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Combined transcriptomic and metabolomic analysis revealed that pH changes affected the expression of carbohydrate and ribosome biogenesis-related genes in *Aspergillus niger* SICU-33

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The process of carbohydrate metabolism and genetic information transfer is an important part of the study on the effects of the external environment on microbial growth and development. As one of the most significant environmental parameters, pH has an important effect on mycelial growth. In this study, the effects of environmental pH on the growth and nutrient composition of *Aspergillus niger* (*A. niger*) filaments were determined. The pH values of the medium were 5, 7, and 9, respectively, and the molecular mechanism was further investigated by transcriptomics and metabolomics methods. The results showed that pH 5 and 9 significantly inhibited filament growth and polysaccharide accumulation of *A. niger*. Further, the mycelium biomass of *A. niger* and the crude polysaccharide content was higher when the medium's pH was 7. The DEGs related to ribosome biogenesis were the most abundant, and the downregulated expression of genes encoding XRN1, RRM, and RIO1 affected protein translation, modification, and carbohydrate metabolism in fungi. The dynamic changes of pargyline and choline were in response to the oxidative metabolism of *A. niger* SICU-33. The ribophorin_I enzymes and DL-lactate may be important substances related to pH changes during carbohydrate metabolism of *A. niger* SICU-33. The results of this study provide useful transcriptomic and metabolomic information for further analyzing the bioinformatic characteristics of *A. niger* and improving the application in ecological agricultural fermentation.

KEYWORDS

pH, *Aspergillus niger*, transcriptomics, metabolomics, carbohydrate metabolism, ribosome biogenesis

1 Introduction

Barley (*Hordeum*) is the fourth leading cereal crop around the world (Dawson et al., 2015), and highland barley (*Hordeum vulgare* L. var. *nudum* hook. f. HB), belonging to the genus *Hordeum* in the family Gramineae, is a grain crop that grows on the plateau under poor soil conditions, distributed mainly in the Qinghai-Tibet Plateau, China (Obadi et al., 2021; Xue et al., 2023). It is the main food for local residents and raw material for related food production and processing (Zeng et al., 2018). At present, the research related to highland barley mainly focuses on food processing, genetic analysis of germplasm resources, determination and analysis of nutrient composition, and the relationship between highland barley cultivation and soil properties (Hadgu et al., 2009; Dou et al., 2021; Nie et al., 2022). Therefore, the isolation of related microorganisms from the soil environment of highland barley will enrich the study of the microbial ecology of highland barley habitat.

Fungi are widely distributed and grow in almost all habitats, including extreme deserts or areas with high salt concentrations, deep sea areas, and other environments (Hawksworth, 2001; Basu et al., 2015; Coleine et al., 2022). In the environment, fungi obtain nutrients by secreting enzymes and absorbing the released molecules (Hawksworth, 2001; Basu et al., 2015). *Aspergillus niger* is a type of fungus that possesses strong metabolic capacity for hydrolyzing carbohydrates and producing proteins and organic acids, secreting a wide variety of different enzymes that allow them to release different nutrients such as biopolymers used in industrial fermentation (Pel et al., 2007; Hossain et al., 2019; Marzo et al., 2019). The extracellular enzymes produced by *A. niger* can degrade various organic materials, decompose soil components, and regulate carbon and nutrient balances, such as β -glucosidase hydrolyzes cellulose disaccharide (Lammirato et al., 2011; Kadri et al., 2017). Because of its enzyme production capacity and high safety, *A. niger* has been widely used in industrial biotechnology (Schuster et al., 2002; Abdel-Azeem et al., 2019). It has been reported that *A. niger* can be used in industry to produce heteropeptides, such as cyclic deposited peptides and itaconic acid (IA), which are widely used in coatings and synthetic resins (Li and van Luijk, 2011; Boecker et al., 2018). Similarly, *A. niger* has also been used in the commercial production of organic acids, e.g., citric and gluconic acids, and industrial proteins, e.g., fungal enzymes and heterologous proteins (Andersen et al., 2008; Krijghsheld et al., 2013; Xie et al., 2018).

In agricultural production, fungi perform various tasks, such as fixing nitrogen, synthesizing hormones, controlling roots, and withstanding drought. They are also crucial for the stability of soil organic matter and the breakdown of residue, and both biotic and abiotic variables can influence their activity (Qiu et al., 2018; Devi et al., 2020). In practical applications, environmental conditions such as nutrient availability, temperature, relative humidity, water activity, and pH are key factors for fungal growth (Silveira et al., 2019; Tai et al., 2020). Previous research has shown that many fungi can grow across a wide pH range and express specific genes depending on the pH of the environment (Peñalva and Arst, 2002; Manteau et al., 2003). The pH of the environment is a crucial indicator of cell activity. It primarily affects growth, morphogenesis, membrane and cell wall stability, and protein function (Prusky and Sionov, 2021; Koza et al., 2022). However, most studies on the

response mechanism of *A. niger* to environmental factors mainly involve industrial, such as the bio adsorption of nano-plastics by *A. niger* (Kadri et al., 2017), citric acid production in industry, and corrosion of metal materials by *A. niger* (Qu et al., 2015; Tong et al., 2019). There are few reports on the effect of pH on the growth and metabolism of *A. niger* in agricultural production. Therefore, it is of great significance to explore the influencing mechanism of environmental pH on the growth and metabolism of *A. niger* to promote its active application in agriculture, such as screening PGPR strains, the production of new biological organic fertilizers, and soil improvement.

Until now, transcriptome and metabolome analysis techniques have been rapidly developed in fungal research (Takano et al., 2019; Huang, 2020; Ma et al., 2021). However, the molecular mechanism of nucleic acid and carbohydrate metabolism of *A. niger* affected by pH is still limited. It is necessary to perform high-throughput investigations of variations in both transcription and expression of genes involved in the changes of nucleic acid and carbohydrate metabolism of *A. niger* in the application of agricultural production. Therefore, the research combined with transcriptomics and metabolomics analysis, and functional gene annotation genes and metabolic pathways were analyzed according to Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis; we discussed the molecular regulatory mechanism of the metabolic process changes of *A. niger* SICU-33 under the influence of different pH. The goals of our study were: (1) to reveal the molecular mechanism of pH change on the expression of genes related to nucleic acid and carbohydrate metabolism in *A. niger*; (2) to characterize the effects of pH changes on the mycelial biomass and polysaccharide content of *A. niger*; and (3) to provide experimental basis for the development of the application of *A. niger* in agroecology and to further study its molecular physiological mechanism under changed relevant environmental conditions.

2 Materials and methods

2.1 Soil samples collection

Samples collection methods are as follows: the isolation details of rhizosphere soil fungi have been referred to in the article of Sun et al. (2022). In 2020, the rhizosphere soil samples from highland barley of Lazi (LZ), Cuomei (CM), Jiangzi (JZ), Longzi4ling (LZ4), Longzi6ling (LZ6), Ali, Zangqing 2000 (ZQ), and Langkazi (LKZ) were collected from eight sites in the Tibetan Plateau for this investigation, and soil fractions from the rhizosphere were brushed and mixed into one sample. The samples were transported in a refrigerated box to the lab, where they were kept at -20°C until the microbes were isolated and purified (Jin et al., 2021).

2.2 Culture and isolation of strains

The rhizosphere soil samples were cultured in a PDA liquid medium, and an appropriate amount of sample suspension was added to the surface of the solid medium. They were cultured at 28°C for 3–5 days using the dilution spread plate method. A single

colony was selected to be transferred to the new PDA medium for purification, and the pollution-free and well-growing strains were obtained as the target strains (Mahadevamurthy et al., 2016). The plant growth promotion (PGP) potential characteristics of the strains were preliminarily evaluated by the following methods: by testing the IAA production (Glickmann and Dessaux, 1995), isolates were grown in liquid PDA medium (with 2 mg/ml l-tryptophan) at 28°C for 3–5 days in a shaker (150 rpm) pathogen resistance; then cultures were centrifuged at 10,000 g for 30 min and 1 ml the supernatant was mixed with 2 ml Salkowski reagent (1 ml of 0.5 M FeCl₃ and 49 ml of 35% HClO₄); finally, the IAA concentration was measured at 530 nm after 30 min reaction at room temperature. The yield of siderophore production was performed using the chrome azurol S (CAS) medium, in which the isolated strain was cultured at 28°C for 3 days, and an orange halo was formed around the colony, indicating that siderophore was clearly produced (Schwyn and Neilands, 1987). The phosphorus-solubilizing activity of the isolated strain was determined by the following method (Chen et al., 2022a), in which the strain was inoculated on Pikovskaya's agar and cultured at 28°C for 5 days. Clear halos appeared around the colony, which was considered to have a phosphorus-solubilizing effect. In addition, cellulose degradation was measured as described earlier (Teather and Wood, 1982). All the above measurements included three replicates.

Following the above methods, we obtained highly effective strains that were primed ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') amplified and sequenced ITS gene fragments, and the phylogenetic tree of SICU-33 was constructed after BLAST in NCBI (Figure 1). Its sequences were deposited in the GenBank of NCBI (<https://www.ncbi.nlm.nih.gov/>) under the accession number MN498286. Then, the conventional PDA liquid culture medium was prepared, and the target pH (5, 7, and 9) was reached by adding 1 mol/L of sodium hydroxide and hydrochloric acid solutions. The strain *A. niger* SICU-33 was activated using a PDA medium at 28°C for 3 days, cut off with a 5 mm hole punch, and inoculated in a liquid medium with different pH. After incubating in a constant temperature oscillating incubator at 28°C, 140 r/min for 3 days to the stable stage, the mycelium was filtered with filter paper, drained of water, and weighed. The pH of the medium was continuously measured to ensure that the pH was maintained at a fixed value during this time. After the liquid medium formed the membrane, the samples were collected, dried with filter paper, quickly frozen with liquid nitrogen, and stored at -80°C for extraction and determination of polysaccharides. The polysaccharide content was determined using the sulfuric acid-phenol method (Huang, 2020). All the analyses were done in three replicates.

