae* Mid2p is a potential cell wall stress sensor and upstream activator of the PKC1-MPK1 cell integrity pathway. J. Bacteriol. *181*, 33303340.

Kim, J.M., Lee, J.G., Yun, S.H., So, K.K., Ko, Y.H., Kim, Y.H., Park, S.M., and Kim, D.H. (2016). A mutant of the Bck1 homolog from *Cryphonectria parasitica* resulted in sectorization with an impaired pathogenicity. Mol. Plant Microbe Interact. *29*, 268–276. 10.1094/MPMI-08-15-0185-R.

Kock, C., Arlt, H., Ungermann, C., and Heinisch, J.J. (2016). Yeast cell wall integrity sensors form specific plasma membrane microdomains important for signalling. Cell Microbiol. *18*, 1251–1267. 10.1111/cmi.12635.

Kovacs, Z., Szarka, M., Kovacs, S., Boczonadi, I., Emri, T., Abe, K., Pocsi, I., and Pusztahelyi, T. (2013). Effect of cell wall integrity stress and RlmA transcription factor on asexual development and autolysis in *Aspergillus nidulans*. Fungal Genet. Biol. *54*, 1–14. 10.1016/j.fgb.2013.02.004.

Krishnan, S., Manavathu, E.K., and Chandrasekar, P.H. (2009). *Aspergillus flavus*: an emerging non-fumigatus *Aspergillus* species of significance. Mycoses *52*, 206–222. 10.1111/j.1439-0507.2008.01642.x.

Krishnan-Natesan, S., Chandrasekar, P.H., Manavathu, E.K., and Revankar, S.G. (2008). Successful treatment of primary cutaneous *Aspergillus ustus* infection with surgical debridement and a combination of voriconazole and terbinafine. Diagn.Microbiol.Infect Dis. *62*, 443–446. 10.1016/j.diagmicrobio.2008.08.003.

Lan, H., Wu, L., Sun, R., Yang, K., Liu, Y., Wu, J., Geng, L., Huang, C., and Wang, S. (2018). Investigation of *Aspergillus flavus* in animal virulence. Toxicon *145*, 40–47. 10.1016/j.toxicon.2018.02.043.

Li, G., Marroquin-Guzman, M., and Wilson, R.A. (2015). Chromatin immunoprecipitation (ChIP) assay for detecting direct and indirect protein -DNA interactions in *Magnaporthe oryzae*. Bio-protocol *5*. 10.21769/BioProtoc.1643.

Lin, P.C., Hu, W.C., Lee, S.C., Chen, Y.L., Lee, C.Y., Chen, Y.R., Liu, L.Y., Chen, P.Y., Lin, S.S., and Chang, Y.C. (2015). Application of an integrated omics approach for identifying host proteins that interact with Odontoglossum ringspot virus capsid protein. Mol. Plant Microbe Interact. *28*, 711–726. 10.1094/MPMI-08-14-0246-R.

Lodder, A.L., Lee, T.K., and Ballester, R. (1999). Characterization of the Wsc1 protein, a putative receptor in the stress response of *Saccharomyces cerevisiae*. Genet.Soc. Am. *152*, 1487–1499.

Maddi, A., Dettman, A., Fu, C., Seiler, S., and Free, S.J. (2012). WSC-1 and HAM-7 are MAK-1 MAP kinase pathway sensors required for cell wall integrity and hyphal fusion in *Neurospora crassa*. PLoS ONE *7*, e42374. 10.1371/journal.pone.0042374.

Mao, K., Wang, K., Zhao, M., Xu, T., and Klionsky, D.J. (2011). Two MAPK-signaling pathways are required for mitophagy in *Saccharomyces cerevisiae*. J.Cell Biol. *193*, 755–767. 10.1083/jcb.201102092.

