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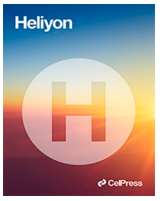

Research article

Physiological, transcriptomic, and metabolomic analyses reveal that *Pantoea* sp. YSD J2 inoculation improves the accumulation of flavonoids in *Cyperus esculentus* L. var. sativus

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ABSTRACT

Plant growth-promoting microorganisms (PGPMs), such as *Pantoea* sp. YSD J2, promote plant development and stress resistance, while their role in flavonoids accumulation still needs to be further understood. To investigate the complex flavonoid biosynthesis pathway of *Cyperus esculentus* L. var. sativus (tigernut), we compared *Pantoea* sp. YSD J2 inoculation (YSD J2) and water inoculation (CK) groups. YSD J2 significantly elevated the content of indole-3-acetic acid (IAA) and orientin. Furthermore, when analyzing flavonoid metabolome, YSD J2 caused increased levels of uralenol, petunidin-3-*O*-glucoside-5-*O*-arabinoside, luteolin-7-*O*-glucuronide-(2 → 1)-glucuronide, kaempferol-3-*O*-neohesperidoside, cyanidin-3-*O*-(2″-*O*-glucosyl)glucoside,

Abbreviations: ANOVA, One-way analysis of variance; ANR, anthocyanidin reductase; ANS, anthocyanidin synthase; BZ1, anthocyanidin 3-O-glucosyltransferase; C. esculentus L., *Cyperus esculen*tus L, var. sativus; CCoAOMT, coffee acyl coenzyme A-O-methyltransferase; CHI, chalcone isomerase; CHS, chalcone synthase; CHR, chalcone reductase; CYP71D9, flavonoid 6-hydroxylase; CYP73A, trans cinnamate 4-monooxygenase; CYP75A, flavonoid 3′,5′-hydroxylase; CYP81E, isoflavone/4′-methoxyisoflavone 2′-hydroxylase; CYP93G1, flavone synthase II; C12RT1, flavanone 7-O-glucoside 2″-O-beta-L-rhamnosyltransferase; DEGs, differentially expressed genes; DEMs, differential metabolites; DFR, bifunctional dihydroflavonol 4-reductase/flavanone 4-reductase; F3H, naringenin 3-dioxygenase; FPKM, fragments per kilobase of transcript per million mapped reads; FLS, flavonol synthase; GT1, anthocyanidin 5,3-O-glucosyltransferase; HCT, shikimate O-hydroxycinnamoyltransferase; HIDH, 2-hydroxyisoflavanone dehydratase; IAA, indole-3-acetic acid; IF7MAT, isoflavone 7-O-glucoside-6″-O-malonyltransferase; LAR, leucoanthocyanidin reductase; MDA, malondialdehyde; MRM, multiple reaction monitoring; MSEA, metabolite sets enrichment analysis; OPLS-DA, orthogonal partial least squaresdiscriminant analysis; PAL, Phenylalanine ammonia-lyase; PGPMs, Plant growth-promoting microorganisms; PTS, pterocarpan synthas; POD, peroxidase; qRT-PCR, quantitative real-time polymerase chain reaction; RNA-seq, RNA sequencing; SOD, superoxide dismutase; TIC, Total ion chromatography; TF, Transcription factor; UPLC-MS/MS, ultra-high performance liquid chromatography coupled with tandem mass spectrometry; UGT73C6, flavonol-3-O-L-rhamnoside-7-O-glucosyltransferase; VIP, variable importance in projection; 5MaT1, anthocyanin 5-O-glucoside-6‴-O-malonyltransferase.

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kaempferol-3-*O*-glucuronide-7-*O*-glucoside, quercetin-3-*O*-glucoside, luteolin-7-*O*-glucuronide-(2 → 1)-(2″-sinapoyl)glucuronide, and quercetin-4′-*O*-glucoside, which further enhanced antioxidant activity. We then performed RNA-seq and LC-MS/MS, aiming to validate key genes and related flavonoid metabolites under YSD J2 inoculation, and rebuild the gene-metabolites regulatory subnetworks. Furthermore, the expression patterns of the trans cinnamate 4-monooxygenase (*CYP73A*), flavonol-3-*O*-L-rhamnoside-7-*O*-glucosyltransferase (*UGT73C6*), shikimate *O*-hydroxycinnamoyltransferase (*HCT*), chalcone isomerase (*CHI*), flavonol synthase (*FLS*), and anthocyanidin synthase (*ANS*) genes were confirmed by qRT-PCR. Additionally, 4 transcription factors (TF) (especially bHLH34, Cluster-37505.3) under YSD J2 inoculation are also engaged in regulating flavonoid accumulation. Moreover, the current work sheds new light on studying the regulatory effect of *Pantoea* sp. YSD J2 on tigernut development and flavonoid biosynthesis.

1. Introduction

Flavonoids, derived from phenylpropanoid compounds, belong to secondary metabolites extensively discovered from plants [1]. According to the degree of oxidation of heterocycles and hydroxyl or methyl group number on benzene ring, flavonoids are categorized as the following 12 groups, including chalcones, stilbenes, aurones, flavanones, flavones, isoflavones, phlobaphenes, dihydroflavonols, flavonols, leucoanthocyanidins, proanthocyanidins, and anthocyanins [2]. Flavonoids have enormous potential in food and medicine fields due to their extensive biological activities, including antioxidant [3], anti-inflammation [4], anti-virus [5], anti-cancer [6], liver protection [7], anti diabetes [8], antidepressant [9], neuroprotective [10], and cardiovascular protection [11]. Furthermore, flavonoids have extensive distribution and vital impacts in plant growth, adaptation, signaling, as well as biotic and abiotic stress responses [12].

Cyperus esculentus L. var. sativus (tigernut) is a kind of traditional perennial C4 plant of the Cyperaceae family [13]. Tigernut contains three parts: aboveground leaves (stems), underground tubers, and roots. Previous studies have indicated that its valuable components in stems, such as lactones, flavonoids, and coumarins, along with the corresponding steroids, glycosides, cardiac glycosides, and triterpenoid [14–16], have pharmaceutical effects such as antioxidation and mitigation of cerebral ischemia injury and are expected to be new drug candidates for treating ischemic stroke [17,18]. Study of tigernut flavonoids is still in the early stages of development. Till the present, efforts to detect the flavonoid compositions of tigernut stems have increased significantly. Nevertheless, only a handful of flavonoid components (such as orientin, isoorientin, and luteolin, etc.) have been successfully detected and isolated [19,20]. Furthermore, such limitations may probably hinder the growth of tigernut utilization in the food industry and medicine field. Therefore, there is a need to obtain more information about flavonoids of tigernut to improve its utilization efficiency.

Flavonoids have broad application prospects due to their various biological activities. However, currently the acquisition of flavonoids is still limited to direct plant extraction and chemical synthesis [21,22]. Traditional chemical synthesis and plant extraction in flavonoid production have been impeded due to a slow dearth of fuel, acute scarcity of land, heightened environmental protection awareness, and rising production expenses. Despite the microbial approach to flavonoid biosynthesis being straightforward and safe, its minimal yield necessitates additional refinement and enhancement. Recently, many studies have been conducted to explore how plant growth-promoting microorganisms (PGPMs) affect the accumulation of active ingredients [23,24]. Long-term host plant-PGPMs interactions and coevolution typically boost plant growth, enhance resistance to pathogens, persistently accelerate plant active ingredient accumulation, and improve plant quality and productivity [25,26]. It has been shown that inoculating PGPMs leads to changes in plant physicochemical characteristics, such as weight, height, antioxidant enzyme activities, and various levels of functional components [27–30]. This is exemplified by using *Pantoea* sp. YSD J2, a PGPMs isolated in tigernut stem, which improved greengrocery growth arrtitubes and antioxidant activity [31]. Several studies have found that PGPMs inoculation results in the accumulation of flavonoids [32,33], and PGPMs-mediated flavonoids mechanisms were also studied [34]. Despite previous research efforts, but different interactions can occur in different plants, the interaction between tigernut and PGPMs to promote the accumulation of flavonoids and their regulatory mechanisms are yet to be explored.

Therefore, to systematically study the pathways, species, and differential genes of PGPMs-mediated secondary metabolite accumulation in medicinal plants, a PGPM, *Pantoea* sp. YSD J2, were selected for this study. *Pantoea* sp. YSD J2 showed good PGP characteristics and growth and metabolism-related abilities [31]. In the current work, flavonoid content and antioxidant activity were analyzed under *Pantoea* sp. YSD J2 inoculation. Then transcriptome combined with metabolome data were used to (1) explore the mechanism underlying gene and metabolite levels in response to *Pantoea* sp. YSD J2 inoculation; (2) screen critical regulatory genes associated with flavonoid biosynthesis pathways in response to *Pantoea* sp. YSD J2 inoculation; and (3) construct a regulatory network based on flavonoid biosynthesis as well as associated gene levels. To some extent, the obtained findings offer the experimental and theoretical foundation for investigating the *Pantoea* sp. YSD J2-mediated regulation on the growth and flavonoid biosynthesis of tigernut.