2.3 RNA extraction, cDNA library construction, and sequencing

RNA was extracted using the Trizol Reagent (Invitrogen Life Technologies, USA). The concentration and quality of extracted RNA were determined using a NanoDrop spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.,

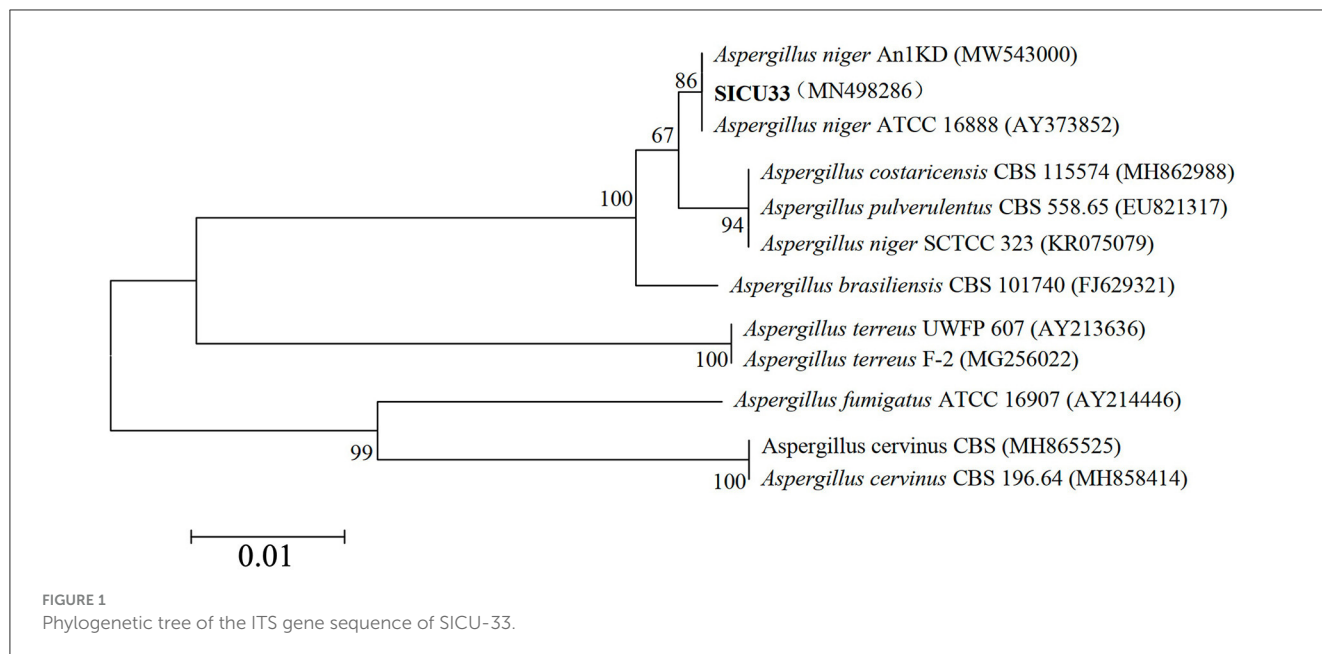
Wilmington, DE, USA). The integrity of the extracted RNA was assessed in 1% agarose by electrophoresis.

Sequencing libraries were generated using the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA) (Jain et al., 2020). Briefly, mRNA was purified from 3 µg RNA using poly-T-oligo-attached magnetic beads (Ozsolak and Milos, 2011). Fragmentation was performed using divalent cations under elevated temperature in an Illumina proprietary fragmentation buffer. First-strand cDNA was synthesized using random oligonucleotides and SuperScript II. Second-strand cDNA was synthesized using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities, and the enzymes were removed. After acetylation of the 3' ends of the DNA fragments, Illumina PE adapter oligonucleotides were ligated to prepare for hybridization (Wang et al., 2009). To select cDNA fragments of the preferred 200 bp in length, the library fragments were purified using the AMPure XP system (Beckman Coulter, Beverly, CA, USA). DNA fragments with ligated adaptors on both ends were selectively enriched using Illumina PCR Primer Cocktail in a 15-cycle PCR reaction. Products were purified using the AMPure XP system and quantified using the Agilent high-sensitivity DNA assay on a Bioanalyzer 2100 system (Agilent). The sequencing library was sequenced on Illumina HiSeq sequencer at Shanghai Personal Biotechnology Co. Ltd, China. The RNA-seq raw data were submitted to the National Center for Biotechnology Information (NCBI) database (<https://www.ncbi.nlm.nih.gov/sra/>; accession number PRJNA880770).

2.4 Transcriptome analysis

Raw data (raw reads) in Fastq format were first processed through in-house Perl scripts. In this step, clean data (clean reads) were obtained by removing reads containing adapter, poly-N, and low-quality reads. At the same time, Q20, Q30, and GC content of the clean data were calculated. All the downstream analyses were based on the clean data assembled using the Trinity (v2.1.1) software with high quality to construct transcript and unigenes sequences. High-quality clean reads were aligned to the assembled transcriptome using HISAT2 (<http://ccb.jhu.edu/software/hisat2/index.shtml>); the criteria for data filtering included the use of Cutadapt to remove sequences with 3' end-band connectors and the removal of reads with an average mass score below Q20. The reference genome (*Aspergillus niger*. SICU_33.scaffolds.fna) for this study was derived from whole genome sequencing of SICU_33. In the differential expression analysis using the DESeq R package (version 1.16.1) (Wang et al., 2010; Love et al., 2014), genes were considered differentially expressed when $|\log_2(\text{Fold Change})| \geq 1$ and $p < 0.05$. p -values were adjusted for multiple testing as described earlier (Mi et al., 2019).

Transcripts were functionally annotated against Universal Protein Knowledgebase 261 (UniProt) (Apweiler et al., 2004), Gene Ontology (GO) (Ashburner et al., 2000), eggNOG (Huerta-Cepas et al., 2016), and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (Kanehisa et al., 2004) using BLAST (Altschul et al., 1997). GO enrichment analysis by Blast2GO software (Minor Release 5.2.5, default parameter) was performed on the annotated



genes. The KEGG pathway enrichment analysis of differentially expressed genes by KAAS (v 2.1, BBH) was performed with the KEGG biological pathways database (<http://www.genome.jp>) to understand the function of the differentially expressed genes. KEGG orthology (KO) analysis of unigenes was performed using the KOBAS 2.0 web server (<http://kobas.cbi.pku.edu.cn/>) (Chen et al., 2011).

2.5 LC-MS analysis of metabolites

The samples were stored at -80°C for metabolomics analysis. After the samples were thawed slowly at 4°C , 80 mg of samples were taken and added to pre-cooled methanol/acetonitrile/aqueous solution (2:2:1, V/V), quickly frozen in liquid nitrogen and ground into fine powder. After adding 1,000 μl methanol/acetonitrile/ H_2O (2:2:1, V/V/V), the mixture was centrifuged for 15 min at 14,000 g and 4°C . The supernatant was collected and dried in a vacuum centrifuge. For LC-MS analysis, the samples were dissolved in 100 μl acetonitrile/water (1:1, V/V).

LC-MS analysis was performed using a 1,290 Infinity LC UHPLC (Agilent Technologies) coupled to an AB Sciex TripleTOF 6600 quadrupole time-of-flight mass spectrometer in Shanghai Applied Protein Technology Co., Ltd, Shanghai, China. The metabolites were determined using both positive and negative mode electrospray injection (ESI). The LC flow rate was 0.3 ml/min at 25°C . Hydrophilic interaction chromatography (HILIC) separation was performed using a 2.1 mm \times 100 mm ACQUITY UPLC BEH 1.7 μm column (Waters, Ireland). The mobile phase included 25 mM ammonium acetate and 25 mM ammonium hydroxide in water (solvent A) and acetonitrile (solvent B). Prior to injecting a sample, the column was equilibrated at 95% B for 5 min. The metabolites in 2 μl aliquot of a sample were eluted with 95% B for 0–0.5 min, a linear gradient to 65% B in 0.5–7 min, followed by a gradient to 40% B in 7–8 min, keeping 40%

B in 8–9 min, followed by a gradient to 95% B in 9–9.1 min, and 95% B maintained for 9.1–12 min. During the analysis process, the samples were placed in a 4°C automatic injector and analyzed continuously in random order.

The ESI source conditions were set as follows: ion source gas1 as 60, gas2 as 60, curtain gas 30, source temperature 600°C , and ion spray voltage floating $\pm 5,500$ V. In the MS-only acquisition, spectra were acquired over the m/z range of 60–1,000 Da, and the accumulation time for a TOF MS scan was 0.20 s/spectra. In the auto MS/MS acquisition, spectra were acquired over the m/z range of 25–1,000 Da, and the accumulation time for a product ion scan was 0.05 s/spectra. The product ion spectra were acquired using information-dependent acquisition (IDA) with high sensitivity mode. The parameters were set as follows: the collision energy was fixed at 35 V with ± 15 eV, declustering potential was 60 V in the positive mode and -60 V in the negative mode, isotopes within 4 Da were excluded, and 10 candidate ions per cycle were monitored. After preprocessing the original data, peak alignment, peak extraction, normalization, deconvolution, and compound identification were done using XCMS software (Smith and Want, 2006).

