Molecular and structural basis of nucleoside diphosphate kinase–mediated regulation of spore and sclerotia development in the fungus Aspergillus flavus

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The fundamental biological function of nucleoside diphosphate kinase (NDK) is to catalyze the reversible exchange of the γ-phosphate between nucleoside triphosphate (NTP) and nucleoside diphosphate (NDP). This kinase also has functions that extend beyond its canonically defined enzymatic role as a phosphotransferase. However, the role of NDK in filamentous fungi, especially in Aspergillus flavus, is not yet known. Here we report that A. flavus has two NDK-encoding gene copies as assessed by qPCR. Using gene-knockout and complementation experiments, we found that AfNDK regulates spore and sclerotia development and is involved in plant virulence as assessed in corn and peanut seed-based assays. An anti-fungal test with the inhibitor azidothymidine suppressed AfNDK activity in vitro and prevented spore production and sclerotia formation in A. flavus, confirming AfNDK’s regulatory functions. Crystallographic analysis of AfNDK, coupled with site-directed mutagenesis experiments, revealed three residues (Arg-104, His-117, and Asp-120) as key sites that contribute to spore and sclerotia development. These results not only enrich our knowledge of the regulatory role of this important protein in A. flavus, but also provide insights into the prevention of A. flavus infection in plants and seeds, as well as into the structural features relevant for future antifungal drug development.

Aspergillus flavus is a notorious saprophytic and pathogenic fungus that is typically found in soil and is distributed worldwide. As an opportunistic filamentous fungus, A. flavus is prone to infect and contaminate important oil-containing agricultural crops with a series of secondary metabolites such as aflatoxin prior to harvest or during storage (1). Accordingly, these natural toxic compounds produced by this fungus can easily lead to severe diseases and death in animals and humans (2). Aflatoxin B1 (AFB1), is the most concerning mycotoxin in A. flavus due to its mutagenic and carcinogenic properties (3). AFB1 is considered to be a potent hepatocarcinogen associated with hepatocellular carcinoma in humans (4). Like other species of Aspergillus, A. flavus is a contributing mediator to aspergillosis diseases (5). Many pathogenic infections are derived from patients’ inhalation or exposure to fungal spores originating from contaminated foods (6), whereas other infections occur in immunocompromised and surgical patients who often have a high risk of open injury infections (7). Thus, A. flavus and its toxic metabolites are not only a threat for agriculture economics, but they are also a significant hazard for animal and human health. Understanding the mechanism of A. flavus growth and aflatoxin synthesis should greatly improve control strategies for fungal contaminations in preharvest and postharvest seed crops.

In recent years, numerous studies of A. flavus have focused on its genomics, taxonomy, pathogenicity, and antifungal properties. The entire A. flavus genome has been sequenced (8, 9), and 30 different aflatoxin biosynthesis-encoding genes have been identified from a crucial gene cluster in A. flavus (10). Our group has previously determined the role of a DNA methyltransferase in growth, conidiation, sclerotial production, aflatoxin biosynthesis, and virulence in this pathogenic filamentous fungi (11). Proteins that have been post-translationally modified with moieties such as succinyl groups or small ubiquitin-like modifiers were verified to regulate fungal development and aflatoxin biosynthesis in A. flavus (12, 13). Furthermore, screening specific compounds to inhibit spoilage organisms has become an area of intense interest in the field of fungal studies (14–17), and some potential candidates against Aspergillus spp. have been described (18, 19). However, the mechanisms regulating growth and aflatoxin production for...
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**Figure 1. Sequence analysis of the NDK protein.** A, sequence alignment of NDKs from *A. flavus* and other species (*A. nidulans, Plasmodium brasilianum, Macrohominia phaseolina, S. cerevisiae, S. pombe, M. musculus* B, and *H. sapiens*). Residues highlighted in colored boxes denote at least half or more identical residues. Sequence alignments were analyzed with DNAMAN software. B, phylogenetic tree of NDK homologous members was constructed with MAGA software.

this kind of fungus have not been clearly illustrated at the molecular level, and knowledge about *A. flavus* infection strategies is still in its infancy, especially in terms of the identification of potential drug targets.

Intracellularly, the NDK family catalyzes and controls the reversible exchange of the γ-phosphate between nucleoside triphosphate (NTP) and nucleoside diphosphate (NDP), using ATP as an intermediate (20–22). Although NDKs are a large family of proteins found in numerous organisms with high homology, their biological functions extend beyond merely being phosphotransferases. In *Neurospora crassa*, NDK acts as a signal transducer for the phosphorylation of downstream target proteins (23, 24), and mutant NDK (P72H) cells have defects in light responses and autophosphorylation features (25). Disruption of the NDK-coding gene in *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe* did not obviously affect cell growth, sporulation, or mating ability, although cellular NDK activities for these two fungi were reduced to 10–20% of WT, respectively (26, 27).

Deletion experiments of the NDK homologous protein swoH1 in *Aspergillus nidulans* suggested that the SwoHp protein was indispensable for hyphal growth at a restrictive incubation temperature, and a deletion strain had only 20% NDK activity compared with WT (28). Two isoforms of NDK, called NDK-A and NDK-B, are found in humans. NDK-A has been considered to be a metastasis suppressor (29), and NDK-B, also known as PuF, was identified as a transcriptional activation factor of the c-myc oncogene (30).

