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Comparison of aflatoxin production of *Aspergillus flavus* at different temperatures and media: Proteome analysis based on TMT

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ABSTRACT

Aflatoxin production of *Aspergillus flavus* is affected by abiotic factors such as temperature, water activity, oxidative stress, etc. These factors likely affect different metabolic pathways and result in altered aflatoxin production. Aflatoxin was determined in liquid media at 28 °C, solid media at 28 °C and solid media at 37 °C. The proteomic method was used to elucidate the mechanism of aflatoxin production in *A. flavus* in liquid media at 28 °C, solid media at 28 °C and solid media at 37 °C. Potential factors affecting aflatoxin production were found by GO and KEGG analysis. *A. flavus* produces more aflatoxin at 28 °C compared to 37 °C. Our study also found that *A. flavus* cultured on solid media produced more aflatoxin than in liquid media. In this study, we identified 5029 proteins from *A. flavus* NRRL3357, in which 1547 differential proteins were identified between liquid media and solid-state media, while 546 differential proteins were identified between 28 °C and 37 °C. Biological informatics analysis showed that these differential proteins were widely involved in a variety of biological processes, molecular functions, and cellular components, and were associated with multiple metabolic pathways. Compared to the liquid media, extracellular hydrolase for nutrient uptake and proteins related to sclerotia development were differentially expressed on solid media ($p < 0.05$). Enzymes involved in oxidative stress showed significantly down-regulated in liquid media and up-regulated at 28 °C ($p < 0.05$). Furthermore, our research also revealed aflatoxin synthesis is a complex process that is affected by a variety of factors such as nutrient uptake, oxidative stress, sclerotia development, G protein signaling pathways and valine, leucine and isoleucine degradation, and a speculative model summarizing the regulation of aflatoxin biosynthesis in *A. flavus* is presented.

1. Introduction

Aspergillus are filamentous fungi that are commonly found in soil, decaying vegetation, seeds and grains where they act as saprophytic fungi to help recover carbon and nitrogen by breaking down dead organic waste (Mousavi et al., 2016). *A. flavus* is a common mold in the genus *Aspergillus*. It is able to cause diseases in economically important crops, such as maize and peanuts, and to produce potent mycotoxins (Hedayati et al., 2007). Aflatoxins (AFs) are a major class of mycotoxins produced primarily by *Aspergillus* (Creppy, 2002). The major classes of AFs are aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2) (Sweeney and Dobson, 1998). When ruminants ingest AFB_1, 1–2% of them are converted to their metabolite AFM_1, and AFM_1 is mainly discharged through milk (Omeiza et al., 2018). High exposure levels of AFB_1 cause acute hepatitis. Chronic exposure leads to liver cancer, lung cancer, and stomach cancer. AFM_1 has the same carcinogenicity as AFB_1 (Marchese et al., 2018). Elucidating the biosynthesis regulatory mechanisms associated with AFs is critical to developing rational biocontrol strategies to relieve the negative effects of AFs contamination of agricultural commodities (Abbas et al., 2017).

At present, the biosynthetic pathway of aflatoxin has been basically clarified (Roze et al., 2013). Aflatoxin biosynthesis genes have also been widely reported (Yu, 2012; Yu et al., 2004). As a secondary metabolite of *A. flavus*, the biosynthesis of aflatoxins is influenced by various environmental factors and various levels of proteins including chromosome, transcription and post-translational modifications in vivo (Calvo and Cary, 2015; Yao et al., 2018). For example, the biosynthesis of aflatoxin is affected by temperature. Aflatoxin has the highest yield at 28–30 °C. As the temperature approaches 37 °C, the yield gradually decreases (Obrian et al., 2007). The production of aflatoxin is highly

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influenced by carbon sources. Sucrose and glucose are most suitable for the synthesis of AFB_1. The inhibition or induction of carbon source on mycotoxin synthesis may be related to its effect on sporulation (Lasram et al., 2016). Oxidative stress may indeed be a pre-requisite for aflatoxin production (Jayashree and Subramanyam, 2000). Solid-state fermentation provides a more near-natural culture state and therefore produces more secondary metabolites (Barrios-González, 2018). *LaeA* encodes a putative methyltransferase involved in chromatin remodeling, which is essential for aflatoxin production, *aflR* expression and *veA* expression (Kale et al., 2008; Keller et al., 2005). *LaeA* deletion mutants show reduced conidial chain elongation, increased production of conidiophores, and decreased colony hydrophobicity. Loss of hydrophobicity and other developmental changes in the *laeA* deletion mutant are responsible for the absence of aflatoxin in fungi (Chang et al., 2012a). As in *A. nidulans*, many new proteins containing methyltransferase domains showed high similarity to *LaeA* in *A. flavus* (Yao et al., 2018). In *Aspergillus*, the G protein signaling pathway typically regulates fungal development, stress response and mycotoxin production (Brodhagen and Keller, 2006).

Over the past decade, mass-spectrometry-based proteomics has matured from a largely technology-driven research field into a mainstream analytical tool for life sciences. Powerful mass-spectrometry-based technologies now provide unprecedented insight into the composition, structure, function, and control of proteomics, revealing complex biological processes and phenotypes (Aebersold and Mann, 2016). It has been reported that proteomic analysis is used to study the relationship between the specific response of *A. flavus* to oxidative stress and aflatoxin production capability (Fountain et al., 2018).

In past studies, people studied the effects of temperature on aflatoxin biosynthesis from the perspective of transcriptomics. A greater number of genes, including aflatoxin biosynthesis clusters, was expressed at 28 °C higher than at 37 °C (Obrian et al., 2007). Subtle changes in the expression level of *aflS* and *aflR* appear to control the transcriptional activation of the aflatoxin cluster (Montalbano et al., 2011). However, the transcriptome did not truly reflect changes in protein levels. To explain the effect of temperature and media type on the biosynthesis of aflatoxin in a molecular perspective, TMT-based (tandem mass tag) quantitative proteomic method was used to detect the change of proteins with different media types and temperatures. And this is the first report of proteomics study related to different media types of *A. flavus*.

2. Materials and methods

2.1. Fungal strains, culture conditions and determination of aflatoxin content

A. flavus NRRL3357 was cultured in Potato Dextrose Broth (PDB) at 28 °C, Potato Dextrose Agar (PDA) at 28 °C, and PDA at 37 °C for 24 h. The volume of PDB media was 100 ml. A small number of mycelia were inoculated into a sterilized liquid media and placed in a shaker for cultivation. The PDA media were paved with mycelia and placed in an incubator for cultivation. The experiment was repeated in triplicate.

The fungi in liquid PDB media were collected by filtration using 4 layers of gauze, and the fungi on solid PDA media were gently scraped with a blade. The fungus was mixed with 65% ethanol at 1:5, shaken vigorously for 3 min, and then settled by filtration to obtain a sample extract. The obtained sample dilutions were used to detect aflatoxin content by using an aflatoxin detection kit (Reveal Q+ kit and AccuScan III reader, Neogen, Lansing, USA). Aflatoxin content in liquid media was determined as the sum of aflatoxin in solution and mycelia of fungi. Dry weight was measured at 80 °C for 15 h.

2.2. Protein extraction, enzymatic hydrolysis of peptides, TMT labeling and high pH reversed-phase peptide fractionation

The experiment was performed in Applied Protein Technology Company in Shanghai. The mycelia were ground with liquid nitrogen, and the disrupted mycelia were extracted with SDT (4% (*w/v*) SDS, 100 mM Tris-HCl pH 7.6, 0.1 M DDT) (Wiśniewski et al., 2009). The BCA method was then used for protein quantification. Each sample was subjected to trypsin digestion using filter aided proteome preparation (FASP) method (Wiśniewski et al., 2009). Peptides were firstly quantified spectrophotometrically at OD280.

100 μg peptide was taken from each sample and labeled according to the TMT Mass Tagging kit (Thermo Fisher Scientific, Waltham, USA). Each group of labeled peptides was mixed in equal amounts and fractionated using a High pH Reversed-Phase Peptide Fractionation Kit (Thermo Fisher Scientific, Waltham, USA). First, the column was equilibrated with acetonitrile and 0.1% trifluoroacetic acid (TFA). Second, the mixed labeled peptide samples and pure water were loaded and then desalted by low-speed centrifugation. Finally, the column was combined with high-pH acetonitrile solution with increasing concentration. The peptides were subjected to gradient elution, and each eluted peptide sample was vacuum dried, and then lyophilized with 12 μl of 0.1% formic acid (FA), and the peptide concentration was determined by OD280.