2. Materials and methods

2.1. Plant materials and treatments

Tigernut used in the present study is a round grain type variety (Zhongyousha I). Tigernut seeds were purchased in Baoding, Hebei

Province of China (115°3′11″E, 38°21′9″N). Seeds that had been sterilized (soaked in 70 % ethanol for 10 min and 5 % NaClO for 5 min) were sprouted in a clean germination container lined with cotton and filter paper. Once the seedlings sprouted their third leaf, carefully select seedlings of the same size and transplant them back into a greenhouse irrigated with tap water without any fertilizer. Tigernut samples were collected every 7 days (five for each timing). During the sampling process, the seedlings of tigernut were carefully dug out, followed by repeated washing of the roots with distilled water. The height of each plant was measured separately. All samples were cut into 5–10 cm fragments and immersed in liquid nitrogen before being sent to the laboratory, followed by preservation under −80 °C.

2.2. Orientin content measurement

Orientin was extracted according to previous description by Wang et al. [35]. Briefly, orientin content in tigernut stem was determined using a high-performance liquid chromatography (HPLC) (Agilent Company, USA). Chromatographic column was the Agilent Zorbax SB-C18 column (particle size, 5 μm; 4.6 mm × 250 mm). Solvent A (0.5 % acetic acid) and solvent B were the mobile phases (acetonitrile, Merck, Germany). Gradient elution: 0 ~ 35 min, A-B (85:15, $V{\cdot}V^{-1}$) to A-B (45:55, $V{\cdot}V^{-1}$). A 10 μL injection volume was injected at the 1.0 mL min^{-1} rate and the 340 nm UV detection wavelength.

2.3. Pot experiments and treatments using Pantoea sp. YSD J2

Strain YSD J2 is an endophytic bacterium isolated from the stem of tigernut in 2019, identified as *Pantea* sp. [31], and the Gen Bank entry number of the 16s rDNA gene is OK090942. After the surface sterilization treatment, the seeds of tigernut were immersed into distilled water for five days under ambient temperature and later positioned in the culture dish covered with filter paper for germination. Seven days later, seedlings exhibiting identical growth patterns were chosen for the pot experiment. In every pot, five seedlings received irrigation using 20 mL of bacterial suspensions at a concentration of 1.25×10^8 CFU/mL, maintained at an average temperature of 28.0 °C and 14 h/10 h light/dark cycle. Water was added every 2–3 d. Moreover, at the end of the experiment (sampling at 102 d), plants were carefully removed from each pot, indole-3-acetic acid (IAA) content was identified. IAA detection was done in line with procedure as previously described [12]. Antioxidant abilities including malondialdehyde (MDA) level, superoxide dismutase (SOD), and peroxidase (POD) activities were identified based on procedure according to the previous description [31,36].

2.4. Metabolite profiling by UPLC-MS/MS

The flavonoid metabolites of seedlings inocubated with YSD J2 and control group (CK) were measured. In this study, the ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) system (UPLC, Shim-pack SHIMADZU Nexera X2, SHIMADZU, Kyoto, Japan; MS/MS, Applied Biosystems 4500 Q TRAP, AB SCIEX, Foster City, CA, USA) was adopted for analyzing flavonoid extracts as follows: UPLC column, Agilent SB-C18 1.8 μm, 2.1 mm × 100 mm; solvent system, ultrapure water (supplemented with 0.1 % formic acid) as phase A, whereas acetonitrile (that contained 0.1 % formic acid) as phase B; gradient program, 95:5 V/V phase B at 0.00 min, 5:95 V/V at 9.00 min, 5:95 V/V at 10.0 min, 95:5 V/V at 11.1 min, and 95:5 V/V at 14 min; temperature, 40 °C; flow rate, 0.35 mL/min; injection volume: 4 μL. In addition, mass spectrometry (MS) data were analyzed as described by Zhou [37].

2.5. Metabolites qualitative and quantitative analyses

According to second-order spectral data, metabolite characterization was performed. We obtained metabolites using multiple reaction monitoring (MRM) methods following previous description [33]. After filtration, metabolite data were exposed to orthogonal partial least squares-discriminant analysis (OPLS-DA) using analyst 1.6.1 software. Subsequently, we used variable importance in the projection (VIP) parameter to check relative importance of different metabolites of OPLS-DA model. By applying R software (www.r-project.org), we analyzed metabolites from each sample using hierarchical cluster analysis. Metabolites under significant regulation in the two groups were analyzed according to the thresholds of $|\log2FC \text{ (fold change)}| \geq 1$ and VIP ≥ 1. Subsequently, based on KEGG compound database, we annotated those detected metabolites and subsequently mapped then into KEGG pathway database. Later, metabolite set enrichment analysis (MSEA) of pathways mapped by significant metabolites was conducted, and p-values from hypergeometric test were determined in order to analyze significance.

2.6. RNA-seq analysis

Total RNAs were isolated from tigernut frozen stems, followed by the construction and sequencing of the mRNA library in every sample using the Illumina HiSeq 2000 platform. Later, adapter and low-quality sequences were eliminated with Fastp under the default parameters [38] to obtain clean reads, which were later assembled in expressed sequence tag clusters (contigs) and *de novo* assembly in transcripts with the trinity. Gene and transcript expression was explored by fragments per kilobase of transcript per million mapped reads (FPKM). In addition, we also detected differentially expressed genes (DEGs) through adopting binom test, and the DESeq function estimated size factors based on the thresholds of FC > 2 or < 0.5 and $p < 0.05$. The raw sequencing data were imported into NCBI BioProject database (project number, PRJNA1003814).

2.7. Quantitative real-time PCR assay

Eight flavonoid biosynthesis-related DEGs (*CYP73A* (Cluster-49537.1), *CHI* (Cluster-37365.0 and Cluster-50548.7), *ANS* (Cluster-49536.4 and Cluster-43695.0), *FLS* (Cluster-25595.0), *HCT*(Cluster-44099.0), and *UGT73C6* (Cluster-51835.5)) were chosen for further verification using qRT-PCR. To this end, we extracted total RNA from tigernut stems following the instructions of the manufacturer (Magen kit, China). cDNA was prepared by total RNA by adopting Thermo Scientific Maxima RT reagent Kit (Thermo Scientific, MA, USA) through reverse transcription. qRT-PCR was conducted with 2X SG Fast qPCR Master Mix (High Rox, BBI, China) using the QuantStudio™ 1 Plus System (Thermo Scientific, MA, USA). In addition, three biological and three technical replicates were used. Table S1 displays the primers used in this study. To be specific, a 20 μL system was prepared for qRT-PCR, containing 10 μL SYBR qPCR Master Mix (2X), 0.4 μL of respective primers (10 μM each), 7.2 μL ddH_2O and 2 μL cDNA template (5 ng/μL). PCR conditions were 3-min denaturation at 95 °C, 15-s amplification at 95 °C, and 30-s at 60 °C for 45 cycles. Then, melting curve measurements were carried out for 15s at 95 °C and 1min at 60 °C, using the 18S rRNA gene being the reference gene. Each sample was analyzed in triplicate. The $2^{-\triangle\triangle Ct}$ approach was adopted for analyzing the quantitative results.

2.8. Statistical analysis

Data were subjected to calculated mean ± standard deviation. Origin 2022 was applied to draw curves and histograms. Statistical analyses were conducted using the SPSS 22.0 software (IBM, Chicago, IL, USA). Differences between samples were determined by one-way analysis of variance (ANOVA) and significant differences were calculated by Duncan's multiple range test at $p < 0.05$.

3. Results

3.1. Improved growth in tigernut inoculated with Pantoea sp. YSD J2

To explore how *Pantoea* sp.YSD J2 might influence flavonoid production in tigernut, both non-inoculated *Pantoea* sp.YSD J2 (CK) and inoculated *Pantoea* sp.YSD J2 (YSD J2) were chosen as experimental materials. Initially, the growth statement and flavonoid level of tigernut without YSD J2-inoculation (CK) was examined to determine the time point at which the maximum amount of flavonoid

