The anti-aflatoxigenic mechanism of cinnamaldehyde in *Aspergillus flavus*

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Aflatoxin B₁ (AFB₁), the predominant and most carcinogenic naturally polyketide, is mainly produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Cinnamaldehyde has been reported for inhibiting the growth and aflatoxin biosynthesis in *A. flavus*. But its molecular mechanism of action still remains largely ambiguous. Here, the anti-aflatoxigenic mechanism of cinnamaldehyde in *A. flavus* was investigated via a comparative transcriptomic analysis. The results indicated that twenty five of thirty genes in aflatoxin cluster showed down-regulation by cinnamaldehyde although the cluster regulators aflR and aflS were slightly up-regulated. This may be due to the up-regulation of the oxidative stress-related genes srrA, msnA and atfB being caused by the significant down-regulation of the diffusible factor FluG. Cinnamaldehyde also inhibited aflatoxin formation by perturbing GPCRs and oxylipins normal function, cell wall biosynthesis and redox equilibrium. In addition, accumulation of NADPH due to up-regulation of pentose phosphate pathway drove acetyl-CoA to lipids synthesis rather than polyketides. Both GO and KEGG analysis suggested that pyruvate and phenylalanine metabolism, post-transcriptional modification and key enzymes biosynthesis might be involved in the suppression of AFB₁ production by cinnamaldehyde. This study served to decipher the anti-aflatoxigenic properties of cinnamaldehyde in *A. flavus* and provided powerful evidence for its use in practice.

*Aspergillus flavus*, as a widely distributed saprotrophic filamentous fungus especially in warmer and moister atmosphere, is the major safety problem in both agricultural and medical products. It can produce an abundance of diverse secondary metabolites including aflatoxins, conidial pigments, cyclopiazonic acid, aflatrem and kojic acid. Of them, aflatoxins are the predominant and most carcinogenic naturally occurring compounds which inevitably result in health complications, including hepatocellular carcinoma, acute intoxication, immune system disorder and growth retardation in children. Therefore, aflatoxin remains a global threat to human and animal health, and is one of the key safety indicators of grain.

Many strategies have been used to reduce aflatoxin contamination. At present, chemical agents still are often used for controlling post-harvest aflatoxin contamination. However, these agents have many disadvantages such as toxicity, residues in food chain, and greater likelihood of resistance. Therefore, facing with a huge burden and threat, people aroused the interest of discovering safe and efficient natural substances for preventing and controlling *A. flavus* growth and aflatoxin production. In previous studies, essential oils such as eugenol, carvacrol, citral and cinnamaldehyde, possessing potent anti-microbial, antioxidant, and other biological activities, were applied to food industry as food additive. Cinnamaldehyde, a major component of Chinese cinnamon oil from *Cinnamomum spp.*, is used as legally flavoring antimicrobial ingredient and referenced as “generally recognized as safe” for mankind and surroundings by the USFDA and FAO/WHO. It has been widely used in food, booze to inhibit the growth of bacteria, yeast and filamentous fungi because of the wider spectrum antimicrobial activities since long time. It was highly efficient for suppressing *Salmonella typhimurium* and *Staphylococcus aureus* in watermelon juice, and *Salmonella enterica* in apple juice. Besides, growth of *Fusarium verticillioides*, *Aspergillus ochraceus*, *Penicillium expansum* and *A. flavus* has been remarkably inhibited by cinnamaldehyde.

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Table 1. GO functional enrichment analysis of DEGs when *A. flavus* was treated with cinnamaldehyde.

| GO ID    | Description                                           | p-value  | q-value  | List hits |
|----------|-------------------------------------------------------|----------|----------|-----------|
| GO:0003723 | RNA binding                                          | 8.31E-03 | 2.07E-01 | 14/149    |
| GO:0016614 | oxidoreductase activity, acting on CH=OH group of donors | 2.47E-02 | 4.32E-01 | 9/149     |
| GO:0004518 | nuclease activity                                    | 1.84E-02 | 3.54E-01 | 6/149     |
| GO:0007470 | translation initiation factor activity                | 1.43E-03 | 2.07E-01 | 3/149     |

particular, it can depress the production of aflatoxin by *A. flavus*\(^{14,16}\). It also stimulates apoptosis and inhibits tumor growth\(^{14}\), and has been reported as an effective agent against several cancers effectively\(^{15,16}\).

Out of 10 essential oils previously studied by our group, cinnamaldehyde was the most effective against fungal growth and aflatoxin production by *A. flavus*\(^{17}\). Production of aflatoxin B\(_1\) (AFB\(_1\)) was completely inhibited by cinnamaldehyde at lower concentration (0.4 mM) without influencing *A. flavus* growth, and at the concentration of 0.8 mM cinnamaldehyde showed complete inhibition of fungal growth and AFB\(_1\) production\(^{11}\). This was similarly consistent to the results reported by Sun\(^8\), which indicated that fungal growth and aflatoxin production were significantly inhibited by cinnamaldehyde in dose-dependent manner by modulating the oxidative stress in *A. flavus*. It takes inhibitory action against bacteria\(^{11,18,19}\), yeast and filamentous molds\(^{10,12}\) by depressing intracellular ATP\(^{23}\), cell wall biosynthesis\(^{12}\), and altering the membrane structure and integrity\(^{14}\). Using qPCR, Yin et al.\(^{24}\) found that cinnamaldehyde (0.005%) significantly inhibited AFB\(_1\) production in *A. flavus* and *A. parasiticus*. The expressions of the majority of aflatoxin gene cluster were down-regulated by more than 4-folds, especially pks\(_A\) (aflC), nor-1 (aflD), and norA (aflE). Our previous studies showed that AFB\(_1\) production was largely reduced in *A. flavus* treated with cinnamaldehyde at the low concentration in YES medium\(^1\). In the presence of cinnamaldehyde (0.4 mM), aflM was significantly down-regulated by more than 5963-fold, following by aflP, aflR, aflD and aflT. The decreased transcription levels of aflatoxin cluster genes subsequently resulted in the reduction of AFB\(_1\) production. Although many researchers develop desire at exploring the anti-aflatoxigenic mechanism of cinnamaldehyde, the detailed molecular mechanism behind in the inhibition of aflatoxin biosynthesis by cinnamaldehyde still remains largely ambiguous.

RNA-seq, a high-throughput sequencing technology used to sequence complementary DNA, has been applied to transcriptomic studies, including anti-fungi response mechanism to essential oils. Wang et al.\(^{15}\) found that cinnamaldehyde inhibited *P. expansum* by modulating the oxidative stress and down-regulating the ergosterol biosynthesis using transcriptional profiling analysis. In another report, the transcriptome profiling of *A. flavus* exposed to antioxidant gallic acid was used in exploring the response mechanism\(^{16}\). The gallic acid played a pivotal role in fungal development via over-expression of *brlA* while the velvet complex didn’t show a significant differential expression. In addition, other regulators were also involved in the inhibitory mechanism of gallic acid. In another transcriptional profiling analysis of *A. flavus* exposure to 5-azaacytidine (5-AC), the up-regulation of *brlA* was also found\(^{25}\).