Mattos, E.C., Silva, L.P., Valero, C., de Castro, P.A., Dos Reis, T.F., Ribeiro, L.F.C., Marten, M.R., Silva-Rocha, R., Westmann, C., da Silva, C., et al. (2020). The *Aspergillus fumigatus* phosphoproteome reveals roles of high-osmolarity glycerol mitogen-activated protein kinases in promoting cell wall damage and caspofungin tolerance. mBio *11*. 10.1128/mBio.02962-19.

Mehrabi, R., Ding, S., and Xu, J.R. (2008). MADS-box transcription factor Mig1 is required for infectious growth in *Magnaporthe grisea*. Eukaryot.Cell *7*, 791–799. 10.1128/Ec.00009-08.

Mishra, R., van Drogen, F., Dechant, R., Oh, S., Jeon, N.L., Lee, S.S., and Peter, M. (2017). Protein kinase C and calcineurin cooperatively mediate cell survival under compressive mechanical stress. Proc. Natl. Acad. Sci. U S A *114*, 13471–13476. 10.1073/pnas.1709079114.

Miyazaki, T., Inamine, T., Yamauchi, S., Nagayoshi, Y., Saijo, T., Izumikawa, K., Seki, M., Kakeya, H., Yamamoto, Y., Yanagihara, K., et al. (2010). Role of the Slt2 mitogen-activated protein kinase pathway in cell wall integrity and virulence in *Candida glabrata*. FEMS Yeast Res. *10*, 343–352. 10.1111/j.1567-1364.2010.00611.x.

Mizuno, T., Masuda, Y., and Irie, K. (2015). The *Saccharomyces cerevisiae* AMPK, Snf1, Negatively regulates the Hog1 MAPK pathway in ER stress response. Plos Genet. *11*, e1005491. 10.1371/journal.pgen.1005491.

Mizuno, T., Nakamura, M., and Irie, K. (2018). Induction of Ptp2 and Cmp2 protein phosphatases is crucial for the adaptive response to ER stress in *Saccharomyces cerevisiae*. Sci. Rep. *8*. 10.1038/s41598-018-31413-6.

Nagayoshi, Y., Miyazaki, T., Minematsu, A., Yamauchi, S., Takazono, T., Nakamura, S., Imamura, Y., Izumikawa, K., Kakeya, H., Yanagihara, K., and Kohno, S. (2014). Contribution of the Slt2-regulated transcription factors to echinocandin tolerance in *Candida glabrata*. FEMS Yeast Res. *14*, 1128–1131. 10.1111/1567-1364.12204.

Nishida, N., Jing, D., Kuroda, K., and Ueda, M. (2014). Activation of signaling pathways related to

cell wall integrity and multidrug resistance by organic solvent in *Saccharomyces cerevisiae*. Curr.Genet. *60*, 149–162. 10.1007/s00294-013-0419-5.

Nobel, H., Ruiz, C., Martin, H., Morris, W., Brul, S., Molina, M., and Klis, F.M. (2000). Cell wall perturbation in yeast results in dual phosphorylation of the Slt2/Mpk1 MAP kinase and in an Slt2-mediated increase in FKS2-lacZ expression, glucanase resistance and thermotolerance. Microbiology *146*, 2121–2132. 10.1099/00221287-146-9-2121.

Ohsawa, S., Yurimoto, H., and Sakai, Y. (2017). Novel function of Wsc proteins as a methanol-sensing machinery in the yeast *Pichia pastoris*. Mol. Microbiol. *104*, 349–363. 10.1111/mmi.13631.

Oliveira-Pacheco, J., Alves, R., Costa-Barbosa, A., Cerqueira-Rodrigues, B., Pereira-Silva, P., Paiva, S., Silva, S., Henriques, M., Pais, C., and Sampaio, P. (2018). The role of *Candida albicans* transcription factor RLM1 in response to carbon adaptation. Front Microbiol. *9*, 1127. 10.3389/fmicb.2018.01127.

Philip, B., and Levin, D.E. (2001). Wsc1 and Mid2 are cell surface sensors for cell wall integrity signaling that act through Rom2, a guanine nucleotide exchange factor for Rho1. Mol.Cell Biol. *21*, 271–280. 10.1128/MCB.21.1.271-280.2001.