2.3. Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis

We determined protein levels in PDB at 28 °C, PDA at 28 °C and PDA at 37 °C by TMT-based LC-MS/MS analysis. Each fractionated sample was separated by HPLC (Easy nLC 1200, Thermo Scientific, Waltham, USA) at a nanoliter flow rate. Buffer A was 0.1% formic acid in water, and buffer B was 0.1% formic acid in acetonitrile (acetonitrile was 84%). The column was equilibrated with 95% of the buffer A. The sample was loaded from the autosampler to the loading column (Thermo scientific Acclaim PepMap100, 100 μm*2 cm, nanoViper C18) and separated by an analytical column (Thermo scientific EASY column, 10 cm, ID75μm, C18-A2) at a flow rate of 300 nl/min.

The sample was chromatographed and subjected to mass spectrometry using a Q-Exactive mass spectrometer (Thermo Scientific, Waltham, USA). The method of mass spectrometry was described by Michalski et al. (2011), with a slight modification. The most abundant precursor ions were dynamically selected from the survey scan (300–1800 *m/z*) for HCD fragmentation. The mass spectrometry resolution was 70,000 at 200 *m/z* and resolution for tandem mass spectrometry was set to 17,500 at 200 *m/z* (TMT 6-plex) or 35,000 at 200 *m/z* (TMT 10-plex).

2.4. Database search and analysis

The resulting MS/MS data were processed using the Mascot search engine (v.2.2.0). Tandem mass spectra were searched against the UniProt *A. flavus* database (http://www.uniprot.org/). Trypsin was specified as a cleavage enzyme, allowing up to max missed cleavages. Carbamidomethyl on Cys was specified as the fixed modification, and oxidation on Met was specified as the variable modification. Mass tolerance was set to 20 ppm for precursor ions and 0.1 Da for fragment ions. For the protein quantification method, the protein ratios are calculated as the median of only unique peptides of the protein. The false-detection rate was adjusted to < 1%. Data correction based on the median of protein quantification ratios and the median protein ratio was 1 after the normalization.

2.5. Bioinformatics analysis

Blast2GO (http://www.blast2go.de) was used for the analysis of gene ontology (GO) annotation: blast searching, mapping, annotation, and InterProScan annotation (Dataset 1 and Dataset 2). Both the protein and gene IDs used in this research were from the UniProt database (http://www.uniprot.org/). The KEGG (Kyoto Encyclopedia of Genes

Table 1
Aflatoxin content in different media types and temperatures.

	PDB 28 °C	PDA 28 °C	PDA 37 °C
Dry weight of *A. flavus* (mg/g)	92.5	16.5	19.0
Aflatoxin B_1 production by *A. flavus* (ng/g)	4.629[a]	22.481	12.334

Data results were the average of three parallel experiments. Error range of all values is between ± 4–10%. The actual error values were omitted in the table.
[a] Aflatoxin content was determined as the sum of aflatoxin in solution and mycelia of fungi.

and Genomes) pathway was interpreted for the target protein set using KAAS (KEGG Automatic Annotation Server). Fisher's exact test was used to compare the distribution of each GO classification or KEGG pathway in the target protein set and the overall protein set, and the target protein set was subjected to enrichment analysis of GO annotation or KEGG pathway annotation. Protein clustering analysis normalizes the quantitative information of the target protein set (−1, 1). Then, Complexheatmap R was used to classify both sample and protein expression levels and generating a hierarchical cluster heat map.

3. Results

3.1. Aflatoxin content

The aflatoxin B_1 concentration was highest in PDA at 28 °C, which was 22.481 ng/g (Table 1). The aflatoxin B_1 concentration was least in PDB at 28 °C, which was 4.629 ng/g. Thus, it proved that *A. flavus* cultured on solid media produced more aflatoxins. It also demonstrated that the yield of aflatoxins at 28 °C was > 37 °C when cultured in the same media type.

3.2. Alteration of proteins in different media types and at different temperatures

It was found that under the same temperature, *A. flavus* produced more aflatoxins on solid media than liquid media. Therefore, it is significant to perform a comprehensive study on *A. flavus* proteome under different temperatures and media conditions. The genome of *A. flavus* encodes 13,655 functional genes. A total of 5029 proteins were identified in *A. flavus* NRRL3357. Differentially expressed proteins (DEPs) were filtered according to a standard of 1.2-fold change in expression fold (up to 1.2-fold or down to < 0.83-fold) and $P < 0.05$.

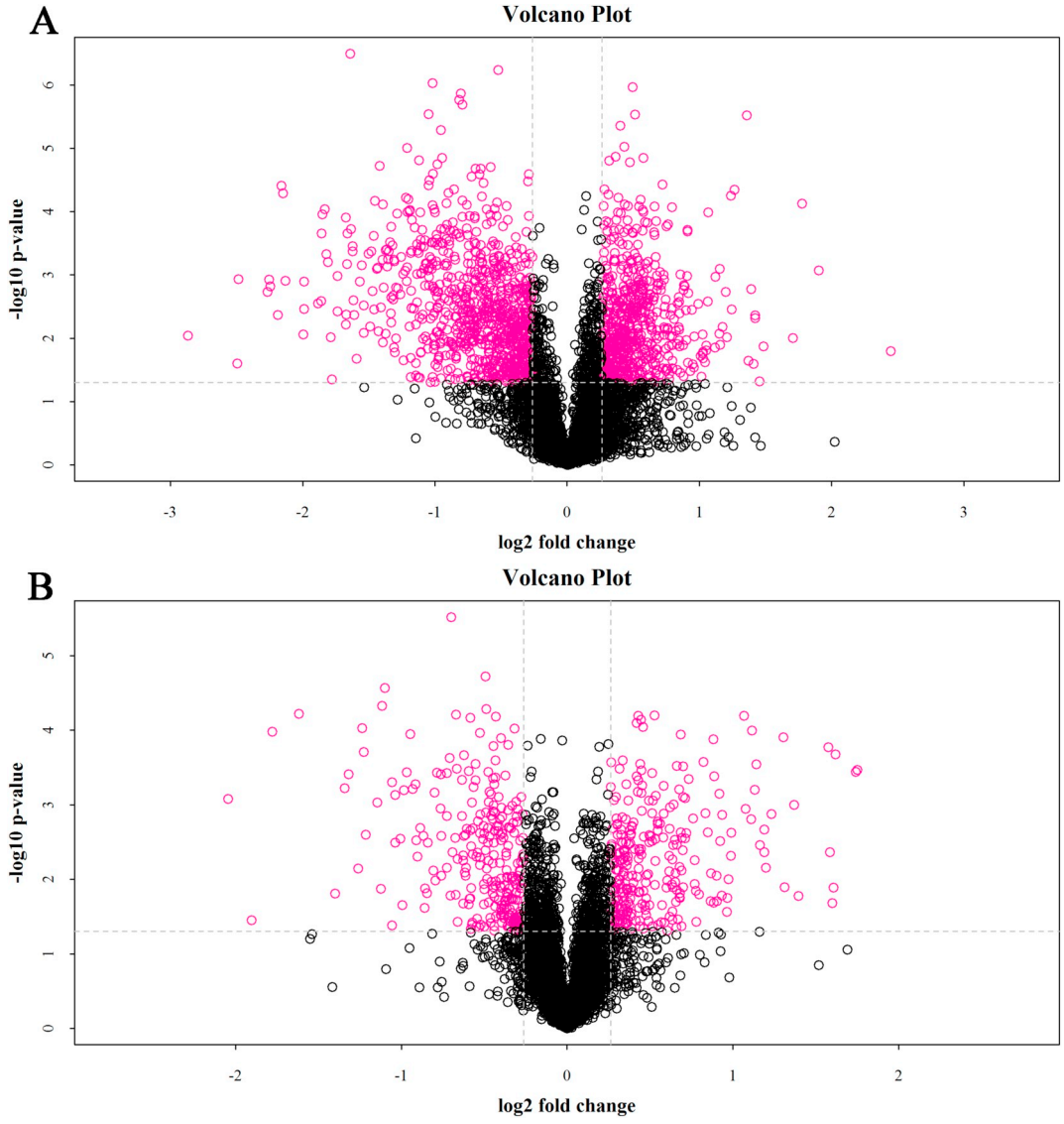


Fig. 1. The volcanic map of all identified proteins. (A) The volcanic map of all identified proteins in different media types (PDB at 28 °C vs PDA at 28 °C). (B) The volcanic map of all identified proteins at different temperatures (PDA at 28 °C vs PDA at 37 °C). Red point: significant DEPs (fold change > 1.2 and p < 0.05). Black point: unchanged protein. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