2.6 Metabolite data analysis

Differentially expressed metabolites were determined using a t -test, fold change (FC) analysis, and partial least squares discriminant analysis (PLS-DA) (Chen et al., 2022b). Differences in metabolomes were visualized using principal component analysis (PCA). In this study, the ion identification data in positive mode were selected. Metabolites were considered differentially expressed metabolites (DEMs) when variable importance of projection (VIP) in the PLS-DA model ≥ 1 , fold-change ≥ 1.5 or ≤ 0.67 , and p -value < 0.05 . In this study, the KEGG database was used to annotate differential metabolites and classify their metabolic pathways.

2.7 qRT-PCR validation of RNA-Seq data

To validate the reliability of the RNA-seq data, the expression levels of 19 differentially expressed genes were assessed using quantitative real-time PCR (qRT-PCR) on a CFX96 Real-Time System (Bio-Rad, California, USA) with SYBR green as the fluorescent dye according to the manufacturer's protocol (Sharma, 2016). Real-Time PCR System with a final volume of 10 μ l containing 3 μ l of cDNA template, 5 μ l of SsoFast™ EvaGreen® Supermix (Bio-Rad, USA), 0.2 μ l of each forward and reverse primer (Supplementary Table S1), and 1.6 μ l of RNase-free water. The qRT-PCR reaction procedure: 94°C 5 min; 94°C 1 min, 53°C 1 min, 72°C 1 min, 40 cycles; 72°C 10 min. The validation included three biological replicates with three technical replicates and ITS as the internal control gene. Genes were considered differentially expressed at $p < 0.05$.

2.8 Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics version 26 (IBM Corporation, New York, USA). Data were presented as mean \pm standard deviation (SD). The significant differences among groups were analyzed using one-way ANOVA and the Duncan test to determine the significant differences based on $p < 0.05$.

3 Results

3.1 Effect of pH on strain growth

Different pH conditions (pH 5, pH 7, and pH 9) affected the growth of *A. niger* SICU-33; the biomass of the strains increased first and then decreased in the liquid medium. It can be seen from Table 1 that the strain grew well at pH 7. Both pH 5 and pH 9 led to the reduction of mycelial biomass and inhibited the growth of *A. niger* SICU-33; pH 7 was the most favorable culture condition for this strain. In addition, the polysaccharide content of *A. niger* strain SICU-33 was the highest at pH 7 (Table 1).

3.2 Gene-expression description under different pH

After Q20 screening, \sim 94% of the reads in the cDNA libraries per sample passed the quality filtering, and close to 98% of the quality filtered reads were mapped to *Aspergillus niger* SICU_33.scaffolds.fna reference genome (Table 2). Approximately 97.57%–97.87% of clean reads were mapped to the reference genome of *A. niger*, 3.14%–7.91% of reads were mapped to multiple positions, and 78.20%–81.11% were mapped to gene, more than 98% of those to exons (Table 2). After different pH treatments, the gene expression density map of *A. niger* SICU-33 showed similar trends in gene abundance and gene expression density (Figure 2A). In addition, PCA analysis (Figure 2B) and correlation heat map (Figure 2C) also showed that there were similarities between repeats of the same treatment and obvious

TABLE 1 The mycelium biomass, growth rate, and polysaccharide content of the *Aspergillus niger* under different pH conditions (pH 5, pH 7, and pH 9).

Sample	Mycelium biomass (g)	Polysaccharide (%)
pH 5	2.93 \pm 0.03b	2.58 \pm 0.32c
pH 7	3.67 \pm 0.02a	3.49 \pm 0.20a
pH 9	3.28 \pm 0.01a	3.05 \pm 0.15b

Data are presented as average \pm SD (n = 3). Different superscript letters a, b, and c in the same row denote statistically significant differences ($p < 0.05$).

differences between treatments, suggesting that the data could be analyzed for differential expression.

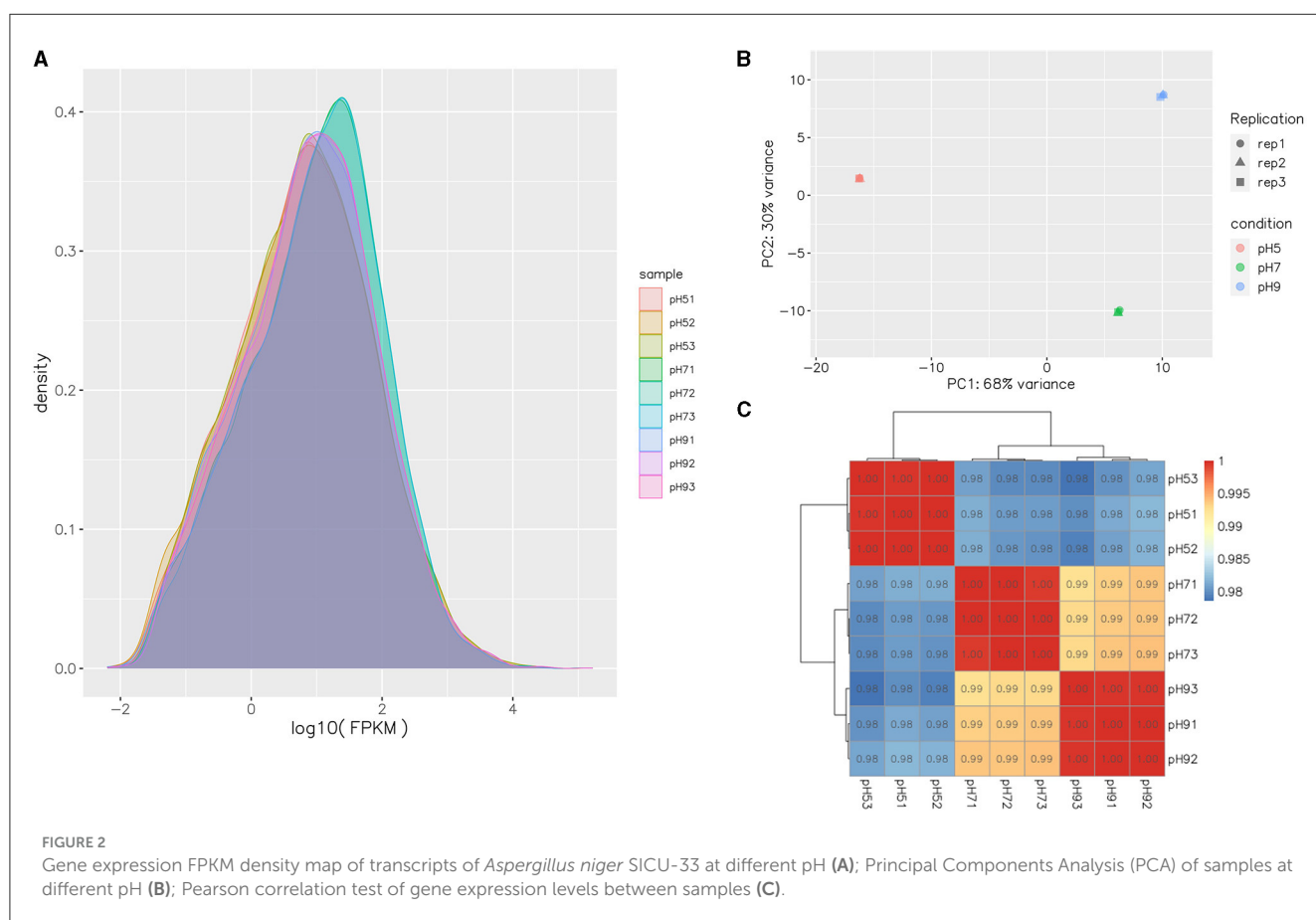
The expression of genes identified in all three groups of *A. niger* SICU-33 was normalized based on the group of pH 7, with the DEGs identified based on $|\log_2(\text{Fold Change})| \geq 1$ and $p < 0.05$ (Figure 3A). Among the differentially expressed genes (DEGs), 1,277 were upregulated, 1,239 were downregulated in the pH 5 medium compared with pH 7 (Figure 3C), 930 were upregulated, and 835 downregulated at pH 9 compared with pH 7 (Figure 3B), and a total of 2,897 DEGs (1,458 upregulated and 1,399 downregulated) were identified between the pH 5 and pH 9 (Figure 3D). A large number of identified DEGs indicated that the gene expression of *A. niger* SICU-33 changed significantly with pH adjustment.

3.3 KEGG and GO enrichment analysis of DEGs

In the KEGG enrichment analysis, the DEGs were assigned to 109 pathways at pH 5 compared with pH 7, and the DEGs were assigned to 105 pathways at pH 9 compared with pH 7 (Supplementary Table S2). KEGG pathways with significant enrichment ($p < 0.05$) of differentially expressed genes mainly include translation, amino acid metabolism, replication and repair, carbohydrate metabolism, amino acid metabolism, and metabolism of cofactors and vitamins in pH 9 compared with pH 7. The translation, replication and repair, folding, sorting and degradation, amino acid metabolism, glycan biosynthesis and metabolism, carbohydrate metabolism, transport and catabolism, and metabolism of other amino acids were enriched in pH 5 compared with pH 7 (Supplementary Table S2). The ribosome biogenesis in the eukaryotes pathway (ang03008) was the highest enriched at pH 9 and contained 27 downregulated DEGs (Figure 4A, Supplementary Table S2), where the *scaffold1.g1067* gene encodes an XRN1 protein with a domain similar to the 5'-3' exoribonuclease and belongs to the exodeoxyribonucleases producing 5'-phosphomonoesters family; the *scaffold4.g566* encodes the RRM_SF protein and belongs to the RNA recognition motif (RRM) superfamily, the *scaffold6.g396* encodes the RIO1_euk protein, which belongs to the catalytic domain of the atypical protein serine kinase. There were 13 downregulated DEGs related to the biosynthesis and metabolism of glycan in pH 5 compared with pH 7, and these genes code for enzymes involved in carbohydrate metabolism, i.e., GH 47 (EC 3.2.1.24), GH 63N (EC 3.2.1.106), and ribophorin_I (EC:2.4.1.119). The

TABLE 2 Statistics of transcriptome sequencing of *Aspergillus niger* SICU-33 under different pH, i.e., pH 5, pH 7, and pH 9, respectively.