Piccirillo, S., Neog, D., Spade, D., Van Horn, J.D., Tiede-Lewis, L.M., Dallas, S.L., Kapros, T., and Honigberg, S.M. (2017). Shrinking Daughters: rlm1-dependent G1/S checkpoint maintains *Saccharomyces cerevisiae* daughter cell size and viability. Genetics *206*, 1923–1938. 10.1534/genetics.117.204206.

Raquel, S., Humberto, M., Antonio, C., and Joaquín, A. (2006). Signaling alkaline pH stress in the yeast *Saccharomyces cerevisiae* through the Wsc1 cell surface sensor and the Slt2 MAPK pathway. J. Biol. Chem. *281*, 39785–39795. 10.1074/jbc.M604497200.

Rocha, M.C., Fabri, J.H., Franco de Godoy, K., Alves de Castro, P., Hori, J.I., Ferreira da Cunha, A., Arentshorst, M., Ram, A.F., van den Hondel, C.A., Goldman, G.H., and Malavazi, I. (2016). *Aspergillus fumigatus* MADS-Box transcription factor rlmA is required for regulation of the cell wall integrity and virulence. G3 (Bethesda) *6*, 2983–3002. 10.1534/g3.116.031112.

Sanz, A.B., Garcia, R., Rodriguez-Pena, J.M., and Arroyo, J. (2017). The CWI pathway: regulation of the transcriptional adaptive response to cell wall stress in yeast. J. Fungi (Basel) *4*. 10.3390/jof4010001.

So, K.K., Ko, Y.H., Chun, J., Kim, J.M., and Kim, D.H. (2017). Mutation of the Slt2 ortholog from *Cryphonectria parasitica* results in abnormal cell wall integrity and sectorization with impaired pathogenicity. Sci. Rep. *7*, 9038. 10.1038/s41598-017-09383-y.

Staleva, L., Hall, A., and Orlow, S.J. (2004). Oxidative stress activates FUS1 and RLM1 transcription in the yeast *Saccharomyces cerevisiae* in an oxidant-dependent manner. Mol. Biol.Cell *15*, 5574–5582. 10.1091/mbc.e04-02-0142.

Straede, A., and Heinisch, J.J. (2007). Functional analyses of the extra- and intracellular domains of the yeast cell wall integrity sensors Mid2 and Wsc1. FEBS Lett. *581*, 4495–4500. 10.1016/j.febslet.2007.08.027.

Sugahara, A., Yoshimi, A., Shoji, F., Fujioka, T., Kawai, K., Umeyama, H., Komatsu, K., Enomoto, M., Kuwahara, S., Hagiwara, D., et al. (2019). Novel antifungal compound Z-705 specifically inhibits protein kinase C of filamentous fungi. Appl. Environ.Microbiol. *85*. 10.1128/AEM.02923-18.

Tong, S.M., Chen, Y., Zhu, J., Ying, S.H., and Feng, M.G. (2016). Subcellular localization of five singular WSC domain-containing proteins and their roles in *Beauveria bassiana* responses to stress cues and metal ions. Environ.Microbiol. Rep. *8*, 295–304. 10.1111/1758-2229.12380.

Tumukunde, E., Li, D., Qin, L., Li, Y., Shen, J., Wang, S., and Yuan, J. (2019). Osmotic-adaptation response of sakA/hogA gene to Aflatoxin biosynthesis, morphology development, and pathogenicity in *Aspergillus flavus*. Toxins (Basel) *11*. 10.3390/toxins11010041.

Valiante, V., Macheleidt, J., Foge, M., and Brakhage, A.A. (2015). The *Aspergillus fumigatus* cell wall integrity signaling pathway: drug target, compensatory pathways, and virulence. Front Microbiol. *6*, 325. 10.3389/fmicb.2015.00325.

Verna, J., Lodder, A., Lee, K., Vagts, A., and Ballester, R. (1997). A family of genes required for maintenance of cell wall integrity and for the stress response in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. U S A *94*, 13804–13809. 10.1073/pnas.94.25.13804.