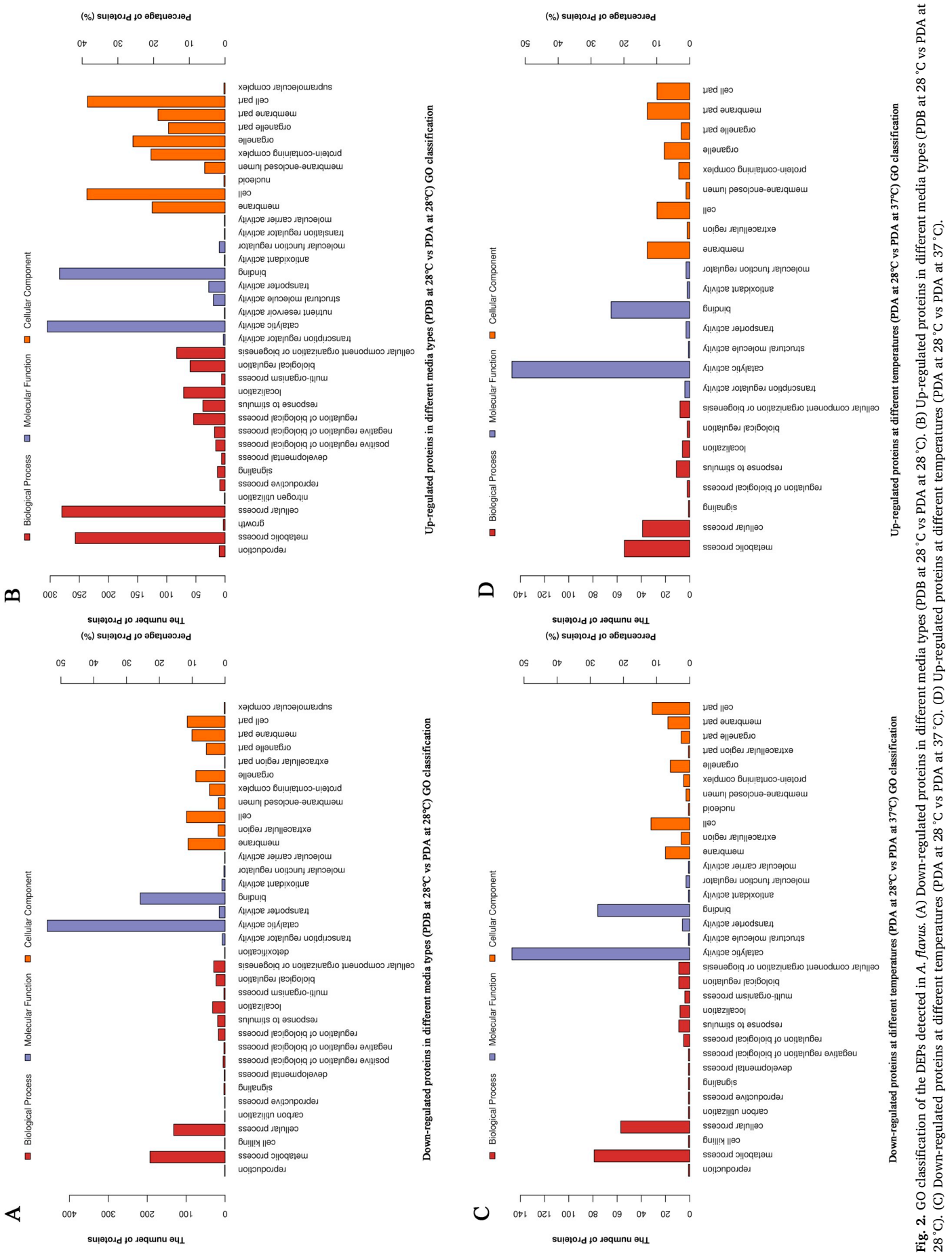
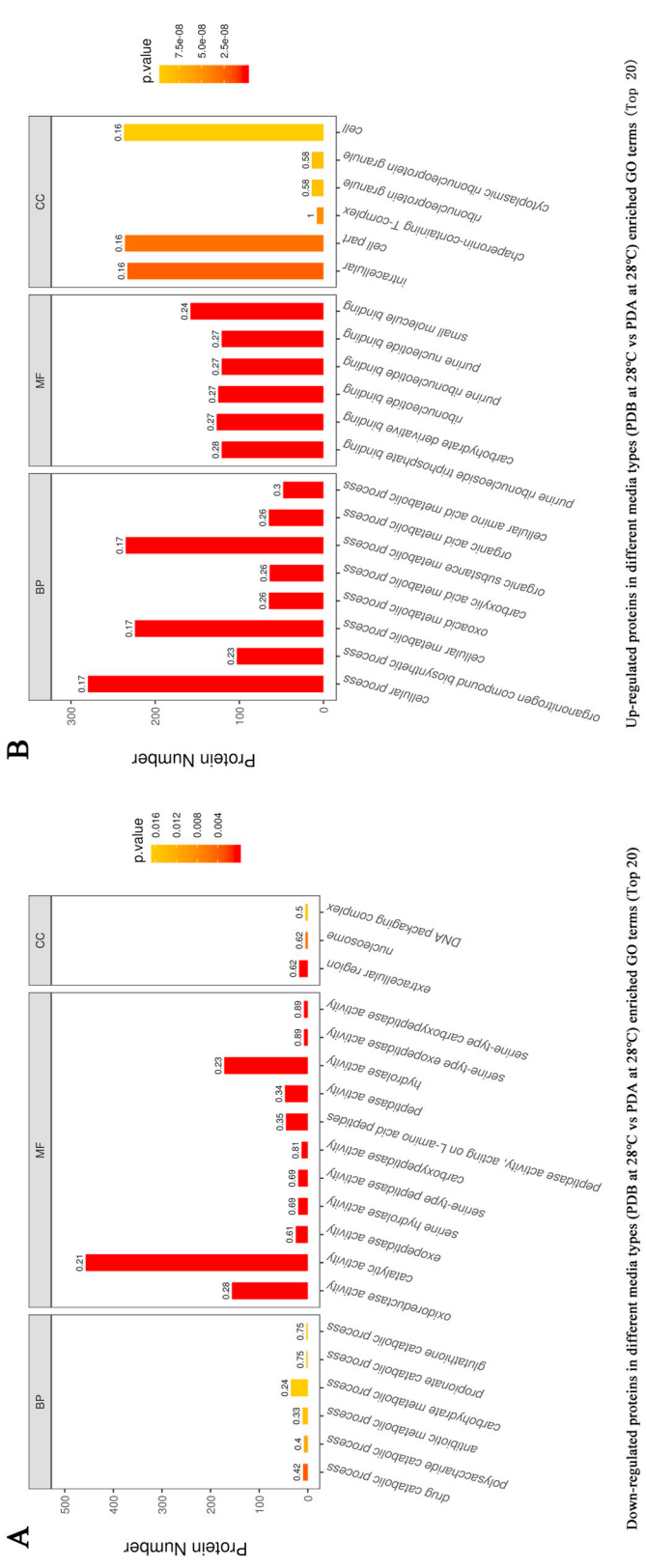
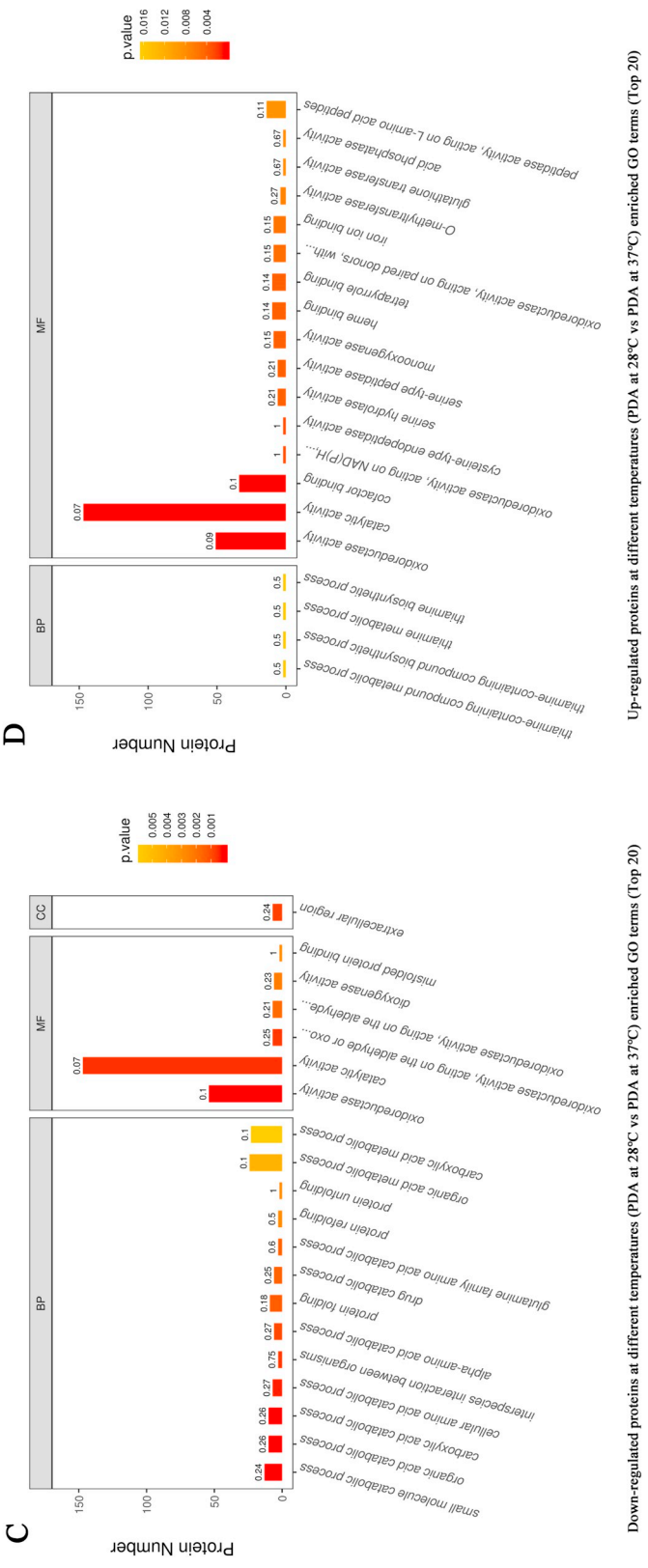


