Transcriptome Insights into the Effect of Sterigmatocystin on Growth and Aflatoxin B₁ Production by *Aspergillus Flavus*

Yarong Zhao (✉ zyr520zyr@163.com)

Institute of Quality Standard and Monitoring Technology for Agro-product of Guangdong Academy of Agricultural Sciences

Rui Zeng

Institute of Quality Standard and Monitoring Technology for Agro-product of Guangdong Academy of Agricultural Sciences

Peirong Chen

Institute of Quality Standard and Monitoring Technology for Agro-product of Guangdong Academy of Agricultural Sciences

Xu Wang

Institute of Quality Standard and Monitoring Technology for Agro-product of Guangdong Academy of Agricultural Sciences

Research Article

Keywords: *Aspergillus flavus*, aflatoxins, sterigmatocystin, transcriptome

Posted Date: July 15th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-701380/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Abstract

Aspergillus flavus (*A. flavus*) is an important fungus that produces aflatoxins, of which aflatoxin B₁ (AFB₁) is the most toxic and prone to contaminating food. This leads to economic losses in agricultural products and human health risks. Sterigmatocystin (STC) is a precursor in the metabolic biosynthesis of AFB₁. In this study growth of *A. flavus* was not affected by the presence of STC, but AFB₁ production was inhibited at certain concentrations. To uncover the mechanism, transcriptomic profiles of *A. flavus* in the presence or absence of STC were evaluated in PDB. A total of 3377 differentially-expressed genes were identified; 1182 were up-regulated and 2195 were down-regulated. GO function and KEGG pathway enrichment analysis indicated that these genes were mainly involved in the organization and biosynthesis of cellular components, organelle part, organelle division, macromolecular compound functions; the main pathway was valine, leucine and isoleucine synthesis and aflatoxin synthesis. The clustered genes responsible for AFB₁ biosynthesis were down-regulated to different degrees; *norB* expression was completely suppressed in the experimental group. This study provides new insights into control of *A. flavus* and the mechanisms regulating mycotoxin production.

Introduction

Aflatoxin B₁ (AFB₁) is one of the most important mycotoxins in the world due to its frequent detection at high concentrations as a contaminant in human food and animal feed¹⁻³. AFB₁ is a polyketide compound synthesized by secondary metabolic pathways in *Aspergillus*. AFB₁ can cause pathophysiological changes in some organisms (e.g. reduced growth rate, disturbed gastrointestinal tract, silenced immune responses and malnutrition); while also inducing a variety of histopathological manifestations in hepatocytes (e.g. proliferation of the bile duct and fatty degeneration of the hepatocytes)⁴⁻⁷. AFB₁ is known to induce hepatocellular carcinoma in various animals including rodents, poultry, non-human primates and fishes⁸⁻⁹. For these reasons, AFB₁ is classified by the International Agency for Research on Cancer (IARC) as a group 1 compound¹⁰.

Another important mycotoxin is sterigmatocystin (STC) which is produced mainly by *A. nidulans* and *A. versicolor*, but also some other species belonging to the genera *Aschersonia*, *Botryotrichum*, *Emericella* and *Chaetomium*¹¹⁻¹². STC is a potential carcinogen, mutagen and teratogen and was categorized by IARC as a group 2 compound [13]. Interestingly, STC shares its biosynthetic pathway with AFB₁, and the relative concentrations of AFB₁ and STC in contaminated foods depends on the invasive species. *Aspergillus versicolor* and *A. nidulans* are not able to metabolize STC into O-methylsterigmatocystin, which is the direct precursor of AFB₁. As a result, food and feed infested by these fungi often contain high concentrations of STC. In contrast, in food and feed invaded by *A. flavus* and *A. parasiticus*, which are able to metabolize STC, concentrations of STC are generally low^{14-16,11}.

The simultaneous occurrence of multiple mycotoxins in an individual product is a common phenomenon, and co-occurrence of AFB₁ and STC has been reported in food commodities (De Saeger, personal communication, 2012)¹⁷⁻²⁰. Exposure to multiple mycotoxins may lead to synergistic, additive or antagonistic toxic effects and the toxicity of mycotoxin combinations cannot always be predicted based on their individual toxicities²¹⁻²⁴. Mycotoxins with similar modes of action would generally be expected to have the least additive effects²⁵. The mechanisms of AFB₁ toxicity is through DNA modification, metabolic activation, cell death/transformation and cell deregulation²⁶⁻²⁹. In contrast, the toxicity of STC is through DNA adducts, inhibition of the cell cycle and mitosis, increases in ROS formation and lipid peroxidation³⁰⁻³³. Thus, there are similarities and differences in the mode of action of AFB₁ and STC.

Methods to remove mycotoxins from contaminated food, or degrade them into less toxic or non-toxic compounds are required. Many methods for degradation of AFB₁ have been reported, including adsorption, physical and chemical reduction³⁴⁻³⁷. In addition, AFB₁ is degraded by microorganisms including bacteria, yeasts and moulds; this has been studied extensively in recent years³⁸⁻⁴².

To our knowledge, there is no information in the literature about interactions between the presence of STC in foods and AFB₁ production. In the present work the effect of STC on *A. flavus* growth and AFB₁ biosynthesis were studied in potato dextrose broth

(PDB).

Results

Effect of STC on mycelial growth and AFB₁ production.

The effect of STC, at five concentrations (0.02, 0.1, 2.0, 10.0, 50.0 µg/mL), on mycelial growth of *A. flavus* and AFB₁ production in PDB medium over 11 days are shown in Table 1. The dry weight of mycelia increasing with increasing time in culture. In the control *A. flavus* grew well in PDB medium with sustained growth over the 11 days. When STC was added to the medium, mycelial dry weights at the highest concentration of STC were significantly different to the control and the other treatments ($p>0.05$) after 3 and 4 days growth (except for 10.0 µg/mL in 3 days); From the 5th day, there was no significant difference in *A. flavus* dry weights amongst any of the STC concentrations and the control except in the 10 µg/mL treatment ($p<0.05$). This indicates that *A. flavus* growth was not affected by the presence of STC, even at high concentrations.