In addition, NDK has become a novel target for drug discovery and design (31). A number of molecules and antibiotics have been reported to inhibit NDK activity efficiently, including flavonoids, 3’-phosphorylated nucleotides, and desdanine (32–34). Some NDK inhibitors have been screened and discovered in screens targeting parasitic inhibitors (35). Typically, NDK proteins have common subunits found in a ββββββ or a “ferredoxin” folding pattern (36–44). The overall structure of NDKs has been found in various oligomeric states, such as dimers (45), tetramers, or hexamers (40, 46). This oligomerization indicates that the NDK protein family can interact with other molecules to perform their function in vivo (47, 48).

Here, we show that NDK plays an important role in spore and sclerotia development, and even in the pathogenicity of *A. flavus*, as verified by functional studies. As an inhibitor, azidothymidine (AZT) retarded conidiation and sclerotia production but not aflatoxin production. We then determined a three-dimensional structure of AfNDK using X-ray crystallography. Based on the AfNDK crystal structure, we constructed a series of AfNDK mutants (R104A, H117A, and D120A). These mutants had significant correlation in many assays with an AfNDK deletion mutation, with the H117A mutant having the most extreme phenotypes of these mutants. These results provide detailed and comprehensive information regarding the regulation mechanism of NDK in *A. flavus*.

**Results**

**Identification of an NDK-encoding gene in *A. flavus***

*A. flavus* NDK proteins were identified using the National Center for Biotechnology Information (NCBI) database with the Reference Sequence (XP_002382830.1). The amino acid sequences of NDK homologous proteins from *Aspergillus niger, A. nidulans, Aspergillus fumigatus, S. cerevisiae, S. pombe, Mus musculus* B, *Homo sapiens* B, and *Escherichia coli* were analyzed by combined clustering and a phylogenetic-tree method. The target AfNDK exhibited high-sequence similarity with other filamentous fungal orthologs and even some similarity with mammalian orthologs (Fig. 1). Also noteworthy was the fact that the majority of eukaryotic NDK homologous proteins did not have the same C-terminal extension that contributes to oligomerization stability and function as these proteins (49, 50). These above results suggested that NDK was a relatively evolutionarily conserved protein family in fungi and mammals.
Multicopy variation of genes has not been explored in detail in fungi, especially in *A. flavus*, but is a commonly known phenomenon in mammalian genomes. Intriguingly, the NDK-coding gene in *A. flavus* had multiple copy numbers, as was confirmed by real-time fluorescent quantitative PCR at the genome level (Table 1 and Table S2). In a WT strain, the number of *Afndk* gene copies was double that compared with the number of copies of the *AfsumO* gene (a single copy control in this study) (13). Furthermore, we cloned another genomic segment from the Δ*Afndk1*/Δ*Afndk2* strain that had one copy of the NDK-coding gene knocked out and contained only one copy of the *Afndk* gene. The sequence alignment results revealed that another genomic segment had 100% homology with *Afndk*, suggesting that there may exist other *Afndk* gene copies but not paralogous *Afndk* genes in a WT strain. Although the entire genome of *A. flavus* has been previously decoded (8, 9), we could not gain additional information on gene copy numbers due to the limitations of Shotgun sequencing methods, nor could we design double-deletion experiments without upstream and downstream information regarding the additional *Afndk* gene copy. To address this, we tried to amplify a full-length copy of the other *Afndk* gene using a reverse PCR method, but we were not successful. In this study, we constructed a deletion strain Δ*Afndk1*/Δ*Afndk2* (containing one copy of the *Afndk* gene) and a complemented strain Δ*Afndk1*/Δ*Afndk2* -com (containing two copies of the *Afndk* gene), which were confirmed by real-time fluorescence quantitative PCR at the genome (Table 1) and transcriptional levels (Fig. 2A). Validation at the protein level is presented in Fig. 2B and Fig. S9B by Western blotting. In the above results, the deletion strain expressed nearly half the amount of the *Afndk* gene compared with a WT or complemented strain at the transcriptional level (Fig. 2A). We speculated that a deletion strain containing one copy of the *Afndk* gene may express half the level of the NDK protein compared with that of a WT or complemented strain. However, the expression level of NDK protein in the Δ*Afndk1*/Δ*Afndk2* strain was much lower than that in either the WT or complemented strains (Fig. S9, B and D), which indicated that the two *Afndk* gene copies were expressed quite differently at the protein level.

**AfNDK is important for spore development and sclerotia production in A. flavus**

To identify the effects of NDK on *A. flavus*, three *A. flavus* strains (WT, Δ*Afndk1*/Δ*Afndk2*-, and Δ*Afndk1*/Δ*Afndk2* -com) were cultured on PDA medium in the dark for 5 days at 37°C. There were no obvious phenotypic differences in terms of colony size or aflatoxin production between these three strains (Fig. 3C and Fig. S2A). However, AfNDK was involved in spore development and sclerotia production in *A. flavus* (Figs. 3 and 4). As determined by microscopy, the Δ*Afndk1*/Δ*Afndk2* strain exhibited worse conidiophore formation and lower growth at the head of the child seat (see Fig. 3A). The spore number of the Δ*Afndk1*/Δ*Afndk2* strain was significantly reduced by 6–7-fold compared with the numbers of the WT and Δ*Afndk1*/Δ*Afndk2* -com strains (Fig. 3B). Moreover, the transcription level of the conidiation regulation gene *brlA* in the Δ*Afndk1*/Δ*Afndk2* strain was lower than that in the other two strains as well, and it was nearly one-third of WT (Fig. 3C). These results indicated that AfNDK may participate in the upstream regulation of the *brlA* gene during spore development (51).