The main aim of this study was to investigate the role of cinnamon in the inhibition of fungal developmental and secondary metabolite biosynthesis of *A. flavus* via RNA-seq approach. The differentially expression genes between cinnamaldehyde treated and untreated *A. flavus* were obtained and further analyzed. Especially, the anti-aflatoxigenic mechanism of cinnamaldehyde was revealed. This work may also contribute to better understanding on the aflatoxin biosynthesis and regulation.

**Results**

**Overall transcriptional response profile of *A. flavus* to natural cinnamaldehyde.** To explore the latent detailed molecular mechanism response to natural cinnamaldehyde on *A. flavus*, a transcriptome analysis was implemented to evaluate the response at mRNA level. Averagely, *A. flavus* YC-15 untreated and treated with cinnamaldehyde generated 10.63 million and 11.11 million raw reads, respectively. From these raw reads, 8.84 million and 9.26 million clean reads were obtained after purity filtering. And, 58.84% and 68.36% of total clean reads from control and treatment groups were mapped to the reference genome sequence while only 0.02% were aligned to rRNA genes. The mRNA data revealed that 1032 genes were significantly differentially transcribed compared with the untreated group. Especially, 113 genes showed up-regulation and 605 genes showed down-regulation in cinnamaldehyde-treated group compared with the untreated group.

**Functional classification and pathway analysis of differential expression genes (DEGs).** The DEGs between the *A. flavus* treated with cinnamaldehyde (R75) and control group (CK) provided a potential anti-aflatoxigenic mechanism of cinnamaldehyde related to *A. flavus*. These 1032 DEGs related to a large quantity of regulatory and metabolic process were identified (with FDR \(\leq 0.05\), log2Ratio \(\geq 1\) or \(\leq -1\)) between R75 and CK according to the FPKM values. In order to analyze the functions of 1032 DEGs, GO functional and KEGG metabolic pathways enrichment analyses were performed. GO analysis revealed that these significantly DEGs were mainly involved in oxidoreductase activity, RNA binding, Nuclease activity and translation initiation factor activity (Table 1, Fig. 1). KEGG analysis revealed that these significantly DEGs were mainly involved in RNA transport, ribosome biogenesis, pyruvate metabolism, phenylalanine metabolism, sulfur relay system and sulfur metabolism (Table 2).
Genes involved in biosynthesis of conidial pigment, aflatrem, aflatoxin and cyclopiazonic acid. The expression profile referred to the biosynthesis of conidial pigment (#10), aflatrem (#15), aflatoxin (#54), and cyclopiazonic acid (#55) were evaluated and shown in Table 3. In pathway #10, O-methyltransferase family protein (AFLA_016120) and a hypothetical protein (AFLA_016130) were down-regulated, but arp1 gene was up-regulated. In pathway #15, the majority of cluster genes showed low-level expression. In pathway #55, MFS multidrug transporter (AFLA_139460) was slightly down-regulated, nevertheless the genes encoding a hybrid PKS/NRPS enzyme (AFLA_139490), FAD dependent oxidoreductase (AFLA_139470), and tryptophan dimethylallyltransferase (AFLA_139480) were up-regulated. Our previous studies confirmed that cinnamaldehyde can repress the aflatoxin production and development in dose-dependent manner1. Based on the transcriptome data, 25 of 34 genes in aflatoxin biosynthetic gene cluster were down-regulated to varying degrees including the key structural genes aflC, aflD, aflE, aflG, aflH, aflI, aflL, aflM, aflO, aflP and aflQ in A. flavus treated with 0.60 mmol/L of cinnamaldehyde. Surprisingly, both transcription regulator genes aflR and aflS in aflatoxin cluster showed a slight up-regulation. However, the lower level of aflS/aflR ratio was observed compared with the untreated group, subsequently resulting in the down regulation of most structural genes. The aflU gene, encoding a P450 monooxygenase and probably involving in the biosynthesis of AFG 1 and AFG2 rather than AFB 1, was up-regulated after treatment of cinnamaldehyde. The aflT gene, encoding a membrane-bound protein presumed to be involved in aflatoxin secretion, was not affected by cinnamaldehyde. Furthermore, genes involved in aflatoxin biosynthesis pathway were analyzed through qPCR and the results were shown in Fig. 2. Meantime, the sugar cluster genes sugR, orf, glcA and hxtA were significantly up-regulated by cinnamaldehyde.

Table 2. KEGG metabolic pathway enrichment analysis of DEGs when A. flavus was treated with cinnamaldehyde.

| ID     | TERM (molecular functions)               | p-value | q-value | List hits |
|--------|-----------------------------------------|---------|---------|-----------|
| afv00620 | Pyruvate metabolism                      | 0.0174  | 0.28691  | 8/58      |
| afv00360 | Phenylalanine metabolism                 | 0.0794  | 0.785848 | 7/58      |
| afv04122 | Sulfur relay system                      | 0.0302  | 0.373098 | 4/58      |
| afv03013 | RNA transport                            | 0.0016  | 0.040238 | 3/58      |
| afv03008 | Ribosome biogenesis in eukaryotes        | 0.0010  | 0.040238 | 2/58      |
| afv00920 | Sulfur metabolism                        | 0.0980  | 0.808409 | 2/58      |

