A Cell Wall Integrity–Related MAP Kinase Kinase Kinase *AflBck1* Is Required for Growth and Virulence in Fungus *Aspergillus flavus*

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Aspergillus flavus represents an important fungal pathogen, causing severe economic losses in crops. The mitogen-activated protein (MAP) kinase signaling pathway contributes to many physiological processes, but its precise role in *A. flavus* is not yet fully understood. In this study, we focused on the *AflBck1* gene, which encodes a MAP kinase kinase kinase of the Slr2-MAPK pathway. Targeted deletion of *AflBck1* led to a significant defect in growth and development, and a *AflBck1*-deleted mutant ($\Delta AflBck1$) showed higher sensitivity to cell-wall stress than wild type (WT). Importantly, we observed that $\Delta AflBck1$ displayed an enhanced ability to produce aflatoxin, a potential carcinogenic mycotoxin. However, the pathogenicity of the $\Delta AflBck1$ mutant was markedly reduced in peanut seeds. We also presented evidence that *AflBck1* was genetically epistatic to *AflMkk2* in the Slr2-MAPK pathway. Finally, we found that loss of the proline-rich region at the N terminus of *AflBck1* affected the reproduction of *A. flavus*. Collectively, this study not only extended the understanding that the MAPK pathway regulated *A. flavus* pathogenicity but also provided a possible strategy to control *A. flavus* contamination.

Keywords: aflatoxin, *Aspergillus flavus*, mitogen-activated protein kinase pathway, pathogenicity

The mitogen-activated protein (MAP) kinase cascade is a critical signal pathway controlling various physiological processes related to growth, adaptation, and pathogenesis in fungal pathogens (Jiang et al. 2018). In general, this cascade consists of three protein kinases: MAP kinase kinase kinase (MAPKKK), MAPKK, and MAPK. A MAPKKK phosphorylates and activates the MAPKK, which in turn activates the downstream

MAPK. In the budding yeast *Saccharomyces cerevisiae*, there are five MAPK pathways that involve mating (Fus3), filamentous growth (Kss1), high osmolarity response (Hog1), cell wall integrity (CWI) (Slr2), and spore formation (Smk1) (Molina et al. 2010). Except for the Smk1-MAPK components, other yeast MAPK members have been more or less characterized in plant-pathogenic ascomycetes (Jiang et al. 2018). In the rice blast fungus *Magnaporthe grisea*, three different orthologs of yeast MAPK have been characterized, two of which play important roles in pathogenicity (Xu 2000). In the wheat scab fungus *Fusarium graminearum*, the Fus3 ortholog (Gpmk1) is essential for infectious growth, while the Slr2 ortholog (Mgv1) is indispensable for hyphal fusion (Zhao et al. 2007). However, in *Aspergillus flavus*, only Hog1-MAPK pathway genes have so far been reported critical for growth and pathogenicity (Tumukunde et al. 2019; Yuan et al. 2018).

A. flavus is an important fungal pathogen of many economically crops, such as corn, cotton, and peanuts. As a soil-borne fungus, *A. flavus* can contaminate pre- and postharvest crops. *A. flavus* is also a human pathogen causing allergic bronchopulmonary aspergillosis (Klich 2007). *A. flavus* grows over the 12 to 48°C range, but growth rate is fastest at 37°C (Hedayati et al. 2007). Water activity (a_w) was also found to be a pivotal factor for growth (Zhang et al. 2014). Under the right conditions, the fungus has the ability to produce mycotoxins on infested crops, notably aflatoxins. Aflatoxins are secondary metabolites that cause acute death, liver cancer, and immune suppression in both animals and humans. It has been estimated that annual economic losses in the corn industry caused by aflatoxin contamination reaches up to \$1.68 billion in the United States (Mitchell et al. 2016). Although the gene cluster for aflatoxin biosynthesis was revealed a long time ago, the precise mechanism of how *A. flavus* regulates the aflatoxin cluster to produce toxin is not completely understood (Nie et al. 2018). In our opinion, this may be the biggest obstacle to control aflatoxin contamination effectively.

The aflatoxin production of *A. flavus* was found to be regulated by several pathways (Georgianna and Payne 2009). Recently, our laboratory has demonstrated that the Hog1-MAPK pathway is involved in the regulation of aflatoxin biosynthesis in *A. flavus* (Yuan et al. 2018; Tumukunde et al. 2019). As a MAPKK of Hog1-MAPK pathway, PbsB positively regulates aflatoxin generation. Interestingly, sakA/hogA, the MAPK of the Hog1-MAPK pathway, plays an opposite role in aflatoxin biosynthesis. This inconsistency suggests the complexity in regulation of aflatoxin synthesis by the Hog1-MAPK pathway.

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However, no element of Slr2-MAPK pathway has been defined in *A. flavus* to date. In this study, we described AflBck1, the most upstream component of the Slr2-MAPK pathway, from the aflatoxin producer *A. flavus*. Our results showed that, like an ortholog of yeast Bck1, AflBck1 played important roles in maintaining CWI and sensing environment stresses. Our study also revealed its functions on aflatoxin biosynthesis and fungal pathogenesis. The present study may be useful to dissect the regulation mechanism of aflatoxin biosynthesis and controlling of *A. flavus* contamination.

RESULTS

AflBck1 encodes a putative MAPKKK of the Slr2-MAPK pathway in *A. flavus*.

