The membrane mucin Msb2 regulates aflatoxin biosynthesis and pathogenicity in fungus *Aspergillus flavus*

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Summary

As a pathogenic fungus, *Aspergillus flavus* can produce carcinogenic aflatoxins (AFs), which poses a great threat to crops and animals. Msb2, the signalling mucin protein, is a part of mitogen-activated protein kinase (MAPK) pathway which contributes to a range of physiological processes. In this study, the roles of membrane mucin Msb2 were explored in *A. flavus* by the application of gene disruption. The deletion of msb2 gene (∆msb2) caused defects in vegetative growth, sporulation and sclerotia formation when compared to WT and complement strain (∆msb2C). Taking together, these results revealed that Msb2 plays key roles in morphological development process, stresses adaptation, secondary metabolism and pathogenicity in fungus *A. flavus*.

Introduction

*Aspergillus flavus* is a famous plant pathogenic fungus, which is notorious as the main producer of aflatoxins (AFs) (Amaike and Keller, 2011). *A. flavus* can contaminate many agricultural crops (such as maize, peanut, cotton and so on) causing huge economic losses (Wu et al., 2014). Studies have shown that food contaminated by low concentration of AFs may lead to hepatocellular carcinoma, while high concentration of AFs can be toxic, and in some cases could be fatal (Amare and Keller, 2014). A 70 kb gene cluster was identified for AFs biosynthesis which encodes about 25 enzymes and other key regulatory proteins in *A. flavus* (Bhatnagar et al., 2003). Previous studies have demonstrated that internal and external factors controlled the AF biosynthesis, such as developmental stage and hyperosmolarity (Tsitigiannis and Keller, 2006; Zhang et al., 2018a,b). These outcomes have indicated that an appropriate response to environmental changes is essential for AF biosynthesis in *A. flavus*.

Environmental stimuli beyond the physiological range can be a matter of life or death for all cells (Aguilera, 2002). Mitogen-activated protein kinases (MAPKs) cascades are evolutionarily conserved signalling units that are utilized for signal transduction in diverse eukaryotic organisms (Chen et al., 2001). Five main MAPK signalling pathways have been identified and characterized in *Saccharomyces cerevisiae*, including the high osmolarity glycerol (HOG) pathway, the mating response pathway, filamentous and invasive growth (FIG) pathway, cell wall integrity (CWI) pathway and pheromone pathway (Saito and Posas, 2012). But in *A. flavus*, only three central MAP kinases orthologous to yeast, including SakA (Hog1), Slt2 (MPKA) and Fus3 (MPKB), have been described (Tumukunde et al., 2019; Zhang et al., 2020; Frawley et al., 2020). These MAP kinases have distinct functions in asexual sporulation, sclerotia formation and aflatoxin production. However, each MAPK module is activated by specific types of stimuli and received signalling by specific sensors (Free, 2013; Tanaka et al., 2014; Chow et al., 2019).

Signalling mucin protein Msb2, as one of the putative osmosensor in *S. cerevisiae*, initiates signalling response in the HOG pathway (Tatebayashi et al., 2007).
Interestingly, the Msb2 homologue protein MsbA in *Aspergillus fumigatus* exhibits different functions, which are essential for stresses adaptation, and in resistance to antifungal drugs through modulating the gene expression of the CWI pathway (Gurgel et al., 2019). Also, the signalling mucin Msb2 protein activates the CEK1-MAPK pathway in human fungal pathogen *Candida albicans* (Puri et al., 2012), Pmk1-MAPK (homologous to Fus3 in *S. cerevisiae*) pathway in the rice blast *Magnaporthe oryzae* (Wang et al., 2015), and the CWI pathway in *Aspergillus nidulans* (Brow et al., 2014). Moreover, Msb2 protein has been extensively characterized as an external sensor activating Fmk1, a MAP kinase in FIG pathway, for cell growth in *Fusarium oxysporum* (Perez-Nadales and Di Pietro, 2015).

Although the roles of Msb2 have been addressed in multiple filamentous fungi, such as involving in plant infection in *F. oxysporum* and *Ustilago maydis*, appressorium formation in *M. oryzae*, and hyphal growth in *Histoplasma capsulatum* (Perez-Nadales and Di Pietro, 2011; Wang et al., 2015; Rodriguez et al., 2019), the function of this protein in *A. flavus* was poorly understood. In this study, *Afmsb2* deletion and complement mutants were generated, and the results demonstrated that *AfMsb2* functioned in fungal growth, asexual development and sclerotia formation in *A. flavus*. The results also showed that *AfMsb2* was crucial for osmotic adaptation and cell wall integrity. Notably, *AfMsb2* was involved in keeping chitinase activity of *A. flavus* and contributed to the caspofungin resistance. Furthermore, this study also provided a first time report of the relationship between mucin protein *AfMsb2* and aflatoxin biosynthesis in *A. flavus*.