Dysfunctional NDK affected the process of sclerotia formation in *A. flavus*. The Δ*Afndk1*/Δ*Afndk2* strain produced about half the number of sclerotia that either the WT or complemented strain (Fig. 4, A and B). In addition, the transcription level of the sclerotia-related gene *nsdD* decreased dramatically in the deletion strain compared with the WT and Δ*Afndk1*/Δ*Afndk2* -com strains (Fig. 4C) (52). These results suggested that AfNDK was important for sclerotial generation, and its coding gene may be involved in the regulation of sclerotia formation and work upstream of *nsdD*.

**AfNDK contributes to the virulence of A. flavus**

To investigate the virulence and toxin production of the deletion strain (Δ*Afndk1*/Δ*Afndk2*-) during *A. flavus* colonization, both maize and peanut seeds were incubated with conidia suspensions from WT, Δ*Afndk1*/Δ*Afndk2*-, and Δ*Afndk1*/Δ*Afndk2* -com strains (Fig. 5A). As the thin-layer chromatography (TLC) results demonstrated, there were no distinctly visible differences in aflatoxin B1 levels from colonized maize seeds or from peanut seeds (Fig. S2B). These unremarkable aflatoxin production changes from infected seeds were consistent with the previous phenotypes from strains harvested on YES medium (Fig. S2A). The virulence of the Δ*Afndk1*/Δ*Afndk2* strain was partially abolished, due to its poor capacity to colonize and sporulate on the surface of host seeds, especially in the case of maize seeds. Furthermore, the spore number from infected seeds was statistically significantly reduced to 30% or lower in the deletion strain compared with the complemented or WT strains (Fig. 5, B and C). From all the aforementioned results, we concluded that AfNDK played a crucial role in the colonization process of *A. flavus*.

**AfNDK has phosphate transferase activity**

Recombinant AfNDK (with a purity of more than 95%) was generated using previously reported procedures (53). The specific kinase activities of this recombinant protein was measured...
with a classically-coupled pyruvate kinase-lactate dehydrogenase method with minor modifications (21). In brief, this assay was divided into two steps. First, the NDK protein converts ATP to ADP using dTDP as a phosphate acceptor. Thus, the second reaction is based on the measurement of the ADP released by an enzyme-coupling assay utilizing pyruvate kinase and lactate dehydrogenase. Enzyme kinetic data indicated that rAfNDK had phosphate transferase activity (Fig. 6A), consistent with it being a member of the NDK family. Additionally, we further measured the nucleotide binding affinity of rAfNDK by performing isothermal titration calorimetry. Four previously reported ligands (CDP, UDP, ADP, and GDP) were used in this titration experiment (44). We observed no significant signal variation in the heat exchange as rAfNDK was titrated with CDP and UDP. In contrast, the nucleotide binding affinity of recombinant rAfNDK was determined by titration with two other nucleotide molecules (ADP and GDP), and rAfNDK had a $K_d$ of 153 and 157 μmol/liter for these nucleotides, respectively. The ITC data (Fig. 7) demonstrated that the binding affinities of rAfNDK with nucleotides were consistent with that of previously reported homologous proteins from Drosophila that were on the same order of magnitude (44).

Azidothymidine is an inhibitor of not only AfNDK but also A. flavus

AZT, also known as zidovudine, was approved by the United States Food and Drug Administration in 1986. This chemical was primarily approved for the treatment of human immunodeficiency virus (HIV) as an antiretroviral drug (54). On the basis of former research, AZT was reported to inhibit mitochondrial NDK activity at a concentration of 10 μM (55). In this study, we also found that this compound inhibited rAfNDK at an IC$_{50}$ value of 5.61 μM (Fig. 6B).

We further investigated whether AZT affected A. flavus growth and secondary metabolite production. Neither colony morphology (colony radius) nor aflatoxin biosynthesis were influenced by increasing concentrations of AZT at the corresponding optimal temperature for A. flavus growth (Figs. S3 and S4). The conidial formation, sclerotial generation, and infection ability of a WT strain were all inhibited by AZT, based on phenotypic observations and statistical analysis (Figs. 8–10).

The morphology of the conidiophore was influenced even at the initial concentration (25 g/ml) of AZT used in these studies (Fig. 8A). As the concentration of AZT reached 250 g/ml, the branches of specialized aerial hyphae became much thinner, and spore tips also shrank in size (Fig. 8A). The number of spores also decreased remarkably at an AZT concentration of 100 g/ml (Fig. 8B). The transcript levels of the sporulation regulatory gene brlA in the WT strain were also depressed after AZT treatment (Fig. 8C). The inhibition effect on sclerotial generation was significant at the 250 μg/ml concentration of AZT, which was reflected by the sclerotia number produced by A. flavus (Fig. 9, A and B). In addition, the transcript levels of the...
sclerotial regulatory gene \( \text{nsdD} \) were also impacted by AZT treatment (Fig. 9C). To investigate the pathology of \( \text{A. flavus} \) infection after AZT treatment, a WT strain was treated with AZT during the invasion of maize and peanut seeds (Fig. 10A). Conidia production on both types of seeds was repressed (Fig. 10, B and C), supporting the notion of AZT inhibiting \( \text{A. flavus} \).
In view of the above results, AZT is an effective inhibitor not only for AfNDK but also for A. flavus. Moreover, both deletion mutant and AZT-inhibited strains had similar phenotypic developments in terms of aflatoxin production, spore number, and sclerotia. We speculated then that NDK in A. flavus acted as an ideal intracellular target for the inhibitor AZT.