Genes involved in fatty acids β-oxidation and pentose phosphate pathway. Fatty acids β-oxidation in peroxisome and mitochondria promoted aflatoxin formation26. And there is a competition in acetyl-CoA between lipid synthesis and polyketides formation27. The transcriptional levels related fatty acids β-oxidation and pentose phosphate pathway were shown in Table S1. A large number of fatty acids β-oxidation-related genes were significantly down-regulated. The most strongly down-regulated gene in peroxisome was AFLA_009410, followed by AFLA_135240, AFLA_091060, and AFLA_090720. However, most of the genes in pentose phosphate pathway were up-regulated in A. flavus exposure to cinnamaldehyde, for example, Znw1 (AFLA_086620), Sol (3AFLA_080390), and Gmd1 (AFLA_036840).
| Cluster ID | Gene ID (AFLA.x) | Untreated (FPKM) | R75 (FPKM) | LOG | Annotated gene function |
|------------|------------------|------------------|------------|-----|-------------------------|
| #10 016130 | 10.58            | 5.31             | -0.99      | O-methyltransferase family protein |
| #10 016130 | 13.25            | 6.91             | -0.94      | hypothetical protein |
| #10 016140 | 14.10            | 20.44            | 0.54       | conidial pigment biosynthesis scytalone dehydratase Arp1 |
| #15 045450 | 37.27            | 47.33            | 0.34       | ankyrin repeat-containing protein, putative |
| #15 045460 | 1.16             | 2.50             | 1.11       | hypothetical protein |
| #15 045470 | 0.10             | 0.07             | -0.43      | nonsense-mediated mRNA decay protein, putative |
| #15 045480 | 0.32             | 1.35             | 2.08       | conserved hypothetical protein |
| #15 045490 | 0.03             | 0.16             | 2.26       | dimethylallyl tryptophan synthase, putative |
| #15 045500 | 0.55             | 0.26             | -1.10      | cytochrome P450, putative |
| #15 045510 | 0.12             | 0.07             | -0.89      | integral membrane protein |
| #15 045520 | 0.00             | 0.00             | /          | integral membrane protein |
| #15 045530 | 0.23             | 0.00             | down       | conserved hypothetical protein |
| #15 045540 | 0.00             | 0.00             | /          | cytochrome P450, putative |
| #15 045550 | 1.24             | 2.57             | 1.05       | hypothetical protein |
| #15 045560 | 1.94             | 3.00             | 0.63       | carboxylic acid transport protein |
| #15 045570 | 1.55             | 0.07             | -4.42      | acetyl xylan esterase, putative |
| #54 139390 | 231.56           | 105.85           | -1.13      | aflD/nor-1/reductase |
| #54 139400 | 84.14            | 39.09            | -1.11      | aflG/hypC/hypothetical protein |
| #54 139260 | 48.69            | 23.00            | -1.08      | aflGI/avrA/ord-1/cytochrome P450 monooxygenase |
| #54 139330 | 192.79           | 92.60            | -1.06      | aflBH/adhA/short chain alcohol dehydrogenase |
| #54 139210 | 92.70            | 45.79            | -1.02      | aflP/ompA/omp-1/O-methyltransferase A |
| #54 139290 | 136.29           | 69.97            | -0.96      | aflM/aflE/hypothetical protein |
| #54 139300 | 496.53           | 274.75           | -0.85      | aflB/M/ver-1/dehydrogenase/ketoreductase |
| #54 139230 | 15.55            | 9.08             | -0.79      | aflI/aflC/cytochrome P450 monooxygenase |
| #54 139240 | 108.16           | 63.18            | -0.78      | aflI/aflH/hypothetical protein |
| #54 139250 | 92.87            | 56.46            | -0.72      | aflI/avfA/desaturase/P450 monooxygenase |
| #54 139140 | 5.53             | 3.38             | -0.71      | aflI/aflH/NADAD oxireductase |
| #54 139160 | 117.97           | 73.17            | -0.69      | aflX/ordB/monooxygenase/oxidase |
| #54 139150 | 101.03           | 63.54            | -0.67      | aflY/hypA/hypothetical protein |
| #54 139310 | 180.51           | 116.23           | -0.64      | aflE/norA/adh-2/NOR reductase/dehydrogenase |
| #54 139180 | 48.91            | 32.57            | -0.59      | aflV/cycX/cytochrome P450 monooxygenase |
| #54 139410 | 37.55            | 25.15            | -0.58      | aflC/pskA/pskL/1/ployketide synthase |
| #54 139170 | 49.07            | 34.09            | -0.53      | aflW/moxY/monooxygenase |
| #54 139320 | 132.91           | 94.02            | -0.50      | aflI/estA/esterase |
| #54 139200 | 12.91            | 9.38             | -0.46      | aflQ/ordA/ord-1/oxireductase/cytochrome P450 monooxygenase |
| #54 139190 | 112.43           | 83.35            | -0.43      | aflQ/avfA/VERB synthase |
| #54 139270 | 572.31           | 434.92           | -0.40      | aflN/aflD/hypothetical protein |
| #54 139220 | 187.22           | 143.20           | -0.39      | aflO/omtB/dmtA/O-methyltransferase B |
| #54 139370 | 35.31            | 28.44            | -0.31      | aflB/fas-1/fatty acid synthase beta subunit |
| #54 139380 | 19.45            | 15.90            | -0.29      | aflA/fas-2/hexA/fatty acid synthase alpha subunit |
| #54 139280 | 34.04            | 31.21            | -0.13      | aflN/verA/monooxygenase |
| #54 139420 | 100.86           | 102.36           | 0.02       | aflT/aflT/transmembrane protein |
| #54 139340 | 177.63           | 195.04           | 0.13       | aflS/pathway regulator |
| #54 139360 | 64.90            | 83.53            | 0.36       | aflR/apa-2/afl-2 /transcription activator |
| #54 139440 | 14.48            | 20.02            | 0.47       | aflF/norB/dehydrogenase |
| #54 139110 | 2.38             | 3.49             | 0.55       | aflT/dhgI/sugR/sugar regulator |
| #54 139100 | 2.96             | 4.78             | 0.69       | aflY/avrA/Ser-Thr protein phosphatase family protein |
| #54 139430 | 20.76            | 35.15            | 0.76       | aflU/cypA/P450 monooxygenase |
| #54 139120 | 1.85             | 3.52             | 0.93       | aflY/cycA/glucosidase |
| #54 139130 | 1.78             | 3.93             | 1.15       | aflP/hxtA/hexose transporter |
| #55 139460 | 1293.63          | 1202.44          | -0.11      | MFS multidrug transporter, putative |
| #55 139470 | 215.54           | 687.52           | 1.67       | FAD dependent oxidoreductase, putative |
| #55 139480 | 243.62           | 522.50           | 1.10       | tryptophan dimethylallyltransferase |
| #55 139490 | 9.14             | 32.13            | 1.81       | hybrid PKS/NRPS enzyme |

Table 3. The expression levels of genes in the biosynthesis of conidial pigment (#10), aflatrem (#15), aflatoxin (#54) and cyclopiazonic acid (#55).
Among the 47 relevant genes, aflatoxin formation. The expression levels concerning oxidative stress related genes are shown in Table S4. aflD were down-regulated. However, none of these was completely suppressed. The most strongly down-regulation of fungal membrane 19,28. The transcriptional levels related ergosterol pathway was shown in Table S2.ing homeostasis, exchanging materials, and transduction of information. And ergosterol is one key constituent of fungal membrane19,28. The transcriptional levels related ergosterol pathway was shown in Table S2. Transcriptional levels of several genes were down-regulated after cinnamaldehyde treatment, for example, sterol delta 5,6-desaturase Erg3 (AFLA_018090), squalene monooxygenase Erg1 (AFLA_061500), and C-14 sterol reductase (AFLA_051080, AFLA_111350).

**Genes involved in fungal development.** The regulation of secondary metabolism is associated with fungal growth and development. From the expression profile data, we found that the expression patterns of some gene referred to conidiophores development were down-regulated when A. flavus was treated with cinnamaldehyde (Table S3). For the velvet complex, veA did not show a significant differential expression while velB, leaA, and vosA, were slightly up-regulated exposure to cinnamaldehyde. fluG (AFLA_039530), encoding a protein comprising an N-terminal amidohydrolase domain and a C-terminal glutamine synthetase domain29, was down-regulated. And esdC, an early sexual development gene, was mildly down-regulated. Nevertheless, development regulator FlbA was up-regulated. BrlA mediating conidiophores, and AbaA controlling phialide differentiation were also up-regulated. In addition, RodA and RodB, conidial hydrophobic genes, both showed strong up-regulation.

**Genes involved in oxidative stress.** In A. flavus, transcriptional factors AtfA, AtfB, AP-1, and MsnA are related to oxidative stress and aflatoxin biosynthesis. And oxylipin synthesis mediates oxidative processes and aflatoxin formation. The expression levels concerning oxidative stress related genes are shown in Table S4. Among the 47 relevant genes, ap-1, aflB and msnA were all up-regulated. The cellular receptors gprC, gprF, gprK, gprM, gprR, gprP, gprS, the oxylipins ppoA, ppoB and ppoC, the MAP kinase genes nkk2, fus3, pbs2, mpkA, sakA, bck1, ste11, sskB and ste7, and catalase gene cat1, catA, and superoxide dismutase gene sod1, mnsod were all up-regulated to varying degrees. AfpXGs, encoding calcium binding protein calceosin, and GPRCs (gprA, gprB, gprD, gprG, gprH) were all down-regulated.

