Differential regulation of mycelial growth and aflatoxin biosynthesis by *Aspergillus flavus* under different temperatures as revealed by strand-specific RNA-Seq

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Abstract
Although several regulatory pathways have been reported for *Aspergillus flavus*, the regulation of aflatoxin production and mycelial growth under different temperatures remains unclear. In this study, *A. flavus* differentially expressed genes (DEGs) and regulatory pathways were analyzed under three temperatures, by strand-specific RNA-Seq. Results show that a total of 2,428 and 1,474 DEGs were identified in fungal mycelia cultured at 20°C and 37°C, respectively, as compared with the control (28°C). Approximately ~79% of DEGs in the 37°C samples were up-regulated genes, while ~63% of DEGs in the 20°C samples were down-regulated genes. Most of the DEG pathways enriched by lower temperatures differed from those enriched by higher temperatures, while only a small portion of the pathways were shared by *A. flavus* grown under different temperatures. Aflatoxin biosynthesis, Butanoate metabolism, oxidation-reduction process, and benzene-containing compound metabolic process were the shared down-regulated pathways, while steroid biosynthesis, oxidoreductase activity, cellular protein modification process, DNA binding, protein complex were the shared up-regulated pathways between lower and higher temperatures. The shared genes and pathways are the key regulatory candidates for aflatoxin biosynthesis with changes of temperature. In addition, the identification of both up-regulated and down-regulated genes provides a useful gene set for further investigation of the aflatoxin biosynthesis among *Aspergillus*.

KEYWORDS
aflatoxin biosynthesis, *Aspergillus flavus* NRRL 3357, pathways, regulation, strand-specific RNA-Seq, temperature

1 | INTRODUCTION

Aflatoxins are a highly toxic secondary metabolite produced by certain molds (e.g., *Aspergillus flavus*, *A. parasiticus* (Chalivendra, DeRobertis, Chang, & Damann, 2017; Klich, 2007). Many important agricultural crops such as maize, rice, wheat, peanuts, and cotton, are prone to contamination by aflatoxins both pre- and postharvest (Bai et al., 2015; Yu et al., 2011). Humans and animals are commonly...
exposed to aflatoxins via the ingestion of contaminated food or products. Several studies have shown that the consumption of food contaminated with aflatoxins can be harmful to the liver, kidney, epidermidis, testis, heart, brain, nervous system and can lead to immune suppression and carcinogenic effects, or even death (Kumar, Mahato, Kamle, Mohanta, & Kang, 2016; Nierman et al., 2015). Although a large number of techniques including physical, chemical, and biological methods have been developed to prevent or reduce the occurrence of aflatoxins in foods, these strategies are not always effective in eliminating grain contamination (Gressel & Polturak, 2018; Klich, 2007; Kumar et al., 2016). To date, the regulatory mechanisms for mycelial growth and aflatoxin biosynthesis under different environmental conditions remain unclear. Studies have also shown that the occurrence of aflatoxins in crops is influenced by various environmental factors, such as nitrogen levels, light, temperature, water, redox status, and pH level (Georgianna & Payne, 2009; Khlangwiset, Shephard, & Wu, 2011). Of these factors, high temperature and drought stress are commonly reported precursors to aflatoxin outbreaks in corn and other crops (Klich, 2007).

Previous investigations have shown that the genes for aflatoxin biosynthesis are clustered in the genome of Aspergillus (Sarma, Bhetaria, Devi, & Varma, 2017; Yu et al., 2004). Twenty-five genes were identified from the 70 kb gene cluster in A. flavus and 82 kb gene cluster in A. parasiticus (Alkhayyat & Yu, 2014; Yu et al., 2004). Of these, the functions of 19 genes have been assigned, while the structures of at least 15 intermediate gene products have been defined (Alkhayyat & Yu, 2014; Yu et al., 2004). Enzymes convert the initial substrate acetate into the four major aflatoxins (B1, B2, G1, and G2) (Yu et al., 2004). It has been also confirmed that the expression of genes in the cluster is regulated by aflR and aflS. The product of aflR is a Gal 4-type 47-kDa polypeptide which binds to the palindromic sequence 5'-TCGN5CGA-3' in the cluster gene promoters, resulting in transcriptional activation of aflatoxin biosynthesis genes (Alkhayyat & Yu, 2014; Yu et al., 2004). AflS can bind AflR inhibitors and act as a transcriptional enhancer to optimize AflR activity (Alkhayyat & Yu, 2014).

The influence of temperature on aflatoxin biosynthesis by A. flavus has been investigated in many studies (Bai et al., 2015; Medina et al., 2017; Yu et al., 2011). The optimum temperature for aflatoxin formation is ~30°C, while the optimum temperature for mycelial growth is ~37°C (Yu et al., 2011). To understand the effects of varying temperatures on aflatoxin biosynthesis, several high-throughput technologies, (e.g., transcriptomic and proteomic analyses) have been introduced. Yu et al., (2011) observed a large number of differently expressed genes between 30°C and 37°C, in mycelia that were harvested 24 hr after inoculation. The average transcription level for the 30 aflatoxin biosynthesis genes increased by ~3,300-fold at 30°C, as compared with 37°C (Yu et al., 2011). Bai et al., (2015) applied transcriptomic and proteomic analyses to identify changes in A. flavus at 37°C for 1.5 days and 28°C for 3 days, showing that post-transcriptional processes play a critical role in regulating the protein level between the two temperatures. Lind, Smith, Saterlee, Calvo, and Rokas (2016) found that 11 temperature-regulated gene clusters, associated with secondary metabolites, were required VeA at 37°C, and LaeA at both 30°C and 37°C. In addition, a large number of gene ontology and KEGG pathways have been identified in maize kernels colonized by A. flavus under different water activities (aw; 0.99 and 0.91) and temperatures (30°C, 37°C) after 10 days (Medina et al., 2017).

With the help of high-throughput technologies, the underlying mechanisms are increasingly being established, although little information is available on the changes in A. flavus mycelial growth and aflatoxin production under low temperature conditions and conflicting results exist on the feasibility of aflatoxin production by A. flavus at 37°C.