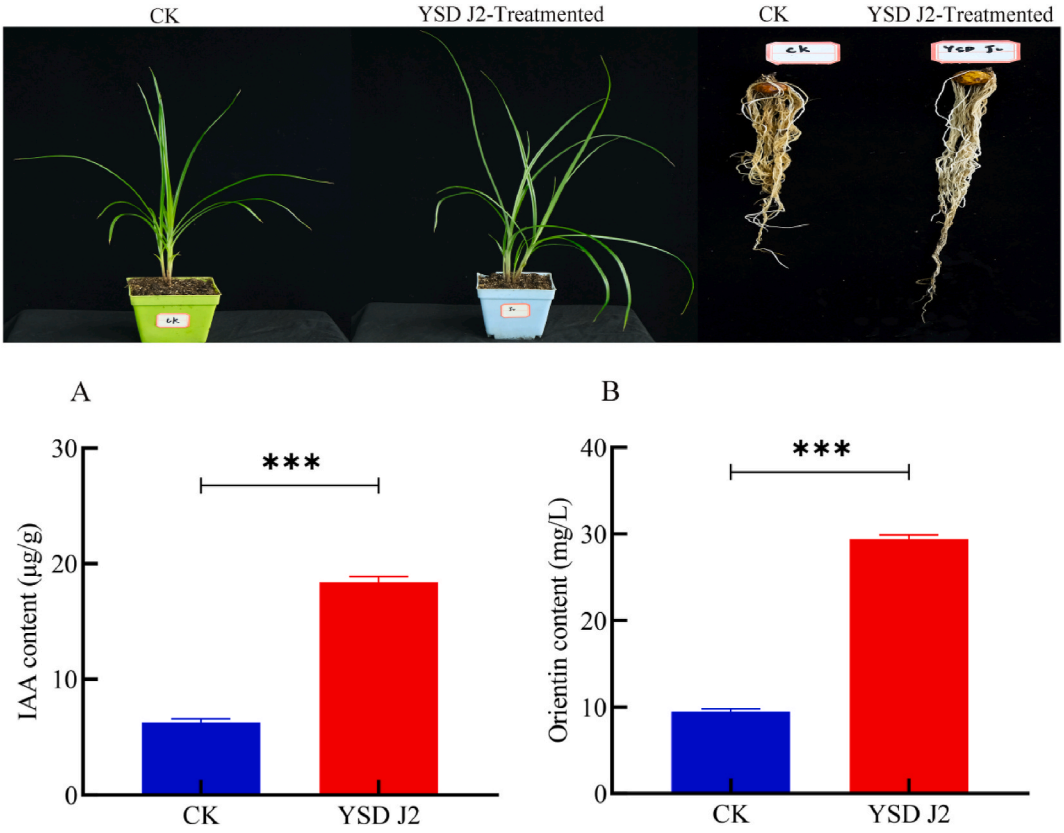

Fig. 1. Phenotype, IAA content, and orientin content of tigernut in response to *Pantoea* sp. YSD J2 inoculation (YSD J2). Values are means ± SD (n = 3). CK is non bacterial inoculated plants. * or ** indicates a significant difference at the 0.05 or 0.01 level, respectively.

synthesized. The present study first analyzed plant growth attributes and orientin level of tigernut at different growth stages (Table S2). As a result, plant height, plant fresh weight, and orientin content of tigernut increased over time. At 102 d, the plant height and plant fresh weight reached 125.80 ± 4.98 cm and 131.40 ± 6.53 g, respectively, and orientin content was 11.07 ± 0.34 mg L^{-1}. From 102 d to 127 d, the orientin content initially decreased and then increased with time. At 127 d, the orientin content reached 8.18 ± 1.20 mg L^{-1}, and the plant height reached the maximum of 131.76 ± 16.33 cm. From 127 d onward, the two indicators remained over time. The plant fresh weight showed a stable upward trend after 102 days and reached the maximum of 145.00 ± 5.70 g at 151 d. Therefore, based on the results of three indicators, 102 nd day was finally determined as the sampling time points of tigernut for exploring how YSD J2 affected flavonoid synthesis of tigernut.

The IAA content, orientin content, and antioxidant activities of tigernut with YSD J2 were tested. YSD J2 elevated the levels of IAA and orientin by 65.74 % (Fig. 1A) and 212.53 % (Fig. 1B) compared to CK, respectively. Additionally, YSD J2 exhibits a significant reduction in MDA levels (Fig. 2C), alongside a marked rise in POD (Fig. 2B) and SOD activities (Fig. 2A) compared to CK. These three enzyme families exhibited patterns akin to those of IAA and orientin, indicating that the elevation in orientin mediated by YSD J2 may be engaged in enhancing the antioxidant activity of tigernut.

3.2. Metabolomic study of tigernut inoculated with Pantoea sp. YSD J2

It has been reported that orientin is the main flavonoid of tigernut [20]. To futher explore the effect of YSD J2 on flavonoid synthesis and possible mechanisms of tigernut, we carried out a widely targeted flavonoids metabolic analysis. Totally 301 flavonoids metabolites were detected by UPLC-MS/MS in tigernut (Table S3). In accordance with the hierarchical cluster analysis (Fig. 3), YSD J2 was clustered into diverse branches compared with CK, and flavonoid distribution and levels in YSD J2 vs. CK groups was also significantly different. Besides, YSD J2 contained significantly accumulated flavonoids (such as naringenin chalcone, naringenin, homoeriodictyol, and orientin, etc.) (Table S3). And the detected DEMs mainly contained flavones and flavonols (Table S4).

Most DEMs displayed significant accumulation in the tigernut incobulated with YSD J2, 55 DEMs were upregulated, and 14 DEMs were notably downregulated in the YSD J2 vs. CK groups (Table S3). Furthermore, cyanidin-3-O-(2″-O-glucosyl) glucoside was the metabolite with the maximum upregulated level, with log2FC value reaching 12.27, and the downregulated expression metabolite was 6-prenylnaringenin, reached −2.47 (Fig. 4A). Additionally, the metabolites in the YSD J2 vs. CK groups was enriched by KEGG, as seen in Fig. 4B, the changes in metabolites treated by YSD J2 mainly concerned these pathways: secondary metabolites biosynthesis, flavonoid biosynthesis, flavone and flavonol biosynthesis. According to the above findings, the unique distribution and accumulation patterns of the above DEMs were the primary factors leading to different flavonoids of tigernut in response to YSD J2 inoculation.

3.3. Correlation analysis in IAA, DEMs and antioxidant activity

The correlations in the IAA content, antioxidant activity indicators (SOD, POD and MDA) of tigernut stems and DEMs in YSD J2 vs. CK groups were explored. The findings demonstrated that the 19 DEMs in tigernut were significantly correlated with IAA and enzymes families ($p < 0.05$). Moreover, those 19 most significant flavonoids were further analyzed, which indicated that uralenol, petunidin-3-O-glucoside-5-O-arabinoside, luteolin-7-O-glucuronide-(2 → 1)-glucuronide, kaempferol-3-O-neohesperidoside, cyanidin-3-O-(2″-O-glucosyl)glucoside, kaempferol-3-O-glucuronide-7-O-glucoside, quercetin-3-O-glucoside, luteolin-7-O-glucuronide-(2 → 1)-(2″-sinapoyl)glucuronide, and quercetin-4′-O-glucoside were positively related to the decrease of MDA content, increase of POD and SOD activities (Fig. 5, $p < 0.01$). Accumulation of nine flavonoids (five flavonols, two flavones, and two anthocyanidins) with YSD J2 inoculation is a crucial factor in enhancing the antioxidant ability of tigernut.

3.4. Transcriptome sequencing analysis by RNA-Seq

For further exploring the flavonoid biosynthesis mechanism in tigernut treated with YSD J2, transcriptome sequencing was performed, and 50.67 Gb clean data were acquired by QC sequencing, with 7.44 Gb clean data per sample. The GC level was 46.3–48.86

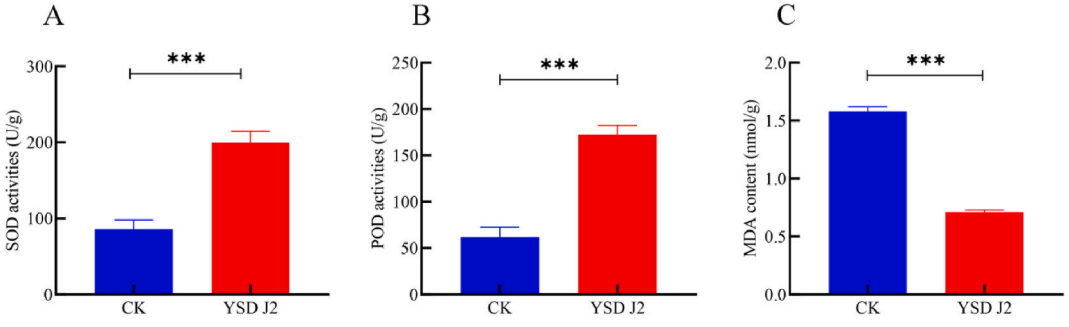

Fig. 2. Antioxidant activities of tigernut in response to *Pantoea* sp. YSD J2 inoculation (YSD J2). Values are means ± SD (n = 3). CK is non bacterial inoculated plants. * or ** indicates a significant difference at the 0.05 or 0.01 level, respectively.

