SfgA Renders *Aspergillus flavus* More Stable to the External Environment

Xiao-Yu Yuan¹, Jie-Ying Li¹, Qing-Qing Zhi¹,², Sheng-Da Chi¹, Su Qu¹, Yan-Feng Luo³ and Zhu-Mei He¹,*

¹ The Guangdong Province Key Laboratory for Aquatic Economic Animals, School of Life Science, Sun Yat-sen University, Guangzhou 510275, China; yuanxy8@mail2.sysu.edu.cn (X.-Y.Y.); lijieyingji@126.com (J.-Y.L.); zhiqq3@mail.sysu.edu.cn (Q.-Q.Z.); sd_chi@163.com (S.-D.C.);
qusu@mail2.sysu.edu.cn (S.Q.)

² College of Agriculture and Biology, Zhongkai University of Agriculture and Engineering, Guangzhou 510225, China

³ Guangdong Jinyinshan Environmental Protection Technology Co., Ltd., Guangzhou 510705, China; kelyna@foxmail.com

* Correspondence: lsshezm@mail.sysu.edu.cn

Abstract: *sfgA* is known as a key negative transcriptional regulator gene of asexual sporulation and sterigmatocystin production in *Aspergillus nidulans*. However, here, we found that the homolog *sfgA* gene shows a broad and complex regulatory role in governing growth, conidiation, sclerotia formation, secondary metabolism, and environmental stress responses in *Aspergillus flavus*. When *sfgA* was deleted in *A. flavus*, the fungal growth was slowed, but the conidiation was significantly increased, and the sclerotia formation displayed different behavior at different temperatures, which increased at 30°C but decreased at 36°C. In addition, *sfgA* regulated aflatoxin biosynthesis in a complex way that was associated with the changes in cultured conditions, and the increased production of aflatoxin in the ∆*sfgA* mutant was associated with a decrease in sclerotia size. Furthermore, the ∆*sfgA* mutant exhibited sensitivity to osmotic, oxidative, and cell wall stresses but still produced dense conidia. Transcriptome data indicated that numerous development- and secondary-metabolism-related genes were expressed differently when *sfgA* was deleted. Additionally, we also found that *sfgA* functions downstream of *fluG* in *A. flavus*, which is consistent with the genetic position in FluG-mediated conidiation in *A. nidulans*. Collectively, *sfgA* plays a critical role in the development, secondary metabolism, and stress responses of *A. flavus*, and *sfgA* renders *A. flavus* more stable to the external environment.

Keywords: *Aspergillus flavus*; *sfgA*; sclerotia; aflatoxin; conidiation; secondary metabolism; stress response; RNA-seq

1. Introduction

*Aspergillus flavus* is an opportunistic filamentous fungus which infects agricultural crops such as maize, peanuts, and cotton [1]. *A. flavus* spores germinate on crops and foods and produce detrimental secondary metabolite mycotoxins, including aflatoxins, which are harmful fungal mycotoxins that cause carcinogenesis in animals and humans, and thus, enormous economic losses [2]. Therefore, exploration of the regulatory mechanism of the development and secondary metabolism of *A. flavus* is vital to control aflatoxin pollution.

The *A. flavus* reproductive cycle involves an asexual growth phase and sexual developmental phase [3,4]. During asexual growth, *A. flavus* differentiates into a variety of structures including spores, which are crucial for genome protection, survival, and proliferation. Additionally, asexual sporulation causes the production of mycotoxin sterigmatocystin or other secondary metabolites [5,6]. In *A. flavus*, the formation of asexual spores is closely related to the production of aflatoxin and the formation of sclerotia [7,8]. Sclerotium, a structure formed in a critical developmental stage, mainly infects crops and
responds to harsh environmental conditions. Extensive studies have reported that the development of sclerotia is closely related to the synthesis of secondary metabolites, and many of them, such as aflatoxins, have been found in sclerotia [9,10]. Hence, morphological development and the secondary metabolism are generally considered to be linked with each other or co-regulated in *A. flavus* and some other fungal species [11–13].

Studies focusing on the conidiation regulatory mechanism in model fungal *Aspergillus nidulans* have been conducted and have provided insight into asexual development and the secondary metabolism [14,15]. According to a report by Park et al., there are three genes, *brlA*, *abaA*, and *wetA*, constructing the central developmental pathway of conidiation in filamentous fungi [16]. Later studies have identified various upstream developmental activators, FluG and Flbs (FlbA, B, C, D, and E), which can activate the essential conidiophore developmental regulator BrlA [17,18]. In addition, FluG and FlbA are interdependent, thereby inhibiting proliferation mediated by the heterotrimeric G protein composed of FadA and SfaD::GpgA [19–21]. FluG has been considered as the most upstream regulator that regulates the growth and development in *Aspergillus*. However, the function of *fluG* and the mechanism of conidiation vary between in *A. nidulans* and *A. flavus*. The *fluG* gene is necessary for the production of conidia and the synthesis of the carcinogenic mycotoxin sterigmatocystin in *A. nidulans*, while the absence of *fluG* in *A. flavus* does not affect the formation of aflatoxin [7,22]. These observations suggest that these two species of *Aspergilli* possess both conserved and divergent signaling pathways associated with the regulation of asexual sporulation and secondary metabolism [23].

According to previous studies, *sfgA* functions downstream of *fluG* but upstream of transcriptional activator genes (\(flbA, flbD, flbC, flbB, \) and *brlA*) necessary for normal conidiation and sterigmatocystin biosynthesis [24]. In *A. nidulans*, *sfgA* was reported to be the key suppressor of *fluG*, because there was conidia formation and sterigmatocystin production in the *fluG*:\(sfgA\) double-deletion strains compared with no conidiation in the *fluG* deletion mutant [25]. Although *SfgA* is conserved among most *Aspergillus* species, which was predicted to be a transcription factor containing the Gal4-type Zn(II)\(_2\)Cys\(_6\) domain [25], sequence conservation does not guarantee the conservation of the functions in other *Aspergillus* spp.

The aim of the present work was to evaluate the functions of the homolog gene *sfgA* in regulating the development and secondary metabolism of *A. flavus*. To examine the role of SfgA, an *sfgA* deletion mutant (\(\Delta sfgA\)) strain was generated, and its phenotypes and transcriptome were analyzed. Our results demonstrate that *sfgA* appeared to be functioning as a global regulator in the development and secondary metabolism of *A. flavus*. This study should contribute to the understanding of the regulatory networks that control fungal development and the production of secondary metabolites.