In this study, an RNA-Seq approach was used to identify differentially expressed genes (DEGs) and regulatory pathways in A. flavus, under three temperature conditions (20°C, 28°C, and 37°C). Furthermore, the results of bioinformatic analysis were verified by experiments. The results of this study assist our understanding of the regulatory mechanisms of aflatoxin formation under different temperature conditions and can help develop strategies to control the production of aflatoxins in the food chain.

2 | MATERIALS AND METHODS

2.1 | Fungal strain and cultivation conditions

An aflatoxin-producing strain, A. flavus NRRL 3357, was provided by Dr. Zhumei He (Sun Yat-sen University, China). This strain can produce high amounts of aflatoxin B1 on YES agar (20 g/L yeast extract, 150 g/L sucrose, 15 g/L agar) under permissive conditions. In order to analyze the mycelial growth and toxin production abilities of A. flavus NRRL 3357 under different temperatures, agar plates were overlaid with sterile 8.5 cellophane sheets and single point inoculated (centrally) with 10 μl of spore suspension (10⁶ spores in sterile water) and incubated at 20°C, 28°C, or 37°C. Three biological replicates were used for all subsequent analyses.

2.2 | Fungal growth and aflatoxin analyses

Fungal mycelia were collected from the cellophane surface using a scraper, for weight and aflatoxin analyses. All fungal mycelia from each plate were transferred into a 50 ml tube containing 5 ml of methanol at room temperature, then incubated with continual agitation at 150 rpm, for 30 min. The supernatant was collected by centrifugation at 3,000 g and filtered through a syringe filter (RC 0.22 μm, Alltech). The presence of aflatoxin B1 was determined by HPLC with fluorescence detection, using a Waters 600 series HPLC equipped with a 600 pump, a 2,707 autosampler, and a 600 column thermostat set at 30°C. Detection was performed using a 2,475 Multi λ fluorescence detector set at 365 nm (λex) and 465 nm (λem), with a Waters Empower Windows xp operating system (Waters). The analytical column was a Luna 3u C18 (2) (150 x 4.6 mm, 3 μm) (Phenomenex) preceded by a SecurityGuard TM precolumn (C18,
The mobile phase consisted of methanol: water (55:45), eluted at a flow rate of 0.6 ml/min, with 20 μl of filtered extract injected into the HPLC per run. Aflatoxin B1 production was measured in μg/g of mycelia.

2.3 | RNA isolation

Total RNA of A. flavus NRRL 3357 was isolated using a RNAiso plus kit (Takara) according to manufacturer’s instructions. The quality and quantity of total RNAs were characterized using an Agilent 2100 and a Nano-Drop 2000c instrument (Thermo Scientific).

2.4 | RNA sequencing

Crude RNA was digested using 10 U DNase I (TaKaRa) at 37°C for 30 min. Ribosomal RNAs was removed using TruSeq Stranded mRNA Sample Preparation Kit (Illumina) according to the manufacturer’s instructions. Strand-specific RNA-sequencing libraries were constructed using the TruSeq RNA Sample Prep Kit v2 (Illumina) according to the manufacturer’s instructions. The fragments of an expected size were purified and amplified by PCR, with the purified PCR products sequenced using an Illumina Hiseq 4000 platform (BGI-shenzhen).

2.5 | Bioinformatics analyses

The quality of 150-bp reads was assessed using FASTQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The paired-end raw reads from RNA-Seq were trimmed with low quality base-calls and adaptor sequences using the pipeline Trimmomatic (v0.33) tool (Bolger, Lohse, & Usadel, 2014). Cleaned reads were mapped to the genome of A. flavus NRRL 3357 via HISAT2 (v2.1.0) (Kim et al., 2013). Uniquely mapped reads were used to quantify the raw counts using HTSeq (v0.9.1) (Anders, Pyl, & Huber, 2015). DEGs were calculated via DESeq2 using the parameters: p < 0.05 and a fold change >2) (Anders & Huber, 2010). Gene functions were annotated via the BLAST pipeline against references of the protein-encoding sequence from the Nr of GenBank, Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Ashburner et al., 2000; Kanehisa & Goto, 2000). Fisher’s exact test was used to obtain enriched functional terms.

2.6 | Real-time quantitative PCR

Real-time quantitative PCR was used to verify the gene expression level calculated from transcriptomic data. DEGs that may regulate fungal growth and aflatoxin production were verified and selected for further investigation. Crude RNA was used to synthesize cDNA using a transScript® first-strand cDNA synthesis superMix kit (Transgen), where the 20 μL reaction system consisted of 10 μl SYBR® Fast qPCR Mix (2x), 0.5 μl of each primer (10 μmol/l) and 1 μl cDNA. The real-time quantitative PCR program was set to the following sequence: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and finally, 60°C for 10 s. The β-tubulin gene was used as an endogenous control, with three biological replicates assessed for each sample. Relative expression levels were calculated using the 2^−ΔΔCT method.

3 | RESULTS

3.1 | Effects of temperature on mycelial growth and aflatoxin production

To assess the effects of temperature on fungal growth and aflatoxin production, three different culture temperatures were assessed. Figure 1a shows that optimal growth of fungal mycelia was observed at 37°C, while the slowest growth occurred at 20°C, as compared with the 28°C control. Cultivation at 28°C resulted in the highest level of aflatoxin B1 production on YES agar, while no aflatoxin B1 was detected by HPLC in all samples grown at 37°C, from 2 days to 7 days (Figure 1b). Cultivation at 20°C formed significantly less aflatoxin B1 than with that of the 28°C control (p < 0.05) (Figure 1b). Large differences were observed in both the fungal biomass and aflatoxin B1 content of A. flavus cultured at varying temperatures at 4 days and as changes in gene expression occurs in advance of changes in fungal growth and aflatoxin production, samples at 3 days were selected for RNA-Seq.

3.2 | Impact of different temperatures on gene expression

The quality and quantity of total RNAs were examined using Agilent 2100 and Nano-Drop 2000c instruments, confirming that

![Figure 1](image_url) Effects of temperature on A. flavus fungal growth (a) and toxin production (b)
the isolated total RNAs were of a good enough quality for cDNA library construction (Table A1 in Appendix 1). Sequencing of all samples yielded a total of 155,177,520 raw paired-end 150-bp reads (Table A2 in Appendix 1). Assessment of the quality of raw reads by FASTQC showed that an overwhelming majority of reads had quality scores above Q30 (Appendix Figure A1), indicating the quality of the raw reads of all samples could be used for further analyses. 95.37% of reads (147,998,105 paired reads) remained as clean reads, following removal of adaptor, unknown, low quality and rRNA sequences (Table A2 in Appendix 1). The clean reads were used for mapping onto the genome of A. flavus NRRL 3357 for measurement of gene expression levels.