Fig. 2. GO classification of the DEPs detected in *A. flavus*. (A) Down-regulated proteins in different media types (PDB at 28 °C vs PDA at 28 °C). (B) Up-regulated proteins in different media types (PDB at 28 °C vs PDA at 28 °C). (C) Down-regulated proteins at different temperatures (PDA at 28 °C vs PDA at 37 °C). (D) Up-regulated proteins at different temperatures (PDA at 28 °C vs PDA at 37 °C).



Down-regulated proteins in different media types (PDB at 28°C vs PDA at 28°C) enriched GO terms (Top 20)

Up-regulated proteins in different media types (PDB at 28°C vs PDA at 28°C) enriched GO terms (Top 20)



Down-regulated proteins at different temperatures (PDA at 28°C vs PDA at 37°C) enriched GO terms (Top 20)

Up-regulated proteins at different temperatures (PDA at 28°C vs PDA at 37°C) enriched GO terms (Top 20)

(caption on next page)

Fig. 3. The top 20 terms of GO enrichment. (A) Down-regulated proteins in different media types (PDB at 28 °C vs PDA at 28 °C) enriched GO terms. (B) Up-regulated proteins in different media types (PDB at 28 °C vs PDA at 28 °C) enriched GO terms. (C) Down-regulated proteins at different temperatures (PDA at 28 °C vs PDA at 37 °C) enriched GO terms. (D) Up-regulated proteins at different temperatures (PDA at 28 °C vs PDA at 37 °C) enriched GO terms. Above the bar graph is rich factor (≤1), which represents the ratio of the number of differentially expressed proteins annotated to a GO functional category to the number of all identified proteins annotated to the GO functional category.

Quantitative results were shown in volcano maps (Fig. 1). Compared with PDA at 28 °C, 1457 differential proteins were identified in PDB at 28 °C, of which 613 proteins were up-regulated, and 844 proteins were down-regulated (Fig. 1A). Compared with PDA at 37 °C, 546 differential proteins were identified on PDA at 28 °C, of which 272 proteins were up-regulated and 274 proteins were down-regulated (Fig. 1B).

3.3. Functional classification of the identified differentially expressed proteins

In different media types and temperatures, differential proteins were mainly enriched in metabolic process and cellular process in biological process. The molecular function of differentially expressed proteins was mainly manifested in catalytic activity and binding. The cellular component of differentially expressed proteins was mainly localized to membrane, organelle and cell (Fig. 2).

3.3.1. Functional classification of down-regulated proteins in different media types (PDB at 28 °C vs PDA at 28 °C)

In biological process, drug catabolic process, carbohydrate metabolic process, polysaccharide catabolic process, etc. showed significant down-regulation. In molecular function, the catalytic activity, oxidoreductase activity, hydrolase activity, etc. showed significant down-regulation. Among the cellular component, the extracellular region, nucleosome, and DNA packaging complex showed significant down-regulation (Fig. 2A). In molecular function, GO analysis indicated that the hydrolyzing active enzymes such as α-amylase, glucoamylase, glucosidase, etc. and response to oxidative stress active enzymes such as superoxide dismutase and peroxidase were significantly down-regulated in liquid media (Table 3).

3.3.2. Functional classification of up-regulated proteins in different media types (PDB at 28 °C vs PDA at 28 °C)

In biological process, cellular process, cellular metabolic process, organic substance metabolic process, etc. showed significant up-regulation. In molecular function, purine ribonucleoside triphosphate binding, carbohydrate derivative binding, ribonucleotide binding, etc. showed significant up-regulation. Among the cellular component, intracellular, cell part, and chaperonin−containing T−complex, etc. showed significant up-regulation (Fig. 2B). In molecular function, the ribonucleoside triphosphate binding and organic metabolism processes played a role in ribosomes, where arginine transferase associated with sclerotia development was significantly up-regulated in liquid media (Table 3).

3.3.3. Functional classification of down-regulated proteins at different temperatures (PDA at 28 °C vs PDA at 37 °C)

In biological process, small molecule catabolic process, organic acid catabolic process, carboxylic acid catabolic process, etc. showed significant down-regulation. In molecular function, catalytic activity, etc. showed significant down-regulation. Among the cellular component, the extracellular region showed significant down-regulation (Fig. 2C).

3.3.4. Functional classification of up-regulated proteins at different temperatures (PDA at 28 °C vs PDA at 37 °C)

In biological process, thiamine−containing compound metabolic process, thiamine−containing compound biosynthetic process, thiamine metabolic process and thiamine biosynthetic process showed significant up-regulation. In molecular function, oxidoreductase activity, catalytic activity, cofactor binding etc. showed significant up-regulation (Fig. 2D). In molecular function, the oxidative stress-related enzymes such as peroxidase and glutathione S-transferase A were significantly up-regulated at 28 °C compared to 37 °C.

3.4. Analysis of the metabolic pathways by the altered proteins

KEGG analysis was used to investigate the enrichment pathways involved in up-regulation and down-regulation of proteins in different media types and temperatures.

3.4.1. Analysis of the metabolic pathways by the down-regulated proteins in different media types (PDB at 28 °C vs PDA at 28 °C)

844 down-regulated proteins in 5037 total proteins were mapped to 32 metabolic pathways ($P < 0.05$) in different media types (Table S1). The *P*-value and all altered proteins of the top 20 enrichment down-regulated pathways were shown in Fig. 3A for different media types. 16 altered proteins in the valine, leucine and isoleucine degradation, which were identified in different media conditions, showed the lowest *P* value in down-regulated pathways.

3.4.2. Analysis of the metabolic pathways by the up-regulated proteins in different media conditions (PDB at 28 °C vs PDA at 28 °C)

613 up-regulated proteins in 5037 total proteins were mapped to 30 metabolic pathways ($P < 0.05$) in different media types (Table S1). The *P*-value and all altered proteins of the top 20 enrichment up-regulated pathways were shown in Fig. 3B for different media types. 13 altered proteins in the aminoacyl-tRNA biosynthesis, which were identified in different media types, showed the lowest *P* value in up-regulated pathways.