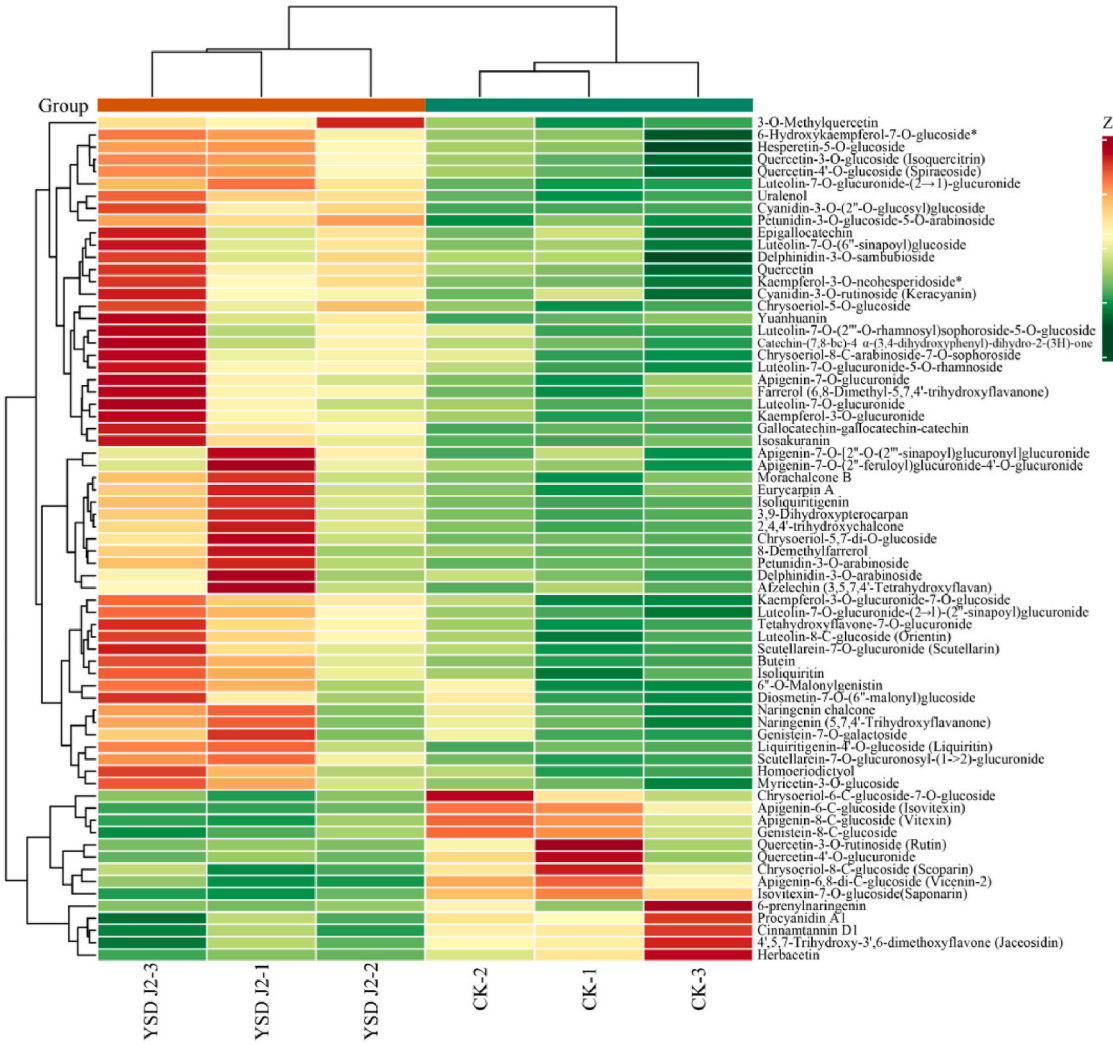

Fig. 3. The total flavonoids/metabolite heat map derived from UPLC-MS/MS profiling. In the above heat map, the red color indicates significant flavonoids accumulation, and the green color indicates significant flavonoids reduction.

%, with Q30 based percentage of > 90.76 % (Table S5). These data revealed that RNA-Seq profiling data were reliable. Altogether, 51344 unigenes more than 1 kb in length were acquired. The obtained N50 and N90 lengths were around 2004 bp and 643 bp, separately (Table S6). Next, the above unigenes were exposed to functional annotation on the basis of the KEGG, NR, SwissProt, GO, COG/KOG, PFAM, and TrEMBL databases, and 98370 unigenes annotations were acquired (Table S7). Clean sample data were found to be assembled and serialized based on the single gene pool.

Additionally, we detected 14391 DEGs, of which 5146 DEGs presented upregulation and 9245 DEGs showed downregulation. The findings are displayed in the volcano map and heatmap (Fig. S1). Meanwhile, we carried out enrichment analysis of DEGs to determine candidate functions and associated biological pathways of possible transcripts. The findings of KOG enrichment analysis suggested that DEGs were mostly enriched in "general function prediction only" (1318), "signal transduction mechanisms" (859), "posttranslational modification, protein turnover, chaperones" (855), "carbohydrate transport and metabolism" (576), "translation, ribosomal structure and biogenesis" (561), and "energy production and conversion" (549; Fig. 6A). Further, the DEGs between the YSD J2 vs. CK groups enriched 148 KEGG metabolic pathways, and 16 remarkably associated pathways were screened ($p < 0.05$) (Table S8), such as "secondary metabolite biosynthesis," "ribosome," "starch and sucrose metabolism," "peroxisome", "phenylpropanoid biosynthesis", and "fatty acid metabolism" (Fig. 6B). According to GO results, DEGs were mainly associated with one subcategory of "cellular component", i.e., "cellular anatanical entity". In addition, numerous DEGs were associated with two subcategories of "biological process," i.e., "cellular process" and "metabolic process". Numberous DEGs were also associated with two subcategories of "molecular function," i.e., "binding" and "catalytic activity" (Fig. 6C).

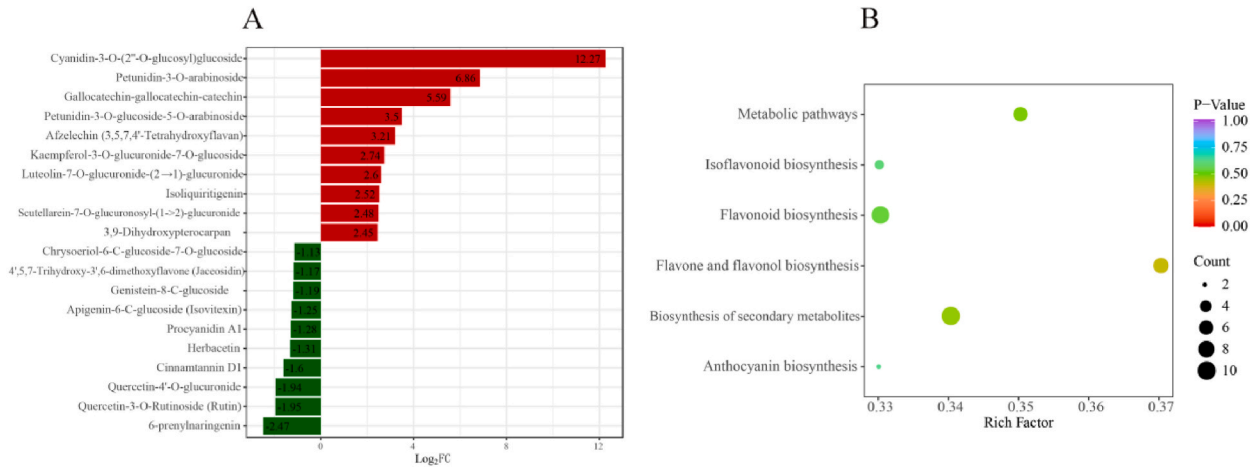

Fig. 4. Metabolomics analysis of tigernut in response to *Pantoea* sp. YSD J2 inoculation. (A) The top 20 metabolites of YSD J2 vs. CK group difference multiple, and in the above difference multiple histogram, red represents up-regulated differential metabolite, green represents down-regulated differential metabolite. (B) KEGG pathway enrichment of DEMs in YSD J2 vs. CK group.

3.5. Transcriptome and metabolome analyses of tigernut

KEGG enrichment analysis indicated that DEGs showed consistent expression profiles with DEMs associated with 6 pathways, including "secondary metabolites biosynthesis," "metabolic pathways," "flavonoid biosynthesis," "isoflavonoid biosynthesis," "flavone and flavonol biosynthesis," and "anthocyanin biosynthesis" (Table S9). Our results revealed consistency and reliability of our metabolome and transcriptome results (Figs. 4 and 6). In addition, 25 critical genes related to isoflavonoid biosynthesis, phenylpropanoid (flavonoid) biosynthesis, anthocyanin biosynthesis, and flavone and flavonol biosynthesis pathways showed remarkably diverse levels within YSD J2 treatment of tigernut (Table S10). Therefore, further studies were conducted to analyze the genes and corresponding metabolites associated with these pathways.

In the YSD J2 vs. CK groups, five of these genes (trans cinnamate 4-monooxygenase (*CYP73A)*, flavonol synthase (*FLS)*, anthocyanin 5-O-glucoside-6‴-O-malonyltransferase (*5MaT1)*, isoflavone 7-O-glucoside-6″-O-malonyltransferase (*IF7MAT)*, and flavonol-3-O-L-rhamnoside-7-O-glucosyltransferase (*UGT73C6)*) were significantly upregulated, while the other eight genes (chalcone synthase (*CHS)*, chalcone reductase (*CHR)*, *caffeoyl-CoA O-methyltransferase* (*CCoAOMT)*, flavone synthase II (*CYP93G1)*, naringenin 3-dioxygenase (*F3H)*, leucoanthocyanidin reductase (*LAR)*, anthocyanidin 3-O-glucosyltransferase (*BZ1)*, and flavonoid 6-hydroxylase (*CYP71D9)*) were significantly downregulated (Fig. 8). In addition, seven chalcone isomerase (*CHI*) coding genes, four anthocyanidin synthase (*ANS*) coding genes, three shikimate O-hydroxycinnamoyltransferase (*HCT*) coding genes, two flavonoids 3′,5′-hydroxylase (*CYP75A*) genes, one anthocyanidin reductase (*ANR*) coding gene, and one bifunctional dihydro flavonol 4-reductase/flavanone 4-reductase (*DFR*) exhibited significant upregulation. These genes were associated with flavonoid synthesis in tigernut.

