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

Xuanli Guan, Yueju Zhao, Xiao Liu, Bo Shang, Fuguo Xing, Lu Zhou, Yan Wang, Chushu Zhang, Deepak Bhatnagar, Yang Liu

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

Shandong Peanut Research Institute, Qingdao 266100, China

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$_2$O$_2$, inhibition was complete at 40 mmol/l. However, in the quantitative analysis by HPLC, the concentration of AFB1 increased with the increased H$_2$O$_2$ 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$_2$O$_2$ 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 AFB1 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*.

© 2018 Published by Elsevier España, S.L.U. on behalf of Asociación Argentina de Microbiología. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
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 carenciales 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 H₂O₂, la inhibición fue completa a 40 mmol/l. Sin embargo, en el análisis cuantitativo por HPLC, la concentración de la aflatoxina AFB₁ aumentó con el aumento de la concentración de H₂O₂ 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 H₂O₂ inhibió completamente el crecimiento de las cepas mutantes en afap1, pero no afectó el crecimiento de la cepa CA14PTS (cepa no mutada). Ademá, la concentración de AFB₁ 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*.

© 2018 Publicado por Elsevier España, S.L.U. en nombre de Asociación Argentina de Microbiología. Este es un artículo Open Access bajo la licencia CC BY-NC-ND (http://creativecommons.org/licenses/by-nc-nd/4.0/)..

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 world³⁹ 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 invasion⁴⁴. In addition, aflatoxin can lead to dysfunction of the immune system, dysplasia in children, and even death due to acute poisoning. 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 products². 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 identified⁹,¹⁰,¹³,³²,⁴¹,⁴⁴. Most gene functions have been clarified⁹,¹⁰,¹³,³²,³³,⁴¹,⁴⁴. AflR and Afs 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 factor². Afs is adjacent to AflR and participates in the regulation of aflatoxin biosynthesis together with AflR. The combination of Afs and AflR forms a complex, which is then bound together in the promoter region of each structural gene in the cluster². In addition to aflR and afs, 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 penicillin⁵,¹¹,¹²,¹⁹.

There is extensive evidence that secondary metabolism is associated with oxidative stress in filamentous fungi and plants¹⁸,³⁷,³⁸. Based on this view, different oxidative stimulants, 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 cerevisiae³⁵,³³.

Like the Yap-1 roles in yeast³⁶, several Yap-1 homologue transcription factors have been identified in filamentous fungi, and they are usually associated with resistance to H₂O₂ or antifungals. In the rice blast fungus Magnaporthe oryzae, Moap1 mediates the oxidative stress response and is necessary for conidia formation, apical growth and pathogenicity³⁹. Afyap1 in *Aspergillus fumigatus* was found to be associated with tolerance to oxidative stress³⁹. 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, AlyapA in *A. parasiticus* also participated in the oxidative stress response and in the modulation of aflatoxin biosynthesis³⁹. 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 (Δku70, ΔniaD) and recipient (Δku70, ΔniaD, ΔpyrG) strains were obtained from Dr. Peng Quang 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 10⁶ 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 MgSO₄.7H₂O and solid medium supplemented with 16 g/l agar) and grown at 28 °C on a rotary incubator in the dark for AFB₁ 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 (10⁶ CFU/ml medium) from each strain were incubated in YES solid medium supplemented with different concentrations of H₂O₂ (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 (10⁶ CFU/ml medium) was cultured in 50 ml YES liquid medium supplemented with the same concentrations of H₂O₂ for AFB₁ concentration and the mycelial dry weight analysis. All experiments were performed in triplicate in three independent experiments.

AFB₁ 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. AFB₁ 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. AFB₁ 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/H₂O (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’-GCTCTTTTTGCTGTCTGTTG-3’) and 18S-R (5’-CGCTATTGGAGCTGGAATTACC-3’) were used based on previous studies in order to cover 154 bp of the 18S RNA gene. 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⁻ΔΔCt 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 DNAsist 2.2.

