The HosA Histone Deacetylase Regulates Aflatoxin Biosynthesis Through Direct Regulation of Aflatoxin Cluster Genes

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Histone deacetylases (HDACs) always function as corepressors and sometimes as coactivators in the regulation of fungal development and secondary metabolite production. However, the mechanism through which HDACs play positive roles in secondary metabolite production is still unknown. Here, classical HDAC enzymes were identified and analyzed in *Aspergillus flavus*, a fungus that produces one of the most carcinogenic secondary metabolites, aflatoxin B₁ (AFB₁). Characterization of the HDACs revealed that a class I family HDAC, HosA, played crucial roles in growth, reproduction, the oxidative stress response, AFB₁ biosynthesis, and pathogenicity. To a lesser extent, a class II family HDAC, HdaA, was also involved in sclerotia formation and AFB₁ biosynthesis. An *in vitro* analysis of HosA revealed that its HDAC activity was considerably diminished at nanomolar concentrations of trichostatin A. Notably, chromatin immunoprecipitation experiments indicated that HosA bound directly to AFB₁ biosynthesis cluster genes to regulate their expression. Finally, we found that a transcriptional regulator, SinA, interacts with HosA to regulate fungal development and AFB₁ biosynthesis. Overall, our results reveal a novel mechanism by which classical HDACs mediate the induction of secondary metabolite genes in fungi.

Keywords: fungal development, fungal effectors

Aspergillus flavus is a widespread saproprobic and pathogenic fungus that is a major contaminant of various crops such as peanut, maize, and cottonseed in both the preharvest and postharvest stages (Amaike and Keller 2011). During colonization, *A. flavus* contaminates host tissue with aflatoxins, including aflatoxin B₁ (AFB₁), which is one of the most potent

carcinogenic natural compounds known (Yu et al. 2005). Additionally, ingestion of AFB₁-contaminated food can result in hepatocellular carcinoma, immunotoxicity, or teratogenic effects on the consumer (Hedayati et al. 2007; Klich 2007a). Consequently, both developed and developing countries have strict regulations regarding the amounts of aflatoxins allowed in food commodities (Van Egmond 2002). Contaminated grains that are above the permitted limit have to be destroyed, resulting in substantial economic losses annually worldwide. Therefore, it is necessary to clarify the regulation of the aflatoxin biosynthesis pathway to control this dangerous threat, thereby preventing economic losses and protecting animal and human health.

Many external environmental factors such as temperature, pH, light, and nutrient levels can affect AFB₁ biosynthesis (Calvo et al. 2002; Klich 2007b; Zhang et al. 2015). In fungal cells, AFB₁ biosynthetic enzymes and direct regulators are encoded by 27 clustered genes located close to the telomere of chromosome III (Yu et al. 2004). Accumulating evidence suggests that AFB₁ biosynthesis is a sophisticated process involving many levels of transcriptional and posttranscriptional control. For example, the velvet family (Baidya et al. 2014), the global regulator LaeA (Amaike and Keller 2009), the basic leucine zipper transcription factor AtfB (Roze et al. 2011), MeaB (Amaike et al. 2013), and G protein signaling kinases (Affeldt et al. 2014) are all involved in regulating morphological development and AFB₁ biosynthesis. In addition, AFB₁ biosynthesis is governed by chromatin remodeling and the covalent modification of histone tails (Pfannenstiel et al. 2017; Roze et al. 2007). Recently, we reported that a DNA methylase, DmtA (Yang et al. 2016), a histone acetyltransferase, GcnE (Lan et al. 2016), and a small ubiquitin-like modifier, AfSumO (Nie et al. 2016), are all involved in AFB₁ biosynthesis in *A. flavus*.

Reversible acetylation and deacetylation are forms of histone modification that are mediated by histone acetylases (HATs) and histone deacetylases (HDACs) (Grunstein 1997). Classical HDACs are grouped into different classes. Class I (Rpd3-like) and class II (Hda1-like) HDACs are sensitive to inhibition by trichostatin A (TSA) and are conserved from yeast to humans. Additionally, they are crucial regulators of gene expression that are responsible for regulating the acetylation state in cells (de Ruijter et al. 2003; Kurdistani and Grunstein 2003; Yang and Seto 2008). HDACs are involved in many biological processes, with their roles varying among various physiological contexts in fungi. The *HDC1* gene of the maize pathogen *Cochliobolus*

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carbonum is required for pathogenicity (Baidyaroy et al. 2001), and an Rpd3-like HDAC from *A. nidulans* termed RpdA plays an essential role in hyphal growth (Tribus et al. 2010). *Candida albicans* *hos2* controls the dimorphic switch during animal infections (Hnisz et al. 2010), and the plant pathogen *Magnaporthe oryzae* *hos2* helps to regulate plant tissue penetration (Ding et al. 2010). HDACs participate in secondary metabolite (SM) biosynthesis. For example, deletion of *A. nidulans* *hdaA* increases sterigmatocystin (the penultimate precursor of aflatoxin B1) biosynthesis (Shwab et al. 2007) whereas down-regulation of *rpdA* decreases production of this mycotoxin (Albright et al. 2015). *A. fumigatus* *hdaA* is involved in regulating gliotoxin biosynthesis (Lee et al. 2009). Deletion of *Fusarium fujikuroi* *FfHda1* or *FfHda2* affects gibberellin biosynthesis (Studt et al. 2013). Generally, HDACs inhibit gene transcription through the deacetylation of promoter-bound histones (Struhl 1998). However, some studies have shown that HDACs have both repressing and activating functions. For example, the *Saccharomyces cerevisiae* Set3/Hos2 HDAC complex inhibits specific meiotic genes while activating others (Hang and Smith 2011; Pijnappel et al. 2001). Hos2 is required for activating *GAL* genes in yeast (Wang et al. 2002), and *Ustilago maydis* Hos2 binds directly to mating-type genes to control their expression (Elías-Villalobos et al. 2015). A large scale metabolomics study of an *A. nidulans* *rpdA* mutant showed that RpdA was involved in positively and negatively regulating hundreds of products, not only sterigmatocystin mentioned above but also new antibiotics such as aspercryptins (Albright et al. 2015; Henke et al. 2016).

Although HDACs have been reported in many fungi, the roles of HDACs in aflatoxin-producing fungi are unstudied. In this study, we systematically characterized the roles of classical HDACs in *A. flavus*. *A. flavus* harbors five classical HDACs, including RpdA, Rpd3, HosA, HdaA, and HosB. RpdA, Rpd3, and HosA belong to the class I HDAC family, and HdaA and HosB belong to the class II HDAC family. An analysis of gene mutants revealed that the class I HDAC HosA is important for various developmental processes and pathogenicity. Additionally, the class II HdaA plays crucial roles in sclerotia formation and AFB1 biosynthesis in *A. flavus*. Furthermore, we revealed that HosA is required for cAMP signaling-induced AFB1 biosynthesis. Last, we found that the HosA/SinA (Swi-independent) complex bound directly to the AFB1 biosynthesis genes to control their expression. These findings provide novel insights into the positive roles that HDACs play in SM biosynthesis in fungi.

RESULTS

Identification of classical HDAC homologs in *A. flavus*.

A protein BLAST and phylogenetic analysis revealed that the *A. flavus* genome harbors five putative HDACs that could be grouped into class I and class II HDACs (Fig. 1A). The five proteins were named according to their closest relative in *A. nidulans*. Thus, the five HDACs in this study were designated RpdA (AFLA_092360), Rpd3 (RpdA-like, AFLA_025120), HosA (AFLA_087410), HdaA (AFLA_006160), and HosB (AFLA_114920) (Fig. 1A). All five putative proteins possess conserved histone deacetyl domains that are characteristic of their types (Fig. 1B).

The expression profiles of *rpdA*, *rpd3*, *hosA*, *hdaA*, and *hosB* were monitored by real-time quantitative (q)PCR during vegetative growth, conidiation, aflatoxin synthesis, and sclerotia development (Fig. 1C). The expression patterns suggest that these classical HDAC genes may have different roles in *A. flavus*.

Histone deacetylation is involved

in hyphal growth and AFB1 production in *A. flavus*.

To investigate the potential roles of classical HDACs in hyphal growth and AFB1 production in *A. flavus*, *A. flavus* strains were treated with the classical HDAC inhibitor TSA. The results showed reduced aerial hyphae and significantly decreased colony growth in the presence of increasing TSA concentrations compared with the control (Fig. 2A and B). Next, we addressed the role of TSA in AFB1 production, and the results showed that it decreased severely (>30% decrease compared with nontreated samples) when liquid yeast extract-sucrose (YES) medium was supplemented with increasing concentrations of TSA (Fig. 2C and D). Additionally, time course studies showed that the effect of TSA was long-lasting (Supplementary Fig. S1).