### 2. Materials and Methods

#### 2.1. Fungal Strains and Media

*Aspergillus flavus* TXZ21.3 (\(\Delta ku70, \Delta argB, \) and *pyrG*) was used as the parental strain to construct \(\Delta sfgA\) and *OE sfgA* mutant strains, and TJE19.1 (\(\Delta ku70\) and *pyrG*) was adopted as a control to exclude the interfering factors of supplemental uracil and uridine in media [10]. The glucose minimum medium (GMM, 10 g/L glucose, 6 g/L NaNO\(_3\), 1.52 g/L KH\(_2\)PO\(_4\), 0.52 g/L KCl, 0.52 g/L MgSO\(_4\)·7H\(_2\)O, and 1 mL of trace elements, pH 6.5), yeast extract–sucrose (YES, 20 g/L yeast extract and 60 g/L sucrose, pH 5.8), yeast extract–glucose (YGT, 5 g/L yeast extract, 20 g/L glucose, and 1 mL of trace elements), potato dextrose agar (PDA, Difco), and potato dextrose broth (PDB, Difco) were used for morphological observations. For transformation, YGT and sorbitol minimal medium (SMM, 10 g/L glucose, 6 g/L NaNO\(_3\), 1.52 g/L KH\(_2\)PO\(_4\), 0.52 g/L KCl, 0.52 g/L MgSO\(_4\)·7H\(_2\)O, 1 mL of trace elements, and 1.2 M sorbitol, pH 6.5) were used. In addition, 1 g/L uracil and 1 g/L uridine (denoted as “UU” when necessary) or 1 g/L arginine (denoted as “\(A\)” when necessary) were adopted to grow auxotroph.
2.2. Fungal Transformation

*A. flavus* protoplast preparation and transformation were carried out according to the protocol of He et al. [26], with some modifications that are described as follows. Briefly, 10^8 spores were inoculated into 100 mL of YGTAUU liquid medium and incubated at 30 °C, 150 rpm for 11 h. Then, the mycelia were harvested and washed with sterile water through centrifugation at 11,000 rpm for 5 min. Protoplasts were prepared with a protoplast solution composed of 20 mM NaH$_2$PO$_4$, 20 mM CaCl$_2$, 200 µL of β-glucuronidase (85,000 U/mL, Sigma, MO, USA), 200 mg of lysis enzymes from *Trichoderma harzianum* (Sigma), and 50 mg of Driselase from *Basidiomycetes* sp. (Sigma) in 1.2 M NaCl. Protoplasting was performed at 80 rpm and 30 °C for 4–6 h. After transformation, the protoplasts were plated on SMM medium plus appropriate supplements.

2.3. Fungal Physiology Experiments

For the morphological observation of colonies, 1 µL of conidia suspension containing approximately 10^3 conidia was point-inoculated on GMMUU and YGTUU solid plates and cultured under light for 5 d at 30 °C. For the spore germination assay, *A. flavus* conidia (10^6 spores) were inoculated in 10 mL of PDBUU media with coverslips at 30 °C. The morphology of germinated conidia and hyphae was visualized under a light microscope (Magnification, 200×) at different time intervals. For the analysis of conidial production, 5 mL of conidia suspension (10^6 spores/mL) dispersed in molten PDBUU medium supplemented with 0.7% agar was overlaid on the PDAUU plates (1.5% agar). Sclerotia production was measured as previously described [27] by counting sclerotia from GMMUU culture plates after incubation for 14 d at 30 °C and 36 °C under darkness. Sclerotia size was photographed using a stereo microscope (SteREO Lumar.V12, ZEISS; magnification: 50×). For the stress test, PDAUU solid plates were supplemented with the following agents: 1.2 M NaCl, 1.2 M KCl and 1.5 M sorbitol for hyperosmotic stress, 6 mM H$_2$O$_2$, 1.8 mM t-BOOH for oxidative stress, and 0.2 mg/mL congo red for cell wall stress.

2.4. Examination of Aflatoxin and Kojic Acid

Aflatoxin B1 (AFB1) production was measured via modified thin-layer chromatography (TLC), as previously described [28]. Each *A. flavus* strain was inoculated on GMMUU, YESUU, YGTUU, and PDBUU at 30 °C and 36 °C, and the same weight of mycelia or the same number of sclerotia was collected for AFB1 extraction. AFB1 on the TLC plates could be visualized using a fluorescent detector with a UV wavelength of 254 nm, and then, the aflatoxin production was quantified using Image J software. Standard AFB1 was purchased from Sigma. Kojic acid production was determined using the colorimetric method, as previously reported [13]. Briefly, *A. flavus* strains were cultured on PDAUU, YGTUU, and YESUU supplemented with 1 mM FeCl$_3$ for 36 h at 30 °C and 36 °C. Kojic acid forms a chelated compound with ferric ions and subsequently generates a red color, allowing for a qualitative comparison between different strains.

2.5. Catalase Activities Measurement

Around 50 mg of mycelia cultured in PDBUU medium for 24 h was suspended in 500 µL of extracting solution, and then, samples were centrifuged at 8000× g for 10 min at 4 °C, and the supernatant was used to measure the catalase activity according to the manufacturer’s instructions. The catalase assay kit (BC0205) was purchased from Solarbio (Beijing, China).

2.6. qRT-PCR Analysis

Spores were inoculated in 30 mL of PDBUU to a final concentration of 3 × 10^5/mL and incubated at 30 °C with shaking (200 rpm) for 48 h. Total RNA was extracted from the harvested mycelia using Trizol Reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized from 1 µg of RNA using the HiScript α Q RT SuperMix cDNA Synthesis kit (Vazyme, Nanjing, China). The qRT-PCR assay was performed using the LightCycler®
480 (Roche, Basel, Switzerland) with SYBR Green (Vazyme, Nanjing, China) detection, as described previously [27]. Gene expression levels were normalized (2−ΔΔCt analysis) to A. flavus β-actin gene expression levels. All analyses were performed in triplicate. The primers used for qPCR are listed in Supplementary Table S2.

2.7. RNA Sequencing and Data Analysis

RNA samples from three independent biological repeats from A. flavus of the TJES19.1 control and ΔsfgA mutant were prepared. Strains were propagated on GMMUU at 30 °C and 36 °C for 48 h, and mycelia were harvested immediately for RNA extraction using Trizol Reagent (Invitrogen). The quality and quantity of isolated RNA were determined using an Agilent 2100 bioanalyzer system, and RNA integrity numbers (RINs) were calculated. RNA samples with a RIN ≥ 8 were used for sequencing library preparation with an Illumina TruSeq RNA Sequencing Kit. The libraries were sequenced on an Illumina Hiseq2500 system (Oebiotech, Shanghai, China).

2.8. Statistical Analysis

All statistical analyses were performed using GraphPad Prism (version 8.0; GraphPad Software), and p < 0.05 was considered a significant difference.

3. Results

3.1. Identification of SfgA in A. flavus

To identify the ortholog of SfgA, the A. flavus NRRL3357 genome was screened by using the protein sequence of the model organism A. nidulans SfgA (XP_681398.1). XP_041146550.1 in A. flavus was 64% identical to A. nidulans SfgA by protein homology. Sequence alignment results showed that the A. flavus SfgA protein displayed 100% similarity to Aspergillus oryzae and 98% similarity to Aspergillus parasiticus. The phylogenetic analysis indicated that the SfgA protein is evolutionarily conserved in Aspergillus species (Figure 1A). The A. flavus sfgA (AFLA_005520) open reading frame (ORF) was predicted to consist of 1882 nucleotides, with two introns, and encodes a putative C6 transcription factor (SfgA) containing 575 amino acids (aa). Additionally, the predicted A. flavus SfgA harbors a GAL4 domain (residues 35–68 aa) and a fungal specific transcription factor domain (residues 166–574 aa). The structural analysis of SfgA proteins from several species showed that all analyzed fungi share a highly conserved GAL4 domain (Figure 1B).