The Slr2-MAPK pathway regulates diverse physiological processes in fungi (Xu 2000). In order to investigate the function of this pathway in *A. flavus*, we selected a Bck1 homolog, the most upstream component of the Slr2-MAPK pathway in yeast, from *A. flavus*. Using the protein sequence of ScBck1 (CAA89389.1) as a query, we identified a Bck1 homolog (RMZ40061) from the *A. flavus* genome. With a similar approach, we obtained the Bck1 homolog from *A. oryzae* (XM_023234981.1), *A. niger* (XP_001399818), *A. fumigatus* (XM_749418.1), *A. nidulans* (XM_657399.1), *Neurospora crassa* (XP_011395112.1), *Magnaporthe oryzae* (XP_003718085.1), and *Candida albicans* (XP_716984.2). Protein sequence alignment results showed that AflBck1 had high sequence similarity to other fungal Bck1 homologs (Fig. 1A). Sequence analysis revealed that AflBck1 contained a highly conserved serine/threonine protein kinase catalytic domain at the carboxy terminus (Fig. 1B). Interestingly, at the N-terminus, AflBck1 possesses a proline-rich region (PRR) from all filamentous fungi, suggesting a conserved function of this region. Based on this information, AflBck1 could be classified into the MAPKK kinase of Slr2-MAPK pathway.

AflBck1 is required for hyphal growth.

In order to gain insight in the biological function of the *AflBck1* gene, the *AflBck1* deletion mutant and complementation strain were constructed using a previous method (Zhang et al. 2018) (Supplementary Fig. S1A). After confirmation by PCR, reverse transcription PCR (RT-PCR), and Southern blot analysis (Supplementary Fig. S1B, C, and D), *AflBck1* deletion mutant (Δ AflBck1), complementation strain (*AflBck1com*), and the wild-type (WT) strain were cultured on yeast extract-sucrose (YES) and potato dextrose agar (PDA) medium. As shown in

Figure 2A, Δ AflBck1 displayed severely retarded growth and a shriveled colony with an irregular margin. A magnified view of the colony margin revealed that hyphae of Δ AflBck1 formed some swollen structures in contrast to the smooth hyphae of WT and *AflBck1com* (Fig. 2B). It has been known that hydrophobicity of the aerial mycelia affects fungal colony morphology (Talbot 1997). When applying 10 μ l of 2.5% bromophenol blue to the mycelial surface, the droplet was partly absorbed by mycelial mats of the Δ AflBck1 mutant but not by WT and *AflBck1com* (Fig. 2C), indicating defective hydrophobicity of the aerial mycelia in mutants. All these results clearly showed that AflBck1 was required for hyphal development.

AflBck1 is important for conidia and sclerotia formation.

For *A. flavus*, asexual conidia are crucial players in infecting hosts. Here, we observed that WT and *AflBck1com* produced an abundance of conidia on YES and PDA medium while Δ AflBck1 nearly lost the ability to produce conidia (Fig. 3A). Conidia of *A. flavus* are produced by the conidiophore, which is formed by the swollen hypha apex (Bojović-Cvetić and Vujičić 1974). As anticipated, the Δ AflBck1 mutant failed to form conidiophore, whereas WT and *AflBck1com* produced bushy conidiophores (Fig. 3B). Both *abaA* and *brlA* are necessary genes for *Aspergillus* spp. to complete conidiophore development (Krijgsheld et al. 2013). Thus, we tested whether the expression of genes *abaA* and *brlA* in the Δ AflBck1 strain was dropped. Unsurprisingly, quantitative PCR results fully confirmed our guess that the expression levels of *abaA* and *brlA* genes in the Δ AflBck1 strain were lower than WT and *AflBck1com* (Fig. 3C).

Apart from conidia, *A. flavus* has the ability to produce sclerotium, which is an important survival structure under adverse conditions. On Wickerham medium (WKM), WT and *AflBck1com* could produce an abundance of sclerotia buried in white hyphae (Fig. 4A and B). On the other hand, the Δ AflBck1 strains failed to produce sclerotium (Fig. 4A and B). Based on the key roles of *nsdC* and *nsdD* in sclerotia formation (Cary et al. 2012), we analyzed the transcription levels of these two genes in *A. flavus*. Our results demonstrated that the Δ AflBck1 strain had fewer transcripts of *nsdC* and *nsdD* than did WT and *AflBck1com* (Fig. 4C). The combined data demonstrated that *AflBck1* was an important regulator in conidia and sclerotia formation.

AflBck1 maintains CWI.

To determine whether *AflBck1* is linked to CWI, we cultured *A. flavus* on YES media supplemented with different cell-wall inhibitors. The results showed that all cell wall-damaging agents were able to inhibit the radial growth of WT, Δ AflBck1,

Fig. 1. Bioinformatic analysis of Bck1 protein from different fungi. **A**, The phylogenetic tree based on all the Bck1 protein sequences from different organisms was constructed, by MEGA7.0 software, using the neighbor-joining method. Bootstrap analysis was performed with 1,000 replicates. **B**, Protein sequence structure of Bck1 was identified by SMART and ScanProsite tools and the domain architectures were visualized using software DOG 2.0.