Phenylalanine ammonia-lyase (PAL) transforms phenylalanine into cinnamoyl-CoA, and the latter is subsequently transformed into p-coumaroyl-CoA under catalysis by CYP73A. The flavonoid were finally synthesized under the catalysis of certain enzymes (such as CHS, CHI, DFR, ANR, and HCT) (Fig. 8). Flavonoids showing differential accumulation were in consistence with the levels of the related genes (Fig. 9A). The 15 DEMs were closely related to the flavonoid synthesis pathway and were upregulated under YSD J2 treatment in tigernut compared to CK. Consequently, these genes were applied to network co-expression and characterization of genes regulating (flavonoids). Such genes such as 2-hydroxyisoflavanone dehydratase (*HIDH)*, anthocyanidin 5,3-O-glucosyltransferase (*GT1)*, *CHI*, *CYP75B1*, *DFR*, *IF7MAT*, pterocarpan synthas (*PTS)*, *CHS*, *CYP75A*, *ANR*, *HCT*, F*LS*, isoflavone/4′-methoxyisoflavone 2′-hydroxylase (*CYP81E)*, anthocyanin 5-O-glucoside-6‴-O-malonyltransferase (*5MaT1)*, *CYP93G1*, and *LAR* were most closely connected with cyanidin-3-O-(2″-O-glucosyl) glucoside, apigenin-6-C-glucoside (isovitexin), 3-O-methylquercetin, apigenin-8-C-glucoside (vitexin), quercetin-3-O-rutinoside (rutin), butein, 3,9-dihydroxypterocarpan, isoliquiritigenin, and luteolin-7-O-glucuronide biosynthesis (Fig. 9B). Three hub metabolites (cyanidin-3-O-(2″-O-glucosyl) glucoside, apigenin-6-C-glucoside (isovitexin), and quercetin-3-O-glucoside (isoquercitrin)) were closely related to critical enzyme genes related to flavonoid synthesis. Moreover, *HCT, CYP73A*, *CHI*, *DFR*, *ANS*, *FLS*, flavanone 7-O-glucoside 2″-O-beta-L-rhamnosyltransferase (*C12RT1)*, and *ANR* gene levels were associated with flavonols (4), flavonoid carbonoside (3), chalcones (3), flavanones (2), isoflavones (1), flavones (1), flavanonols (1), and anthocyanidins (1) synthesis in tigernut. Based on the above findings, such structural genes were identified as essential regulators of flavonoid biosynthesis in tigernut.

Orientin, or luteolin-8-C-glucoside, whose structure is 3′, 4′, 5, 7-tetrahydroxyyellow keto-8-D-glucopyranoside, is a flavonoid carbon glycoside monomer compound. Previous research has shown that orientin possesses several pharmacological effects, including anti-inflammatory, antioxidant, anticancer, cardioprotective, neuroprotective, and analgesic properties [39–43]. Up to now, two flavonoid C-glycoside biosynthetic pathways have been identified [44]. This study found that tigernut with YSD J2 treatment significantly accumulated orientin compared to CK (Fig. 1B). Based on transcriptomic and metabonomic analyses, we generated a

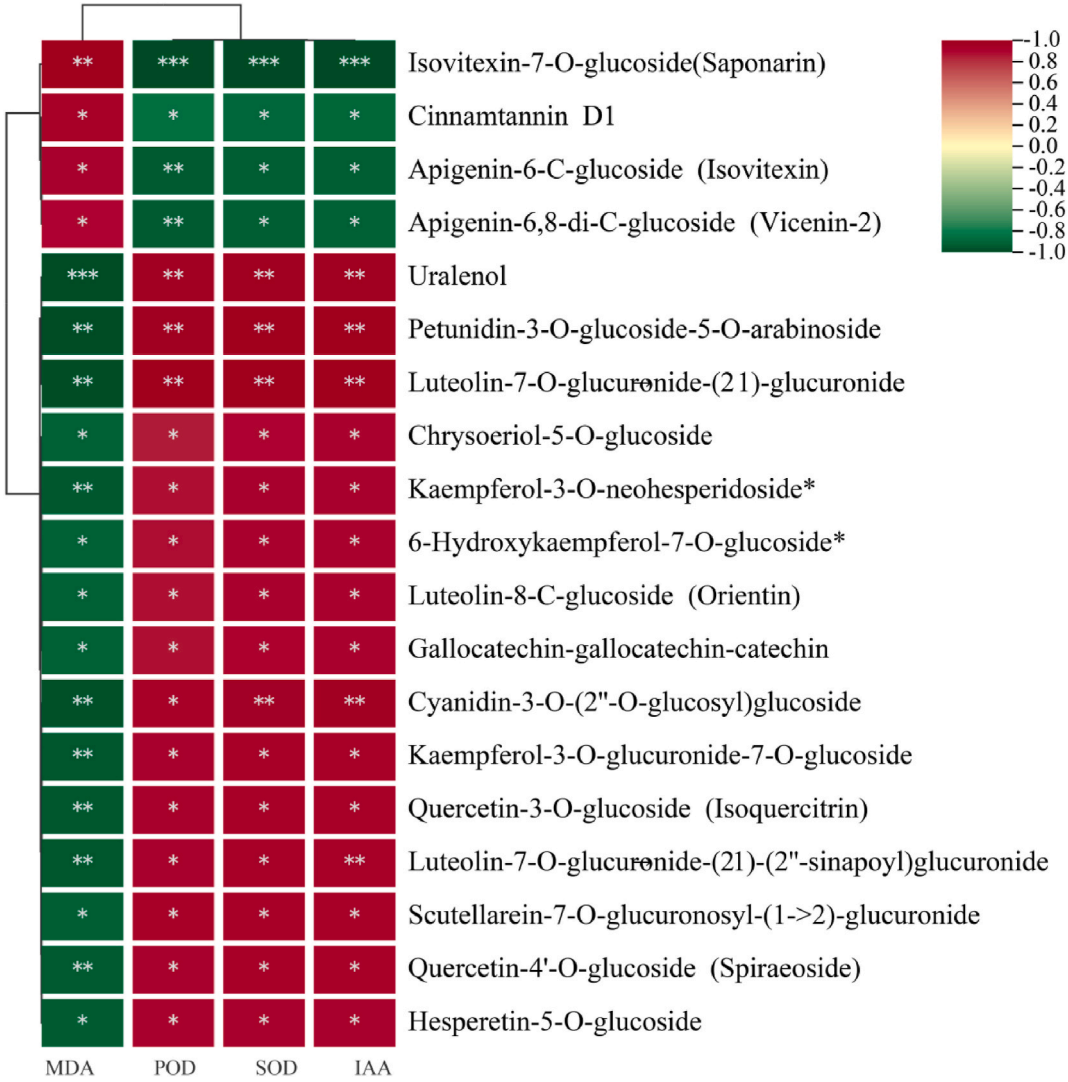

Fig. 5. Correlation analysis of differential expressed metabolites (DEMs), IAA content, and antioxidant activity parameters (SOD, POD and MDA). * or ** indicates a significant difference at the 0.05 or 0.01 level, respectively.

proposed biosynthetic pathway for orientin in tigernut (Fig. 7).

3.6. Quantitative real-time PCR verification

In order to ensure that our transcriptome data at a gene level were valid, a qRT-PCR experiment was carried out on eight essential genes (Fig. 10). These genes included *CYP73A* (Cluster-49537.1), *CHI* (Cluster-37365.0 and Cluster-50548.7), *ANS* (Cluster-49536.4 and Cluster-43695.0), *FLS* (Cluster-25595.0), *HCT* (Cluster-44099.0), and *UGT73C6* (Cluster-51835.5). As a result, these genes under YSD J2 treatment were significantly upregulated by 2.81, 9.64, 1.73, 1.82, 12.90, 2.19, 2.70, and 18.10 folds, compared to CK, separately. These findings conformed to RNA-Seq data, suggesting that these data can be adopted for evaluating the upregulation and downregulation of the gene expression. Finally, YSD J2 treatment could increase the expression of flavonoid synthesis-associated genes to different degrees. Such critical genes in tigernut participated in flavonoid biosynthesis and were potential genes used in further cloning and gene function verification.