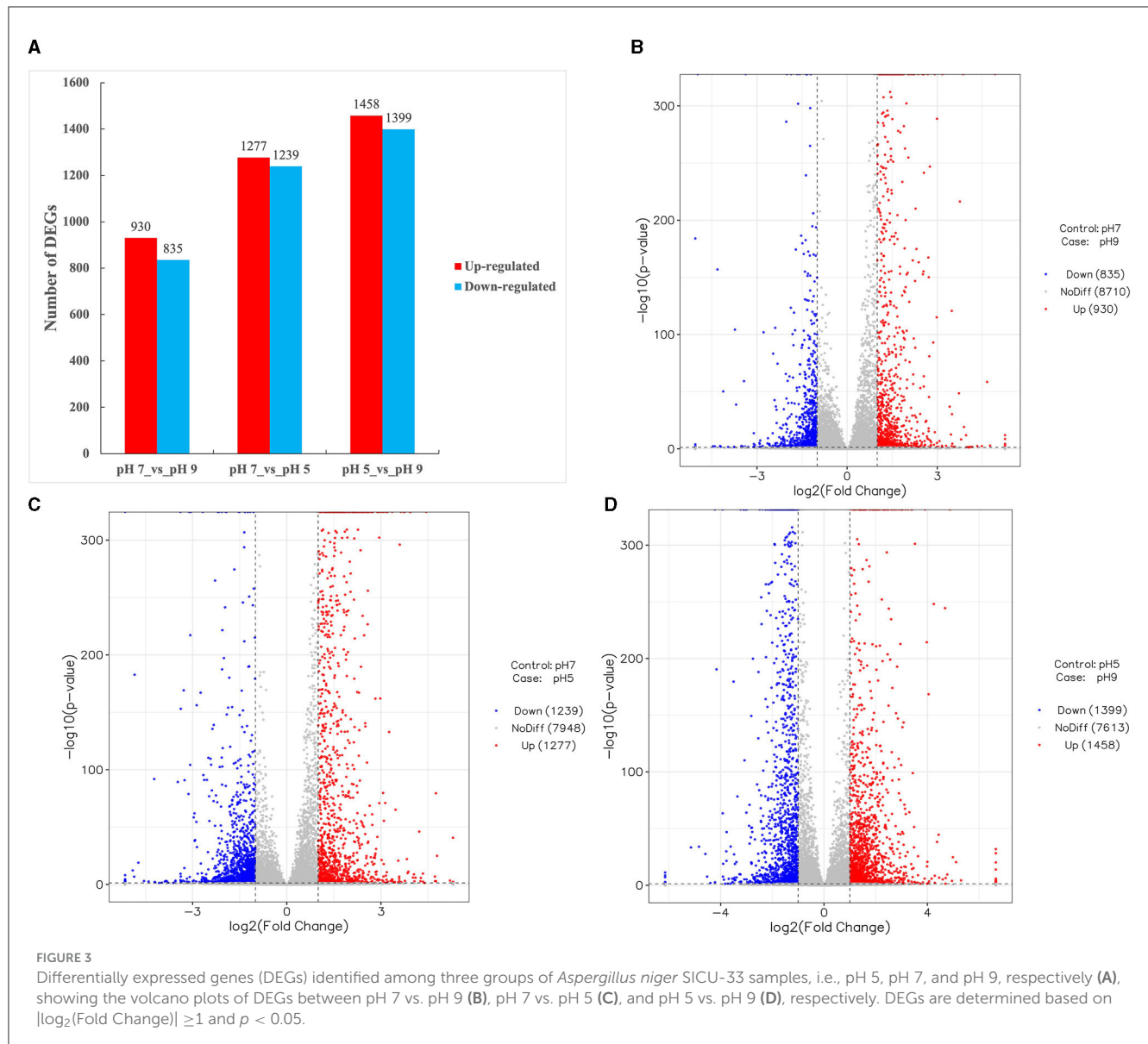
Sample	Raw reads	Clean reads	Clean reads (%)	Total mapped	Multiple mapped	Mapped to gene	Mapped to exons
pH51	48,681,446	45,469,224	93.40%	44,468,066 (97.80%)	3.14%	81.11%	98.75%
pH52	55,126,132	51,699,122	93.78%	50,444,361 (97.57%)	3.64%	81.09%	98.77%
pH53	47,869,964	44,809,794	93.60%	43,765,568 (97.67%)	3.17%	80.87%	98.75%
pH71	46,105,010	43,238,062	93.78%	42,309,522 (97.85%)	3.42%	80.65%	98.91%
pH72	44,174,234	41,511,620	93.97%	40,624,565 (97.86%)	3.71%	80.37%	98.91%
pH73	53,079,524	49,760,440	93.74%	48,639,252 (97.75%)	3.87%	80.28%	98.90%
pH91	56,249,262	52,603,422	93.51%	51,435,628 (97.78%)	7.91%	78.20%	98.88%
pH92	43,841,744	41,169,696	93.90%	40,293,614 (97.87%)	6.78%	78.95%	98.88%
pH93	51,008,940	47,931,428	93.96%	46,900,660 (97.85%)	7.75%	78.96%	98.88%



pathways associated with genetic information processing included the ribosome (ang03010), which contained 66 upregulated DEGs in pH 5 compared with pH 7 (Figure 4B, Supplementary Table S2). Among them, *scaffold1.g1336* (CAK45721.1), *scaffold1.g820* (CAK37009.1), *scaffold1.g333* (CAK43220.1), *scaffold8.g739* (CAL00567.1), *scaffold2.g1279* (XP_020061647.1), and *scaffold4.g1193* (CAK38984.1) encodes different types of ribosomal protein, which plays an important role in the cellular ribosome and participates in protein synthesis.

The functions of DEGs were further explored using GO annotation analysis, and the results showed that the top

three GO terms with the highest number of DEGs were the heterocycle metabolic process (GO:0046483; 115 upregulated and 234 downregulated), nucleus (GO:0090304; 69 upregulated and 192 downregulated), and nucleic acid metabolic process (GO:0005634; 61 upregulated and 175 downregulated) in pH 9 vs. pH7 (Figure 5A, Supplementary Table S3). In addition, DEGs in the following three GO terms of ribosomal large subunit biogenesis, preribosome, and preribosome, large subunit precursor were only downregulated, including six identical genes (*scaffold1.g583*, *scaffold2.g729*, *scaffold4.g751*, *scaffold5.g785*, *scaffold6.g295*, and *scaffold6.g908*) that appear in



all above three GO terms (Supplementary Table S3). Moreover, we found that these six genes were involved in ribosome biosynthesis, and they encoded related proteins or provided precursor substances such as *erb1* protein (A2QPZ4.1), *brx1* protein (GAQ33823.1), and *MAK16* (CAL00962.1), etc. In pH 5 vs. pH 7, GO terms such as ribosome (GO:0005840), cytosolic ribosome (GO:0005840), cytosolic small ribosomal subunit (GO:0022627), small ribosomal subunit (GO:0015935), and ribosomal subunit (GO:0044391), including 158 DEGs were upregulated, indicating that ribosome metabolism of *Aspergillus niger* was more active at pH 5 (Figure 5B, Supplementary Table S3). Twelve genes appear together in all five of the above GO terms, and these genes also encode different types of ribosomal proteins similar to the KEGG results, i.e., 40S ribosomal protein S24 (CAK40980.1), 60S ribosomal protein L8 (CAK45721.1), and 40S ribosomal protein S7 (CAL00638.1).

3.4 qPCR validation of DEGs

The expression levels of 19 DEGs were randomly validated for real-time qPCR analysis. Among them, 17 genes passed the differential abundance criteria in both RNAseq and qPCR analysis ($p < 0.05$, Table 3), and the melting curve of the qPCR experiment is shown in Supplementary Figure S1: in pH 5 vs. pH 7, two out of seven genes assigned to the GO term are integral components of ribosome metabolism, three out of seven genes assigned to the GO term are related to carbohydrate metabolic processes, and three genes assigned to the GO term double-strand break repair via homologous recombination; In pH 9 vs. pH 7, five out of 10 genes assigned to the GO terms are related to nucleic acid metabolic process, and the remaining four genes are assigned to the GO terms with carbohydrate metabolic process. Specifically, the qPCR results revealed three genes, including *Scaffod1.g1067* (XRN1), *Scaffod4.g566* (RRM), and *Scaffod6.g396* (RIO1) were significantly