Table 1

Effect of different sterigmatocystin concentrations on dry weight of mycelium

Added concentration µg/mL	mycelial weights mg					
	3d	4d	5d	7d	9d	11d
CK	25.32±1.92 ^a	26.97±3.42 ^b	42.62±4.22 ^{bc}	53.49±6.28 ^b	60.27±3.77 ^{ab}	75.81±8.16 ^a
0.02	27.75±1.64 ^a	33.63±2.87 ^a	46.29±2.23 ^{bc}	55.94±6.86 ^{ab}	56.05±2.51 ^b	78.91±3.57 ^a
0.1	28.75±2.08 ^a	35.30±2.33 ^a	40.63±3.06 ^c	61.21±4.13 ^{ab}	65.83±6.40 ^a	77.36±3.69 ^a
2.0	20.10±2.31 ^b	19.76±2.47 ^c	44.59±3.95 ^{bc}	61.18±1.57 ^{ab}	69.29±1.70 ^a	80.91±5.78 ^a
10	16.54±2.58 ^b	20.76±1.34 ^c	54.90±4.29 ^a	60.85±2.74 ^{ab}	63.73±2.44 ^{ab}	71.51±7.84 ^a
50	18.55±0.64 ^b	18.98±1.51 ^c	50.32±1.20 ^{ab}	65.84±2.20 ^b	68.82±4.66 ^a	77.48±4.13 ^a

AFB₁ production increased with increasing culture time in all treatments and the control, however the rate varied (Table 2). The relative ability to produce toxin did not show a linear trend in relation to STC concentration. For example, after 3 days AFB₁ production was 17.18 µg/L, 0.38 µg/L, 2.94 µg/L and 33.36 µg/L at STC concentrations of 0.02 mg/L, 0.1 mg/L, 2 mg/L and 10 mg/L, respectively, while in the control it was 61.62 µg/L. Maximum AFB₁ production reached 545.70 µg/L, 95.65 µg/L, 366.71 µg/L and 804.90 µg/L in the same four STC concentrations, and 1145.67 µg/L in the control. Hence, a certain concentration of STC inhibited AFB₁ production, and at an STC concentration of 0.1 µg/mL AFB₁ production was significantly different than the control ($p>0.05$). It is noteworthy that at an STC concentration of 50 mg/L AFB₁ production was not significantly different to the control ($p<0.05$) between days 4 and 7 of growth.

Table 2

Effect of different sterigmatocystin concentrations on aflatoxin B1 production of *Aspergillus flavus*

Added concentration µg/mL	AFB ₁ production µg/L					
	3d	4d	5d	7d	9d	11d
CK	61.62±8.52 ^a	255.79±9.98 ^a	372.35±89.93 ^a	759.72±61.02 ^a	1199.85±203.76 ^a	1145.67±71.44 ^a
0.02	17.18±1.22 ^c	108.06±22.45 ^b	203.17±69.28 ^b	419.09±32.51 ^b	414.90±42.27 ^c	545.70±155.63 ^{bc}
0.1	0.38±0.10 ^d	27.23±2.64 ^c	39.00±3.97 ^c	77.02±11.11 ^d	66.68±10.18 ^d	95.65±46.30 ^d
2.0	2.94±0.79 ^d	22.57±8.65 ^c	81.27±27.23 ^c	233.39±73.04 ^c	365.99±82.04 ^c	366.71±60.23 ^c
10	33.36±6.12 ^b	140.09±13.21 ^b	310.18±19.74 ^{ab}	674.41±52.34 ^a	783.96±120.74 ^b	804.90±131.15 ^b
50	69.29±9.91 ^a	131.09±22.47 ^b	308.93±29.37 ^{ab}	479.80±56.80 ^b	966.24±138.27 ^{ab}	1264.37±189.36 ^a

Summary of RNA-seq data sets

Data output statistics and quality control.

Transcriptome sequencing of the six samples (two groups, three duplicates) of *A. flavus* CGMCC 3.2980 generated a total of 30.31 million raw reads; amongst the total read pairs, 29.38 million passed purity filtering standards. Through base composition and mass analysis, it was apparent that there were few low quality bases. This met the quality control standard of sequencing and requirements for subsequent bioinformatic analysis.

Reference sequence alignment analysis.

Comparative analysis of the genome of *A. flavus* was done using clean reads after filtering with TopHat 2.0 software. The results showed that over 85% of reads were uniquely mapped to the *A. flavus* genome and multiple mapped reads or fragments coincided with the standard of half-point ratio below 10% in multiple localization-sequencing. This meant that the reference genome was appropriate and there was no pollution in the experiment.

Gene expression level analysis.

Gene expression level is reflected in transcription abundance; the lower the transcription abundance, the lower the level of gene expression. The overall transcriptional activity of genes in our data were quantified by calculating the number of reads per kilobase of exon per million mapped reads (FPKM); it is generally recognized that an FPKM ≥ 1 means that the gene is expressed. Gene expression levels are shown in Table 3.

Table 3

Proportion of genes at different expression levels

Samples	FPKM Interval				
	0~1	1~3	3~15	15~60	≥60
CK_1	233	399	1041	833	872
CK_2	220	407	1032	818	853
CK_4	251	423	1048	829	788
TJ_1	697	489	722	637	614
TJ_2	754	463	728	655	587
TJ_4	594	473	753	673	684

Identification and analysis of DEGs

Differential expression analysis.

To identify differences in molecular responses (based on read count) between experimental groups and the control group, we identified 3377 differentially transcribed ($FDR \leq 0.05$, $|\log_2 FC| \geq 1$) genes, of which 1182 genes (35.00%) were up-regulated and 2195 (65.00%) genes were down-regulated.

GO and KEGG analysis of DEGs.

Functional assignments were defined by Gene Ontology (GO) terms (<http://www.genontology.org>), which provided a broad functional classification of genes and gene products for various biological processes (BP), cellular components (CC) and molecular functions (MF). GO functional enrichment analysis revealed that: 13 MF terms were enriched, mainly involved with oxidoreductase activity, catalytic activity, structural constituent of ribosome and monooxygenase activity; 18 CC terms were enriched including intracellular organelle part, intracellular part, cell part and macromolecular complexes; 65 BP terms were enriched including cellular component organization or biogenesis, oxidation-reduction processes, single-organism metabolic processes, single-organism processes and cellular protein metabolic processes (Table 4).