Western blotting was performed to determine the histone acetylation levels in TSA-treated samples and an untreated control using antibodies against acetylated histone H3 (ac-H3) and ac-H4. The results showed that the total acetylation level of ac-H3 increased in the TSA-treated samples compared with that of the untreated samples (Fig. 2E), and that the ac-H4 level of the TSA-treated samples increased dramatically (Fig. 2F). These results indicate that the changes in morphology and AFB1 production might have resulted from changes in the levels of histone acetylation after TSA treatment in *A. flavus*.

HosA is required for fungal vegetative growth and asexual development.

To further characterize the function of HDACs in *A. flavus*, we attempted to generate five single classical HDAC gene deletion mutants using a homologous recombination strategy, as described previously Supplementary Fig. S2 (Chang et al. 2010; Lan et al. 2016). Four mutants were constructed successfully, and we named them $\Delta rpd3$, $\Delta hosA$, $\Delta hdaA$, and $\Delta hosB$. However, despite many attempts, we failed to delete the *rpdA* gene in *A. flavus*. However, it was reported previously that *rpdA* is essential for growth in *A. nidulans* (Tribus et al. 2010), *A. oryzae* (Kawauchi et al. 2013), and *A. fumigatus* (Bauer et al. 2016), suggesting that *rpdA* may also be essential for growth in *A. flavus* and, thus, cannot be deleted. Selective mutants were confirmed by diagnostic PCR and Southern blot. Next, reverse-transcription PCR (RT-PCR) was used to examine gene expression in each mutant. We first tested the growth of these mutant strains, and the $\Delta rpd3$, $\Delta hdaA$, and $\Delta hosB$ mutants showed similar phenotypes to a wild-type (WT) strain. In contrast the $\Delta hosA$ mutant strain displayed a defect in development. Therefore, we generated the complementation strain $\Delta hosA.C$ to confirm that the growth defect was the result of deleting the *hosA* gene. A growth assay showed that the $\Delta hosA$ strain displayed a fluffy phenotype on potato dextrose agar (PDA), complete medium (CM), and glucose minimum medium (GMM), and the $\Delta hosA$ strain grew more slowly than the WT and $\Delta hosA.C$ strains (Fig. 3A and B). In addition, the $\Delta hosA$ strain appeared to produce more pigments in GMM (Fig. 3A). Furthermore, microscopic examination revealed that only the $\Delta hosA$ mutant displayed sparse mycelia and generated fewer branches compared with the WT and $\Delta hosA.C$ strains (Fig. 3C).

Next, we analyzed the roles of HDACs in asexual production. There were no differences in conidial production among the WT, $\Delta rpd3$, $\Delta hdaA$, and $\Delta hosB$ strains, whereas a significant decrease (>2.8-fold, $P < 0.01$) was observed in the $\Delta hosA$ strain (Fig. 3D). No differences in the conidiophores were observed among the WT, $\Delta rpd3$, $\Delta hdaA$, and $\Delta hosB$ strains by microscopic examination (Fig. 3E) but the conidiophores of the $\Delta hosA$ strain were sparser and exhibited smaller conidial heads compared with those of the WT and $\Delta hosA.C$ strains (Fig. 3E). To confirm these findings, two key regulatory genes, *brlA* and

abaA, which are required for the activation of conidiation, were examined for gene expression (Barrales et al. 2012). Transcription levels of *brlA* and *abaA* were both downregulated significantly in the Δ *hosA* mutant relative to those of the WT and Δ *hosA.C* strains (Fig. 3F), suggesting that HosA is required for conidia formation upstream of *brlA* and *abaA*. All of these results indicate that, of all the HDACs, only the class I HDAC

HosA is required for growth and asexual development in *A. flavus*.

HosA and HdaA play opposite roles in sclerotial formation.

Sclerotia are alternative reproductive and survival structures that are used to adapt to unsuitable environmental conditions

Fig. 1. Phylogenetic trees, protein domains, and expression profiles of classical histone deacetylases RpdA, Rpd3, HosA, HdaA, and HosB in *Aspergillus flavus*. **A**, Phylogenetic analysis of putative classical histone deacetylase (HDAC) proteins in different fungi, *Homo sapiens*, *Mus musculus*, and *Arabidopsis thaliana*. The five classical HDACs from *Aspergillus flavus* are marked with a circle. **B**, Domain structure analysis of HDAC in different species. Different domains are indicated by different shades. **C**, Expression patterns of classical HDAC genes of *A. flavus* were tested by quantitative real-time PCR at four different stages, including vegetative growth (VG), conidial development (CON), aflatoxin synthesis (AS), and sclerotial development (SD). Results from each condition were presented with β -actin as reference gene and calibrated against the transcript level in VG.

and, when two *A. flavus* strains of opposite mating type are present, the sexual spores (ascospores) will form in the sclerotium (Horn et al. 2009). To investigate the roles of the HDAC genes in sclerotia formation, all of the mutant and WT strains of *A. flavus* were incubated in sclerotia-inducing Wickerham (WKM) media in the dark at 37°C for 7 days. The results revealed that sclerotia production in the $\Delta hdaA$ mutant strain decreased significantly to 10% ($P < 0.01$) of the level of the WT (Fig. 4A and B), whereas the complemented $\Delta hdaA.C$ strain restored the defect (data not shown). In contrast, the $\Delta hosA$ mutant produced abundant sclerotia (>2.2 -fold increase, $P < 0.01$) compared with the WT and $\Delta hosA.C$ strains (Fig. 4A and B). However, there were no differences in sclerotia production among the $\Delta rpd3$, $\Delta hosB$, and WT strains (Fig. 4A and B). Similar results were obtained when we used sclerotia-inducing yeast-peptone-dextrose (YPD) agar medium (Fig. 4A and B). To further confirm these findings of the altered production of sclerotia in the $\Delta hdaA$ and $\Delta hosA$ strains, we performed qRT-PCR to determine the expression levels of *nsdC*

(Cary et al. 2012) and *sclR* (Jin et al. 2011), which encode transcription factors that are involved in sclerotium formation. Our results revealed that *nsdC* expression was downregulated (>2.5 -fold, $P < 0.05$) in the $\Delta hdaA$ strain but upregulated (>2 -fold, $P < 0.05$) in the $\Delta hosA$ strain (Fig. 4C), which is consistent with the reduced sclerotia production in the $\Delta hdaA$ mutant strain and the increased sclerotia formation in the $\Delta hosA$ strain. Furthermore, *sclR* gene expression decreased substantially (>7.1 -fold, $P < 0.05$) in the $\Delta hdaA$ strain, whereas it increased significantly (>2.3 -fold, $P < 0.05$) in the $\Delta hosA$ strain (Fig. 4C). All of these results indicate that the class II family HDAC gene *hdaA* is required for normal levels of sclerotium formation and that the class I family HDAC gene *hosA* functions as a repressor during sclerotium formation.

HosA negatively regulates oxidative stress responses through mediation of reactive oxygen species.

To characterize the roles of classical HDACs in environmental stress responses the sensitivity of the WT, $\Delta rpd3$,

Fig. 2. Morphology and aflatoxin B₁ (AFB₁) production of *Aspergillus flavus* when treated with trichostatin A (TSA). **A**, Colony morphology after 3 and 5 days of incubation with different concentrations of TSA on yeast extract-sucrose media. UT indicates untreated samples, and dimethyl sulfoxide (DMSO) was used as a control to exclude the possibility of solvent. T1 μM, T5 μM, T10 μM, and T20 μM indicate different concentrations of TSA (1, 5, 10, and 20 μM, respectively). **B**, Colony diameter was measured from 24 to 120 h as in A. **C**, Thin-layer chromatography indicated that the increasing concentration of TSA leads to decreased AFB₁ production. **D**, Quantification of AFB₁ in C by densitometric analyses. **E**, TSA-treated samples showed an increased level of total H3 acetylation as measured by acetylated histone H3 (ac-H3) antibody, while histone H3 was used as loading control. **F**, TSA-treated samples showed an increased level of total H4 acetylation as measured by ac-H4 antibody, while histone H4 was used as loading control.