Watanabe, C.M., Wilson, D., Linz, J.E., and Townsend, C.A. (1996). Demonstration of the catalytic roles and evidence for the physical association of type I fatty acid synthases and a polyketide synthase in the biosynthesis of aflatoxin B1. Chem. Biol. *3*, 463–469. 10.1016/S1074-5521(96)90094-0.

Weaver, M.A., Mack, B.M., and Gilbert, M.K. (2019). Genome sequences of 20 georeferenced *Aspergillus flavus* isolates. Microbiol. Resour.Announc. *8*. 10.1128/MRA.01718-18.

Widmann, C., Gibson, S., Jarpe, M.B., and Johnson, G.L. (1999). Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol. Rev. *79*, 143–180. 10.1152/physrev.1999.79.1.143.

Wilk, S., Wittland, J., Thywissen, A., Schmitz, H.P., and Heinisch, J.J. (2010). A block of endocytosis of the yeast cell wall integrity sensors Wsc1 and Wsc2 results in reduced fitness in vivo. Mol. Genet. Genomics *284*, 217–229. 10.1007/s00438-010-0563-2.

Yang, G., Hu, Y., Fasoyin, O.E., Yue, Y., Chen, L., Qiu, Y., Wang, X., Zhuang, Z., and Wang, S. (2018). The *Aspergillus flavus* Phosphatase CDC14 regulates development, aflatoxin biosynthesis, and pathogenicity. Front Cell Infect.Microbiol. *8*, 141. 10.3389/fcimb.2018.00141.

Yu, J., Chang, P.K., Ehrlich, K.C., Cary, J.W., Bhatnagar, D., Cleveland, T.E., Payne, G.A., Linz, J.E., Woloshuk, C.P., and Bennett, J.W. (2004). Clustered pathway genes in aflatoxin biosynthesis. Appl. Environ.Microbiol. *70*, 1253–1262. 10.1128/aem.70.3.1253-1262.2004.

Zeng, F., Gong, X., Hamid, M.I., Fu, Y., Jiatao, X., Cheng, J., Li, G., and Jiang, D. (2012). A fungal cell wall integrity-associated MAP kinase cascade in *Coniothyrium minitans* is required for conidiation and mycoparasitism. Fungal Genet. Biol. *49*, 347–357. 10.1016/j.fgb.2012.02.008.

Zhang, F., Xu, G., Geng, L., Lu, X., Yang, K., Yuan, J., Nie, X., Zhuang, Z., and Wang, S. (2016). The stress response regulator AflSkn7 influences morphological development, stress response, and pathogenicity in the fungus *Aspergillus flavus*. Toxins (Basel) *8*. 10.3390/toxins8070202.

Zhang, X., Jia, X., Tian, S., Zhang, C., Lu, Z., Chen, Y., Chen, F., Li, Z., Su, X., Han, X., et al. (2018). Role of the small GTPase Rho1 in cell wall integrity, stress response, and pathogenesis of *Aspergillus fumigatus*. Fungal Genet. Biol. *120*, 30–41. 10.1016/j.fgb.2018.09.003.

Zhang, F., Geng, L., Deng, J., Huang, L., Zhong, H., Xin, S., Fasoyin, O.E., and Wang, S. (2020a). The MAP kinase AflSlt2 modulates aflatoxin biosynthesis and peanut infection in the fungus *Aspergillus flavus*. Int. J. Food Microbiol. *322*. 10.1016/j.ijfoodmicro.2020.108576.

Zhang, F., Huang, L., Deng, J., Tan, C., Geng, L., Liao, Y., Yuan, J., and Wang, S. (2020b). A cell wall integrity-related MAP kinase kinase kinase AflBck1 is required for growth and virulence in fungus *Aspergillus flavus*. Mol. Plant Microbe Interact. *33*, 680–692. 10.1094/MPMI-11-19-0327-R.