Fig. 2. Effects of *AflBck1* on hyphal growth. **A**, Colonies morphology of wild type (WT), $\Delta AflBck1$, $AflBck1^{K1330R}$, and $AflBck1com$ strains cultured on yeast extract-sucrose (YES) and potato dextrose agar (PDA) solid media at 37°C for 4 days. **B**, Mycelial tips of WT, $\Delta AflBck1$, $AflBck1^{K1330R}$, and $AflBck1com$ strains observed by light microscope after incubation for 12 h. White arrows indicate swollen structures at the tip of hyphae. **C**, Hydrophobicity tests of WT, $\Delta AflBck1$, $AflBck1^{K1330R}$, and $AflBck1com$ strains cultured on YES solid media.

Fig. 3. Conidiation analysis of *Aspergillus flavus* strains. **A**, Quantitative analysis of conidia on yeast extract-sucrose (YES) and potato dextrose agar solid media at 37°C for 4 days. **B**, Microscopic analysis of conidiophores on YES media after incubation for 12 h, bars = 100 μ m. **C**, Quantitative reverse transcription PCR analysis of conidiation-related genes *brlA* and *abaA* on YES solid media after incubation for 48 h at 37°C. Different letters above the bars represent significant difference ($P < 0.001$).

and *AflBck1com* (Fig. 5A and B). However, we observed that the growth inhibition rate of $\Delta AflBck1$ in additive media was markedly higher than those of WT and *AflBck1com* (Fig. 5A and B). As glucan and chitin are two major components of the cell wall, we hypothesize that AflBck1 impacts on glucan and chitin synthesis. To test this hypothesis, we quantified the expression levels of one glucan synthase (AFLA_023460) and four chitin synthase (AFLA_114760, AFLA_013690, AFLA_136030, and AFLA_060590) genes. As shown in Figure 5C, under Congo red stress, the expression levels of five cell wall synthesis-related genes in $\Delta AflBck1$ were significantly lower than those in WT and *AflBck1com*. These results demonstrated that the *AflBck1* knockout strain was compromised in maintaining CWI.

AflBck1 has an adverse effect on oxidation tolerance.

Considering that oxidative stress is another important environment stimuli during fungi survival (Moye-Rowley 2003), we questioned whether AflBck1 participated in responding to this stress. To address this question, WT, $\Delta AflBck1$, and *AflBck1com* strains were equally inoculated into YES medium amended with different concentrations of hydrogen peroxide for 2 days. As anticipated, the WT and *AflBck1com* grew slower when the concentration of hydrogen peroxide rose (Fig. 6A). Otherwise, no significant difference of vegetative growth was

observed when the $\Delta AflBck1$ strain was grown on YES medium with different concentration of hydrogen peroxide (Fig. 6A). In agreement, the inhibitory effect of hydrogen peroxide on the growth of the WT and *AflBck1com* was stronger than that of the $\Delta AflBck1$ strain (Fig. 6B). In trying to understand this phenomenon, we tested the relative expression of two reactive oxygen species (ROS) scavenging enzymes (CAT2 and SOD) in all three strains. The result showed that the transcription levels of both genes in the $\Delta AflBck1$ strain were higher than that of the WT and *AflBck1com* (Fig. 6C). By summarizing these results, we conclude that AflBck1 plays a negative role in the regulation of oxidative response.

AflBck1 negatively regulates aflatoxin biosynthesis.

To examine whether the disruption of *AflBck1* affects aflatoxin biosynthesis, conidial suspensions of WT, $\Delta AflBck1$, and *AflBck1com* were inoculated into YES broth. Thin-layer chromatography (TLC) analysis results showed a significantly increased aflatoxin production in $\Delta AflBck1$ when compared with WT and *AflBck1com* (Fig. 7A and B). *AflC*, *AflD*, *AflM*, *AflO*, *AflP*, *AflQ*, *AflK*, *AflS*, and *AflR* are essential genes in aflatoxin biosynthesis pathway (Yu et al. 2004). When we tested the expression levels of these genes, $\Delta AflBck1$ showed the highest expression levels of these genes among all three strains (Fig. 7C).

Fig. 4. Sclerotial formation of *Aspergillus flavus* strains. **A**, Sclerotial formation of different strains on Wickerham solid media (WKM) after grown for 7 days at 37°C. **B**, Quantitative analysis of the sclerotia shown in A. **C**, Expression levels of sclerotia-related genes *nsdC* and *nsdD* in different strains on WKM solid media for 48 h at 37°C. ND = not detectable. Different letters above the bars represent significant difference ($P < 0.001$).

These results lead us to conclude that *AflBck1* influences aflatoxin biosynthesis through the downregulation of aflatoxin pathway genes.

AflBck1 is indispensable for fungal pathogenicity.

A. flavus is a detrimental pathogen for many crops, which prompts us to test if lack of *AflBck1* influences pathogenicity of *A. flavus*. Our pathogenicity assay showed that loss of *AflBck1* sharply reduced the colonization of *A. flavus* on peanuts (Fig. 8A). At the same time, the amount of conidia produced by $\Delta AflBck1$ on seeds was far fewer than that by WT and *AflBck1com* (Fig. 8B). To investigate whether this defect in infection affects aflatoxin biosynthesis on seeds, we determined mycotoxin accumulation of the contaminated seeds. As evidence from the data in Figure 8C and D, the $\Delta AflBck1$ produced more aflatoxin than WT and *AflBck1com*. Thus, we favor the idea that *AflBck1* is indispensable for colonization of *A. flavus* on seeds.

AflBck1 is involved in the activation of the Slt2-MAPK pathway.