Table 4

GO functional enrichment analysis of differentially expressed genes

GO ID	Number	Pop number	P value_uncorrected	FDR	Description
MF					
GO:0016491	548	1573	4.81297E-10	0	Oxidoreductase activity
GO:0003824	1480	5098	6.51185E-10	0	catalytic activity oxidoreductase activity, acting on paired donors,
GO:0016705	119	287	9.20882E-09	0	with incorporation or reduction of molecular oxygen
GO:0003735	6	113	1.43745E-08	0	structural constituent of ribosome
GO:0004497	119	291	1.88615E-08	0	monooxygenase activity
BP					
GO:0071840	53	413	2.58209E-10	0	cellular component organization or biogenesis
GO:0055114	543	1538	4.10448E-10	0	oxidation-reduction process
GO:0044710	918	2926	5.87454E-10	0	single-organism metabolic process
GO:0044699	1217	4145	6.47599E-10	0	single-organism process
GO:0044267	76	504	1.37828E-09	0	cellular protein metabolic process
CC					
GO:0044446	98	667	3.2679E-10	0	intracellular organelle part
GO:0044422	98	672	3.71546E-10	0	organelle part
GO:0032991	168	996	4.08468E-10	0	macromolecular complex
GO:0044424	456	2232	5.59664E-10	0	intracellular part
GO:0044464	538	2507	9.22963E-10	0	cell part
Note: FDR: P value_correctedNote: FDR: P value_corrected					

To further investigate the biological functions and interactions amongst genes, pathway-based analysis was done using the Kyoto Encyclopedia of Genes and Genomes (KEGG, <http://www.genome.ad.jp/kegg>) pathway database. Results showed that 3377 DEGs were involved in 110 pathways. The top ten down- and up-regulated genes enriched in KEGG is shown in Table 5. For down-regulated genes, the most enriched were involved in aflatoxin biosynthesis, followed by starch and sucrose metabolism and tyrosine metabolism; this explains why gene expression involved in aflatoxins synthesis and glucose metabolic pathway was suppressed in the experiment group. For up-regulated genes, valine, leucine and isoleucine biosynthesis was the most enriched, followed by alanine, aspartate and glutamate metabolism; this accounts for why genes participating in amino acid metabolism in the experimental groups were highly expressed. KEGG metabolic pathway analysis showed that enrichment of valine, leucine and isoleucine biosynthesis and aflatoxin biosynthesis was significant at $p \leq 0.05$.

Table 5

Top 10 KEGG enrichments for down- and up-regulated genes

Ko id	Term	Number	P value_uncorrected
Genes down -regulated			
map00254	Aflatoxin biosynthesis	13	0.0000
map00500	Starch and sucrose metabolism	20	0.0011
map00350	Tyrosine metabolism	18	0.0012
map00650	Butanoate metabolism	12	0.0018
map00380	Tryptophan metabolism	15	0.0024
map00071	Fatty acid degradation	12	0.0058
map02010	ABC transporters	5	0.0066
map04213	Longevity regulating pathway - multiple species	8	0.0070
map00591	Linoleic acid metabolism	3	0.0114
map00061	Fatty acid biosynthesis	7	0.0125
Genes up-regulated			
map00290	Valine, leucine and isoleucine biosynthesis	14	0.0000
map00250	Alanine, aspartate and glutamate metabolism	16	0.0002
map04113	Meiosis - yeast	18	0.0003
map00770	Pantothenate and CoA biosynthesis	11	0.0003
map00970	Aminoacyl-tRNA biosynthesis	15	0.0004
map00670	One carbon pool by folate	8	0.0007
map00300	Lysine biosynthesis	8	0.0011
map03008	Ribosome biogenesis in eukaryotes	18	0.0015
map00230	Purine metabolism	23	0.0020
map00260	Glycine, serine and threonine metabolism	18	0.0036

Analysis of gene expression in secondary metabolism gene clusters of *A. flavus*.

To investigate the effect of STC on secondary metabolism synthesis of *A. flavus*, we used CIFR (Center for Integrated Fungal Research) information (<http://weir.statgen.ncsu.edu/aspergillus/chromosomes.php>) and SMURF (<http://www.jcvi.org/smurf>) software to analyze 55 secondary metabolic gene clusters from *A. flavus*. Expression of most gene clusters was not affected by STC. Comparative analysis of secondary metabolism gene expression demonstrated that in 639 secondary metabolism genes there were 162 genes with significantly different expression ($q\text{-value} \leq 0.001$), of which 17 were backbone genes: AFLA_004450, AFLA_010020, AFLA_064560, AFLA_069330 and AFLA_139490 encoded mainly non-ribosomal peptide synthetase (NRPS); AFLA_006170, AFLA_10545, AFLA_128060, AFLA_137870 and AFLA_139410 are responsible for polyketide synthase, PKS; AFLA_017840, AFLA_023020, AFLA_079400, AFLA_105190 encode NRPS-like synthase and AFLA_028660, AFLA_066890 and AFLA_139480 encode for mitochondrial translation initiation factor IF-2, cytochrome P450 and hybrid PKS/NRPS enzymes, respectively.

Most secondary metabolic gene clusters were down-regulated, with only the minority being up-regulated. In the 2[#] gene cluster, backbone gene AFLA_004450, which is responsible for encoding dimethylallyl transferase, was up-regulated, but AFLA_004370 and AFLA_004400 were down-regulated. In the 11[#] gene cluster, only AFLA_028660 was up-regulated while there was no significant

difference in expression of other genes in this cluster. Similarly, the genes AFLA_082220, AFLA_082230 and AFLA_082250 in the 27[#] gene cluster and AFLA_101690 and AFLA_101710 in the 33[#] gene cluster were all up-regulated to varying degrees. Amongst the up-regulated genes, AFLA_112890 and AFLA_066740 encode major facilitator superfamily transporters (MFS), which have an important role in transfer of some metabolites. Furthermore, genes involved in amino acid synthesis were differentially expressed to varying degrees; for example, AFLA_005510, AFLA_041550, AFLA_083270 and AFLA_126970, which encode amino acid permease, cysteine β -lyase, GABA permease and arginine permease, respectively, were up-regulated, but AFLA_038620 (encoding branched amino acid transferase) and AFLA_062910 (encoding specific proline permease) were down-regulated.

AFB₁ synthesis genes were located on the 54[#] gene cluster. In this study, 30 genes were all down-regulated to varying degrees; *norB* was completely repressed in the experimental group (Table 6). However, there was no difference in expression of the global regulatory genes of secondary metabolite, *laeA* and *veA*. Also, *brlA*, which is involved with growth was down-regulated.