3.7. Transcription factors (TFs) related to flavonoid biosynthesis

TFs can influence plant flavonoid biosynthesis. YSD J2 inoculation can activate TFs related to flavonoid biosynthesis in tigernut. The TFs associated with flavonoid biosynthesis were AP2/ERF-ERF, bHLH, GRAS, C2H2, NAC, MYB, bZIP, MYB-related, WRKY, AUX/IAA, and TRAF TFs (Fig. S2). The most abundant TFs were AP2/ERF-ERF and bHLH TFs. We obtained twenty-one AP2/ERF-ERF and

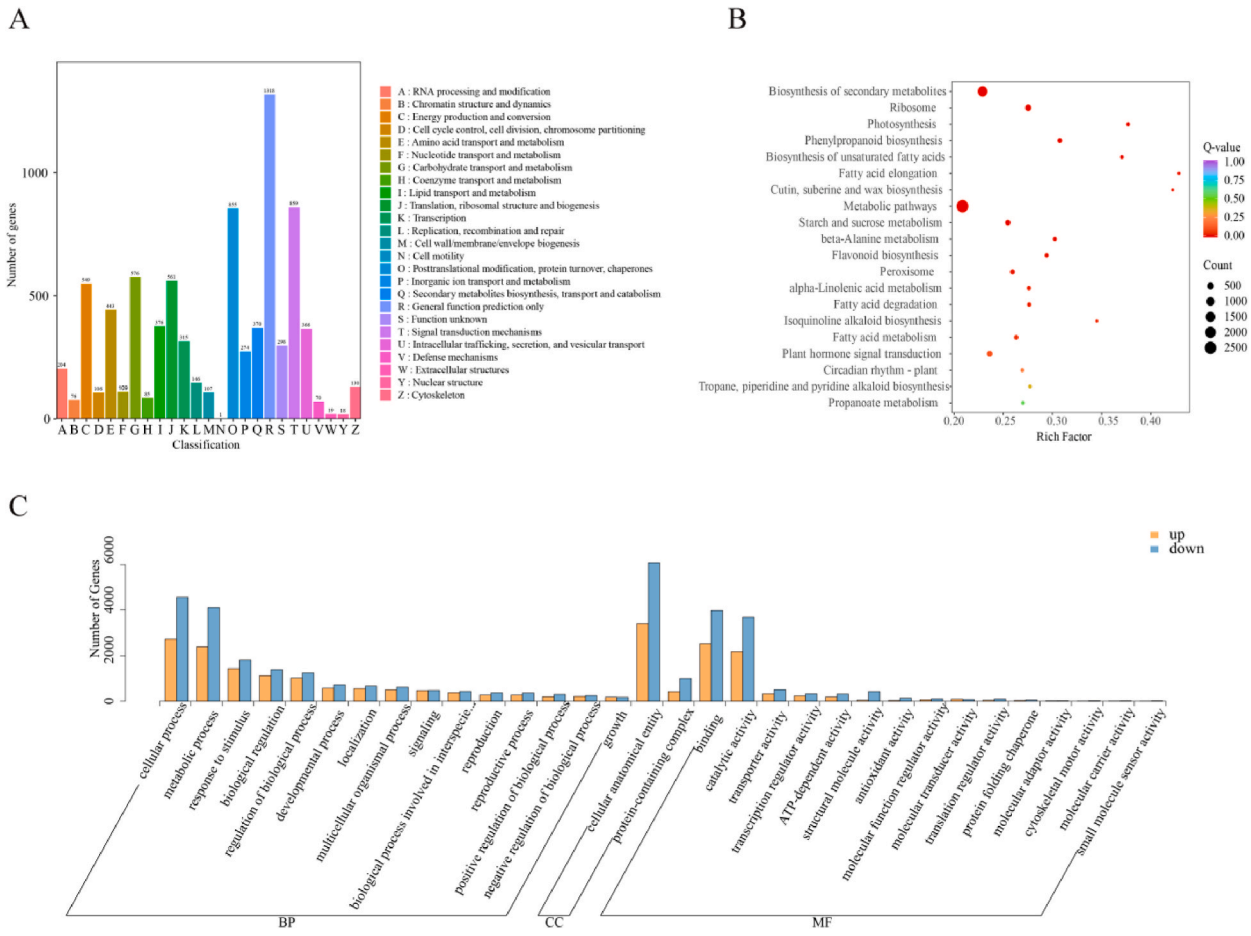

Fig. 6. Transcriptomic analysis of tigernut in response to *Pantoea* sp. YSD J2 inoculation. (A) Bar graph of KOG classification. The horizontal coordinate indicates the functional classification (code) of KOG ID, and the vertical coordinate indicates the number of DEGs included, different classifications are indicated by different colors. The legend shows the code plus its functional description information. (B) KEGG pathway enrichment of DEGs in YSD J2 vs. CK. (C) Clusters of Gene Ontology (GO). The orange and blue colors represent the amount of up-regulated DEGs, and the amount of down-regulated DEGs, respectively. BP: Biological Process; CC: Cellular Component; MF: Molecular Function.

five bHLH TFs (FPKM >10) (Fig. S3).

bHLH34 (Cluster-37505.3) was significantly upregulated under YSD J2 treatment and showed a positive correlation with tetahydroxyflavone-7-O-glucuronide, orientin, kaempferol-3-O-glucuronide and scutellarin, which indicates that the TF bHLH34 (Cluster-37505.3) may positively regulate flavonoid biosynthesis. Additionally, AP2/ERF-ERF (Cluster-10150.7 and Cluster-32230.0) were positively correlated with orientin. In contrast, the level of bHLH16 (Cluster-11980.2) showed significant negative correlation with those of tetahydroxyflavone-7-O-glucuronide, orientin, and kaempferol-3-O-glucuronide, and AP2/ERF-ERF (Cluster-22211.0) was significantly negatively related to that of tetahydroxyflavone-7-O-glucuronide, luteolin-7-O-glucuronide, and kaempferol-3-O-glucuronide, which indicated that AP2/ERF-ERF (Cluster-22211.0) may negatively regulate flavonoid accumulation in tigernut (Fig. S4).

4. Discussion

Tigernut, known as chufas, is the second most abundant species within Cyperaceae family. *Cyperus* is the type genus and core taxon of the Cyperaceae family [45]. Recently, tigernut has been recognized for its high-quality oils, similar to olive oil, and its rich components like lactones, flavonoids, coumarins, steroids glycosides, cardiac glycosides, and triterpenoids [15,16]. These elements offer various pharmaceutical benefits, including antioxidation, antibacterial, and anticoagulation effects, and they help improve blood microcirculation and prevent ischemic stroke [17,18]. Given the numerous pharmacological effects of tigernut, it is crucial to study its secondary metabolites and their synthesis mechanisms.

Auxin critically affects plant growth through concentration changes that regulate multiple processes. IAA functions as the predominant natural auxin in plants [46]. Our results indicate that IAA levels in tigernut significantly increased under the YSD J2 treatment compared to the CK. This was in consistence with previous research results, IAA not only promotes growth, but also induces

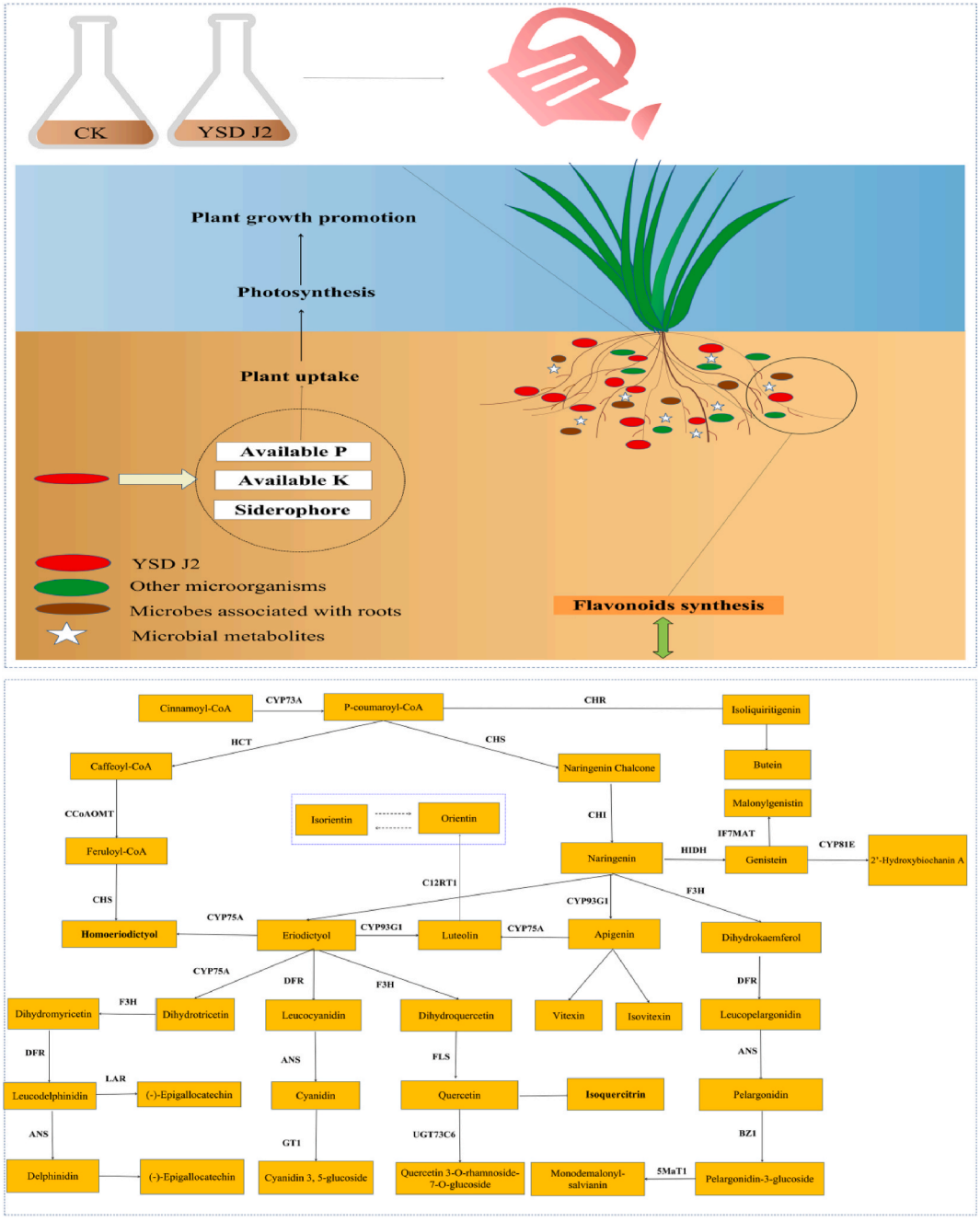

Fig. 7. Speculative pathways of flavonoid synthesis of the tigernut. The pathway was constructed based on the KEGG pathway and the scientific literature.

