Rev Argent Microbiol. 2019;51(4):292–301

asociación argentina de microbiología

REVISTA ARGENTINA DE MICROBIOLOGÍA

www.elsevier.es/ram

ORIGINAL ARTICLE

The bZIP transcription factor Afap1 mediates the oxidative stress response and aflatoxin biosynthesis in *Aspergillus flavus*

Xuanli Guan[a,◊], Yueju Zhao[a,◊], Xiao Liu[a], Bo Shang[a], Fuguo Xing[a], Lu Zhou[a], Yan Wang[a], Chushu Zhang[a,b], Deepak Bhatnagar[c,*], Yang Liu[a,*]

[a] *Institute of Food Science and Technology, Chinese Academy of Agricultural Sciences/Key Laboratory of Agro-products Quality and Safety Control in Storage and Transport Process, Ministry of Agriculture, Beijing 100193, PR China*
[b] *Shandong Peanut Research Institute, Qingdao 266100, China*
[c] *Food and Feed Safety Research, USDA/ARS, Southern Regional Research Institute, New Orleans, LA 70124, USA*

Received 16 March 2018; accepted 15 July 2018
Available online 22 March 2019

KEYWORDS
Aflatoxin;
Oxidative stress;
Afap1;
Aspergillus flavus

Abstract Aflatoxin is a carcinogenic secondary metabolite produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus*, which can seriously endanger the health of humans and animals. Oxidative stress is a common defense response, and it is known that reactive oxygen species (ROS) can induce the synthesis of a series of secondary metabolites, including aflatoxin. By using mutants lacking the *afap 1* gene, the role of *afap 1* gene in oxidative stress and aflatoxin synthesis was assessed. The growth of the mutant strains was significantly inhibited by the increase in the concentration of H_2O_2, inhibition was complete at 40 mmol/l. However, in the quantitative analysis by HPLC, the concentration of AFB1 increased with the increased H_2O_2 until 10 mmol/l. Following an analysis based on the information provided by the NCBI BLAST analysis, it was assumed that Afap1, a basic leucine zipper (bZIP) transcription factor, was associated with the oxidative stress in this fungus. Treatment with 5 mmol/l H_2O_2 completely inhibited the growth of the mutant strains in *afap 1* but did not affect the growth of the CA14PTs strain (non-mutant strain). In addition, the concentration of AFB_1 in the mutant strains was approximately ¼ of that observed in the CA14PTs strain. These results suggested that Afap1 plays a key role in the regulation of oxidative stress and aflatoxin production in *A. flavus*.

* Corresponding authors.
E-mail addresses: deepak.bhatnagar@ars.usda.gov (D. Bhatnagar), liuyang01@caas.cn (Y. Liu).
◊ These authors contributed equally to this work.

https://doi.org/10.1016/j.ram.2018.07.003

PALABRAS CLAVE
Aflatoxina;
Estrés oxidativo;
Afap1;
Aspergillus flavus

El factor de transcripción bZIP Afap1 afecta al estrés oxidativo y la biosíntesis de aflatoxinas en *Aspergillus flavus*

Resumen La aflatoxina es un metabolito secundario cancerígeno producido principalmente por *Aspergillus flavus* y *Aspergillus parasiticus*, que pone en riesgo grave a la salud de los humanos y los animales. El estrés oxidativo es una respuesta de defensa común, y es sabido que las especies reactivas de oxígeno (ROS) pueden inducir la síntesis de una serie de metabolitos secundarios, incluida la aflatoxina. Empleando mutantes carentes del gen *afap1* se evaluó el papel de Afap1 en el estrés oxidativo y la síntesis de aflatoxinas. El crecimiento de las cepas mutadas se vio significativamente inhibido con el aumento de la concentración de H_2O_2, la inhibición fue completa a 40 mmol/l. Sin embargo, en el análisis cuantitativo por HPLC, la concentración de la aflatoxina AFB_1 aumentó con el aumento de la concentración de H_2O_2 hasta 10 mmol/l. Tras un análisis apoyado en la información provista por la herramienta NCBI BLAST, se supuso que Afap1, un factor de transcripción de la cremallera de leucina básica (bZIP), estaba asociado con el estrés oxidativo en este hongo. El tratamiento con 5 mmol/l de H_2O_2 inhibió completamente el crecimiento de las cepas mutantes en *afap1*, pero no afectó el crecimiento de la cepa CA14PTs (cepa no mutada). Además, la concentración de AFB_1 en las cepas mutadas fue de aproximadamente 1/4 de la observada en CA14PTs. Estos resultados sugieren que Afap1 juega un papel clave en la regulación del estrés oxidativo y la producción de aflatoxinas en *A. flavus*.

Introduction

Aflatoxin is a secondary metabolite produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus*. As the most potent naturally-occurring toxic and carcinogenic substance, aflatoxin causes an estimated 28% of hepatocellular carcinoma (HCC), while HCC is the most common form of liver cancer in the world[39] and the case rate is very high in sub-Saharan Africa, the Western Pacific region and Southeast Asia, as well as in Central America. Individuals with liver damage due to hepatitis B virus (HBV) infection are particularly vulnerable to aflatoxin invasion[14]. In addition, aflatoxin can lead to dysfunction of the immune system, dysplasia in children, and even death due to acute poisoning[10,18]. Aflatoxin contamination occurs in a wide range of food and feed commodities, including wheat, maize, peanuts, rice, peanut oil, cotton seed, milk, nuts and dairy products[2]. Therefore, aflatoxin not only poses a serious threat to human and animal health, but also causes huge economic losses.

The gene cluster involved in aflatoxin biosynthesis has been identified[3,5,9,13,22,41,44]. Most gene functions have been clarified[1,9]. AflR and AflS are two key transcription factors. The aflatoxin regulatory gene *aflR* activates the transcription of other structural genes in the aflatoxin biosynthesis pathway by encoding a positive regulatory factor[6]. AflS is adjacent to AflR and participates in the regulation of aflatoxin biosynthesis together with AflR. The combination of AflS and AflR forms a complex, which is then bound together in the promoter region of each structural gene in the cluster[7]. In addition to *aflR* and *aflS*, there are many regulatory genes involved in aflatoxin biosynthesis regulation outside the aflatoxin gene cluster. *laeA* and *veA* encode global transcription factors that regulate the biosynthesis of many secondary metabolites, such as aflatoxin, sterigmatocystin, and penicillin[4,11,12,19].

There is extensive evidence that secondary metabolism is associated with oxidative stress in filamentous fungi and plants[16,27,36]. Based on this view, different oxidative stimuli, such as peroxides and diamide, can activate a variety of transcription factors, and many transcription factors have been proved to be involved in regulating secondary metabolism in yeast, fungi and plants. Within this network, the well-known Ap-1 transcription factor Yap-1 participated in the cellular response to oxidative stress signal in *Saccharomyces cerevisiae*[25,33].