**Discussion**

Cinnamaldehyde is gradually regarded as safer food additive in food processing and manufacturing comparing to chemical fungicides. The inhibitory effects and mechanism of cinnamaldehyde on fungal growth and mycotoxin production have been reported by many researchers.14,18 In our previous study, 0.4 mM cinnamaldehyde inhibited AFB1 production with the rate of 68.9%, and 0.8 mM cinnamaldehyde could completely suppress A. flavus growth1. In this study, the mechanism of A. flavus growth and aflatoxin formation dysfunction exposure to cinnamaldehyde was investigated by RNA-seq analysis. Moreover, the anti-fungal and anti-aflatoxigenic properties of cinnamaldehyde were discussed and conclusions were drawn based on the results of the previous studies and this study.

Aflatoxin synthesis is supported by the action of enzymatic cascade and involves 21 steps30. In A. flavus, this process is managed by a gene cluster in which aflR and aflS serve as regulators31,32. In our RNA-seq data, 25 genes of the aflatoxin biosynthesis cluster were down-regulated after treatment with cinnamaldehyde although aflR and aflS were up-regulated. With the exception of an up-regulated result for aflF, all the structural genes in the cluster were down-regulated. However, none of these was completely suppressed. The most strongly down-regulation gene was aflD, followed by the key structural genes aflG, aflH, aflP, aflM, aflI, aflJ and aflE. The expression levels of all genes in the cluster were confirmed by q-PCR (Fig. 2). The aflF, encoding a dehydrogenase, is involved in the conversion of NOR to AVR33. The expression level of aflF was up-regulated, but its two homology protein genes aflD and aflE both were down-regulated. The gene aflT, encoding a membrane-bound protein presumed to be involved in aflatoxin secretion, was not modulated after treatment with cinnamaldehyde. And similar results were also reported in A. flavus treated with piperine19 and eugenol34,35. These findings indicated that cinnamaldehyde suppressed aflatoxin biosynthesis by down-regulating the transcript levels of most structural genes.

An astonishing result is that the transcriptional factor aflR and cofactor aflS showed a mild up-regulation in A. flavus treated with cinnamaldehyde. In our previous study32, the expression level of aflR and aflS showed slight up-regulation in A. flavus treated with eugenol although aflatoxin production was significantly inhibited by
Acetyl-CoA, the fundamental structure element of all known fungal polyketides, is mainly produced from fatty acids β-oxidation and glycolysis of sugars. For aflatoxin biosynthesis, fatty acids β-oxidation is a major contributor to acetyl-CoA synthesis. It was reported that fatty acids were down-regulated in the pentose phosphate pathway. Incubated in aflatoxin inhibitory medium, *A. flavus* pentose phosphate pathway was accelerated leading to NADPH accumulation. Ultimately, acetyl-CoA was converted into lipid biosynthesis and ethylene reduced the oxidative stress in leading to the decrease of aflatoxin content. GPCRs and enolase are steroidogenic enzymes which are positively regulated by aflatoxin cluster regulators *aflR* and *aflS*, which were up-regulated in *A. flavus* treated with cinnamaldehyde. The similar result was obtained in *A. flavus* treated with eugenol. The *LaeA* and *veA* genes were down-regulated, which were positively regulated by *aflR* and *aflS*. The similar result that the aflatoxin cluster regulators *aflR* and *aflS* were up-regulated although most structural genes were down-regulated in *A. flavus* treated with different anti-aflatoxigenic natural compounds, suggesting the stable expression of *aflR* and *aflS*.

The velvet complex was critical for conidiation and aflatoxin formation in *A. flavus*. In the deletion mutant of *veA*, the expression of key aflatoxin genes including *aflR*, *aflD*, *aflM* and *aflP* was completely suppressed. Consequently, aflatoxin was halted. However, *veA* did not show significant differential expression although almost all structural genes were down-regulated. The oxidative stress-related genes such as *msnA*, *srrA*, *atfB* and *pacC*, which were positively regulated by *veA*, were up-regulated after cinnamaldehyde treatment. The similar result was obtained in *A. flavus* treated with eugenol. The *LaeA* and *veA* genes, encoding the other two proteins of velvet complex, were slightly up-regulated. Interestingly, a velvet-related gene *FluG* were significantly down-regulated in *A. flavus* treated with cinnamaldehyde. FluG, composed of an N-terminal amidohydrolase domain and C-terminal glutamine synthetase domain, was assumed for synthesizing a diffusible factor. Chang et al. (2013) reported that *VeA*, *VeB*, and *LaeA*, combined with *FluG*, were indispensable to maintaining conidiation program, sclerotial formation, and aflatoxin biosynthesis in *A. flavus*. These results suggested that FluG may play an important role in the anti-aflatoxigenic mechanism by cinnamaldehyde.

Cinnamaldehyde was considered to make its anti-fungal effects on perturbing cell wall biosynthesis, ergosterol biosynthesis and ATPase. The 4 genes associated with cell wall, *AFLA* 009830, *AFLA* 008360, *AFLA* 014260 and *AFLA* 100100, were down-regulated. The similar phenomenon had been reported that cinnamaldehyde caused several genes involved in cell wall biosynthesis dysfunction. Ergosterol is one of the principal sterol ingredients in the fungal membrane and is crucial for survival due to the ability in maintaining cell membrane fluidity, permeability, and pheromone signaling. In the present work, the transcriptional level of several genes related ergosterol was down-regulated, for example squalene monooxygenase *Erg1* (*AFLA* 061500), C-14 sterol reductase (*AFLA* 051080 and *AFLA* 111350). The *Erg1* gene of *S. cerevisiae* encodes squalene epoxidase, a key enzyme in the ergosterol pathway. Disruption of the gene resulted in a lethal phenotype when cells grew under aerobic conditions, even in the presence of ergosterol. The *ergosterol biosynthesis* which resulted in the disruption of the intracellular ATP, and some essential iron equilibrium. In *E. coli* and *Listeria monocytogenes*, cinnamaldehyde inhibited the membrane-bound ATPase activity. In the present study, some genes related to mitochondrial ATPase activity were repressed, for example, mitochondrial F1F0 ATP synthase subunit (*AFLA* 129660, *AFLA* 032070 and *AFLA* 043330).

RNA-binding was found to be the most dysregulated function after cinnamaldehyde treatment using GO enrichment analysis. Our previous study found the similar results in *A. flavus* treated with eugenol. Therefore, similar with eugenol, the post-transcriptional regulation may play an important role in the anti-aflatoxigenic mechanism of cinnamaldehyde. KEGG metabolic pathway analysis showed that pyruvate metabolism and phenylalanine metabolism were dysregulated. In *A. flavus* treated with eugenol, 2-phenylethanol reduced the oxidative stress in *A. flavus* leading to the decrease of aflatoxin content. GPCRs and pentose phosphate pathway locates intersection of intermediary metabolism, which refers to multiple metabolic processes covering glucose metabolism, lipogenesis and energy production. As a metabolic switch, the pyruvate dehydrogenase complex (PDH) was considered for carbon metabolism because of turning pyruvate into acetyl-CoA. Acetyl-CoA and malonyl-CoA are precursor substances in aflatoxin formation. Besides, PDH was crucial for morphology and pathogenicity in different fungal species. Amino acid metabolism plays an important role in aflatoxin biosynthesis. It was reported that phenylalanine metabolism was dysregulated in *A. flavus* treated with 2-phenylethanol. In addition, phenylalanine was slightly incorporated into aflatoxin in *A. flavus*. These results suggested that pyruvate metabolism and phenylalanine metabolism dysfunction might result in the reduction of aflatoxin biosynthesis.