To test the potential biological function of sfgA in A. flavus, the sfgA deletion mutant (ΔsfgA) and over-expression mutant (OEsfgA) were generated using the argB gene as a selection marker to complement the arginine auxotrophy of Aspergillus flavus TXZ21.3 (Aku70, ΔargB, and pyrG–). The schematic diagram of the homologous recombination strategy is shown in Figure 2A,B, and the primers used are shown in Supplementary Table S1. Afterward, the mutants were characterized by PCR (Supplementary Figure S1) and qPCR (Figure 2C) to confirm successful gene manipulation. Then, the loss-of-function strain ΔsfgA-1 and gain-of-function strain OEsfgA-13 were selected for further study.

Figure 1. Cont.
Figure 1. Summary of SfgA from different fungi. (A) A phylogenetic tree of the SfgA homologs identified in different species including *A. flavus* NRRL3357 (AFLA_005520). The tree was generated using MEGA 7 software with neighbor-joining and bootstrap method. (B) Domain analysis of the SfgA homologs in species. Protein structure was characterized using SMART and drawn using DOG 2.0 software.

Figure 2. Generation of the ∆sfgA and OEsfgA mutants in *A. flavus*. (A, B) The scheme of sfgA deletion and over-expression strategy, respectively. (C) qPCR analysis for the sfgA gene expression in ∆sfgA and OEsfgA strains. TXZ21.3 (∆ku70, ∆argB, pyrG−) is the transformation recipient strain. ND: not detected. *** p ≤ 0.001.

3.2. sfgA Influences Growth and Conidiophore Development in *A. flavus*

To investigate the roles of sfgA in the fungal growth of *A. flavus*, the control (TJES19.1) and mutant strains were inoculated to YGTUU and GMMUU media and incubated for 5 d. As shown in Figure 3A,B, the results show that the colony growth of the ∆sfgA mutant...
was inhibited on both media when compared with TJES19.1 and OEsfgA strains. Moreover, microscopic observations revealed that conidiophore stipes were significantly shorter and denser in ΔsfgA and OEsfgA strains, resulting in a somewhat flat colony phenotype in contrast to the typical floccose appearance of the control strain (Figure 3C). Additionally, the absence of sfgA resulted in hyperactive conidiation, evidenced by the formation of conidiophores in liquid shake culture (Figure 3C). Additionally, 75% of ΔsfgA conidia germinated after 8 h of incubation, while only about 40% of control conidia were germinated (Supplementary Figure S2), indicating that sfgA may negatively regulate conidial germination.

**Figure 3.** Fungal growth and conidiophore development of the ΔsfgA mutant. (A) The colony phenotype of TJES19.1, ΔsfgA, and OEsfgA strains point-inoculated on solid YGTUU and GMMUU media and propagated at 30 °C for 5 days. (B) Quantitative analysis of colony diameter shown in (A). **p ≤ 0.01; ***p ≤ 0.001. (C) Conidia formation of all strains were observed under a light microscope (magnification, 200×) at 48 h post-inoculation onto solid YGTUU and 14 h after inoculation into liquid YGTUU, respectively.

sfgA was reported as the suppressor of fluG (SFGs) that bypasses the need of fluG in conidiation in *A. nidulans* [24]. To identify the relationship between FluG and SfgA in *A. flavus*, the ΔsfgAΔfluG double mutant was generated. As shown in Supplementary Figure S3, both ΔsfgA and ΔsfgAΔfluG mutations showed identical phenotypes in growth and conidiation, indicating that SfgA functions downstream of FluG in *A. flavus*, which is consistent with the genetic position of sfgA in the FluG-mediated conidiation in *A. nidulans* [25].

3.3. Roles of sfgA in Sclerotia Formation

Sclerotia is commonly considered to be a survival structure of *A. flavus* against unfavorable conditions. To investigate the impact of the sfgA gene on sclerotia formation in *A. flavus*, the TJES19.1, ΔsfgA, and OEsfgA strains were point-inoculated on GMMUU medium and cultured at 30 °C and 36 °C for 14 d under dark conditions. After being sprayed with 75% ethanol, the number of sclerotia on each plate was counted. The result suggests that sfgA plays a complex role in sclerotia production in different conditions. A lack of sfgA significantly increased the production of sclerotia, and the over-expression of sfgA clearly decreased sclerotia production versus the control strain when cultured at 30 °C (Figure 4A,B). When cultured at 36 °C, to our astonishment, the sclerotial number of the
ΔsfgA mutant declined sharply and was less than that of the control strain (Figure 4A,B). Furthermore, the sclerotia size produced in the ΔsfgA mutant at 30 °C was much smaller than that of the TJES19.1 and OEsfgA strains; however, this change was partly restored at 36 °C (Figure 4C). The weight of a single sclerotia in the ΔsfgA mutant was also lighter than that in the control strain at both temperatures (Figure 4D).

Interestingly, we found that the sclerotia formation, quantitated in size under different temperatures mediated by sfgA, was associated with aflatoxin accumulation. The accumulation of aflatoxin in the sclerotia of the ΔsfgA mutant was accompanied by a decrease in their sclerotia size (Figure 4E), which was previously reported in A. parasiticus by Chang et al. [29]. Aflatoxins were weakly produced in variant strains at 36 °C in GMMUU medium (data not shown), the variations of which were difficult to compare with.

3.4. sfgA Affects Secondary Metabolite Production of A. flavus

Filamentous fungi can produce numbers of small bioactive molecules as part of their secondary metabolism, which is closely related with fungal developmental programs. The sfgA deletion strain showed different AFB1 levels, a crucial metabolite in A. flavus, in different culture conditions. When propagated on solid GMMUU media for 48 h, sfgA deletion resulted in elevated AFB1 levels at both 30 °C and 36 °C (Figure 5A,C). When cultured on solid YESUU media for 48 h, sfgA deletion resulted in reduced AFB1 levels at both 30 °C and 36 °C (Figure 5A,C). Inexplicably, when cultured in YGTUU media (Figure 5A,C) and PDBUU media (Figure 5A–C), the AFB1 level was increased in the ΔsfgA mutant at 30 °C, while the AFB1 level was decreased at 36 °C, from which it is
evident that the mode of aflatoxin biosynthesis in ΔsfgA would vary in accordance with the external environmental factors. The results of qPCR (Figure 5D) showed that sfgA affected the aflatoxin production through regulating transcription in aflatoxin cluster genes. Furthermore, as the antioxidant enzyme catalase is linked with reactive oxygen species (ROS) stress response with aflatoxin biosynthesis, analysis of the activity measured in mycelia samples cultivated in PDBUU for 24 h demonstrated that the catalase activity was inhibited in the ΔsfgA mutant at 30 °C while it was increased at 36 °C compared with the control strain (Figure 5E). This result indicated that sfgA would be involved in intracellular oxidative stress balance and takes part in regulating aflatoxin biosynthesis in *A. flavus*.