Zhu, Z., Yang, M., Bai, Y., Ge, F., and Wang, S. (2020). Antioxidant-related catalase CTA1 regulates development, aflatoxin biosynthesis, and virulence in pathogenic fungus *Aspergillus flavus*. Environ.Microbiol. *22*, 2792–2810. 10.1111/1462-2920.15011.

Zu, T., Verna, J., and Ballester, R. (2001). Mutations in WSC genes for putative stress receptors result in sensitivity to multiple stress conditions and impairment of Rlm1-dependent gene expression in *Saccharomyces cerevisiae*. Mol. Genet. Genomics *266*, 142–155. 10.1007/s004380100537.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-HA	Abcam	Cat# ab9110: RRID:AB_307019
Rabbit polyclonal anti- Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	Cell Signaling Technology	Cat# 9101: RRID:AB_331646
Goat polyclonal anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, HRP	Thermo Fisher Scientific	Cat# G-21234: RRID:AB_2536530
Goat polyclonal anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, HRP	Thermo Fisher Scientific	Cat# G-21040: RRID:AB_2536527
Mouse polyclonal anti-AflSlt2	Zhang et al., 2016.	N/A
Critical commercial assays		
Phos-tag™ Acrylamide AAL-107 5mM Aqueous Solution	NARD	Cat# 304-93526: Lot# 18L-01
Magna ChIP™ Protein A+G Magnetic Beads	Merck	Cat# 16-663: Lot# 3436986
Deposited data		
Aspergillus flavus six-sample RNA-seq raw data	This paper	CNGBdb: CNP0002169
Experimental models: organisms/strains		
Aspergillus flavus CA14 PTS strains	Chang et al., 2006.	N/A
Aspergillus flavus Δ*aflrlm1* strains	This paper	N/A
Aspergillus flavus Δ*aflwsc1* strains	This paper	N/A
Aspergillus flavus Δ*aflwsc2* strains	This paper	N/A
*Aspergillus flavus*Δ*aflwsc1*Δ*aflwsc2* strains	This paper	N/A
Aspergillus flavus aflrlm1-HA strains	This paper	N/A
*Aspergillus flavus*Δ*aflslt2/aflrlm1-HA* strains	This paper	N/A
Aspergillus flavus aflrlm1-eGFP strains	This paper	N/A
Aspergillus flavus aflwsc1-mCherry strains	This paper	N/A
Aspergillus flavus aflwsc2-mCherry strains	This paper	N/A
Aspergillus flavus Δ*aflrlm1*^{C} strains	This paper	N/A
Aspergillus flavus Δ*aflwsc1*^{C} strains	This paper	N/A
Aspergillus flavus Δ*aflwsc2*^{C} strains	This paper	N/A
Oligonucleotides		
For all oligonucleotides used in this study	See Table S2	N/A
Software and algorithms		
Gene tool	Zhang et al., 2016.	https://www.syngene.com/software/genetools-automatic-image-analysis/
MEGA	Zhang et al., 2016.	https://www.megasoftware.net/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for data should be directed to and will be fulfilled by the lead contact, Shi-Hua Wang (wshyyl@sina.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

All data and methods necessary to reproduce this study are included in the manuscript and Supplemental Information. RNA sequencing raw data were deposited to the CNGBdb (China National GeneBank DataBase) under accession number CNP0002169.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Strains and culture conditions

In this study, the wide-type strain (WT, CA14 PTS Δ*ku70*) was used as the control strain and CA14 strain (CA14 PTS Δ*ku70*Δ*pyrG*) as the parental strain to construct the mutants (Chang et al., 2006). All *A. flavus* strains used in this study were listed in Supplementary Table S1. PDA medium (Difco, USA) was used for sporulation and stress experiments, PDB medium (Difco, USA) for aflatoxin production, and YES medium for fluorescence observation. WKM medium (2 g/L Yeast extract, 3 g/L Tryptone, 5 g/L Corn steep liquor, 2 g/L Glucose, 30 g/L Sucrose, 2 g/L $NaNO_3$, 0.5 g/L KCl, 1 g/L $K_2HPO_4 \cdot 3H_2O$, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.01 g/L $FeSO_4 \cdot 7H_2O$ (pH 5.5), and 15 g/L Agarose), YPD medium (10 g/L Yeast extract, 20 g/L Tryptone, 20 g/L Glucose, and 15 g/L Agarose) and CM (6 g/L Tryptone, 6 g/L Yeast extract, 10 g/L Sucrose, and 15 g/L Agarose) medium were used for sclerotia formation (Zhang et al., 2016).