Like the Yap-1 roles in yeast[26], several Yap-1 homologue transcription factors have been identified in filamentous fungi, and they are usually associated with resistance to H_2O_2 or antifungals. In the rice blast fungus *Magnaporthe oryzae*, Moap1 mediates the oxidative stress response and is necessary for conidia formation, apical growth and pathogenicity[15]. Afyap1 in *Aspergillus fumigates* was found to be associated with tolerance to oxidative stress[29]. NapA and RsmA affect stress response, sexual development and secondary metabolism in *Aspergillus nidulans*[42]. In *Aspergillus ochraceus*, Aoyap1 not only participated in the oxidative stress response, but also regulated ochratoxin A biosynthesis. Similarly, to Aoyap1, ApyapA in *A. parasiticus* also participated in the oxidative stress response and in the modulation of aflatoxin biosynthesis[30]. These findings suggested a probable similar link between the oxidative stress response and mycotoxin biosynthesis. However, under the oxidative stress condition, the mechanism of *yap-1* homologue gene in the regulation of aflatoxin biosynthesis in *A. flavus* is not clear.

In this paper, *afap1*, the homologue of *yap-1*, was suggested to encode protein containing conserved bZIP domains based on the NCBI BLAST analysis. We engineered genetically modified strains of *A. flavus* lacking *afap1* and showed the key role played by *afap1* in response to oxidative stress and in the regulation of aflatoxin biosynthesis.

Materials and methods

Strains and growth conditions

The toxigenic *A. flavus* CA14PTs (Δ*ku70*, Δ*niaD*) and recipient (Δ*ku70*, Δ*niaD*, Δ*pyrG*) strains were obtained from Dr. Perng Kuang Chang, United States Department of Agriculture, New Orleans, USA. The strain *A. nidulans* WJAO1 was obtained from Prof. Shihua Wang, Fujian Agriculture and Forestry University, Fuzhou, China.

Strains were activated on potato dextrose agar (PDA) plates (20 g/l dextrose, 200 g/l peeled potatoes and 20 g/l agar) at 28 °C in the dark for 3 days for conidia production. Conidial suspensions were collected from sporulated cultures of fungi on PDA plates by surface washing with sterile deionized water containing 0.1% Tween-20. The number of conidia in the suspensions was counted using a hemocytometer and diluted to 10^6 CFU/ml with 0.1% Tween-20 solution. Conidia were cultivated in 50 ml YES medium (150 g/l sucrose, 20 g/l yeast extract and 1 g/l $MgSO_4 \cdot 7H_2O$ and solid medium supplemented with 16 g/l agar) and grown at 28 °C on a rotary incubator in the dark for AFB_1 concentration detection and mycelia collection. The recipient strain was grown in broth containing yeast, glucose, trace element solution, uracil, uridine (YGTUU) (20 g/l glucose, 5 g/l yeast extract, 1 ml trace element solution per liter of medium, 1 g/l uracil and 1 g/l uridine and solid medium supplemented with 15 g/l agar) at 28 °C for mycelial growth and conidia production. Czapek-Dox medium (Difco) supplemented with 3% sucrose was used for mutant selection.

Hydrogen peroxide sensitivity analysis

Five microliters of conidia (10^6 CFU/ml medium) from each strain were incubated in YES solid medium supplemented with different concentrations of H_2O_2 (0, 5, 10, 20, and 40 mmol/l) for the oxidative stress response assay. All plates were cultivated in the dark at 28 °C for 5 days, and colonies were photographed. Meanwhile, 5 ml of conidial suspension (10^6 CFU/ml medium) was cultured in 50 ml YES liquid medium supplemented with the same concentrations of H_2O_2 for AFB_1 concentration and the mycelial dry weight analysis. All experiments were performed in triplicate in three independent experiments.

AFB_1 concentration and fungi mycelial dry weight analysis

Mycelia were collected and mycelial dry weight was measured after drying in a dryer (HASUC, Inc., Shanghai, China) at 65 °C for 72 h. AFB_1 concentration in the culture filtrate was determined by extracting metabolites from the filtrate using methanol, followed by purification using an immunoaffinity column (Romer Labs, Inc., Tulln, Austria) according to the manufacturer's instructions. AFB_1 concentration was detected by high-performance liquid chromatography (HPLC; Agilent Series 1260; Agilent Technologies, Santa Clara, CA, USA). HPLC was performed on an Agilent C18 Zorbax XDB column (150 mm × 4.6 mm × 5 mm, Agilent Technologies), and detection was performed using a fluorescence detector (Agilent 1260; Agilent Technologies) with an excitation wavelength of 360 nm and an emission wavelength of 440 nm at 30 °C. The mobile phase consisted of methanol/H_2O (7:3, v/v) injected at a flow rate of 1 ml/min.

RNA extraction and quantification of gene expression by quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from fungal mycelia collected from YES liquid medium using a RNeasy mini kit (Qiagen, Germany) according to the manufacturer's instructions. RNA samples were treated with DNA-free DNase. The purity and concentrations of RNA were determined by measuring the absorbance of samples at 260 and 280 nm using spectrophotometric quantification in a Beckman DU800 spectrophotometer (Beckman, USA). qRT-PCR was carried out in triplicate in 20-μl volumes using Power SYBR green master mix (Applied Biosystems, USA) in an ABI 7500 Real-Time PCR System (Applied Biosystems, USA). The thermal-cycling program was set as follows: 95 °C for 5 min, followed by 40 cycles of 95 °C for 25 s, 55 °C to 60 °C for 25 s (optimized for each primer pair), and 72 °C for 35 s, with a melting-curve stage at 95 °C for 15 s, 60 °C for 1 min, 95 °C for 30 s, and 80 cycles of 60 °C for 15 s. Gene-specific primers were designed for each target gene using Primer 5.0 (http://www.premierbiosoft.com/primerdesign/) (Table 1). As an endogenous control, primers 18S-F (5′-GCTCTTTTGGGTCTCGTAATTGG-3′) and 18S-R (5′-CGCTATTGGAGCTGGAATTACC-3′) were used based on previous studies in order to cover 154 bp of the 18S RNA gene[21]. Samples from each of the three biological replicates were assayed in triplicate, and data were analyzed using the ABI 7500 SDS program (Applied Biosystems, USA) by the $2^{-\Delta\Delta Ct}$ method.

Identification of Afap1

The sequence of Afap1 and its homologues in *S. cerevisiae*, *A. parasiticus*, *A. nidulans* and *Aspergillus fumigatus* were used as input for BLAST in the National Center for Biotechnology Information (NCBI) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi), to identify sequences with high similarities in the translated genome of *A. flavus*. Multiple sequence alignments were carried out using DNAssist 2.2.