Construction of the mutant strain

The deletion-mutant strain (Δafap1) was constructed as previously described. A homologous transformation system for A. flavus with the pyrG gene as selection marker was used in this study. The pyrG gene encodes an orotidine-5′-phosphate decarboxylase, which is a key gene
Table 1  Primers used in this study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Nucleotide position$^a$</th>
<th>Sequence (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>aflD-F</td>
<td>469</td>
<td>ATGCTCCCGTCTCATCTTT</td>
</tr>
<tr>
<td>aflD-R</td>
<td>555</td>
<td>ATGGTGGTATGTTGCTGA</td>
</tr>
<tr>
<td>aflB-F</td>
<td>2553</td>
<td>ATGCACTTGGTACGCCAGTGA</td>
</tr>
<tr>
<td>aflB-R</td>
<td>2657</td>
<td>TGCTCAATTACCGCTCACATCT</td>
</tr>
<tr>
<td>aflR-F</td>
<td>1236</td>
<td>CTTTTCTCATACTCGGGTTT</td>
</tr>
<tr>
<td>aflR-R</td>
<td>1303</td>
<td>GCAGGTAAATATAATGCGG</td>
</tr>
<tr>
<td>aflJ-F</td>
<td>486</td>
<td>GGAGCAACCGCCTCACC</td>
</tr>
<tr>
<td>aflJ-R</td>
<td>640</td>
<td>CCGGTGACTGCTCTTCAAAT</td>
</tr>
<tr>
<td>aflM-F</td>
<td>−102</td>
<td>GAGCCCAAGTGGTGGTAGAC</td>
</tr>
<tr>
<td>aflM-R</td>
<td>−22</td>
<td>GCTTGGATGGGAGGTCGTC</td>
</tr>
<tr>
<td>aflO-F</td>
<td>411</td>
<td>GACATCTGGGACCTGTC</td>
</tr>
<tr>
<td>aflO-R</td>
<td>579</td>
<td>ACGGCTTACAGGATTTA</td>
</tr>
<tr>
<td>aflP-F</td>
<td>41</td>
<td>CAGGGTTTCCAGAGAGG</td>
</tr>
<tr>
<td>aflP-R</td>
<td>131</td>
<td>TCCGGTGAGAGGGAGGT</td>
</tr>
<tr>
<td>aflX-F</td>
<td>282</td>
<td>ACCACGCTGCTGCAAT</td>
</tr>
<tr>
<td>aflX-R</td>
<td>417</td>
<td>ACCACGCTGCTGCAAT</td>
</tr>
<tr>
<td>LaeA-F</td>
<td>685</td>
<td>AGAAAGAAAGGTTTCTGGTGA</td>
</tr>
<tr>
<td>LaeA-R</td>
<td>810</td>
<td>TTGGTTGAAGCGCTCGACTGAG</td>
</tr>
<tr>
<td>AtfA-F</td>
<td>840</td>
<td>TGGCGTGGTTTCAATGCTGTCAA</td>
</tr>
<tr>
<td>AtfA-R</td>
<td>977</td>
<td>TCTTTCTGCTCGTGACGGTTT</td>
</tr>
<tr>
<td>Afap1-F</td>
<td>56</td>
<td>AGACCTTCTTGTCCGCCTC</td>
</tr>
<tr>
<td>Afap1-R</td>
<td>219</td>
<td>CATCATTGCGTCAACCAATCCACC</td>
</tr>
<tr>
<td>AtfB-F</td>
<td>399</td>
<td>TCCAGTATTGACGGGTGTC</td>
</tr>
<tr>
<td>AtfB-R</td>
<td>583</td>
<td>TCCGATGGTACTGGGGT</td>
</tr>
<tr>
<td>TcsA-F</td>
<td>874</td>
<td>GGGCAAGAGCGCGTTAGT</td>
</tr>
<tr>
<td>TcsA-R</td>
<td>1063</td>
<td>CAGAGCTTCCCCGATAGCGTTCATAC</td>
</tr>
<tr>
<td>Bos1-F</td>
<td>1613</td>
<td>TGTAAGACCTGCGGATCGTCTT</td>
</tr>
<tr>
<td>Bos1-R</td>
<td>1800</td>
<td>TCTTTGCGACGCGATCTG</td>
</tr>
<tr>
<td>SrrA-F</td>
<td>1141</td>
<td>TCGCAGACAATCGGAAGAGG</td>
</tr>
<tr>
<td>SrrA-R</td>
<td>1459</td>
<td>GGGCAATGCTGGGGTCATCAAAT</td>
</tr>
<tr>
<td>Up-F</td>
<td>−1424</td>
<td>TTGTCCAGCCTGACATCCAAG</td>
</tr>
<tr>
<td>Up-R</td>
<td>−265</td>
<td>AGGATAAGAGCATTGTCGCTGCCGCCGTTGTT</td>
</tr>
<tr>
<td>Down-F</td>
<td>+866</td>
<td>CATTGTAGTACGTTCTCTCATCATCGACCACTCTC</td>
</tr>
<tr>
<td>Down-R</td>
<td>+1912</td>
<td>AGCCCTACCTCAGGCAGAAC</td>
</tr>
<tr>
<td>Middle-F</td>
<td>218</td>
<td>CGGTGGATTGGTATCAGGATG</td>
</tr>
<tr>
<td>Middle-R</td>
<td>922</td>
<td>CCTGGAACTTGGAGGACTGTT</td>
</tr>
<tr>
<td>pyrG-F</td>
<td>−453</td>
<td>CAGACAATGCTTCTCATC</td>
</tr>
<tr>
<td>pyrG-R</td>
<td>+280</td>
<td>GGAACCTGACTTACAAATG</td>
</tr>
</tbody>
</table>