### Results

#### Identification and analysis of Msb2 in *A. flavus*

To identify orthologs of the *S. cerevisiae* Msb2 in *A. flavus*, the protein sequence of *S. cerevisiae* (NP_011528.3) was used with a basic local alignment search tool algorithm (BLAST), and an orthologs gene was identified in *A. flavus* named *Afmsb2* (AFLA_114450). *Afmsb2* was predicted to encode 959 amino acids that contains a conserved transmembrane region domain at the C-terminal (Fig. 1A). The phylogenetic analysis of signalling mucin *AfMsb2* showed a high conservation to Msb2 orthologue proteins from different fungi (Fig. 1B). Among them, Msb2 protein sequence of *A. flavus* exhibited the strongest similarity to that of *A. oryzae* which has been identified as a transmembrane mucin.

#### Msb2 is involved in vegetative growth

To evaluate the function of *Afmsb2* in *A. flavus*, *Afmsb2* deletion (Δ*msb2*) and complement (Δ*msb2*C) mutants were constructed by homologous recombination. The transformants were confirmed by PCR analysis with three pairs of primers, and the results showed that the AP and BP fragments were contained in both Δ*msb2* and Δ*msb2*C mutant strains, but absent in wild type (WT), indicating that the *A. fumigatus* pyrG gene had completely replaced the *msb2* gene in *A. flavus* (Fig. 2A). Expression levels of *msb2* in the WT, Δ*msb2* and Δ*msb2*C were confirmed by RT-PCR and qRT-PCR respectively, and the results shown in Figure 2B and C.
proved that Atflmsb2 gene was existed in the WT and Δmsb2, but was missing in Δmsb2C. Ultimately, the deletion strain was further verified by Southern blot, which indicated that Atflmsb2 was successfully disrupted in the mutants (Fig. 2D). Colony morphology was observed after incubation at 37°C in dark for 4 days, and the result indicated a reduction in the growth rate of the Δmsb2 strains as compared to the WT and Δmsb2C strains in minimal medium (MM), complete medium (CM) and glucose minimal medium (GMM) (Fig. 2E and F), suggesting a defect in vegetative growth.

Msb2 is involved in conidiation

Conidia is produced at the hypha tip, which is one of the most important asexual reproduction in A. flavus (Amaike and Keller, 2011). For analysis of the function of Msb2 in conidiation, the WT and mutant strains were grown on PDA medium for 4 days. Following microscopic observations, Δmsb2 strain exhibited a growth reduction on PDA medium plates (Fig. 3A and B) and produced less and shorter conidiophores in comparison to the WT and Δmsb2C mutant strains (Fig. 3C). With respect to defect in conidiophores, we also found that the number of conidia was significantly decreased in Δmsb2 mutant, as compared to the WT and Δmsb2C strains (Fig. 3D). We further studied the expression levels of regulatory genes for conidial formation (brlA and abaA) and global regulator gene (veA), and the result showed that the expression levels of brlA, abaA and veA genes were all down-regulated in the Δmsb2 strain in comparison to Δmsb2C and WT strains (Fig. 3E). All above results demonstrated that AflMsb2 is involved in conidial formation and vegetative growth in A. flavus.

Msb2 positively regulates sclerotia formation in A. flavus

Different to conidia, sclerotium is one of the alternative reproduction to survive in the adverse environments in A. flavus (Dyer and O’Gorman, 2012). To validate the function of Msb2 in formation of sclerotium, WT, Δmsb2 and Δmsb2C strains were cultured on sclerotia-inducing WKM solid medium, at 37°C under dark condition for 7 days. As shown in Figure 4A and B, Δmsb2 mutant barely able to generate sclerotium, whereas WT and Δmsb2C could produce similar amounts of sclerotia. In addition, we had detected the expression levels of nsdC and sclR genes, which are indispensable for sclerotia generation (Cary et al., 2012). As expected, the
transcript levels of sclR and nsdC were both declined sharply in the Δmsb2 strain when compared to Δmsb2C and WT strains, and the expression level of global regulator veA gene had also decreased (Fig. 4C). All these observed results strongly indicated that Msb2 plays a key role in sclerotia production of A. flavus.
**Effect of msb2 on aflatoxin biosynthesis**

