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Multi-Omics Analysis of the Enhancement of Bioactive Components in *Glycyrrhiza uralensis* Fisch. through Combined Inoculation with *Rhizobium* and PGPR under Salt Stress

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Abstract

Background and objective Licorice, an important forage crop widely cultivated worldwide, faces increasing scarcity of wild resources, making artificial cultivation the primary means of market supply. Salt stress negatively impacts the quality and yield of licorice, while microbial inoculation can not only effectively alleviate the damage caused by salt stress but also enhance its quality. To elucidate the response mechanism of licorice to salt stress, this study investigated the physiological, transcriptomic, and metabolomic changes under salt stress.

Results The results showed that the combined inoculation group exhibited significant increases in osmotic substance and chlorophyll content. Transcriptome and metabolome sequencing analysis, along with pathway validation, identified 34, 76, and 54 differentially expressed genes (DEGs) in the CK vs CKNaCl, GM9ZNaCl vs CKNaCl, and GM10NaCl vs CKNaCl groups, respectively. These genes were primarily enriched in pathways such as ABC transporters, oxidative phosphorylation, plant hormone signal transduction, and jasmonic acid biosynthesis. The contents of metabolites including linoleic acid, amino acids, and jasmonic acid in licorice were significantly increased.

Conclusion It is inferred that combined inoculation may enhance the osmotic regulation capacity and antioxidant defense ability of *G. uralensis* roots under salt stress, activate phytohormone signaling pathways such as jasmonic acid, and thereby coordinately regulate the expression of related functional genes, effectively improving the salt tolerance of *G. uralensis* and promoting the synthesis of secondary metabolites. This study provides an important theoretical basis for utilizing microbial agents to improve the growth of medicinal plants in saline-alkali soils.

Keywords: *Rhizobium*; PGPR; *Glycyrrhiza uralensis*(*G. uralensis*); Salt stress; Transcriptomics; Metabolomics

Introduction

The genus *Glycyrrhiza*, a perennial herb of the Fabaceae family, is widely distributed across

global flora. China is a core region for the geographical distribution of *G. uralensis*, its largest consumer, and holds a dominant position in the international *G. uralensis* trade^[1]. In traditional Chinese medicine, *G. uralensis* is a commonly used bulk medicinal material with effects such as clearing heat and detoxifying^[2]. Internationally, it is also frequently used for medicinal purposes in countries such as the United States, Japan, and South Korea^[3,4]. However, with the sharp increase in global market demand for *G. uralensis*, wild resources have been subjected to predatory exploitation. Over-harvesting has damaged the ecological balance, leading to the gradual depletion of wild *G. uralensis* resources^[5]. Currently, cultivated *G. uralensis* has become the main source for market supply^[6]. However, due to environmental constraints, the content of active ingredients in cultivated licorice is generally low, often failing to meet the quality control standards of pharmacopoeias. As a result, most cultivated resources are diverted to the food processing industry, severely restricting the sustainable development of the medicinal *G. uralensis* industry chain^[7].

Salt stress is a critical environmental factor limiting plant growth and development. When plants experience salt stress, it triggers a cascade of reactions including osmotic imbalance and ion toxicity, thereby interfering with their physiological and metabolic processes^[8]. In response to stress signals, the plant's own defense mechanisms are rapidly activated. Transcriptome sequencing technology can comprehensively analyze gene expression dynamics and accurately identify differentially expressed genes^[9]. To adapt to environmental pressure, plants have evolved interconnected regulatory networks that maintain homeostasis by restructuring transcriptional regulation and metabolic pathways, ultimately adjusting their growth state to a stress adaptation mode^[10]. However, the plant's inherent salt tolerance regulation often suffers from slow response speed and limited regulatory scope^[11]. In contrast, microbial fertilizers show significant potential in alleviating salt stress in plants. Inoculation with specific microorganisms can quickly improve the rhizosphere microecological environment, reducing direct damage to roots from soil salinity. Furthermore, they promote plant nutrient absorption, regulate plant hormone levels, and enhance plant stress resistance through various pathways, providing additional support for plant growth under salt stress^[12-14].

Multi-omics analysis involves the integration of multiple omics technologies, including genomics, transcriptomics, proteomics, and metabolomics. A single omics approach can only analyze medicinal plants from a single level, making multi-dimensional research difficult and

hindering the in-depth exploration of its multi-target and multi-pathway mechanisms, presenting significant limitations^[15]. In contrast, the combination of multiple omics techniques enables a comprehensive and systematic exploration of the physiological processes and regulatory mechanisms of organisms from multiple levels^[16-17]. Liu Caixia et al.^[18] found that combining transcriptomic and metabolomic analyses not only helps identify more superior and potential genes and elucidate the metabolic regulation mechanisms during the growth stages of medicinal plants, but also reveals the genetic material and synthesis mechanisms of medicinal plants in response to stress. Salt stress makes the biosynthesis of flavonoids, carbohydrates, glutathione, and salicylic acid in alfalfa key pathways in its stress response^[19]. Chen Lu's study showed that under saline-alkali stress, inoculation with bacterial strains regulated alfalfa growth by improving soil chemical properties, enhancing soil enzyme activities, and promoting the secretion of beneficial metabolites from rhizosphere microorganisms^[20]. Gao Hong^[21] investigated the effect of co-inoculation on the metabolites of alfalfa under saline-alkali stress. The results showed that microbial inoculation could regulate the metabolic pathways of compounds such as flavonoids, carbohydrates, and amino acids, thereby enhancing the plant's saline-alkali tolerance. Therefore, combined transcriptomic and metabolomic analysis of plants under salt stress can reveal changes in gene expression and metabolite accumulation, identify functional genes and metabolic pathways associated with saline-alkali stress, and thus lay a theoretical foundation for improving the stress resistance of medicinal plants such as *G. uralensis*.

1 Materials and Methods

1.1 Test Materials

The test material was *Glycyrrhiza uralensis*. Seeds were collected in 2023 from three-year-old *G. uralensis* plants at the Lianqiang Licorice Base in Changji City, Xinjiang Uygur Autonomous Region (44°17'3"N, 87°6'9"E, altitude 464.1 m). The test rhizobial strain 78 was *Mesorhizobium temperatum*, originally designated CCBAU 0157^[22]. PGPR included M9Z and M10. M9Z was *Paenibacillus illinoisensis*, and M10 was *Ensifer* sp.^[23]. Both were kindly provided by Associate Professor Chen Wenfeng from China Agricultural University.

1.2 Test Reagents

Agarose, methanol, formic acid, ammonium acetate, etc.

1.3 Test Instruments

Covaris ultrasonic homogenizer (Covaris M220), nucleic acid electrophoresis apparatus (DYCP-32C horizontal electrophoresis apparatus), mass spectrometer (Q Exactive™ HF/Q Exactive™ HF-X), chromatograph (Vanquish UHPLC), low-temperature centrifuge (D3024R), etc.

2 Methods

2.1 Pot Experiment

Uniformly sized, healthy, and plump *G. uralensis* seeds were selected. They were first soaked in concentrated sulfuric acid for 15 min, rinsed with sterile water, then soaked in 75% ethanol for 1 min and 10% sodium hypochlorite for 10 min, followed by rinsing with sterile water^[24]. The treated seeds were soaked in single or combined bacterial suspensions (6.84×10^8 CFU·mL⁻¹) for 2 h, then sown in pots with an upper diameter of 17.5 cm, a lower diameter of 13.0 cm, and a height of 20.0 cm. The test substrate was sterilized a mixture of river sand and vermiculite (1:1)^[25]. When the *G. uralensis* seedlings reached the three-leaf stage, they were irrigated weekly with a specific concentration of NaCl solution, low-nitrogen nutrient solution, and corresponding bacterial suspension (10 mL·plant⁻¹) once a week for 4 weeks^[26]. Roots were collected for metabolomic analysis. Samples were immediately frozen in liquid nitrogen and stored at -80°C until RNA and metabolite extraction. Seven treatments were established: control (CK), alone salt stress (CKNaCl), *Rhizobium* treatment under salt stress (GNaCl), PGPR treatments under salt stress (M9ZNaCl, M10NaCl), and combined *Rhizobium* and PGPR treatments under salt stress (GM9ZNaCl, GM10NaCl).

2.2 Determination of Biochemical Indicators

Proline content was determined using the ninhydrin method; soluble sugar content was determined using the anthrone colorimetric method; soluble protein content was determined using the Coomassie Brilliant Blue method^[27]; chlorophyll content was determined using the acetone extraction colorimetric method; nitrate nitrogen content was determined using the salicylic acid method.

2.3 Transcriptomics Detection and Analysis

2.3.1 RNA Extraction and Quality Control

Total RNA suitable for transcriptome sequencing was extracted from *G. uralensis* roots using the DP441 RNAprep Pure Plant Total RNA Extraction Kit (polysaccharide and polyphenol-rich).

Strict quality control was performed on the RNA samples. An Agilent 2100 Bioanalyzer was used to accurately assess RNA integrity.

2.3.2 Library Construction and Quality Inspection

The starting RNA for library construction was total RNA, with a total amount $\geq 1 \mu\text{g}$. The library was constructed using Illumina's NEBNext® Ultra™ RNA Library Prep Kit. mRNA with polyA tails was enriched using Oligo(dT) magnetic beads and then randomly fragmented using divalent cations in NEB Fragmentation Buffer. Using the fragmented mRNA as a template and random oligonucleotides as primers, first-strand cDNA was synthesized in the M-MuLV reverse transcriptase system. Subsequently, the RNA strand was degraded using RNaseH, and second-strand cDNA was synthesized in the DNA Polymerase I system using dNTPs as substrates. The purified double-stranded cDNA underwent end repair, A-tailing, and ligation to sequencing adapters. cDNA fragments of approximately 250–300 bp were selected using AMPure XP beads. After PCR amplification, the PCR products were purified again using AMPure XP beads to obtain the final library.