$\Delta hdaA$, $\Delta hosB$, $\Delta hosA$, and $\Delta hosA.C$ strains to oxidative stress, hyperosmotic stress, cell-wall-damaging stress, and genome stability stress were examined. The results showed that the $\Delta rpd3$, $\Delta hdaA$, and $\Delta hosB$ strains did not respond to exogenous oxidative, osmotic, cell wall, and genotoxic stresses (Supplementary Fig. S3). However, despite the fact that the $\Delta hosA$ mutant did not show altered sensitivities to exogenous osmotic, cell wall, and genotoxic stresses, it exhibited increased

tolerance to oxidative stress generated by hydrogen peroxide (H_2O_2) compared with the WT and $\Delta hosA.C$ strains (Fig. 5A and B). Therefore, we tested oxidative stress generated by tert-butyl hydroperoxide. Similarly, decreased sensitivity of the $\Delta hosA$ mutant was observed compared with the WT and $\Delta hosA.C$ strains (Fig. 5A and B), suggesting that HosA plays negative roles in the oxidative stress response. Because the $\Delta hosA$ strain exhibited greater tolerance in the presence of

Fig. 3. HosA is involved in fungal vegetative growth and asexual development. **A**, Colony morphology of wild-type (WT), $\Delta rpd3$, $\Delta hdaA$ and $\Delta hosB$, $\Delta hosA$, and $\Delta hosA.C$ strains cultured on potato dextrose agar (PDA), complete medium (CM), and glucose minimum medium (GMM) plates at 37°C for 7 days. **B**, Growth assays of WT, $\Delta rpd3$, $\Delta hdaA$ and $\Delta hosB$, $\Delta hosA$, and $\Delta hosA.C$ strains. **C**, Hyphal tip growth and branching patterns of WT, $\Delta rpd3$, $\Delta hdaA$ and $\Delta hosB$, $\Delta hosA$, and $\Delta hosA.C$ strains. Bars = 20 μ m. **D**, Conidial amounts of WT, $\Delta rpd3$, $\Delta hdaA$ and $\Delta hosB$, $\Delta hosA$, and $\Delta hosA.C$ strains. Conidia were extracted from each plate, completely suspended into the 5 ml of sterilized water, and counted by a microscope. **E**, Microscopic observation of asexual development. Conidiophores was observed after constant light induction for 12 h. Bars = 20 μ m. **F**, Quantitative real-time PCR results showed that two regulatory genes for conidiation were downregulated in $\Delta hosA$. β -actin was used as a reference. Asterisks (**) represent a significant difference level of $P < 0.01$.

drugs that generate free radicals, we wondered whether intracellular reactive oxygen species (ROS) were altered within $\Delta hosA$ cells. To address this question, mycelia of the WT, $\Delta hosA$, and $\Delta hosA.C$ strains were stained with dihydroethidium (DHE), a redox-sensitive probe that has been used widely for detecting intracellular ROS (Luo et al. 2002). The fluorescence intensity of the hyphae of the $\Delta hosA$ strain decreased compared with that of the WT and $\Delta hosA.C$ strains, indicating that knocking out the *hosA* gene decreased the ROS level in fungal cells (Fig. 5C). All of these results suggest that HosA acts as a repressor in response to oxidative stress.

HosA and HdaA are involved in aflatoxin biosynthesis.

In addition to fungal development, previous studies have demonstrated the importance of HDACs in regulating SM production in fungi (Albright et al. 2015; Shwab et al. 2007). However, the functions of HDACs in aflatoxin biosynthesis are poorly understood. After growth in YES liquid medium at 28°C for 72 h, aflatoxins from the WT, $\Delta rpd3$, $\Delta hdaA$, $\Delta hosB$, $\Delta hosA$, and $\Delta hosA.C$ strains were extracted and examined by thin-layer chromatography (TLC). The TLC results showed that the production of AFB1 by the $\Delta hdaA$ strain was lower than that of the WT strain (Fig. 6A). Furthermore, the $\Delta hosA$ strain produced little detectable AFB1 compared with the WT and $\Delta hosA.C$ strains, whereas deleting the other HDAC genes had

only minor effects on AFB1 biosynthesis (Fig. 6A). High-performance liquid chromatography (HPLC) was performed to determine the AFB1 yield, and the results showed that AFB1 production by the $\Delta hdaA$ strain was almost 50% lower than that of the WT strain (Fig. 6B and C). In addition, AFB2 production decreased considerably (>90%, $P < 0.01$) in the $\Delta hosA$ strain (Fig. 6B and C) compared with the WT and $\Delta hosA.C$ strains. However, the $\Delta rpd3$ and $\Delta hosB$ strains did not exhibit altered AFB1 yields relative to the WT strain (Fig. 6B and C).

Consistently, the qRT-PCR data showed that the transcript levels of the AFB1 cluster genes *aflD*, *aflM*, and *aflP* all decreased drastically in the $\Delta hosA$ and $\Delta hdaA$ strains compared with those in the WT strain (Fig. 6D). The transcript level of the regulatory gene *aflR* was also reduced significantly in the $\Delta hdaA$ strain. However, the *aflR* transcript level did not obviously change in the $\Delta hosA$ strain (Fig. 6D), which indicates that HosA affects AFB1 biosynthesis independently of *aflR*. These results indicate that HdaA and HosA play critical roles in regulating the transcription of AFB1 biosynthesis genes in *A. flavus*.

Because the $\Delta hosA$ strain generated more pigments (Fig. 3A; Supplementary Fig. S4A) and little AFB1 (Fig. 6B), we determined whether the production of other SMs was altered in the $\Delta hosA$ strain. Using a broad-spectrum ultraviolet light detector, the HPLC profiles showed that the $\Delta hosA$ strain

Fig. 4. Effects of classical histone deacetylases on sclerotia formation in *Aspergillus flavus*. **A**, Wild-type (WT), $\Delta rpd3$, $\Delta hdaA$ and $\Delta hosB$, $\Delta hosA$, and $\Delta hosA.C$ strains were cultured on the Wickerham (WKM) and yeast-peptone-dextrose (YPD) media at 37°C for 7 days, and the images were taken after the colony was washed with 75% ethanol to expose the sclerotia. **B**, Sclerotia were weighed after culturing for 7 days. **C**, Expression levels of *nsdC* and *sclR* genes among WT, $\Delta hdaA$, and $\Delta hosA$ strains. The β -actin was used as a reference. Asterisks ** and * represent significant difference levels of $P < 0.01$ and 0.05, respectively.

produced increased amounts of two unidentified products at peaks with 20.32- and 23.68-min retention times, as well as decreased amounts of two unknown products at peaks with 3.25- and 28.75-min retention times, suggesting that *HosA* is important for regulating the production of other SMs.

Rpd3, HdaA, and HosB do not act redundantly with *HosA* in development and aflatoxin biosynthesis.

It was reported that HDACs have redundant roles in several organisms (Haberland et al. 2010; Robbins et al. 2012) although this did not appear to be the case in *A. nidulans* with regard secondary metabolism (Shwab et al. 2007). To partially address whether there is a redundant function of *A. flavus* HDACs, we constructed double mutants in the $\Delta hosA$ background. Examination of the double mutants $\Delta rpd3/\Delta hosA$, $\Delta hdaA/\Delta hosA$, and $\Delta hosB/\Delta hosA$ revealed that neither the asexual development conidial formation, sclerotia production, nor aflatoxin biosynthesis were significantly affected (Supplementary Fig. S5). This indicates that the *A. flavus* Rpd3, HdaA, and HosB do not act redundantly with *HosA* in these processes.

HosA is required for plant infection.

In infection assays, two different susceptible crops (peanut seed and maize kernels) were infected with the WT, $\Delta rpd3$, $\Delta hdaA$, $\Delta hosB$, $\Delta hosA$, or $\Delta hosA.C$ strains. After 5 days of infection, macroscopically, the $\Delta rpd3$, $\Delta hdaA$, and $\Delta hosB$

mutants caused almost the same severe symptoms as the WT strain (Supplementary Fig. S6). In contrast to the full colonization of the entire surface of the host seed by the WT and $\Delta hosA.C$ strains, the $\Delta hosA$ strain appeared to produce less vigorous mycelia, which crippled its ability to infect and sporulate on host seed (Fig. 7A and B). The results were also reflected in the conidia production from the infected peanut and maize seed, because very few conidia were generated by the $\Delta hosA$ strain on both peanut seed and maize kernels compared with the WT and $\Delta hosA.C$ strains (Fig. 7C). However, there were no significant differences in the conidial amounts among the WT, $\Delta rpd3$, $\Delta hdaA$, and $\Delta hosB$ strains, indicating that the $\Delta hosA$ strain is defective in plant infection or colonization. Aflatoxins in colonized seed were extracted and assayed subsequently by TLC. The $\Delta hosA$ strain showed strikingly reduced AFB1 production in both peanut and maize seed (Fig. 7D), and moderately decreased AFB1 production was observed in the $\Delta hdaA$ strain compared with the WT strain. In contrast, AFB1 production was not altered in the $\Delta rpd3$ and $\Delta hosB$ mutants. These results indicate that *HosA* of *A. flavus* plays an important role in seed colonization.

Subcellular localization of *HosA* and *HdaA*.

Thus far, we observed that only the class I HDAC *HosA* and the class II HDAC *HdaA* played roles in fungal development and SM production. To determine the intracellular localization of *HosA* and *HdaA*, we generated *HosA*-mCherry and

Fig. 5. Roles of *HosA* in response to oxidative stresses. **A**, Wild-type (WT), $\Delta hosA$, and $\Delta hosA.C$ strains were incubated on potato dextrose agar plates supplemented with oxidative-stress-inducing molecules. Strains grown on media supplemented with H₂O₂ (10 and 20 mM) or tert-butyl hydroperoxide (tBOOH) (0.5 and 1 mM) at 37°C for 3 days. **B**, Inhibition of growth rate of strains in media supplemented with H₂O₂ or tBOOH. **C**, Reactive oxygen species within hyphae of WT, $\Delta hosA$, and $\Delta hosA.C$ strains were stained by dihydroethidium (DHE) and visualized as red fluorescence using fluorescence microscopy (DIC). Bars = 100 μ m.