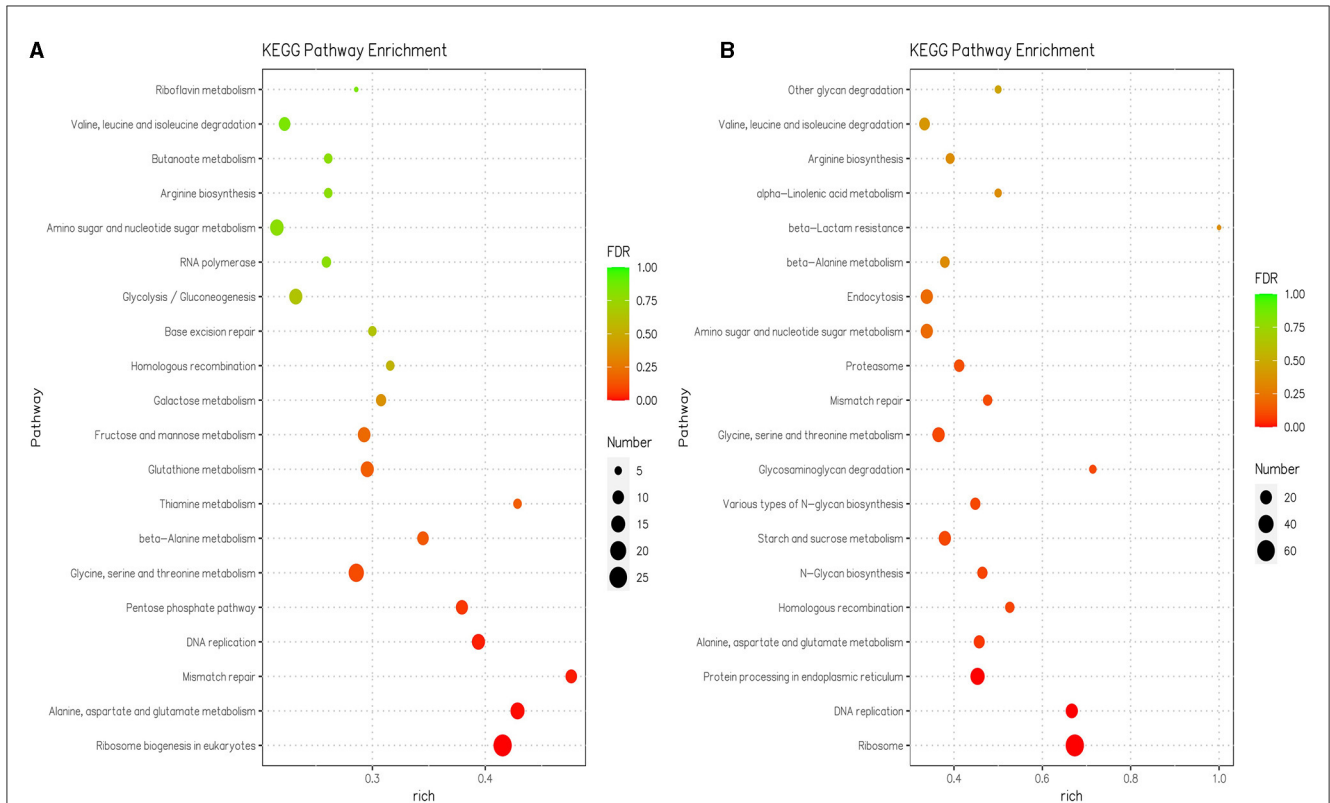


FIGURE 4 The 20 most significant KEGG pathways in the KEGG enrichment analysis were in pH 9 vs. pH 7 (A) and pH 5 vs. pH 7 (B) of *Aspergillus niger* SICU-33, respectively. Genes were considered differentially expressed when fold change (FC) ≥ 1.5 or ≤ 0.667 and $p < 0.05$.

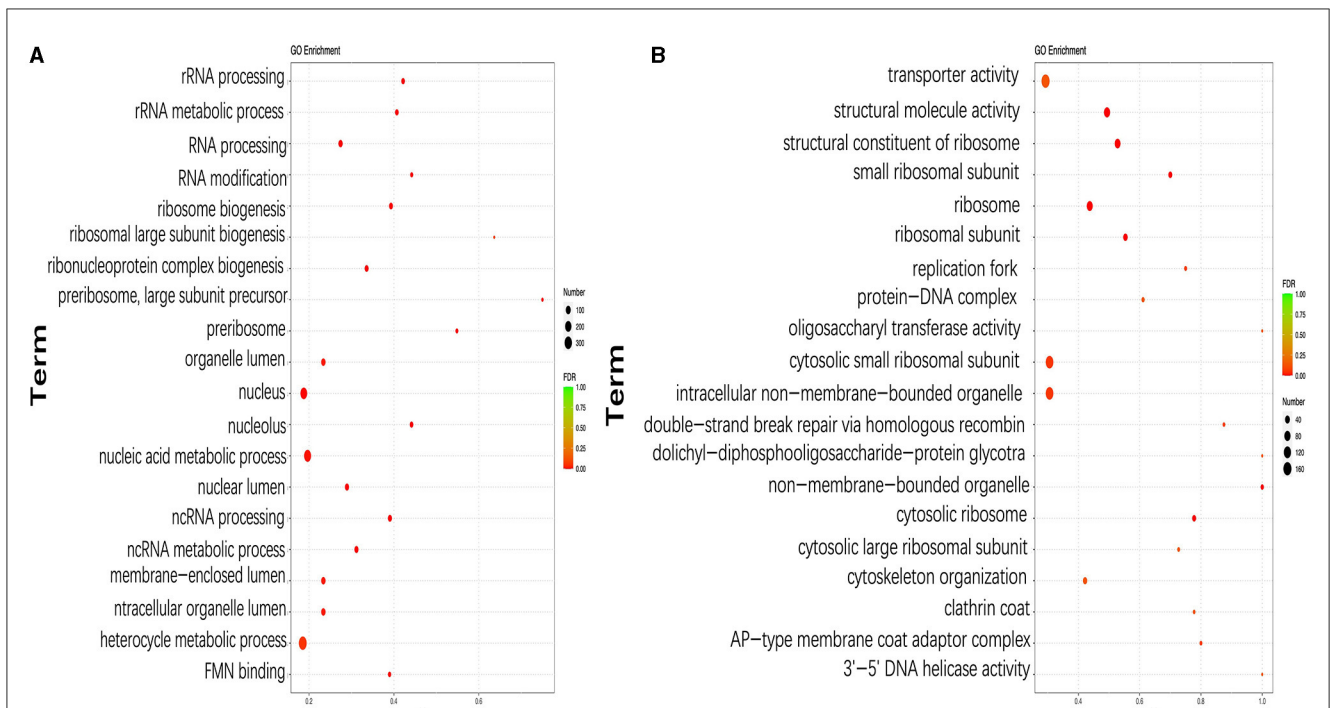


FIGURE 5 Top 20 most significantly enriched GO terms based on GO annotation of differentially expressed genes (DEGs) in pH 9 vs. pH 7 (A), pH 5 vs. pH 7 (B) of *Aspergillus niger* SICU-33. Genes were considered differentially expressed when fold change (FC) ≥ 1.5 or ≤ 0.667 and $p < 0.05$.

TABLE 3 Validation by qRT-PCR of a total of 19 differentially expressed genes (DEGs) identified by transcriptome analysis of *Aspergillus niger* under group pH 5 vs. pH 7 and pH 9 vs. pH 7.

Group	GO term	Gene ID	Gene name	RNA seq		qPCR	
				log ₂ (FC)	p-value	log ₂ (FC)	p-value
pH 5 vs. pH 7	Structural constituent of ribosome	<i>Scaffold8.g739</i>	<i>An17g02240</i>	1.53	0.00*	1.68	0.04
	Ribosome	<i>Scaffold2.g820</i>	<i>An01g04740</i>	1.91	0.00*	1.87	0.03
	Carbohydrate metabolic process	<i>Scaffold6.g1141</i>	<i>ostA</i>	-1.55	0.00*	-1.59	0.01
		<i>Scaffold1.g1425</i>	<i>An06g01100</i>	-1.41	0.00*	-2.79	0.11
		<i>Scaffold7.g486</i>	<i>An09g05880</i>	-1.44	0.00*	-1.94	0.02
		<i>Scaffold8.g662</i>	<i>An16g08570</i>	-2.41	0.00*	-2.03	0.01
	Double-strand break repair via homologous recombination	<i>Scaffold7.g389</i>	<i>An09g04640</i>	-1.59	0.00*	-1.42	0.03
		<i>Scaffold2.g970</i>	<i>An01g02720</i>	-1.45	0.00*	-1.51	0.01
		<i>Scaffold5.g1219</i>	<i>An15g07150</i>	-1.09	0.00*	-1.22	0.01
pH 9 vs. pH 7	Nucleic acid metabolic process	<i>Scaffold7.g1014</i>	<i>An14g05390</i>	-1.11	0.00*	-1.18	0.03
	Nucleic acid binding	<i>Scaffold3.g363</i>	<i>ANOM_001814</i>	-1.28	0.00*	-0.96	0.02
	ncRNA processing	<i>Scaffold1.g1067</i>	<i>An08g06790</i>	-1.09	0.00*	-1.27	0.01
	Nucleoside phosphate binding	<i>Scaffold4.g566</i>	<i>An04g00570</i>	-1.30	0.00*	-1.15	0.03
	Glucose metabolic process	<i>Scaffold4.g953</i>	<i>An04g05300</i>	1.81	0.00*	1.64	0.03
	Ribonucleotide binding	<i>Scaffold6.g396</i>	<i>An02g04830</i>	-1.45	0.00*	-1.83	0.02
	Carbohydrate metabolic process	<i>Scaffold6.g249</i>	<i>An02g02930</i>	1.88	0.00*	2.92	0.14
		<i>Scaffold9.g46</i>	<i>ANOM_009576</i>	1.36	0.00*	1.73	0.02
	Polysaccharide catabolic process	<i>Scaffold4.g1067</i>	<i>aglU</i>	1.16	0.00*	1.44	0.01
Ribose phosphate metabolic process	<i>Scaffold6.g1128</i>	<i>hxx</i>	1.77	0.00*	1.59	0.01	

Two genes not verified by RT-qPCR are highlighted in bold. * denote statistically significant differences ($P < 0.05$).

downregulated at pH 9 relative to pH 7 (Supplementary Figure S2), which was consistent with the transcriptomic data.