Studies in *S. cerevisiae* have established that Bck1 is involved in the activation of Slt2-MAPK pathway (Widmann et al. 1999). To confirm the relationship between *AflBck1* and Slt2-MAPK pathway, we measured the phosphorylation levels

of Slt2 in the WT, $\Delta AflBck1$, and *AflBck1com* strains under Congo red stimulation. As illustrated in Figure 9C, the phosphorylation levels of Slt2 in WT and *AflBck1com* were enhanced under Congo red induction. As a comparison, no phosphorylation of Slt2 occurred in the mutant $\Delta AflBck1$, neither with nor without Congo red treatment (Fig. 9C). To exclude the possibility that *AflBck1* affects the expression of Slt2, we checked the expression levels of Slt2 in the WT, $\Delta AflBck1$, and *AflBck1com* strains. Figure 9C shows that the Slt2 levels in all three strains had no difference whether Congo red was present or not. In view of the results shown above, we believe that *AflBck1* is involved in the activation of Slt2-MAPK pathway.

In *Pneumocystis carinii*, the kinase activity of Bck1 was abolished by changing the Lys⁵⁷⁴ to arginine (Thomas et al. 2003). Based on protein sequence alignments, we found that Lys⁵⁷⁴ is a highly conserved residue on Bck1 homologs from *S. cerevisiae* to *A. flavus* (Fig. 9A). To determine whether this residue on *AflBck1* is a functional site, we created a point mutant (*AflBck1*^{K1330R}) by replacing the conserved lysine to an arginine (Fig. 9B). When we examined the phosphorylation levels of Slt2 in *AflBck1*^{K1330R} mutant, the results indicated that the phosphorylated Slt2 was vanished in *AflBck1*^{K1330R} mutant under unstressed condition (Fig. 9C). Interestingly, the mutant *AflBck1*^{K1330R} had a slightly phosphorylated Slt2 under stress

Fig. 5. Response of *AflBck1* to cell wall stress. **A**, Colony morphology of the wild type (WT), $\Delta AflBck1$, *AflBck1*^{K1330R}, and *AflBck1com* strains grown on yeast extract-sucrose (YES) solid media supplemented with sodium dodecyl sulfate (SDS), Congo red (CR), and Calcofluor white (CFW) at 37°C for 3 days. **B**, The inhibition rate of radial growth of strains shown in **A**. **C**, Relative transcript levels of cell wall synthesis-relevant genes in different strains on YES solid media containing 60 μg of CR per milliliter for 72 h at 37°C. Different letters above the bars represent significant difference ($P < 0.001$).

conditions (Fig. 9C). Although not entirely as expected, these results confirmed that the Lys-1330 residue of AflBck1 is an important site for the phosphorylation of Slr2. Like $\Delta AflBck1$, we found that the $AflBck1^{K1330R}$ mutant displayed defects in

growth, development, response to stresses, aflatoxin biosynthesis, and fungal pathogenicity (Figs. 2, 3, 4, 5, 6, 7, and 8). Taken as a whole, we found that Lys¹³³⁰ in AflBck1 was an essential residue for activation and full function in *A. flavus*.

Fig. 6. Sensitivity of *Aspergillus flavus* strains to H_2O_2 . **A**, Colony morphology of the wild type (WT), $\Delta AflBck1$, $AflBck1^{K1330R}$, and $AflBck1com$ strains grown on solid media of yeast extract-sucrose (YES) (Control) and YES containing the different concentrations of H_2O_2 at 37°C for 2 days. **B**, The inhibition rate of radial growth of strains shown in **A**. **C**, Relative transcript levels of two antioxidant genes in different strains on YES solid media containing 10 mM H_2O_2 medium for 72 h at 37°C. Different letters above the bars represent significant difference ($P < 0.001$).

Fig. 7. Aflatoxin analysis of *Aspergillus flavus* strains. **A**, Thin-layer chromatography assay of AFB1 produced by wild type (WT), $\Delta AflBck1$, $AflBck1^{K1330R}$, and $AflBck1com$ strains in yeast extract-sucrose (YES) liquid media cultured at 29°C for 7 days. **B**, Quantification analysis of aflatoxin B1 (AFB1) from **A**. **C**, Relative transcript levels of nine aflatoxin biosynthesis-related genes in different strains cultured in YES liquid media for 72 h at 29°C. Different letters above the bars represent significant difference ($P < 0.001$).

***AflBck1* is genetically epistatic to *AflMkk2* in the Slit2-MAPK pathway.**

Bearing in mind that *Mkk2* is located downstream of *Bck1* and upstream of *Slit2* in yeast (Levin 2005), we wondered

whether a constitutive activation of *Mkk2* mutant could rescue the functional deficiency of $\Delta AflBck1$ mutant. For that, two constitutive-activation of *Mkk2* mutants ($\Delta AflBck1/Mkk2^{DD-3}$ and $\Delta AflBck1/Mkk2^{DD-12}$) were generated by replacing the

Fig. 8. Pathogenesis of *Aspergillus flavus* strains to peanuts. **A**, Colonization of wild type (WT), $\Delta AflBck1$, *AflBck1*^{K1330R}, and *AflBck1com* strains on peanuts after 7 days of inoculation at 29°C. **B**, Conidia production on peanuts shown in **A**. **C**, Thin-layer chromatography measurements of aflatoxin B1 (AFB1) extracted from peanuts shown in **A**. **D**, Quantification analysis of AFB1 shown in **C**. Different letters above the bars represent significant difference ($P < 0.001$).