$^a$ 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 (∆ku70, ∆niaD, ∆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. 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 WJA01 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. Ten µg genomic DNA was digested
Figure 1  Colony growth and aflatoxin production after 0, 5, 10, 20 and 40 mmol/l \( \text{H}_2\text{O}_2 \) 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 AFB1 concentration per unit of mycelial weight by HPLC in response to \( \text{H}_2\text{O}_2 \). Different letters indicate that there were statistically significant differences (\( p = 0.05 \)). (C) Relative expressions of aflatoxin synthesis genes. Samples were harvested after \( \text{H}_2\text{O}_2 \) 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.
with HindIII (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’-AACGTGGTTTATTTGCCC-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$_2$O$_2$ 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$_2$O$_2$ and were completely inhibited at 40 mmol/l (Fig. 1A). The AFB$_1$ concentration in YES broth was increased after the treatment of H$_2$O$_2$ at the concentration of 5, 10 and 20 mmol/l, respectively (Fig. 1B). There is no
obvious increase for AFB₁ concentration at 20 mmol/l H₂O₂ compared with 10 mmol/l. In addition, the expression levels of key aflatoxin biosynthetic structural genes (afI, afM, afO, afP and afX) were up-regulated by the treatment of 10 mmol/l H₂O₂ 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), AlyapA (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 Δafap1 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/pyrG-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 HindIII, 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 H₂O₂ (Fig. 4A). The growth rates of the CA14PTs and Δafap1 mutants were similar on YES plates without H₂O₂. However, the growth of Δafap1 mutants was completely inhibited by 5 mmol/l H₂O₂. In contrast, the growth of CA14PTs was not inhibited even at 10 mmol/l H₂O₂. Meanwhile, AFB₁ concentration of the Δafap1 mutants was significan-