### 3.3 Identification of DEGs

The genome sequence and related annotation files of A. flavus NRRL 3357 were obtained from the J. Craig Venter Institute (https://www.jcvi.org/). More than 61% of paired clean reads could be uniquely mapped to the A. flavus NRRL 3357 genome via pipeline HISAT2 (Table A2 in Appendix 1). 81.22% (10,953 out of 13,486) of putative protein-coding genes could be detected throughout all samples and at the cut-off count of \( \geq 10 \) in at least one sample. Further analysis between samples showed a high correlation of the gene expression levels in the replicates of each treatment (Figure 2).

Due to A. flavus NRRL 3357 producing more aflatoxin at 28°C than at 20°C or 37°C, gene expression following mycelial growth at 28°C was selected as the control. Figure 3 and Table A3 in Appendix 1 show that a total of 2,428 and 1,474 more DEGs were identified in fungal mycelia grown at 20°C and 37°C, respectively, as compared with that of the 28°C control. It is of note that ~79% of the DEGs in 37°C samples belonged to up-regulated genes, while only ~21% of the DEGs were down-regulated. Conversely, ~63% of the DEGs in 20°C samples belonged to down-regulated genes (Table A3 in Appendix 1). The Venn diagram shows that 7.1% of DEGs (137 genes) were shared between up-regulated genes, and 4.4% of DEGs (77 genes) were shared between down-regulated genes (Figure 4).

### 3.4 GO term and KEGG pathway enrichment analyses of the down-regulated DEGs

The enriched GO terms of the down-regulated DEGs impacted by high temperatures were apparently less than that of lower temperatures. The GO terms of the down-regulated DEGs impacted by lower temperatures were enriched in serine-type carboxypeptidase activity, oxidoreductase activity, nitrate metabolic process, fatty acid catabolic process, catalytic complex, cytoskeletal part, benzene-containing compound metabolic process, etc. while oxidoreductase activity, aflatoxin biosynthetic process, austinol biosynthetic process, benzene-containing compound metabolic process, protein complex, among others, were the enriched terms under higher temperature conditions (Figure 5).

KEGG analysis showed that the down-regulated DEGs enriched by lower temperatures were involved in carbon metabolism, nitrogen metabolism, amino acid metabolism, fatty acid degradation, peroxisome, among others (Figure 6). The enriched pathways of down-regulated the DEGs by higher temperatures were involved in aflatoxin biosynthesis, butanone metabolism, C5-branched dibasic acid metabolism, biosynthesis of amino acids, etc (Figure 6). Aflatoxin biosynthesis, butanone metabolism, oxidation-reduction process, and benzene-containing compound metabolic process were the shared down-regulated pathways between lower and higher temperatures.

### 3.5 GO term and KEGG pathway enrichment analyses of up-regulated DEGs

The enriched GO terms of the up-regulated DEGs impacted by high temperatures were apparently more than that at lower
temperatures. The GO terms of the up-regulated DEGs impacted by lower temperatures were enriched in iron ion homeostasis, amino acid transport, oxidoreductase activity, monooxygenase activity, carboxylic acid transmembrane transporter activity, steroid metabolic process, cellular protein modification process, oxidation-reduction process, cellular protein modification process, protein complex, etc., while nitrate metabolic process, melanin biosynthetic process, glycosaminoglycan catabolic process, asexual spore wall assembly, O-methyltransferase activity, galactosidase activity, among others were the enriched terms under higher temperature (Figure 7).

KEGG analysis showed that the up-regulated DEGs enriched by lower temperature were involved only in steroid biosynthesis (Figure 7). The enriched pathways of up-regulated the DEGs by higher temperatures were involved in steroid biosynthesis, glycosphingolipid biosynthesis, Nitrogen metabolism, Amino sugar and nucleotide sugar metabolism, etc. (Figure 8). Steroid biosynthesis, oxidoreductase activity, cellular protein modification process, DNA binding, protein complex were the shared up-regulated pathways between lower and higher temperatures.

3.6 | Aflatoxin biosynthesis processes

A. flavus aflatoxin biosynthesis genes were first analyzed using the SMURF informatics tool. Thirty genes were annotated in the aflatoxin biosynthetic cluster, with twenty-two aflatoxin biosynthetic genes down-regulated by lower temperatures, while all 30 genes were down-regulated by higher temperatures, as compared to the control conditions (Table 1).

3.7 | Oxidoreductase activity

Oxidoreductase activity is driven by laccase or multicopper oxidase. Although the expression levels of the two genes encoding oxidoreductase activity were significantly higher at 37°C than at 28°C, the total expression levels were ~9% lower at 37°C than at 28°C (Table 2). It is of note, that the total expression level was highest at 20°C, suggesting that oxidation-reduction (redox) reactions are influenced by temperature.

3.8 | The DEGs shared by lower and higher temperature

It can be seen that the 77 down-regulated genes shared by lower and higher temperatures were involved in aflatoxin biosynthetic process, and sterigmatocystin biosynthetic process (Table A4 in Appendix 1, Figure 9). The 137 up-regulated genes shared by lower and higher temperatures were involved in cellular macromolecule biosynthetic process, gene expression, nucleic acid metabolic process, and gliotoxin biosynthetic process (Table A5 in Appendix 1, Figure 9). The functions of many DEGs are still unclear.

3.9 | Real-time PCR verification and analysis of several DEGs

To verify the reliability of DEGs identified by RNA-Seq, the relative expression levels of several DEGs were further investigated via real-time PCR. Results show that a similar expression pattern was observed between RNA-Seq and real-time PCR data (Appendix Figure A2), indicating that the relative expression level identified via RNA-Seq was reliable.