Fig. 9. Construction and confirmation of *AflBck1*^{K1330R} strains. **A**, Alignment of partial sequence of *Bck1* homologs. Orange arrow indicates the conserved lysine residue. **B**, Sequencing confirmation of *AflBck1*^{K1330R} strains. **C**, Western blot confirmation of *AflBck1*^{K1330R} strains. Proteins were extracted from wild type (WT), $\Delta AflBck1$, *AflBck1*^{K1330R}, and *AflBck1com* strains grown in yeast extract-sucrose liquid medium for 12 h at 37°C and treated with or without Congo red for 15 min before harvesting. Phosphorylated *Slit2* (P-*Slit2*), *Slit2*, and β -actin (as loading control) were detected with antiphospho-p44/42, anti-*Slit2*, and anti- β -actin antibodies, respectively.

Thr³⁶⁸ and Thr³⁷⁴ of AflMkk2 in Δ AflBck1 mutant with Asp (Fig. 10A) (Yin et al. 2016). As shown in Figure 10B, the phosphorylated Slt2 reappeared in both Δ AflBck1/Mkk2^{DD} mutants, although the intensity of Slt2 phosphorylation detected in the Δ AflBck1/Mkk2^{DD} mutants was not as strong as that in WT. On a similar note, resistance to Congo red was partially recovered in the Δ AflBck1/Mkk2^{DD} strains, in comparison with the Δ AflBck1 strain (Fig. 10C and D). Taken together, these results indicate that AflBck1 is epistatic to AflMkk2 in the Slt2-MAPK pathway.

To a better understanding of AflBck1 epistatic to AflMkk2, we examined the phenotypic changes in Δ AflMkk2 mutants. By applying a similar approach to disrupt AflBck1 gene, we obtained the AflMkk2 deletion and complementary strains (Supplementary Fig. S2). The Δ AflMkk2 mutants showed a grievous fault in colony growth with respect to the WT and AflMkk2 complemented strains (Fig. 11A). The Δ AflMkk2 mutants displayed shriveled colony margins, which was consistent with the results from the Δ AflBck1 mutant. Further, we found that the ability to produce conidia in the Δ AflMkk2 mutants was destroyed, in contrast to the conidiation of the WT and AflMkk2 complemented strains (Fig. 11B). We also compared the ability to produce sclerotia of Δ AflMkk2 mutants with that of the WT and AflMkk2 complemented strains. As showed in Figure 11C, the sclerotia formation in Δ AflMkk2 mutants was completely abolished, while the WT and AflMkk2 complemented strains had the normal capacity to produce sclerotia. By contrast, the production of aflatoxin was upregulated in Δ AflMkk2 mutants (Fig. 11D). All above results also supported that AflBck1 was genetic epistasis of AflMkk2 in Slt2-MAPK pathway.

The PRR of AflBck1 influences conidia and sclerotia formation.

As results from sequence analysis in Figure 1A, we found that all selected filamentous fungi harbored a PRR at the N

terminus of Bck1 protein. As far as we are aware, the function of this structure has not been explored in Bck1 homologs of filamentous fungi. In a manner analogous to the AflBck1 knockout, we constructed a strain omitting the PRR of AflBck1 and checked whether PRR was of functional importance for AflBck1. As can be seen in Supplementary Fig. S3, AflBck1^{ΔPRR} mutant displayed a WT phenotype in growth, response to stress, and aflatoxin biosynthesis. To our surprise, the AflBck1^{ΔPRR} mutant showed an increase in conidia production but an abolition in sclerotia formation as compared with the WT strain (Fig. 12). These results point to the PRR of AflBck1 influencing conidia and sclerotia formation in *A. flavus*.

DISCUSSION

MAPK cascades are conserved signal pathways that transduce signals from the cell surface to the nucleus. This allows them to participate in numerous biological processes, such as development, response to environmental stimuli, and pathogenesis (Zhao et al. 2007). In a previous study, we have investigated two components of the Hog1-MAPK pathway and found that both kinases functioned in morphogenesis differentiation, aflatoxin biosynthesis, and plant infection in *A. flavus* (Tumukunde et al. 2019; Yuan et al. 2018). This arouses our interest in the biological functions of Slt2-MAPK. For this purpose, we disrupted AflBck1, the MAPKKK in the Slt2-MAPK pathway from *A. flavus*, using a split-marker strategy.

Slt2-MAPK pathway is believed to be essential for the maintenance of CWI in fungi (Xu 2000). For example, the Δ Slt2 mutant displays serious defects in response to cell wall-disturbing agents in various fungi, such as *Alternaria alternata* (Yago et al. 2011), *Fusarium graminearum* (Hou et al. 2002), *A. fumigatus* (Valiante et al. 2008), *Cryphonectria parasitica* (So et al. 2017), and *Trichoderma reesei* (Wang et al.