Construction of the mutant strain

The deletion-mutant strain (Δ*afap1*) was constructed as previously described[8,37]. A homologous transformation system for *A. flavus* with the *pyrG* gene as selection marker was used in this study[35]. The *pyrG* gene encodes an orotidine-5′-phosphate decarboxylase, which is a key gene

Table 1 Primers used in this study

Primer name	Nucleotide position[a]	Sequence (5′–3′)
aflD-F	469	ATGCTCCCGTCCTACTGTTT
aflD-R	555	ATGTTGGTGATGGTGCTGAT
aflB-F	2553	ATGCATTTGTTCAGCCGACGTGAC
aflB-R	2657	TGTCCAATTACCGCCTCCACATCT
aflR-F	1236	CCTTTCTCACTACTCGGGTTT
aflR-R	1303	GCAGGTAATCAATAATGTCGG
aflJ-F	486	GAGAACCAACCGCTCATCC
aflJ-R	640	CCGTGTACTGCTCCTTCAAAT
aflM-F	−102	GAGCCAAAGTCGTGGTGAAC
aflM-R	−22	GCCTGGATTGCGATAGCGTC
aflO-F	411	GACATCTCGGGACCTTGC
aflO-R	579	ACGCTCCAGAGCCATTAA
aflP-F	41	CACGCTTTCAGAGCAGGTAA
aflP-R	131	TTCGGTGGAGGAGGGAGTT
aflX-F	282	ACCAATGCCGTGCTCAAT
aflX-R	417	ACCAGCGGCTATGTTGAG
LaeA-F	685	ACGAAAGAAAGGTTGCTCGCTGGTA
LaeA-R	810	TTTGTTGAACGCCTCCGACTTGAC
AtfA-F	840	TTCGCTGTTTCCAATCAGTCGTCAA
AtfA-R	977	TTCTTCCCTTTCGTGGCAGGTTTT
Afap1-F	56	AGACCTTCTTCTTGCCGCTCTTTCC
Afap1-R	219	CATCATCTCCGTAACCCAATCCACC
AtfB-F	399	TCCAGTATTGACCGGTGTGC
AtfB-R	583	TTCGATGGCATTGGGTTGGA
TcsA-F	874	GGGCAGAGGCCGTTAGGCTAGTGA
TcsA-R	1063	CACAGGCTTTCCCAGATAGTCGTTCATAC
Bos1-F	1613	TGTGAAACTCGCCGATCAGTCTTTG
Bos1-R	1800	TCCTTGCCAGACGGATCTTGGAT
SrrA-F	1141	TCGCAGACAATCGGAAGAAGAGTAACCA
SrrA-R	1459	GGCAATGATCGGCGTCCTATCAAACT
Up-F	−1424	TGTTTCACGCTGACATCCATCCG
Up-R	−265	AGGATAGAGAGCATTGTCTGGCTTCCCGCCCGTTCGTTCTAT
Down-F	+866	CATTGTAGTAGTCAGGTTCCTTCATCATCGCACCATCTTCATCTCA
Down-R	+1912	AGCCTCAACCTCAGCAGGGACAA
Middle-F	218	CGGTGGATTGGGTTACGGAGATG
Middle-R	922	CCTGGAACTTGGGAGTCGCTTGT
pyrG-F	−453	CAGACAATGCTCTCTATCCT
pyrG-R	+280	GGAACCTGACTACTACAATG

[a] The meaning of Nucleotide position is the position of primer. ''+'', the number of nucleotides downstream of the sequence; ''−'', the number of nucleotides upstream of the sequence; the other numbers are the number of nucleotides from the initiation codon ''ATG'' of the sequence.

in the synthesis of uracil nucleotides. The recipient strain CA14PTs (Δ*ku70*, Δ*niaD*, Δ*pyrG*) with the *pyrG* deletion cannot grow on the transformant selection medium Czapek-Dox without adding uracil and uridine, while homologous transformants carrying the *pyrG* gene instead of *afap1* could survive on the selection medium. The detailed procedure is as follows: Genomic DNA was extracted from mycelia grown for 5 days in 50 ml of YES liquid medium using benzyl chloride[38,45]. For the homologous fragments, the 5′ and 3′ regions of *afap1* (1159 and 1046 bp, respectively) were amplified with specific primer pairs Up-F/R and Down-F/R (Table 1), which contain sequences that overlap the marker gene, and were verified by sequencing. The 1600 bp *pyrG* gene was amplified with primer pairs *pyrG*-F/R from *A. nidulans* WJAO1 genomic DNA. The PCR-fusion product was constructed and transformed into recipient strain protoplasts using polyethylene glycol buffer (15mM KCl, 20 mM CaCl2 and 1M Tris-HCl buffer, pH 7.5), and 500 g/l PEG 4000. The cell suspension was plated on Czapek-Dox medium at 28 °C in the dark for 5 days. Putative mutants were confirmed by PCR using primer pairs Middle-F/R and Up-F/*pyrG*-R and sequencing analysis.

Southern blot

Transformants that passed the PCR pre-screening were further checked by Southern blot analysis, using the DIG system (Roche, Germany) in accordance with a previously described protocol[24]. Ten μg genomic DNA was digested