3.4.3. Analysis of the metabolic pathways by the down-regulated proteins at different temperatures (PDA at 28 °C vs PDA at 37 °C)

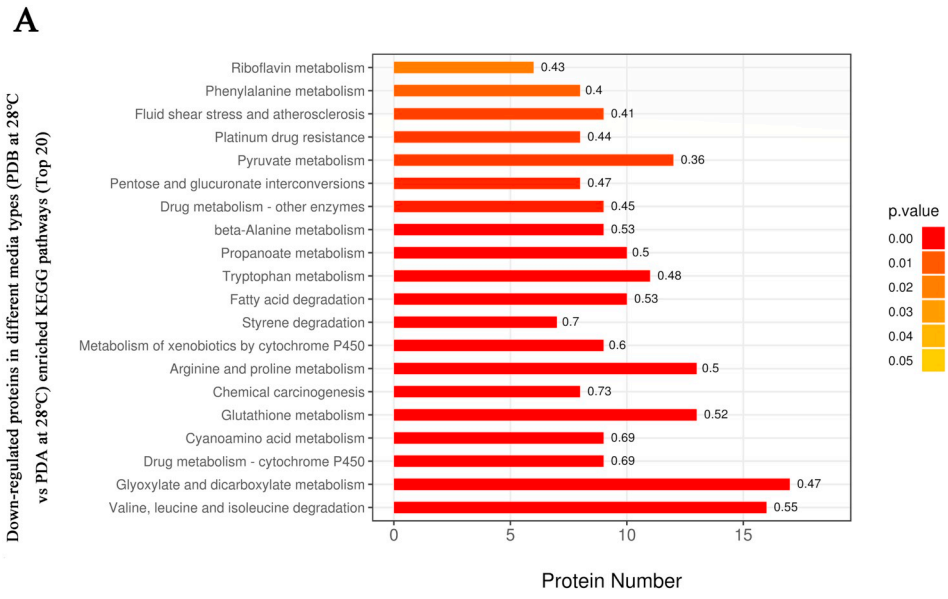
274 down-regulated proteins in 5037 total proteins were mapped to 22 metabolic pathways ($P < 0.05$) at different temperatures (Table S2). The enriched pathways evaluated by *P*-value and the number of altered proteins were shown in Fig. 3C for different temperatures. 11 altered proteins in the valine, leucine, and isoleucine degradation pathway, which were identified at different temperatures, show the lowest *P* value among all of the identified pathways.

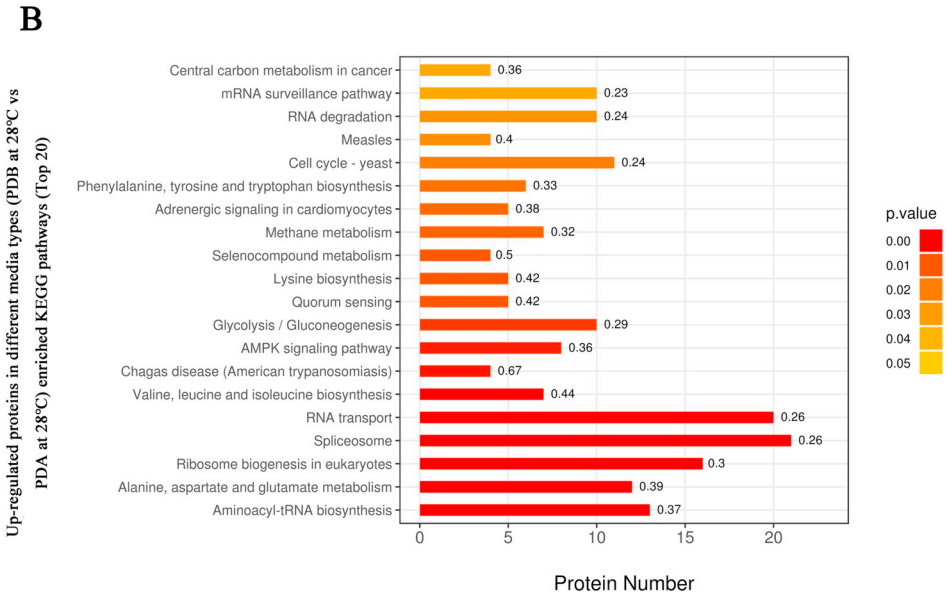
3.4.4. Analysis of the metabolic pathways by the up-regulated proteins at different temperatures (PDA at 28 °C vs PDA at 37 °C)

272 up-regulated proteins in 5037 total proteins were mapped to 2 metabolic pathways ($P < 0.05$) at different temperatures (Table S2). Thiamine metabolism and riboflavin metabolism were significantly up-regulated.

3.5. Potential roles of valine, leucine and isoleucine degradation pathway-related proteins in A. flavus NRRL3357 in different media types (PDB at 28 °C vs PDA at 28 °C)

Based on KEGG pathway analysis, the expression levels of some proteins involved in the degradation of valine, leucine, and isoleucine in *A. flavus* were changed compared to the solid media (Fig. 4). The DEPs involved in valine, leucine, and isoleucine was summarized in Table 2. Valine, leucine, and isoleucine degradation pathways in liquid media were significantly down-regulated compared to solid media. The final products of valine, leucine and isoleucine degradation were acetyl-





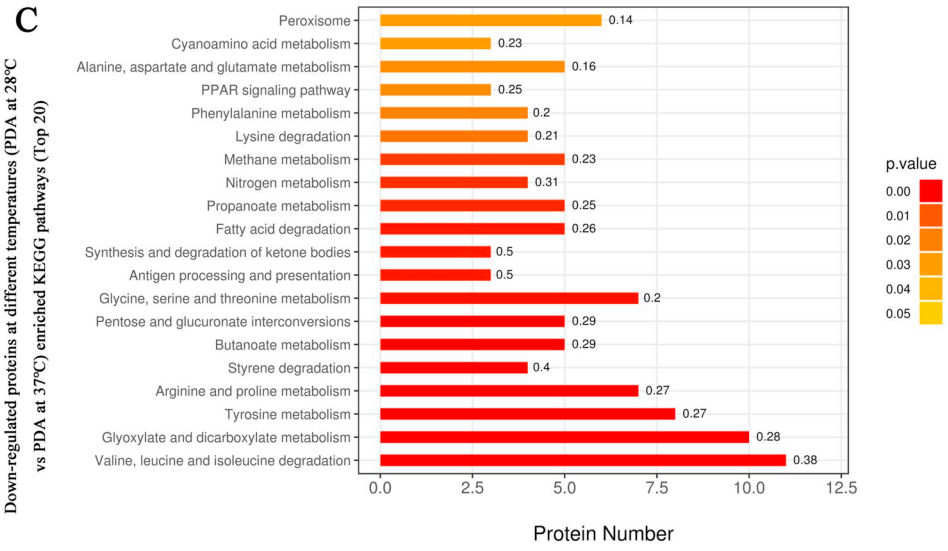


Fig. 4. KEGG pathway (Top 20) enrichment analysis of the DEPs detected in *A. flavus*. (A) Down-regulated proteins in different media types (PDB at 28 °C vs PDA at 28 °C) enriched KEGG pathways. (B) Up-regulated proteins in different media types (PDB at 28 °C vs PDA at 28 °C) enriched KEGG pathways. (C) Down-regulated proteins at different temperatures (PDA at 28 °C vs PDA at 37 °C) enriched KEGG pathways. (D) Up-regulated proteins at different temperatures (PDA at 28 °C vs PDA at 37 °C) enriched KEGG pathways. The tab above the bar graph shows the rich factor (rich factor ≤ 1), which indicates the ratio of the number of DEPs involved in a KEGG pathway to the number of proteins involved in the pathway in all identified proteins.

Table 2
DEPs involved in valine, leucine and isoleucine degradation.