**Structural basis for the role of AfNDK in A. flavus**

To investigate the structural basis of NDK regulation of spore and sclerotia development in A. flavus, purified rAfNDK was harvested and then crystallized according to previously published methods (53). The diffraction data were scaled and processed using the CCP4i2 DIALS package (56). The crystal was diffracted to a resolution of 2.17 Å in the space group C121 with large cell parameters (a = 1100.5 Å, b = 1100.5 Å, and c = 146.0 Å). The overall crystal structure of AfNDK (Protein Data Bank code 6K3H) was packed with 24 monomers per asymmetric unit after being resolved by molecular replacement (Fig. 11, A and B). The overall protein assembled like a saddle with four subunits, and each subunit con-
tained a hexamer (Fig. 11C). In addition, each subunit was formed from three dimers (Fig. 11C).

The protein structure model was refined to final $R_{\text{factor}}$ of 23% and $R_{\text{free}}$ of 28%. Good stereochemistry, with root mean square deviations in bond length and bond angles of 0.008 Å and 0.96, respectively, was obtained. In a Ramachandran plot, 95.8% of the residues were in the most favored Ramachandran region and 4.2% in the additional region (Table S3).

The NDK monomeric subunit consisted of four antiparallel strand $\beta$-sheets and several $\alpha$-helices (Fig. 11E), in which all the internal strands were covered by $\alpha$-helices on the protein surface like other NDK family structures (36–44). A linker region

Figure 7. ITC profiles of recombinant AfNDK binding to nucleotides. Heat changes (microcalories) plotted versus time (minutes) are shown in the top panels, and the integrated binding isotherms versus the molar ratio of the ligand are plotted in the bottom panels. A, recombinant AfNDK titrated with ADP had a $K_d$ of 153 μmol/liter; B, recombinant AfNDK titrated with GDP had a $K_d$ of 157 μmol/liter.

Figure 8. Effect of AZT on conidia production in A. flavus. A, morphology of WT strains inhibited with AZT (0–250 μg/ml). Top row: morphological character of conidiophores and conidium peduncles. Bottom row: features on head of child seat for A. flavus. B, number of conidia produced by a WT strain treated with different concentrations of AZT. C, AZT affects the transcription level of the conidia biosynthesis gene $brlA$. * and *** indicate significance levels of $p < 0.05$ and $p < 0.001$, respectively, as analyzed using Tukey's multiple-comparisons test from three replicates.
named the Kpn loop (57) was between α3 and β4 and contained about 20 residues (Fig. 11E). To identify the active residues on AfNDK, we tried to cultivate the rAfNDK–AZT complex crystals but were not successful. An alternative approach was to use a molecular simulation docking method that may predict this structure and provide interaction information between an AZT molecule and the AfNDK protein. With MVD software analysis, an AZT molecule was surrounded with NDK residues (Lys-11, Gly-86, Arg-87, Leu-90, Gly-91, Ala-92, Thr-93, Arg-104, Asn-114, Cys-116, His-117, Gly-118, Ser-119, Asp-120, and Glu-128). In the AfNDK–AZT structure, the polar pyrimidine ring of AZT was exposed to solvent, whereas the charged group inserted into the activity pocket of AfNDK (Fig. 11F). In addition, the AZT molecule was sandwiched between Arg-104 and Asp-120 and formed a strong hydrogen bond with these two residues, respectively (Fig. 11F). There was another hydrogen bridge between the azido group (N3) of AZT and the N atom of AfNDK His-117 (Fig. 11F). Therefore, we selected these above three residues (Arg-104, Asp-120, and His-117) and mutated them into alanine for follow-up investigations.

Recombinant AfNDK (H117A and D120A) displays low activity in vitro

The point mutation plasmids for recombinant AfNDK expression in E. coli were constructed using a one-step point mutation method. An experiment was carried out to verify the
protein expression levels of these mutated recombinant AfNDK proteins. SDS-PAGE results showed that R104A existed primarily in inclusion bodies, and sufficient soluble protein for downstream analysis was not obtained (Fig. S5). The phosphatase enzyme activity of the H117A and D120A mutant rAfNDK enzymes was measured and compared with that of WT rAfNDK. Using the specific activity of the WT rAfNDK as a benchmark, the activity value of H117A and D120A declined remarkably by comparison, especially the His-117 mutant, which had nearly half the rAfNDK activity (Fig. 12).

Arg-104, His-117, and Asp-120 AfNDK affect conidia and sclerotia production in A. flavus

Morphological characteristics, such as spore development and sclerotia formation, of the three mutants (AfNDK<sup>R104A</sup>, AfNDK<sup>H117A</sup>, and AfNDK<sup>D120A</sup>), were similar to those observed in the deletion strain or the inhibited WT strain. Colony diameter and toxin production in these mutant strains were not affected either (Fig. S6). All mutant strains displayed visible effects on sporulation, such as the atrophy of spore heads and stalks (Fig. 13A). Compared with the WT strain, the amount of conidia production decreased in the AfNDK<sup>R104A</sup>, AfNDK<sup>H117A</sup>, and AfNDK<sup>D120A</sup> mutant strains, particularly the AfNDK<sup>H117A</sup> strain (Fig. 13B). Meanwhile, the transcript levels of the conidia-regulating gene <i>brlA</i> in these mutant strains declined as well (Fig. 13C). Based on the above data analysis, we deduced that these residues (Arg-104, His-117, and Asp-120) in AfNDK played important roles in conidia development of <i>A. flavus</i>, especially the His-117 site.