*Aspergillus flavus* is notorious for its ability to produce aflatoxins (AFs), which are one of the most toxic and carcinogenic natural contaminants (Amare and Keller, 2014). In order to determine the specific function of Msb2 in AFs biosynthesis, WT, Δmsb2 and Δmsb2<sup>C</sup> strains were cultured in liquid YES medium for 7 days, and AF production was tested by TLC and HPLC, respectively. TLC results indicated that AFB<sub>1</sub> production in Δmsb2 mutant strains was significantly decreased when compared to that of the WT and Δmsb2<sup>C</sup> mutants (Fig. 5A and C). Quantitative analysis by HPLC further confirmed that AFB<sub>1</sub> production was down-regulated in Δmsb2 mutants (Fig. 5B). Additionally, to explore the possible reasons for the resultant decrease in AF production in Δmsb2 mutant strain, we detected transcript levels of aflatoxin biosynthesis related genes (*aflC*, *aflR* and *aflQ*) and secondary metabolism regulator genes (*veA* and *laeA*) (Cary et al., 2015). The results revealed that the expression levels of *aflC*, *aflR*, *aflQ*, *veA* and *laeA* were all decreased significantly in Δmsb2 in comparison to Δmsb2<sup>C</sup> and WT strains (Fig. 5D). In overall, these data demonstrated that Msb2 was involved in the regulation of aflatoxin biosynthesis in *A. flavus* through dominating the transcription of global regulators and aflatoxin-producing related genes.

**Msb2 contributes to the pathogenicity**

Since *msb2* gene exhibited a variety of functions in growth, asexual conidia production, sclerotia formation and AFB<sub>1</sub> biosynthesis, we anticipated that *msb2* might influence strain colonization on plant seeds. So peanut seeds and Chinese chestnut were inoculated with the WT, Δmsb2 and Δmsb2<sup>C</sup> mutant strains. Visually, the WT and Δmsb2<sup>C</sup> strains showed stronger ability to infect and sporulate on peanut and Chinese chestnut surface in comparison to Δmsb2 mutant (Fig. 6A and B). Then, the amount of conidia in the infected seeds was measured, and we found that conidia number was significantly decreased in Δmsb2 mutant compared to Δmsb2<sup>C</sup>.
and WT (Fig. 6E). Further determination of AFB$_1$ production from the infected seeds by TLC indicated that the AFB$_1$ production in $\Delta msb2$ mutant was also significantly reduced as compared with the WT and $\Delta msb2C$ mutant (Fig. 6C, D and F). These data confirmed that msb2 is crucial for A. flavus maintaining full pathogenicity to plant seeds.

Msb2 positive regulates phosphorylation of Hog1 in response to osmostress

Previous studies have reported Msb2 protein as a part of HOG-MAPK pathway, played roles in various stress responses in fungi (Puri et al., 2012; Gurgel et al., 2019). Due to the osmosensor role of Msb2 in signal transduction in S. cerevisiae (Saito and Posas, 2012), we are especially interested in the role of AflMsb2 when facing hyperosmotic pressure. Then, osmotic stress (water activity, 0.95Aw and NaCl, 1.2M/L) were used in the YES solid medium, and the results showed that $\Delta msb2$ mutant was more sensitive to the osmotic stress when compared to $\Delta msb2C$ mutant and WT (Fig. 7A and B). Furthermore, cell growth upon high osmotic stress was also significantly inhibited in $\Delta msb2$ strain (Fig. 7C). At 8 and 12 h, growth of $\Delta msb2$ was also obviously inhibited upon high osmotic stress (Fig. 7C). To confirm the relationship between Aflmsb2 and HOG-MAPK pathway, we detected the phosphorylation levels of Hog1 kinase in the WT and $\Delta msb2$ mutant under osmotic stress (1.2 M l$^{-1}$ NaCl) at different times (0, 10, 30, 60 min). The WT and $\Delta msb2$ strains showed increased Hog1 phosphorylation after exposed to 1.2 M l$^{-1}$ NaCl for 10 min. However, the phosphorylation level of Hog1 in $\Delta msb2$ mutant was lower than that in the WT. At 30 min, phosphorylation levels of Hog1 were both decreased in the WT and $\Delta msb2$ mutant as compared to 10 min, but the Hog1 phosphorylation in WT was still higher than that in the $\Delta msb2$ mutant (Fig. 7D). The protein expression levels of Hog1 were determined in the WT and $\Delta msb2$ mutant as well, and the result showed that Hog1 expressed no difference among these indicated strains under different conditions (Fig. 7D and E). In overall, these results suggested that AflMsb2 plays an important role in osmotic stress and involves in the phosphorylation of Hog1 in response to osmotic stress.