3.5 DEMs description of *Aspergillus niger*

In our research, 348 metabolites were identified after the combination of positive and negative ion modes, and the proportion of various metabolites is shown in Figure 6A and Table 4, among which 236 metabolites were identified under the positive ion mode. In particular, the metabolites related to organic acids and derivatives accounted for the highest proportion of 16.954% (Figure 6A). PCA analysis (Figure 6B) and total ion chromatogram (TIC, Figure 6C) in positive ion patterns were performed to analyze the mass spectrometry data of the treatment group. The results showed that the response intensity and retention time of each chromatographic peak overlapped, and the six biological repeat data points in the treatment group could be collected together. The data points of the samples of the three varieties of the treatment groups could be clearly distinguished in space, indicating that the metabolites of each group of samples were different in terms of species, concentration, and quantity. In addition, the interpretation of the PLS-DA model was good; the samples were relatively dispersed among the groups, and the aggregation was obvious within the groups (Figures 7A, C).

The permutation test results showed that R^2 and Q^2 of the random model gradually decreased with the gradual reduction of replacement retention, indicating that the model was reasonable and effective (Figures 7B, D).

Conditions for screening differentially expressed metabolites (DEMs): (1) $VIP \geq 1$; (2) p -value < 0.05 . In two groups (pH 5 vs. pH 7 and pH 9 vs. pH 7), respectively, 19 and 20 significant DEMS were detected; most DEMS were uniquely associated with a specific pH treatment (Supplementary Table S4). Among the metabolites annotated based on chemical classification, four substances, including pargyline, trans-4-hydroxy-L-proline, ergothioneine, l-leucine, and d-ornithine, were downregulated at pH 5. Moreover, cyclohexylamine, diacetyl, and erythritol metabolites were upregulated at this pH value (Figure 8A). In pH 9 vs. pH 7, choline, trans-4-hydroxy-l-proline, and l-leucine were upregulated, but diacetyl was the opposite (Figure 8B). Interestingly, the change of pargyline at pH 9 is similar to pH 5, showing different degrees of downregulation.

Correlation analysis can help to measure the metabolic proximities of significantly different metabolites, which is conducive to further understanding the mutual regulatory relationship between metabolites in the process of biological state change. The results showed that there was a significant negative correlation between pargyline and diacetyl metabolites based on the metabolites annotated by the detection, indicating that

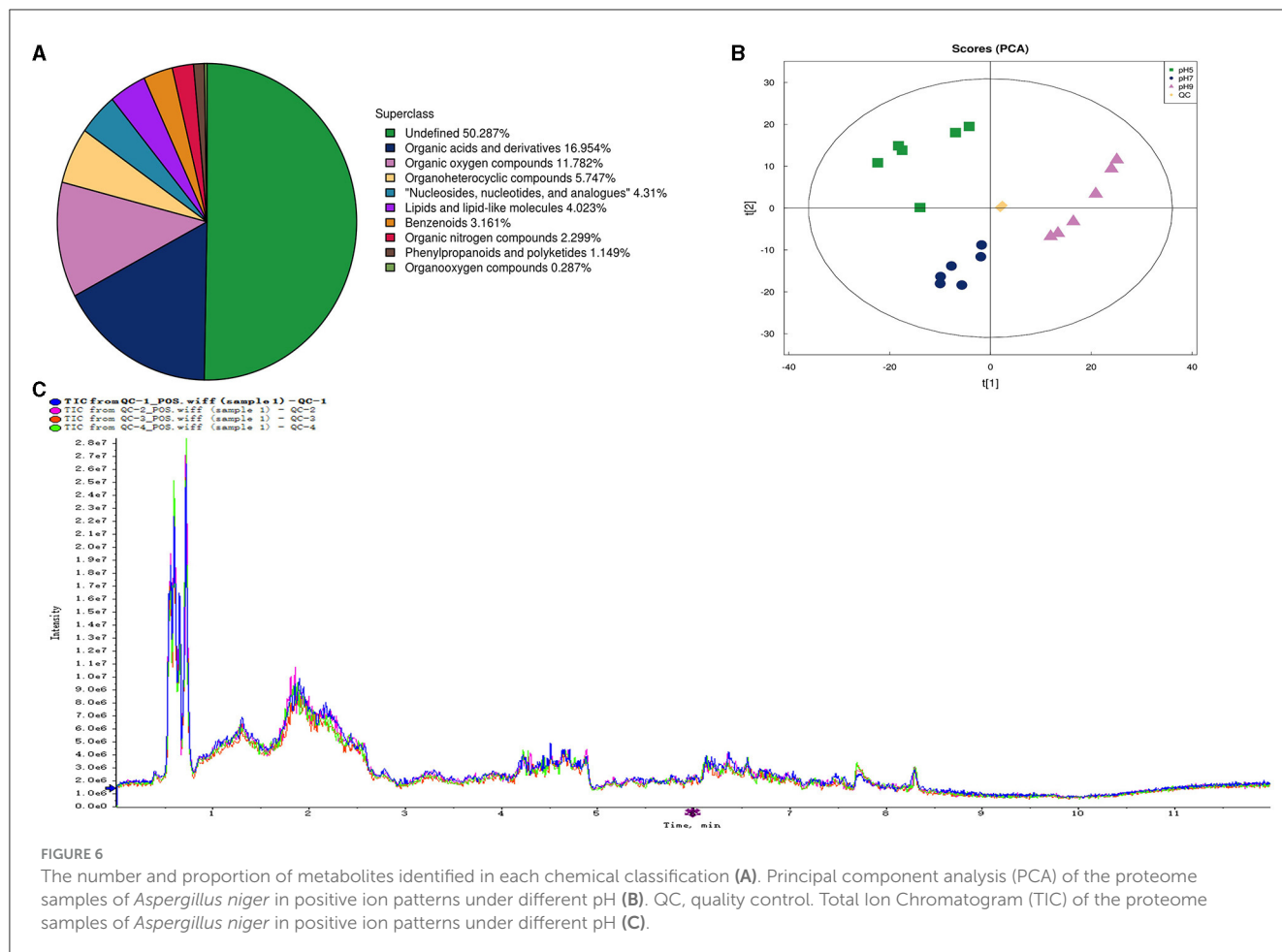


TABLE 4 The number of metabolites identified in each chemical classification.

Superclass	No. of identified metabolites
Organic acids and derivatives	59
Organic oxygen compounds	41
Organoheterocyclic compounds	20
Nucleosides, nucleotides, and analogs	15
Lipids and lipid-like molecules	14
Benzenoids	11
Organic nitrogen compounds	8
Phenylpropanoids and polyketides	4
Organoxygen compounds	1
Undefined	175

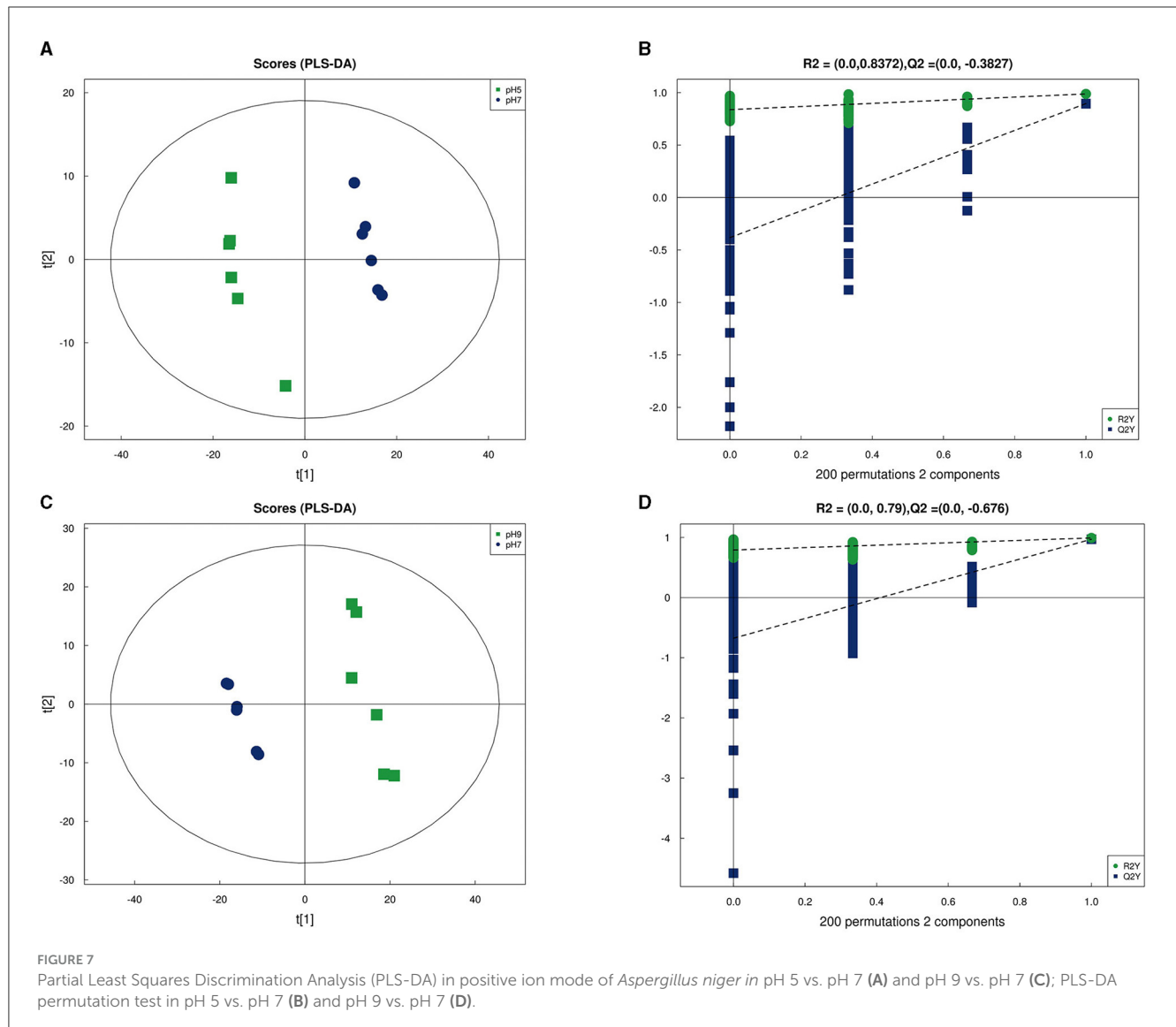
there may be a synthetic transformation relationship between the above two substances at pH 5 (Figure 9A). In pH 9 vs. pH 7, choline and trans-4-hydroxy-L-proline were significantly negatively correlated with diacetyl and pargyline, indicating that the above four metabolites may synthesize and transform each other at pH 9 (Figure 9B). The enrichment analysis of the