![Figure 5](image-url)

**Figure 5.** Aflatoxin and kojic acid production of ΔsfgA in *A. flavus*. (A) TLC analyses of AFB1 production of mycelia extracts cultured on GMMUU, YESUU, YGTUU, and PDBUU media, respectively. (B) TLC analyses of AFB1 production of culture extracts from PDBUU media. Sd represents the AFB1 standard. (C) Relative quantitative analyses of AFB1 from *A. flavus* by Image J software. (D) qPCR analysis of transcriptional levels of the aflatoxin cluster genes cultured in PDBUU at 30 °C for 48 h. Gene expression levels at each time point were normalized to β-actin by $2^{-\Delta\Delta CT}$ analysis. *p ≤ 0.1; **p ≤ 0.01; ***p ≤ 0.001. (E) Catalase activity of mycelia cultured in PDBUU at 30 °C and 36 °C for 24 h. (F) Determination of kojic acid production in solid PDAUU medium for 36 h via the colorimetric method.

In addition, the production of kojic acid, an important chemical material utilized to manufacture various cosmetics and pharmaceutics, was positively affected by sfgA at both temperatures under all media tested (Figure 5F and Supplementary Figure S4). The aforementioned results indicated that sfgA exerts a vital and complex role in *A. flavus* secondary metabolite biosynthesis.
3.5. The Effect of sfgA on Response to Environmental Stress

Control and mutant strains were inoculated on PDAUU with several compounds that are related with osmotic stress (NaCl, KCl, and sorbitol), oxidative stress (H₂O₂ and t-BOOH), and cell wall stress (Congo red). The ΔsfgA mutant strain displayed more sensitivity to all stressors tested and could produce more conidia under various environmental stress than the control strain (Figure 6A,B), which indicated that the deletion of sfgA conferred A. flavus more sensitivity to various stress conditions.

Figure 6. Phenotypes of the ΔsfgA mutant in various stress conditions. (A) TJES19.1, ΔsfgA, and OE_sfgA strains were point-inoculated on solid PDAUU media containing various compounds including NaCl, KCl, sorbitol, H₂O₂, t-BOOH, and CR at 30 °C for 5 days. (B) Quantitative analysis of colony diameter shown in (A). (C) qPCR analysis of transcriptional levels of oxidative and cell-wall-related genes of mycelia cultured on PDAUU at 30 °C for 24 h. Gene expression levels at each time point were normalized to β-actin by 2^−ΔΔCT analysis. *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

In addition, we detected the relative expression of two ROS scavenging enzymes (SOD and CAT2) encoding genes, three cell-wall-related genes (AFLA_013690, AFLA_060590, and AFLA_078300, which encode different chitin synthases), and one cell wall integrity regulator gene, AFLA_016890. As shown in Figure 6C, the transcriptional levels of all of
the above genes in the ΔsfgA strain were significantly lower than those in the control strain. These results suggest that the response of sfgA to environmental stress resistance may be through affecting the cell wall integrity and intracellular oxidative stress balance.

3.6. Transcriptome Analysis of the ΔsfgA strain

To investigate how sfgA affected the overall gene expression in A. flavus, RNA sequencing analysis was carried out between the sfgA deletion mutant (named group sfgA) and the TJES19.1 strain (named group CK) cultured at 30 °C and 36 °C for 48 h on GM-MUU media. The results are highly reproducible and reliable (Supplementary Table S3 and Supplementary Figure S5). Alignments were prepared with DESeq2 [30] for a genome-wide analysis of differential gene expression. When propagated at 30 °C, the expression of 1038 genes in the ΔsfgA strain (sfgA-30) were significantly up-regulated, while the expression of 1016 genes were down-regulated compared with the TJES19.1 strain (CK-30) (fold change ≥ 2, q-value ≤ 0.05). When cultured at 36 °C, the expression of 1837 genes in the ΔsfgA strain (sfgA-36) were clearly up-regulated, while the expression of 2375 genes were down-regulated compared to the TJES19.1 strain (CK-36) (fold change ≥ 2, q-value ≤ 0.05) (Figure 7A).

![Figure 7](image_url)

**Figure 7.** Transcriptome analysis of sfgA in A. flavus. (A) Venn diagram of common and differentially expressed gene number among different comparison groups. (B) Heat map showing transcript abundance of genes of the parental and ΔsfgA strains associated with aflatoxin production at 30 °C and 36 °C, respectively. The heat map is color-coded and represents the log10 value of the FPKM values of each gene in ΔsfgA and control samples. (C) Impact of sfgA on the expression of stress-related genes in A. flavus at 30 °C.

The transcriptional activities of genes involved in fungal development are shown in Supplementary Table S4. The brlA, con-6, con-10, and rodB genes related to conidiation in the ΔsfgA strain were up-regulated at both 30 °C and 36 °C, which is consistent with the result that showed the ΔsfgA strain produced more conidiophores. Remarkably, the genes encoding the Cys2His2 (C2H2) zinc finger transcription factor NsdC and the sexual development transcription factor NsdD were up-regulated at 30 °C but down-regulated at 36 °C. Moreover, the expression of the ppoA gene which induced sexual reproduction was also up-regulated at 30 °C and down-regulated at 36 °C. Additionally, the expression of the ppoC gene which induced asexual development was down-regulated at 30 °C and exhibited no variation at 36 °C. These data may provide some clues for the contradicting phenotypes of sclerotial number in the ΔsfgA strain cultured at different temperatures.

As to the transcriptional changes in secondary metabolism genes induced by sfgA deletion, it was found that 42 out of the predicted 56 secondary metabolite gene clusters [31–33] were differentially expressed, including the asparasones cluster (#27), aflavarins cluster (#39), aflatoxin cluster (#54), and kojic acid cluster (#56) (Supplementary Table S5). For example, at least 24 out of the 34 aflatoxin cluster genes were significantly up-regulated
in the ∆sfgA strain at both temperatures (Figure 7B), which was consistent with the result of aflatoxin detection. Additionally, kojA (AFLA_096040) in the kojic acid cluster was obviously down-regulated in the ∆sfgA strain cultured on GMMUU media at 30 °C.

After analyzing the expression of genes involved in the environmental stresses, we found that the absence of sfgA resulted in the significant deviation of the expression levels of approximately 113 genes related to stress response from the control strain (Figure 7C and Supplementary Table S6).