4 | DISCUSSION

The results of the present study demonstrate the complexity of aflatoxin biosynthesis regulation and fungal mycelial growth, under different temperatures. As compared to the control, 1,539 genes were significantly down-regulated by the reduced temperature of 20°C, while only 303 genes were significantly down-regulated by the higher temperature of 37°C, in mycelia at 3 days (Table A3 in Appendix 1). It is also very interesting that a majority of the down-regulated genes at higher temperatures related to secondary metabolic processes (Figures 5 and 6), while a majority of genes up-regulated by higher
temperatures were related to primary metabolic processes (Figures 7 and 8) related to fungal growth (Wiseaver, Slot, & Rokas, 2014). It also has been established that sterigmatocystin compounds are the precursor substances for aflatoxin synthesis (Georgianna & Payne, 2009). Majority of the genes related to sterigmatocystin biosynthesis and aflatoxin synthesis were down-regulated by the higher temperature of 37°C, which was consistent with the observation that no aflatoxin B1 was detected by HPLC in all samples grown at 37°C.
 Majority of the enriched pathways at lower temperatures and higher temperature were different. Only a small portion of the pathways were shared by mycelium grown under different temperatures. Aflatoxin biosynthesis, butanoate metabolism, oxidation-reduction process, and benzene-containing compound metabolic process were the shared down-regulated pathways, while steroid biosynthesis, oxidoreductase activity, cellular protein modification process, DNA binding, protein complex were the shared up-regulated pathways between lower and higher temperatures. Secondary metabolic process (toxin metabolic process) and oxidoreductase activity were also shared by the absence of two key members (VeA and LaeA) of the Velvet protein complex (Lind et al., 2016). The results of the present study are in accordance with previous investigation, which implied that the enriched pathways in this study should be reliable and can be used for further investigation. The results of the present study also demonstrate that the total expression level of genes related to oxidoreductase activity was lowest at 37°C and highest at 20°C. These results indicate that fast growth mycelia may be less affected by oxidative stress at 37°C, while slow growth mycelia might be affected by oxidative stress at 20°C. Previous studies have shown that oxidative...
### Table 1: The expression levels of genes in the Aflatoxin biosynthetic cluster

| CDS_ID     | Protein_id | Annotation                                                                 | 20°C (FPKM) | 28°C (FPKM) | 37°C (FPKM) |
|------------|------------|-----------------------------------------------------------------------------|-------------|-------------|-------------|
| AFLA_139200| EED51155   | afiQ/ordA/ord-1/oxidoreductase/cytochrome P450 monoxygenase                 | 20.68       | 57.68       | 10.21       |
| AFLA_139210| EED51156   | afIP/omtA/omt-1/O-methyltransferase A                                       | 55.92       | 162.99      | 25.22       |
| AFLA_139220| EED51157   | afIO/omtB/dmtA/O-methyltransferase B                                        | 336.20      | 592.08      | 85.29       |
| AFLA_139230| EED51158   | afI/avfA/cytochrome P450 monoxygenase                                       | 15.42       | 21.13       | 4.11        |
| AFLA_139240| EED51159   | afILA/hypB/hypothetical protein                                             | 41.56       | 102.93      | 21.14       |
| AFLA_139250| EED51160   | afIL/verB/desaturase/P450 monoxygenase                                      | 81.25       | 192.50      | 36.76       |
| AFLA_139260| EED51161   | afIG/avnA/ord-1/cytochrome P450 monoxygenase                               | 19.68       | 46.12       | 10.72       |
| AFLA_139270| EED51162   | afINa/hypD/hypothetical protein                                             | 1517.30     | 1451.73     | 303.44      |
| AFLA_139280| EED51163   | afIN/verA/monoxygenase                                                      | 57.34       | 77.57       | 19.94       |
| AFLA_139290| EED51164   | afIMa/hypE/hypothetical protein                                             | 121.35      | 320.65      | 59.98       |
| AFLA_139300| EED51165   | afIM/ver-1/dehydrogenase/ketoreductase                                     | 551.98      | 1105.51     | 163.80      |
| AFLA_139310| EED51166   | afIE/norA/aad/adh-2/NOR reductase/dehydrogenase                            | 320.36      | 450.69      | 64.53       |
| AFLA_139320| EED51167   | afJ/estA/esterase                                                          | 368.31      | 631.40      | 94.41       |
| AFLA_139330| EED51168   | afIH/adhA/short-chain alcohol dehydrogenase                               | 163.41      | 274.31      | 38.44       |
| AFLA_139340| EED51169   | afI5/pathway regulator                                                     | 215.12      | 275.53      | 207.27      |
| AFLA_139360| EED51170   | afIR/apa-2/afl-2/transcription activator                                   | 24.44       | 22.74       | 20.89       |
| AFLA_139370| EED51171   | afIB/fas-1/fatty acid synthase beta subunit                                | 32.57       | 57.55       | 11.88       |
| AFLA_139380| EED51172   | afIA/fas-2/hexA/fatty acid synthase alpha subunit                          | 11.35       | 40.30       | 10.40       |
| AFLA_139390| EED51173   | afID/nor-1/reductase                                                       | 76.12       | 348.68      | 47.04       |
| AFLA_139400| EED51174   | afICa/hypC/hypothetical protein                                            | 29.85       | 195.80      | 35.97       |
| AFLA_139410| EED51175   | afIC/pksA/pksL1/polyketide synthase                                        | 30.04       | 278.49      | 61.46       |
| AFLA_139420| EED51176   | afIT/aflT/transmembrane protein                                            | 54.71       | 78.62       | 48.04       |
| AFLA_139430| EED51177   | afIU/cypA/P450 monoxygenase                                                | 0.03        | 0.06        | 0.04        |
| AFLA_139440| EED51178   | afin/norB/dehydrogenase                                                    | 8.88        | 7.63        | 6.94        |
| AFLA_139450| EED51179   | Conserved hypothetical protein                                              | 0.00        | 0.15        | 0.10        |
| AFLA_139460| EED51180   | MFS multidrug transporter putative                                          | 495.81      | 344.31      | 289.30      |
| AFLA_139470| EED51181   | FAD-dependent oxidoreductase putative                                       | 1108.93     | 570.24      | 233.71      |
| AFLA_139480| EED51182   | Dimethylallyl tryptophan synthase putative                                 | 1118.70     | 682.57      | 248.59      |
| AFLA_139490| EED51183   | Hybrid PKS/NRPS enzyme putative                                            | 168.29      | 103.42      | 45.36       |
| AFLA_139500| EED51184   | Conserved hypothetical protein                                              | 0.50        | 0.29        | 0.10        |