After library construction, initial quantification was performed using a Qubit 2.0 Fluorometer. The library was diluted to 1.5 ng/ μL , and the insert size was checked using an Agilent 2100 Bioanalyzer. Once the insert size met expectations, the effective concentration of the library was accurately quantified by qRT-PCR (effective concentration $> 2 \text{ nM}$) to ensure library quality.

2.3.3 Sequencing

After passing quality inspection, different libraries were pooled according to the required effective concentration and target data output, followed by Illumina sequencing to generate 150 bp paired-end reads. The sequencing principle was sequencing by synthesis (SBS). On the flow cell, four types of fluorescently labeled dNTPs, DNA polymerase, and adapter primers were added for amplification. As each fluorescently labeled dNTP was incorporated during the extension of complementary strands in each sequencing cluster, a corresponding fluorescence signal was released. The sequencer captured these signals and converted them into sequencing peaks using computer software, thereby obtaining the sequence information of the target fragments.

2.4 Metabolomics Detection and Analysis]

2.4.1 Metabolite Extraction

100 mg of tissue sample ground in liquid nitrogen was placed in an EP tube, and 500 μL of

80% methanol aqueous solution was added. The mixture was vortexed, incubated on ice for 5 min, and centrifuged at 15,000 g for 20 min at 4°C. An appropriate amount of supernatant was taken and diluted with mass spectrometry-grade water to a methanol content of 53%. After centrifugation at 15,000 g for 20 min at 4°C, the supernatant was collected and injected into LC-MS for analysis.

2.4.2 Instrument Parameters

Chromatographic column: Hypersil Gold column (C18), column temperature: 40°C, flow rate: 0.2 mL/min, mobile phase A: 0.1% formic acid, mobile phase B: methanol. Gradient elution was performed according to the table below.

Tab.1 Gradient Elution Table

Time	A%	B%
0	98	2
1.5	98	2
3	15	85
10	0	100
10.1	98	2
11	98	2
12	98	2

2.4.3 Mass Spectrometry Conditions

Scan range: m/z 100–1500; ESI source settings were as follows: Spray Voltage: 3.5 kV; Sheath gas flow rate: 35 psi; Aux Gas flow rate: 10 L/min; Capillary Temp: 320°C; S-lens RF level: 60; Aux gas heater temp: 350°C; Polarity: positive, negative; MS/MS scans were data-dependent scans.

2.4.4 Data Preprocessing and Metabolite Identification

Raw data files were converted to mzXML format using ProteoWizard. Peak extraction and quantification were performed using XCMS. Peaks were aligned across different samples based on parameters such as retention time and m/z. Peak area calibration was performed using the first sample for more accurate quantification. Metabolites were identified by comparing the data (with settings including 10 ppm mass deviation and adduct ion information) against high-quality secondary spectral databases. Background ions were removed using blank samples. The original quantitative results were normalized using the formula: sample original value / (sum of metabolite quantitative values in the sample / sum of metabolite quantitative values in the first sample) to obtain relative peak areas.

2.4.5 Statistical Data Analysis

After obtaining read counts for transcripts, analysis of gene expression differences between sample groups was performed to identify differentially expressed genes (DEGs), followed by functional analysis of the DEGs. To obtain comprehensive genetic information, five databases were queried: KEGG (Kyoto Encyclopedia of Genes and Genomes), NR (NCBI non-redundant protein database), SwissProt (Swiss-Prot protein sequence database), Pfam (Protein family database), and GO (Gene Ontology database).

For metabolomics data, identified metabolites were annotated using the KEGG database (<https://www.genome.jp/kegg/pathway.html>), HMDB database (<https://hmdb.ca/metabolites>), and LIPID Maps database (<http://www.lipidmaps.org/>).

3 Results and Analysis

Tab.2 *G.uralensis* oxydative stress index after combined vaccination

Treatment	SOD	CAT	POD	MDA
CK	131.72±2.32 ^e	442.67±3.51 ^f	853.00±11.27 ^e	5.8030±0.0714 ^d
CKNaCl	164.87±0.32 ^d	398.67±4.05 ^g	948.97±8.05 ^d	10.2617±0.1014 ^a
GNaCl	169.30±0.80 ^{cd}	482.67±3.51 ^d	1 020.00±5.57 ^c	6.4824±0.0869 ^b
M9ZNaCl	167.47±1.92 ^{cd}	473.67±4.51 ^e	1 047.03±8.34 ^c	6.3587±0.0546 ^{bc}
M10NaCl	171.73±3.21 ^c	519.33±2.08 ^c	1 051.37±9.27 ^c	6.2301±0.0401 ^c
GM9ZNaCl	200.60±3.73 ^b	543.33±6.11 ^b	1 626.57±67.02 ^a	5.5720±0.0742 ^e
GM10NaCl	206.65±6.25 ^a	559.00±2.65 ^a	1 234.67±11.93 ^b	5.5587±0.0740 ^e

Note: Different lowercase letters indicate significant differences ($P<0.05$). The same applies below.

As shown in Table 2, compared to the CKNaCl treatment, SOD, CAT, and POD activities were significantly increased in all *G.uralensis* plants inoculated with microbial agents. Specifically, SOD increased by 2.62%, 1.6%, and 4% in the GNaCl, M9ZNaCl, and M10NaCl treatments, respectively, while it increased by 17.81% and 20.22% in the combined inoculation treatments GM9ZNaCl and GM10NaCl. CAT increased by 17.4%, 15.83%, and 23.23% in GNaCl, M9ZNaCl, and M10NaCl, respectively, and by 26.63% and 28.68% in GM9ZNaCl and GM10NaCl. POD increased by 6.9%, 9.4%, and 9.7% in GNaCl, M9ZNaCl, and M10NaCl, respectively, and by 41.66% and 23.14% in GM9ZNaCl and GM10NaCl. Compared to the CKNaCl treatment, MDA content was significantly decreased in all inoculated treatments. MDA decreased by 36.83%, 38.03%, and 39.29% in GNaCl, M9ZNaCl, and M10NaCl, respectively, and by 45.7% and 45.83% in the combined inoculation treatments GM9ZNaCl and GM10NaCl.

3.1 Content of Osmotic Adjustment Substances

Tab.3 Content of *G.uralensis* penetrant substances after combined vaccination

Treatment	Soluble polysaccharides	Proline	Nitrate nitrogen	Soluble protein
CK	0.0288±0.0078 ^e	26.2222±0.53374 ^d	0.0402±0.0011 ^e	7.1051±0.3392 ^d
CKNaCl	0.0758±0.0175 ^d	28.2735±0.6453 ^e	0.0447±0.0003 ^d	8.5837±1.2131 ^d
GNaCl	0.1227±0.0162 ^c	39.2991±0.8242 ^d	0.0504±0.0008 ^c	16.4955±0.1188 ^c
M9ZNaCl	0.1165±0.0043 ^c	39.2137±1.1843 ^d	0.0518±0.0021 ^{bc}	10.8664±1.4123 ^d
M10NaCl	0.1030±0.0085 ^c	48.2735±1.1562 ^c	0.0514±0.0012 ^c	24.1997±1.4895 ^b
GM9ZNaCl	0.3984±0.0114 ^a	78.1880±1.8549 ^b	0.0552±0.0003 ^a	33.2529±4.7317 ^a
GM10NaCl	0.1796±0.0343 ^b	94.3419±5.0398 ^a	0.0538±0.0028 ^{ab}	30.3476±4.6410 ^a

As shown in Table 3, compared to the CKNaCl treatment, soluble polysaccharide content was significantly increased in all treatments. Specifically, soluble polysaccharide in GM9ZNaCl and GM10NaCl increased by 92.77% and 83.96%, respectively, compared to CKNaCl. Proline content was significantly increased in all treatments compared to CK and CKNaCl. Compared to CKNaCl, proline content in GM9ZNaCl and GM10NaCl increased by 66.46% and 72.21%, respectively. Compared to CK, proline in GM9ZNaCl and GM10NaCl increased by 63.84% and 70.03%. Compared to the CKNaCl treatment, nitrate nitrogen content in GM9ZNaCl and GM10NaCl was significantly higher by 27.36% and 25.60%, respectively. Compared to CK, nitrate nitrogen in GM9ZNaCl and GM10NaCl was significantly higher by 19.02% and 17.07%. Compared to CKNaCl, soluble protein content in the combined inoculation treatments GM9ZNaCl and GM10NaCl was significantly higher by 78.63% and 76.59%, respectively. Compared to CK, soluble protein in GM9ZNaCl and GM10NaCl was higher by 74.19% and 71.72%.