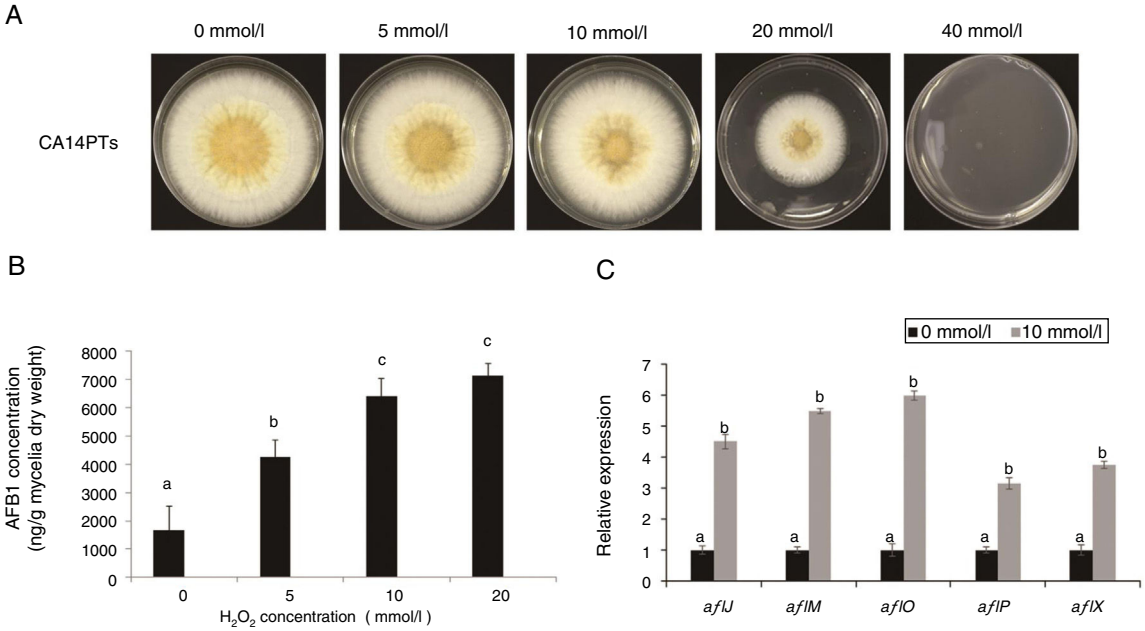

Figure 1 Colony growth and aflatoxin production after 0, 5, 10, 20 and 40 mmol/l H_2O_2 treatment. The *A. flavus* toxigenic strain CA14PTs was grown for 5 d at 28 °C in darkness. (A) View from top of colony. (B) Determination of AFB_1 concentration per unit of mycelial weight by HPLC in response to H_2O_2. Different letters indicate that there were statistically significant differences ($p = 0.05$). (C) Relative expressions of aflatoxin synthesis genes. Samples were harvested after H_2O_2 treatment (10 mmol/l), and gene expression was measured by quantitative real-time PCR. Standard errors of the mean are shown (n = 3). Different letters indicate that there were statistically significant differences ($p = 0.05$).

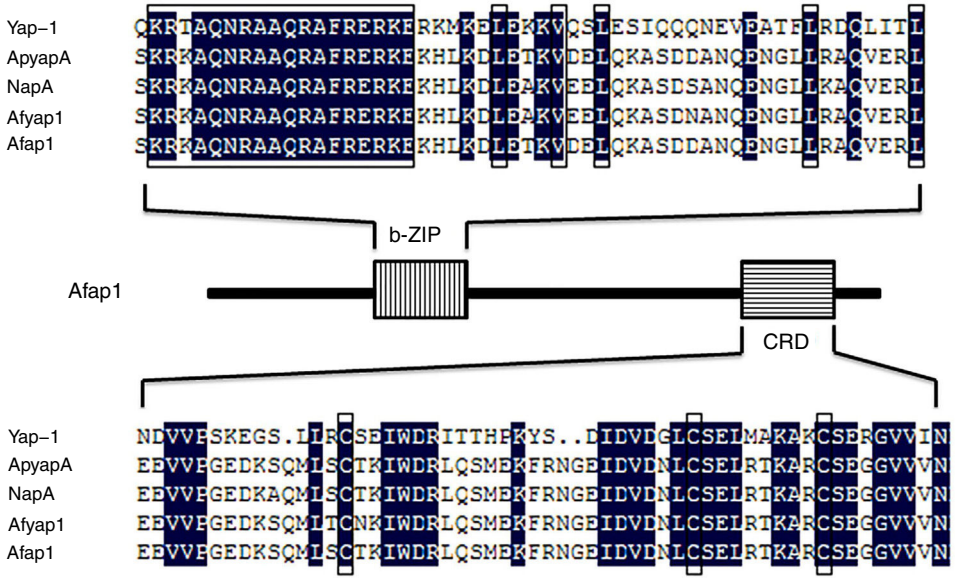

Figure 2 Amino acid sequence alignment of the characteristic domain. Yap-1 is from *S. cerevisiae* S288c, ApyapA is from *A. parasiticus* SU-1, NapA is from *A. nidulans* FGSC A4, Afyap1 is from *A. fumigatus* Af293, Afap1 is from *A. flavus*. The bZIP domain is shown as a vertical line box, and the cysteine-rich domain (CRD) as a horizontal line box. The amino acid sequence of these domains of Afap1 is aligned with those of Yap-1, ApyapA, NapA, and Afyap1. Boxes indicate conserved regions of different functional domains.