KEGG pathways showed that most of the DEMs were mainly enriched in carbohydrate metabolism; amino acid metabolism, biosynthesis of other secondary metabolites in pH 5 vs. pH 7, fructose and mannose metabolism (ko00051), and propanoate metabolism (ko00640) were the most significant carbohydrate metabolism pathways (Figure 9C, Supplementary Table S5). Specifically, DL-lactate was detected in two metabolic pathways associated with carbohydrates, and both were upregulated in pH 5 vs. pH 7 (Supplementary Table S6). Moreover, vitamin B6 metabolism (ko00750), beta-alanine metabolism (ko00410), and vitamin digestion and absorption (ko04977) were the top three significant metabolic pathways in pH 9 vs. pH 7 (Figure 9D, Supplementary Table S5). We also found that pyridoxal (vitamin B6) was upregulated in the corresponding metabolic process in pH 9 vs. pH 7 (Supplementary Table S6).

4 Discussion

4.1 Effects of pH change on physiological characteristics of *Aspergillus niger* SICU-33

Converting various organic and inorganic raw materials into valuable metabolites is a viable process in fungal systems, which offers great potential for the development of biotechnology production. For instance, filamentous fungi play an important

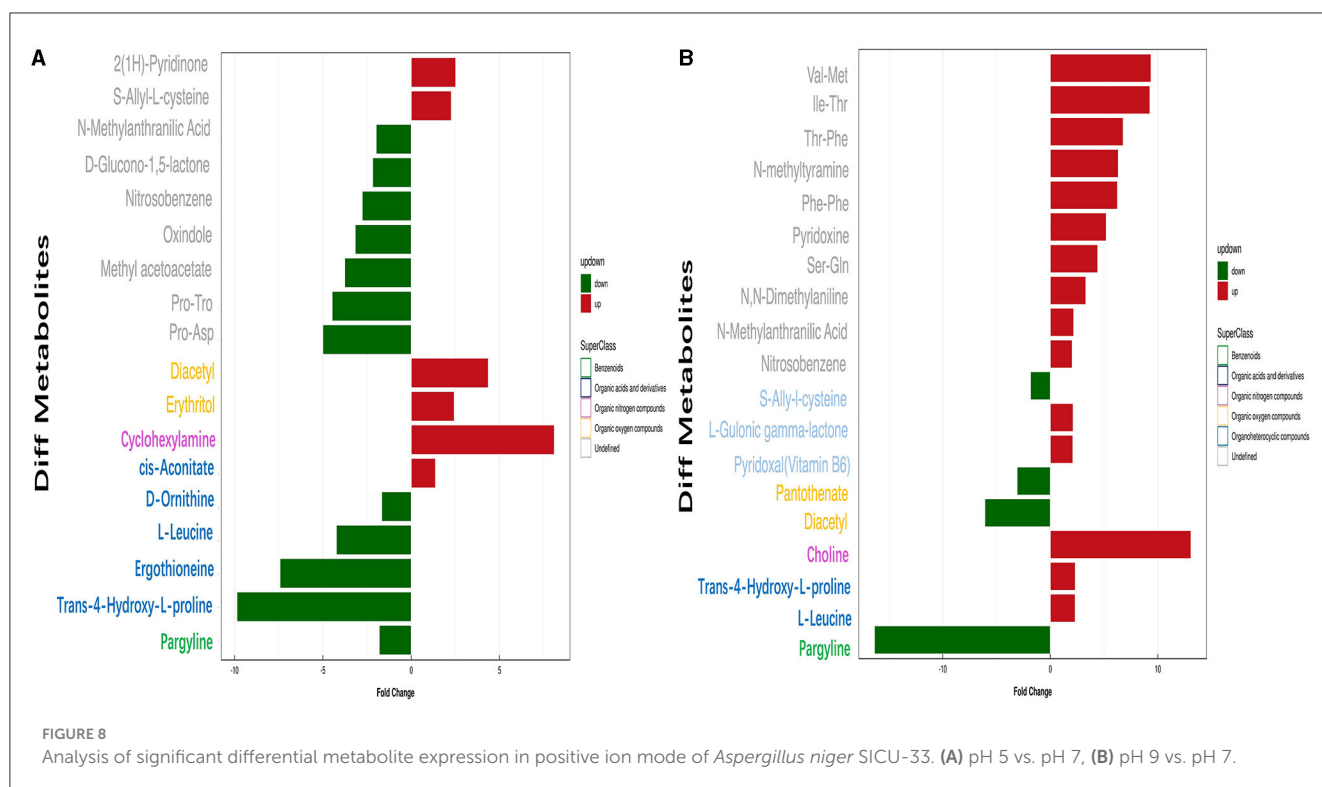


role in the biological manufacture of primary and secondary metabolites (Meyer et al., 2020; Vorapreeda et al., 2021). During the process, microorganisms optimize growth conditions for their cellular functions. Metabolic disorders, and even cell death, can be caused by changes in the external environment, such as pH (Beales, 2004; Guan and Liu, 2020). More systematic studies on *A. niger* are needed to better understand the effects of pH on mycelial growth, biological metabolism, and polysaccharide metabolism. In the present study, the effect of different ambient pH on *A. niger* growth and biological synthesis process (*in vitro*) were studied. Our results show that, at pH 5 and pH 9, *A. niger* spore germination and mycelium biomass and growth were significantly inhibited, whereas the mycelium polysaccharide content reached the highest at pH 7. It has been reported that environmental pH can affect spore germination, transcriptional expression, and metabolism-related pathways of fungi pathogens (Li et al., 2010; Walaszczyk et al., 2018; Pang et al., 2020; Jimdjio et al., 2021). Relevant studies also showed that environmental burden had significant effects on the biochemical, macroscopic,

and microscopic morphological characteristics of the strains (Šimonovičová et al., 2013). Furthermore, *A. niger* mycelium biomass and growth increased significantly at pH 7; the reason may be that strong acid or alkali conditions destroy the chromosome and protein, DNA structure, thus inhibiting the growth and metabolism of *A. niger* (Jimdjio et al., 2021). Some studies have shown that patulin was more stable at a pH range of 2.5 to 5.5 than at a more basic pH (Tannous et al., 2016). In our study, the content of polysaccharides in *A. niger* mycelium was correlated with pH. Similarly, Calvo et al. (2002) speculated that the production of aflatoxin and sterigmatocystin may be affected by the pH of the culture medium.

4.2 Ribosome biogenesis responds to pH changes of *Aspergillus niger* SICU-33

Previous studies have shown that pH value, as one of the important environmental parameters, has a crucial impact