METHOD DETAILS

Sequence analysis

Rlm1 protein sequences were searched from National Center for Biotechnology Information (NCBI), including *S. cerevisiae*, *A. flavus*, *A. nidulans*, *A. niger*, *A. fumigatus*, *A. oryzae*, *C. albicans*, *M. robertsii*, *F. oxysporum*, *B. bassiana* and *P. digitatum*. The WSC1 and WSC2 sequences of *S. cerevisiae* were used for Blast search against *A. flavus* NRRL3357. The neighbor-joining method of MEGA7.0 software was used to construct phylogenetic tree. The proteins conserved domains were analyzed on the SMART website (http://smart.embl-heidelberg.de/). At the same time, DOG 2.0 was used to record the domain structure (Zhang et al., 2016).

Construction of knockout strain and complementary strain

Homologous recombination was used in the construction of *aflrlm1* knockout mutant strain (Δ*aflrlm1*) and complementary strain (Δ*aflrlm1*^{C}) of *A. flavus* according to the previously described method (Zhang et al., 2016). For the homologous fragments, the 5′ and 3′ regions of *aflrlm1* (1179 and 1587 bp, respectively) were amplified with primer pairs (Table S2), which contain sequences that overlap the marker gene. The marker gene was amplified with the primer pair pyrG/F-pyrG/R (Table S2). The resulting PCR products were purified and then linked by overlapping PCR using an overlap primer pair. The hyphae are treated with protoplast lysis buffer (0.02 mol/L NaH2PO4 (pH 5.8), 70 g/L NaCl, 0.02 mol/L CaCl2, 10 mL/L β- glucorinidase (Roche, USA), 10g/L Lysing Enzymes (Sigma, Germany), 2.5 g/L Driselase (Sigma, Germany)) to obtain protoplasts. The protoplasts were treated with STC buffer (1.2 mol/L Sorbitol, 0.05 mol/L Tris-HCl (pH 7.5), 0.05 mol/L CaCl2) and PEG buffer (500 g/L PEG-4000, 0.05 mol/L CaCl2, 0.6 mol/L KCl, 0.02 mol/L Tris-HCl (pH 7.5)) to make the overlap PCR products enter the protoplasts, and the whole process was performed on ice to keep a low temperature. Complementary strains are constructed by two steps include selection marker knockout and gene complementation, and *pyrG* knockout strains were screened using uracil, uridine, and 5-Fluoroorotic Acid (5-FOA) supplemental medium. The transformants were selected by not adding uracil and uridine to the medium, and verified by PCR and Southern blot. The same method was used to obtain *aflwsc1* and *aflwsc2* knockout and complementary strains.

Morphological analysis

The morphological analysis was carried out based on our previous work (Zhang et al., 2016). In short, 1 μL conidia suspension of all *A. flavus* were spotted onto PDA plates and cultivated at 37°C for 5 days. Subsequently, the colony diameter and the number of conidia were counted. In the same way, the same amount of conidia suspension was inoculated onto WKM/YPD/CM medium and cultured at 37°C for 7 days to form sclerotia. All experiments were repeated at least three times.

Stress response analysis

The 10^4 spores were inoculated in 7.5 mL PDA medium plates with cell wall stress agents (300 μg/mL CR, 200 μg/mL CFW, or 100 μg/mL SDS). All the plates were incubated at 37°C for 3–4 days (Zhang et al., 2020b). The inhibition rate is equal to the diameter of the control group minus the diameter of the inhibition group as a percentage of the diameter of the control group. The stress response experiments were repeated three times.