The sclerotia formation of all of the mutant strains had significant differences compared with that of the WT strain, as was reflected in the sclerotia number and the expression of the regulating gene <i>nsdD</i> (Fig. 14). These three sites (Arg-104, His-117, and ASP-120) were critical for the reduction in sclerotia number (Fig. 14, A and C), and the expression level of the <i>nsdD</i> gene in these three mutant strains was remarkably low (Fig. 14B). Sclerotia formation in <i>A. flavus</i>, it seemed, required these three residues (Arg-104, His-117, and ASP-120) in AfNDK, and the His-117 site seemed to play a leading role in this process.

We additionally investigated whether AZT affects conidia production in these genetically modified strains (Fig. S7). After
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**Figure 13. Differences between AfNDK mutant strains during the development of conidiation.** 
*A*, development of conidiation in different mutants of *A. flavus* by microscopic observation. *Top row*: morphological character of conidiophores and conidium peduncles. *Bottom row*: features on the head of conidiophores. *B*, amounts of conidia produced by AfNDK mutant strains. *C*, transcriptional level of the conidia biosynthesis gene *brlA* in different mutant strains. ** and *** indicate significance levels of *p* < 0.01 and *p* < 0.001, respectively, as analyzed using Tukey's multiple-comparisons test.

**Figure 14. Differences between AfNDK mutant strains during the development of sclerotia in *A. flavus*.** 
*A*, morphological analysis of sclerotia of AfNDK mutant strains. *B*, amounts of sclerotia produced by AfNDK mutant strains. *C*, transcription level of the sclerotia biosynthesis gene *nsdD* expressed in different mutant strains. *, **, and *** indicate significance levels of *p* < 0.05, *p* < 0.01, and *p* < 0.001, respectively, as analyzed by Tukey's multiple-comparisons test.
AZT treatment, the data showed that there was significant difference in spore number in both the mutant and complemented strains, but not as compared with the /H9004Afndk1/H11001Afndk2 strain. These results demonstrated once again that the above three residues (Arg-104, His-117, and Asp-120) played the key roles in AfNDK phosphatase activity. Arg-104, His-117, and Asp-120 contribute to the virulence of A. flavus

We further analyzed the differences in virulence between the three mutant strains. The results suggested that most of the strains had a reduced ability to invade a host (Fig. 15A), as reflected by statistical differences in the conidiophore counts. In infected peanut seeds, AfNDK\textsuperscript{R104A}, AfNDK\textsuperscript{H117A}, and AfNDK\textsuperscript{D120A} produced much lower numbers of conidiophores than did a WT strain. For the number of conditions on infected corn seeds, they were significantly reduced to a relatively low level in the AfNDK\textsuperscript{H117A} and AfNDK\textsuperscript{D120A} strains compared with the level in WT (Fig. 15, B and C). Most notably, the conidiophore counts from these two crop seeds infected with AfNDK\textsuperscript{H117A} were less than half that of a WT strain. However, in the case of the AfNDK\textsuperscript{R104A} strain, there was no significant difference in the spore number from infected corn seeds compared with the WT strain. The lack of an obvious phenotype in the AfNDK\textsuperscript{R104A} strain could be explained by various factors during the infection by the pathogen. All of the strains, including each of the mutants and the WT strain, had similar results in terms of toxin production during A. flavus invasion of crop seeds (Fig. S8).

Discussion

There are two copies of Afndk-coding genes in A. flavus

We used a real-time fluorescence quantitative PCR method to investigate the gene-copy number of ndk in WT, deletion, and complemented strains. As this is a quantitative technology, we recommend it as an alternative to Southern blotting, due to the advantages of high-throughput screening measurements, minimal amounts of DNA sample required, and the large time savings (58). We applied this technology and found that the ndk gene existed as two copies in WT and complemented strains, whereas only one copy was found in the deletion strain.

Copy number variation is a common phenomenon in higher organisms, such as mammals, and can be associated with physiology, evolution, and disease (59). Other organisms, such as bacteria (60) and yeast (61), have been reported to be in possession of genes with multiple copy numbers much more frequently. Although current studies have regularly focused on the consequence of copy number variations in human phenotypic diversity, less is known about the result of this in other organisms, especially in A. flavus.

Izumiya et al. (26) speculated that they may have another gene encoding an NDK homologous protein that was expressed at much higher levels than the deleted one, leading to no obvious phenotypes in other fungi. Western blot analysis showed that AfNDK was still expressed in the Afndk1\textsuperscript{+}/Afndk2\textsuperscript{−} strain, but at a much lower level than in a WT A. flavus strain. We hypothesized that the different protein expression level was due to the two copies of the ndk gene being controlled by different strength promoters, respectively. The promoter regula-
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The expression of the *Afndk* gene was similar to the specific alcohol oxidase promoter mediating *Pichia pastoris* protein expression (63).

**AfNDK is a crucial factor regulating development in A. flavus**

NDK family proteins are ubiquitous enzymes that exchange the γ-phosphate between NTP and NDP, preserving NTPs in the cell. In *A. flavus*, AfNDK plays an important role in spore development, sclerotia production, and pathogenicity on seeds. AfNDK also regulated the gene *brlA* for conidia development and the gene *nsdD* for sclerotia development, respectively. These results indicated that the sophisticated role of AfNDK in multiple developmental stages of *A. flavus* was associated with its phosphotransferase activity. In addition, it appeared that NDK deletion strains, mutation strains, and inhibited strains could all produce aflatoxin normally, suggesting that the AfNDK-coding gene was not involved in aflatoxin synthesis.