Msb2 involves in CWI pathway

CWI-MAPK pathway was first discovered and identified in yeast, for it responses to cell wall stress maintaining normal life activities (Sanz et al., 2017). SH2, MAP...
kinase, is the core component of CWI pathway which transmits a signal to the nucleus when cell surface sensors perceive the cell wall stress (Sanz et al., 2018). To explore the function of Msb2 in CWI, we cultured all strains on PDA media supplemented with cell wall-damaging agent Congo red (CR) and cell membrane inhibitor Sodium Dodecyl Sulfate (SDS). The results showed that the growth inhibition rate of Δmsb2 mutant in different

Fig. 7. Deletion of msb2 affects osmotic stress response in A. flavus. A. Colony morphology of the WT, Δmsb2 and Δmsb2C strains cultured on YES medium under osmotic stress (0.95Aw and 1.2 M NaCl) at 37°C for 4 days. B. Growth inhibition rate of WT, Δmsb2 and Δmsb2C strains was calculated based on A. C. Microscopic observation of WT, Δmsb2 and Δmsb2C strains on YES liquid medium with osmotic stress (1.2 M NaCl) (bars = 50µm). D. Phosphorylation levels of Hog1 in strains were measured by phosphor-p38 MAPK antibody under NaCl stress, while Hog protein was used as loading control. E. Optical density semi-quantitative analysis of the phosphorylation levels of Hog1 in the WT and Δmsb2 strains. **, denotes P < 0.01.
additive media was significantly increased as compared to that of the WT and Δmsb2C mutant (Fig. 8A and B). To test the phosphorylation level of Slt2, WT and Δmsb2 mutant were cultured in YES liquid media and treated with CR for 15 min. Western blotting results showed that the levels of phosphorylated Slt2 in indicated strains were significantly increased when induced by CR. However, there is a lower Slt2 phosphorylation level in Δmsb-2 strain than that in the WT under the stress (Fig. 8C and D), suggesting that Msb2 is able to impact the phosphorylation of Slt2 and involves in CWI pathway.

**Msb2 mutant has cell wall defects**

The cell wall components mainly contain β-glucan and chitin in fungi (Gow et al., 2017). Δmsb2 mutant from A. flavus exhibits hypersensitivity to cell wall stress, so we suspected that Msb2 may have some effects on glucan synthase and chitinase activity. To test these hypotheses, all strains were cultured on YES liquid media supplemented with caspofungin which is an inhibitor of β-1,3-glucan synthase. As shown in Figure 9A, the Δmsb2 mutant was more sensitive to caspofungin when compared to the WT and Δmsb2C. Interestingly, the chitinase activity of Δmsb2 mutant was also significantly decreased (Fig. 9B). Then, the expression levels of two chitin synthase genes (AFLA_114760 and AFLA_060590) and one β-glucan synthase gene (AFLA_023460) were quantified. Under CR stress, the expression levels of three relative genes in Δmsb2 mutant were markedly reduced in comparison with that in the WT and Δmsb2C (Fig. 9C). The aforementioned results suggested that Msb2 plays a crucial role in maintaining cell wall integrity.

**Discussion**

MAPK cascades are essential for signal transduction in diverse eukaryotes including yeast and other fungi (Martínez-Soto and Ruiz-Herrera, 2017). HOG is one of the best understood MAPK pathways in yeast which play important roles in response to the stimuli from outside environments (Saito and Posas, 2012). The mucin-like protein Msb2 is located on the cell membrane, acting as...
an osmosensor upstream the HOG pathway in *S. cerevisiae* (Tatebayashi et al., 2007). In the plant and animal pathogenic fungus *A. flavus*, our previous works suggested that three kinases of the HOG pathway play crucial roles in plant infection, aflatoxin biosynthesis and morphogenesis (Yuan et al., 2018; Tumukunde et al., 2019). But the role of *AflMsb2* which has a typical transmembrane domain was yet to be identified (Fig. 1). In this study, we committed to investigate the biological function of Msb2 in *A. flavus*.