Tab.4 Chlorophyll content in *G.uralensis* leaves after combined vaccination

Treatment	Chlorophyll a	Chlorophyll b	Carotenoid	Total Chlorophyll
CK	3.4361±0.0076 ^f	1.1103±0.0534 ^d	1.0731±0.0567 ^d	5.6195±0.0845 ^f
CKNaCl	2.6741±0.0010 ^g	0.8352±0.0073 ^f	0.7488±0.0017 ^f	4.2581±0.0045 ^g
GNaCl	4.9224±0.0073 ^c	0.9761±0.0300 ^e	1.2449±0.0144 ^c	7.1435±0.0103 ^c
M9ZNaCl	3.4754±0.0380 ^e	1.2086±0.0465 ^c	1.3675±0.0247 ^b	6.0515±0.0352 ^e
M10NaCl	3.5859±0.0054 ^d	1.7916±0.0091 ^a	0.9820±0.0097 ^e	6.3594±0.0061 ^d
GM9ZNaCl	6.2158±0.0010 ^a	1.4315±0.0075 ^b	1.4491±0.0017 ^a	9.0964±0.0045 ^a
GM10NaCl	5.9133±0.0054 ^b	1.4691±0.0091 ^b	1.4026±0.0050 ^b	8.7850±0.0018 ^b

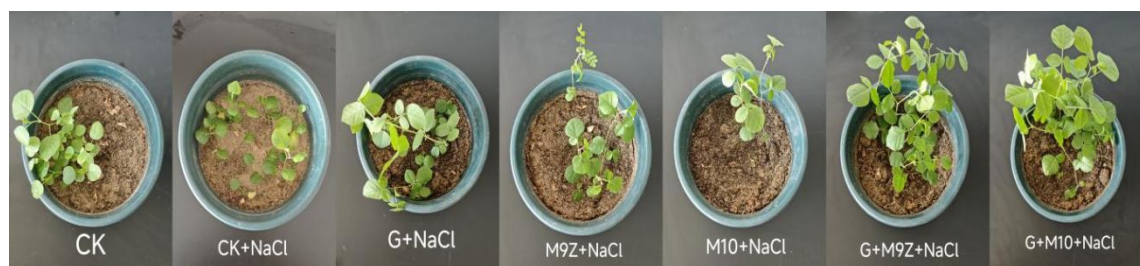


Fig.1 pot experiment

Chlorophyll is an essential substance for plant photosynthesis and an indispensable component of this process. As shown in Figure 1 and Table 4, chlorophyll content in the CKNaCl treatment was significantly different from that in other treatments, indicating that salt stress inhibits chlorophyll synthesis, leading to a decrease in chlorophyll content in licorice seedling leaves. Compared to the CKNaCl treatment, the contents of chlorophyll a, chlorophyll b, and carotenoids were significantly increased in the GM9ZNaCl and GM10NaCl treatments. Total chlorophyll content increased by 53.19% and 51.53%, respectively; chlorophyll a increased by 56.98% and 54.78%; chlorophyll b increased by 41.66% and 43.15%; carotenoids increased by 48.32% and 46.61%. Compared to CK, total chlorophyll content in the GM9ZNaCl and GM10NaCl treatments was significantly higher by 38.22% and 36.03%, respectively; chlorophyll a increased by 44.72% and 41.89%; chlorophyll b increased by 22.44% and 24.42%; carotenoids increased by 25.95% and 23.49%. These results indicate that under salt stress, combined inoculation with rhizobia and PGPR can slow down chlorophyll degradation, reduce damage caused by adverse factors such as salt stress, and increase the content of various chlorophyll types in *G. uralensis* seedling leaves.

Osmotic adjustment substances and chlorophyll in the CKNaCl group were significantly lower than those in the CK group, indicating that salt stress inhibited the normal growth of *G. uralensis*. The above indicators in the single inoculation groups were significantly different from the CK group and showed some improvement compared to the CKNaCl group, but the alleviation effect was limited. The alleviation effect of combined inoculation was significantly better than that of single inoculation, indicating that the combined inoculation group was more effective in alleviating salt stress and maintaining physiological homeostasis.

3.2 Transcriptome Analysis

3.2.1 Sample Repeatability Correlation Analysis

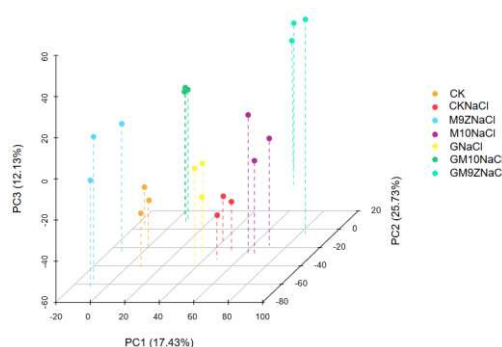


Fig.2 PCA analysis of the transcriptome data of *G. uralensis* after inoculation

Principal component analysis (PCA) was performed on the transcriptome data of the samples. The results, as shown in the figure, indicated ideal clustering of the three replicates for each sample, and samples from different treatment groups showed significant separation. In summary, the sample selection in this study was reasonable, the experimental repeatability was good, and the data reliability was fully verified.

3.2.2 Gene Expression Level Analysis

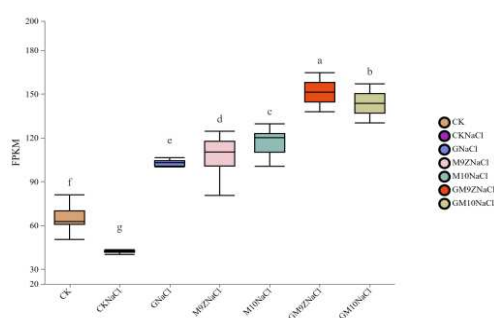


Fig.3 Box plot of *G. uralensis* gene expression levels after inoculation treatment

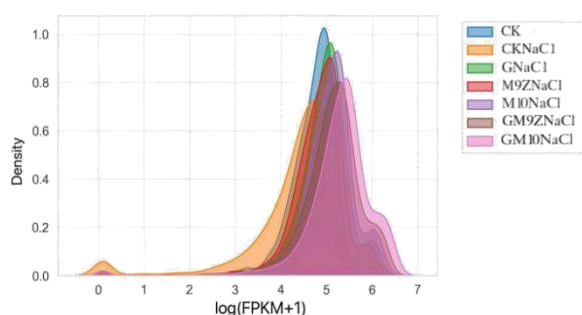


Fig.4 Density plot of *G. uralensis* gene expression levels after inoculation treatment

Based on samples from *G. uralensis* co-inoculated with rhizobia and PGPR under salt stress conditions, a boxplot of gene expression levels was generated (Figure 3). The results showed significant differences in gene expression characteristics among treatment groups. The median expression level of genes in the CKNaCl group was significantly lower than that in the CK group, while the median expression level in the combined treatment groups significantly exceeded that of the CK group. Further analysis using a density plot (Figure 4) revealed that the gene expression peak in the CK group was mainly concentrated in the higher $\log_{10}(\text{FPKM}+1)$ interval, whereas the expression peak in the CKNaCl group shifted left to the low-expression region. This indicates that salt stress reduces the overall gene expression activity in *G. uralensis*. The expression peaks of the GM9ZNaCl and GM10NaCl combined inoculation groups were similar to those of the CK group, suggesting that the combined treatment can mitigate the negative impact of salt stress on the overall gene expression level in *G. uralensis* roots.

3.2.3 Differential Expression Gene Analysis

Based on previous research results, single inoculation with microorganisms had a certain alleviating effect on salt stress in *G. uralensis* roots, but the combined inoculation effect was more

prominent. Therefore, subsequent transcriptomic analysis focused on three core comparisons (CK vs CKNaCl, GM9ZNaCl vs CKNaCl, GM10NaCl vs CKNaCl) to analyze the molecular regulatory mechanism of combined inoculation on *G. uralensis*'s response to salt stress.

Tab.5 Statistical results of differences in expression of *G. uralensis* after combination

vaccination			
DEG Set	DEGs Number	up-regulated	down-regulated
CK vs CKNaCl	34	15	19
GM9ZNaCl vs CKNaCl	76	40	36
GM10NaCl vs CKNaCl	54	24	30

DESeq2 software was used to analyze differential genes between groups based on gene expression quantitative data. The thresholds for screening differential genes were set as $|\text{Fold Change}| \geq 2$ and $\text{FDR} < 0.01$. As shown in Table 5, differentially expressed genes (DEGs) showed significant dynamic changes among treatment groups. A total of 34 DEGs were detected in the CK vs CKNaCl group (15 up-regulated, 19 down-regulated); 76 DEGs were detected in the GM9ZNaCl vs CKNaCl group (40 up-regulated, 36 down-regulated); and 54 DEGs were detected in the GM10NaCl vs CKNaCl group (24 up-regulated, 30 down-regulated).

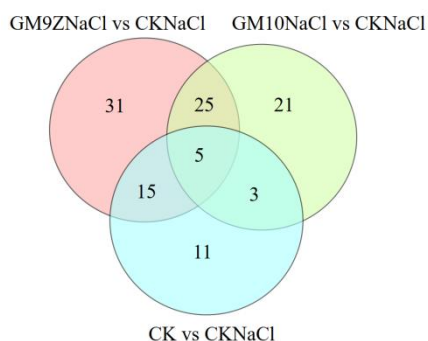
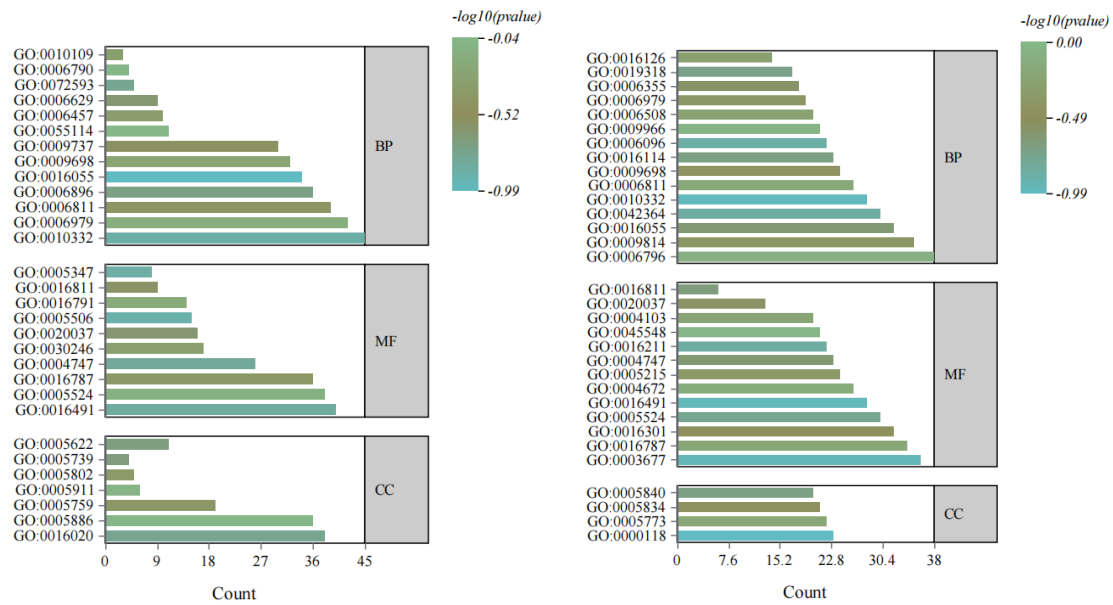