### Table 2: The expression levels of members of oxidoreductase activity

| CDS_ID    | Protein_id | Seq. Description                   | 20°C (FPKM) | 28°C (FPKM) | 37°C (FPKM) |
|-----------|------------|-----------------------------------|-------------|-------------|-------------|
| AFLA_084170| EED57720   | Laccase                            | 2.78        | 2.34        | 4.34        |
| AFLA_089660| EED48886   | Iron transport multicopper oxidase | 0.00        | 0.09        | 0.12        |
| AFLA_000890| EED47448   | Laccase                            | 0.00        | 0.00        | 0.02        |
| AFLA_006620| EED48018   | Iron transport multicopper oxidase | 241.78      | 96.82       | 80.56       |
| AFLA_120890| EED45860   | Extracellular dihydrogeodin oxidase | 3.77        | 4.06        | 8.24        |
stress can cause changes in cytosolic and mitochondrial calcium concentrations in *A. nidulans* (Greene, Cao, Schanne, & Bartelt, 2002). Several investigations have also implied that antioxidants can significantly inhibit aflatoxin production, while oxidants enhanced aflatoxin production (Kim et al., 2008; Narasaiah, Sashidhar, & Subramanyam, 2006; Reverberi et al., 2005). According to our results and previously reported studies, oxidative stress resulting from reactive oxygen species might be involved in instigating aflatoxin biosynthesis. The formation of aflatoxin should be regulated via different pathways while being independent from fungal growth.

Previous studies have observed that spore and pigment production is accompanied by the formation of toxins (Chang, 2008; Georgianna & Payne, 2009). In the present study, genes for asexual spore wall assembly were significantly up-regulated under higher temperatures (Figure 7), suggesting that spores are associated or involved in the process of syntheses. Some previous studies have not detected aflatoxins in growth medium at 37°C, while other studies have detected aflatoxins under the same temperatures. According to the enriched pathways, it may be speculated that higher temperatures may block the formation of aflatoxin by delaying the synthesis of its precursor substances during the fast growth stage of fungal mycelia. According to this theory, we cultivated the fungus again as described in the materials and methods section, incubating samples at 37°C for a longer period of time and at 10 days, aflatoxin concentrations reached as high as 10 μg/g. These results support the proposed theory and explain the contrasting results reported by previous studies.

Several genes have been reported to be involved in the production of aflatoxin. Members of the Velvet protein complex coding genes (VeA, LaeA) and homeobox gene (*hbx1*) were proved to be required for the formation of aflatoxin (Cary et al., 2019; Lind et al., 2016). All of them were not the down-regulated genes shared by lower and higher temperatures (Table A4 in Appendix 1). Only the expression of VeA was down-regulated by lower temperature, which suggested that the regulation of aflatoxin biosynthesis by different temperatures is very complex. It is worth noting that a few down-regulated genes shared by lower and higher temperatures were related to toxin biosynthesis, several shared DEGs belong to transcription factor and methyltransferase. In addition, the functions of many DEGs are unclear and still need further investigation. Among the 77 down-regulated genes shared by lower and higher temperature (Table A4 in Appendix 1), and the 137 up-regulated genes shared by lower and higher temperature (Table A5 in Appendix 1), majority of the genes are likely involved in the direct aflatoxin biosynthesis or indirect regulation of aflatoxin biosynthesis. The shared DEGs list provides a useful gene set for further investigation of the aflatoxin biosynthesis among *Aspergillus*.

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**CONFLICT OF INTERESTS**

The authors declare no conflict of interest.

**AUTHOR CONTRIBUTIONS**

Fang Tao conceived and designed experiments. Guomin Han carried out the bioinformatics studies, and Kai Zhao conducted experiments. Guomin Han and Fang Tao wrote the manuscript. Xiaodan Yan, Fangzhi Xiang, and Xuede Li helped draft the manuscript.

**ETHICS STATEMENT**

None required.

**DATA AVAILABILITY STATEMENT**

The raw paired-end sequence data are available in SRA under accession number SRP159671, https://www.ncbi.nlm.nih.gov/sra/SRP159671.
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### APPENDIX 1

#### TABLE A1  The quality and quantity of isolated total RNAs

| Sample  | Concentration (ng/μL) | Volume (μL) | Total amount (μg) | OD$_{260/280}$ | OD$_{260/230}$ | RIN  | 28S/18S Class |
|---------|-----------------------|-------------|------------------|----------------|----------------|-------|--------------|
| 20T_1   | 882                   | 40          | 35.28            | 1.99           | 2.34           | 7.1   | 2            | A            |
| 20T_2   | 786                   | 40          | 31.44            | 1.98           | 2.35           | 7.1   | 1.9          | A            |
| 20T_3   | 1,416                 | 40          | 56.64            | 2.03           | 2.39           | 7.1   | 2            | A            |
| 28T_1   | 1,014                 | 100         | 101.4            | 1.96           | 2.12           | 9.9   | 1.7          | A            |
| 28T_2   | 750                   | 20          | 15               | 2.01           | 1.95           | 9.2   | 2            | A            |
| 28T_3   | 1,464                 | 100         | 146.4            | 1.85           | 2.09           | 7.2   | 1.6          | A            |
| 37T_1   | 1,521                 | 150         | 228.15           | 2.05           | 2.27           | 8.5   | 1.7          | A            |
| 37T_2   | 1,370                 | 100         | 137              | 1.95           | 2.14           | 8.9   | 1.7          | A            |
| 37T_3   | 1,834                 | 100         | 183.4            | 1.81           | 2.02           | 8.3   | 1.7          | A            |

#### TABLE A2  Statistical analysis of RNA-Seq reads mapping results

| Sample  | Number of raw Seq  | Number of clean Seq | Overall read mapping rate (%) | Uniquely mapped reads (%) |
|---------|-------------------|---------------------|-------------------------------|---------------------------|
| 20T_1   | 17,197,487        | 16,355,506          | 76.22                         | 68.14                     |
| 20T_2   | 17,154,192        | 16,259,858          | 75.41                         | 66.97                     |
| 20T_3   | 17,383,718        | 16,616,781          | 77.96                         | 69.77                     |
| 28T_1   | 17,251,932        | 16,455,704          | 71.4                          | 66.14                     |
| 28T_2   | 17,221,767        | 16,446,529          | 68.74                         | 62.68                     |
| 28T_3   | 17,255,279        | 16,484,140          | 67.13                         | 61.59                     |
| 37T_1   | 17,221,828        | 16,449,309          | 67.45                         | 62.45                     |
| 37T_2   | 17,188,718        | 16,316,984          | 67.95                         | 62.58                     |
| 37T_3   | 17,302,599        | 16,553,294          | 68.17                         | 63.16                     |

Note: The number of reads was expressed in pairs.