Msb2 is important for vegetative growth in many filamentous fungi. In *A. fumigatus*, MsbA is required for the conidiation and hyphal growth (Gurgel et al., 2019), and the homologous protein MsbA in *A. nidulans* regulates not only conidiation process but also vegetative growth (Brow et al., 2014). In *Cryptococcus neofor mans* and *C. albicans*, Msb2 plays essential roles during vegetative growth (Román et al., 2009; So et al., 2018). The current study demonstrated retarded growth, and a substantial reduction in conidial production in Δmsb2 mutant in comparison to the WT. Following the similar trend, the expression levels of key regulator genes *braA* and *abaA*, which control asexual development, were also markedly reduced. In addition to conidiation, Msb2 also engages in sclerotia formation that helps the fungus survival in adverse environments. The sharp decrease of sclerotia formation in Δmsb2 mutant was also verified by the down-regulation of *sclF* and *nsdC* (Fig. 4). Interestingly, qRT-PCR results also showed the decreased expression level of *veA* in ΔAflmsb2 in different culture conditions. In filamentous fungus *A. niger*, *veA* regulated conidia production through the development gene *brlA* which mediates the budding growth of conidiophores (Zhang et al., 2018a,b). As a global regulator, *veA* is necessary for sclerotia formation in *A. flavus* (Gary et al., 2007; Duran et al., 2007). Therefore, it could be speculated that Msb2 regulates conidial and sclerotia formation related genes, further influences the conidia and sclerotia production in *A. flavus*.

Secondary metabolites synthesis in filamentous fungi is complicated (Yu et al., 2004a,b). In *S. cerevisiae*, Msb2 regulated the activity of p21-activated protein kinases (PAKs) Ste20 and Cla4 (Tanaka et al., 2014). Similarly, the report from previous study further verified that the loss of PAKs *Aflste20* would lead to the impaired colonization and reduced AFB1 production (Li...
et al., 2019). It hints that genes in HOG pathway are important for AFB1 production in *A. flavus*. In this study, the AFB1 production was significantly decreased in ΔAffmsb2 mutant. To further study the mechanism of Affmsb2 in AFB1 synthesis, we also detected the expression levels of structural genes (*atfC* and *atfQ*) and regulatory gene (*atfR*) in aflatoxin biosynthesis gene cluster, and we found that they were all down-regulated in ΔAffmsb2 mutant (Fig. 5). Based on the functional genomics and transcriptome analysis of *A. flavus*, the results reveal that veA regulated secondary metabolite gene clusters (Cary et al., 2007, 2015). Especially, veA controls aflatoxin synthesis cluster and directly impact the *atfR, atfD, atfM* and *atfP* transcripts (Duran et al., 2007). Previous study also showed that both laeA and veA genes are required for AFB1 production in *A. flavus* (Kale et al., 2008). In this study, expression levels of laeA and veA in ΔAffmsb2 were both significantly lower than that in WT. Therefore, we conclude that msb2 is involved in AFB1 production and regulation of aflatoxin synthesis gene expression in *A. flavus*, most likely through veA and laeA.

Plant infection is a complex process and changes in the composition of cell wall of pathogenic fungus can reduce its ability to penetrate plant tissues (Geoghegan et al., 2017). In *A. flavus*, pathogenicity defect of msb2 mutant occurred in seeds infection experiments, and the growth retardation and decreased conidiation in ΔAffmsb2 mutant could explain the defect of pathogenicity. On the other hand, the defective in cell wall integrity may strongly affect the infection ability of ΔAffmsb2. In *A. fumigatus* and *A. nidulans*, it also showed the greatest reduction in mortality rate of mice for msb2 deletion mutant compared to that for WT (Brow et al., 2014; Gurgel et al., 2019). msb2 deletion mutant of *U. maydis* showed reduced colonization and appressorium in the plant surface (Lanver et al., 2010). It also reported that the mucin protein Msb2 is a broad range protectant against antimicrobial peptides in *C. albicans* (Swidergall et al., 2013). All these results indicated the important roles of msb2 in self-protection and pathogenicity in fungi.