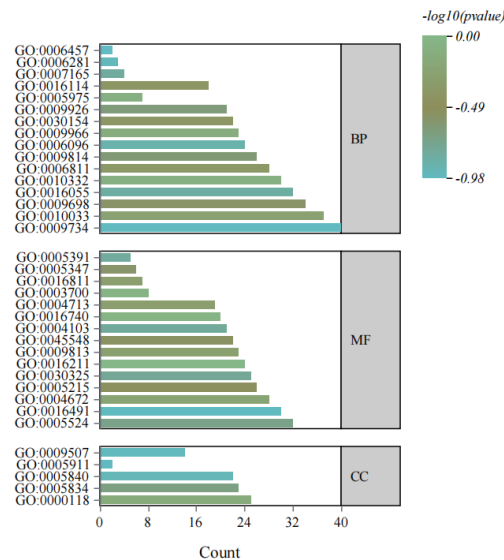
Fig.5 Venn diagram of differentially expressed genes in *G. uralensis* root after co-inoculation

3.2.4 GO Functional Annotation and Enrichment Analysis of DEGs



(a) CK vs CKNaCl

(b) GM9ZNaCl vs CKNaCl



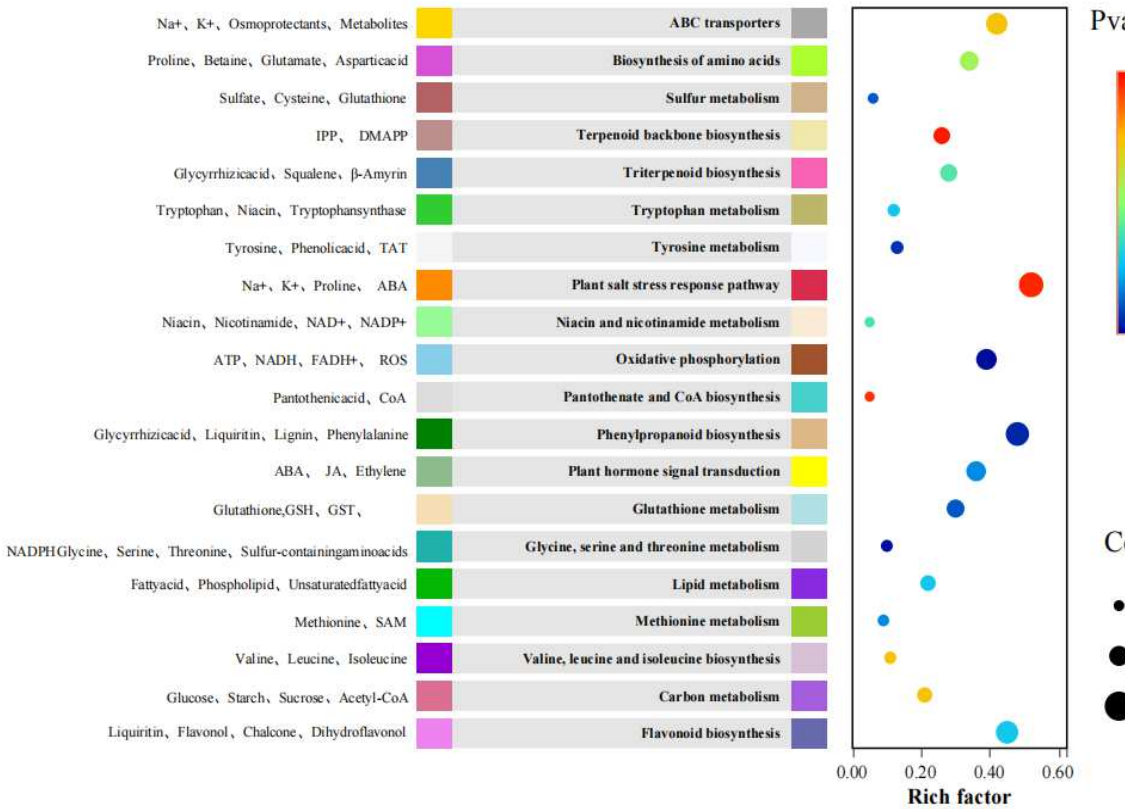
(c) GM10NaCl vs CKNaCl

Fig.6 GO enrichment analysis of differential genes in *G. uralensis* root after co-vaccination

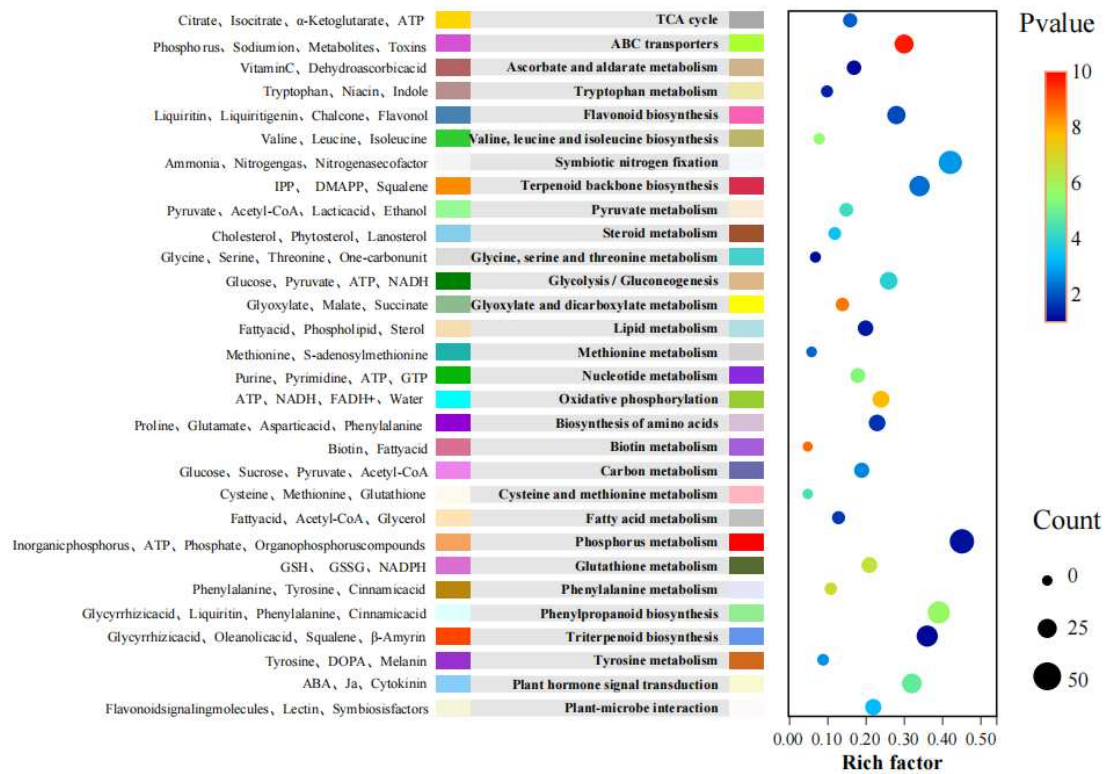
GO enrichment analysis was performed on the DEGs in licorice roots from different treatment groups. The results showed that in the CK vs CKNaCl group, the DEGs were mainly involved in biological processes such as osmoregulation, lipid metabolic process, oxidation-reduction process, sulfur metabolic process, response to abscisic acid, and ion transport. Molecular functions mainly included terpene synthase activity, ion transmembrane transporter activity, ATP binding, hydrolase activity, and carbohydrate binding. Cellular components mainly included the cytoskeleton, cell, and plasma membrane. In the GM9ZNaCl vs CKNaCl group, DEGs were mainly involved in biological processes such as phosphorus metabolic process, ion

transport, glycolysis, hormone-mediated signaling pathway, response to oxidative stress, and mevalonate metabolic process. Molecular functions mainly included oxidoreductase activity, protein kinase activity, phenylalanine ammonia-lyase activity, terpene synthase activity, and squalene synthase activity. Cellular components mainly included the mitochondrion and transcription factor complex. In the GM10NaCl vs CKNaCl group, DEGs were mainly involved in biological processes such as protein folding, carbohydrate metabolic process, signal transduction, regulation of cell division, flavonoid biosynthetic process, and hormone-mediated signaling pathway. Molecular functions mainly included transporter activity, Na⁺/K⁺-ATPase activity, flavonol synthase activity, and phenylalanine ammonia-lyase activity. Cellular components mainly included the cytoskeleton and transcription factor complex.