Fig. 10. Rescue assay of Δ AflBck1 strain. **A**, Alignment of the phosphorylation consensus motif of Mkk2 homologs. The phosphorylated residues of Mkk2 were written in red. **B**, Western blot analysis of Slt2 phosphorylation. Proteins were extracted from wild type (WT), Δ AflBck1, Δ AflBck1/Mkk2^{DD}-3, and Δ AflBck1/Mkk2^{DD}-12 strains grown in yeast extract-sucrose (YES) liquid medium for 12 h at 37°C and treated with or without Congo red (CR) for 15 min before harvesting. Phosphorylated Slt2 (P-Slt2) and Slt2 (as loading control) were detected with antiphospho-p44/42 and anti-Slt2 antibodies, respectively. **C**, Colony morphology of the WT, Δ AflBck1, Δ AflBck1/Mkk2^{DD}-3, and Δ AflBck1/Mkk2^{DD}-12 strains grown on YES solid media supplemented with or without CR at 37°C for 3 days. Δ AflBck1/Mkk2^{DD}-3 and Δ AflBck1/Mkk2^{DD}-12 are two clones isolated from the same transformation. **D**, The inhibition rate of radial growth of the strains shown in C. Different letters above the bars represent significant difference ($P < 0.001$).

2014). As expected, deletion of *Bck1* from *A. flavus* leads to hypersensitivity to cell-wall stress, which is similar to the orthologs in other pathogenic fungi including *M. oryzae* (Jeon et al. 2008), *C. parasitica* (Kim et al. 2016), *Coniothyrium minitans* (Zeng et al. 2012), and *Fusarium verticillioides* (Zhang et al. 2015). Peculiarly, lack of *Bck1* does not result in higher sensitivity to cell wall-damaging chemicals in the milk yeast *Kluyveromyces lactis* (Jacoby et al. 1999). Yet, heterologous complementation of *K. lactis Bck1* into a *S. cerevisiae Bck1* null mutant restored resistance to caffeine, a cell wall-damaging agent. Similarly, *P. carinii Bck1* salvaged the CWI defect in the *S. cerevisiae ΔBck1* mutant (Thomas et al. 2003). These studies imply that the function of *Bck1* in CWI is conserved among fungi.

The cell wall is essential for cell division and morphological development in fungi. Thus, it is not surprising that the *ΔAflBck1* mutant exhibited severely retarded growth compared with WT and complemented strain. This outcome is in line with the mutant characteristics of *Bck1* orthologs, including *C. parasitica* (Kim et al. 2016), *F. verticillioides* (Zhang et al. 2015), and *A. fumigatus* (Valiante et al. 2008). Interestingly, we noticed that the *ΔAflBck1* mutant formed swollen structures at the tip of hyphae, which is also observed in the *Bck1* deletion mutant of *F. verticillioides* (Zhang et al. 2015). In *A. fumigatus*, Valiante and coworkers (2008) found that the *ΔBck1* mutant had excessive branching hyphae in comparison with WT. Most interestingly, mutation in *C. parasitica Bck1* resulted in a sporadic sectorization colony (So et al. 2018). In addition, the mycelia of *C. parasitica ΔBck1* mutant showed an impaired hydrophobicity (So and Kim 2017), which is in agreement with our findings. Although the *Bck1* orthologs of *M. oryzae* and *Coniothyrium minitans* are not required for hyphal growth, hyphal autolysis was sharply enhanced in the *ΔBck1* mutants (Jeon et al. 2008; Zeng et al. 2012). Maybe this lysis contributes to the drastic decrease in conidiation (Jeon et al. 2008).

However, this autolysis phenotype was not observed in *A. flavus ΔBck1* mutant, indicating a specie-specific role of *Bck1* in morphological development.

According to previous publications, aflatoxin biosynthesis is highly interconnected with the oxidative status of *A. flavus* (Zhang et al. 2016, 2018). In our data, we observed that loss of *AflBck1* improved resistance to hydrogen peroxide. The upregulated expression of oxidative-stress defense genes in the *ΔAflBck1* mutant provides compelling evidence. However, different behavior was observed in the *F. verticillioides ΔBck1* mutant under oxidative stresses (Zhang et al. 2015). In yeast, significant progress has been made in the understanding of the role of *Bck1* in response to hydrogen peroxide. Transcriptional repressor cyclin C regulates many stress response genes in yeast. Under low oxidative stress, activated *Bck1* induces cyclin C translocation and degradation, whereas *Bck1* is not essential to destruction of cyclin C under high stress (Jin et al. 2015). Many studies have revealed that aflatoxin generation can alleviate damage by ROS accumulation (Kenne et al. 2018; Roze et al. 2015). We observed here that the *ΔAflBck1* mutant displayed an augmented aflatoxin production, which seems to provide an answer to a better tolerance to H_2O_2 of the mutant. However, the exact mechanism by which *A. flavus Bck1* negatively controls oxidative response remains to be further investigated.

In *A. flavus*, aflatoxin is the major culprit that threatens animal and human health. Considering this, it is necessary to survey whether *AflBck1* relates to aflatoxin production. Here, we observed that disruption of *AflBck1* resulted in increased aflatoxin generation in YES broth. This result completely agrees with aflatoxigenic characteristics exhibited by the *A. flavus Δsaka/hogA* mutant, which also shows increased aflatoxin generation (Tumukunde et al. 2019). This consistency may be explained by similar regulation mechanisms of aflatoxin biosynthesis between *Slt2*-MAPK and *Hog1*-MAPK.

Fig. 11. Phenotypes of *ΔAflMkk2* mutants. **A**, Colony morphology of wild type (WT), *ΔAflMkk2*, and *AflMkk2* complemented strains, on potato dextrose agar (PDA) solid media after 4 days of inoculation. **B**, Conidia production collected from samples shown in **A**. **C**, Sclerotial formation of different strains on Wickerham solid media after growth for 7 days at 37°C. **D**, Thin-layer chromatography measurements of aflatoxin B1 (AFB1). All strains were cultured in yeast extract-sucrose liquid medium for 7 days at 29°C. ND = not detectable.