In cells, genes change expression models to adapt the rapidly varying in environmental conditions (Karpińska and Foy, 2005). MAPK cascades regulate many biological processes and the most striking aspect is the role of MAP kinases (Lee et al., 2016). Msb2, as an osmosensor, was already identified in *S. cerevisiae* (Tanaka et al., 2014). In *C. albicans*, Msb2 can regulate adaptation to thermal stress, cell wall stress, oxidative stress and other stimuli (Román et al., 2009; Puri et al., 2012; Swidergall et al., 2013). Our results showed that under osmotic stress, Δmsb2 mutant became more sensitive and had decreased phosphorylation level of Hog1 than WT (Fig. 7). The similar results were also occurred in *C. albican* and *F. oxysporum*, but needs hyperosmotic stress (minimum 1.5 M l⁻¹ NaCl) (Pérez-Nadales and Di Pietro, 2011; Puri et al., 2012). We also noticed that the phosphorylation of Hog1 in *C. neoformans* msb2 deletion mutant was not affected when responding to osmotic shock (1 M l⁻¹ NaCl). This outcome could be as a result that sho1 has an overlapping function with msb2 in *C. neoformans* HOG pathway (So et al., 2018). Surprisingly, at least five osmosensors (Msb2, Sho1, Sin1, Opy2 and Hkr1) were found to adapt hyperosmolarity in *S. cerevisiae*. There results suggested that Msb2 is important for full activation of the HOG pathway and positively regulate the Hog1 phosphorylation in *A. flavus*. But the exact role of Msb2 in the HOG pathway signal transduction needs further investigation.

The cell wall of fungi has a crucial role in cell division and hyphal development (Geoghegan et al., 2017). It has been reported that Msb2 homologues in *A. nidulans*, *C. albican* and *F. oxysporum* regulated the Slt2 (Mkc1) phosphorylation which was important for the cell wall biogenesis (Román et al., 2009; Brow et al., 2014; Perez-Nadales and Di Pietro, 2015). Therefore, we are not surprised that ΔAffmsb2 mutant exhibited increased sensitivity to CR, SDS and caspofungin (Figs 8 and 9). As expected, the expression levels of cell wall chitin synthase genes and β-glucan synthase gene were significantly decreased in ΔAffmsb2 mutant. The CR-induced Slt2 phosphorylation level was also reduced in the ΔAffmsb2 mutant, indicating that AffMsb2 positively regulates the phosphorylation level of Slt2 in *A. flavus*. At the same time, our results also showed the reduced chitinase activity in ΔAffmsb2 mutant of *A. flavus*. Similarly, in *A. fumigatus*, MsbA positively modulates expression level of the chitin synthases (*chsA, chsB*), β-1,3-glucan synthase (*fksA*) and other related genes in the CWI pathway (Gurgel et al., 2019). MsbA interferences the chitin production in the cell wall and biofilm formation in *A. nidulans* (Brow et al., 2014). Interestingly, not only Δmsb2 mutant but also Δsho1 mutant has cell wall defects in *C. albican*. Collectively, AffMsb2 plays a positive role in the activation of the phosphorylation of Slt2 and is important for cell wall biogenesis.

Overall, these results lead us to understand the role of msb2 in reproduction, aflatoxins biosynthesis and pathogenicity in *A. flavus*. Our findings also provide a novel insight into the controlling of AF production and *A. flavus* in oil crops and food during storage.

**Experimental procedures**

**Strains and media**

*Aspergillus flavus* CA14 PTS (Δku70ΔpyrG, uracil auxotrophic) (Chang et al., 2010) was used as the parental

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strain for transformation. For pyrG auxotroph, the solid medium was supplied with uracil and uridine (1 g l⁻¹ for each). The strains in this study were cultured on yeast extract-sucrose (YES), minimal medium (MM), potato dextrose agar (PDA), glucose minimal medium (GMM) and complete medium (CM) for conidiation and mycelia growth assays, respectively (Ren et al., 2019). The sclerotia-inducing Wickerham (WKM) medium was used for sclerotia assays. YES liquid medium was used for AFB1 assays. Each experiment was repeated at least 3 times.

Domain and phylogenetic tree analysis
BLAST was carried out with the S. cerevisiae Msb2 protein sequence (NP_011528.3) to obtain A. flavus Msb2 (XP_002385498.1) and other Aspergillus sequence. MEGA 7.0 software was used to create the phylogenetic tree. The protein domains were predicted by SMART (http://smart.embl-heidelberg.de/) and drawn by IBS 2.0 software (http://dog.biocuckoo.org/).

Construction of mutant strains
The method of homologous recombination was used to construct msb2 knockout mutant (\(\Delta\)msb2). The primers used in this study were listed in Table 1. Double-joint polymerase chain reaction (PCR) method (Yu et al., 2004a,b) was used to generate gene-deletion cassettes which amplified by specialized primers. Then, overlap PCR products were transformed into the A. flavus CA14 Pts protoplasts. Positive transformants were screened by diagnostic PCR and Southern blot (Thermo Fisher Scientific, Waltham, MA, USA). To construct the \(\Delta\)msb2⁻² complement strain (\(\Delta\)msb2⁻²), similar strategy was used and the pyrG marker was replaced by pyrithiamine resistance (prtA) marker. Then the gene complement cassettes were re-introduced into \(\Delta\)msb2 protoplasts. The positive transformants were screened by PCR.