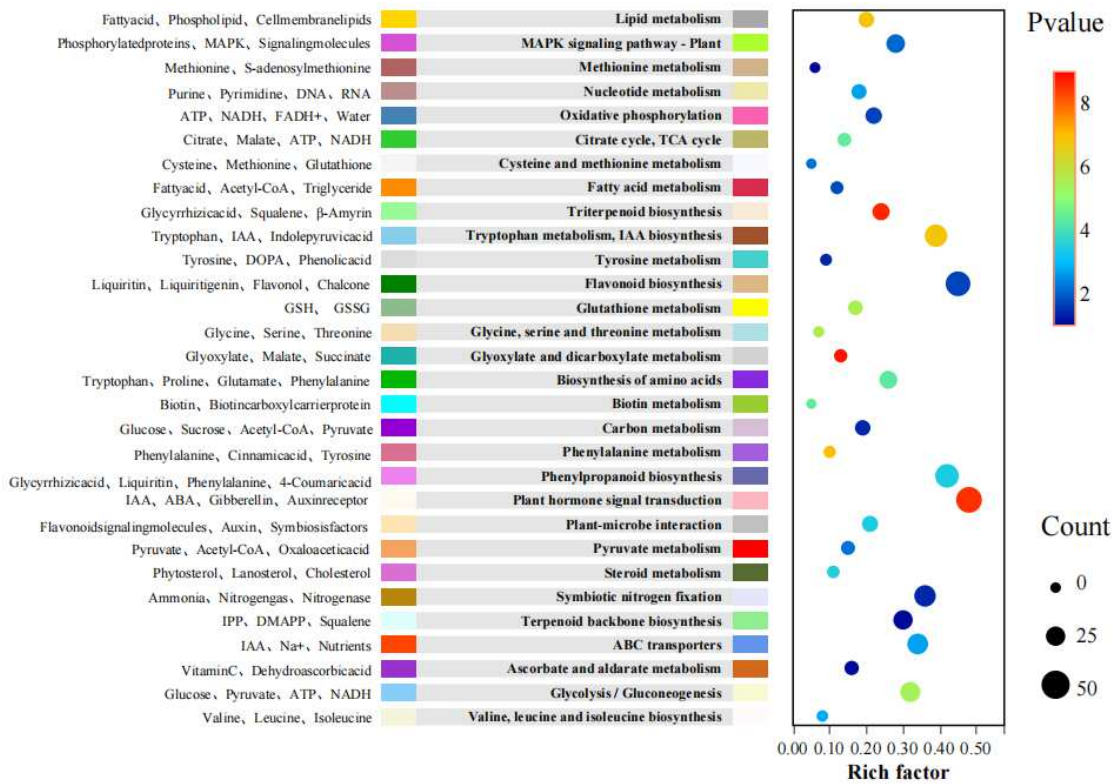
3.2.5 KEGG Functional Pathway Enrichment Analysis of DEGs



(a) CK vs CKNaCl



(b) GM9ZNaCl vs CKNaCl



(c) GM10NaCl vs CKNaCl

Fig.7 KEGG enrichment analysis of differential genes in *G. uralensis* root system after combined

inoculation

KEGG functional pathway enrichment analysis was performed on the differential genes from different comparison groups, and the top 20 significantly enriched KEGG terms were statistically analyzed, as shown in Figure 7. In the CK vs CKNaCl group, the main enriched metabolic pathways in *G. uralensis* roots were ABC transporters, biosynthesis of amino acids, carbon metabolism, terpenoid backbone biosynthesis, tryptophan metabolism, and oxidative phosphorylation. In the GM9ZNaCl vs CKNaCl group, the main enriched pathways were the TCA cycle, ABC transporters, ascorbate and aldarate metabolism, flavonoid biosynthesis, pyruvate metabolism, carbon metabolism, and oxidative phosphorylation. In the GM10NaCl vs CKNaCl group, the main enriched pathways were the MAPK signaling pathway – plant, oxidative phosphorylation, TCA cycle, cysteine and methionine metabolism, triterpenoid biosynthesis, tryptophan metabolism, and carbon metabolism.

3.3 Metabolomic Data Examination

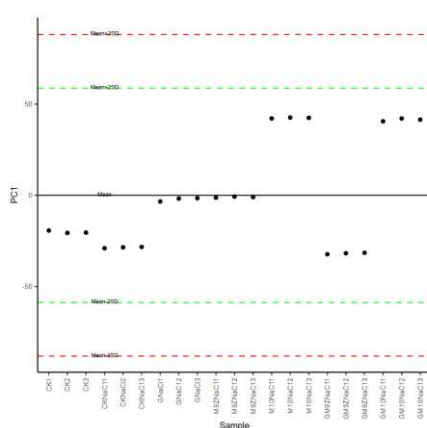


Fig.8 PC1 distribution of each sample point

The distribution of PC1 values for all sample points was used to assess whether the laboratory sample preparation and measurement processes were under control. Sample points exceeding the control limit (3 times the standard deviation) were considered outliers. As shown in Figure 8, most points fluctuated around the mean axis within 2 times the standard deviation, and all points were within the control limits. This indicates good experimental repeatability and high data reliability, laying a data foundation for subsequent metabolomic analysis.

3.2 Multivariate Statistical Analysis

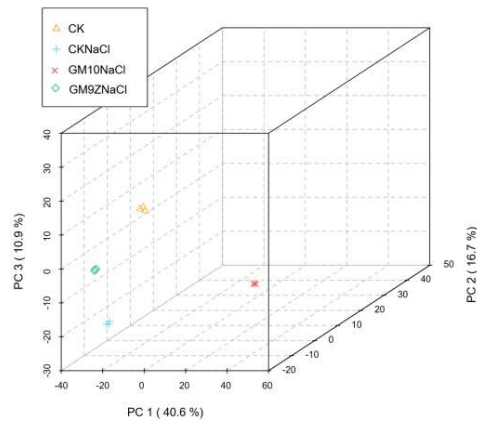


Fig.9 PCA analysis of *G.uralensis* metabolite after inoculation

Principal component analysis (PCA) of samples (including quality control samples) was conducted to analyze the degree of variation in metabolites among fruit samples from different comparison groups. The cumulative variance contribution rates of PC1, PC2, and PC3 were 29.87%, 19.50%, and 11.09%, respectively (see Figure 9); the cumulative contribution rate reached 68.2%, indicating significant metabolic differences between treatment groups.

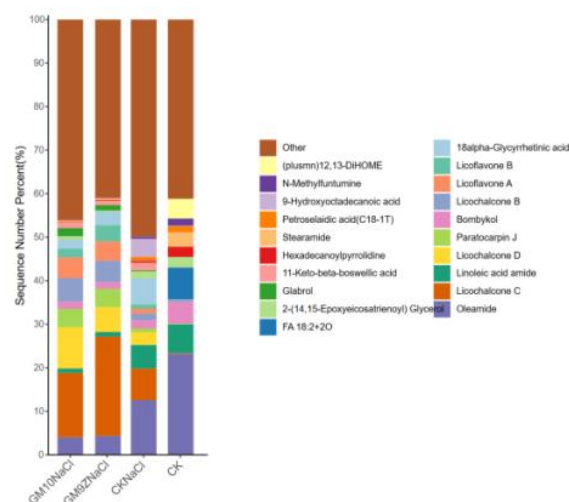


Fig.10 Classification diagram of metabolite composition

Figure 10 shows the percentage distribution of the top 20 metabolites in total abundance across the seven different experimental groups in the non-targeted metabolomic analysis. These mainly include alkaloids, fatty acids, triterpenes, flavonoids, polyphenols, and chalcones. The figure shows that components such as Licochalcone C, Licochalcone D, Licoflavone B, Licoflavone A, and Glabrol were significantly enriched in the GM9ZNaCl and GM10NaCl groups.