#### TABLE A3  Number of up-regulated and down-regulated genes

| Sample comparison | Up     | Down   | Total |
|-------------------|--------|--------|-------|
| 20°C vs. 28°C     | 889    | 1,539  | 2,428 |
| 37°C vs. 28°C     | 1,171  | 303    | 1,474 |
| CDS Id    | Protein Id | Seq. description                                           |
|-----------|------------|------------------------------------------------------------|
| AFLA_072350 | EED56541   | Conserved hypothetical protein                             |
| AFLA_073450 | EED56651   | Amine oxidase                                              |
| AFLA_074340 | EED56740   | Uncharacterized oxidoreductase Ygbj                       |
| AFLA_074510 | EED56757   | Short-chain dehydrogenase family                           |
| AFLA_082440 | EED57547   | Methyltransferase domain-containing protein                |
| AFLA_084250 | EED57728   | Ketose-bisphosphate aldolase class-ii family protein       |
| AFLA_087620 | EED58063   | nmra family transcriptional regulator                     |
| AFLA_024510 | EED55180   | MFS quinate transporter                                    |
| AFLA_033970 | EED55180   | Conserved hypothetical protein                             |
| AFLA_013220 | EED54070   | Beta-lactamase family protein                              |
| AFLA_016350 | EED54383   | Nadph-dependent fmn reductase                              |
| AFLA_016620 | EED54410   | Tryptophanyl-trna synthetase                               |
| AFLA_109300 | EED53553   | fad synthetase                                             |
| AFLA_110220 | EED53645   | mgs207 protein                                             |
| AFLA_038420 | EED52141   | Chitosanase                                                |
| AFLA_039270 | EED52225   | Carboxylesterase family protein                            |
| AFLA_039280 | EED52226   | Carboxylesterase family protein                            |
| AFLA_039650 | EED52263   | Nadh-cytochrome b5 reductase                               |
| AFLA_041280 | EED52426   | Dimethylaniine monooxygenase                               |
| AFLA_061120 | EED51849   | Polyamine transporter                                      |
| AFLA_061760 | EED51913   | Conserved hypothetical protein                             |
| AFLA_062700 | EED52007   | Mitochondrial carrier                                      |
| AFLA_062890 | EED52026   | Hypothetical protein AFLA_062890                          |
| AFLA_136790 | EED50916   | Acetolactate synthase                                      |
| AFLA_138150 | EED51051   | Hypothetical protein AFLA_138150                          |
| AFLA_138870 | EED51122   | Cyanovirin-n family protein                                |
| AFLA_138920 | EED51127   | Dimethylaniine monooxygenase                               |
| AFLA_139170 | EED51152   | Sterigmatocystin biosynthesis monooxygenase stcw           |
| AFLA_139200 | EED51155   | Cytochrome p450 monooxygenase                              |
| AFLA_139210 | EED51156   | o- partial                                                 |
| AFLA_139240 | EED51159   | Partial                                                    |
| AFLA_139250 | EED51160   | aflf verb desaturase p450 monooxygenase                    |
| AFLA_139260 | EED51161   | aflg avna ord-1 cytochrome p450 monooxygenase              |
| AFLA_139290 | EED51164   | Hypothetical e partial                                     |
| AFLA_139380 | EED51172   | Fatty acid synthase alpha subunit                          |
| AFLA_139390 | EED51173   | Norsolorinic acid partial                                  |
| AFLA_139400 | EED51174   | duf1772-domain-containing protein                          |
| AFLA_139410 | EED51175   | Polyketide synthase                                        |
| AFLA_064900 | EED49669   | Nadh-ubiquinone oxidoreductase kda mitochondrial            |
| AFLA_066480 | EED49826   | Cyclase                                                    |
| AFLA_066970 | EED49875   | Endonuclease exonuclease phosphatase family protein         |
| AFLA_066980 | EED49876   | Polyketide synthase                                        |
| AFLA_067740 | EED49952   | Conserved hypothetical protein                             |

(Continues)
### TABLE A4  (Continued)

| CDS Id     | Protein Id | Seq. description                      |
|------------|------------|---------------------------------------|
| AFLA_067880 | EED49966   | MFS transporter                       |
| AFLA_069030 | EED50081   | Conserved hypothetical protein         |
| AFLA_093890 | EED49308   | Conserved hypothetical protein         |
| AFLA_096130 | EED49531   | clavata3 esr-like protein              |
| AFLA_124290 | EED48206   | c6 transcription                       |
| AFLA_124300 | EED48207   | MFS general substrate transporter      |
| AFLA_124970 | EED48274   | Ankyrin domain protein                 |
| AFLA_125000 | EED48277   | MFS multidrug transporter              |
| AFLA_125330 | EED48310   | Ankyrin repeat-containing protein      |
| AFLA_128540 | EED48631   | Proline oxidase                        |
| AFLA_005040 | EED47863   | Phenazine biosynthesis-like            |
| AFLA_005570 | EED47915   | Short-chain dehydrogenase reductase family |
| AFLA_052610 | EED47207   | Succinyl-3-ketoacid:coenzyme a transferase |
| AFLA_053540 | EED47299   | fad-dependent oxidoreductase           |
| AFLA_054060 | EED47351   | atp gtp-binding protein                |
| AFLA_054520 | EED47397   | 1-aminoalkylcyclopropane-1-carboxylate synthase |
| AFLA_054530 | EED47398   | Synaptic vesicle transporter svop      |
| AFLA_054550 | EED47400   | myo-inositol 2-dehydrogenase           |
| AFLA_097300 | EED46067   | Metal-activated pyridoxal enzyme       |
| AFLA_097340 | EED46071   | Transmembrane amino acid transporter protein |
| AFLA_097530 | EED46090   | duf1857 domain-containing protein      |
| AFLA_098140 | EED46151   | Monocarboxylate                        |
| AFLA_101930 | EED46529   | Succinate-semialdehyde dehydrogenase   |
| AFLA_101990 | EED46535   | Hexose carrier protein                 |
| AFLA_116550 | EED45426   | Glycoside hydrolase family 24 protein  |
| AFLA_117000 | EED45471   | RNA                                   |
| AFLA_118420 | EED45613   | Hypothetical protein AFLA_118420      |
| AFLA_118450 | EED45616   | Six-hairpin glycosidase                |
| AFLA_118740 | EED45645   | Xylose isomerase tim barrel            |
| AFLA_120990 | EED45870   | o-methyltransferase                   |
| AFLA_122140 | EED45985   | Acetyltransferase                     |
| AFLA_009040 | EED45020   | 3-Isopropylmalate dehydrogenase        |