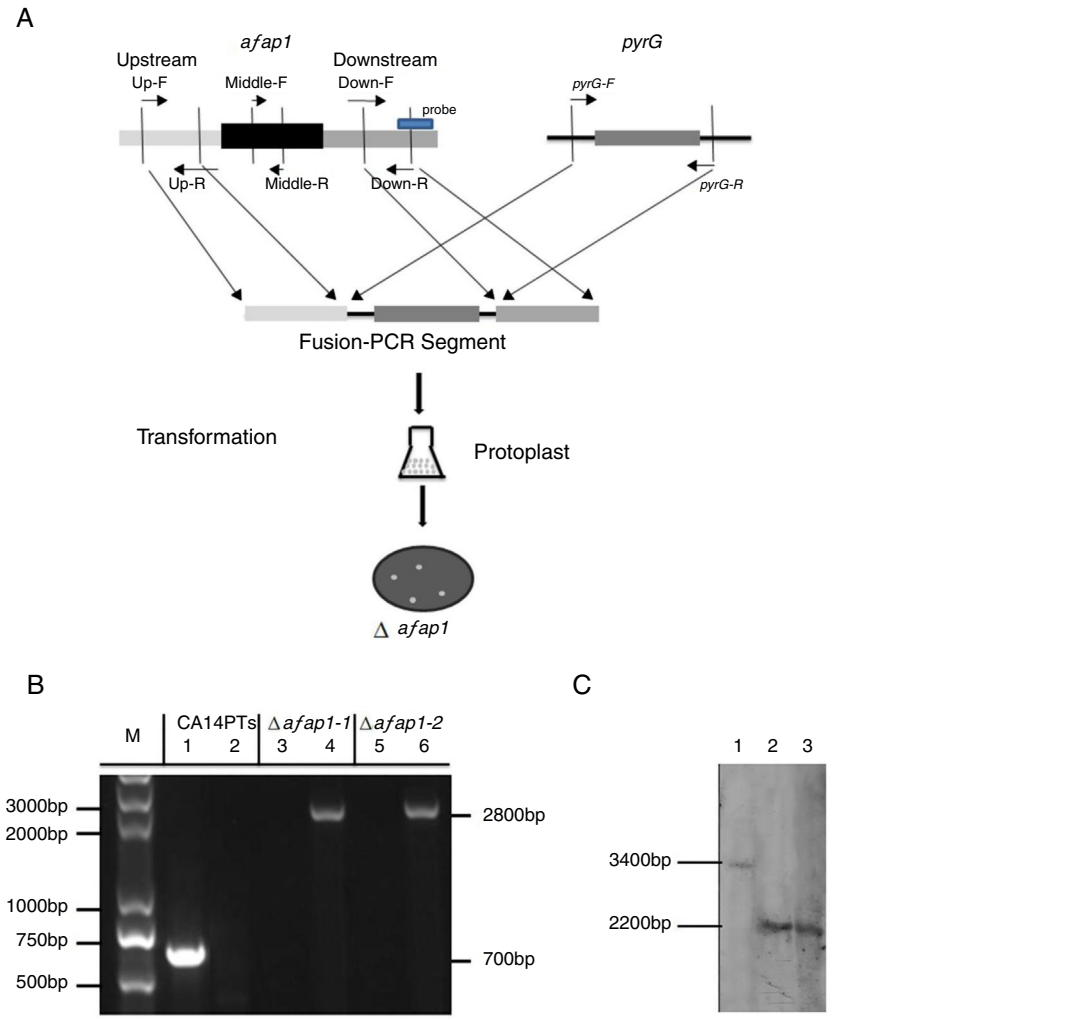

Figure 3 Construction and verification of the *afap1*-deletion mutants. (A) *Afap1* gene-replacement strategy. Primers are shown in Table 1. (B) PCR confirmation of the Δ*afap1* mutants. Lanes 1, 3 and 5 results are from the use of the Middle-F and Middle-R primers, and lanes 2, 4 and 6 results are from the use of the Up-F and *pyr*G-R primers. M, DNA marker; lanes 1 and 2, CA14PTs; lanes 3 and 4, Δ*afap1-1*; lanes 5 and 6, Δ*afap1-2*. (C) Southern blot hybridization. Lanes 1, CA14PTs; lanes 2, Δ*afap1-1*; lanes 3, Δ*afap1-2*. In CA14PTs, probe reveals a fragment of 3.4 kb when digested with *Hind*III. In the mutant strains Δ*afap1*, probe reveals a fragment of 2.2 kb when digested with *Hind*III.

with *Hind*III (Takara, Japan), and then electrophoresed on a 1% agarose gel to separate by size. A sheet of nylon membranes (Hybond N^+, Pharmacia, USA) was placed on top of the gel for DNA transference. The hybridization probe with DIG-labeled was synthesized with PCR DIG probe synthesis kit (Roche, Germany) by following the manufacturer's protocol. The probe matched the downstream sequence of the homology arm in the homologous recombination fragment was generated by PCR amplification using primers 5′-AACGTGGTTGTATTTGCCCC-3′ and 5′-GCTCTTGGACAATGCTCTCG-3′.

Statistical analysis

Statistical analyses of the obtained data were performed using SPSS 21.0 software (IBM, Chicago, IL, USA). Differences between the means were evaluated by a one-way analysis of variance (ANOVA), and in all cases, statistical significance was established at $p < 0.05$.

Results

Effect of different oxidative stress on *A. flavus* growth and AFB_1 production

Oxidative stress is a very important environmental stimulus for fungi. To evaluate the effect of oxidative stress on the growth and AFB_1 production of *A. flavus*, H_2O_2 solutions at different concentrations (0, 5, 10, 20, and 40 mmol/l) were added to YES plates and liquid culture medium, respectively. The growth of *A. flavus* CA14PTs was significantly inhibited by the increased H_2O_2 and were completely inhibited at 40 mmol/l (Fig. 1A). The AFB_1 concentration in YES broth was increased after the treatment of H_2O_2 at the concentration of 5, 10 and 20 mmol/l, respectively (Fig. 1B). There is no

obvious increase for AFB_1 concentration at 20 mmol/l H_2O_2 compared with 10 mmol/l. In addition, the expression levels of key aflatoxin biosynthetic structural genes (*aflJ*, *aflM*, *aflO*, *aflP* and *aflX*) were up-regulated by the treatment of 10 mmol/l H_2O_2 according to qRT-PCR (Fig. 1C). The data based on the results indicated that oxidative stress could affect strain growth and stimulate aflatoxin biosynthesis.

Identification of Afap1, a Yap-1 homologue in *A. flavus*

By the NCBI BLAST analysis, Afap1 was identified as a putative bZIP transcription factor. Alignment of the Afap1 protein sequence to those of Yap-1 (*S. cerevisiae* S288c), ApyapA (*A. parasiticus* SU-1), NapA (*A. nidulans* FGSC A4) and Afyap1 (*A. fumigatus* Af293) (Fig. 2) showed two conserved domains: A C-terminal nuclear export signal (NES) embedded in a characteristic cysteine-rich domain (c-CRD) and a N-terminal basic leucine zipper domain (bZIP domain). Afap1 has lower homology (16.22% similarity) with its yeast orthologues, but it has higher homology with other filamentous ascomycetes. The conserved bZIP domain and cystein-rich domain (CRD) suggested that Afap1 has a similar role in response to oxidative stress and toxin biosynthesis in *A. flavus* as other homologue proteins.

Confirmation of Δ*afap1* deletion mutants

To analyze the role of *afap1* in oxidative-stress response and aflatoxin biosynthesis, *Δafap1* mutants were generated using homologous recombination (Fig. 3A). Two transformants (*i.e.*, *Δafap1-1* and *Δafap1-2*) were selected for further PCR verification. A 700 bp fragment, encoding for partial ORF of *afap1*, could be amplified with primers Middle-F/R in CA14PTs but not in the positive transformants. A 2800 bp fragment, encoding for *pyrG* and upstream of *afap1*, could only be amplified with primers Up-F/*pyr*G-R in positive transformants. As shown in Figure 3B, only the 2800 bp fragment was observed in *Δafap1-1* and *Δafap1-2*. Further sequencing analysis showed that the gene *afap1* was exactly replaced by *pyrG* in these two mutants. Additionally, southern blot hybridization revealed a 3.4 kb fragment and a 2.2 kb fragment in CA14PTs and the *afap1* mutants when digested with *Hind*III, respectively (Fig. 3C). It is confirmed that there are sequence differences between the *afap1* mutants and CA14PTs as expected. Combined with the above homologous recombination strategy and PCR analysis, it was shown that *afap1* was properly deleted and mono-copy.

Effects of *afap1* deletion on sensitivity to oxidative stress

CA14PTs and the *Δafap1* mutants were incubated in YES plates supplemented with different concentration of H_2O_2 (Fig. 4A). The growth rates of the CA14PTs and *Δafap1* mutants were similar on YES plates without H_2O_2. However, the growth of *Δafap1* mutants was completely inhibited by 5 mmol/l H_2O_2. In contrast, the growth of CA14PTs was not inhibited even at 10 mmol/l H_2O_2. Meanwhile, AFB_1 concentration of the *Δafap1* mutants was significantly decreased by around 75%, which compared to CA14PTs ($p < 0.01$) (Fig. 4B). In addition, the expression levels of the key transcription factors genes (*tcsA*, *bos1*, *atfB* and *srrA*) related to the oxidative-stress response and aflatoxin biosynthetic genes (*aflD*, *aflB*, *aflR*, *aflS*, *aflM* and *aflP*) in YES liquid medium were detected using qRT-PCR. The expression of *tcsA*, *bos1*, *srrA*, *aflB* and *aflR* was significantly up-regulated in the *Δafap1* mutants compared to CA14PTs, and the expression of *aflM* and *aflP* were significantly down-regulated. The expression of *atfB* was up-regulated in the *Δafap1* mutants, although the difference was not significant (Fig. 5).