Gene IDs	EC	Protein name	Fold change (PDB 28 °C vs PDA 28 °C)
AFLA_107590	1.2.4.4	3-Methyl-2-oxobutanoate dehydrogenase	0.62[a]
AFLA_028090	2.3.1.168	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	0.70[b]
AFLA_049020	1.3.8.7	Acyl-CoA dehydrogenase family protein	0.81
AFLA_043610	4.2.1.17	Enoyl-CoA hydratase/isomerase family protein	0.80
AFLA_099250	6.4.1.4	3-Methylcrotonyl-CoA carboxylase subunit alpha	0.74
AFLA_031150	4.2.1.18	Methylglutaconyl-CoA hydratase, mitochondrial	0.77
AFLA_052610	2.8.3.5	Succinyl-CoA:3-ketoacid-coenzyme A transferase	0.68
AFLA_079190	6.2.1.16	Acetoacetyl-CoA synthase	0.60
AFLA_008310	2.3.1.9	Acetyl-CoA-acetyltransferase	0.74
AFLA_119960	1.1.1.31	Oxidoreductase, putative	0.50
AFLA_109320	1.1.1.31	3-Hydroxyisobutyrate dehydrogenase	0.70
AFLA_056670	2.6.1.22	4-Aminobutyrate transaminase GatA	0.75
AFLA_050600	1.2.1.3	Betaine-aldehyde dehydrogenase, putative	0.81
AFLA_073320	2.3.1.16	3-Ketoacyl-CoA thiolase (POT1), putative	0.59

[a] Significantly upregulated proteins were > 1.2-fold and *P* value was < 0.05.
[b] Significantly downregulated proteins were < 0.83-fold and *P* value was < 0.05.

CoA and propionyl-CoA. Valine, leucine, and isoleucine biosynthetic pathways were significantly up-regulated in liquid media. Thus, less acetyl-CoA and propionyl-CoA were produced in liquid media compared to solid media (Fig. 5).

4. Discussion

Previous studies show that temperature is an important factor affecting aflatoxin biosynthesis in *Aspergillus*. Studies report that *A. flavus* produces the most aflatoxins at 28 °C and barely at 37 °C (Bai et al., 2015; Liu et al., 2017; Montalbano et al., 2011; Obrian et al., 2007). Most studies stood in the perspective of the transcriptome to explain why aflatoxin produced at 28 °C > 37 °C (Liu et al., 2017; Montalbano et al., 2011; Obrian et al., 2007). In the study by Bai et al. (2015), transcriptome analysis of *A. flavus* showed that growth temperature changes altered amino acid metabolism at 28 °C and 37 °C. This result was consistent with the results of our proteome data. We found valine, leucine and isoleucine degradation, tyrosine metabolism, arginine and proline metabolism, glycine, serine and threonine metabolism and alanine, aspartate and glutamate metabolism changed significantly. In a transcriptome study in which water activity and temperature interact to produce aflatoxin B_1, the author found that *rolA* was significantly down-regulated at 30 °C compared to 37 °C (Medina et al., 2017). In the study by Kong et al. (2014), *rolA*, which was involved in the adhesion of hydrophobic surfaces in fungi, was a potential target for controlling aflatoxin biosynthesis. In our study RolA (B8NTJ8) was also significantly down-regulated at 28 °C compared to 37 °C. Interestingly, we found that the dimethylallyl tryptophan synthase (B8NI11) involved in the synthesis of cyclopiazonic acid (CPA) in our study was significantly down-regulated at 28 °C. The production of CPA was related to the production of aflatoxin (Horn and Dorner, 1999). These are consistent with the transcriptome results of Bai et al. (2015) and Medina et al. (2017). Due to the effects of post-transcriptional modifications, gene expression levels were inconsistent with protein abundance. Studies already showed low correlation between transcriptome and proteome of aflatoxin biosynthesis at different temperatures in *A. flavus* (Bai et al., 2015). Solid-state fermentation provides a more near-natural culture state and therefore produces more secondary metabolites (Barrios-González, 2018). Our study found that *A. flavus* produced more aflatoxin on solid media than liquid media (Table 1). In this study, the effects of temperature and media conditions on aflatoxin biosynthesis at protein levels were studied by proteomic analysis of *A. flavus*. Results revealed that these differentially expressed proteins ($P < 0.05$) mainly exhibited catalytic activity involved in metabolic processes and cell development.

For better nutrient intake, *A. flavus* produces a variety of extracellular hydrolases (Mellon et al., 2005). Regulation of hydrolase secretion is complicated and relates to substrates, which plays a critical role in the induction of aflatoxin biosynthesis (Mellon et al., 2007). In our study, GO analysis showed that compared with the liquid, the carbohydrate metabolism process and polysaccharide catabolism process was significantly up-regulated on solid media, and these up-regulated differential protein showed hydrolase activity. Proteins involved in nutrient uptake significantly increased on solid media (Table 3). Enzymes involved in starch hydrolytic activity and proteolysis were significantly up-regulated on solid media, including alpha-amylase (B8N0S9), glucoamylase (B8NX52), alkaline protease A (B8N106), neutral protease B (B8NJB2), autophagic serine protease (B8NUE0), acetylxylan esterase A (B8NBI2), beta-glycosidase (B8N151, B8N3J2) and chitinase (B8NQH7). Studies show that the reduction of extracellular hydrolase affects the fungus-host interaction, including penetration and colonization of seed and ultimately results in a reduction in toxin production in *laeA* mutants (Lv et al., 2018). Glycosylphosphatidylinositol-anchored proteins (GPI-APs) are important for fungal cell wall integrity and viability. Ecm33 is a protein encoded by GPI-AP gene family in *A. flavus*. Defects in ecm33 lead to slow growth, development and aflatoxin production in *A. flavus*, which may be related to glucose uptake and complete activation of nutrient-responsive TOR kinase complex-1 signaling pathway (Chang et al., 2018; Umekawa et al., 2017). Compared to liquid media, we found that Ecm33 (B8NA16) was significantly up-regulated on solid media.

Glyoxylate and dicarboxylate, pyruvate metabolism and interconversion of pentose and glucuronic acid metabolism on solid media were significantly up-regulated. Upregulation of carbohydrate metabolism further promotes nutrient uptake in *A. flavus*. Carbohydrate metabolism linked other nutrients such as lipids to glucose metabolism, producing more acetyl-CoA as a precursor to produce aflatoxins. Compared to liquid media, this was one of the important reasons for *A. flavus* to produce more aflatoxin on solid media.

Although LaeA was not differentially expressed in different media types (PDB at 28 °C vs PDA at 28 °C), S-adenosylmethionine synthase (SasA, B8NE22) was found to be significantly up-regulated on solid media. LaeA carries a conserved SAM (S-adenosylmethionine)-binding site typical for nuclear protein methyltransferases (Bok and Keller, 2004). LaeA has the highest sequence similarity to histone and arginine transferase. This protein may be involved in chromatin modification, perhaps reminiscent of the expression of the mating-type locus in yeast (Keller et al., 2005). Over-expression of SasA has a strong influence on the development and secondary metabolism of *A. nidulans*. The transient interaction between SasA and the VelB-VeA-LaeA complex might be responsible for the secondary metabolism and developmental coordination defects caused by over-expression of SasA (Gerke et al.,