4. Discussion
Previous studies have shown that SfgA in A. nidulans is a negative regulator of conidiation, functioning downstream of FluG but upstream of other key developmental activators, including FlbD, FlbC, FlbB, and BrlA, which are necessary for normal conidiation and sterigmatocystin biosynthesis [25]. In this paper, we found that sfgA in A. flavus has broad regulatory roles, including in growth, conidiation, sclerotia formation, secondary metabolism, and environmental stress responses. sfgA exhibits differential effects in sclerotia production at different temperatures. sfgA in A. flavus also displays the regulation of environmental stress responses and secondary metabolism in a complex way. Our research indicated that the regulatory function of the sfgA gene in A. flavus may be alterable with changes in external environmental factors, which was further illuminated via a comparative transcriptomic study of ∆sfgA mutant.

The formation of conidia in A. flavus requires the concerted activity of a number of signaling proteins and transcription factors. For example, the brlA gene encodes a Cys2His2 (C2H2) zinc finger transcription factor which regulates the developmental switch from vegetative cells to conidiophores [14]. con-6, together with con-10, is involved in desiccation stress and conidial germination in A. nidulans [34]. Based on our experimental data, sfgA in A. flavus could negatively influence the conidia formation, which was consistent with the result in A. nidulans [25]. We also found that the transcription levels of the conidial-specific genes brlA, con-6, con-10, and rodB/hypB were up-regulated significantly, but the transcription levels of the fluG and flbs genes related to mycelia development exhibited no difference when the sfgA deletion mutant of A. flavus was propagated at 30 °C, which indicates that the sfgA-mediated repression of the conidia formation in A. flavus may be realized by affecting the expression of conidiophore development genes but not by altering the expression of flbs. These results were distinct from those in A. nidulans on the mechanism of conidia development [18,25].

Sclerotia is a sexual structure for survival under harsh environments in A. flavus [4]. Sexual reproduction in fungi requires the presence of many specific genes in the genome. In this study, we found that the sclerotia formation of the ∆sfgA mutant was significantly different from the control strain when cultured at both 30 °C and 36 °C. This difference was recorded in the sclerotia amount and the expression of the regulating genes nsdC [35] and nsdD [36], which were essential for sclerotia production. The differential expression of ppoA and ppoC genes, which both encode putative fatty acid oxygenases, can balance sexual and asexual spore development [37,38]. The deletion of ppoA in A. nidulans resulted in a fourfold rise in the ratio of asexual to sexual spores due to a decrease in psiBα levels (precocious sexual inducer). The over-expression of ppoA on the other hand, resulted in elevated levels of psiBα and a sixfold reduction in the ratio of asexual to sexual spore amounts [39]. An increased ratio of sexual to asexual spore amounts was also observed after the deletion of ppoC [39]. Alterations in the sexual sclerotia production in the ∆sfgA mutant at 30 °C and 36 °C were concomitantly reflected in mRNA levels of ppoA and ppoC genes in A. flavus. The deletion of sfgA increased sexual sclerotal numbers with the up-regulated expression of the ppoA gene as well as down-regulated ppoC gene expression at 30 °C. What is interesting is that the deletion of sfgA decreased the sclerotal numbers with the down-regulated expression of the ppoA gene at 36 °C, while the expression of the ppoC did not display any variation. Thus, it is clear that sfgA of A. flavus, in our study,
plays different roles in sexual sclerotia production by affecting the expression of \textit{ppoA} and \textit{ppoC} genes.

In addition, the change in secondary metabolite production was correlated with conidiophore formation and sclerotia production [40]. In \textit{A. flavus}, several genetic co-regulators, which activate the genes involved in secondary metabolite production and the formation of spores and sclerotia, were identified [41]. For example, in the \textit{A. flavus ΔveA} strain, more conidia but no sclerotia were produced, and more importantly, \textit{veA} was required for the production of aflatoxin, cyclopiazonic acid, and asparasone, which have been isolated from the sclerotia [42]. In our study, we found that \textit{sfgA} regulated AFB1 biosynthesis in a complex way in response to the changes in culture conditions (Figure 5A–C). Additionally, through the qPCR (Figure 5D) and RNA-seq (Figure 7B) analyses, we found that the crucial regulator gene \textit{aflR} was slightly activated, accompanied with significant activation in the expression of aflatoxin structural genes, leading to a consecutive increase in the ability to synthesize aflatoxin and its intermediates when cultured in PDBUU and GMMUU media at 30 °C, which suggests that \textit{sfgA} regulated aflatoxin biosynthesis by affecting the aflatoxin cluster genes. It has been reported that ROS induces aflatoxin synthesis, and catalase can remove ROS to protect cells from oxidative stress [43,44]. In our experiment, we also found that catalase activity changed at different temperatures. The loss of the \textit{sfgA} gene resulted in different aflatoxin production levels at different temperatures, which may have been caused by changes in the ROS clearance system in \textit{A. flavus}. In fact, aflatoxin production is a very complex process and is susceptible to external factors [45]. Temperature is one of the most important factors affecting growth and aflatoxin biosynthesis in \textit{A. flavus}. A number of studies have reported that temperature may affect the expression of aflatoxin cluster structural genes by regulating the specific regulatory factors \textit{AflR} and \textit{AflS}, leading to changes in aflatoxin biosynthesis [46]. So far, other transcription factors or related receptors that regulate the response to temperature change have not been found, and how to transmit the signals of temperature change is still unknown. Thus, the observations made regarding \textit{sfgA} can be insightful. Furthermore, aflatoxin production is also influenced by nutritional conditions, including carbon sources, amino acids, trace elements, pH, and so on [41,47], and the effect of pH depends on the composition of the medium [48]. Taken together, \textit{sfgA} plays a complex role in aflatoxin production.

In addition, the size of sclerotia produced by the \textit{ΔsfgA} mutant was much smaller compared to the control strain when propagated at 30 °C, which is consistent with the research reported by Chang et al. [29]. They described that the increase in toxin production coincided with a decrease in sclerotia size and an alteration in sclerotia shape, together with an increase in sclerotial numbers in some cases, and they suggested that these alterations could be caused by competition for a common substrate such as acetate. Our result confirmed that \textit{sfgA} in \textit{A. flavus} is a co-regulator of the secondary metabolism and sclerotia production, which is similar to the function of \textit{A. flavus aswA} which regulates sclerotial development and the biosynthesis of sclerotium-associated secondary metabolites [49].

\textit{sfgA} plays a major role in the secondary metabolism. Apart from aflatoxin, dozens of other secondary metabolism gene clusters, including kojic acid, asparasones, and aflavarins, were influenced by deleting \textit{sfgA} in \textit{A. flavus} according to our transcriptome data. Our kojic acid detection results confirmed the positive regulation of \textit{sfgA} on kojic acid formation in \textit{A. flavus}, and the transcription level of \textit{kojA} involved in the kojic acid biosynthesis pathway was also down-regulated in the \textit{ΔsfgA} mutant. In fact, different secondary metabolic pathways are usually co-regulated to maintain cellular homeostasis and promote cell survival under stress conditions [50].