3.3 Differential Metabolite Screening

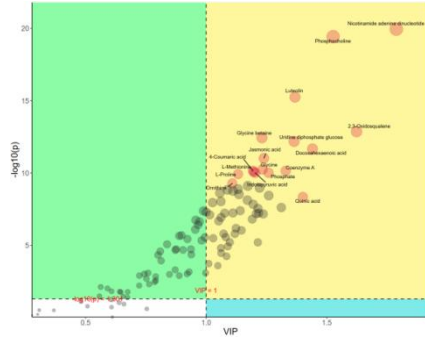


Fig.11 Combined screening of VIP-p

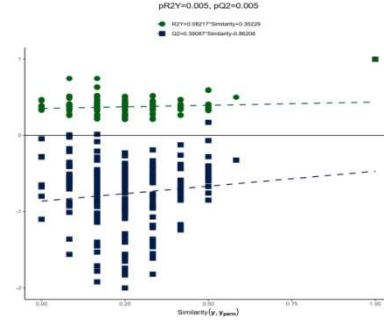


Fig.12 PLS-DA permutation test diagram

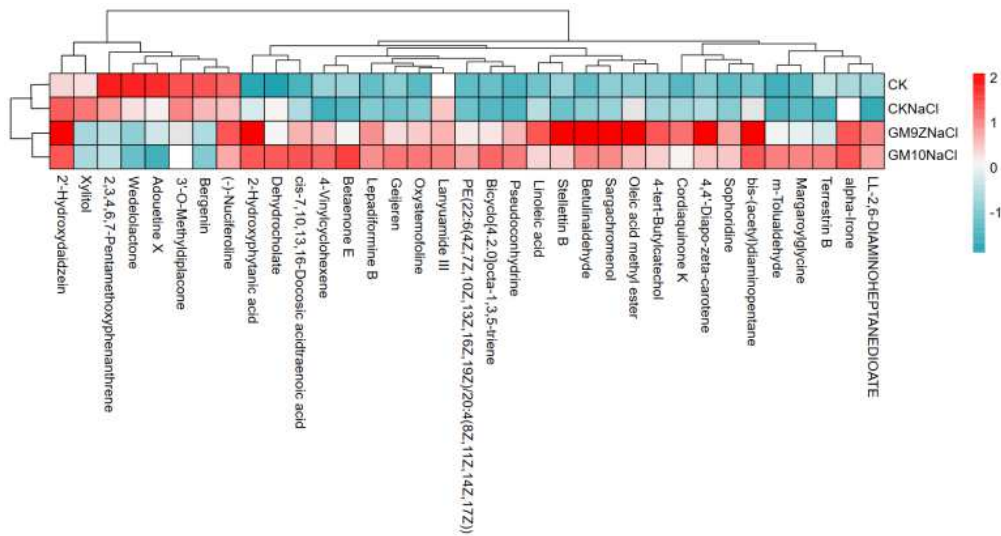


Fig.13 Heat map of differential metabolite clustering analysis

Combining the Variable Importance in Projection (VIP) from the PLS-DA model and the p-value from univariate analysis, differential metabolites with $VIP > 1$ and $p < 0.05$ were selected in multiple group comparisons. Seventeen differential metabolites were identified under salt stress, mainly including flavonoids, amino acids, coenzymes, phenylpropanoids, terpenoids, and fatty acids. The permutation test diagram showed $R^2Y = 0.005$ and $Q^2 = 0.005$, both less than 0.05, indicating that the R^2Y and Q^2 of the multi-group PLS-DA model were significantly higher than those of the randomly permuted model. Moreover, the R^2Y and Q^2 corresponding to the original data (Similarity=1) were significantly higher than the "post-permutation data," and as data similarity decreased, R^2Y/Q^2 decreased synchronously. This indicates no overfitting in the multi-group PLS-DA model, and the output VIP values are reliable.

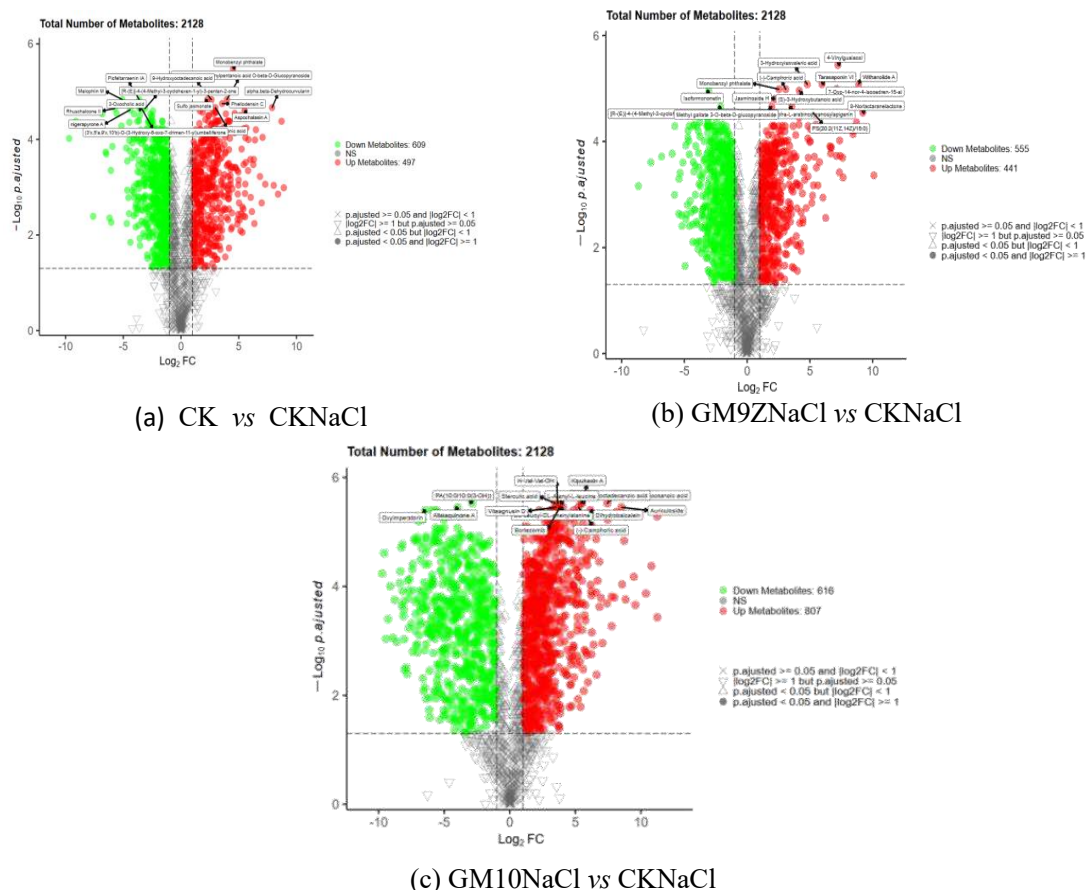
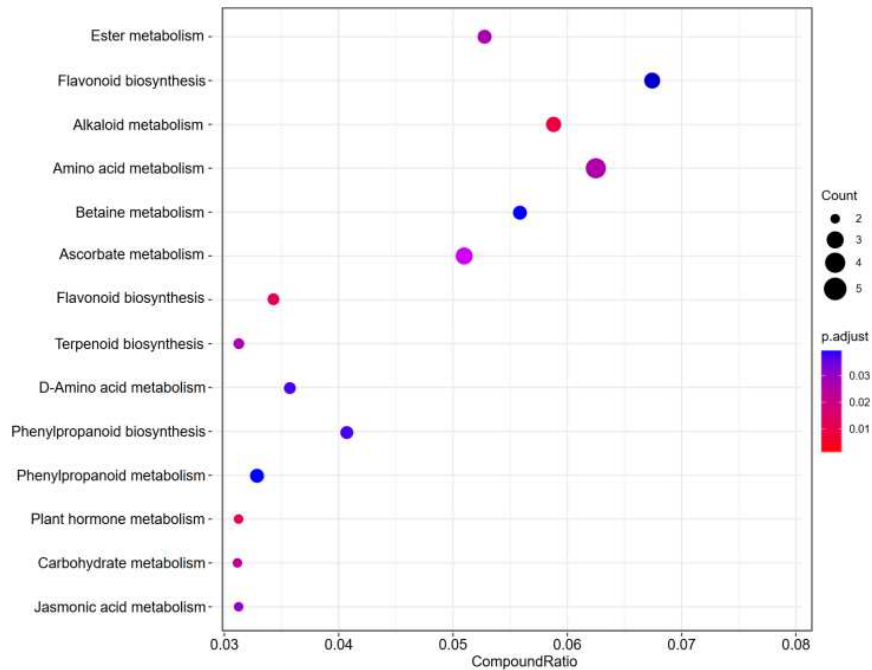


Fig.14 Volcano plot of DEMs in *G.uralensis* in different comparison groups

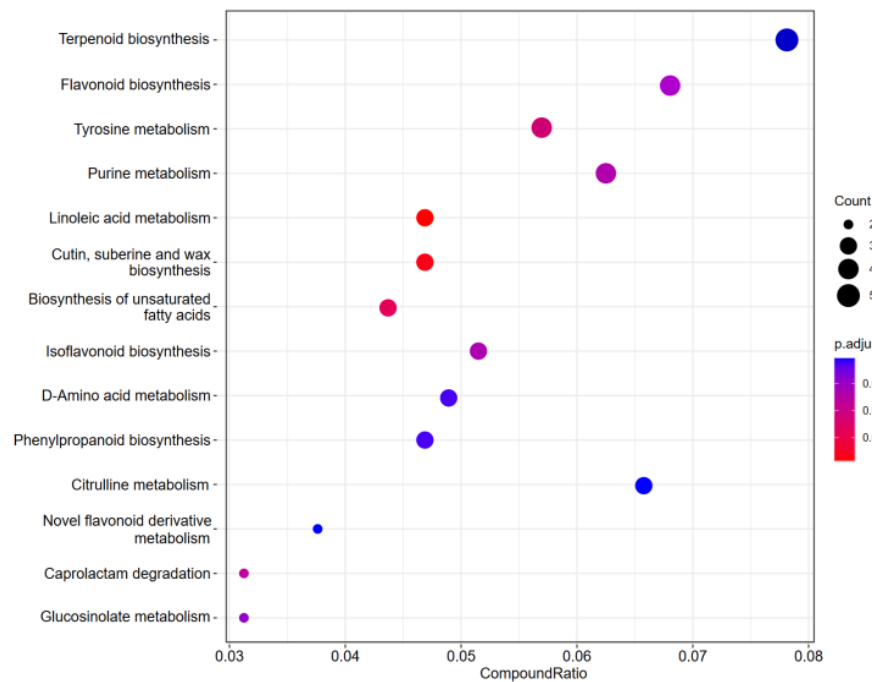
Using non-targeted metabolomics technology, a total of 2128 metabolites were identified in *G.uralensis* samples. Volcano plot analysis of DEMs was performed to visually present the variation patterns and expression level differences of metabolites in fruits from different comparison groups (Figure 14). The results showed that in the CK vs CKNaCl group, 1106 DEMs changed significantly, including 497 up-regulated DEMs and 609 down-regulated DEMs (a). Examples include Phellodendrin C, Sulfu jasmonate, (3',5',9',10')-O-(3-Hydroxy-5-oxo-7-drimen-11-yl) umbelliferonic acid, Rhuschalcone II, and (R)-E-1-(4-(4-Methyl-3-cyclohexen-1-yl)-3-penten-2-one. In the GM9ZNaCl vs CKNaCl group, 996 DEMs changed significantly, including 441 up-regulated DEMs and 555 down-regulated DEMs (b). Examples include 4-Vinylguaiaicol, Tarasaponin VI, Withanolide A, PS(20:2(1Z,14Z)/15:0), (R)-(E)-4-(4-Methyl-3-cyclohexen-1-yl)butanoic acid, Jasminoside H, and Methyl gallate 3-O-beta-D-glucopyranoside. In the GM10NaCl vs CKNaCl group, 1423 DEMs changed significantly, including 807 up-regulated DEMs and 616 down-regulated DEMs (c). Examples include Kipukasin A, Auriculoside, Dihydrobaicalin, Oxyimperatorin, Viteagnusin D, H-V

al-Val-OH, and L-Carnitine-L-leucinolactate.