Different stress can perturb cellular redox equilibrium, resulting in enhancive reactive oxygen species (ROS) levels named oxidative stress. Excessive accumulation of ROS can jeopardize DNA, proteins and lipids, leading to cellular dysfunction. Several researchers have thought that oxidative stress is a pre-condition for aflatoxin biosynthesis in *A. flavus* and *A. parasiticus*. The hypothesis is associated with the tentative that aflatoxin biosynthesis protects the fungus against oxidative stress. Reverteri et al. introduced a P33 gene into *A. flavus* resulting in enhanced ROS accompanying aflatoxin accumulation. On the contrary, antioxidants such as gallic acid and ethylene reduced the oxidative stress in *A. flavus* leading to the decrease of aflatoxin content. Acetyl-CoA is a precursor substance in aflatoxin formation.
oxylipins are tied in oxidative process. The expression levels in regard to oxidative-related genes were shown in Table S3. After cinnamaldehyde exposure, 7 GPCRs and 2 oxylipins genes showed significant differential expression. In this study, we found that gprG, gprF, gprK, gprM and gprS were significantly up-regulated with AFB1 inhibition in A. flavus treated with cinnamaldehyde. Similar results were obtained in A. flavus treated with eugenol in our previous study. The genes, gprG, gprF, gprK, gprM and gprS were also up-regulated after eugenol treatment. Caceres et al. also reported that over-expressed gprK accompanied with lower content of AFB1. Oxylipins pathway includes four genes, ppoA, ppoB, ppoC, and aFXG in A. flavus. Affeldt et al. reported that high content of oxylipins was associated with lower levels of aflatoxins. Simultaneous silencing via RNAi of ppoA, ppoB and ppoC and aFXG resulted in an increase of aflatoxin biosynthesis. Caceres et al. also found that over expression of ppoB and ppoA was correlated with AFB1 inhibition by piperine. In present study, the expression levels of ppoA, ppoB and ppoC were all up-regulated, suggesting the decreased oxylipins genes expression was associated with AFB1 inhibition by cinnamaldehyde. All these results suggest that the up-regulation of GPCRs and oxylipins genes was involved in AFB1 inhibition by cinnamaldehyde.

In A. flavus and A. parasiticus, there were several bZIP transcription factors referring to aflatoxin biosynthesis and oxidative stress response. Among these, SrrA, AtfB, AP-1, and MsnA were characterized as co-regulators. In this study, we found that genes belonging to bZIP-type family were involved in the anti-aflatoxicogenic mechanism of cinnamaldehyde. SrrA, an orthologue of S. cerevisiae Skn7 and Saccharomyces pombe Prr1, controlled key functions in response to osmotic and oxidative stress and was considered as a regulator in aflatoxin biosynthesis. AP-1, a highly conserved protein in mammalian, yeast and fungi. AP-1 may play crucial roles in sensing ROS because of high cysteine content in N- and C-terminal. Over-expression of napA, an ortholog of AP-1, resulted in secondary metabolite inhibition in A. nidulans which implied napA was a negative regulator in secondary metabolite synthesis. In A. parasiticus, the napA disruption resulted in more aflatoxin production. This study, the ap-1 showed up-regulation accompanying with aflatoxin inhibition in A. flavus. Similar results were also obtained by Caceres et al. They found that the AP-1 was up-regulated with aflatoxin inhibition in A. flavus after piperine treatment. AtfA mediates several processes in vegetative hyphae, contributes to stress tolerance and changes secondary metabolism in A. nidulans. A. oryzae, and A. fumigatus. AtfB, an orthologue of AtfA, is an important regulator referring to aflatoxin production and oxidative stress via binding to CER sites of aflatoxin biosynthesis genes promoter. This CRE binding site was found in 7 genes promoter regions. In the present study, AtfA did not show significant differential expression while AtfB was up-regulated by cinnamaldehyde. Caceres et al. also found AtfB was up-regulated with decreased production of aflatoxin after piperine treatment. MsnA has an important effect on fungal growth, aflatoxin and kójic acid formation, and oxidative stress. In A. flavus and A. parasiticus, MsnA disruption resulted in aflatoxin and ROS accumulation. In our previous study, we also found that transcript factor MsnA played a negative role in aflatoxin biosynthesis. Taken together, srrA, atfB, ap-1, and msnA were all up-regulated after cinnamaldehyde exposure. These results implied that bZIP transcription factors SrrA, AtfB, AP-1, and MsnA up-regulation played a direct negative role in aflatoxin formation after cinnamaldehyde treatment.

Antioxidant enzymes SOD and CAT which were regulated by the bZIP transcription factors make crucial effect on defense against ROS. Many publications have reported that some inhibitors could suppress aflatoxin formation via positive regulating the antioxidant enzymes activities. However, different aflatoxin inhibitors act on different type of antioxidant enzymes. For example, piperine and -glucans from Lentinula edodes led to lower AFB1 production with higher CAT activity. Oppositely, eugenol and ascorbic acid sharply depressed the AFB1 biosynthesis accompanying with high SOD activity. In addition, gallic acid may equilibrium ROS by activating the glutathione- and thioredoxin-dependent antioxidant system instead of changing CAT and SOD activities. Taken together, antioxidant enzymes catalase gene (sod1, sod2) and superoxide dismutase gene (sod1, and mmSOD) were all up-regulated in A. flavus treated with cinnamaldehyde. However, Sun et al. reported that exposure to cinnamaldehyde only resulted in higher SOD activity using the hydroxylamine analysis. The different results may imply that (1) reveals a dose effect; (2) exists a post-translational modification of CAT. These results made it clear that cinnamaldehyde enhanced CAT and SOD activities as part of its anti-aflatoxicogenic mechanism.

Figure 3 shows the hypothetical gene modulation mode of action on aflatoxin formation and fungal growth in A. flavus treated with cinnamaldehyde at transcription levels. The signal transduction disorder happens when cinnamaldehyde regulates the expression of GPCRs and oxylipins genes. Velvet complex together with FluG modulates conidiation, sclerotial production, and aflatoxin biosynthesis. However, the differential expression of LaeA, veA, and VelB was not significant. The down-regulation of FluG may trigger the expression of stress response transcription factor gene srrA, which results in up-regulation of bZIP transcriptional factor ap-1, zinc finger transcriptional factor msnA, and CREB/ATF family member atfB. Ultimately, the redox system is perturbed and then antioxidant enzymes are activated. In addition, AP-1, MsnA, AtfB, as negative regulatory factors, modulate aflatoxin biosynthesis gene cluster. For conidia development, early asexual development factor FlbA is modulated by velvet complex and FluG. Up-regulation of FlbA activates FadA and SfoD which play a negative role in the expression of esdC. Besides, FlbA causes the up-regulation of BrlA which triggers over-expression of AbaA and wetA. Taken together, down-regulation of esdC and over-expression of BrlA, AbaA, and wetA facilitate asexual development.