Table 6

Analysis of genes expressed in the biosynthesis of aflatoxins

Gene name	RPKM		Log ₂	q value	DGE	Annotated gene function
	CK	TJ	TJ/CK			
AFLA_139200	152.1003	0.0890	-10.7389	1.076E-83	down	aflQ/ ordA/ ord-1/ oxidoreductase / cytochrome P450 monooxygenase
AFLA_139210	917.6813	0.2213	-12.0176	7.73E-142	down	aflP/ omtA/ omt-1/ O-methyltransferase A
AFLA_139220	2925.1377	0.5760	-12.3101	5.21E-174	down	aflO/ omtB/ dmtA/ O-methyltransferase B
AFLA_139230	99.5160	0.1137	-9.7740	1.589E-45	down	aflI/ avfA/ cytochrome P450 monooxygenase
AFLA_139240	1044.9193	0.2383	-12.0981	2.354E-65	down	aflLa/ hypB/ hypothetical protein
AFLA_139250	799.4160	0.1230	-12.6661	2.64E-125	down	aflL/ verB/ desaturase/ P450 monooxygenase
AFLA_139260	786.0533	0.1383	-12.4723	3.22E-117	down	aflG/ avnA/ ord-1/ cytochrome P450 monooxygenase
AFLA_139270	1428.8010	1.4530	-9.9416	5.055E-67	down	aflNa/ hypD/ hypothetical protein
AFLA_139280	227.1293	0.0113	-14.2907	5.76E-61	down	aflN/ verA/ monooxygenase
AFLA_139290	821.2367	0.1913	-12.0675	7.899E-43	down	aflMa/ hypE/ hypothetical protein
AFLA_139300	6524.7297	1.3467	-12.2423	1.72E-134	down	aflM/ ver-1/ dehydrogenase/ ketoreductase
AFLA_139310	1071.7900	0.2320	-12.1736	3.66E-130	down	aflE/ norA/ aad/ adh-2/ NOR reductase/ dehydrogenase
AFLA_139320	1900.3200	0.4837	-11.9399	2.15E-124	down	aflJ/ estA/ esterase
AFLA_139330	3153.5033	1.8543	-10.7318	7.72E-131	down	aflH/ adhA/ short chain alcohol dehydrogenase
AFLA_139340	560.8290	3.6423	-7.2666	3.33E-124	down	aflS/ pathway regulator
AFLA_139360	92.4453	4.6113	-4.3253	5.357E-37	down	aflR / apa-2 / afl-2 / transcription activator
						aflB / fas-1 / fatty acid synthase beta subunit

AFLA_139370	143.3517	3.2357	-5.4694	2.01E-161	down	
AFLA_139380	117.8963	5.8397	-4.3355	3.961E-70	down	aflA / fas-2 / hexA / fatty acid synthase alpha subunit
AFLA_139390	2307.8407	5.6377	-8.6772	1.382E-74	down	aflD / nor-1 / reductase
AFLA_139400	2940.4380	1.0157	-11.4994	1.607E-86	down	aflCa / hypC / hypothetical protein
AFLA_139410	196.8083	4.3753	-5.4913	4.01E-164	down	aflC / pksA / pksL1 / polyketide synthase
AFLA_139420	333.8647	4.3997	-6.2457	1.04E-112	down	aflT / aflT / transmembrane protein
AFLA_139430	178.0950	0.3763	-8.8864	1.018E-60	down	aflU / cypA / P450 monooxygenase
AFLA_139440	25.7907	0.0000	–	1.613E-06	down	aflF / norB / dehydrogenase
AFLA_139450	11.8450	0.0000	–	3.169E-06	down	conserved hypothetical protein
AFLA_139460	558.1760	1.0897	-9.0007	1.47E-48	down	MFS multidrug transporter, putative
AFLA_139470	320.0510	1.5767	-7.6653	1.088E-14	down	FAD dependent oxidoreductase, putative
AFLA_139480	493.3330	3.8730	-6.9930	2.24E-48	down	dimethylallyl tryptophan synthase, putative
AFLA_139490	8.2883	0.0330	-7.9725	3.695E-43	down	hybrid PKS/NRPS enzyme, putative
AFLA_139500	0.8250	0.0257	-5.0064	3.439E-05	down	conserved hypothetical protein

Discussion

Much research has been devoting to controlling the occurrence of aflatoxins. Some natural substances with inhibitory effects have been found e.g. onions, garlic extract, eugenol, khellin, caffeine, piperlongumine, luteolin, saccharol, resveratrol and tannic acid⁴³, but these are all exogenous substances. This study found that particular concentrations of STC can inhibit the synthesis of AFB₁, while not affecting fungal growth. Transcriptome analysis showed that 30 genes in the AFB₁ biosynthetic gene cluster were down-regulated to varying degrees. Meanwhile, KEGG analysis showed that most DEGs were enriched in branched-chain amino acids (BCAAs).

Aspergillus flavus reproduces asexually depending on conidia production for growth and development, which is precisely controlled by multiple genes. The genes *wetA*, *brlA* and *abaA* are the main regulatory proteins involved in the sporulation process and play important roles in the different stages of conidia production. Activation of the *brlA* gene is a key step in conidia production by *Aspergillus*⁴⁴; knockout of *brlA* makes *Aspergillus* isolates form quill-like structures with slender stems that cannot produce conidia, while over-expression interrupts isolate growth⁴⁵. Generally, aflatoxin synthesis is always associated with slow growth⁴⁶. In our study, *brlA* gene expression was down-regulated meaning that growth of *A. flavus* remained active, leading to the synthesis of

AFB₁. However, there was no significant difference in expression of the other two genes, *wetA* and *abaA*; in combination with results of the phenotypic study, we speculate that STC cannot affect the growth of *A. flavus*. Lin et al (2013) found that 5-azacytidine induced up-regulation of *brlA*, which blocked sporulation, resulted in a fluffy phenotype, and influenced AFB₁ synthesis⁴⁷.

Secondary metabolic pathways in fungi are relatively complex, involving polyketone synthase, epoxide hydrolase, methyl transferase, reductase, dehydrogenase, cytochrome P450 monooxygenase and fatty acid synthase. However, one type of enzyme can be produced as a result of more than one gene, and it is difficult to determine whether a gene is involved in secondary metabolism. Using metabolic analysis tools and SMURF software, Georgianna et al., (2010) found 55 secondary metabolic pathways in *A. flavus*, including 27 NRPS, 22 PKS, ciproanilic acid synthesis, spore pigment synthesis and aflatoxin synthesis metabolic pathways that perform specific biological functions^{48–49}. *AflR* is a transcriptional regulatory gene for aflatoxin synthesis and is responsible for activation of almost all structural genes. As the transcription factor auxiliary gene, *AflS* cooperates with *aflR* to prevent inhibition and guarantee aflatoxin synthesis. LaeA and veA are global regulatory factors involved in regulation of aflatoxin synthesis and were down-regulated in our study.