HdaA-mCherry strains, as described previously (Lan et al. 2016). For each gene, at least two mCherry fusion transformants were obtained. The HosA-mCherry and HdaA-mCherry strains showed a WT phenotype, and they exhibited normal growth and conidia production (data not shown). We selected one of the mCherry fusion transformants from each gene for subsequent analyses. However, only a very weak mCherry fluorescence signal was observed in the HosA-mCherry strains (data not shown). Thus, we generated a HosA-mCherry strain using the strong promoter *gpdA* from *A. nidulans*. During vegetative mycelial growth stages, mCherry fluorescence mainly exhibited a punctate appearance, pointing to a likely nuclear localization of the HosA protein (Supplementary Fig. S7). Staining with the nuclear marker 4',6-diamidino-2-phenylindole (DAPI) indicated that the fluorescence of HosA-mCherry overlapped with the DAPI-labeled nuclei. Detailed three-dimensional fluorescence images showed that HosA-mCherry localized mainly in the nuclei but also in the cytoplasm. Additionally, HdaA-mCherry fluorescence was found not only in the nuclei but also in the cytoplasm.

A. flavus HosA and HdaA are indeed histone deacetylases.

It has been reported that lysine 16 of histone H4 (H4K16) is the canonical target of Hos2 in yeast (Wang et al. 2002) and that H3K9 is the target of Hda1 (Vogelauer et al. 2000). Considering the histone deacetylation roles of HosA and HdaA, Western blotting was performed to determine the histone acetylation levels across the genome. Compared with the WT strain, the Δ *hosA* strain exhibited higher acetylation levels of H4 and, particularly, H4K16 (Supplementary Fig. S8), indicating that

H4K16 is the deacetylated target of HosA in *A. flavus*. Additionally, both the H3 and H3K9 acetylation levels in the Δ *hdaA* strain were higher than those in the WT strain, showing that H3K9 might be the deacetylated target of HdaA in *A. flavus*. All of these results revealed that HosA and HdaA function as histone deacetylases *in vivo* and are involved in regulating the acetylation levels of histones H3 and H4 in *A. flavus*.

HosA from *A. flavus* is a deacetylase that is sensitive to TSA *in vitro*.

Because the Δ *hosA* strain exhibited the most severe phenotypes, we further characterized HosA. *A. flavus* HosA is similar to *S. cerevisiae* Hos2 (a HosA homolog) (NP_011321.1; 58% identity) and HosA of *A. nidulans* (AAF80490; 79% identity). Compared with the three-dimensional protein structures among *S. cerevisiae*, *A. nidulans*, and *A. flavus*, HosA is highly conserved in fungi (Fig. 8A). To determine the HDAC activity of HosA, we cloned and sequenced the *hosA* coding sequence from *A. flavus* cDNA, and sequencing results revealed that the *hosA* gene contains a 1,461-bp open reading frame (ORF). Then, a pET28a-*hosA* plasmid was constructed and transformed into *Escherichia coli* AD494 (DE3). After the protein was successfully expressed, the recombinant HosA protein was purified. A sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed an obviously visible protein of approximately 54 kDa in the lane containing the recombinant HosA protein (Fig. 8B). To determine the HDAC activity of HosA, an HDAC activity kit was used to assay the purified, recombinant HosA protein. Compared with the HDAC activity of the positive control (HeLa cell nuclear extracts,

Fig. 6. Effects of histone deacetylase gene in aflatoxin biosynthesis. **A**, Thin-layer chromatography plates showed aflatoxin production extracted from wild-type (WT), Δ *rpd3*, Δ *hdaA*, Δ *hosB*, Δ *hosA*, and Δ *hosA.C* strains in yeast extract-sucrose liquid media for 3 days. **B**, High-performance liquid chromatography profiles showed aflatoxins production of WT, Δ *rpd3*, Δ *hdaA*, Δ *hosB*, Δ *hosA*, and Δ *hosA.C* strains. **C**, Yield of aflatoxin B₁ (AFB1) production among WT, Δ *rpd3*, Δ *hdaA*, Δ *hosB*, Δ *hosA*, and Δ *hosA.C*. **D**, Quantitative real-time PCR results indicated transcripts level of the aflatoxin cluster genes. The β -actin was used as a reference. Asterisks ** and * represent significant difference levels of $P < 0.01$ and 0.05 , respectively; ns = no difference.

539.2 ± 8.7 nM/min/ml), the HDAC activity of HosA was lower (172.0 ± 5.6 nM/min/ml) but it was much higher than that of the negative control (Fig. 8C). Previous studies revealed that TSA selectively inhibits classic HDACs of the Rpd3 and Hda1 families (Finnin et al. 1999). To test the sensitivity of HosA to TSA, TSA was added to the recombinant HosA protein. The results showed that the HDAC activity of HosA was diminished (>30%) by the addition of 1 nM TSA, whereas it was decreased considerably (>50%) when the TSA concentration was increased to 5 nM. However, the HDAC activity did not change significantly when the control dimethyl sulfoxide was added (Fig. 8D). All of these results show that HosA is an *A. flavus* HDAC that is sensitive to TSA in vitro.

HosA is required for cAMP signaling-induced AFB1 biosynthesis.

Aflatoxin biosynthesis is also controlled by the cAMP-PKA signaling pathway (Roze et al. 2004a,b). Previous studies reported that homologs of the yeast Set3/Hos2 histone deacetylase complex attenuate the cAMP signaling pathway to regulate morphogenesis in *C. albicans* (Hnisz et al. 2010) and, in *U. maydis*, *hos2* is required for the cAMP-induced expression of mating-type genes (Elías-Villalobos et al. 2015). Therefore, we wanted to determine whether HosA might control the expression of aflatoxin biosynthesis genes in response to activation of the cAMP pathway. To do so, we assayed AFB1 production in the WT and Δ *hosA* strains grown in YES liquid medium, with or without the addition of exogenous cAMP in the WT. The addition of exogenous cAMP drastically increased AFB1 production (Fig. 9A); however, there was no such

increase in the Δ *hosA* strain. Furthermore, the addition of cAMP significantly induced the expression of the AFB1 biosynthesis genes *aflD*, *aflM*, and *aflP* in the WT strain whereas, even in the presence of cAMP, the expression of these genes was still markedly reduced in the Δ *hosA* mutant (Fig. 9B), suggesting a requirement for HosA processes in cAMP signaling.

HosA binds to AFB1 biosynthesis genes to control their expression.

In many fungi, a homolog of Hos2 is recruited to gene sequences in a transcription-dependent manner (Elías-Villalobos et al. 2015; Hnisz et al. 2012; Wang et al. 2002). We observed that deleting the *hosA* gene significantly reduced the expression of the aforementioned AFB1 biosynthesis genes (Fig. 6D). To investigate whether HosA directly affects AFB1 biosynthesis gene expression, we performed chromatin immunoprecipitation (ChIP) assays, followed by qRT-PCR. We generated the strains HosA-HA3, in which the HosA protein is tagged with three copies of the hemagglutinin (HA) epitope and introduced into a Δ *hosA* deletion strain. These strains had a WT phenotype (Fig. 10A), and Western blotting verified the expression of HosA-HA, indicating that the HosA-3HA allele was functional (Fig. 10B). Next, we determined whether HosA bound directly to the AFB1 biosynthesis genes. Compared with the HA ChIP signal obtained in untagged strains, there was a significant enrichment of HosA-3HA within the *aflD*, *aflM*, and *aflP* ORFs; however, *aflR* binding was unaffected compared with the untagged controls (Fig. 10C). As a negative control, the binding of HosA to the housekeeping β -actin gene

Fig. 7. Pathogenicity analysis of wild-type (WT), Δ *hosA*, and Δ *hosA.C* strains. Morphology of *Aspergillus flavus* on **A**, peanut cotyledons and **B**, maize kernels after 5 days of inoculation. Mock represents seed inoculated with sterile water as control. **C**, Conidia production from seed. **D**, Thin-layer chromatography measurement of aflatoxin B₁ (AFB1) extracted from the seed. Asterisks (**) represent a significant difference level of $P < 0.01$.

did not change significantly compared with the untagged control (Fig. 10C). Therefore, our ChIP experiments revealed that HosA binds directly to AFB1 biosynthesis genes, where it most likely acts to regulate their expression.

HosA associates with SinA to control fungal development and aflatoxin biosynthesis.