We also found that the deletion of \textit{sfgA} increased the sensitivity of \textit{A. flavus} when the \textit{ΔsfgA} mutant was challenged by osmotic, oxidative, and cell wall stresses, which was confirmed with the transcriptome result shown in Supplementary Table S6. The RNA-seq data demonstrated that some of differently expressed genes in the MAPK pathway [51] play a pivotal role in the osmotic stress response in \textit{Aspergillus}. These genes were consistently down-regulated, including sensor histidine kinase TcsB, MAP kinase.
kinase Ste7, Ste20-like serine, protein tyrosine phosphatase Pps1, and Mst3-like protein kinase (Supplementary Table S6). Our finding suggested that sfgA responses to the osmotic pressure may occur through the MAPK pathway.

Some studies have found that five complexes (I–V) are involved in oxidative stress and phosphorylation [52,53]. As shown in Supplementary Figure S6, our RNA-seq data showed that the expression levels of some genes encoding NADH dehydrogenase, succinate dehydrogenase, cytochrome oxidase, and ATPase in the complexes were down-regulated to different degrees in the ∆sfgA mutant, which suggests that sfgA compromises fungal oxidative stress tolerance, which maybe mediated by altering mitochondrial respiration [54].

The cell wall is not only essential for the survival of fungi during development and reproduction, but it also acts as a protective barrier for fungi against environmental factors [55]. According to our RNA-seq data, the transcript of chitin synthase gene chs3 [56] was moderately down-regulated in the ∆sfgA mutant. The regulatory subunit of the rho family of GTPases is essential to the cell wall integrity signaling pathway, and it has been confirmed that the deletion of the rho protein resulted in cytoplasmic leakage in Aspergillus fumigatus [57]. In our study, the Rho GTPase activator Lrg11 was down-regulated in the sfgA deletion mutant, and the important component of fungal cell wall, the alpha−1,3-glucan synthase encoded by ags1 and ags3 [58], was also significantly up-regulated in the sfgA deletion mutant. These results suggested that the deletion of sfgA may affect the main components of the fungal cell wall of A. flavus, including chitin and structural polysaccharides. Consequently, the cells will generate a defensive response to by over-expressing alpha-1,3-glucan synthase genes to overcome stimulation [59]. In general, it is possible that the response of sfgA to environmental stress resistance could be linked to the differential expression of these genes.

5. Conclusions

In this study, we explored the diversified roles of the sfgA gene in fungal pathogen A. flavus. We verified that sfgA can regulate the growth, conidiation, sclerotia formation, secondary metabolism, and environmental stresses responses in A. flavus in a complex way. Our findings shed light on the roles of sfgA in the regulatory mechanisms of morphogenesis and the secondary metabolism in filamentous fungi.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jof8060638/s1, Table S1: Primers used for constructing sfgA deletion and over-expression strains; Table S2: Primers used for qPCR; Table S3: Reads and reference genome comparison; Table S4: Transcript abundance of genes involved in A. flavus development; Table S5: Transcript abundance of genes that are involved in secondary metabolism; Table S6: Transcript abundance of genes that are involved in environmental stresses; Figure S1: PCR confirmation of the sfgA deletion (A, B, C) and over-expression (D, E, F) transformants; Figure S2: sfgA affects conidia growth and spore germination of A. flavus; Figure S3: Phenotypes of ∆sfgA∆fluG in A. flavus; Figure S4: Determination of kojic acid production in solid YGTUU and YESUU medium for 36 h via colorimetric method; Figure S5: Overview of RNA-seq results; Figure S6: The diagram of oxidative phosphorylation including complexes I, II, III, IV, and V.