Aflatoxins are highly oxidative metabolic products and oxidative stress is a necessary condition for their formation⁵⁰. Transcriptome analysis demonstrated that Cu–Zn superoxide dismutase expression was raised, which is consistent with previous research⁴³. Thirty genes involved in aflatoxin synthesis, were all down-regulated demonstrating that STC directly inhibits the genes encoding enzyme in this gene cluster which inhibits aflatoxin production. Some antioxidant enzymes encoded by gene updated in the presence of STC can also inhibit synthesis of aflatoxins.

The role of amino acid metabolism in aflatoxin synthesis is complex. Some amino acids can be used as carbon or nitrogen sources in growth and aflatoxin synthesis. In *A. flavus*, phenylalanine, tyrosine, tryptophan, proline and arginine can be used in this way for aflatoxin synthesis⁵¹. Arginine is necessary for aflatoxin synthesis in *A. parasiticus*, and can replace aspartate and alanine^{52–53}. KEGG enrichment analysis found that, in 67 metabolic pathways, BCAAs metabolic activity was associated with biosynthesis of aflatoxins, and other basic ammonia acids and acidic amino acid metabolism mainly related to the fungal growth. Eighteen ribosome biosynthesis genes were up-regulated showing that STC may promote the growth of *A. flavus* to a certain extent, but inhibited the synthesis of AFs. This result is consistent with previous studies⁵⁴.

STC is a precursor of aflatoxin synthesis and, theoretically, an increase in STC should enhance the ability of isolates to synthesize aflatoxins. However, cell metabolism and microbial secondary metabolism are sophisticated processes. Although precursor substances can inhibit metabolic production, this has not been reported before for aflatoxin synthesis; there is a large body of research on synthesis of antibiotics for example. In general, exogenous precursor added during synthesis of antibiotics can control synthetic direction and increase antibiotics production. Epothilone is a type of polyketide secondary metabolite, and when precursors are added they have an inhibitory effect on epothilone synthesis⁵⁵. Similarly, FK506 output is significantly improved during fermentation in the presence of precursors, but only at low concentrations⁵⁶. Aflatoxin synthesis in *A. flavus* CGMCC declined in the presence of STC this study. However, under the same conditions, aflatoxin production by other isolates increased. This confirms that aflatoxin synthesis is isolate specific, and can vary depending on the nutrient medium, external environment and the degree of tolerance to particular substances⁵⁷.

Synthesis of secondary metabolites is closely related to primary metabolism because cell energy, precursors and co-factors can potentially limit secondary metabolism. Many enzymes involved in aflatoxin biosynthesis and catalytic reactions are NADPH dependent. Glucose may control aflatoxin biosynthesis by NADPH generated carbon metabolism repression and the tricarboxylic acid cycle. Therefore, NADPH/NADP⁺ levels in cells may also affect aflatoxin synthesis^{58–59}. Lipid metabolism is also closely associated with aflatoxin synthesis. NADPH/NADP determines whether acetyl-CoA enters fatty metabolism or aflatoxin anabolism. It is possible that a high proportion of NADPH/NADP enables acetyl-CoA to enter fatty metabolic pathways, while low levels encourage its entry into other pathways⁶⁰. The pentose phosphate pathway is the main source of intracellular NADPH in organisms, some important gene expressed significant difference may be the other possible influencing factors in aflatoxin synthesis.

Conclusion

In order to discuss the influence of STC on secondary metabolite synthesis in *A. flavus*, we used transcriptome sequencing analysis to identify 3377 differentially expressed genes of which 1182 genes were up-regulated and 2195 genes were down-regulated. GO function and KEGG pathway enrichment analysis indicated these genes mainly participated in cellular component organization and biosynthesis of components, organelle part, organelle division, macromolecular compound functions, and the main pathway was valine, leucine and isoleucine synthesis and aflatoxin synthesis. We also showed that STC had least influence on 55 secondary metabolic gene cluster, but that 30 genes on the aflatoxin synthesis gene cluster were expressed to varying degrees; *norB* was completely suppressed in the experimental group, suggesting that STC probably increases oxidoreduction enzyme activity, facilitating growth of isolates, and improving branched chain amino acid biosynthesis, thus inhibiting synthesis of aflatoxins.

Materials And Methods

Apparatus.

Equipment used in this study included: an autoclave MLS-3750 (Labo, Janpa); drying oven DHG-9070A (DHG, China); centrifuge 5804R (Eppendorf, Germany); clean Bench KLC (KLC, China); vortex mixer vx200 (Labnet, USA); incubator (Boxun, China); ultrasonic instrument (Kunshan, China); and [optical microscope](#) Nikon E100 (Nikon, Japan). Water was ultra-purified in a MilliQ® system (Millipore, USA) for use throughout the study.

Reagents and media.

AFB₁ and STC standards were obtained from Sigma (St. Louis, MO, USA). The C₁₈ column was purchased from Agilent (California, USA). All reagents used were purchased from Sigma and were of analytical grade. HPLC solvents were of HPLC grade. The [potato dextrose agar](#) (PDA) and [potato dextrose](#) broth (PDB) were from Huankai (Guangzhou, China).

Preparation of spore inoculum.

The aflatoxigenic isolate, *A. flavus* (CGMCC 3.2980) was purchased from China General Microbiological Culture Collection Center. This isolate was maintained on PDA slants at 4°C until activated. Inoculum was prepared by culturing *A. flavus* on PDA for 7 days at 30°C; conidia were harvested aseptically and suspended homogeneously in sterile distilled water containing 0.05% Tween 80. Conidial concentration was determined using a haemocytometer and adjusted to 1×10^7 conidia ml⁻¹ before use in experiments.

Inoculation.

Each replicate flask containing 100 mL PDB medium was inoculated with 1 mL of conidial suspension (see section 2.3) and different quantities of STC (0.02, 0.1, 2.0, 10, 50 µg/mL); there were three replicate flasks for each STC concentration and the control. Control flasks contained only conidia of *A. flavus* and no STC. The flasks were incubated at 30°C in darkness. On days 3, 4, 5, 7, 9, 11 after inoculation a set of 18 tubes were autoclaved (121°C, 30 min) for safety reasons and mycelial growth and AFB₁ concentration of each flask measured.

Determinations of fungal dry weights and AFB₁ concentration.