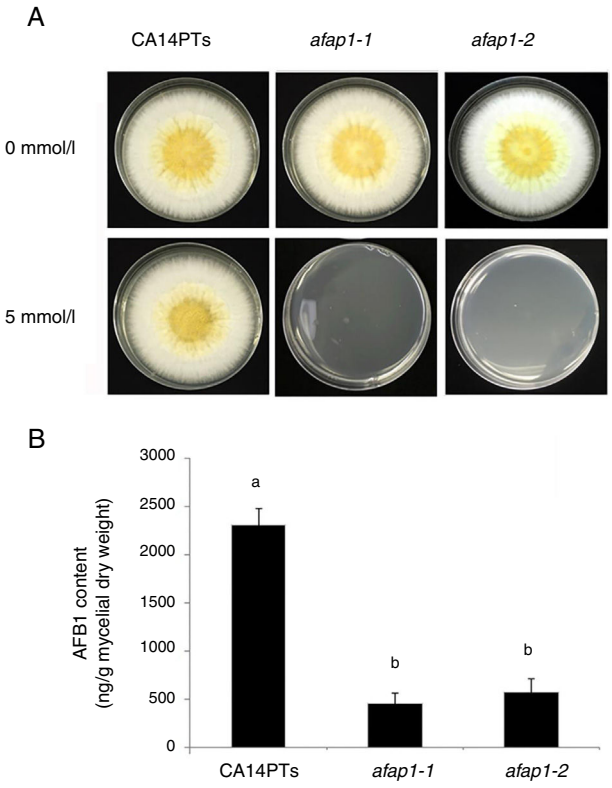

Figure 4 Defects of CA14PTs and *Δafap1* mutants in response to different oxidative stress. (A) CA14PTs and *Δafap1* mutants were separately incubated in YES medium under different oxidative-stress conditions for 5 days. (B) HPLC analyses of AFB_1 concentration per unit of mycelial weight. Different letters indicate that there were statistically significant differences ($p = 0.05$).

Discussion

Oxidative stress is one of the earliest responses and a common cell defense mechanism in living things. Cellular response to oxidative stress plays a crucial role in plants, vertebrates and fungi; it enables the cell to survive a variety of extra- and intracellular oxidative stressors. The classical review of the oxidative stress response in fungi was developed based on research in yeast which showed that regulation of defense-related antioxidant genes contributed to the survival of the organism. The regulation of secondary metabolism is closely linked to the cellular response

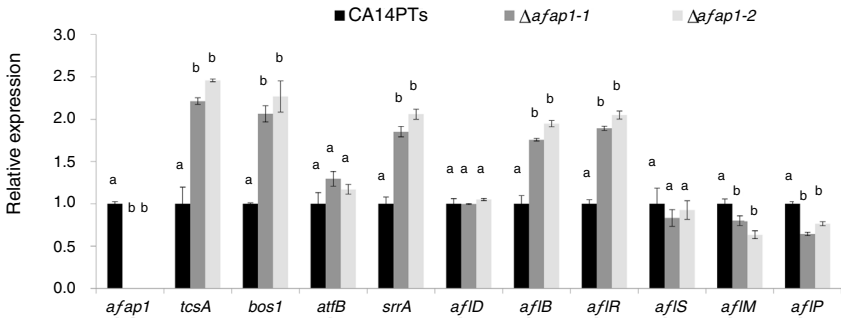

Figure 5 Quantitative real-time PCR analyses of genes related to oxidative stress and aflatoxin biosynthesis in the Δ*afap1* mutants as compared with those in CA14PTs. All data represent the means of three independent samples, and standard errors of the means are shown (n = 3). Different letters indicate that there were statistically significant differences ($p = 0.05$).

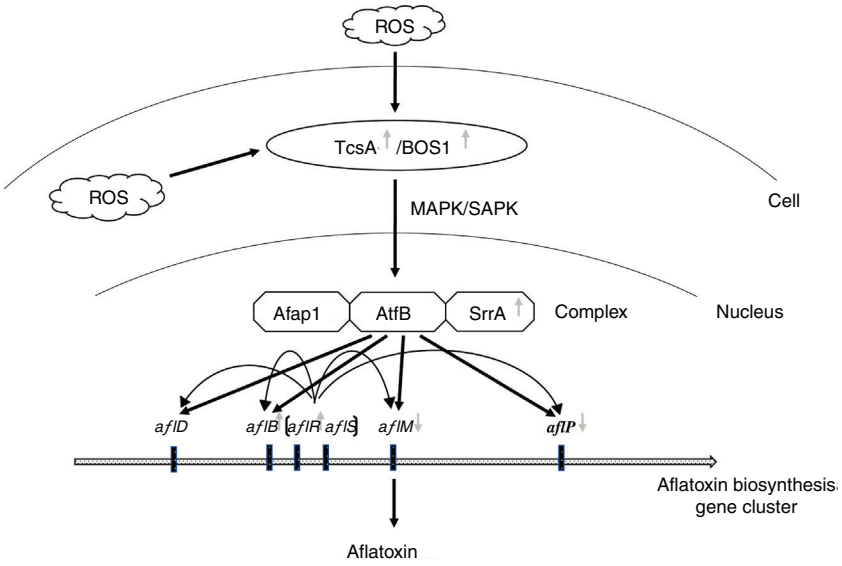

Figure 6 Hypothetical oxidative stress-activated signaling pathway in *A. flavus*. Based on available experimental evidence, we speculate that exposure of the fungal cell to intra- or extracellular ROS activates signaling cascade.

to oxidative stress in filamentous fungi and contributes to the complexity of the response[16]. However, this response is most complicated and robust than that of yeast in response to various environmental conditions.

Previous reports strongly suggested that several transcription factors associated with the Stress Activated Protein Kinase/Mitogen-Activated Protein Kinase (SAPK/MAPK) pathway coordinate the transcriptional level of secondary metabolism genes and antioxidant enzymes, thereby controlling the metabolic processes in cellular stress response. Ap-1 family is one of the most important transcription factors. Ap-1 family have many homologous proteins in *S. cerevisiae*[33] and *Aspergillus* spp.[30,31]; however, its role in toxin biosynthesis and virulence is divergent[28]. In *A. parasiticus* and *A. ochraceus*, deletion of *apyapA* and *aoyap1* resulted in increases of aflatoxin and ochratoxin, respectively[30,32]. In *Fusarium graminearum*, the *Δfgap1* mutant showed higher sensitivity to oxidative stress (H_2O_2) and higher level of trichothecene concentration associated with overexpression of *TRI* genes. However, the activation mechanism of toxin accumulation in response to oxidative stress was not observed[25]. In contrast, in *A. nidulans*, deletion or overexpression of *napA* led to a decreased tolerance to oxidative stress and sterigmatocystin synthesis[42]. In this study, the growth of CA14PTs was significantly inhibited following treatment with 20 mmol/l H_2O_2, whereas growth of the knockout mutants was completely inhibited following treatment with only 5 mmol/l H_2O_2 due to the lack of a key transcription factor Afap1 related to the oxidative-stress response. The effect of deletion of *afap1* is similar with *napA* and is contrary to *aoyap1*, *apyapA* and *fgap1*.