### TABLE A5  Annotation of up-regulated genes shared by lower and higher temperature

| CDS Id     | Protein Id | Seq. description                                      |
|------------|------------|-------------------------------------------------------|
| AFLA_078540 | EED57159   | Immediate early response protein ier                   |
| AFLA_082090 | EED57512   | Fungal-specific transcription factor domain-containing protein |
| AFLA_082720 | EED57575   | Fatty acid elongase                                   |
| AFLA_083800 | EED57683   | Endonuclease exonuclease phosphatase family protein    |
| AFLA_087280 | EED58029   | Alpha-ketoglutarate-dependent taurine dioxygenase      |
| AFLA_022820 | EED55021   | Conserved hypothetical protein                         |
| AFLA_022830 | EED55022   | Conserved hypothetical protein                         |
| AFLA_023350 | EED55064   | Beta-glucosidase m                                     |

(Continues)
| CDS Id      | Protein Id | Seq. description                                                                 |
|------------|------------|----------------------------------------------------------------------------------|
| AFLA_023870 | EED55116   | Transmembrane domain of the epidermal growth factor receptor family of protein tyrosine kinase |
| AFLA_023880 | EED55117   | Hsp70 family chaperone                                                            |
| AFLA_023890 | EED55118   | Conserved hypothetical protein                                                     |
| AFLA_025190 | EED55248   | Lipase                                                                           |
| AFLA_027990 | EED55527   | Conserved hypothetical protein                                                     |
| AFLA_028640 | EED55992   | Cytochrome p450 61                                                                |
| AFLA_028890 | EED5617    | Tartrate dehydrogenase                                                            |
| AFLA_030430 | EED5771    | Fatty acid oxygenase                                                              |
| AFLA_032440 | EED5972    | Conserved hypothetical protein                                                     |
| AFLA_034810 | EED6209    | Hypothetical protein AFLA_034810                                                  |
| AFLA_035890 | EED6317    | Acyl- n-acyltransferase                                                            |
| AFLA_036370 | EED6365    | Phosphoenolpyruvate carboxykinase                                                 |
| AFLA_037250 | EED6451    | Cyanide hydratase                                                                 |
| AFLA_040430 | EED5415    | Hypothetical protein AFLA_040430                                                  |
| AFLA_041310 | EED5417    | Sodium bile acid symporter family protein                                          |
| AFLA_041530 | EED5440    | Beta-galactosidase                                                                |
| AFLA_041760 | EED5496    | Sun domain protein                                                                |
| AFLA_046700 | EED5524    | Phenol 2-                                                                         |
| AFLA_046800 | EED5583    | 4-coumarate-- ligase-like 7                                                       |
| AFLA_048740 | EED5622    | Cora family metal ion transporter                                                 |
| AFLA_050960 | EED5844    | Copper resistance-associated p-type atpase                                         |
| AFLA_051000 | EED5906    | Monoxygenase                                                                       |
| AFLA_052500 | EED5153    | Conserved hypothetical protein                                                     |
| AFLA_106900 | EED5315    | Major facilitator superfamily general substrate transporter                         |
| AFLA_109230 | EED53546   | 2-hydroxyacid dehydrogenase                                                       |
| AFLA_109460 | EED5369    | Family taurine                                                                    |
| AFLA_113430 | EED5966    | Transcription factor subunit 5                                                    |
| AFLA_038530 | EED52152   | Elastinolytic metalloproteinase mep                                                |
| AFLA_039540 | EED5252    | Conserved hypothetical protein                                                     |
| AFLA_040140 | EED5239    | Aquaporin                                                                         |
| AFLA_040580 | EED5236    | Serine threonine protein kinase                                                    |
| AFLA_041410 | EED52439   | Aldehyde dehydrogenase family protein                                             |
| AFLA_046740 | EED52972   | nadp-dependent malic enzyme                                                        |
| AFLA_057600 | EED51497   | Heat shock protein                                                                 |
| AFLA_057800 | EED5105    | Beta-n-                                                                           |
| AFLA_057710 | EED5108    | Oxaloacetate acetylhydrolase                                                       |
| AFLA_057810 | EED51518   | Glutamine-serine-proline rich                                                      |
| AFLA_059990 | EED51736   | flavin-dependent halogenase o-methyltransferase bifunctional protein              |
| AFLA_060020 | EED51739   | Polyketide synthase                                                                |
| AFLA_060770 | EED51814   | Protein alcs                                                                      |
| AFLA_062630 | EED52000   | hyp effector                                                                       |
| AFLA_062820 | EED52019   | Polyketide synthase                                                               |
| AFLA_063040 | EED52041   | Glycosyl hydrolase family 3 n terminal domain-containing protein                  |
| CDS Id     | Protein Id | Seq. description                                      |
|------------|------------|-------------------------------------------------------|
| AFLA_063240| EED52061   | Hypothetical protein AFLA_063240                     |
| AFLA_063250| EED52062   | Glutaminyl cyclase                                    |
| AFLA_063260| EED52063   | Lwamide neuropeptide partial                          |
| AFLA_063270| EED52064   | Hypothetical protein AFLA_063270                     |
| AFLA_063280| EED52065   | Conserved hypothetical protein                        |
| AFLA_063290| EED52066   | Conserved hypothetical protein                        |
| AFLA_063300| EED52067   | Conserved hypothetical protein                        |