Like aflatoxin produced by *A. flavus*, riboflavin is a product of the secondary metabolism in *Ashbya gossypii*. Earlier study showed that the $\Delta Bck1$ mutant of *Ashbya gossypii* displayed a strong increase in riboflavin biosynthesis (Lengeler et al. 2013). A similar result was found when *A. fumigatus* lacked *Bck1*, which produced more pyomelanin than the WT (Valiante et al. 2008). However, loss of *Bck1* has an inhibitory effect on mycotoxin fumonisin B1 production in *F. verticillioides* (Zhang et al. 2015). These results might reflect absolutely different roles played by *Bck1* in specific secondary metabolism. Unfortunately, the exact role of *Bck1* in the regulation of secondary metabolism is still an unsolved question.

In addition to producing aflatoxin, *A. flavus* is capable of colonizing numerous crop seeds. Pathogenicity assays showed that the deletion of *AflBck1* dramatically ablated the ability to infect plant hosts. A similar phenomenon on attenuated pathogenicity of $\Delta Bck1$ mutant has also been reported in *F. verticillioides* (Zhang et al. 2015), *C. parasitica* (Kim et al. 2016), and *M. oryzae* (Jeon et al. 2008). In *M. oryzae*, deletion

of *Bck1* reduces the turgor pressure of the infection structure, weakening the ability to penetrate plant tissues (Jeon et al. 2008). The *Bck1* homolog in *F. verticillioides* regulates the expression of catalase and peroxidase, which prevents fungi from oxidative damage by the host (Zhang et al. 2015). The fungal cell wall acts as the first line of defense against host challenges, whose integrity is extremely important for fungal infection. As depicted in Figure 5, lack of *AflBck1* lends to augmented sensitivity to several cell wall-damaging compounds, implicating that *AflBck1* abrogates the ability to cope with the stresses during infection. Again, the deficiencies in growth and development are also fatal for $\Delta AflBck1$ colonization. We observed here that AFB₁ production of the $\Delta AflBck1$ mutant on peanuts was much more than WT and *AflBck1*com strains. In fact, similar results were obtained from phosphatase CDC14 loss mutant, which displayed impaired colonization but enhanced aflatoxin generation on seeds (Yang et al. 2018). These results seem to implicate no direct connection between pathogenicity and AFB₁ production. However, all presently

Fig. 12. Defects of *Aspergillus flavus* strains lacking the proline-rich region of *AflBck1*. **A**, Sclerotial formation of the wild type (WT), $\Delta AflBck1^{\Delta PRR-5}$, and $\Delta AflBck1^{\Delta PRR-6}$ strains on Wickerham medium (WKM) and yeast extract peptone dextrose solid media after growth for 7 days at 37°C. **B**, Quantitative analysis of the sclerotia shown in A. **C**, Conidia production of the WT, $\Delta AflBck1^{\Delta PRR-5}$, and $\Delta AflBck1^{\Delta PRR-6}$ strains on yeast extract-sucrose solid media at 37°C for 4 days. $\Delta AflBck1^{\Delta PRR-5}$ and $\Delta AflBck1^{\Delta PRR-6}$ are two clones isolated from the same transformation. ND = not detectable.

available data indicate that a correlation indeed exists between *AflBck1* and pathogenesis in *A. flavus*.

Another interesting finding of this work is the critical function of a PRR of *AflBck1* in conidia and sclerotia formation. As reported previously, the PRR is frequently found in eukaryotic proteins, which plays versatile roles in protein function (Williamson 1994). In one example, the PRR in huntingtin protein determines toxic properties of this protein (Dehay and Bertolotti 2006). In another example, the PRR of human p53 protein is necessary for promoting apoptosis of tumor cells (Baptiste et al. 2002). In this study, we have presented evidence for the first time that the PRR of *AflBck1* is involved in conidia and sclerotia formation. Although the mechanisms involved are currently unknown, many elegant studies have demonstrated that PRR mainly contributes the interaction of signaling proteins with their ligands (Kay et al. 2000). Deletion of the PRR of *Fbx7* disrupts the combination of *Fbx7* and HURP (Hsu et al. 2004). When *NoxA1* lacks the N-terminal PRR, *Tks* proteins cannot bind *NoxA1* and mediate *Nox1*-dependent ROS generation (Gianni et al. 2011). Thus, we infer that the PRR of *AflBck1* may influence interaction between *AflBck1* and reproduction-related proteins. Future studies will be necessary to clarify the underlying mechanisms for PRR of *AflBck1* regulating *A. flavus* reproduction.

In summary, we characterized a CWI-related MAPKKK, which exhibited a pivotal function in the growth, development, aflatoxin biosynthesis, and pathogenesis in *A. flavus*. When the activation site of this kinase was mutated, the phosphorylation of MAPK was almost abolished. In addition, we have presented evidence, for the first time, that the PRR of *Bck1* plays a significant role in conidia and sclerotia formation. This work further underlined the functional importance of MAPK pathway in *A. flavus*, suggesting the possibility of developing novel fungicides to control *A. flavus* contamination.

MATERIALS AND METHODS

Strains and culture conditions.

Aspergillus flavus *PTSΔku70ΔpyrG* (Chang et al. 2010) was used as an original strain for gene knockout and *PTSΔku70ΔpyrG::AfpyrG* was used as the WT strain. All *A. flavus* strains used in this study are listed in Supplementary Table S1. Growth and development experiments were performed as described in the previous studies (Zhang et al. 2016, 2018).