Fungal dry weights of *A. flavus* in each treatment and at each sampling time were determined by filtering out the mycelium from each flask using Whatman No 4 filter paper, washing in sterilized water, and then freeze-drying for 24 h at 65°C before weighing.

The filtrate from each flask was used for AFB₁ analysis. Quantification of AFB₁ was done following the method described by Guo et al., with some modification⁶¹. For extraction of AFB₁ from media, 2 mL filtrate was transferred by pipette to a centrifuge tube

before centrifugation at 11000 rpm for 10 min. Then 1.5 mL of the supernatant and 4.5 mL acetonitrile aqueous solution containing 1% formic acid were placed in a 10 mL centrifuge tube, vortexed for 1 min and then dried with nitrogen. To each centrifuge tube, 0.1 mg PSA and 1.5 mL acetonitrile aqueous solution (3:7, v:v) containing 0.1% formic acid were added to achieve a constant volume; the mixture was vortexed for 1 min and then subjected to ultrasound in an ultrasonic generator for 5 min prior to centrifugation at 10000 rpm for 5 min. Then 1 mL of the supernatant was filtered through a 0.22 µm nylon filter before analysis using HPLC-MS/MS.

The LCMS-8050 system was a UFLC connected to a triple quadrupole MS analyzer with an electrospray ionization (ESI) interface (Shimadzu, Japan). The separation was run using a Shimadzu Shim-pack XR-ODS II (75mm×2.0 mm×1.6 µm) column maintained at 40°C with a flow rate of 300 µL/min. The mobile phases were (A) 0.1% aqueous formic acid solution and (B) MeCN. The elution gradient started with 30% B (0 - 3 min), 50% B - 80% B for 5 min, and finally 30% B for 7 min, isocratic for 1 min. The injection volume was 1 µL.

RNA extraction and quality testing.

Total RNA was extracted from cultivated PDB media containing 10 µg/mL STC using TRIzol® reagent according to the manufacturer's instructions (Invitrogen) and genomic DNA was removed using DNase I (TaKara). Then RNA quality was determined using a 2100 Bioanalyser (Agilent) and quantified using the ND-2000 (NanoDrop Technologies).

RNA-seq library construction and sequencing.

An RNA-seq transcriptome library was prepared using the TruSeq™ RNA sample preparation kit from Illumina (San Diego, CA) with 5 µg of total RNA. Messenger RNA was isolated according to the polyA selection method using oligo (dT) beads and then fragmented by fragmentation buffer firstly. Secondly double-stranded cDNA was synthesized using a SuperScript double-stranded cDNA synthesis kit (Invitrogen, CA) with random hexamer primers (Illumina). The synthesized cDNA was subjected to end-repair, phosphorylation and 'A' base addition according to Illumina's library construction protocol. Libraries were size selected for cDNA target fragments of 200–300 bp on 2% Low Range Ultra Agarose followed by PCR amplification using Phusion DNA polymerase (NEB) and 15 PCR cycles. After quantification using TBS380, the paired-end RNA-seq sequencing library was sequenced using the Illumina HiSeq xten (2 × 150bp read length).

Mapping reads and sequence assembly.

The raw paired-end reads were trimmed and quality controlled by SeqPrep (<https://github.com/jstjohn/SeqPrep>) and Sickle (<https://github.com/najoshi/sickle>) with default parameters. Clean reads were separately aligned to reference genomes using the orientation mode of TopHat (<http://tophat.cbcb.umd.edu/version 2.0.0>) software⁶². The mapping criteria of bowtie were as follows: sequencing reads should be uniquely matched to the genome allowing up to two mismatches, without insertions or deletions. The regions of genes were expanded following depths of sites and the operon obtained. In addition, the whole genome was split into multiple 15 kbp windows that shared 5 kbp. Newly transcribed regions were defined as having more than two consecutive windows without overlapped regions of the gene, where at least two reads mapped per window in the same orientation.

Differential expression analysis and functional enrichment.

To identify DEGs (differentially-expressed genes) amongst paired samples, the expression level of each transcript was calculated according to the fragments per kilobase of exon per million mapped reads (FRKM) method. RSEM (<http://deweylab.biostat.wisc.edu/rsem/>) was used to quantify gene abundances⁶³. The R statistical package software EdgeR (Empirical analysis of Digital Gene Expression in R [<http://www.bioconductor.org/packages/2.12/bioc/html/edgeR.html>]) was used for differential expression analysis⁶⁴. In addition, functional enrichment analysis including GO and KEGG were done to identify which DEGs and metabolic pathways were significantly enriched in GO terms compared with the whole-transcriptome background

(using the Bonferroni-corrected P-value ≤ 0.05 to determine significance). GO functional enrichment and KEGG pathway analysis were achieved using Goatools (<https://github.com/tanghaibao/Goatools>) and KOBAS (<http://kobas.cbi.pku.edu.cn/home.do>)⁶⁵.

Declarations

Data availability

All data generated or analysed during this study are included in this article.

Acknowledgements

This work was funded by the President Funding of Guangdong Academy of Agricultural Sciences (202008).

Author Contributions

Yarong Zhao conceived and designed the experiments; Peirong Chen performed the experiments; Rui Zeng analyzed the data. All authors discussed, edited and approved the final manuscript.

Competing interests

The authors declare no competing interests.