To sum up in Figs 3 and 4, cinnamaldehyde inhibits the aflatoxin biosynthesis and fungal growth of A. flavus via (1) reducing the fatty acid oxidation level by modulating several oxidation-related genes which leads to marked reduction of aflatoxin precursor acetyl-CoA; (2) increasing the NADPH accumulation by HMP which competes with aflatoxin biosynthesis; (3) weakening ergosterol synthesis which does damage to cell membrane integrity accompanied with altering the intracellular ATP and some indispensable iron equilibrium; (4) disturbing the redox system and then activating antioxidant enzymes which are deemed as key elements for
regulating aflatoxin-related genes. These results uncovered in this study play a critical role in understanding the anti-aflatoxigenic mechanism of cinnamaldehyde in *A. flavus* and may accelerate its use in practice. Moreover, these results should assist further studies on the mechanism of action of inhibitor against fungal growth and mycotoxin production.

**Conclusion**

The results of this study put forward a mechanism to explain the transcription regulation concerning the inhibitory effect of cinnamaldehyde on aflatoxin biosynthesis via RNA-seq. On basis of early studies, we draw a conclusion that (1) the decline in aflatoxin biosynthesis is on account of the down-expression of most of structural genes of aflatoxin cluster after treatment with cinnamaldehyde; (2) accumulation NADPH drives acetyl-CoA to lipid synthesis rather than polyketide formation; (3) the down-expression of diffusible factor FluG working with the velvet complex and the concomitant up-regulation of the oxidative stress-related genes srrA, msmA, and atfB; (4)
dysfunction of GPCRs and oxylipins genes; (5) post-transcriptional modification and key enzymes biosynthesis may be involved in the suppression of AFB, formation by cinnamaldehyde.

Materials and Methods
Natural compound, strain, and growth conditions. Natural cinnamaldehyde (99%) was purchased from Jiangxi Xue Song Natural Medicinal Oil Co., Ltd. (Ji’an City, Jiangxi, China). The strain A. flavus YC-15 was inoculated in PDA medium (200 g boiled potato, 20 g dextrose, 20 g agar, 1 L) in the dark. The conidia from a PDA culture grown for 7 d at 28 °C were washed with 0.01% Tween-20 solution, counted and added into YES liquid medium (20 g yeast extract, 150 g sucrose, 0.5 g MgSO4·7H2O, 1 L) at a final concentration of 10⁶ conidia/mL. The cinnamaldehyde was added into the YES cultures at a final concentration of 0.60 mM. As the control group, cinnamaldehyde was absent. All cultures were incubated at 28 °C for 5 d in the dark. Then the mycelia of A. flavus were collected from YES cultures for the extraction of total RNA.

Preparation of fungal total RNA, Illumina sequencing and bioinformatics analysis. The extraction of fungal total RNA, the preparation of cDNA libraries and RNA sequencing were conducted according to the methods described by Lv35. Total RNA was extracted with a Fungal RNA Kit (Omega, Norcross, GA, USA). The cDNA libraries were made using an Illumina® TruSeq® RNA Sample Preparation Kit (Illumina Inc., San Diego, CA, USA) using an Illumina® HiSeq 4000™ system (Illumina Inc., San Diego, CA, USA). The clean reads were obtained by filtering the raw reads and used for subsequent analysis. Then they were mapped to the A. flavus genome, the EST sequencing and rRNA sequencing33,35,36, and assembled using programs TopHat 1.3.1, Bowtie and Cufflinks, respectively. The FPKM values were counted to calculate and normalize the transcription levels of genes in A. flavus35,37.

Identification and analysis of differentially expressed genes. The difference in expression level between A. flavus genes treated with and without cinnamaldehyde was evaluated to be significant and a gene was identified as a differentially expressed gene when FDR value was ≤0.0536. For annotated genes, GO (gene ontology) functional analysis and KEGG (Kyoto Encyclopedia of Genes and Genome) pathway analysis were performed using FungiFun (https://sbi.hki-jena.de/FungiFun/FungiFun.cgi) and KAAS (KEGG Automatic Annotation Sever) annotation file, respectively24,78–80.

RT-PCR and q-PCR analysis of aflatoxin biosynthesis genes. The isolation of RNA, synthesis of first-strand cDNA, RT-PCR and q-PCR were performed according to the methods described by Lv35. First-strand cDNA synthesis was carried out by RT-PCR using the Takara RNA Kit (AMV) ver. Q-3.0. (Takara Bio inc. Japan). All genes of aflatoxin cluster were analyzed. q-PCR was carried out using an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

Availability of RNA-seq data. The raw RNA-Seq data of A. flavus discussed in this work have been deposited in the NCBI Sequence Read Archive with accession number of SRP132641.

References
1. Liang, D. et al. Inhibitory effect of cinnamaldehyde, citral, and eugenol on aflatoxin biosynthetic gene expression and aflatoxin B1 biosynthesis in. Aspergillus flavus. J Food Sci 80, M2917–M2924 (2015).
2. Bennett, J. W. & Klich, M. Mycotoxins. Clin Microbiol Rev 16, 497–516 (2009).
3. Hoffmeister, D. & Keller, N. P. Natural products of filamentous fungi: enzymes, genes, and their regulation. Nat Prod Rep 24, 393–416 (2007).
4. Groopman, J. D., Kersel, T. W. & Wild, C. P. Protective interventions to prevent aflatoxin-induced carcinogenesis in developing countries. Annu Rev Public Health 29, 187–203 (2008).
5. Hua, H. et al. Inhibitory effect of essential oils on Aspergillus oryzae growth and ochratoxin a production. PLoS One 25, e108285 (2014).
6. Issac, S. What is the mode of action of fungicides and how do fungi develop resistance? Mycologia 13, 38–39 (1999).
7. Ceker, S., Agar, G., Alpsoy, L., Nardemir, G. & Kizil, H. E. Antagonistic effects of Satureja hortensis against AFB, on human lymphocytes. In vitro. Cytol and Genet 48, 327–332 (2014).
8. Sun, Q., Shang, B., Wang, L., Lu, Z. & Liu, Y. Cinnamaldehyde inhibits fungal growth and aflatoxin B1 biosynthesis by modulating the oxidative stress response of Aspergillus flavus. Appl Microbiol Biotechnol 100, 1355–1364 (2015).
9. Li, H. et al. Nanocapsular dispersion of cinnamaldehyde for enhanced inhibitory activity against aflatoxin production by Aspergillus flavus. Molecules 20, 6022–6032 (2015).
10. Ooi, L. S., Li, Y., Kam, S. L., Wong, E. Y. & Vincent Ooi, V. E. Antimicrobial activities of cinnamon oil and cinnamaldehyde from the Chinese medicinal herb Cinnamomum cassia Blume. Am J Chin Med 34, 511–522 (2006).
11. Friedman, M., Henika, P. R. & Mandrell, R. E. Bacterial activities of plant essential oils and some of their isolated constituents against Campylobacter jejuni, Escherichia coli, Listeria monocytogenes, and Salmonella enter. J Food Prot 65, 1545–1560 (2002).
12. Wang, Y. et al. Effect of cinnamaldehyde and citral combination on transcriptional profile, growth, oxidative damage and patulin biosynthesis of Penicillium expansum. Front Microbiol 9, 597 (2018).
13. Xing, F. et al. Growth inhibition and morphological alterations of Fusarium verticillioides by cinnamon oil and cinnamaldehyde. Food Control 46, 343–350 (2014).
14. Liao, B. C. et al. Cinnamaldehyde inhibits the tumor necrosis factor-α-induced expression of cell adhesion molecules in endothelial cells by suppressing NFκB activation: effects upon Inh and Nrf2. Toxicol Appl Pharm 229, 161–171 (2008).
15. Hong, S. H., Ismail, I. A., Kang, S. M., Han, D. C. & Kwon, B. M. Cinnamaldehyde in cancer chemotherapy. Phytother Res 30, 754–767 (2016).
16. Wu, C. et al. Cinnamaldehyde induces apoptosis and reverses epithelial-mesenchymal transition through inhibition of Wnt/β-catenin pathway in non-small cell lung cancer. Int J Biochem Cell Biol 84, 58–74 (2017).
17. Yuan, Y., Xing, F. & Liu, Y. Role of essential oils in the inhibition of fungal growth and mycotoxin accumulation. J Nuclear Agri Sci 27, 1168–1172. (In Chinese), https://doi.org/10.11869/hnxb.2013.08.1168 (2013).
18. Chang, S. T., Chen, P. F. & Chang, S. C. Antibacterial activity of leaf essential oils and their constituents from Cinnamomum osmophloeum. J Ethnopharmacol 77, 123–127 (2001).
60. Zhang, Z., Qin, G., Li, B. & Tian, S. Effect of cinnamic acid for controlling gray mold on table grape and its possible mechanisms of action. *Microb Pathog* **49**, 75–82 (2010).

