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

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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
Article

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

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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., 2013). 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 pck1 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 pck1 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 Δrlm1 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

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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 (Staleta 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 Sit2 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). 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: EFT79711.1), 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 (Δaflrlm1) and complementary strain (Δaflrlm1Δaflrlm1) were constructed according to the homologous recombination strategy (Figure S2A). Δaflrlm1 and Δaflrlm1 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 Δaflrlm1 by RT-PCR. PCR analysis (Figure S2C) showed that open reading frame fragment could not be amplified from Δaflrlm1, whereas AP and BP fragments (from upstream or downstream homologous fragments to
afuypyrG partial fragments) were amplified from Δaflrlm1 and Δaflrlm1C. At the same time, we also verified Δaflrlm1 and Δaflrlm1C by Southern blot, and the result gave the right detection bands (Figure S2D). All these results confirmed that Δaflrlm1 and Δaflrlm1C 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], Δaflrlm1, and Δaflrlm1C) were cultured on Potato Dextrose Agar medium (*Figure 1*A). Statistical analysis showed that the absence of AflRlm1 significantly inhibited conidia production (*Figure 1*B). Furthermore, the transcription levels of conidia formation-related genes, *aflabaA* and *afibrA*, in Δaflrlm1 were notably lower than those in WT and Δaflrlm1C (*Figure 1*C), suggesting that AflRlm1 was beneficial to hyphal growth and conidia formation. The result also showed that the Δaflrlm1 strain could not form sclerotia at all (*Figures 1*D and 1*E). In order to find out the cause of this phenomenon, the expression level of sclerota-formation-related genes (*nsdC* and *nsdD*) was further detected by qPCR, and the result showed that the expression levels of *nsdC* and *nsdD* in Δaflrlm1 were significantly decreased when compared to WT and Δaflrlm1C (*Figure 1*F), 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
The amount of aflatoxin AFB1 was decreased significantly in \( \Delta afluim1 \), when compared to WT and \( \Delta afluim1C \) (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 \( \Delta afluim1 \) were significantly lower than those in WT and \( \Delta afluim1C \) (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 \( \Delta afluim1C \) was covered with dense conidia, but seeds infected by \( \Delta afluim1 \) were not completely covered with conidia (Figure 3A). According to statistical analysis, the conidia amount on the surface of \( \Delta afluim1 \) was sharply decreased when compared to WT and \( \Delta afluim1C \) (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 \( \Delta afluim1 \) was far less than that by WT and \( \Delta afluim1C \). 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 \( \Delta afluim1 \) (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 \( \Delta afluim1 \) when compared to those in WT and \( \Delta afluim1C \) (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 \( \Delta aflslt2 \) 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...
that AflRlm1 was in a phosphorylation state in a normal condition (Figure 5A). Interestingly, the phosphorylation level of AflRlm1 was decreased in \( \text{D}_{\text{aflslt2}} \) strain. These results showed that AflSlt2 was important for the phosphorylation of AflRlm1. To test whether AflSlt2 was associated with AflRlm1 \( \text{in vivo} \), 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 \( \text{in vivo} \). All the results indicated that AflSlt2 played an important role in phosphorylation of AflRlm1 and was physically associated with AflRlm1 in \( A. \text{flavus} \).

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 \( A. \text{flavus} \).

Transcriptome analysis of \( \Delta_{\text{aflrlm1}} \) mutant

Here, RNA-seq was used to further explore the function of aflrlm1 in \( A. \text{flavus} \). The total RNA of WT strains and \( \Delta_{\text{aflrlm1}} \) mutants was extracted for transcriptome experiments. The quality control documents showed that...
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 Δaflrlm1 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 Δaflrlm1 strains, including ribosome and aminoacyl-tRNA. Partial genes of some metabolic pathways were downregulated significantly in Δaflrlm1 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 Δaflrlm1 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 Δaflrlm1. 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
synthesis pathway, and all these 9 genes were downregulated in Δaflrlm1 (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 Δaflrlm1 (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 Δaflwsc1 and Δaflwsc2 were more significant (Figures 10A and 10B). At the same time, the
formation of conidiophores in double-knockout strains is also significantly reduced (Figure 10A). While observing the mycelium tip, it was found that the \textit{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 \textit{D aflwsc1} and \textit{D aflwsc2} (Figures 10E and 10F), showing that WSCs play an important role in the pathogenicity of \textit{A. flavus}. Overall, WSCs play important roles in the growth and pathogenicity of \textit{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 \textit{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 \textit{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 \textit{A. flavus}. In \textit{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 \textit{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 \textit{C. albicans}, \textit{Δrml1} mutant was very sensitive to CR, CFW, and caspofungin, and the mannann content of the mutant was decreased significantly, but the chitin content was
opposite (Delgado-Silva et al., 2014). Under the condition of cell wall stress, Δaflrml also showed the same trend in A. flavus (Figure 4). It was reported that A. nidulans ΔrlmA showed sensitivity to cell wall stress (CR and CFW) and oxidative stress (H2O2) (Kovacs et al., 2013). A. niger Δrlm1 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 ΔrlmA 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
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 \( \text{D}_{\text{aflrlm1}} \) 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 \( \text{aflA}, \text{aflB}, \) and \( \text{aflC} \) 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 8. Transcriptome analysis shows that aflrlm1 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 \( \Delta_{\text{aflrlm1}} \) and WT strains.
(D) Transcriptome analysis of Co-A metabolism.
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 Δwsc 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 Δwsc mutants (Zu et al., 2001). This phenomenon was also shown in aflbck1 knockout strains (Zhang et al., 2020b), and the mycelial tip of Δaflslt2 also appears to be abnormal (Zhang et al., 2012).

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) AflSlit2 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)
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 *D. aflbck1* and *D. aflslt2* strains were sensitive to cell wall stress, and the expression of cell wall-related genes was decreased (Zhang et al., 2020a, 2020b). The *slt2* of *P. digitatum* was also sensitive to cell wall stress reagents (Gandía et al., 2019). In *C. glabrata*, *Ds*lt2 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

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**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)
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/Mkk2DD 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 5Q). 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 Δaflrlm1 during crop infection. The same situation also occurred in other pathogenic fungi, such as B. bassiana (He et al., 2020), M. grisea (Mehrabii 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 Δaflbck1 and Δaflslt2 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
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**SUPPLEMENTAL INFORMATION**

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

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**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.

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## 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 Δaflrm1 strains | This paper | N/A |
| Aspergillus flavus Δaflwsc1 strains | This paper | N/A |
| Aspergillus flavus Δaflwsc2 strains | This paper | N/A |
| Aspergillus flavusΔaflwsc1Δaflwsc2 strains | This paper | N/A |
| Aspergillus flavus aflrm1-HA strains | This paper | N/A |
| Aspergillus flavusΔaflslk2/aflrm1-HA strains | This paper | N/A |
| Aspergillus flavus aflrm1-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 flavusΔaflrm1Tc+ strains | This paper | N/A |
| Aspergillus flavus Δaflwsc1Tc strains | This paper | N/A |
| Aspergillus flavus Δaflwsc2Tc 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 wild-type strain (WT, CA14 PTS Δku70) was used as the control strain and CA14 strain (CA14 PTS Δku70ΔpyrG) 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. PDB 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 NaNO3, 0.5 g/L KCl, 1 g/L K2HPO4•3H2O, 0.5 g/L MgSO4•7H2O, 0.01 g/L FeSO4•7H2O (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. TheWSC1 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 (Δaflrlm1) and complementary strain (Δaflrlm1ΔpyrG) 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. ΔSlt2/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 Δaflrlm1 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^-ΔΔCT 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.