References

1. Mottaghianpour, E., Nazari, F., Mehrasbi, M. R. & Hosseini, M. Occurrence of aflatoxin B1 in baby foods marketed in Iran. *Journal of the Science of Food and Agriculture*, **97**, 2690–2694 (2016).
2. Ehsania, A., Baranib, A. & Nasiric, Z. Occurrence of aflatoxin B1 contamination in dairy cows feed in Iran. *Toxin Reviews*, **35**, 54–57 (2016).
3. Njoroge, S. M. *et al.* Aflatoxin B1 levels in groundnut products from local markets in Zambia. *Mycotoxin Res*, **33**, 113–119 (2017).
4. Bakheet, S. A. *et al.* β -1,3-glucan reverses aflatoxin B1-mediated suppression of immune responses in mice. *Life Sci*, **152**, 1–13 (2016).
5. Mukumu, C. K. & Macharia, B. N. Effects of aflatoxin b1 on liver, testis, and epididymis of reproductively mature male pigs: Histopathological evaluation. *East Afr. Med. J*, **94**, 95–99 (2017).
6. Mughal, M. J., Peng, X., Kamboh, A. A., Zhou, Y. & Fang, J. Aflatoxin B1 induced systemic toxicity in poultry and rescue effects of selenium and zinc. *Biol. Trace Elem. Res*, **178**, 292–300 (2017).
7. Rieswijkab, L. *et al.* Aflatoxin B1 induces persistent epigenomic effects in primary human hepatocytes associated with hepatocellular carcinoma., **350**, 31–39 (2016).
8. Robens, J. F. & Richard, J. L. (1992). Aflatoxins in animal and human health. In: *Reviews of Environmental Contamination and Toxicology*. **69–94**, Springer-Verlag.
9. Chu, Y. J. *et al.* Aflatoxin B1 exposure increases the risk of cirrhosis and hepatocellular carcinoma in chronic hepatitis B virus carriers. *International Journal of Cancer*, **141**, 711–720 (2017).
10. IARC (1972). Some inorganic substances, chlorinated hydrocarbons, aromatic amines, N-nitroso compounds and natural products, in: *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*, **101**, 1-184.
11. Bertuzzi, T., Romani, M., Rastelli, S., Mulazzi, A. & Pietri, A. Sterigmatocystin occurrence in paddy and processed rice produced in Italy in the years 2014–2015 and distribution in milled rice fractions. *Toxins (Basel)*, **9**, 86 (2017).
12. Rank, C. *et al.* Distribution of sterigmatocystin in filamentous fungi. *Fungal Biology*, **115**, 406–420 (2011).

13. IARC (1987). Monographs on the evaluation of the carcinogenic risk of chemicals to human. overall evaluations of carcinogenicity: an updating of IARC monographs. World Health Organization, Volumes 1–42.
14. EFSA CONTAM Panel. Scientific opinion on the risk for public and animal health related to the presence of sterigmatocystin in food and feed. *EFSA Journal*, **11**, 3254 (2013).
15. Yu, J. *et al.* Clustered pathway genes in aflatoxin biosynthesis. *Applied and Environmental Microbiology*, **70**, 1253–1262 (2004).
16. Sweeney, M. J. & Dobson, A. D. W. Molecular biology of mycotoxin biosynthesis. *FEMS Microbiology Letters*, **175**, 149–163 (1999).
17. Saxena, J. & Mehrotra, B. S. Screening of spices commonly marketed in India for natural occurrence of mycotoxins. *Journal of Food Composition & Analysis*, **2**, 286–292 (1989).
18. Yogendrarajah, P. *et al.* Mycological quality and mycotoxin contamination of Sri Lankan peppers (*Piper nigrum* L.) and subsequent exposure assessment., **41**, 219–230 (2014a).
19. Yogendrarajah, P., Jacxsens, L., De Saeger, S. & De Meulenaer, B. Co-occurrence of multiple mycotoxins in dry chilli (*Capsicum annum* L.) samples from the markets of Sri Lanka and Belgium., **46**, 26–34 (2014b).
20. Yoshinari, T., Suzuki, Y., Sugita-Konishi, Y., Ohnishi, T. & Terajima, J. (2016). Occurrence of beauvericin and enniatins in wheat flour and corn grits on the Japanese market, and their co-contamination with type B trichothecene mycotoxins. Food Additives & Contaminants. Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment, **33**, 1620–1626.
21. Kowalska, A., Walkiewicz, K., Kozie, P. & Muc-Wierzgón, M. Aflatoxins: characteristics and impact on human health. *Postępy Higieny Medycyny Doswiadc Zalnej*, **71**, 315–327 (2017).
22. Ji, J. *et al.* The antagonistic effect of mycotoxins deoxynivalenol and zearalenone on metabolic profiling in serum and liver of mice. *Toxins (Basel)*, **9**, 28 (2017).
23. Oswald, I. *et al.* Co-exposure to low doses of the food contaminants deoxynivalenol and nivalenol has a synergistic inflammatory effect on intestinal explants. *Archives of Toxicology*, **91**, 1–11 (2016).
24. Vejdovsky, K., Hahn, K., Braun, D., Warth, B. & Marko, D. Synergistic estrogenic effects of fusarium and alternaria mycotoxins in vitro. *Archives of Toxicology*, **91**, 1447–1460 (2017).
25. Speijers, G. J. A. & Speijers, M. H. M. Combined toxic effects of mycotoxins. *Toxicol. Lett*, **153**, 91–98 (2004).
26. Jayaraj, A. & Richardson, R. Metabolic activation of aflatoxin B₁ by liver tissue from male fischer F344 rats of various ages. *Mechanisms of Ageing and Development*, **17**, 163–171 (1981).
27. Alassane-Kpembi, I. *et al.* Mycotoxins co-contamination: Methodological aspects and biological relevance of combined toxicity studies. *Critical Reviews in Food Science and Nutrition*, **57**, 3489–3507 (2017).
28. D'Andrea, A. D. & Haseltine, W. A. (1978). Modification of DNA by aflatoxin B₁ creates alkali-labile lesions in DNA at positions of guanine and adenine. Proceedings Of The National Academy Of Sciences Of The United States Of America, **75**, 4120–4124.
29. Heidrun, E.-Z., Barry, S., Brad, S., Werner, B. & Hans-Jurgen, A. Characteristic expression profiles induced by genotoxic carcinogens in rat liver. *Toxicol. Sci*, **1**, 19–34 (2004).
30. Essigmann, J. M., Donahue, P. R., Story, D. L., Wogan, G. N. & Brunengraber, H. Use of the isolated perfused rat liver to study carcinogen-DNA adduct formation from aflatoxin B₁ and sterigmatocystin. *Cancer Res*, **40**, 4085–4091 (1980).
31. Sivakumar, V., Thanissar, J., Niranjali, S. & Devaraj, H. Lipid peroxidation as a possible secondary mechanism of sterigmatocystin toxicity. *Human and Experimental Toxicology*, **20**, 398–403 (2001).
32. Ueno, Y. *et al.* Induction of apoptosis by T-2 toxin and other natural toxins in HL-60 human promyelotic leukemia cells. *Nat. Toxins*, **3**, 129–137 (1995).
33. Xing, X. *et al.* Involvement of MAPK and PI3K signaling pathway in sterigmatocystin-induced G(2) phase arrest in human gastric epithelium cells. *Molecular Nutrition and Food Research*, **55**, 749–760 (2011).
34. Chen, R. *et al.* Effect of ozone on aflatoxins detoxification and nutritional quality of peanuts. *Food Chem*, **146**, 284–288 (2014).
35. Iram, W. *et al.* Structural elucidation and toxicity assessment of degraded products of aflatoxin B₁ and B₂ by aqueous extracts of *trachyspermum ammi*. *Frontiers in Microbiology*, **7**, 1–16 (2016).