NDK family proteins belong to one category of multifunctional proteins. During the *Pseudomonas aeruginosa* infection process, NDK mediates the expression of proinflammatory cytokines (64). In *Mycobacterium tuberculosis*, NDK was identified as a virulence factor for macrophages, contributing to the pathogenesis of bacterial infections (65). However, most of NDK deletion fungi strains display no obvious phenotypes in terms of cell growth, sporulation, mating ability, or morphology (26, 27). NDKs have also been suggested to be dispensable during asexual development (26, 27), except for the SwoHp protein (an NDK homologous protein), which was shown to be a temperature-sensitive factor in the polar growth of *A. nidulans* (28). On the contrary, our work indicates that one major function of AfNDK is in spore development and sclerotia production in *A. flavus*, rather than being involved in growth or aflatoxin production. Interestingly, the kinase activity of NDK has been linked to the virulence of *A. flavus*, which is dissimilar to how this protein behaves in *P. aeruginosa* (66). It thus appeared that the balance between NTP and NDP is interrupted in the AfNDK deletion or mutant strains and that the observed phenotypes were a consequence of this imbalance.

NDK has become a popular drug target in *E. coli* and protozoan parasites, and special inhibitors of NDK have been exploited by other researchers (35, 67). Few NDK inhibitors for pathogenic fungi have been described in the literature. *A. flavus* infection contributes heavily to economic loss in terms of crop yields, and it also impacts human health through diseases like aspergillosis. As *A. flavus* reproduces asexually (68), its spores and sclerotia are the main sources of pathogenicity in its route of infectious transmission. On the basis of our deletion and complementation experiments, AfNDK may be considered a potential drug target for its role in regulating the spore and sclerotia development of *A. flavus*.

**AZT is a potential fungicide and antifungal drug**

AZT has not just been proposed for the treatment of acquired immune deficiency syndrome (AIDS), it also has other uses. This drug has been applied as a potential antineoplastic agent for the treatment of gastrointestinal cancers, pancreatic cancer, and advanced malignant tumors (69). The antimicrobial properties of AZT have been demonstrated in that it has been shown to have biological activity against *E. coli*, *Vibrio cholerae*, and the fish pathogen *Vibrio anguillarum* (70). Moreover, AZT is routinely administered with other antifungal agents to treat patients who have serious fungal infections (71). But its antifungal activity without the assistance of other agents has rarely been mentioned in other studies. In this study, AZT was shown to be a potent inhibitor of AfNDK *in vitro*, targeting either the development of the spore or the sclerota in *A. flavus*. Moreover, AZT may be rapidly advanced into clinical settings as an antifungal agent, as it has been safely used on HIV patients for a long time.

Thus, it seems that increased management of *A. flavus* could be accomplished through the following: 1) high-throughput screening for more effective candidate molecules targeting NDK kinases, such as nucleotide analogs or derivatives, or 2) a mixture of AZT in combination with other antifungal drugs targeting *A. flavus*.

**Oligomeric state of AfNDK**

To our surprise, there were 24 monomers in the three-dimensional structure of AfNDK. In previous studies of crystal structures, NDKs typically fold into dimers (45), tetramers, or hexamers (40, 46). The oligomer conversion may imbue AfNDK with diverse interactions, allowing it to interact with different molecules for different functional purposes (47, 48).

In this uncommon AfNDK structure, we sought evidence of strong interactions between four subunits (hexamers), but it seemed that only a few salt-bridge interactions occurred between each hexamer (Fig. 6D). Moreover, using the assembly analysis generated by PISA (an interactive tool for the exploration of macromolecular interfaces), the quaternary structure of this protein was hypothesized to be a hexamer. In our previous study, the oligomeric state of AfNDK had been checked in solution, and it was determined to be a trimer by molecular-exclusion chromatography analysis (53). Thus, the physiological quaternary structure of AfNDK in solution may be a trimer, and in crystal structure AfNDK may fold into a hexamer. In addition, NDK family proteins from eukaryotes usually tend to form into hexamers rather than the tetramers found in prokaryotes. This may, in part, be because they seem to have a longer C-terminal region that contributes to hexamer stability, which is similar to our results (Figs. 1A and 11C) (49). We reasoned that the uncommon observation in the AfNDK crystal structure may have come from crystal packing effects.

**Arg-104, His-117, and Asp-120 are the key sites on AfNDK**

In this study, we demonstrated that three residues (Arg-104, His-117, and Asp-120), especially His-117, played important roles in the bioactivity of AfNDK *in vitro*. Moreover, they were also crucial sites in AfNDK mutation strains, as reflected in their phenotypic characterizations.