Detection of aflatoxin production

Each 10^6 spores were inoculated in 10 mL PDB medium respectively and cultured at 29°C for 6 days in dark. Equal volume of dichloromethane was used to extract aflatoxin from culture medium. Aflatoxin was detected by TLC and quantified by Gene Tool software (Zhang et al., 2020b).

Seeds infection of *A. flavus*

The seeds were sterilized in ethanol and sodium hypochlorite solution, then washed by Triton X-100 solution for three times. The seeds were put on a moist double-layer sterile filter paper in a petri dish after infection with 10^4 spores/mL suspension. Then, those seeds were cultured at 29°C for 5 days. The conidia on the surface of peanut were washed by sterile water and the yield of spores was counted. At the same time, the aflatoxin was extracted and quantified (Zhang et al., 2016).

Construction of HA-tagged strains

For constructing the HA-tagged strains, homologous recombination method was used. HA-tag and *AfupyrG* gene sequences were integrated behind the *aflrlm1* gene. Δ*Slt2*/Rlm1-HA strain was based on AflRlm1-HA, and *ptrA* was used as a selection marker to replace *aflslt2* gene. The HA-tagged strains were verified by PCR and Western blot testing (Zhu et al., 2020).

Transcriptome analysis

A. flavus WT and Δ*aflrlm1* were used for transcriptome sequencing by the Berry-Genomics company (Beijing, China). Transcriptome sequencing was completed using the Illumina NovaSeq6000 sequencing platform, and the sequencing mode is 150PE. Differentially expressed genes were screened with fold change more than 1.2, and all data were preliminarily screened with a p value < 0.05. Transcriptome analysis was performed using the recommended steps of OmicsBox 1.4. Data visualization was completed by python 3.7 and Microsoft Office 2019 (Zhu et al., 2020).

Co-IP(Co-immunoprecipitation) analysis

Co-IP (Co-immunoprecipitation) analysis was carried out according to the revised edition based on previous publication (Li et al., 2015; Lin et al., 2015). 2× 10^7 fresh spores suspend were cultured in PDB, then the hyphae were harvested and ground to fine powder in liquid nitrogen. 10 mL ECB (25 mM Tris-HCl, 100 mM NaCl, 0.25% Triton X-100, 1 mM PMSF, 1 tablet proteinase inhibitor cocktail (Roche, (CHE) per 50 mL) was added for every 2 g powder to extract protein. 5 μg anti-HA (rabbitsource, Abcam, UK) and 20 μL Protein A + G Magnetic Beads (Meck, USA) were added for each 5 mL whole cell extract for immunoprecipitation.

qPCR analysis

Real-time fluorescent quantitative PCR (qPCR) was performed according to the previous method (Zhang et al., 2016). RNA was extracted by total RNA extraction kit (Tianmo biotech, Beijing, China). The cDNA was synthesized by reverse transcription PCR (RT-PCR) with First-Strand cDNA Synthesis Kit (TransGen Biotech, Beijing, China). The transcript levels of the related genes were detected by qPCR. The primers used for qPCR were shown in Table S2. The total qPCR system was 10 μL, containing 5 μL of 2× SYBR Green qPCR Mix (Dongsheng Biotech, Guangzhou, China), 1 μL of cDNA template, 0.3 μL of each forward and reverse primers (10 μM), and the remained volume was supplemented using ultrapure water. The qPCR results of related genes were calculated using the $2^{-\Delta\Delta CT}$ method with the reference gene, and the relative transcription level was obtained by comparing with WT. All qPCR experiments were repeated three times.

QUANTIFICATION AND STATISTICAL ANALYSIS

For statistical data visualization and significance analysis, GraphPad Prism 5 and SPSS 22 were used in this study. In this study, the significant difference of single-factor or two-factor analysis was verified by the Bonferroni method, and the different lowercase letters indicated significant differences at $p < 0.01$, while the capital letters represented significant differences at $p < 0.05$. The pathway model was drawn using Adobe Illustrator 2020.