flavonoid accumulation and enhances the expression of flavonoid pathway enzyme genes [47–51]. Antioxidants belong to plant stress tolerance indicators [52,53]. POD and SOD represent key plant antioxidant enzymes families, and the alterations can show plant's stress resistance [54]. Under normal conditions, enzymes in plants are coordinated mutually, while enzyme activity within this defense system is the decisive factor in controlling damage, better reflecting the adaptability of plants to adversity. This study evaluated the activity of antioxidants in both uninoculated and inoculated plants and found that compared with CK, tigernut inoculated with YSD J2 showed higher SOD and POD activities, and lower MDA content. The results indicate that YSD J2 inoculation significantly promotes plant growth and development.

Plant flavonoids exhibit appropriate biochemical activity and additional bioactivities in different diseases [55,56]. Flavonoids are important components in the tigernut stem, including 7-hydroxyl-6-methoxy radical coumarin, isoorientin, and orientin. Orientin

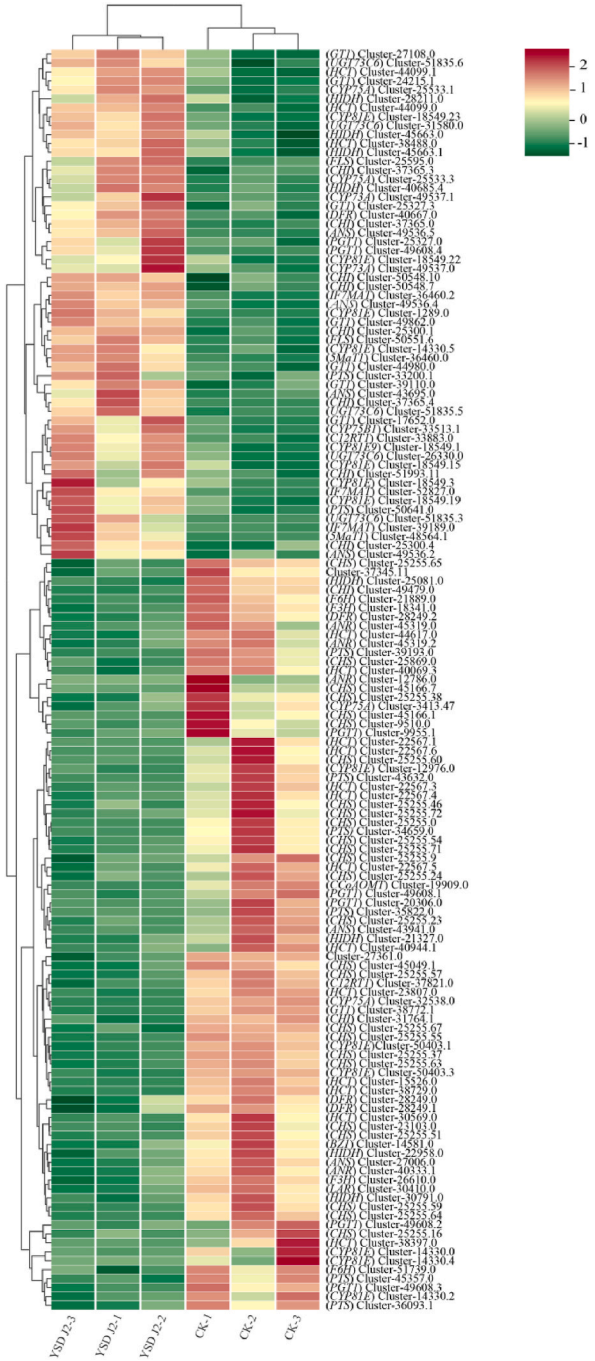

Fig. 8. Heat map of gene expression levels (FPKM values) of key enzyme sites of the flavonoid synthesis pathway. Enzymes encoded by the differentially expressed genes were marked in red and the heatmaps showed log2 (FPKM+1) values of each DEGs. Each row of the heatmap represented one gene and each column represented one a group (from right to left, CK, YSD J2-treated). In the above heat map, the red color indicates the expression of genes significantly up-regulated, and the green color indicates the expression of genes significantly down-regulated.

accounts for 65.4 % (mass percentage) of the total three monomers and is the main component of the monomer compounds [20]. The biosynthesis of active ingredients in medicinal crops results from the interaction between crop genotypes and specific environmental conditions, with certain genes playing a crucial role. Critical enzyme genes related to secondary metabolic pathways are fully expressed only under specific ecological conditions. However, most studies have focused on external environmental effects on biosynthesis [57], neglecting the internal environment of the plants.

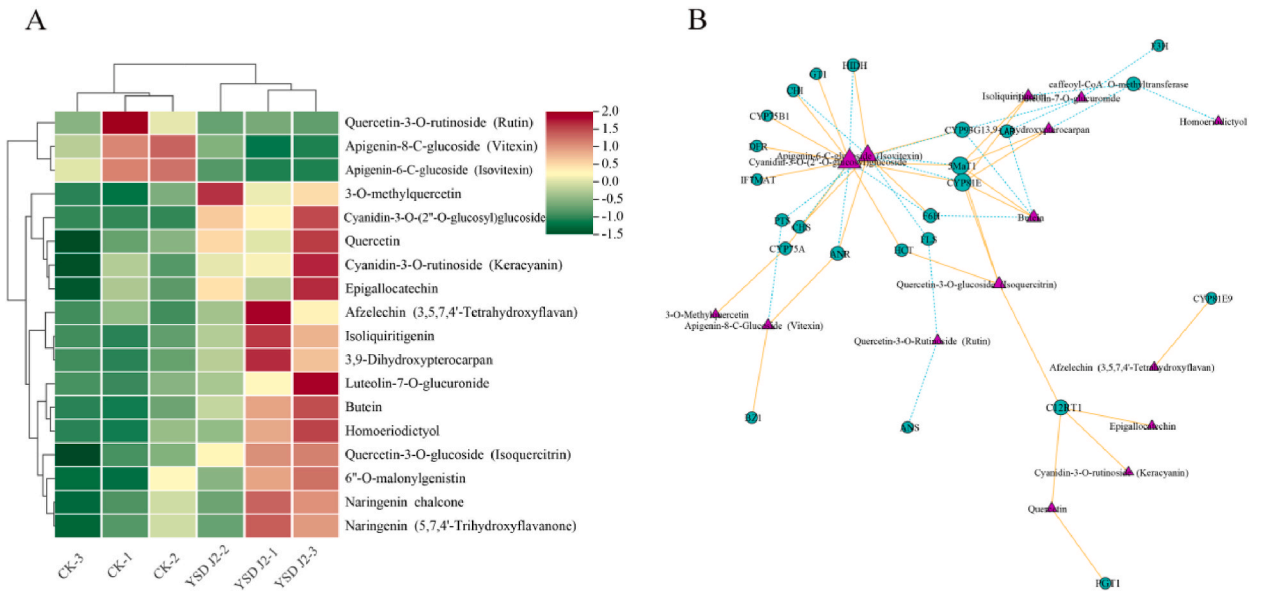

Fig. 9. (A) The heat map of significantly accumulated flavonoids that are enriched in flavonoid biosynthesis pathway. (B) Transcriptome and metabolome combined analysis network in tigernut in response to *Pantoea* sp. YSD J2 inoculation. In the above heat map, the red color indicates the expression of genes significantly up-regulated, and the green color indicates the expression of genes significantly down-regulated. In the network, the tiffany blue circles represent the DEGs and the purple triangle represent the corresponding metabolites.