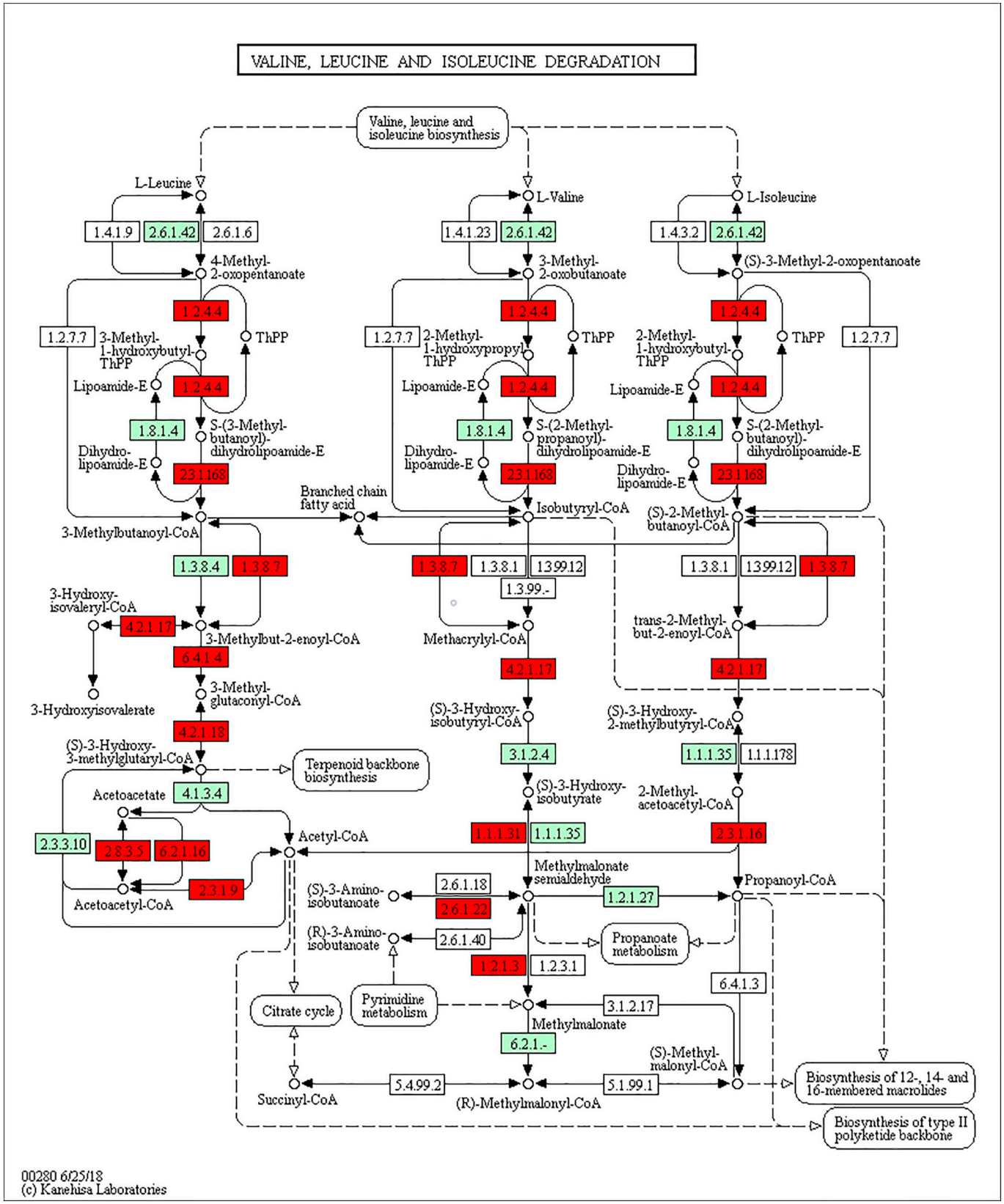


Fig. 5. The DEPs involved in valine, leucine and isoleucine degradation in different media types (PDB at 28 °C vs PDA at 28 °C). Quantitative proteomics identifiable proteins in *A. flavus* are indicated with red and green boxes. The significantly up-regulated proteins on solid media compared to liquid media are shown by the red boxes. The green ones mean no change or no detected in protein production level. The numbers in the red boxes correspond to the EC numbers listed in Table 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Screened differentially expressed proteins associated with aflatoxin synthesis.

Protein IDs	Protein name	Protein function	log_2 ratios	
			PDB 28 °C vs PDA 28 °C	PDA 28 °C vs PDA 37 °C
B8N0S9	Alpha-amylase (AmyA)	Nutrient uptake	0.59[a]	0.81[b]
B8NXN3	Alpha-amylase	Nutrient uptake	1.05	1.29
B8NX52	Glucoamylase (GlaA)	Nutrient uptake	0.59	1.01
B8N106	Alkaline protease A (AlpA)	Nutrient uptake	0.49	0.46
B8NUE0	Autophagic serine protease (Alp2)	Nutrient uptake	0.68	1.36
B8NJB2	Neutral protease B (NptB)	Nutrient uptake	0.45	0.73
B8NBI2	Acetylxylan esterase A (AxeA)	Nutrient uptake	0.79	0.33
B8N151	Beta-glycosidase (CB)	Nutrient uptake	0.60	0.84
B8N3J2	Beta-glycosidase	Nutrient uptake	0.76	0.90
B8NQH7	Chitinase (Cfc1)	Nutrient uptake	0.57	1.75
B8NA16	Ecm33	Nutrient uptake	0.78	0.88
B8N419	Superoxide dismutase (SodM)	Oxidative stress	0.50	0.78
B8N9C3	Peroxidase (Ccp1)	Oxidative stress	0.81	0.97
B8NX24	Peroxidase (CpeB)	Oxidative stress	0.55	1.72
B8N244	Catalase (Cat)	Oxidative stress	0.59	1.00
B8NTU4	Catalase (Cat)	Oxidative stress	1.89	0.91
B8N3U8	Glutathione S-transferase A (GstA)	Oxidative stress	0.51	1.26
B8MX61	Glutathione S-transferase B (GstB)	Oxidative stress	0.54	1.05
B8N2F6	Peroxisomal biogenesis factor (PEX11)	Oxidative stress	0.71	0.88
B8NE22	S-Adenosylmethionine synthase (SasA)	Sclerotia development	0.53	1.06
B8NNC6	RmtA	Sclerotial development	1.43	0.92
B8MY33	Arginine methyltransferase RmtB	Sclerotial development	1.29	1.14
B8NU21	FluG	Sclerotial development	0.72	1.18
		G protein signaling pathway		
B8N4X5	FadA	G protein signaling pathway	1.20	0.99
B8N1D7	Ste18	G protein signaling pathway	0.68	1.06
B8NT05	CpcB	G protein signaling pathway	1.91	0.88
B8NHN8	FlbC	G protein signaling pathway	0.51	1.14
B8NEG2	Toxin biosynthesis protein	Toxin biosynthesis	0.81	0.91
B8NRQ4	Toxin biosynthesis protein	Toxin biosynthesis	0.73	1.27
B8NWU6	Methyltransferase LaeA-like (laeA1)	Global regulator	0.93	0.74

[a] Significantly upregulated proteins were > 1.2-fold and *P* value was < 0.05.
[b] Significantly downregulated proteins were < 0.83-fold and *P* value was < 0.05.

2012). The secondary metabolic regulation pattern of SasA in *A. flavus* has never been reported. Methyltransferase LaeA-like (B8NWU6) was significantly down-regulated at 28 °C compared to 37 °C. In the study by Yao et al., a new LaeA-like methyltransferase (B8NWU6) was identified to play a specific role in the regulation of AF biosynthesis. In contrast to LaeA, LaeA-like methyltransferase negatively regulates the biosynthesis of aflatoxins (Yao et al., 2018).

Studies report that oxidative stress stimulates *A. flavus* to produce aflatoxins (Georgianna and Payne, 2009). A knockout of a superoxide dismutase gene in *A. flavus* has been shown to decrease aflatoxin production (He et al., 2007). In *Aspergillus*, aflatoxin biosynthesis is accompanied by an increase in the activities of catalase, superoxide dismutase, peroxidase, and glutathione S-transferase (Jayashree and Subramanyam, 2000). There is a positive correlation between glutathione S-transferase activity and aflatoxin formation in *A. flavus* (Saxena et al., 1988). In our study, superoxide dismutase (B8N419), peroxidase (B8N9C3, B8NX24), catalase (B8N244), glutathione S-transferase A (GstA, B8N3U8) and glutathione S-transferase B (GstB, B8MX61) were shown significant up-regulated on solid media compared to liquid media. However, one catalase (B8NTU4) showed down-regulated. Compared to 37 °C, peroxidase (B8NX24) and glutathione S-transferase A (GstA, B8N3U8) were shown significant up-regulation at 28 °C. The Pex11 peroxisomal membrane proteins are only factors known to promote peroxisome division in multiple species (Li and Gould, 2002). In a study by Reverberi et al., mammalian peroxisome proliferator enhanced *pex11* expression and triggered aflatoxin biosynthesis in *A. flavus* (Reverberi et al., 2012). Our study found that *pex11* (B8N2F6) was significantly up-regulated on solid media compared to liquid media, which was consistent with results of Reverberi et al. The results in this study showed the synthesis of aflatoxin in different media types and at different temperatures was affected by oxidative stress. When the culture environment changed, oxidative stress kinases changed at the same time in *A. flavus*, and production of these oxidative kinases stimulated the production of aflatoxin.