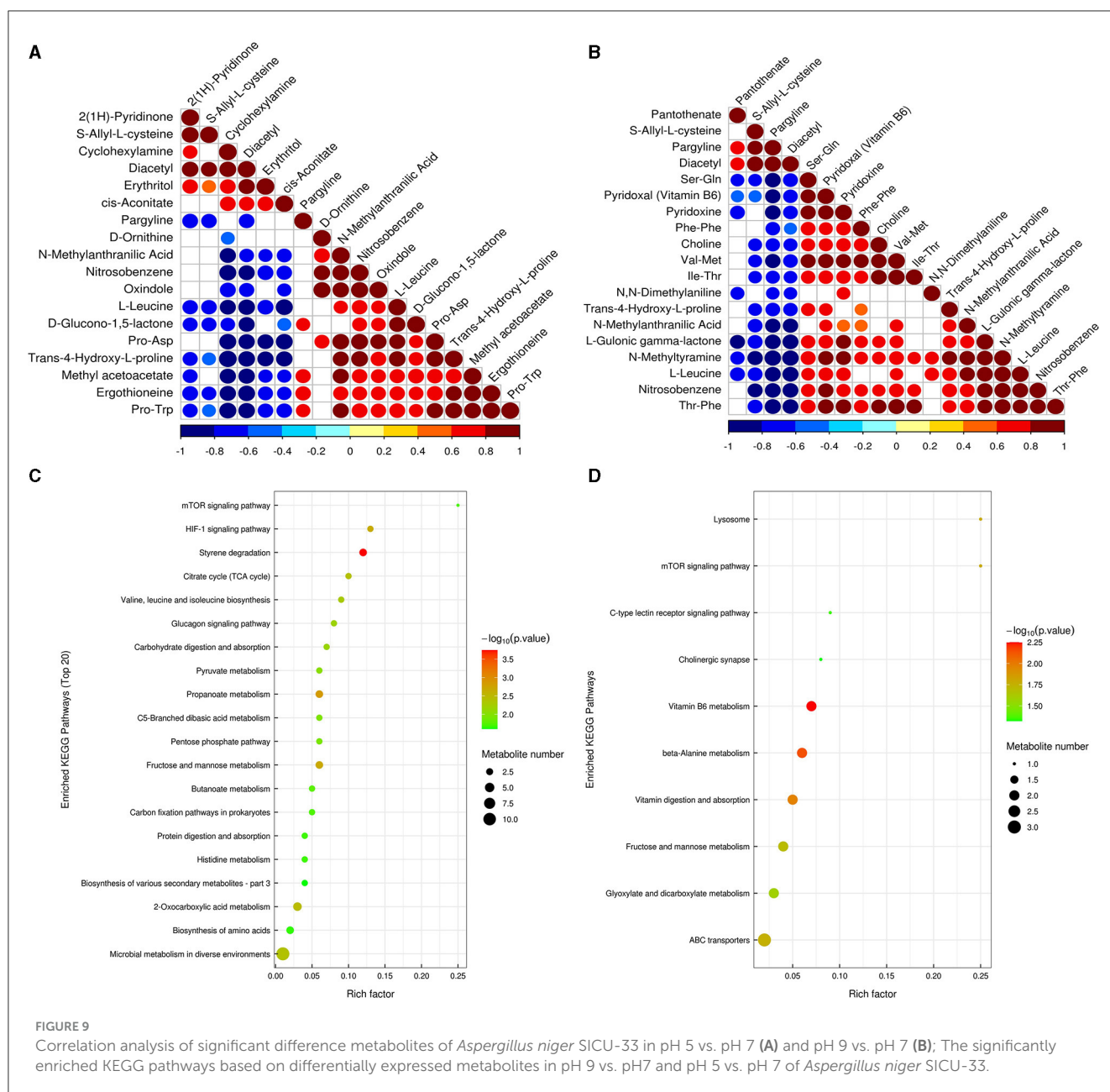
on the growth and metabolism of microorganisms (Li et al., 2010). Meanwhile, a few studies have also pointed out that some microorganisms can respond to the pH value of the growth environment and adjust the expression of genes in a certain pH range (Peñalva and Arst, 2002). For example, PacC was found to encode a transcription factor in *Aspergillus nidulans*, which showed activation under alkaline conditions and inhibition under acidic conditions, and related proteins were also confirmed to be associated with pH signal transduction and gene expression (Peñalva et al., 2008). In our study, KEGG analysis of the transcriptome showed that ribosome biogenesis in *A. niger* SICU-33 was more active at pH 9, which involved many biochemical reactions such as translation, protein modification, and carbohydrate metabolism. In particular, we found that the downregulated genes in this condition encode the XRN1 protein, RRM (RNA recognition motif) protein, and RIO1_euk protein, which is involved in translation while transmitting genetic information. Studies have shown that RRM was a very rich domain in eukaryotes and was present in proteins involved in RNA processing, export, and RNA stability; it is a key regulator of related metabolic processes in response to abiotic stress conditions in studies of plants (Ambrosone et al., 2012; Muthusamy et al., 2021).

Similarly, the zinc finger domain protein PacC in *Aspergillus nidulans* mediates the ambient pH regulatory pathway (Picazo et al., 2020); another RIO1 protein in our results is essential for eukaryotic survival and is required for 18S rRNA processing, normal cell cycle progression, and chromosome maintenance (LaRonde-LeBlanc and Wlodawer, 2005). In addition, Iacovella et al. (2018) found in their studies of *Saccharomyces cerevisiae* that the RIO1 protein directly regulates important signaling networks at the protein, chromatin, and RNA levels through a

series of regulatory factors. Therefore, we speculate that RIO1 and RRM proteins in ribosome biogenesis may have a similar role in *Aspergillus niger*, responding to changes in external pH to maintain their normal metabolic processes, and alkaline conditions cause DEGs for both two proteins to be downregulated. However, ribosome biogenesis is still active, but genes encoding ribosome-related proteins are upregulated at pH 5, which seems to differ from the conclusion of alkaline conditions. The reason for this situation may be that this part of the protein has dual functions, which are acid-expressed and alkaline-expressed genes (Espeso and Arst, 2000). Acid-expressed genes are inhibited under alkaline conditions. Still, they positively regulate related genes under acidic conditions and acid and alkaline phosphatase in organisms illustrate this opposite effect (Caddick et al., 1986).

4.3 Characteristics of DEMs of *Aspergillus niger* SICU-33

Various studies have examined the metabolic network underlying aspergillus growth and metabolism using metabolomics (Xu et al., 2018; Li et al., 2019). In our study, several metabolites were found in different pH control groups. We noted that the pargyline was significantly downregulated at both acidic and alkaline conditions, while cyclohexylamine and choline were significantly upregulated at pH 5 and pH 9, respectively. It has been reported that the pargyline belongs to an inhibitor specific to flavin-containing amine oxidases in the study of filamentous fungi; the substance inhibits the activity of flavin-containing amine oxidases (EC 1.4.3.4.) (Frébert et al., 1997), which have



been reported to be involved in oxidative metabolic processes in *Aspergillus niger* (Frederick et al., 2014). Therefore, under acidic and alkaline conditions, the downregulation of pargyline can lead to changes in the activity of flavin-containing amine oxidases, further affecting the oxidative metabolism of strain SICU-33.

Moreover, choline is negatively correlated with pargyline in alkaline conditions, indicating that there may be a relationship between the synthesis and transformation of these two substances in the metabolic process. Choline is an alkaloid that can be used as an enzyme inhibitor to combine with oxidase in cells, similar to pargyline (Luhová et al., 1996). In addition, D and L amino acids as external nitrogen sources have been shown to promote hyphal growth similar to D-ornithine and L-leucine (Mardon et al., 1969; Hara and Kino, 2020). Still, in our study, the expression of these two amino acids was downregulated by pH, indicating that the

amino acid metabolism of *A. niger* may be inhibited under acidic or alkaline culture conditions. To sum up, pH affects the dissociation state of some nutrients in the medium, the physiological function of the cell membrane, and the activities of various enzymes in the cell, and then affects the growth of microorganisms and the synthesis of products (Guan and Liu, 2020).

4.4 Characteristics of carbohydrate metabolism with pH

Carbohydrate metabolism is an important process of microbial growth and development, and the accumulation of microbial nutrients can be improved by studying this process (Zhang et al.,

2020). We found that the genes encoding GH 47, GH 63N, and ribophorin_I, which are related to the biosynthesis and metabolism of glycan, were downregulated under acidic conditions. These results suggest that acidic conditions may inhibit the expression of these genes and affect the normal carbohydrate metabolism of *Aspergillus niger*. In particular, ribophorin_I is an essential subunit of oligosaccharyltransferase (OST), which is also known as dolichyl-diphosphooligosaccharide-protein glycosyltransferase (EC: 2.4.1.119), catalyzing the transfer of oligosaccharides into the lumen of the endoplasmic reticulum (Pathak et al., 1995). In our analysis of DEMs related to carbohydrates, we found that DL-lactate was upregulated in acidic conditions, primarily in the fructose and mannose metabolism and propanoate metabolism metabolic pathways. Some studies have shown that lactic acid can inhibit the growth and metabolism of microorganisms due to its uncoupling and intracellular anion accumulation or chelation (Yáñez et al., 2008). Similarly, the accumulation of DL-lactate metabolites in our study may inhibit the growth of *Aspergillus niger*. In summary, ribophorin_I enzymes and DL-lactate may be important substances in response to pH changes during carbohydrate metabolism of *A. niger* SICU-33.

5 Conclusion

We used transcriptomic and metabolic methods to study *A. niger* cultured under different pH conditions (pH 5, pH 7, pH 9), focusing on ribosomal biogenesis and carbohydrate metabolism. The results showed that the biomass, growth rate, and polysaccharide content of *A. niger* in pH 7 were higher than those in acidic and alkaline medium. Transcriptome analysis showed that out of the 1,765 annotated genes, 450 and out of 1,516 annotated genes, 714 were differentially expressed in pH 9 vs. pH 7 and pH 5 vs. pH 9, respectively; DEGs related to ribosome biogenesis were the most abundant. The downregulated expression of genes encoding XRN1, RRM, and RIO1 affected protein translation, modification, and carbohydrate metabolism in fungi. The dynamic changes of pargyline and choline were in response to the oxidative metabolism of *A. niger*. The ribophorin_I enzymes and DL-lactate may be important substances related to pH changes during carbohydrate metabolism of *A. niger* SICU-33. In all, acidic and alkaline conditions can affect the growth and polysaccharide metabolism of *A. niger*, indicating that in the actual production and application of *A. niger*, in addition to the influence of environmental factors, we should also consider the change of related enzymes, the accumulation of enzyme inhibitors or increase the research on stress-tolerance related genes. As far as we know, this study revealed the molecular variation of *A. niger* at different pH through joint analysis of transcriptome and metabolome, providing a basic molecular mechanism for the identification of key genes of *A. niger* and further providing a strong experimental basis for the development and application of *A. niger* in agriculture.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material,

further inquiries can be directed to the corresponding author.

Author contributions

RZ: Writing – review & editing, Writing – original draft, Validation, Formal analysis. YC: Writing – review & editing, Data curation, Formal analysis. WW: Writing – review & editing, Investigation, Formal analysis, Data curation. JC: Writing – review & editing, Resources, Investigation, Data curation. DL: Writing – review & editing, Supervision, Software, Formal analysis. LZ: Writing – review & editing, Methodology, Formal analysis, Data curation. QX: Writing – review & editing, Visualization, Software, Methodology, Formal analysis. KZ: Writing – review & editing, Software, Methodology, Formal analysis. MM: Writing – review & editing, Validation, Supervision, Formal analysis, Data curation. XY: Writing – review & editing, Formal analysis, Software, Methodology. QC: Writing – review & editing, Validation, Methodology, Conceptualization. PP: Formal analysis, Writing – review & editing, Software, Methodology. YG: Software, Resources, Project administration, Methodology, Funding acquisition, Conceptualization, Writing – review & editing, Writing – original draft.

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Conflict of interest

JC and DL are employed by Liangshan Tobacco Corporation of Sichuan Province, China.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2024.1389268/full#supplementary-material>

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