The distribution and accumulation of PGPMs in medicinal plants are tightly associated with external environmental factors, such as humidity, temperature, and other climatic conditions, as well as the growth period of medicinal plants, which are critical internal factors that influence medicinal plant growth and metabolism. To systematically study the pathway, species, and differential genes of endophyte-mediated secondary metabolite accumulation in medicinal plants, metabolome (LC-MS/MS) analysis plus transcriptome analysis was conducted to explore the mechanism of flavonoid biosynthesis of tigernut with YSD J2. In the current study, 301 flavonoids were found in tigernut and 55 significantly upregulated DEMs w associated with flavonoid synthesis pathway were identified. Therefore, such unique DEMs distribution and accumulation modes accounted for the primary factor leading to different flavonoids of tigernut under YSD J2. PGPMs can directly produce various metabolites and plant hormones, such as GA, ABA, SA, and IAA, to promote plant development [58] and produce flavonoids as antioxidant regulators. In addition, horizontal gene exchange between plants and microorganisms may lead to changes in plant secondary metabolites [59]. Many studies have shown that some PGPMs "reprocesses" plant metabolites into other compounds, leading to degradation of certain toxic compounds or other plant functions [60]. PGPMs may interact differently in different plants species, which requires further in-depth research. The present study found that YSD J2 treatment significantly enriched nine compounds, namely uralenol, petunidin-3-O-glucoside-5-O-arabinoside, luteolin-7-O-glucuronide-(2 → 1)-glucuronide, kaempferol-3-O-neohesperidoside, cyanidin-3-O-(2″-O-glucosyl)glucoside, kaempferol-3-O-glucuronide-7-O-glucoside, quercetin-3-O--glucoside, luteolin-7-O-glucuronide-(2 → 1)-(2″-sinapoyl)glucuronide, and quercetin-4′-O-glucoside, which futher enhanced antioxidant activity. Our previous research has shown that YSD J2 exhibited multiple growth- and metabolism-related abilities [31]. It was speculated that the IAA produced by YSD J2 significantly activated the expression of CHS, CHR, and CHI, which are widely distributed in the phloem of vascular bundle cells of the cell wall, resulting in a significant accumulation of flavonoids. In the present study, levels of IAA in tigernut significantly increased in comparison to CK under YSD J2 treatment. The qRT-PCR results revealed a significant upregulation of *CHI* gene expression in the YSD J2 treatment groups, confirming the above speculation. At the same time, the YSD J2 treatment significantly reduced the content of vitexin and isovitexin, which led to the accumulation of apigenin, causing the reaction to enter flavone and flavonol biosynthesis or form flavanonols to accumulate orientin further.

In tigernut, flavonoid accumulation was markedly related to phenylpropanoid (flavonoid) biosynthesis, anthocyanin biosynthesis, isoflavonoid biosynthesis, and flavone and flavonol biosynthesis pathways, which specifically target hundreds of genes related to flavonoid biosynthesis. Flavonoid accumulation modes of tigernut were significantly different under YSD J2 treatments, associated genes closely related to this pathway also exhibited differential expression. Consequently, this study analyzed different flavonoid biosynthesis-related transcriptional reprogramming cascades and metabolite synthesis flow in tigernut. According to our results, PAL is responsible for catalyzing phenylalanine transformation into cinnamoyl-CoA. Subsequently, CYP73A catalyzes cinnamoyl-CoA isomerization into p-coumaroyl-CoA. *CYP73A*-encoding genes of tigernut were significantly upregulated under YSD J2 treatments. Moreover, we found that the above gene levels and upstream metabolic compounds accumulation facilitated downstream flavonoid biosynthesis within tigernut. Moreover, eleven *CHI* coding genes (Cluster-25300.1, Cluster-25300.7, Cluster-25300.8, Cluster-37365.0, Cluster-37365.3, Cluster-37365.4, Cluster-50548.1, Cluster-50548.10, Cluster-50548.5, Cluster-50548.6, Cluster-50548.7) were significantly upregulated by YSD J2 treatment. CHI is responsible for the isomerization of naringenin chalcone into naringenin, the frequently adopted precursor for synthesizing diverse plant flavonoids, such as chalcones (isoliquiritigenin, butein), flavanones

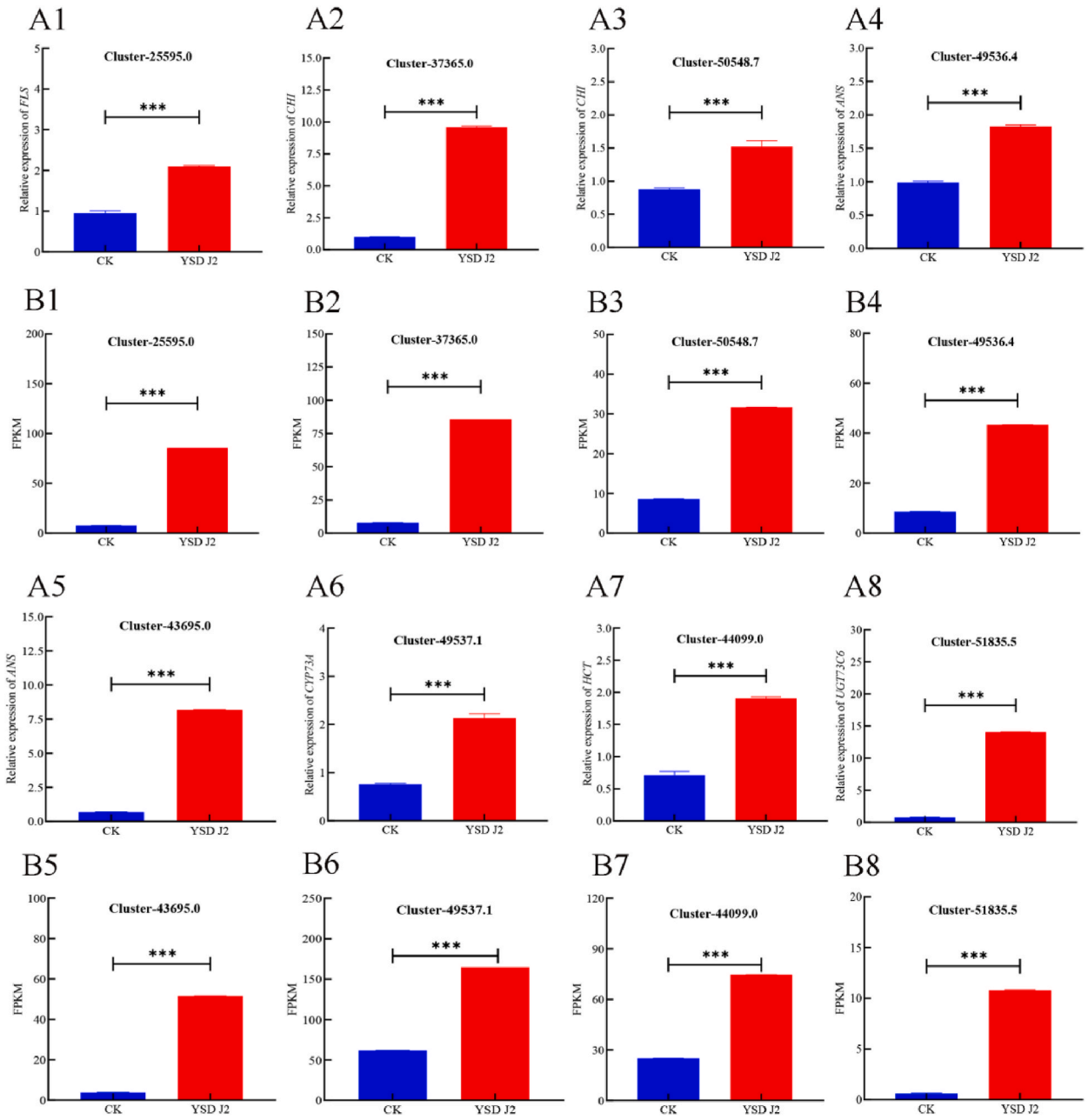

Fig. 10. Expression levels of flavonoids biosynthesis genes of tigernut in response to *Pantoea* sp. YSD J2 inoculation. Cluster-xxxxx.x represents the ID of gene. The y-axis indicates the relative expression level (A: by qRT-PCR; B: FPKM values) of the genes. Each value is the mean of three replicates, and error bars indicate standard deviations. Statistical analysis of the data was performed by independent samples *t*-test using the SPSS 22.0 software (IBM, Chicago, IL, USA). *** above the columns are significantly different at $p \leq 0.001$.

(homoeriodictyol), flavanols (afzelechin, epigallocatechin, luteoforol), flavonols (quercetin, isoquercitrin, 3-O-methyl quercetin), flavanonols (dihydro kaempferol), anthocyanidins (cyanidin-3-O-rutinoside, cyanidin-3-O-sophoroside), flavones (luteolin-7-O-glucuronide), isoflavones (3,9-dihydroxypterocarpan, 6″-O-malonyl genistin) detected in this study. These DEMs possess potent anti-inflammatory and antioxidative effects, which can regulate some signal transducers to alleviate relevant disorders [61,62].

5. Conclusions

The physiological, transcriptomic and flavonoid metabolomic analyses of tigernut were compared under *Pantoea* sp. YSD J2 inoculation. *Pantoea* sp. YSD J2 considerably improved IAA and orientin content. This work indicated flavonoid metabolites related to

enhancing tigernut antioxidant capacity upon *Pantoea* sp. YSD J2 inoculation, and several candidate genes related to DEMs were detected. In conclusion, the findings provide a foundation for investigating the regulatory effect of *Pantoea* sp. YSD J2 on tigernut development and flavonoid biosynthesis.

Data availability statements

All relevant data are within the manuscript and its Additional files.

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CRediT authorship contribution statement

Saisai Wang: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Yanna Huang:** Writing – review & editing, Formal analysis, Data curation, Conceptualization. **Yu Sun:** Writing – review & editing, Formal analysis, Data curation, Conceptualization. **Jinbin Wang:** Writing – review & editing, Formal analysis, Data curation, Conceptualization. **Xueming Tang:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

All authors declares that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e35966.

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