Transcription factor Sin3 interacts with HDACs and forms a complex to regulate many cellular processes (Barnes et al. 2014; Hassig et al. 1997; Laherty et al. 1997). By blasting with yeast Sin3, we found a Sin3 homolog (AFLA_078390) in *A. flavus* and designated it SinA. We showed that *A. flavus* HosA could interact with SinA through yeast two-hybrid assays

(Fig. 11A). To investigate whether SinA plays roles in *A. flavus* development and aflatoxins biosynthesis, we generated $\Delta sinA$ deletion mutants and overexpressed strains *OE::sinA*. The results showed that $\Delta sinA$ mutants showed a severe defect in growth, conidiation, and AFB biosynthesis (Fig. 11B to E), whereas *OE::sinA* strains significantly increased conidial formation and AFB production (Fig. 11B to E).

Considering the yeast literature (Grzenda et al. 2009; Laherty et al. 1997), we hypothesized that SinA recruited HDAC enzymes to chromatin, including the region of the aflatoxin gene cluster. To assess this hypothesis, an *OE::sinA*/ $\Delta hosA$ strain was constructed where *sinA* was overexpressed in combination with a *hosA* gene deletion. The results showed that the

Fig. 8. Histone deacetylase (HDAC) activity analysis of *Aspergillus flavus* HosA in vitro. **A**, Computer generated three-dimensional structure of *Saccharomyces cerevisiae*, *A. nidulans*, and *A. flavus* HosA orthologous proteins were obtained using CPH model 3.2 Server and PyMol-viewer. **B**, Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the expressed and purified recombinant HosA protein. Lane 1 = maker, lane 2 = total protein without isopropyl- β -D-thiogalactoside (IPTG) induced, lane 3 = total protein of IPTG induced, lane 4 = supernatant of IPTG induced, and lane 5 = purified recombinant HosA protein. **C**, HDAC activities of recombinant HosA protein. PC = positive control (HeLa Nuclear Extracts) and NC = negative control. **D**, HosA protein was sensitive to different concentration of trichostatin A (TSA) in vitro. DMSO = dimethyl sulfoxide. Asterisks ** and * represent significant difference levels of $P < 0.01$ and 0.05, respectively; ns = no difference.

AFB1 synthesis was restored in *OE::sinA/ΔhosA* strains compared with that of in *ΔhosA* strains (Fig. 11D and E), and it also revealed that *OE::sinA/ΔhosA* strains restored partial conidial formation relative to *ΔhosA* strains (Fig. 11B and C). As a consequence, the expression level of *aflD*, *aflM*, and *aflP* in *OE::sinA/ΔhosA* strains were upregulated when compared with *ΔhosA* strains (Fig. 11F). Taken together, these results suggested HosA associates with SinA to control fungal development and aflatoxin biosynthesis.

DISCUSSION

Growing evidence has shown that HDACs play crucial roles in controlling fundamental processes such as growth, differentiation, development, and metabolism in fungi (Baidyaroy et al. 2001; Shwab et al. 2007; Tribus et al. 2005, 2010). However, studies of HDACs in *Aspergillus* spp. are limited, and no previous studies have reported the functions of HDACs in *A. flavus*. Our aim was to ascertain the functions of classical HDACs in *A. flavus*. Among all five classical HDACs in

A. flavus, we showed that only HosA played significant roles in growth, conidiation, sclerotia formation, AFB1 production, and virulence. To a lesser extent, HdaA played roles in sclerotia formation and AFB1 production. Moreover, HosA was required for cAMP signaling-induced AFB1 biosynthesis. Importantly, we confirmed that HosA controlled AFB1 biosynthesis by binding directly to the AFB1 gene sequences. Finally, by integrating the master transcriptional scaffold regulator Sin3, we found that HosA associates with SinA in the regulation of fungal development and aflatoxin biosynthesis and, unexpectedly, overexpression of SinC could partially remediate the loss of aflatoxin synthesis in a *hosA* deletion strain, possibly due to recruitment of other HDACs.

Homologs of HDACs are evolutionarily conserved among fungi. *S. cerevisiae* contains five classical HDAC genes, including *hda1*, *hos1*, *hos2*, *hos3*, and *rdp3* (Robyr et al. 2002). Four *A. nidulans* HDAC genes have been reported: *hdaA*, *rdpA*, *hosA*, and *hosB* (Shwab et al. 2007; Tribus et al. 2005; Trojer et al. 2003). Most recently, *A. nidulans* HosA was reported to be very important for morphology and secondary metabolisms

Fig. 9. HosA is required for cAMP signaling induced aflatoxins biosynthesis. **A**, Measurement of aflatoxin production by thin-layer chromatography analyses between wild-type (WT) and HosA strains with or without addition of exogenous 5 mM cAMP. Strains were grown in yeast extract-sucrose liquid media for 3 days. Std = standard. **B**, Quantification of aflatoxin B₁ (AFB1) in **A** by densitometric analyses. **C**, Expression level of the aflatoxin genes between WT and Δ hosA strains with or without addition of exogenous 5 mM cAMP. The β -actin was used as a reference. Asterisks ** and * represent significant difference levels of $P < 0.01$ and 0.05 , respectively; ns = no difference.

(Pidroni et al. 2018). Here, five classical HDAC genes were identified in *A. flavus*, including three class I HDAC genes (*rpda*, *rp3* [*rpda*-like], and *hosA*) and two class II genes (*hdaA* and *hosB*) (Fig. 1A), similar to what has been reported previously in *A. oryzae* (Kawauchi et al. 2013). Previously, *rpda* was reported to be an essential HDAC gene in *A. nidulans* (Tribus et al. 2010), *A. oryzae* (Kawauchi et al. 2013), and *A. fumigatus* (Bauer et al. 2016), which is consistent with *rpda* possibly being essential for survival in *A. flavus*. Although we successfully deleted another class I HDAC Rpd3-type gene, *rp3*, we did not find any evidence that *rp3* plays a role in development and SM production. Surprisingly, only the class I HDAC *HosA* played crucial roles in fungal development, aflatoxin biosynthesis, and pathogenicity. Compared with *HosA*, the other class II HDAC genes (*hdaA* or *hosB*) appeared to be less important in *A. flavus*. The $\Delta hdaA$ and $\Delta hosB$ strains exhibited normal growth, and *HdaA* was required for sclerotium formation and AFB1 synthesis. In addition, examination of the double mutants $\Delta rp3/\Delta hosA$, $\Delta hdaA/\Delta hosA$, and $\Delta hosB/\Delta hosA$ also indicated that neither conidial formation, sclerotia production, nor aflatoxin biosynthesis were significantly affected when compared with single a $\Delta hosA$ mutant. *A. nidulans hdaA* was initially reported to be the major HDAC gene in this species (Tribus et al. 2005) until *rpda* was

characterized (Tribus et al. 2010). Without considering the essential roles of *RpdA*, in this study, we propose that *HosA* is the primary HDAC in *Aspergillus* spp.

Previous studies illustrated that treatment with HDAC inhibitors such as TSA and suberoylanilide hydroxamic acid (SAHA) increased the production of various SMs, and this may be a strategy for activating SMs in fungi (Albright et al. 2015; Williams et al. 2008). This is easy to understand because HDACs often function as transcriptional corepressors (de Ruijter et al. 2003). Inhibiting HDACs increases gene expression and can promote cryptic SM production (Albright et al. 2015; Henke et al. 2016). However, it is now accepted that HDACs also act as activating complexes and research has revealed that TSA treatment decreased gibberellin biosynthesis in *F. fujikuroi* (Studt et al. 2013), SAHA treatment of *A. flavus* led to decreased AFB1 synthesis (Basimia et al. 2013), and *rpda* repression resulted in decreased sterigmatocystin production (Albright et al. 2015; Henke et al. 2016). Similarly, in the present study, AFB1 production decreased significantly with increasing TSA concentrations (Fig. 2C and D). Here, TSA treatment caused the hyperacetylation of histones H3 and H4, indicating that they are the main substrates of HDAC in this fungus (Fig. 2E and F). One explanation for the TSA-mediated decreases in growth and AFB1 production is that TSA inhibits

Fig. 10. *HosA* directly binds to aflatoxin biosynthesis genes. **A**, Phenotype of wild-type (WT) and *HosA*-HA3 strains. *HosA*-HA3-5 and *HosA*-HA3-7 were two transformants of *HosA*-hemagglutinin (HA) strains, grown in potato dextrose agar for 7 days. **B**, Western blotting results showed the expression of *HosA*, while β -actin protein was used for control. **C**, Chromatin immunoprecipitation (IP) analysis by an anti-HA antibody on chromatin extracts from either an untagged strain or a *HosA*-HA3 strains. Immunoprecipitated DNA was analyzed by quantitative real-time PCR. Amplifying specific regions within the open reading frames (5' or 3') indicated below the graph. Values correspond to the amount of DNA recovered in the HA IP divided by the amount of DNA in the corresponding input extract. Asterisks ** and * represent significant difference levels of $P < 0.01$ and 0.05 , respectively; ns = no difference.