Hong et al. (2013) proposed that SrrA (SrrA recruits AP-1) and AtfB combined with the promoter regions of aflatoxin biosynthetic genes to help their induction by transcription factor AflR[17]. Moreover, AtfB has been proved to combine with the promoter regions of aflatoxin biosynthetic genes including *aflB (fas-1)*, *aflD (nor-1)*, *aflM (ver-1)* and *aflP (omtA)*, which carry CRE sites[17,34]. In addition, the sensor kinases TcsA transmit oxidative stress signals through SrrA and/or SskA response regulators[23], and then cooperate with Ap-1 against oxidative stress in other *Aspergillus* spp.[16]. Therefore, a similar pathway may have *A. flavus* since oxidative stress signals were transmitted through sensor kinases (ortholog of TcsA or Bos1 in yeast) to AtfB-SrrA-Afap1 homo-

logous complex, and then induced aflatoxin biosynthesis (Fig. 6). In this study, the expression of *tcsA*, *bos1*, *srrA* and *aflR* was up-regulated in the *Δafap1* mutants compared to CA14PTs. *aflB*, encoding fatty acid synthase and being close to *aflR* in the aflatoxin gene cluster, was also up-regulated in the *Δafap1* mutants. However, the expression of *aflM* and *aflP*, two downstream structural genes, were significantly down-regulated in the *Δafap1* mutants, which resulted in a down-regulation of aflatoxin production. On the other hand, interestingly, the gene expression of *aflS* was down-regulated in the knockout mutants. Down-regulation of *aflS* led to the decreased production of AflS protein, which is beneficial for some potential suppressors to bind to AflR in place of AflS. Consequently, the transcription of the aflatoxin biosynthesis gene, which relies on AflS-AflR, would be reduced and aflatoxin biosynthesis would decrease. There were similar findings in some previous studies[20,40,43].

The present study revealed that oxidative stress inhibited the growth of toxigenic strains and was completely inhibited at 40 mmol/l H_2O_2. However, the AFB_1 concentration was increased until 10 mmol/l. According to the NCBI BLAST analysis, transcription factor Afap1 has the conserved protein domains of other AP-1 homologue proteins. Deletion of *afap1* resulted in an increase in sensitivity to oxidative stress and a decrease in aflatoxin production in *A. flavus*. These results suggested that *afap1* plays a key role in tolerance to oxidative stress and promoted aflatoxin production in *A. flavus*.

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

We gratefully acknowledge the financial support of National Key R&D Program of China (2017YFC1600903), National Program of China Basic Science and Technology Research (2013FY113400).

References

1. Amaike S, Keller NP. *Aspergillus flavus*. Annu Rev Phytopathol. 2011;49:107–33.
2. Bhatnagar D, Brown R, Ehrlich K, Cleveland TE. Mycotoxins contaminating cereal grain crops: their occurrence and toxicity. Appl Mycol Biotechnol. 2002;2:171–96.
3. Calvo AM. The VeA regulatory system and its role in morphological and chemical development in fungi. Fungal Genet Biol. 2008;45:1053–61.
4. Calvo AM, Bok J, Brooks W, Keller NP. VeA is required for toxin and sclerotial production in *Aspergillus parasiticus*. Appl Environ Microbiol. 2004;70:4733–9.
5. Calvo AM, Wilson RA, Bok JW, Keller NP. Relationship between secondary metabolism and fungal development. Microbiol Mol Biol Rev. 2002;66:447–59.
6. Chang PK, Ehrlich KC, Yu JJ, Bhatnagar D, Cleveland TE. Increased expression of *Aspergillus parasiticus* aflR, encoding a sequence-specific DNA-binding protein, relieves nitrate inhibition of aflatoxin biosynthesis. Appl Environ Microbiol. 1995;61:2372–7.
7. Chang PK. The *Aspergillus parasiticus* protein AFLJ interacts with the aflatoxin pathway-specific regulator AFLR. Mol Gen Genomics. 2003;268:711–9.
8. Chang PK, Scharfenstein LL, Wei Q, Bhatnagar D. Development and refinement of a high-efficiency gene-targeting system for *Aspergillus flavus*. J Microbiol Methods. 2010;81:240–6.
9. Cleveland TE, Yu J, Fedorova N, Bhatnagar D, Payne GA, Nierman WC, Bennett JW. Potential of *Aspergillus flavus* genomics for applications in biotechnology. Trends Biotechnol. 2009;27:151–7.
10. Diaz DE, Hagler WM, Hopkins BA, Whitlow LW. Aflatoxin Binders I: in vitro binding assay for aflatoxin B_1 by several potential sequestering agents. Mycopathologia. 2002;156:223–6.
11. Duran RM, Cary JW, Calvo AM. Production of cyclopiazonic acid, aflatrem, and aflatoxin by *Aspergillus flavus* is regulated by *veA*, a gene necessary for sclerotial formation. Appl Microbiol Biotechnol. 2006;73:1158–68.
12. Duran RM, Cary JW, Calvo AM. The role of veA in *Aspergillus flavus* infection of peanut, corn and cotton. Open Mycol J. 2009;3:27–36.
13. Georgianna DR, Payne GA. Genetic regulation of aflatoxin biosynthesis: from gene to genome. Fungal Genet Biol. 2009;46:113–25.
14. Groopman JD, Kensler TW, Wild CP. Protective interventions to prevent aflatoxin-induced carcinogenesis in developing countries. Annu Rev Public Health. 2008;29:187–203.
15. Guo M, Chen Y, Du Y, Dong Y, Guo W, Zhai S, Zhang H, Dong S, Zhang Z, Wang Y, Wang P, Zheng X. The bZIP transcription factor MoAP1 mediates the oxidative stress response and is critical for pathogenicity of the rice blast fungus *Magnaporthe oryzae*. PLoS Pathog. 2011;7:e1001302.
16. Hong SY, Roze LV, Linz JE. Oxidative stress-related transcription factors in the regulation of secondary metabolism. Toxins. 2013;5:683–702.
17. Hong SY, Roze LV, Wee J, Linz JE. Evidence that a transcription factor regulatory network coordinates oxidative stress response and secondary metabolism in *Aspergilli*. MicrobiologyOpen. 2013;2:144–60.
18. Jegorov A, Šimek P, Heydová A, Cvak L, Minář J. Applications of fungal ecology in the search for new bioactive natural products. In: Environmental and microbial relationships. Berlin/Heidelberg: Springer; 2007. p. 257–83.
19. Kato N, Brooks W, Calvo AM. The expression of Sterigmatocystin and Penicillin genes in *Aspergillus nidulans* is controlled by *veA*, a gene required for sexual development. Eukaryotic Cell. 2003;2:1178–86.
20. Kong Q, Chi C, Yu J, Shan S, Li Q, Guan B, Nieman WC, Bennett JW. The inhibitory effect of *Bacillus megaterium* on aflatoxin and cyclopiazonic acid biosynthetic pathway gene expression in *Aspergillus flavus*. Appl Microbiol Biotechnol. 2014;98:5161–72.
21. Liang D, Xing F, Selvaraj JN, Liu X, Wang L, Hua H, Zhou L, Zhao Y, Wang Y, Liu Y. Inhibitory effect of cinnamaldehyde, citral, and eugenol on aflatoxin biosynthetic gene expression and aflatoxin B_1 biosynthesis in *Aspergillus flavus*. J Food Sci. 2015;80:2917–24.
22. Lind AL, Wisecaver JH, Smith TD, Feng X, Calvo AM, Rokas A. Examining the evolution of the regulatory circuit controlling secondary metabolism and development in the fungal genus *Aspergillus*. PLoS Genet. 2015;11:e1005096.
23. Miskei M, Karanyi Z, Pocsi I. Annotation of stress-response proteins in the *Aspergilli*. Fungal Genet Biol. 2009;46:105–20.
24. Montibus M, Ducos C, Bonnin-Verdal MN, Bormann J, Ponts N, Richard-Forget F, Barreau C. The bZIP transcription factor Fgap1 mediates oxidative stress response and trichothecene biosynthesis but not virulence in *Fusarium graminearum*. PLoS One. 2013;8:e83377.
25. Montibus M, Pinson-Gadais L, Richard-Forget F, Barreau C, Ponts N. Coupling of transcriptional response to oxidative stress and