61. Taguchi, Y. et al. Therapeutic effects on murine oral candidiasis by oral administration of cassis (*Cinnamomum cassia*) preparation. *Nippon Ishinkin Gakkai Zasshi* **51**, 13–21 (2010).

62. Usta, J., Kreydiyeh, S., Barnabe, P., Bou-Moughlabay, Y. & Nakash-Chmaisse, H. Comparative study on the effect of cinnamon and clove extracts and their main components on different types of ATPases. *Hum Exp Toxicol* **22**, 355–362 (2003).

63. Bang, K. H., Lee, D. W., Park, H. M. & Rhee, Y. H. Inhibition of fungal cell wall synthesizing enzymes by trans-cinnamaldehyde. *Biochem Biophys Res Commun* **364**, 1061–1063 (2000).

64. Yin, H. B., Chen, C. H., Kollanoor-Johny, A., Darre, M. J. & Venkitaranayan, K. Controlling *Aspergillus flavus* and *Aspergillus parasiticus* growth and aflatoxin production in poultry feed using carvacrol and trans-cinnamaldehyde. *Poult Sci* **94**, 2183–2190 (2015).

65. Zhao, X., Zhi, Q. Q., Li, J. Y., Keller, N. P. & He, Z. M. The antioxidant gallic acid inhibits aflatoxin formation in *Aspergillus flavus* by modulating transcription factors FarB and CreC. *Toxins* **10**, 270 (2018).

66. Lin, I. Q., Zhao, X. X., Zhi, Q. Q., Zhao, M. & He, Z. M. Transcriptomic profiling of *Aspergillus flavus* in response to 5-azacytidine. *Fungal Genet Biol* **56**, 78–86 (2013).

67. Maggio-Hall, A. L., Wilson, R. A. & Keller, N. P. Fundamental contribution of 5-oxidation to polyketide mycotoxin production in planta. *Mol Plant Microbe Interact* **18**, 783–793 (2005).

68. Kiser, R. C. & Nittrouer, W. G. Jr. Purification and kinetic characterization of manno-l-1-phosphate dehydrogenase from *Aspergillus niger*. *Arch Biochem Biophys* **211**, 613–621 (1981).

69. Georgopapadou, N. H. & Walsh, T. J. Human mycoses: drugs and targets for emerging pathogens. *Fungal Genet Biol* **37**, 641–651 (1994).

70. Bhatnagar, D., Ehrlich, K. C. & Cleveland, T. E. Molecular genetic analysis and regulation of aflatoxin biosynthesis. *Appl Microbiol Biotechnol* **61**, 83–93 (2003).

71. Georgianna, D. R. et al. Beyond aflatoxin: four distinct expression patterns and functional roles associated with *Aspergillus flavus* secondary metabolism gene clusters. *Mol Plant Pathol* **11**, 213–226 (2010).

72. Georgianna, D. R. & Payne, G. A. Genetic regulation of aflatoxin biosynthesis from gene to genome. *Fungal Genet Biol* **46**, 113–125 (2009).

73. Yu, J. et al. Clustered pathway genes in aflatoxin biosynthesis. *Appl Environ Microbiol* **70**, 1253–1262 (2004).

74. Caceres, I. et al. Piperine inhibits aflatoxin B<sub>1</sub> production in *Aspergillus flavus* by modulating fungal oxidative stress response. *Fungal Genet Biol* **107**, 77–85 (2017).

75. Iv, C. et al. Large-scale comparative analysis of euugenol-induced/repressed genes expression in *Aspergillus flavus* using RNA-seq. *Front Microbiol* **9**, 1116 (2018).

76. Amaike, S. & Keller, N. P. Distinct roles for VeA and LaeA in development and pathogenesis of *Aspergillus flavus*. *Eukaryot Cell* **8**, 1051–1060 (2009).

77. Bayram, O. et al. VeR/VeB/VeA/LaeA complex coordinates light signal with fungal development and secondary metabolism. *Science* **320**, 1504–1506 (2008).

78. Duran, R. M., Curry, J. W. & Calvo, A. M. Production of cyclopiazonic acid, aflatrem, and aflatoxin by *Aspergillus flavus* and *Nidulans*. *Can J Microbiol* **37**, 1253–1262 (2004).

79. Chang, P. K., Scharfenstein, L. L., Li, P. & Ehrlich, K. C. *Aspergillus flavus* VeRB acts distinctly from VeA in conidiation and may coordinate with FluG to modulate sclerotial production. *Fungal Genet Biol* **58–59**, 71–79 (2013).

80. Yan, S., Liang, Y., Zhang, I. & Liu, C. M. *Aspergillus flavus* grown in peptone as the carbon source exhibits spore density- and peptone concentration-dependent aflatoxin biosynthesis. *BMC Microbiol* **12**, 106 (2012).

81. Shreaz, S. et al. Cinnamaldehyde and its derivatives, a novel class of antifungal agents. *Fitoterapia* **112**, 116–131 (2016).

82. Lees, N. D., Skaggs, B., Kirsch, D. R. & Bard, M. Cloning of the late genes in the ergosterol biosynthetic pathway of *Saccharomyces cerevisiae*—a review. *Lipids* **30**, 221–226 (1995).

83. Galli-Kienle, M., Anastasia, M., Cighetti, G., Galli, G. & Fiechti, A. Studies on the 14 alpha-demethylase mechanism in cholesterol biosynthesis. *Eur J Biochem* **110**, 93–105 (1980).

84. Ouyang, Q., Tao, N. & Jing, G. Transcriptional profiling analysis of *Penicillium digitatum*, the causal agent of citrus green mold, unravels an inhibited ergosterol biosynthesis response in *P. digitatum*. *G3 (Bethesda)* **7**, 641–651 (2016).

85. Landl, K. M., Klösch, B. & Turnowsky, F. EFG1, encoding squalene epoxidase, is located on the right arm of chromosome VII of *Saccharomyces cerevisiae*. *Yeast* **12**, 609–613 (1996).