Mycelial growth, conidiation and sclerotia analysis
The phenotypes of the WT, \(\Delta\)msb2 and \(\Delta\)msb2⁻² strains were observed using different medium. Assays for mycelial growth, fungal conidia and sclerotia formation were carried out according to our previously described methods (Yuan et al., 2018). Each experiment was repeated at least 3 times.

Stress assay
For the stress assays, 1 l of conidial suspension (10⁷ conidial ml⁻¹) were inoculated at the centre of the YES and PDA medium plates supplemented with the following agents: 1.2 M l⁻¹ NaCl, 0.95% water activity (95Aw:

Table 1. PCR primer sets used in this study.

| Primer | Sequence (5’–3’) | Characteristics |
|--------|------------------|-----------------|
| msb2-P1 | TCTCCAGGCACGCAACA | For amplifying |
| msb2-P3 | GACGTTGGGACGACGCAACA | 5’UTR of msb2 in \(\Delta\)msb2 |
| pyrG-F | GGTGTAAGCGATTCGAGAGACGACGCAACA | For amplifying |
| pyrG-R | CTCCTCCAGGCACGCAACA | A. fumigatus pyrG |
| msb2-P6 | GACTATGTCCTCTTGAGACGACGCAACA | For amplifying |
| msb2-P8 | CTCCTCCAGGCACGCAACA | 3’UTR of msb2 in \(\Delta\)msb2 |
| msb2-P2 | GCTGTAAGCGATTCGAGAGACGACGCAACA | For fusion PCR of \(\Delta\)msb2 |
| msb2-P7 | GACTATGTCCTCTTGAGACGACGCAACA | For ORF verification |
| msb2-P9 | GCTGTAAGCGATTCGAGAGACGACGCAACA | For \(\Delta\)msb2 mutant screening |
| msb2-P10 | GCTGTAAGCGATTCGAGAGACGACGCAACA | 5’UTR of \(\Delta\)msb2⁻² |
| pyrG-907-F | ATGACGCGGATTCGAGAGACGACGCAACA | For amplifying |
| pyrG-919-R | ATGACGCGGATTCGAGAGACGACGCAACA | PrA |
| msb2-C-P1 | TACGCGCAGCGATTCGAGAGACGACGCAACA | For \(\Delta\)msb2 mutant screening |
| msb2-C-P3 | TACGCGCAGCGATTCGAGAGACGACGCAACA | 5’UTR of \(\Delta\)msb2⁻² |
| PnA-F | CTGGTGACGATTCGAGAGACGACGCAACA | For amplifying |
| PnA-R | CACGTTGGGACGACGCAACA | For \(\Delta\)msb2⁻² mutant screening |
| msb2-C-P6 | CACGTTGGGACGACGCAACA | For amplifying |
| msb2-C-P8 | CACGTTGGGACGACGCAACA | For \(\Delta\)msb2⁻² mutant screening |
| msb2-C-P2 | CACGTTGGGACGACGCAACA | For \(\Delta\)msb2⁻² mutant screening |
| msb2-C-P7 | CACGTTGGGACGACGCAACA | For \(\Delta\)msb2⁻² mutant screening |
| msb2-C-AP-F | CAGTGGTGTACGAGTTCGAGAGACGCAACA | For \(\Delta\)msb2⁻² mutant screening |
| msb2-C-AP-R | TACGCGCAGCGATTCGAGAGACGACGCAACA | For \(\Delta\)msb2⁻² mutant screening |
| msb2-C-BP-F | TACGCGCAGCGATTCGAGAGACGACGCAACA | For \(\Delta\)msb2⁻² mutant screening |
| msb2-C-BP-R | TACGCGCAGCGATTCGAGAGACGACGCAACA | For \(\Delta\)msb2⁻² mutant screening |

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glycerol was used as an osmotressor to adjust the water availability), 300 μg ml⁻¹ CR, 10 μg ml⁻¹ caspofungin and 100 μg ml⁻¹ glycerol was used to inoculate the same aliquots of conidial suspension. After 1–24 h inoculation at 37°C on shaker, Leica confocal SP8 microscope (Leica, Germany) with a 20× objective was used to record the hyphae growth, and Latexphochol cotton blue was used to make the hyphae morphology more clear. The experiments were repeated 3 times, using 3 independent batches of conidia.