Author Contributions: Conceptualization, Z.-M.H. and Y.-F.L.; investigation, X.-Y.Y., J.-Y.L., Q.-Q.Z., S.-D.C. and S.Q.; writing—original draft preparation, Z.-M.H. and X.-Y.Y.; writing—review and editing, Z.-M.H. and X.-Y.Y.; supervision, Z.-M.H.; funding acquisition, Z.-M.H., Q.-Q.Z. and Y.-F.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Natural Science Foundation of China (Grant No. 31870031), the Basic and Applied Basic Research Fund of Guangdong Province, China (Grant No. 2020A1515010243), and the Science and Technology Planning Project of Guangzhou City, China (Grant No. 201903010089).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.
Data Availability Statement: Data is contained within the article or Supplementary Materials.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Yu, J. Current Understanding on Aflatoxin Biosynthesis and Future Perspective in Reducing Aflatoxin Contamination. *Toxins* 2012, 4, 1024–1057. [CrossRef] [PubMed]
2. Rushing, B.R.; Selim, M.I. Aflatoxin B1: A review on metabolism, toxicity, occurrence in food, occupational exposure, and detoxification methods. *Food Chem. Toxicol.* 2019, 124, 81–100. [CrossRef] [PubMed]
3. Dyer, P.S.; Gorman, C.M.O. A fungal sexual revolution: Aspergillus and Penicillium show the way. *Curr. Opin. Microbiol.* 2011, 14, 649–654. [CrossRef] [PubMed]
4. Amaike, S.; Keller, N.P. Aspergillus flavus. *Annu. Rev. Phytopathol.* 2011, 49, 107–133. [CrossRef]
5. Calvo, A.M.; Wilson, R.A.; Bok, J.W.; Keller, N.P. Relationship between Secondary Metabolism and Fungal Development. *Microbiol. Mol. Biol. Rev.* 2002, 66, 447–459. [CrossRef]
6. Yu, J.-H.; Keller, N. Regulation of Secondary Metabolism in Filamentous Fungi. *Crit. Rev. Microbiol.* 2005, 31, 437–458. [CrossRef]
7. Chang, P.; Scharfenstein, L.L.; Mack, B.; Ehrlich, K.C. Deletion of the Aspergillus flavus Orthologue of A. nidulans FluG reduces conidiation and promotes production of sclerotia but does not abolish aflatoxin biosynthesis. *Appl. Environ. Microb.* 2012, 78, 7557–7563. [CrossRef] [PubMed]
8. Gqaleni, N.; Smith, J.E.; Lacey, J. Co-production of aflatoxins and cyclopiazonic acid in isolates of Aspergillus flavus. *Food Addit. Contam.* 1996, 13, 677–685. [CrossRef]
9. Frawley, D.; Greco, C.; Oakley, B.; Alhussain, M.M.; Fleming, A.B.; Keller, N.P.; Bayram, Ö. The tetrameric pheromone module SteC-MkkB-MpkB-SteD regulates asexual sporulation, sclerotia formation and aflatoxin production in Aspergillus flavus. *Cell Microbiol.* 2020, 22, 1–14. [CrossRef] [PubMed]
10. Zhao, X.; Spraker, J.E.; Bok, J.W.; Velk, T.; He, Z.-M.; Keller, N.P. A Cellular Fusion Cascade Regulated by LaeA Is Required for Sclerotial Development in Aspergillus flavus. *Front. Microbiol.* 2017, 8, 1925. [CrossRef] [PubMed]
11. Shwab, E.K.; Keller, N.P. Regulation of secondary metabolite production in filamentous ascomycetes. *Mycol. Res.* 2008, 112, 225–230. [CrossRef]
12. Calvo, A.M.; Cary, J.W. Association of fungal secondary metabolism and sclerotal biology. *Front. Microbiol.* 2015, 6, 62. [CrossRef]
13. Zhi, Q.-Q.; He, L.; Li, J.-Y.; Li, J.; Wang, Z.-L.; He, G.-Y.; He, Z.-M. The Kinetochore Protein Spc105, a Novel Interaction Partner of LaeA, Regulates Development and Secondary Metabolism in Aspergillus flavus. *Front. Microbiol.* 2019, 10, 1881. [CrossRef]
14. Ojeda-López, M.; Chen, W.; Eagle, C.; Gutiérrez, G.; Jia, W.; Swilaaman, S.; Huang, Z.; Park, H.-S.; Yu, J.-H.; Cánovas, D.; et al. Evolution of asexual and sexual reproduction in the aspergilli. *Stud. Mycol.* 2018, 91, 37–59. [CrossRef]
15. Etxebeste, O.; Otamendi, A.; Garzia, A.; Espeso, E.A.; Cortese, M.S. Rewiring of transcriptional networks as a major event leading to the diversity of asexual multicellularity in fungi. *Curr. Rev. Microbiol.* 2019, 45, 548–563. [CrossRef]
16. Park, H.-S.; Yu, J.-H. Genetic control of asexual sporulation in filamentous fungi. *Curr. Opin. Microbiol.* 2012, 15, 669–677. [CrossRef]
17. Etxebeste, O.; Garzia, A.; Espeso, E.A.; Ugalde, U. Aspergillus nidulans asexual development: Making the most of cellular modules. *Trends Microbiol.* 2010, 18, 569–576. [CrossRef]
18. Lee, M.-K.; Kwon, N.-J.; Lee, I.-S.; Jung, S.; Kim, S.-C.; Yu, J.-H. Negative regulation and developmental competence in Aspergillus. *Sci. Rep.* 2016, 6, 28874. [CrossRef]
19. Yu, J.H.; Wieser, J.; Adams, T.H. The Aspergillus FlbA RGS domain protein antagonizes G protein signaling to block proliferation and allow development. *Embo J.* 1996, 15, 5184–5190. [CrossRef]
20. Lee, B.N.; Adams, T.H. FluG and flbA function interdependently to initiate conidiophore development in Aspergillus nidulans through brIA activation. *Embo J.* 1996, 15, 299–309. [CrossRef]
21. Park, H.-S.; Kim, M.-J.; Yu, J.-H.; Shin, K.-S. Heterotrimeric G-protein signalers and RGSs in *Aspergillus fumigatus*. *Pathogens* 2020, 9, 902. [CrossRef]
22. Lee, B.N.; Adams, T.H. The Aspergillus nidulans fluG gene is required for production of an extracellular developmental signal and is related to prokaryotic glutamine synthetase I. *Genes Dev.* 1994, 8, 641–651. [CrossRef]
23. Jun, S.-C.; Kim, J.-H.; Han, K.-H. The Conserved MAP Kinase MpkB Regulates Development and Sporulation without Affecting Aflatoxin Biosynthesis in *Aspergillus flavus*. *J. Fungi* 2020, 6, 289. [CrossRef]
24. Seo, J.-A.; Guan, Y.; Yu, J.-H. Suppressor Mutations Bypass the Requirement of fluG for Asexual Sporulation and Sterigmatocystin Production in *Aspergillus nidulans*. *Genetics* 2003, 165, 1083–1093. [CrossRef]
25. Seo, J.; Guan, Y.; Yu, J. FluG-dependent asexual development in Aspergillus nidulans occurs via derepression. *Genetics* 2006, 172, 1535–1544. [CrossRef]
26. He, Z.-M.; Price, M.S.; Obrian, G.R.; Georgianna, D.R.; Payne, G.A. Improved protocols for functional analysis in the pathogenic fungus Aspergillus flavus. *BMC Microbiol.* 2007, 7, 104. [CrossRef]
27. Zhi, Q.-Q.; Li, J.-Y.; Liu, Q.; He, Z.-M. A cytosine methyltransferase ortholog dmtA is involved in the sensitivity of Aspergillus flavus to environmental stresses. *Fungal Biol.* 2017, 121, 501–514. [CrossRef]
28. Lin, J.-Q.; Zhao, X.-X.; Wang, C.-C.; Xie, Y.; Li, G.-H.; He, Z.-M. 5-Azacytidine inhibits aflatoxin biosynthesis in Aspergillus flavus. *Ann. Microbiol.* **2012**, *62*, 763–769. [CrossRef]

29. Chang, P.; Bennett, J.W.; Cotty, P.J. Association of aflatoxin biosynthesis and sclerotial development in Aspergillus parasiticus. *Mycopathologia* **2002**, *153*, 41–48. [CrossRef] [PubMed]

30. Love, M.I.; Huber, W.; Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **2014**, *15*, 550. [CrossRef] [PubMed]

31. Geogrianna, D.R.; Fedorova, N.D.; Burroughs, J.L.; Dolezal, A.L.; Bok, J.W.; Horowitz-Brown, S.; Woloshuk, C.P.; Yu, J.J.; Keller, N.P.; Payne, G.A. Beyond aflatoxin: Four distinct expression patterns and functional roles associated with Aspergillus flavus secondary metabolism gene clusters. *Mol. Plant Pathol.* **2010**, *11*, 213–226.

32. Khaldi, N.; Seifuddin, F.T.; Turner, G.; Haft, D.; Nierman, W.C.; Fedorova, N.D. SMURF: Genomic mapping of fungal secondary metabolite gene clusters. *Fungal Genet. Biol.* **2010**, *47*, 736–741. [CrossRef]

33. Marui, J.; Yamane, N.; Ohashi-Kunihiro, S.; Ando, T.; Terabayashi, Y.; Sano, M.; Ohashi, S.; Ohshima, E.; Tachibana, K.; Higa, Y.; et al. Kojic acid biosynthesis in Aspergillus oryzae is regulated by a Zn(II)Cys6 transcriptional activator and induced by kojic acid at the transcriptional level. *J. Biosci. Bioeng.* **2011**, *112*, 40–43. [CrossRef]

34. Suzuki, S.; Sariyaka Bayram, O.; Bayram, O.; Braus, G.H. conf and confb contribute to conidia germination and stress response in the filamentous fungus Aspergillus nidulans. *Fungal Genet. Biol.* **2013**, *56*, 42–53. [CrossRef]

35. Kim, H.-R.; Chae, K.-S.; Han, K.-H.; Han, D.-M. The nsdD gene encodes a putative GATA-type transcription factor necessary for sexual development of Aspergillus nidulans. *Genetics* **2009**, *182*, 771–783. [CrossRef]

36. Han, K.; Yu, J.; Chae, K.; Jahng, K.; Han, D. The nsdD gene encodes a putative GATA-type transcription factor necessary for sexual development of Aspergillus nidulans. *Mol. Microbiol.* **2001**, *41*, 299–309. [CrossRef]