Fig. 11. HosA associates with SinA to regulate development and aflatoxin B (AFB) synthesis in *Aspergillus flavus*. **A**, Yeast two-hybrid assay with SinA as the bait and HosA as the prey. Yeast transformants diluted to specified concentrations (cells per milliliter) were plated onto SD-Ade-Leu-Trp-His addition of 40 mM 5-bromo-4-chloro-3-indoxyl- α -D-galactopyranoside media to examine the HIS3 reporter gene expression and β -galactosidase activity. The interaction of BD-SinA and PGADT7 was used as the negative control to rule out self-activation. The interaction of pGBKT7-P53 and pGADT7-T was used as the positive control. Plasmid pairs pGBKT7-Lam/pGADT7-T and pGADT7/pGBKT7 served as the negative control. **B**, Analysis of conidia production by each strain. WT = wild type. **C**, Each strain was grown on potato dextrose agar (PDA) plates for 4 days and then photographed. **D**, Analysis of aflatoxin B₁ (AFB₁) production by each strain. **E**, Quantitative analysis of AFB₁ production by each strain. **F**, Gene expression analysis of aflatoxin biosynthesis related genes by each strain. Asterisks ** and * represent significant difference levels of $P < 0.01$ and 0.05 , respectively; ns = no difference.

HDACs in vivo, which mimics the deletion of classical HDACs genes. These results are consistent with the deletion results in this study, because the Δ *hosA* strain exhibited significantly decreased growth and AFB1 production, which phenocopied the treatment with TSA. We also recognize that TSA inhibits RpdA function in *A. nidulans* (Bauer et al. 2016) and speculate that the ortholog in *A. flavus* also contributes to aflatoxin production.

Classical HDAC genes are involved in morphogenesis in fungi. Among the *A. flavus* HDAC genes, our results showed that *HosA* is the only classical HDAC gene that is required for growth, and similar results were obtained during studies of *HosA* homologs such as *A. oryzae hdaD* (Kawauchi et al. 2013), *M. oryzae hos2* (Ding et al. 2010), and *F. graminearum hdf1* (Li et al. 2011). Previously, it was shown that *A. nidulans* *HdaA* affects growth under oxidative stress (Tribus et al. 2005), and that *A. fumigatus* *HdaA* is required for normal germination and vegetative growth (Lee et al. 2009). To our surprise, we did not observe similar growth effects in the Δ *hdaA* strain under oxidative stress conditions. In contrast, significantly increased growth was found in the Δ *hosA* strain, and this was attributed to decreased ROS in fungal cells and reduced expression of catalase and peroxidase genes (Fig. 5D).

Reproduction is an important event for fungal life cycles; thus, *A. flavus* produces more conidia in suitable conditions but generates more sclerotia in a harsh environments (Horowitz Brown et al. 2008). Our results showed that the class I HDAC *HosA* increased conidiation (Fig. 3D); however, it repressed the formation of sclerotia (Fig. 4A and B). The class II HDAC *HdaA* was also required for sclerotia formation (Fig. 4A and B), although it was not involved in conidia formation (Fig. 3D), whereas the *rpd3* and *hosB* genes were not involved in reproduction. These observations on sclerotial formation suggest a similar role for *HdaA* in *A. flavus* as in *A. nidulans*, where loss of *hdaA* decreased sexual development in that fungus (Palmer et al. 2010). Similar results were found after deleting the *HosA* homolog *hdaD*, which significantly decreased conidia formation in *A. oryzae* (Kawauchi et al. 2013), as well as after deleting *M. oryzae hos2*, which also caused conidiation defects (Ding et al. 2010). In *F. graminearum*, a Δ *hdf1* mutant is defective in sexual reproduction (Li et al. 2011) and an *A. fumigatus* Δ *hdaA* strain did not exhibit altered conidia production (Lee et al. 2009). In *C. albicans*, *CaHos2* and *CaHda1* also play opposite roles in regulating morphological changes (Haberland et al. 2010). Unlike in our study, in *U. maydis*, *Hda1* is the only HDAC that is required for spore formation (Reichmann et al. 2002). These results suggest that classical HDACs regulate growth and reproduction in a species-specific manner to some degree.

Although the roles of HDACs in SM production have been characterized in many fungi, it is unknown why HDACs sometime play negative roles in SM biosynthesis whereas, at other times, they play positive roles. Generally, HATs act as activators whereas, at first, HDACs were thought to act primarily as repressors in regulating SM biosynthesis (Strauss and Reyes-Dominguez 2011; Yu and Keller 2005). This was first shown when the inactivation of *hdaA* upregulated the expression of SM genes and increased the production of several metabolites (sterigmatocystin, penicillin) in *A. nidulans* (Shwab et al. 2007). This broad effect was further confirmed in other fungi (e.g., *Alternaria* and *Penicillium* spp.), indicating a conserved mechanism for SM gene expression by chromatin acetylation (Williams et al. 2008). However, emerging evidence has shown that inactivating HDACs reduces SM production (Henke et al. 2016; Lee et al. 2009; Li et al. 2011; Studt et al. 2013). Here, the addition of the HDAC inhibitor TSA decreased AFB1 production, and single deletions of *hosA* or *hdaA*

exhibited significantly decreased instead of increased AFB1 production (Fig. 6). How can HDACs play negative roles in SM production? In the present study, considering the roles of HDACs in histone deacetylation, Western blotting results showed that the targets of *HosA* and *HdaA* were H4K16 and H3K9, respectively, thereby confirming the roles of HDAC activity in vivo. However, our ChIP experiment results strongly suggest that *HosA* binds directly to AFB1 biosynthesis gene sequences to control their expression (Fig. 10), suggesting that *HosA* is recruited to genes to promote AFB1 cluster gene activation. These findings support a novel mechanism by which HDACs can directly act as activators to regulate gene expression during SM biosynthesis. Our study also demonstrated that, by integrating with *HosA*, the transcriptional scaffold regulator *SinA* is also involved in fungal development and aflatoxin biosynthesis (Fig. 11). However, *HosA* and *SinA* harbor no DNA-binding domain; thus, it is unclear how *HosA* can bind directly to these genes. We propose that *HosA* might act as a member of a functional complex such as the Set3/Hos2 complex (Ding et al. 2010; Hnisz et al. 2010) or together with RNA polymerase II, although further studies are needed to confirm this.

cAMP signal transduction pathways participate in a variety of developmental processes in fungi (Hnisz et al. 2010; Hu et al. 2014; Jiang et al. 2016; Shimizu and Keller 2001). In *Aspergillus* spp., cAMP/PKA signaling pathways control conidiation and aflatoxin biosynthesis (Fillinger et al. 2002; Roze et al. 2004a; Shimizu and Keller 2001). A previous study demonstrated that homologs of the Set3/Hos2 complex are involved in cAMP signaling to control filamentation-induced signals in *C. albicans* (Hnisz et al. 2010). In *U. maydis*, *Hos2* acts as a downstream component of the cAMP-PKA pathway to control mating-type genes (Elías-Villalobos et al. 2015). In the present study, because of the effects of deleting *hosA* on AFB1 production, as well as the association of *HosA* with the cAMP-PKA pathway, we assumed that *HosA* was required for cAMP signaling-induced AFB1 biosynthesis. Indeed, the addition of exogenous cAMP significantly increased AFB1 production (Fig. 9); however, the addition of exogenous cAMP was unable to complement the AFB production defect in the Δ *hosA* strain (Fig. 9). These results confirmed that *A. flavus* *HosA* acts downstream of the cAMP signaling pathway to control the expression of AFB1 biosynthesis genes. One explanation for these observations is that *HosA* directly targets aflatoxin biosynthesis genes in response to cAMP signaling. Because a previous study showed that a cAMP response element (Cre1) mediated aflatoxin biosynthesis (Roze et al. 2004b), the relationship between *HosA* and *Cre1* should be investigated further.

A. flavus spores have the potential to infect crops. Subsequently, they produce aflatoxins in the host, resulting in huge economic losses, and *A. flavus* has become a threat to human health. Although a variety of genetic and environmental cues relevant to fungal virulence have been reported, unfortunately, there is no effective and economical strategy to control these factors (Amaike and Keller 2011; Amare and Keller 2014). Over the past few decades, studies have paid attention to the potential of HDACs as targets to treat various human diseases such as neurodegenerative disorders (Abel and Zukin 2008; Chuang et al. 2009) and cancers (West and Johnstone 2014; Witt et al. 2009). Here, our results demonstrated that deleting *hosA* significantly decreased plant pathogenicity (Fig. 7). Additionally, micromolar concentrations of the HDAC inhibitor TSA significantly inhibited the development and AFB1 biosynthesis of *A. flavus*. Furthermore, the HDAC activity of *HosA* decreased substantially at nanomolar TSA concentrations. These results strongly suggest that HDACs and *HosA* could be

targets for controlling *A. flavus*; an antifungal strategy suggested by other research groups (Bauer et al. 2016; Lamoth et al. 2015).