| AFLA_063310| EED52068   | tat pathway signal sequence protein                   |
| AFLA_132470| EED50485   | Toxin biosynthesis protein                            |
| AFLA_133640| EED50602   | Cell wall cysteine-rich protein                       |
| AFLA_133810| EED50619   | Conserved hypothetical protein                        |
| AFLA_137770| EED51013   | uma methyltransferase family protein                 |
| AFLA_138060| EED51042   | c-24 sterol reductase                                 |
| AFLA_138400| EED51076   | nad-dependent epimerase dehydratase                  |
| AFLA_138760| EED51111   | trx2p                                                 |
| AFLA_064380| EED49617   | radh flavin-dependent halogenase                     |
| AFLA_064390| EED49618   | Cytochrome p450                                       |
| AFLA_064400| EED49619   | Cytochrome p450                                       |
| AFLA_064440| EED49623   | Heavy metal tolerance protein                         |
| AFLA_064450| EED49624   | 1-aminocyclopropane-1-carboxylate synthase           |
| AFLA_064460| EED49625   | Toxin biosynthesis protein                            |
| AFLA_064470| EED49626   | Cytochrome p450                                       |
| AFLA_064480| EED49627   | Thioredoxin reductase                                 |
| AFLA_064490| EED49628   | Methyltransferase                                     |
| AFLA_064510| EED49630   | Thioredoxin reductase                                 |
| AFLA_064530| EED49632   | Glutathione s-transferase                             |
| AFLA_064540| EED49633   | Cytochrome p450 monooxygenase                         |
| AFLA_064550| EED49634   | Membrane dipeptidase                                  |
| AFLA_064560| EED49635   | Nonribosomal peptide synthase-like                    |
| AFLA_064570| EED49636   | ncs1 nucleoside transporter                           |
| AFLA_064580| EED49637   | Dimeric dihydrodiol                                   |
| AFLA_064590| EED49638   | o-methyltransferase                                   |
| AFLA_064600| EED49639   | Major facilitator superfamily domain                  |
| AFLA_066050| EED49783   | DNA repair family protein                              |
| AFLA_066710| EED49849   | Oxoglutarate iron-dependent dioxygenase              |
| AFLA_070280| EED50206   | Siderophore esterase -like protein                    |
| AFLA_070400| EED50218   | aaa family atpase                                     |
| AFLA_070420| EED50220   | Siderochrome-iron transporter                         |
| AFLA_090590| EED48978   | Alpha-1 subfamily                                     |
| AFLA_093580| EED49277   | Integral membrane protein                             |
| AFLA_095310| EED49450   | Conserved hypothetical protein                        |
| AFLA_096180| EED49536   | duf636 domain protein                                 |
| AFLA_096650| EED49583   | Conserved hypothetical protein                        |
| AFLA_096660| EED49584   | Conserved hypothetical protein                        |
| AFLA_122840| EED48082   | Conserved hypothetical protein                        |
| CDS Id  | Protein Id | Seq. description                                      |
|---------|------------|------------------------------------------------------|
| AFLA_123700 | EED48147  | Extracellular proline-rich protein                   |
| AFLA_125620 | EED48339  | dj-1-type                                            |
| AFLA_125760 | EED48353  | Squalene cyclase                                     |
| AFLA_126510 | EED48428  | Copper-transporting atpase                            |
| AFLA_127490 | EED48526  | Hypothetical protein AOR_1_770164                    |
| AFLA_128040 | EED48581  | Major facilitator superfamily transporter            |
| AFLA_128050 | EED48582  | Serine hydrolase fsh                                 |
| AFLA_128110 | EED48588  | Aquaglyceroporin                                     |
| AFLA_129750 | EED48752  | mt-A70 family                                        |
| AFLA_000850 | EED47444  | Isoamyl alcohol                                      |
| AFLA_003960 | EED47755  | Hypothetical protein AFLA_003960                    |
| AFLA_004270 | EED47786  | Protein kinase-like domain                           |
| AFLA_004870 | EED47846  | Cytochrome p450                                      |
| AFLA_005760 | EED47934  | Conserved hypothetical protein                       |
| AFLA_007170 | EED48073  | Pumilio-family rna binding repeat protein            |
| AFLA_048390 | EED46786  | S-Adenosyl-l-methionine-dependent methyltransferase  |
| AFLA_049160 | EED46863  | Cyclopentanone-monooxygenase                         |
| AFLA_049210 | EED46868  | Integral membrane protein                            |
| AFLA_049520 | EED46899  | Integral membrane protein pth11                     |
| AFLA_052520 | EED47198  | Hypothetical protein AFLA_052520                    |
| AFLA_054360 | EED47381  | Methyltransferase type 11                            |
| AFLA_099110 | EED46248  | Fibronectin type iii domain-containing protein       |
| AFLA_099750 | EED46311  | Epoxide hydrolase                                    |
| AFLA_100260 | EED46362  | t5orf172 domain protein                              |
| AFLA_101540 | EED46490  | Protein                                              |
| AFLA_116390 | EED45410  | Amino acid transporter                               |
| AFLA_120630 | EED45834  | Formate dehydrogenase                                |
| AFLA_121190 | EED45890  | Zinc-binding oxidoreductase                          |
| AFLA_121730 | EED45944  | Alpha-galactosidase c                                |
| AFLA_121740 | EED45945  | Hypothetical protein AFLA_121740                    |
| AFLA_122040 | EED45975  | Oleate delta-12 desaturase                           |
| AFLA_007600 | EED44877  | Oligopeptide transporter opt superfamily            |
| AFLA_009910 | EED45107  | Membrane fusion mating protein Figure 1             |
| AFLA_010590 | EED45175  | Siderophore biosynthesis lipase                     |
| AFLA_010610 | EED45177  | enoyl-hydration isomerase family protein            |
| AFLA_010620 | EED45178  | amp-dependent synthetase ligase                     |
| AFLA_010630 | EED45179  | abc multidrug transporter                            |
| AFLA_010640 | EED45180  | Siderophore iron transporter                         |
| AFLA_010740 | EED45190  | Carboxypeptidase s1                                 |
| AFLA_011540 | EED45270  | Multiple drug resistance protein                     |
APPENDIX 2

**FIGURE A1** Quality of raw reads of two arbitrarily selected samples

**FIGURE A2** Relative expression of several DEGs via Real-time PCR