37. Tsitsigiannis, D.I.; Zarnowski, R.; Keller, N.P. The Lipid Body Protein, PpoA, Coordinates Sexual and Asexual Sporulation in Aspergillus nidulans. *J. Biol. Chem.* **2004**, *279*, 11344–11353. [CrossRef]

38. Tsitsigiannis, D.I.; Kowieski, T.M.; Zarnowski, R.; Keller, N.P. Three putative oxylipin biosynthetic genes integrate sexual and asexual development in Aspergillus nidulans. *Microbiology* **2005**, *151*, 1809–1821. [CrossRef]

39. Suzuki, S.; Sariyaka Bayram, O.; Bayram, O.; Braus, G.H. Coordinate of secondary metabolism and development in fungi: The velvet family of regulatory proteins. *Fems. Microbiol. Rev.* **2012**, *36*, 1–24. [CrossRef]

40. Bayram, O.; Braus, G.H. Coordination of secondary metabolism and development in fungi: The velvet family of regulatory proteins. *Fems. Microbiol. Rev.* **2012**, *36*, 1–24. [CrossRef]

41. Caceres, I.; Al Khoury, A.; El Khoury, R.; Lorber, S.; Oswald, I.P.; El Khoury, A.; Atoui, A.; Puel, O.; Bailly, J. Aflatoxin biosynthesis and methods of measurement. *Int. J. Mol. Med.* **2016**, *37*, 638–650. [CrossRef] [PubMed]

42. Duran, R.M.; Cary, J.W.; Calvo, A.M. Production of cyclopiazonic acid, aflatem, and aflatoxin by Aspergillus flavus is regulated by kojic acid at the transcriptional level. *J. Biosci. Bioeng.* **2011**, *112*, 40–43. [CrossRef]

43. Buitimea-Cantuia, G.V.; Gutierrez, H.M.L.; Buitimea-Cantuia, N.E.; Rocha-Pizaña, M.D.R.; Garcia-Triana, A.; Hernandez-Morales, A.; Magana-Barajas, E.; Molina-Torres, J. The aflatoxin inhibitors capsacidin and piperine from *Capsicum chinense*. *J. Biol. Chem.* **2007**, *282*, 33451–33459. [CrossRef]

44. Zhu, Z.; Yang, M.; Bai, Y.; Ge, F.; Wang, S. Antioxidant-related catalase CTA1 regulates development, aflatoxin biosynthesis, and virulence in pathogenic fungus *Aspergillus flavus*. *Microbiology* **2020**, *22*, 2792–2810. [CrossRef] [PubMed]

45. Zhi, Q.Q.; Xie, Y.Y.; He, Z.M. Genome Mining for Aflatoxin Biosynthesis. *Fungal Genom. Biol.* **2015**, *3*, 1000108. [CrossRef]

46. Yu, J.; Fedorova, N.D.; Montalbano, B.G.; Bhatnagar, D.; Cleveland, T.E.; Bennett, J.W.; Nierman, W.C. Tight control of mycotoxin biosynthesis gene expression in Aspergillus flavus as regulated by kojic acid at the transcriptional level. *J. Biosci. Bioeng.* **2011**, *112*, 40–43. [CrossRef]

47. Wilkinson, J.R.; Yu, J.; Bland, J.M.; Nierman, W.C.; Bhatnagar, D.; Cleveland, T.E. Amino acid supplementation reveals differential regulation of aflatoxin biosynthesis in *Aspergillus flavus* NRRL 3357 and Aspergillus parasiticus SRRC 143. *Appl. Microbiol. Biotechnol.* **2007**, *74*, 1308–1319. [CrossRef]

48. Muriel, C.; Claude, G. pH signaling in human fungal pathogens: A new target for antifungal strategies. *Eukaryot. Cell* **2014**, *13*, 342–352.

49. Chang, P.-K.; Scharfenstein, L.L.; Li, R.W.; Arroyo-Manzanares, N.; De Saeger, S.; Di Mavungu, J.D. Aspergillus flavus aswA, a gene homolog of *Aspergillus nidulans* oefC, regulates sclerotial development and biosynthesis of sclerotium-associated secondary metabolites. *Fungal Genet. Biol.* **2017**, *104*, 29–37. [CrossRef]

50. Tian, F.; Lee, S.Y.; Woo, S.Y.; Choi, H.Y.; Heo, S.; Nah, G.; Chun, H.S. Transcriptomic responses of *Aspergillus flavus* to temperature and oxidative stresses during aflatoxin formation. *Sci. Rep.* **2021**, *11*, 2803. [CrossRef]

51. Du, C.; Sarfati, J.; Latge, J.; Calderone, R. The role of the sakA (Hog1) and tcsB (shl1) genes in the oxidant adaptation of *Aspergillus fumigatus*. *Med. Mycol.* **2006**, *44*, 211–218. [CrossRef]

52. Nolfi-Donegan, D.; Braganza, A.; Shiva, S. Mitochondrial electron transport chain: Oxidative phosphorylation, oxidant production, and methods of measurement. *Redox Biol.* **2020**, *37*, 101674. [CrossRef]

53. Zhao, R.; Jiang, S.; Zhang, L.; Yu, Z. Mitochondrial electron transport chain, ROS generation and uncoupling. *Int. J. Mol. Med.* **2019**, *44*, 3–15. [CrossRef]

54. Madrigal-Pérez, L.A.; Ramos-Gómez, M. Resveratrol Inhibition of Cellular Respiration: New Paradigm for an Old Mechanism. *Int. J. Mol. Sci.* **2016**, *17*, 368. [CrossRef]
55. Lee, M.J.; Sheppard, D.C. Recent advances in the understanding of the Aspergillus fumigatus cell wall. *J. Microbiol.* 2016, 54, 232–242. [CrossRef]

56. Yang, J.; Zhang, K. Chitin synthesis and degradation in fungi: Biology and enzymes. In *Advances in Experimental Medicine and Biology*; Springer: Singapore, 2019; Volume 1142, pp. 153–167.

57. Dichtl, K.; Helmschrott, C.; Dirr, F.; Wagener, J. Deciphering cell wall integrity signalling in Aspergillus fumigatus: Identification and functional characterization of cell wall stress sensors and relevant Rho GTPases. *Mol. Microbiol.* 2012, 83, 506–519. [CrossRef]

58. Henry, C.; Latgé, J.-P.; Beauvais, A. α1,3 Glucans Are Dispensable in Aspergillus fumigatus. *Eukaryot. Cell* 2012, 11, 26–29. [CrossRef]

59. Pan, L.; Chang, P.; Jin, J.; Yang, Q.; Xing, F. Dimethylformamide Inhibits Fungal Growth and Aflatoxin B1 Biosynthesis in *Aspergillus flavus* by Down-Regulating Glucose Metabolism and Amino Acid Biosynthesis. *Toxins* 2020, 12, 683. [CrossRef]