Arg-104, His-117, and Asp-120 are all conserved positions in both prokaryotic and eukaryotic NDKs (Fig. 1). In the AfNDK crystal structure, all of the above amino acids were determined to be in the important part of the protein catalytic region. An acidic amino acid (Asp-120) was located at the entrance of the NDK-binding pocket, and alkaline amino acids (His-117 and Arg-104) were positioned on the bottom of this active center.
Table 2

| Strains used in this study | Genotype | Refs. |
|---------------------------|----------|-------|
| rAfNDK                    | ndk      | 53    |
| R104A                     | ndk (Arg-104 → Ala), E. coli BL21 | This study |
| H117A                     | ndk (His-117 → Ala), E. coli BL21 | This study |
| D120A                     | ndk (Asp-120 → Ala), E. coli BL21 | This study |
| CA14 PTS                  | Δku70, ΔpyrG | 62    |
| WT                        | Δku70, ΔpyrG; pyrG | This study |
| ΔAfndk1"/Afndk2"-         | Δku70, ΔpyrG; ΔAfndk::pyrG | This study |
| ΔAfndk1"/Afndk2"-com      | Δku70, ΔpyrG; ΔAfndk::pyrG::Afndk | This study |
| AfNDKH117A                | Δku70, ΔpyrG; ΔAfndk::pyrG::Afndk | This study |
| AfNDKΔ104A                | Δku70, ΔpyrG; ΔAfndk::pyrG::AfndkR104A | This study |
| AfNDKΔ117A                | Δku70, ΔpyrG; ΔAfndk::pyrG::AfndkH117A | This study |
| AfNDKΔ120A                | Δku70, ΔpyrG; ΔAfndk::pyrG::AfndkD120A | This study |

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(Fig. 11G). The basic hole composed of His-117 and Arg-104 is likely to attract nucleotide derivatives with high efficiency. His-117 was essential for the enzyme catalysis reaction during the ligand recognition processes (72), and mutation of this site abolished both autophosphorylation of NDK and its kinase activity in previous reports (65, 73). Another alkaline amino acid (Arg-104) was involved in either phosphotransferase or autophosphorylation activity as well (73). Although a series of studies have highlighted the roles of His-117 and Arg-104 on NDK, the function of Asp-120 has been rarely reported in other species. Therefore, it is a noteworthy implication from our study that the Asp-120 site on NDK may be important in NDKs from other species as well.

**Conclusion**

At present, numerous studies have attempted to explore the function of NDK during organismal development. It seems that the diversity of biological functions for NDK has been under-emphasized, particularly in Eumycophyta. We found that AflNDK appears to play an intricate role in spore and sclerotia development in *A. flavus*, in addition to its canonical role as a phosphotransferase. Moreover, the molecule AZT has inhibitory effects on AflNDK and *A. flavus*, and it should be considered as a potential antinymycotic candidate for further agricultural and medical pharmacology investigations.

**Experimental procedures**

**Strains and culture conditions**

The *E. coli* strains DH5α and BL21 (DE3) were used for plasmid DNA preparation and recombinant AflNDK expression, respectively. *A. flavus* strains used in this study are listed in Table 2. The strains were cultured at 37 or 28 °C for study of the growth on PDA media (BD Biosciences) and for the analysis of sclerotia production on CM media (complete medium: 6 g/liter yeast extract, 6 g/liter peptone, 10 g/liter sucrose). To investigate aflatoxin production, cultivating conditions were established using YES (yeast extract/sucrose, 2% yeast extract, 150 g/liter sucrose, 1 g/liter MgSO₄·7H₂O) liquid culture at 28 °C (13, 74).

**Mutant-strain construction**

For the preparation of rAflNDK mutant strains, a DpnI enzyme-mediated site-directed mutagenesis method was used. The prokaryotic expression vector pRSFDuet-1 containing the NDK protein-coding gene was used as template plasmid for the amplification of mutant DNA (53). After PCR, the parental DNA was digested with the enzyme DpnI and then was transformed into *E. coli* DH5α. Finally, correctly constructed plasmids were confirmed by Sanger sequencing and were then transformed into *E. coli* BL21 (DE3)-competent cells (Fig. S1A).

For obtaining a ΔAfndk1"/Afndk2" deletion strain, we followed previously described methods (13, 75). An ndk deletion cassette was fused with an ndk gene upstream fragment, an *A. fumigatus* pyrG and an ndk gene downstream fragment by overlap extension PCR (Fig. S1B). The PCR product was then transformed into the CA14 PTS strain, and a ΔAfndk1"/Afndk2" deletion strain was verified by real-time fluorescent quantitative PCR at the genomic and transcriptional levels. The expression level of the NDK protein in deletion strains was determined by Western blot analysis. The complemented strain (ΔAfndk1"/Afndk2"-com) was constructed based on a previously described protocol (76, 77). In the first step, the nutrition marker (pyrG) in the ΔAfndk1"/Afndk2" strain was replaced by the ndk gene fragment. PCR was conducted to confirm this intermediate strain. This intermediate strain was also used in the construction of a AflNDK mutation strain. In the second step, the pyrG marker was introduced into the above strains by homologous recombination for complemented strain selection (Fig. S1C) (76).

Point mutations in *A. flavus* strains were constructed using a similar construction method (Fig. S1D). The ndk gene fragment (genomic DNA) was cloned into the pET-28a vector. The point mutation ndk genes were obtained using a site-directed mutagenesis method. The AflNDK mutation cassette carrying mutations in the ndk gene fragment, a selection marker gene (pyrG), and an ndk gene downstream fragment were amplified by fusion PCR and transformed into the intermediate strain that was created during complemented strain construction.

Semiquantitative real-time PCR and Western blotting were conducted to confirm the constructed strains. All the primers used in this study can be found in Table S1.

**Detection of Afndk gene copy number in strains**

Genomic DNA from each strain was extracted from frozen mycelia using phenol/chloroform extraction. Commercial SYBR Green qPCR mix (Takara TaKaRa Biototechnology, Japan) was used in real-time experiments. Primers were designed to amplify short DNA segments in the range of 50–150 bp to build calibration curves for the ndk and SumO genes. The SumO gene, which has been previously reported as a single copy gene in *A. flavus* (13), was selected as a reference gene. qRT-PCR was performed using a Pikoreal 96 real-time PCR system (Thermo Fisher Scientific). Three tests were used for each gene from each experimental strain to ensure good reproducibility.