Aflatoxins analysis
For aflatoxins (AFs) production, 10 μl conidial suspension (10⁷ conidial ml⁻¹) of WT, Δmsb2 and Δmsb2C strains were inoculated into 7.5 ml YES liquid medium respectively and incubated in 29°C under dark condition for 7 days. AFs production was detected by thin-layer chromatography (TLC) (Haiyang Chemical, Qingdao, China). For quantitative analysis of aflatoxins production, methanol was used to resuspend the aflatoxin extracts. After filtration (0.22 μm), the samples were analysed by high-performance liquid chromatography (HPLC) (Waters, Milford, MA, USA) on a Mycotox™ column (Water, Milford, USA) at 42°C. After the column was equilibrated in running solvent (water:methanol:acetonitrile, 56:22:22), 10 μl samples were injected, and isocratic runs were conducted for 15 min in 100% running solvent at a flow rate of 1.0 ml min⁻¹. Aflatoxins were checked using a fluorescent detector (Water, Milford, USA) with excitation and emission wave lengths of 365 and 455 nm, respectively. Each experiment was repeated 3 times.

Seeds infections
The ability of WT, Δmsb2 and Δmsb2C strains to infect plant seeds was assayed as described previously (Yang et al., 2019). Seeds were inoculated with conidial suspension and cultured at 29°C, then 700 μl sterile water was added to keep the filter paper almost moist. After 6 days, seeds were harvested in 50 ml centrifuge tubes (one plate corresponds to one centrifuge tube) which contained 15 ml sterile water and 7.5 μl Tween 20. In order to release conidia on seeds surface, tubes were vigorously mixed for 5 min. The number of conidia was counted and AF was extracted according to the method described above. Each experiment was repeated at least 3 times.

Western blot analysis
The conidia (6 × 10⁶) of WT, Δmsb2 and Δmsb2C strains were inoculated in YES liquid medium respectively and cultivated for 48 h. Whole-cell extraction and Western blot were performed as described previously (Lan et al., 2019). Phospho-p38 MAPK antibody (Cell Signaling Technology, Boston, MA, USA) and Anti-phospho-p44/42 MAPK (Erk 1/2) antibody (Cell signaling Technology, MA, USA) against target proteins were used to detect the specific phosphorylated proteins. Monoclonal antibody Hog1 (Santa Cruz Biotechnology, Dallas, Texas, USA) and anti-AFSt2 antibody (prepared by our laboratory) were used as loading controls respectively. Enhanced chemiluminescence (ECL) substrate was used for immunoblot analysis and chemiluminescence was measured by G-BOX Chemi XT4 (Syngene, HK, China).

Chitinase activity assay
The conidia (6 × 10⁶) of WT, Δmsb2 and Δmsb2C strains were grown in YES liquid medium in a rotary shaker (180 r min⁻¹) for 72 h. The mycelia were removed with glass filter, and ultrasonic crusher (Sientz, Zhangjiang, China) was used for protein extraction. Chitinase activity was measured using a chitinase activity detection reagent (Solarbio Science, Beijing, China) as described by Yanai et al. (1992). Chitinase hydrolyses chitin to produce n-acetylglucosamine, which is further combined with 3, 5-dinitrosalicylic acid to produce brownish red compounds. There is a characteristic absorption peak at 540 nm detected by enzyme-labelled instrument (Molecular Devices, San Jose, CA, USA), and the increase of absorption reflects the activity of chitinase. The experiment was repeated 3 times.

Quantitative RT-PCR analysis
For qRT-PCR, the mycelia of WT, Δmsb2 and Δmsb2C strains were harvested at WKM, PDA and YES medium at 37°C. The total RNA was got from 100 mg ground mycelium through the TRIzol reagent (Biomarker Technologies, Beijing, China), and the First-strand cDNA was obtained by the cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). qRT-PCR was performed on the Thermo Fisher Scientific Real-time PCR System (Finland) using SYBR Green qPCR Mix (DongSheng Biotech, Guangzhou, China). The actin gene of A. flavus was utilized as the reference gene, and the relative expression of the target gene was calculated using the 2⁻ΔΔCt.
method (Livak and Schmittgen, 2001). The qRT-PCR primers were listed in Table 2. All assays of qRT-PCR were conducted with technical triplicates for each sample, and each experiment was repeated at least 3 times.

**Statistical analysis**

**Graphpad PRISM 7** (https://www.graphpad.com) was used to analyse the statistics and significance. Student’s *t* test was performed for comparison of two different groups, while multiple groups comparisons were through One-way analysis of variance (ANOVA) test.

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**Conflict of interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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