In conclusion, TSA inhibited HDACs, resulting in a severe effect on development and AFB1 biosynthesis in *A. flavus*. Characterization of the HDACs revealed that deleting the class I HDAC *hosA* gene resulted in a phenotype that was similar to that observed after the addition of TSA, suggesting that HosA might be the major target of TSA in vivo. Importantly, our data demonstrated that HosA acts downstream of the cAMP signaling pathway to control AFB1 biosynthesis, and that it bound directly to AFB1 biosynthesis genes to control their expression. We proposed a model for the roles of HDACs in *A. flavus* and how HosA regulates fungal development and AFB1 biosynthesis (Fig. 12). Furthermore, we believe that these findings increase our understanding of how HDACs promote SM production via a conserved signaling pathway, which might be highly relevant to other fungi.

MATERIALS AND METHODS

Fungal strains and growth conditions.

Strains and plasmid used in this study are listed in Supplementary Table S1. Strains of *A. flavus* generated in this study were cultured on PDA, CM, and GMM agar plates at 37°C, and growth rate on PDA, CM, and GMM and conidiation on PDA medium were assayed as described previously (Lan et al. 2016). For sclerotial reproduction, strains were grown on WKM solid medium as described previously (Lan et al. 2016). For DNA and RNA extraction, mycelia were harvested from PDA, WKM solid, and YES (2% yeast extract, sucrose at 150 g/liter, and MgSO₄·7H₂O at 1 g/liter) liquid cultures (Yang et al. 2016). Protoplast preparation and fungal transformations were performed as described before (Cary et al. 2012).

To test fungal growth under various stress conditions, conidial suspensions (10⁶ conidia) of different strains were inoculated onto a Petri plate that contained solid PDA medium to which the following compounds were added: the HDAC inhibitor TSA (Cayman Chemical, Ann Arbor, MI, U.S.A.), the hyperosmotic stress mediators NaCl, the cell-wall-damaging agent Congo red, and the genome stability damaging agent

methyl methanesulfonate (at various concentrations, as indicated in the figures). The plates were cultured in dark at 37°C for 3 days. Each strain was grown on five plates, and each experiment was repeated at least three times.

HDAC sequence resources, phylogenetic and domain analysis.

The HDAC sequences in *A. flavus* were identified by BlastP searches with yeast Rpd3, Hda1, Hos2 and Hos3 which were downloaded from the Broad Institute or NCBI websites, and sequences from other organisms were obtained from the NCBI resources using BlastP searches. A neighbor-joining tree was constructed with MEGA 5.0 software (Tamura et al. 2011) based on amino acid sequences. Domain structures were made by DOG 2.0 software (Ren et al. 2009). An alignment of *A. flavus* classical HDAC genes was performed online.

Construction of gene engineered mutants.

The construction of gene deletion and gene overexpressed transformations of *A. flavus* were carried out using previously described methods (Yang et al. 2016; Yao et al. 2017). The primers used to amplify the sequences for each gene are listed in Supplementary Table S2. Gene deletion mutant strains were identified by diagnostic PCR and Southern blot and further confirmed by RT-PCR (Yang et al. 2016). All mutants constructed in this study were kept in 15% glycerol at -80°C.

Assays for growth, conidiation, and sclerotium formation.

Growth rate on PDA, CM, and GMM were measured after incubation for 7 days. For conidiation assays, conidia were harvested from 7-day-old PDA cultures and sclerotia were harvested from 7-day-old WKM and YPD agar plates. Each strain was examined on five plates, and each experiment was repeated three times.

Determination of AFB production and HPLC procedures.

In all, 10⁶ conidia of each strain were incubated in 20 ml of YES liquid medium at 28°C. After 3 days, aflatoxins were extracted using previously described protocols (Lan et al. 2016). The yield of AFB production in each sample was determined by HPLC systems (Waters, Milford, MA, U.S.A.)

Fig. 12. Deduced model, based on presented results, of histone deacetylase function in *Aspergillus flavus* and how HosA regulates its development and aflatoxin biosynthesis.

using a MYCOTOXTM column (catalog number 1612124, 5-m-long column, 25 × 4.6 mm), as described previously (Lan et al. 2016). To analyze other SMs, the WT, Δ *hosA*, and Δ *hosA.C* strains were grown on yeast-glucose-trace agar medium at 37°C for 10 days. Extracts from the medium were filtered (0.22-mm filter) and examined by an HPLC instrument (Waters) using a Sun Fire C18 column (catalog number 186002560, 250 × 4.6 mm column) at 42°C, and SMs were detected by a UV light detector (broad spectrum, wavelength = 200 to 600 nm). Each experiment was repeated three times.

Infection assays.

Virulence assays on peanut and maize seed were performed as previously described (Yang et al. 2016), with modifications. Peanut seed ($n = 20$) and maize seed ($n = 20$) were surface sterilized and inoculated with each strain at 10^6 spores/ml for 30 min, with shaking at 50 rpm at 37°C, and a mock-inoculated control was incubated in sterile water. Seed attached to conidia were incubated at 28°C. Filter papers were moistened daily to maintain the humidity. After 5 days, the conidia generated on the seed were harvested and counted as previously described and AFB was extracted from the seed and quantified as previously described (Lan et al. 2016).

Fluorescent observation.

To analyze ROS within hyphae, 10 μ l of conidia (10^6 conidia/ml) of each strain were coated on petri dishes containing 15 ml of PDA media (with a 1-cm² hole in the center), with coverslips on each plate at 37°C, overnight. Subsequently, 5 μ M DHE (Beyotime, Beijing) was added to each coverslip for 30 min. Mycelia were observed with a fluorescence microscope (Leica).

To observe the subcellular localization of HosA, methods were performed as described before (Lan et al. 2016; Wong et al. 2008). The confirmed strains were verified using a Leica confocal laser-scanning microscope. Conidia (20 μ l at 10^6 conidia/ml) were incubated in YES liquid medium for 24 h at 28°C for observing the cells during vegetative growth stage. Samples were nuclear stained with DAPI at 10 μ g/ml for 20 min before observation.

Purification of recombinant protein and detection of its HDAC activity.

The HDAC gene *hosA* was cloned by PCR using *A. flavus* cDNA and a set of primers. The PCR products were purified and ligated into a T-vector (Takara, Shiga, Japan); then, the plasmid was digested with *Eco*RI and *Hind*III (Thermo Fisher Scientific, Waltham, MA, U.S.A.). The *hosA* fragment was ligated into pET28a (Takara) that had been digested with the same restriction enzymes. *E. coli* BL21(DE3) cells were transformed with the recombinant plasmid and grown in Luria-Bertani (LB) medium containing kanamycin sulfate at 50 μ g/ml for 12 h, and a confirmed transformant was transferred into another flask containing 100 ml of LB medium and grown at 37°C for 2 h. After optical density of the culture reached 0.5, isopropyl- β -D-thiogalactoside (0.5 mM) was added into the medium, and the culture was incubated at 16°C overnight. Cells were harvested in 20 ml of 20 mM potassium phosphate (pH 8.0) and disrupted by sonication. The sonicate was centrifuged at 10,000 rpm for 15 min, and the supernatant was passed through a column containing Ni²⁺-NTA agarose, and washed five times with buffer A (50 mM NaH₂PO₄, 30 mM NaCl, 10 mM imidazole, and 10% glycerol) and five times with buffer B (50 mM NaH₂PO₄, 30 mM NaCl, 20 mM imidazole, and 10% glycerol). His-tagged HosA was eluted with buffer C (50 mM NaH₂PO₄, 30 mM NaCl, 250 mM imidazole, and 10% glycerol), and the purified protein was visualized by 12%

SDS-PAGE, followed by staining with Coomassie brilliant blue. To determine HDAC activity of the purified, recombinant HosA protein, HDAC activities were measured by the HDAC Fluorometric Assay/Drug Discovery kit (BML-AK500; Enzo Life Science, Farmingdale, NY, U.S.A.) according to the manufacturer's instructions. Briefly, approximately 15 μ l of purified protein (0.5 mg/ml) was added to the assay buffer, and the HDAC reaction was initiated by adding substrate (25 μ l) at 25°C for 10 min in a 96-well microplate; then, the reaction was stopped by adding developer (50 μ l) at 25°C for 10 min. The samples' signals were read in a microtiter-plate-reading fluorometer (BioTek Instruments, Winooski, VT, U.S.A.) at an excitation wavelength of 350 nm and an emission wavelength of 450 nm.

qRT-PCR analysis.

For qRT-PCR assays, RNA was isolated with the TRIzol reagent. First-strand cDNA was synthesized with the TransScript One-Step gDNA Removal and cDNA Synthesis Super-Mix kit (TransScript, Beijing) according to the manufacturer's instructions. Amplification and detection of genes were performed with the TransScriptGreen One-Step qRT-PCR Super-Mix kit (TransScript) using the Real-time PCR PikoReal 96 system (Thermo Fisher Scientific). The relative quantification of each transcript was calculated using the $2^{-\Delta\Delta CT}$ method. All transcripts were normalized relative to that of the β -actin housekeeping gene. The qRT-PCR primers are listed in Supplementary Table S3. All qRT-PCRs were performed in triplicate for each sample, and each experiment was repeated three times.