secondary metabolism regulation in filamentous fungi. Crit Rev Microbiol. 2013;41:295–308.
26. Moye-Rowley WS, Harshman KD, Parker CS. Yeast YAP1 encodes a novel form of the jun family of transcriptional activator proteins. Genes Dev. 1989;3:233–92.
27. Moye-Rowley WS. Regulation of the transcriptional response to oxidative stress in fungi: similarities and differences. Eukaryotic Cell. 2003;2:381–9.
28. Nagao KTY, Arioka M, Kadokura H, Takatsuki A. Bfr1^{+}, a novel gene of *Schizosaccharomyces pombe* which confers brefeldin A resistance, is structurally related to the ATP-binding cassette superfamily. J Bacteriol. 1995;177:1536–43.
29. Qiao J, Liu W, Li R. Truncated Afyap1 attenuates antifungal susceptibility of *Aspergillus fumigatus* to voriconazole and confers adaptation of the fungus to oxidative stress. Mycopathologia. 2010;170:155–60.
30. Reverberi M, Gazzetti K, Punelli F, Scarpari M, Zjalic S, Ricelli A, Fabbri AA, Fanelli C. Aoyap1 regulates OTA synthesis by controlling cell redox balance in *Aspergillus ochraceus*. Appl Microbiol Biotechnol. 2012;95:1293–304.
31. Reverberi M, Zjalic S, Ricelli A, Punelli F, Camera E, Fabbri C, Picardo M, Fanelli C, Fabbri AA. Modulation of antioxidant defense in *Aspergillus parasiticus* is involved in aflatoxin biosynthesis: a role for the apyapA gene. Eukaryotic Cell. 2008;7:988–1000.
32. Reverberi M, Zjalic S, Punelli F, Ricelli A, Fabbri AA, Fanelli C. Apyap1 affects aflatoxin biosynthesis during *Aspergillus parasiticus* growth in maize seeds. Food Addit Contam. 2007;24:1070–5.
33. Rodrigues-Pousada C, Menezes RA, Pimentel C. The Yap family and its role in stress response. Yeast. 2010;27:245–58.
34. Roze LV, Chanda A, Wee J, Awad D, Linz JE. Stress-related transcription factor AtfB integrates secondary metabolism with oxidative stress response in *Aspergilli*. J Biol Chem. 2011;286:35137–48.
35. Van HW, Mattern IE, van Zeijl CM, Pouwels PH, van den Hondel CA. Development of a homologous transformation system for *Aspergillus niger* based on the *pyrG* gene. Mol Gen Genet. 1987;206:71.
36. Vivancos AP, Jara M, Zuin A, Sanso M, Hidalgo E. Oxidative stress in *Schizosaccharomyces pombe*: different H_2O_2 levels, different response pathways. Mol Genet Genomics. 2006;276:495–502.
37. Wang L, Wang Y, Wang Q, Liu F, Selvaraj JN, Liu L, Xing F, Zhao Y, Zhou L, Liu Y. Functional characterization of new polyketide synthase genes involved in Ochratoxin A biosynthesis in *Aspergillus Ochraceus* fc-1. Toxins. 2015;7:2723–38.
38. Wei D, Zhou L, Selvaraj JN, Zhang C, Xing F, Zhao Y, Wang Y, Liu Y. Molecular characterization of atoxigenic *Aspergillus flavus* isolates collected in China. J Microbiol. 2014;52:559–65.
39. Wu F. Perspective time to face the fungal threat. Nature. 2014;516:S7.
40. Xing F, Wang L, Liu X, Selvaraj JN, Wang Y, Zhao Y, Liu Y. Aflatoxin B_1 inhibition in *Aspergillus flavus* by *Aspergillus niger* through down-regulating expression of major biosynthetic genes and AFB_1 degradation by atoxigenic *A. flavus*. Int J Food Microbiol. 2017;256:1–10.
41. Yabe K, Nakajima H. Enzyme reactions and genes in aflatoxin biosynthesis. Appl Microbiol Biotechnol. 2004;64:745–55.
42. Yin WB, Reinke AW, Szilagyi M, Emri T, Chiang YM, Keating AE, Pocsi I, Wang CC, Keller NP. bZIP transcription factors affecting secondary metabolism, sexual development and stress responses in *Aspergillus nidulans*. Microbiology. 2013;159:77–88.
43. Yu J, Fedorova ND, Montalbano BG, Bhatnagar D, Cleveland TE, Bennett JW, Nierman WC. Tight control of mycotoxin biosynthesis gene expression in *Aspergillus flavus* by temperature as revealed by RNA-Seq. FEMS Microbiol Lett. 2011;322:145–9.
44. Yu J, Bhatnagar D, Cleveland TE. Completed sequence of aflatoxin pathway gene cluster in *Aspergillus parasiticus*. FEBS Lett. 2004;564:126–30.
45. Zhang CS, Xing FG, Selvaraj JN, Yang QL, Zhou L, Zhao YJ, Liu Y. The effectiveness of ISSR profiling for studying genetic diversity of *Aspergillus flavus* from peanut-cropped soils in China. Biochem Syst Ecol. 2013;50:147–53.