Sequence analysis and phylogenetic tree construction.

The *Bck1* protein sequences of *A. flavus*, *S. cerevisiae*, *Candida albicans*, *M. oryzae*, *A. oryzae*, *A. nidulans*, *A. fumigatus*, *A. niger*, and *N. crassa* were downloaded from National Center for Biotechnology Information resources. The *Bck1* protein domain was identified by SMART and ScanProsite and was drawn by DOG 2.0 software. The phylogenetic tree was constructed, by clustalw alignment comparison of MEGA7.0 software, using the neighbor-joining method.

Construction of the gene knockout and complementation strains.

To construct gene knockout strains, a homologous recombination method was used as described previously (Zhang et al. 2016). The primers used in this study are listed in Supplementary Table S2. Positive transformants were verified by diagnostic PCR and Southern blot (North2SouthChemiluminescent Hybridization and Detection Kit, Thermo scientific). For constructing the complementation strain, gene-coding sequence and its native promoter were cloned into the *Aspergillus* chromosome integrating pPTRI vector (Takara), and then, the

confirmed vectors were used to transform *ΔAflBck1* protoplasts of *A. flavus*. PCR and RT-PCR were used to confirm the positive transformants.

Stress assays.

For stress assays, 10^4 conidia were inoculated onto the center of YES agar medium plates supplemented with the following agents: oxidative stress agent hydrogen peroxide (H_2O_2 , 3, 6, 9, or 12 mM), cell-wall stress agents Calcofluor white (8 μ g/ml) and Congo red (60 μ g/ml), cell-membrane stress agent sodium dodecyl sulfate (45 μ g/ml). Each plate was incubated at 37°C for 4 days under dark conditions and colony diameters were measured daily. The experiment was conducted with technical triplicates and were repeated three times.

RNA extraction and quantitative RT-PCR.

The mycelia of WT and all mutant strains were harvested at growth stages (48 and 72 h, incubated on YES or WKM). Total RNA extraction and a quantitative real-time PCR assay were performed according to the previously reported protocols (Zhang et al. 2016). In the quantitative real-time PCR, *actin* gene was used as the endogenous reference gene, and other primers of target genes are listed in Supplementary Table S3. The relative transcript level of each gene was calculated by the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001). All experiments were carried out three times.

Determination of aflatoxin production.

WT, *ΔAflBck1*, *AflBck1^{K1330R}*, and *AflBck1com* strains (10^6 spores) were incubated in 15 ml of YES liquid medium in the dark at 29°C for 7 days. Chloroform was used to leach aflatoxin, and the extraction method was followed as previously described (Zhang et al. 2018). TLC was used to analyze aflatoxin, and results were shown under the irradiation of 365 nm ultraviolet rays. Then aflatoxin production was quantified by Gene Tools software.

Seed infections.

The peanut infection experiment of various strains was carried out according to previously reported steps (Zhang et al. 2018). Briefly, peanut cotyledons were sterilized with 0.05% sodium hypochlorite and 75% anhydrous ethanol; 30 ml of sterile water and 10^6 spores were added to every 50 peanut cotyledons and were placed in a 29°C shaker at 80 rpm for 1 h. Peanuts were then cultured in a 29°C incubator for 7 days. The peanut cotyledons were then harvested in 50-ml centrifuge tubes and 10 ml of sterile water with 0.05% Tween-20 was added to wash conidia. The number of conidia was counted using a hemocytometer and microscope, and aflatoxin was extracted as described above. Each experiment was repeated three times.

Generation of *AflBck1^{K1330R}*, *ΔAflBck1/Mkk2^{DD}*, and *AflBck1^{ΔPRR}* strains.

To generate an *AflBck1^{K1330R}* strain, a 1,561-bp fragment of the *AflBck1* gene including the mutation site (1,330, replacing lysine with arginine), a 1,476-bp downstream fragment of *AflBck1* gene, and the *A. fumigatus* *pyrG* cassette were fused and transformed into the original strain (Szewczyk et al. 2007). The *ΔAflBck1/Mkk2^{DD}* mutant bearing residues T368 and T374 to Asp was generated using an analogous strategy, but a pyriithiamine resistance gene derived from pPTRI vector served as a selectable marker and the *ΔAflBck1* mutant as a parental strain. For the generation of an *AflBck1^{ΔPRR}* strain, the coding sequence of *AflBck1* containing amino acids 1 to 10 was fused to the *AflBck1* coding sequence including amino acids 100 to 1,592, with the application of homologous recombination

method as mentioned above (Szewczyk et al. 2007). All positive constructs were verified by PCR and sequencing. The primers used in this section are listed in Supplementary Table S4.

Western blot analysis.

Each strain (5×10^6 spores) was inoculated into 50 ml of YES liquid medium, respectively, and was shaken at 180 rpm for 2 days (or another 15 min with 150 μ g of Congo red per milliliter) in a 37°C shaker. Protein preparation and Western blot experiments were performed as described previously (Lan et al. 2019). The antibodies (Cell Signaling Technology) against target proteins were added and were incubated with a polyvinylidene difluoride membrane, and chemiluminescence was detected using the Gene Imaging System (Syngene).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

SMART: <http://smart.embl-heidelberg.de>
ScanProsite: <https://prosite.expasy.org/scanprosite>

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