Western blot analysis.

Total protein was extracted from 3-day-old mycelia grown in YES liquid medium at 28°C with protein extraction buffer and RIPA lysis buffer; then, the mycelia were put in liquid nitrogen for protein extraction. Equal amounts of proteins were separated on 15% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, U.S.A.) using an electroblotting apparatus (Bio-Rad, Hercules, CA, U.S.A.). After blocking, the membrane was incubated with following primary antibodies and a secondary goat antirabbit horseradish peroxidase (HRP)-conjugated antibody (1:10,000 dilution; Abgent, San Diego, CA, U.S.A.). Primary antiacetyl-histone H3 (lot number 2556299, 1:2,000 dilution; EMD Millipore) and antiacetyl-histone H4 (lot number 2510372, 1:2,000 dilution; EMD Millipore) were used to detect acetylation of H3 and H4, respectively. Antiacetyl-histone H3K9 (1:2,000 dilution; PTM Biolabs, China) and antiacetyl-histone H4K16 (1:2000 dilution; PTM Biolabs) antibodies were used for detect acetylation of H3K9 and H4K16, respectively. Antihistone H3 (1:750 dilution; Abcam, Cambridge, U.K.) and antihistone H4 (1:1,000 dilution; Abcam) antibodies were used as loading controls. The WesternBright Quantum chemiluminescent HRP substrate kit was used for Western blot detection (Advansta; Menlo Park, CA, U.S.A.), and chemiluminescence detection was performed using a Tanon 5200 chemiluminescence instrument (Tanon, Shanghai, China).

Yeast two-hybrid assay.

To examine the possible interaction between HosA and SinA, we used the MATCHMAKER GAL4 Two-Hybrid System 3 (Clontech) to determine protein-protein interactions. The HosA cDNA was amplified with primers HosA-YF and HosA-YR and inserted into the yeast vector pGADT7, to create the vector AD-HosA as the prey in the two-hybrid assay. The Sin3 cDNA was amplified from cDNA of WT and cloned into the pGBKT7 vector, which was named BD-Sin3 and used as the

bait in yeast two-hybrid assay. The resulting yeast two-hybrid plasmids BD-Sin3 and AD-HosA, confirmed by sequencing, were cotransformed into the yeast strain AH109 (Clontech) according to the LiAc/SS-DNA/PEG transformation procedure (Schiestl and Gietz 1989). The pairs of plasmids pGBKT7-P53/pGADT7-T and pGBKT7-Lam/pGADT7-T were used as the positive and the negative controls, respectively.

ChIP.

Reagents and buffers were used as following: fix buffer (0.4 M sucrose, 10 mM Tris-HCl, [pH 8.0], 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1% formaldehyde, on ice), low-salt wash buffer (50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES] [pH 7.5], 150 mM NaCl, and 1 mM EDTA, on ice), high-salt wash buffer (45 mM HEPES [pH] 7.5, 135 mM NaCl, 0.9 mM EDTA, and 500 mM NaCl, on ice), ChIP lysis buffer 1 (CLB1, 45 mM HEPES [pH 7.5], 135 mM NaCl, 0.9 mM EDTA, 500 mM NaCl, 1% TritonX-100, 10% glycerol, and 1 pill cocktail, with 12 μ l of β -mercaptoethanol added per 35 ml), ChIP lysis buffer 2 (CLB2, 45 mM HEPES [pH 7.5], 135 mM NaCl, 0.9 mM EDTA, 500 mM NaCl, 1% TritonX-100, 10% glycerol, and 1 pill cocktail), LiCl wash buffer (0.25 mM LiCl, 0.5% NP-40, 1 mM EDTA, and 10 mM Tris-HCl [pH 8.0]), elution buffer (100 mM NaHCO₃, and 100 mM 10% SDS), and 1 $\times$ Tris-EDTA (TE) buffer (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA [pH 8.0]).

Conidia (10⁶ conidia/ml) of each strain were grown in 20 ml of YES liquid medium at 28°C for 72 h. Approximately 3 g of mycelia were cross-linked by adding 100 ml of Fix buffer (freshly, on ice) and incubated for 7 min with a vacuum machine (>25 KPa) at room temperature, twice. Fresh glycine was added to a final concentration of 100 mM and cultures were incubated for another 7 min. Samples were then washed by 1 $\times$ phosphate-buffered saline three times and dried with blotting papers. Next, samples were completely ground into powder by liquid nitrogen. Then, samples were collected in 50-ml Falcon tubes, and 20 ml of CLB1 buffer (on ice) was added and vortexed immediately, with shaking for 30 min at 4°C. A Falcon cell strainer (BD, 100 μ m) was used to filter the above samples to a new tube. Then, the samples were transferred into another Falcon cell strainer (BD, 40 μ m) and filtered a second time. Samples were then centrifuged for 20 min at 4,000 rpm and 4°C. The supernatant was removed and the pellet resuspended by 700 μ l of CLB2. The samples were then centrifuged for 20 min at 4,000 rpm at 4°C. The supernatant was discarded, 1 ml of Micrococcal Nuclease buffer was added to resuspend the samples, 2 μ l of Micrococcal Nuclease (Thermo Scientific) was added, and the samples were incubated at 37°C for 10 min. Then, 20 μ l of 0.5 mM EDTA (pH 8.0) was added to stop the reaction. The above sample was then centrifuged for 10 min at 4,000 rpm and 4°C. The above supernatant was transferred into a new tube, 50 μ l of Protein A agarose beads were added, CLB2 was added up to 2 ml, and the samples were incubated at 4°C for 2 h, with shaking. The samples were then centrifuged for 2 min at 5,000 rpm and 4°C. Under these conditions, the chromatin extract was in the supernatant. Then, 10% of the chromatin extract was kept as input and frozen at -20°C. A total chromatin extract was used for immunoprecipitation (IP) in a final volume of 850 μ l. For IP, to each 850- μ l sample, 3 μ l of anti-HA antibody HA (ab9110; Abcam) were used. For negative control, immunoglobulin G antibody was used. Then, samples were incubated at 4°C for 16 h on a rotating platform. Next, 60 μ l of Protein A agarose beads (Millipore) were added into each sample and incubated at 4°C for 1 h on a rotating platform. The above samples were then centrifuged for 2 min at 500 rpm and 4°C. The supernatant was discarded, and 1 ml of low-salt wash buffer added and the sample was incubated at

4°C for 5 min on a rotating platform. Then, the above samples were centrifuged for 2 min at 500 rpm and 4°C. The supernatant was discarded, 1 ml of high-salt wash buffer was added, and samples were incubated at 4°C for 5 min on a rotating platform. Next, the above samples were centrifuged for 2 min at 500 rpm and 4°C. The supernatant was discarded, 1 ml of LiCl buffer was added, and samples were incubated at 4°C for 5 min on a rotating platform. The above samples were then centrifuged for 2 min at 500 rpm and 4°C. The supernatant was discarded, 1 ml of TE buffer was added, and samples were incubated at 4°C for 5 min on a rotating platform. The above samples were then centrifuged for 2 min at 500 rpm and 4°C. The supernatant was discarded and 250 μ l of elution buffer added and incubated at 65°C for 15 min, with shaking. Samples were then centrifuged for 2 min at room temperature and 500 rpm. The supernatant was transferred into new tubes and 20 μ l of 5M NaCl was added to release the cross-linking. The above input samples also received 20 μ l of 5M NaCl. Both the samples and negative-control samples were incubated overnight at 65°C, with shaking at 300 rpm. Phenol-chloroform (500 μ l) was used for chromatin DNA extracts, centrifuged for 10 min at room temperature and 12,000 rpm. The supernatant was removed; 1 ml of anhydrous ethanol, 50 μ l of NaAc, and 1 μ l of glycogen at 15 mg/ml were added; and samples were incubated at -20°C for 4 h. The above samples were centrifuged for 20 min at 12,000 rpm. The supernatant was discarded and 300 μ l of 70% ethanol was added. The above samples were then centrifuged for 10 min at 12,000 rpm. After removing the ethanol, the tubes were dried at room temperature for 15 min. Finally, 30 μ l of TE buffer was added to each sample and incubated for 10 min at room temperature. For qRT-PCR, a 3-fold dilution of each immunoprecipitated sample and a 200-fold dilution of input (10%) samples were used. The qRT-PCR primers used in this study are listed in Supplementary Table S4.

Statistical analysis.

Data were presented as means $\pm$ standard deviation of at least three biological replicate samples in figures. Statistical and significance analyses were performed using the GraphPad Prism 5/6 and regarded as significant if *P* values were <0.05. Student's *t* test was used when comparing two means for differences. For multiple comparisons, Turkey's multiple comparison test was used for significance analysis.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Broad Institute: https://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html
NCBI: <https://www.ncbi.nlm.nih.gov/>
DOG 2.0 software: https://embnet.vital-it.ch/software/BOX_form.html

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