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
to oxidative stress in filamentous fungi and contributes to the complexity of the response\textsuperscript{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 \textit{S. cerevisiae}\textsuperscript{13} and \textit{Aspergillus} spp.\textsuperscript{30,31}, however, its role in toxin biosynthesis and virulence is divergent\textsuperscript{34}. In \textit{A. parasiticus} and \textit{A. ochraceus}, deletion of \textit{apyapA} and \textit{arypap1} resulted in increases of aflatoxin and ochratoxin, respectively\textsuperscript{30,32}. In \textit{Fusarium graminearum}, the \textit{Δfgap1} mutant showed higher sensitivity to oxidative stress (H\textsubscript{2}O\textsubscript{2}) and higher level of trichothecene concentration associated with overexpression of \textit{TRI} genes. However, the activation mechanism of toxin accumulation in response to oxidative stress was not observed\textsuperscript{35}. In contrast, in \textit{A. nidulans}, deletion or overexpression of \textit{napA} led to a decreased tolerance to oxidative stress and sterigmatocystin synthesis\textsuperscript{12}. In this study, the growth of CA14PTs was significantly inhibited following treatment with 20 mmol/l H\textsubscript{2}O\textsubscript{2}, whereas growth of the knockout mutants was completely inhibited following treatment with only 5 mmol/l H\textsubscript{2}O\textsubscript{2} due to the lack of a key transcription factor \textit{Afap1} related to the oxidative-stress response. The effect of deletion of \textit{afap1} is similar with \textit{napA} and is contrary to \textit{apyap1}, \textit{apyapA} and \textit{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\textsuperscript{17}. Moreover, AtfB has been proved to combine with the promoter regions of aflatoxin biosynthetic genes including \textit{aflB} (\textit{fas}-1), \textit{aflD} (\textit{nor}-1), \textit{aflM} (\textit{ver}-1) and \textit{aflP} (\textit{omtA}), which carry CRE sites\textsuperscript{17,34}. In addition, the sensor kinases TcsA transmit oxidative stress signals through SrrA and/or SskA response regulators\textsuperscript{23}, and then cooperate with Ap-1 against oxidative stress in other \textit{Aspergillus} spp.\textsuperscript{16}. Therefore, a similar pathway may have \textit{A. flavus} since oxidative stress signals were transmitted through sensor kinases (ortholog of TcsA or Bos1 in yeast) to AtfB-SrrA-Afap1 homo-

![Figure 5](image-url) Quantitative real-time PCR analyses of genes related to oxidative stress and aflatoxin biosynthesis in the \textit{Δafap1} 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](image-url) Hypothetical oxidative stress-activated signaling pathway in \textit{A. flavus}. Based on available experimental evidence, we speculate that exposure of the fungal cell to intra- or extracellular ROS activates signaling cascade.

\textbf{The bZIP transcription factor \textit{Afap1}}

\textit{Figure 5}  Quantitative real-time PCR analyses of genes related to oxidative stress and aflatoxin biosynthesis in the \textit{Δafap1} 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).

\textbf{Figure 6}  Hypothetical oxidative stress-activated signaling pathway in \textit{A. flavus}. Based on available experimental evidence, we speculate that exposure of the fungal cell to intra- or extracellular ROS activates signaling cascade.
logous complex, and then induced aflatoxin biosynthesis (Fig. 6). In this study, the expression of tcsA, bos1, srA and aflR was up-regulated in the ∆aflP1 mutants compared to CA14Pfs. aflB, encoding fatty acid synthase and being close to aflR in the aflatoxin gene cluster, was also up-regulated in the ∆aflP1 mutants. However, the expression of aflM and aflP, two downstream structural genes, were significantly down-regulated in the ∆aflP1 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 AFs protein, which is beneficial for some potential suppressors to bind to AflR in place of AFs. Consequently, the transcription of the aflatoxin biosynthesis gene, which relies on AFs-AflR, would be reduced and aflatoxin biosynthesis would decrease. There were similar findings in some previous studies.\(^{3,40,43}\)

The present study revealed that oxidative stress inhibited the growth of toxigenic strains and was completely inhibited at 40 mmol/l \(\text{H}_2\text{O}_2\). However, the AFB1 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 aflP1 resulted in an increase in sensitivity to oxidative stress and a decrease in aflatoxin production in \(A.\ flavus\). These results suggested that aflP1 plays a key role in tolerance to oxidative stress and promoted aflatoxin production in \(A.\ flavus\).\(^{4,15}\)

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

25. Montibus M, Pinson-Gadala L, Richard-Forget F, Barreau C, Ponts N. Coupling of transcriptional response to oxidative stress and