36. Vekiru, E. *et al.* (2015). *In vitro* binding assessment and *in vivo* efficacy of several adsorbents against aflatoxin B₁. *World Mycotoxin Journal*, **8**, 477–488.
37. Wajih, I., Tehmina, A., Mazhar, I., Abdul, G. & Mateen, A. Mass spectrometric identification and toxicity assessment of degraded products of aflatoxin B₁ and B₂ by corymbia citriodora aqueous extracts. *Sci. Rep*, **5**, 14672 (2015).
38. Chena, Y., Konga, Q., Chia, C., Shanb, S. & Guana, B. (2015). Biotransformation of aflatoxin B₁ and aflatoxin G₁ in peanut meal by anaerobic solid fermentation of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. *International Journal of Food Microbiology*, **211**, 1–5.
39. Das, A., Bhattacharya, S., Palaniswamy, M. & Angayarkanni, J. (2014). Aflatoxin B₁ degradation during co-cultivation of *Aspergillus flavus* and *Pleurotus ostreatus* strains on rice straw. *Biotechnology*, **5**, 279–284.
40. Elsanhoty, R. M., Al-Turki, I. A. & Ramadan, M. F. Application of lactic acid bacteria in removing heavy metals and aflatoxin B₁ from contaminated water. *Water Science & Technology*, **74**, 625–638 (2016).
41. Farzaneh, M. *et al.* Aflatoxin B₁ degradation by *Bacillus subtilis* UTBSP1 isolated from pistachio nuts of Iran., **23**, 100–106 (2012).
42. Hackbart, H. C. S., Machado, A. R., Christ-Ribeiro, A., Prietto, L. & Badiale-Furlong, E. Reduction of aflatoxins by *Rhizopus oryzae* and *Trichoderma reesei*. *Mycotoxin Res*, **30**, 141–149 (2014).
43. Wang, H. *et al.* Deep sequencing analysis of transcriptomes in *Aspergillus flavus* in response to resveratrol. *BMC Microbiology*, **15**, 182 (2015).
44. Etxebeste, O., Garzia, A., Espeso, E. A. & Ugalde, U. *Aspergillus nidulans* asexual development: making the most of cellular modules. *Trends in Microbiology*, **18**, 569–576 (2010).
45. Boylan, M. T., Timberlake, W. E. & Adams, T. H. *brlA* is necessary and sufficient to direct conidiophore development in *Aspergillus nidulans*., **54**, 353–362 (1988).
46. Keller, N. P., Turner, G. & Bennett, J. W. Fungal secondary metabolism — from biochemistry to genomics. *Nature Reviews Microbiology*, **3**, 937–947 (2005).
47. Lin, J., Zhao, X., Zhi, Q., Zhao, M. & He, Z. Transcriptomic profiling of *Aspergillus flavus* in response to 5-azacytidine. *Fungal Genetics and Biology*, **56**, 78–86 (2013).
48. Georgianna, D. R. & Fedorova, N. D. Burroughs. J. L., *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).
49. Payne, G. A. Beyond aflatoxin: four distinct expression patterns and functional roles associated with *Aspergillus flavus* secondary metabolism gene clusters. *Mol. Plant Pathol*, **11**, 213–226 (2010).
50. Jayashree, T. & Subramanyam, C. Oxidative stress as a prerequisite for aflatoxin production by *Aspergillus parasiticus*. *Free Radical Biology Medicine*, **29**, 981–985 (2000).
51. Adye, J. & Mateles, R. Incorporation of labelled compounds into aflatoxins. *Biochimica et Biophysica Acta, General Subjects*, **86**, 418–420 (1964).
52. Buchanan, R. L., Applebaum, R. S. & Conway, P. Effect of theobromine on growth and aflatoxin production by *Aspergillus parasiticus*. *Journal of Food Safety*, **1**, 211–216 (1978).
53. Reddy, T. V., Viswanathan, L. & Venkatasubramanian, T. A. Factors affecting aflatoxin production by *Aspergillus parasiticus* in a chemically defined medium. *Journal of General Microbiology*, **114**, 409 (1979).
54. Peng-Kuang, C., Sui, H., Siov, S. & Robert, L. 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).
55. Wen Rui, C. *et al.* Optimization of epothilone B production by *Sorangium cellulosum* using multiple steps of the response surface methodology. *African Journal of Biotechnology*, **10**, 11058–11070 (2011).
56. Turo, J. *et al.* Enhancement of tacrolimus productivity in *Streptomyces tsukubaensis* by the use of novel precursors for biosynthesis. *Enzyme & Microbial Technology*, **51**, 388–395 (2012).
57. Lafont, P. & Debeaupuis, J. P. Effect of sterigmatocystin on the toxinogenesis of the *Aspergillus flavus* group., **69**, 187 (1979).
58. Bhatnagar, D., Ehrlich, K. C. & Cleveland, T. E. (1992). Oxidation-reduction reactions in biosynthesis of secondary metabolites. In: Bhatnagar D, Lillehoj EB, Arora DK (eds). *Handbook of Applied Mycology, Vol V. Mycotoxins in Ecological Systems*. Dekker,

New York.

59. Dutton, M. F. Enzymes and aflatoxin biosynthesis. *Microbiological Review*, **52**, 274–295 (1988).
60. Kiser, R. C. & Niehaus, W. G. Purification and kinetic characterization of mannitol-1-phosphate dehydrogenase from *Aspergillus niger*. *Archives of Biochemistry & Biophysics*, **211**, 613–621 (1981).
61. Guo, L. Q. *et al.* Determination of aflatoxin and aflatoxin-like compounds in aspergilli metabolites by high-performance liquid chromatography-mass spectrometry. *Chinese Journal of Analytical Laboratory*, **34**, 677–682 (2015).
62. Trapnell, C., Pachter, L. & Salzberg, S. TopHat: discovering splice junctions with RNA-Seq., **25**, 1105–1111 (2009).
63. Li, B. & Dewey, C. N. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics*, **12**, 323 (2011).
64. Robinson, M. D., McCarthy, D. J. & Smyth, G. K. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data., **26**, 139–140 (2010).
65. Xie, C. *et al.* KOBAS 2.0: a web server for annotation and identification of enriched pathways and diseases. *Nucleic Acids Res*, **39**, 316–322 (2011).