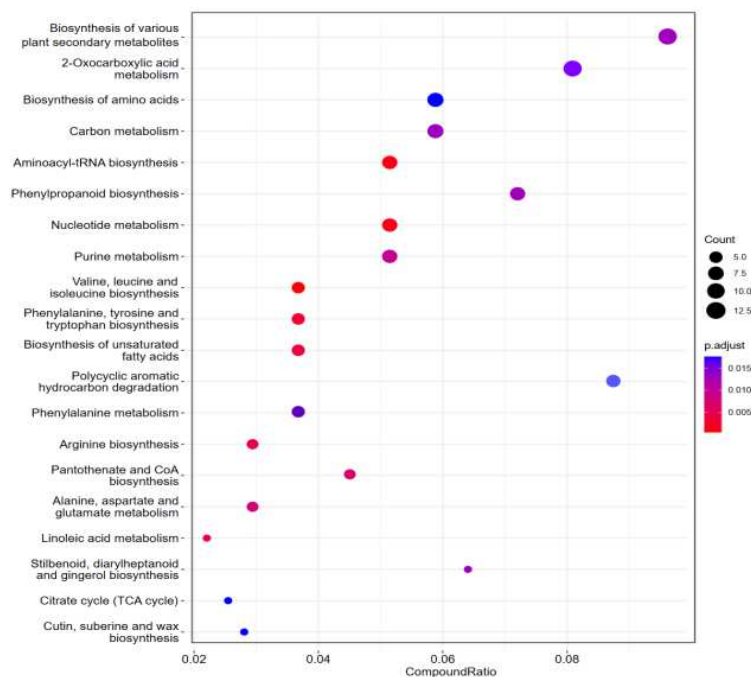
3.4 KEGG Metabolic Pathway Functional Annotation and Enrichment Analysis of DEMs



(a) CK vs CKNaCl



(b) GM9ZNaCl vs CKNaCl



(c) GM10NaCl vs CKNaCl

Fig.15 KEGG enrichment analysis of DEMs in *G. uralensis* in different comparison groups

KEGG annotation analysis of DEMs showed that in the CK vs CKNaCl group, the top 14 significantly enriched metabolic pathways of DEMs mainly included lipid metabolism, flavonoid biosynthesis, alkaloid metabolism, amino acid metabolism, betaine metabolism, and ascorbate metabolism pathways (a). In GM9ZNaCl vs CKNaCl, the top 14 significantly enriched pathways mainly included terpenoid biosynthesis, flavonoid biosynthesis, tyrosine metabolism, purine metabolism, linoleic acid metabolism, and cutin, suberine and wax biosynthesis pathways (b). In the GM10NaCl vs CKNaCl group, the top 14 significantly enriched pathways mainly included biosynthesis of various plant secondary metabolites, 2-oxocarboxylic acid metabolism, amino acid biosynthesis, carbon metabolism, phenylpropanoid biosynthesis, and pantothenate and CoA biosynthesis pathways (c). All three groups showed significant enrichment in the flavonoid biosynthesis pathway.

4 Discussion

4.1 Transcriptomics

In recent years, with the continuous advancement of high-throughput sequencing technologies, transcriptome sequencing has been widely applied in the field of gene discovery. These technologies enable in-depth analysis of whether microbial inoculation can effectively induce plant response mechanisms to abiotic stresses such as salinity, drought, and high

temperature^[28]. By comparing the GO and KEGG enrichment analysis results of DEGs in this study, it was found that the functional annotations from different databases collectively reveal key features of metabolic regulation during strain interaction^[29], uncovering the possible mechanisms by which combined inoculation of *Rhizobium* and PGPR mediates *G.uralensis*'s response to salt stress.

Beneficial bacteria act on the synthesis pathways of secondary metabolites, regulating their synthesis and accumulation^[30,31]. Natural glycosides are primarily derived from plant secondary metabolism and play important roles in plant growth regulation^[32]. Most β -glucosidase genes directly influence the levels of secondary metabolites by modulating substrate specificity^[33]. Niu et al.^[34] found that overexpression of the *SpBGLU25* gene significantly increased the accumulation of lignin precursors and flavonoids in *Arabidopsis thaliana* and enhanced its drought resistance. Manatchanok^[35] demonstrated the crucial functions of β -glucosidase in rice defense, hormone activation, and the synthesis of phenoxazine and isoflavonoids. Hannemann et al.^[36] showed that LgGLU can hydrolyze various phenoxazine glycosides (DIMBOA-Glc, GDIMBOA-Glc) and cyanogenic glycosides, regulating the activity and content of these defensive secondary metabolites. The Heme Oxygenase 1 gene catalyzes the rate-limiting step in heme degradation, producing biliverdin and bilirubin to scavenge ROS and reduce oxidative damage^[37]; the released Fe^{2+} can participate in iron metabolism balance or regulate the activity of other stress-related proteins through iron-sulfur cluster assembly^[38]. This aligns with the conclusions of this study. In this study, the significant expression of BGLU5 and HO1 genes in the CK vs CKNaCl group suggests that combined inoculation with *Rhizobium* and PGPR might enhance licorice's tolerance to salt stress by regulating the synthesis of secondary metabolites and the oxidative stress response in *G.uralensis* roots.

The LEA gene family (Late Embryogenesis Abundant Protein Family) is a typical plant stress resistance-related gene family, divided into several subfamilies based on amino acid sequence characteristics and functions. LEA5 is typically classified as a Group 5 LEA protein, primarily involved in plant adaptation to abiotic stress. It enhances plant salt tolerance by scavenging ROS to reduce oxidative damage caused by salt stress, stabilizing cell structure, protecting biological macromolecules, and maintaining cellular osmotic pressure^[39]. In this study, transcriptome sequencing results showed that in the CK vs CKNaCl group, LEA5 expression was significantly

up-regulated, influenced by transcription factors like DREB2 and AREB and regulated by the ABA signaling pathway. This is consistent with previous studies^[40-42].

The BAM1 gene belongs to the LRR-RLK receptor kinase family. Takahashi et al.^[43] first discovered that dehydration up-regulates CLE25 expression; the CLE25 peptide moves upward through the vasculature to leaves, binds to BAM1/BAM3 receptors, induces NCED3 expression, and increases ABA content in leaves. Hu^[44] et al. found that CLE25/26/45 together with BAM1/3 activate CIK protein phosphorylation during root primordium differentiation. Kang^[45] et al. demonstrated that the CLE25-BAM1/3-ABA mechanism, along with rhizosphere microbial pathways, is a hub for root response to soil water deficit. Zhu^[46] et al. showed that overexpression of the IbBAM1.1 gene significantly enhances plant stress resistance under salt stress, improves the cellular osmotic environment, helps roots maintain normal ion channel function, and thereby enhances nutrient ion absorption efficiency. Zhao et al.^[47] found that under salt stress, root-derived CLE25 still up-regulates NCED3 via BAM1/3, increasing ABA and helping plants maintain ion balance and stomatal regulation. In the GM9ZNaCl vs CKNaCl group of this study, the BAM1 gene was significantly up-regulated. KEGG enrichment analysis revealed that the main enriched pathways in *G.uralensis* roots included the ABA signaling pathway, indicating that the response of licorice roots to salt stress after combined inoculation with rhizobia and PGPR might be regulated through the CLE25-BAM1-ABA signaling pathway.

RPL29 (Ribosomal Protein L29) is a gene encoding a constituent protein of the 60S large subunit of the ribosome, belonging to the L29E ribosomal protein family. Moin et al.^[48] performed transcriptome sequencing on rice under dehydration conditions; the results showed significantly increased expression of PRL29 and increased accumulation of lignin precursors and flavonoids. Cao Guangying et al.^[49] cloned the PRL29-1 gene, constructed overexpression and antisense vectors, and used qPCR to detect its expression changes in different tissues and under *Ralstonia solanacearum* pathogen stress. The results showed that PRL29-1 was expressed in roots, stems, and leaves, and its expression increased after pathogen infection. In the GM9ZNaCl vs CKNaCl group of this study, the PRL29 gene was significantly up-regulated. Previous research results also indicated an increase in flavonoid content. This suggests that combined inoculation with *Rhizobium* and PGPR might enhance the osmotic regulation ability and antioxidant defense capacity of *G.uralensis* roots under salt stress by regulating osmoregulators and secondary

metabolites, thereby alleviating the inhibitory effect of salt stress on *G.uralensis* growth.