86. Bresser, S. J., Merriman, B., Grahl, N., Chung, D. & Cramer, R. A. Two C4-sterol methyl oxidasies (Erg25) catalyse ergosterol intermediate demethylation and impact environmental stress adaptation in *Aspergillus fumigatus*. *Microbiology* **160**, 2492–2506 (2014).

87. Gill, A. O. & Holley, R. A. Disruption of *Escherichia coli*, *Listeria monocytogenes* and *Lactobacillus sakei* cellular membranes by plant oil aromatics. *Int J Food Microbiol* **108**, 1–9 (2006).

88. Gill, A. O. & Holley, R. A. Inhibition of membrane bound ATPases of *Escherichia coli* and *Listeria monocytogenes* by plant oil aromatics. *Int J Food Microbiol* **111**, 170–174 (2006).

89. Blass, J. P. Disorders of pyruvate metabolism. *Neurology* **29**, 280–286 (1979).

90. Kolobova, E., Tuganova, A., Boulantikov, I. & Popov, K. M. Regulation of pyruvate dehydrogenase activity through phosphorylation at multiple sites. *Biochem J* **358**, 69–77 (2001).

91. Minto, R. E. & Townsend, C. A. Enzymology and molecular biology of aflatoxin biosynthesis. *Chem Rev* **97**, 2537–2556 (1997).

92. Gao, T., Chen, J. & Shi, Z. *Fusarium graminearum* pyruvate dehydrogenase kinase 1 (FgPDK1) is critical for conidiation, mycelium growth, and pathogenicity. *PLoS One* **11**, e0158077 (2016).

93. Rice, L. N. A. et al. The *Aspergillus nidulans* pyruvate dehydrogenase kinases are essential to integrate carbon source metabolism. *G3 (Bethesda)* **8**, 2445–2463 (2018).

94. Chang, P. K., Hu, S. S., Sarreal, S. B. & Li, R. W. Suppression of aflatoxin biosynthesis in *Aspergillus flavus* by 2-phenylethanol is associated with stimulated growth and decreased degradation of branched-chain amino acids. *Toxins* 7, 3887–3902 (2015).

95. Adye, J. & Mateles, R. I. Incorporation of labelled compounds into aflatoxins. *Biochem Biophys Acta* **86**, 418–420 (1964).

96. Loshchak, V. I. Adaptive response to oxidative stress: Bacteria, fungi, plants and animals. *Comp Biochem Physiol C Toxicol Pharmacol* **153**, 175–190 (2011).

97. Zhang, Z., Qin, G., Li, B. & Tian, S. Effect of cinnamic acid for controlling gray mold on table grape and its possible mechanisms of action. *Curr Microbiol* **71**, 396–402 (2015).
61. Reverberi, M. et al. Modulation of antioxidant defense in *Aspergillus parasiticus* is involved in aflatoxin biosynthesis: a role for the ApyA1 gene. *Eukaryot Cell* **7**, 988–1000 (2008).
62. Roze, L. V. et al. Aflatoxin biosynthesis is a novel source of reactive oxygen species—a potential redox signal to initiate resistance to oxidative stress? *Toxins* **7**, 1411–1430 (2015).
63. Brown, S. H. et al. Oxygenase coordination is required for morphological transition and the host–fungus interaction of *Aspergillus flavus*. *Mol Plant Microbe Interact* **22**, 882–894 (2009).
64. Affeldt, K. I., Brodhagen, M. & Keller, N. P. *Aspergillus* oxylin signaling and quorum sensing pathways depend on G protein-coupled receptors. *Toxins* **4**, 695–717 (2012).
65. Hong, S. Y., Roze, L. V., Wee, J. & Linz, J. E. Evidence that a transcription factor regulatory network coordinates oxidative stress response and secondary metabolism in aspergilli. *Microbiology Open* **2**, 144–160 (2013).
66. Reverberi, M., Zsáli, S., Punelli, F., Ricelli, A. & Fabbri, A. A. ApyA1 affects aflatoxin biosynthesis during *Aspergillus parasiticus* growth in maize seeds. *Food Addit Contam Part A* **24**, 1070–1075 (2007).
67. Reverberi, M., Zsáli, S., Ricelli, A., Fabbri, A. A. & Fanelli, C. Oxidant/antioxidant balance in *Aspergillus parasiticus* affects aflatoxin biosynthesis. *Mycotoxicin Res* **22**, 39–47 (2006).
68. Roze, L. V., Chanda, A., Wee, J., Awad, D. & Linz, J. E. Stress-related transcription factor AtfB integrates secondary metabolism with oxidative stress response in aspergilli. *J Biol Chem* **286**, 35137–35148 (2011).
69. Toone, W. M. & Jones, N. Stress-activated signaling pathways in yeast. *Genes Cells* **3**, 14 (1998).
70. Wu, A. L. & Moye-Rowley, W. S. GSH1, which encodes gamma-glutamylcysteine synthetase, is a target gene for γAP-1 transcriptional regulation. *Mol Cell Biol* **14**, 5832–5839 (1994).
71. Toone, W. M., Morgan, B. A. & Jones, N. Redox control of AP-1-like factors in yeast and beyond. *Oncogene* **20**, 2336–2346 (2001).
72. Yin, W. B. et al. bZIP transcription factors affecting secondary metabolism, sexual development and stress responses in *Aspergillus nidulans*. *Microbiology* **159**, 77–88 (2013).
73. Lara-Rojas, F., Sánchez, O., Kawasaki, L. & Aguirre, J. *Aspergillus oryzae* oryzae atfA controls conidial germination and stress tolerance. *Fungal Genet Biol* **46**, 887–897 (2009).
74. Hag iwara, D., Suzuki, S., Kamei, K., Gono i, T. & Kawamoto, S. The role of AtfA and HOG MAPK pathway in stress tolerance in conidia of *Aspergillus fumigatus*. *Fungal Genet Biol* **73**, 138–149 (2014).
75. Chang, P. K. et al. Loss of msnA, a putative stress regulatory gene, in *Aspergillus parasiticus* and *Aspergillus flavus* increased production of conidia, aflatoxins and kojic acid. *Toxins* **3**, 82–104 (2011).
76. Trapnell, C., Pachter, L. & Salzberg, S. L. Tophat: discovering splice junctions with RNA-seq. *Bioinformatics* **25**, 1105–1111 (2009).
77. Mortazavi, A., Williams, B. A., McCue, K., Schaeffer, L. & Wold, B. Mapping and quantifying mammalian transcriptomes by RNA-seq. *Nature Methods* **5**, 621–628 (2008).
78. Kanehisa, M. et al. KEGG for linking genomes to life and the environment. *Nucleic Acids Res* **36**, D480–484 (2008).
79. Priebe, S., Linde, J., Albrecht, D., Guthke, R. & Brakhage, A. A. FungiFun: A web-based application for functional categorization of fungal genes and proteins. *Fungal Genet Biol* **48**, 353–8 (2011).

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**Author Contributions**

Conceived and designed the experiments: F.-G.X. and Y.L. Performed the experiments: P.W., L.-X.M., M.-M.Z. and L.P. Analyzed the data: P.W., J.J., Y.-J.Z. and F.-G.X. Wrote the paper: P.W. and F.-G.X. Revised the paper: F.-G.X. and X.-L.S.

**Additional Information**

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