The development of *A. flavus* has a profound effect on the production of aflatoxins (Kale et al., 2008). FluG-deficient strain reduced conidia and promoted sclerotia development in *A. flavus*. When LaeA is present, FluG interacts with LaeA to regulate the size of sclerotia (Chang et al., 2012b). Studies report that the synthesis of ochratoxin, aflatoxin and asexual spores in *Aspergillus* is negatively controlled by FadA (the alpha-subunit of the heterotrimeric G protein) (Hicks et al., 1997). In *Aspergillus* and *Fusarium*, sporulation and mycotoxin production are regulated by G protein signaling pathways (Brodhagen and Keller, 2006). The heterotrimeric G protein (G protein) is composed of an α, β, γ subunit (FadA, CpcB, Ste18) and binds to a membrane-bound G protein-coupled receptor (GPCR), resulting in a GDP-GTP exchange dissociation of the Gα subunit. Dissociated GTP-Gα, Gβγ or two units can amplify and propagate signals by modulating the activity of effector proteins (Fig. 6B) (Shin et al., 2009). FluG inactivates the FadA G protein signaling pathway in fungi, leading to ST production and allowing sexual and asexual development (Fig. 6B) (Calvo and Cary, 2015). *flbC* encoding C2H2 transcription factor, which is involved in asexual development, sexual development and germination. Deletion of *flbC* causes a delay/reduction in conidiation, *brlA* and *vosA* expression, and conidial germination (Kwon et al., 2010). In our study, FluG, Ste18 and FlbC were significantly up-regulated, while FadA and CpcB were significantly down-regulated on solid media compared to liquid media. Histone H4 arginine methyltransferase (RmtA, B8NNC6) and arginine methyltransferase (RmtB, B8MY33) were significantly down-regulated on solid media. It is reported that overexpression of RmtA is accompanied by an increase in sclerotia affecting the biosynthesis of aflatoxin (Satterlee et al., 2016). In *A. parasiticus*, the production of aflatoxin is

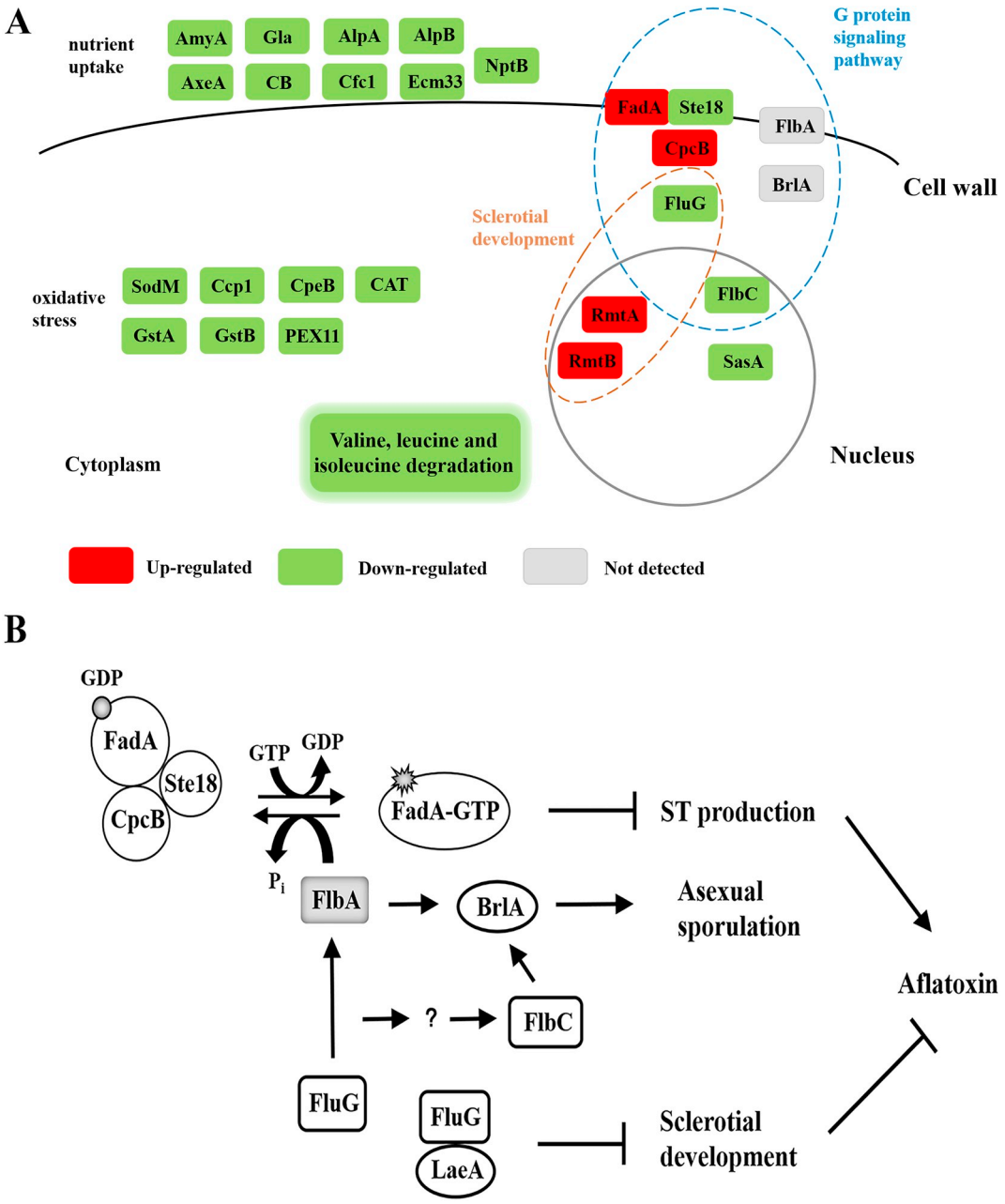


Fig. 6. The aflatoxin synthesis regulation patterns of *A. flavus* in different media types (PDB at 28 °C vs PDA at 28 °C) is shown at the cellular (A) and molecular levels (B). (A) Regulatory model of enzymes of *A. flavus* in liquid media compared to solid media. Boxes indicate DEP (1.2-fold change; $p < 0.05$) specified by the name of the homolog in *Aspergillus*. (B) A model of regulatory pathway for fungal development and secondary metabolites in *A. flavus* linked by components of the G protein signaling pathway in different media types (PDB at 28 °C vs PDA at 28 °C).

often accompanied minification in sclerotia (Chang et al., 2002). Sclerotial development and changes in G protein signaling pathways may be important pathways affecting the biosynthesis of aflatoxins in *A. flavus*.

Amino acid metabolisms have a complex effect on the biosynthesis of aflatoxins. Amino acids play different roles in primary and secondary metabolism. Valine, leucine, and isoleucine degradation are associated with aflatoxin biosynthesis, while tryptophan and phenylalanine metabolism are primarily associated with fungal growth (Chang et al., 2015). Valine, leucine and isoleucine degradation, tryptophan metabolism and phenylalanine metabolism were significantly up-regulated on solid media compared to liquid media in our research. Degradation of isoleucine and valine results in the production of propionate, which produces severe damage to asexual conidia (Brock, 2005; Roze et al., 2010). The production of aflatoxin requires a higher activity of catabolism of branched chain amino acids (valine, leucine, isoleucine). It is possible that the end products of this degradation pathway, such as acetate and propionate, are not only used as precursors for aflatoxin biosynthesis, but also for energy regeneration (Chang et al., 2015). The effect of amino acid metabolism on the production of aflatoxin by *A. flavus* is complex. Different amino acid metabolisms have different effects on fungal development and toxin synthesis.

The factors affecting the biosynthesis of *A. flavus* are not controlled by a single variable. Synthesis of aflatoxins is likely to be affected by a variety of factors such as nutrient uptake, sclerotia development, G protein signaling pathway and other metabolisms. In different media types, nutrient uptake regulates the biosynthesis of aflatoxins from the source, while some proteins (Table 3) identified indirectly regulate the

synthesis of aflatoxins by altering oxidative stress, sclerotia development and G protein signaling pathway (Fig. 6A).

Proteins encoded by genes associated with aflatoxin biosynthesis were not detected. Due to technical deficiencies, a present limitation is that most studies that analyze protein abundance do not reach the depth that is required to fully address the status of low-abundance proteins (Harper and Bennett, 2016). Some unresolved issues such as the relationship between glyoxylate and dicarboxylate metabolism and amino acid metabolism and aflatoxin biosynthesis were found in our research. Some significantly differentially expressed proteins were also found to be uncharacterized proteins (Table S3). These issues warrant further study for their potential to affect aflatoxin biosynthesis and/or fungal growth directly or indirectly.

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Notes

The authors declare no competing financial interest.

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