Jasmonic acid (JA) is a lipid-derived plant hormone that helps plants resist diseases and enhance stress tolerance^[50]. LOX4 and LOX3 are key enzymes in JA biosynthesis, catalyzing the synthesis of the JA precursor 12-oxo-phytodienoic acid^[51]. Zhang et al.^[52] used qPCR to detect the expression of the AfLOX4 gene under salt stress. The results indicated that AfLOX4 is a positive regulator of salt stress; its overexpression can enhance plant tolerance to salinity and might regulate the secondary metabolic network through JA/oxylin and photosynthesis pathways. Hou et al.^[53] showed that in persimmon, oxidation products like JA, MDA, and volatile aldehydes/ketones produced via LOX4 participate in antioxidant defense, indirectly improving its salt stress tolerance. Yang et al.^[54] demonstrated that salt stress induces LOX4 expression, oxidizing α -linolenic acid in cell membranes to 13-HPOT (13-hydroperoxide), promoting the rapid synthesis of JA precursors, thereby helping cells regulate osmotic pressure and antioxidant defense. LOX4 activation can be triggered by membrane depolarization and Ca^{2+} influx, indicating its upstream position in the membrane potential- Ca^{2+} -LOX4-JA signal cascade. In this study, compared to the CKNaCl group, the LOX4 gene showed a significant expression level in the GM10NaCl group. Previous research confirmed phenomena such as increased chlorophyll content, enhanced antioxidant enzyme activities, and increased secondary metabolite content in *G.uralensis*. KEGG enrichment analysis results indicated that the main enriched metabolic pathways in *G.uralensis* roots included the MAPK signaling pathway and triterpenoid biosynthesis. As a key member of the lipoxygenase family, the significant expression of LOX4 suggests that combined inoculation with *Rhizobium* and PGPR might initiate the salt stress response by catalyzing membrane lipid oxidation and regulate the synthesis of secondary metabolites through the jasmonic acid signaling network, thereby enhancing the salt tolerance of *G.uralensis*.

4.2 Metabolomics

The dynamic changes of metabolites in plants are closely related to their growth environment, physiological state, etc. This study analyzed the metabolites of *G.uralensis* under salt stress and found 1106 DEMs changed significantly in the salt treatment group, consistent with results from studies on honeysuckle responding to salt stress^[55]. This suggests that *G.uralensis* might enhance its stress resistance by regulating more metabolic pathways. The GM9ZNaCl treatment group had

996 DEMs changing significantly, and the GM10NaCl treatment group had 1423 DEMs changing significantly, indicating that after microbial inoculation, the metabolic regulation capacity of *G.uralensis* under salt stress was further enhanced. This aligns with results from studies on *Cajanus cajan* seedlings inoculated with arbuscular mycorrhizal fungi under salt stress^[56]. Furthermore, whether in the CKNaCl group or the combined inoculation treatment groups, the number of down-regulated metabolites was higher than that of up-regulated ones. This might suggest that under salt stress, plants suppress certain metabolic pathways to reduce unnecessary energy consumption, thereby maintaining their growth and development under saline conditions^[57].

Under salt stress, amino acids, as the basic building blocks of proteins and precursors for metabolites produced in response to various biotic and abiotic stresses, play roles in osmotic regulation and maintaining membrane stability^[58]. This study shows that under equivalent sodium ion stress, the contents of proline, soluble polysaccharides, soluble protein, nitrate nitrogen, and chlorophyll in *G.uralensis* significantly decreased, while microbial inoculation significantly increased the accumulation of these components. This is consistent with previous research^[59-61]. Inoculation with *Franconibacter* not only increased proline accumulation but also enhanced photosynthetic efficiency by up-regulating genes related to chlorophyll biosynthesis^[62]. Sharma et al.^[63] showed that *Salicornia* resists salinization stress by increasing the content of the intracellular osmoregulatory substance proline and activating the antioxidant enzyme system, enhancing CAT and SOD activities.

Oxygen-containing organic compounds and organic heterocyclic compounds mainly include numerous substances such as nucleotides, coumarins, alkaloids, and flavonoids. These are generated through complex plant metabolic processes and are crucial components in plant stress resistance^[64]. In this study, combined inoculation significantly affected the composition of organic heterocyclic compounds and oxygen-containing organic compounds in *G.uralensis*. This indicates that combined inoculation might enhance the plant's ability to cope with adversity by influencing the synthesis and transformation of organic heterocyclic compounds in plants. Plant responses to environmental stress are also regulated by the metabolism of organic acids and their derivatives, especially the metabolism of linoleic acid, linolenic acid, and unsaturated fatty acids^[65]. The increase in the content of organic acids and their derivatives under combined inoculation might be

one of *G.uralensis*'s responses to salt stress, consistent with previous studies^[66-69].

This study shows that combined inoculation affects the metabolites in *G.uralensis* roots. These affected metabolites are mostly concentrated in specific cellular metabolic pathways, including the biosynthesis of phenylpropanoids, isoflavonoid biosynthesis, linoleic acid and linolenic acid metabolism, starch and sucrose metabolism, and some amino acid metabolism pathways. Among these, isoflavonoid biosynthesis and linoleic acid metabolism showed significant up-regulation. Linoleic acid and linolenic acid are involved in the synthesis of fatty acids, cell membranes, and thylakoids. The stability of their metabolic levels helps maintain normal cell membrane fluidity, material transport, and chloroplast photosynthesis^[70], which aligns with the measured changes in chlorophyll content. Ullah et al.^[71] demonstrated that *Pseudomonas fluorescens* B8 can synthesize arginine to resist salt stress. Bacteria from the genera *Sphingomonas* and *Pseudomonas* can help plants resist stress by promoting the interconversion of linoleic acid and lipids^[72-74], all consistent with the findings of this study.

Jasmonic acid, as a lipid-derived plant hormone, not only enhances plant stress resistance but also resists diseases^[75]. Gomez^[76] et al. showed that inoculation with *Bradyrhizobium japonicum* IRAT FA3 stimulated an increase in endogenous jasmonate levels in *Arabidopsis thaliana* and regulated hydrogen peroxide production in response to salt stress. Dere's^[77] research indicated that combined inoculation with AMF and PGPR activated the jasmonic acid signaling pathway in pepper, thereby alleviating the negative effects of salt stress. These findings are consistent with the results of this study. The above research results indicate that jasmonic acid plays a key role in enhancing plant abiotic stress resistance. This suggests that *G.uralensis* also responds to salt stress through the jasmonic acid synthesis pathway.

5 Conclusion

This chapter, through transcriptomic and metabolomic analysis of *G.uralensis* treated with combined inoculation of *Rhizobium* and PGPR, revealed the molecular mechanism by which combined inoculation regulates *G.uralensis* tolerance under salt stress. Under equivalent Na⁺ concentration conditions, the increase in content of osmotic adjustment substances such as soluble polysaccharides, soluble protein, proline, nitrate nitrogen, and chlorophyll in the roots of the combined inoculation group was more significant. Transcriptome sequencing results showed that 34, 76, and 54 DEGs were identified in the CK vs CKNaCl, GM9ZNaCl vs CKNaCl, and

GM10NaCl vs CKNaCl groups, respectively. These DEGs are primarily involved in processes like ion transport, redox reactions, and osmotic regulation, mainly enriched in signaling pathways such as ABC transporters, oxidative phosphorylation, plant hormone signal transduction, and jasmonic acid synthesis. Metabolomic sequencing analysis and metabolic pathway validation indicated that salt stress increased the content of metabolites like oxygen-containing organic compounds and organic heterocyclic compounds, linoleic acid, amino acids, and jasmonic acid in *G.uralensis*. In summary, combined inoculation likely enhances the osmotic regulation ability and antioxidant defense capacity of *G.uralensis* roots under salt stress, activates plant hormone signaling pathways such as jasmonic acid, and coordinately regulates the expression of related functional genes, thereby effectively improving the salt stress tolerance of *G.uralensis* and promoting the synthesis of secondary metabolites. This discovery provides a new perspective for further research on the function of plant-microbe interactions in stress adaptation, offers a theoretical basis for using microbial agents to improve the growth conditions of medicinal plants in saline-alkali soil, and provides potential technical support for enhancing crop salt tolerance in agricultural production.

Declarations

Abbreviations ABA: Absciscic acid, ABC:ATP-binding cassette, AMF:Arbuscular mycorrhizal fungi, ATP:Adenosine triphosphate, bp:Base pair(s), CAT:Catalase, cDNA:Complementary DNA, CFU:Colony-forming unit, DEGs:Differentially expressed genes, DEMs:Differentially expressed metabolites, dNTPs:Deoxyribonucleoside triphosphates, ESI:Electrospray ionization, FC:Fold change, FDR:False discovery rate, FPKM:Fragments per kilobase of transcript per million mapped reads, GO:Gene Ontology, HMDB:Human Metabolome Database, JA:Jasmonic acid, KEGG:Kyoto Encyclopedia of Genes and Genomes, LC-MS:Liquid chromatography – mass spectrometry, LRR-RLK:Leucine-rich repeat receptor-like kinase, MAPK : Mitogen-activated protein kinase, MDA: Malondialdehyde, mRNA: Messenger RNA, mRNA: Messenger RNA, MS/MS:Tandem mass spectrometry, m/z:Mass-to-charge ratio, NCBI:National Center for Biotechnology Information, NR:NCBI non-redundant protein database, PCA:Principal component analysis, PCR:Polymerase chain reaction, Pfam:Protein families database, PGPR:Plant growth-promoting rhizobacteria, PLS-DA: Partial least squares discriminant analysis, POD: Peroxidase, qRT-PCR: Quantitative reverse transcription PCR, RNA: Ribonucleic acid, RNaseH: Ribonuclease H, ROS: Reactive oxygen species, SBS: Sequencing by synthesis, SOD: Superoxide dismutase, SwissProt: Swiss-Prot protein sequence database, TCA: Tricarboxylic acid, UHPLC: Ultra-high performance liquid chromatography, VIP: Variable importance in projection, AREB: ABA-responsive element binding protein, BAM1: BARELY ANY MERISTEM 1, BGLU5: β -glucosidase 5, CLE25 : CLAVATA3/EMBRYO SURROUNDING REGION 25 , DREB2 : Dehydration-responsive element-binding protein 2, HO1:Heme oxygenase 1, LEA: Late embryogenesis abundant, LOX:Lipoxygenase, NCED3: 9-cis-epoxycarotenoid dioxygenase 3, RPL29: Ribosomal protein L29

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Availability of data and materials

Competing Interests The authors declare no competing interests.

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