**Growth, conidia, sclerotia, aflatoxin, and seed infection analysis**

For colony diameter evaluation, PDA media plates were point inoculated with 10⁶ conidia and then incubated at 29 or 37 °C for 4 days. For comparing the production of conidia, 10⁴ conidia were inoculated onto PDA media plates at 29 or 37 °C for 5 days. Three 7-mm diameter plugs from each colony from each plate were collected and homogenized in 3 ml of a 0.05%
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Tween 20 solution for conidia harvesting. The number of conidia was counted using a hemocytometer (75). For sclerotia production analysis, the strains were cultured on CM medium at 37 °C in the dark for 7 days. To visualize sclerotia, the media plates were washed with 75% ethanol to remove conidia and mycelia. Aflatoxin (AF) extraction and examination followed protocols described previously (75). Briefly, all the strains in this study were incubated in 15 ml of YES media at 29 °C in the dark for 5 days. AF was then extracted with chloroform and detected using TLC with an UV light source. Crop seed (peanut and maize) infections and AF extraction from these infected seeds followed previously described procedures for pathogenicity analysis (78). All trials were repeated three times.

Enzymatic assay of recombinant AfNDK

Recombinant WT and mutant proteins (rAfNDK) were prepared according to previous publications (53). The activity of rAfNDK was measured using a two-step assay (21). First, a protein sample was incubated with reaction mixture (50 mM Tris-HCl buffer with 150 mM NaCl, 5 mM dTDP, 100 mM ATP, and 25 mM MgCl₂) at 30 °C for 5 min, and then the first reactions were terminated with the addition of 10% TCA. In the second assay, reactions were initiated in another reaction mixture containing 0.3 mM NADH, pyruvate kinase (2.6 units/ml), lactate dehydrogenase (11 units/ml), 3 mM phosphoenolpyruvate, 25 mM MgCl₂, and 0.1 mM KCl. Reactions were measured using a microplate reader at a detection wavelength of 340 nm, and the yield of ADP from the previous reaction was measured to detect the activity of rAfNDK.

ITC

ITC determinations were performed using a MicroCal™ ITC200 instrument (MicroCal Inc.) equilibrated to a temperature of 25 °C. The receptor protein (rAfNDK), at a concentration of 100 μM in a 200-μl sample cell, was titrated with 1 mM ligand (ADP and GDP). The protein solution was injected with 2 μl of the ligand until a total volume of 40 μl was in the syringe. Titration of the ligand into buffer was performed in order to obtain the buffer correction. The equilibrium association constant (Kₐ) and the reaction enthalpy (∆H) were calculated by fitting the integrated titration peaks using a one-binding–site model in the ITC ORIGIN7 program package. The Gibb's free energy change (∆G) during the reaction was calculated with the formula: ∆G = -RT lnKₐ. The reaction entropy (∆S) was calculated with the formula: ∆G = ∆ - T∆S.

Crystal structure determination and molecular docking with inhibitor

X-ray diffraction data of the rAfNDK protein crystal was collected at 100 K on beam line 17U of the Shanghai Synchrotron Radiation Facility. The diffraction data were scaled and processed using the CCP4i2 DIALS package (56). The crystal structure of rAfNDK was automatically built by the molecular replacement method with the phenix program package, and then manual refinement and model building was performed with COOT. With multiple rounds of manual refinement, the final refined structure was analyzed and displayed using PyMOL software (79). Details of data collection and structural refinement statistics are given in Table S3. Docking of the AZT molecule with rAfNDK was performed using Molegro Virtual Docker (MVD) software. Docking running was guided using default search algorithms (MolDock Optimizer). MolDock Score (GRID) was selected for evaluating AZT molecular poses. The predicted detailed interactions between protein and ligand are shown using PyMOL software.

Animal immunization and Western blotting

For preparation of AfNDK antibody serum, 6-week-old female BALB/c mice (Wushi Animal Laboratory, Shanghai, China) were injected with 60 μg of purified rAfNDK mixed with 150 μl of Quick Adjuvant and 0.9% saline solution twice at 2-week intervals (80). After 4 weeks, blood samples of the immunized mice were harvested. The effect of antibody serum was evaluated by Western blotting (Fig. S9A). All animal experiments obeyed the protocols approved by the Animal Ethics Committee of the Fujian Agriculture and Forestry University.

For total protein extraction, the mycelia of A. flavus were treated by liquid nitrogen freezing, and the protein extracts were dissolved in ice-cold RIPA buffer. The protein extracts or purified recombinant NDK were separated on a 15% SDS-polyacrylamide gel until the dye reached the bottom of the gel. After electrophoresis, the proteins were then transferred to polyvinylidene difluoride (PVDF) membranes. After transfer, the PVDF membranes were incubated with blocking buffer containing 5% skim milk powder in TBS for 1 h at room temperature. The membranes were then incubated with AfNDK antibody serum as a primary antibody at 4 °C overnight. After several washes, the membranes were incubated with horseradish peroxidase–conjugated secondary antibody. In addition, monoclonal rabbit anti-actin antibodies were used as an internal control. Finally, the membranes were washed with TBST extensively, and proteins were detected using a chemiluminescence reagent and visualized with a G:BOX Chemi XT4 (Syngene, UK) (13).

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