TRANSCRIPTOME ANALYSIS OF RESPONSE TO Pb STRESS IN ENDOPHYTE INFECTED RICE ROOT TISSUE

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Abstract. To study the molecular mechanism of endophyte infected rice root tissues response under Pb stress, transcriptome sequencing was performed on the root tissues of endophyte infected and uninfected rice seedlings for 1 d and 5 d after 100 μMPb$(NO_3)_2$ stress, and differentially expressed genes(DEGs) were analyzed. GO enrichment and KEGG pathway were used to annotate the function of DEGs. The results showed that endophytic infection increased plant height, above-ground dry weight, chlorophyll content and net photosynthetic rate under Pb stress, but shortened root length. After 1 d of treatment, 9022 DEGs were screened, with 4641 being up-regulated and 4381 being down-regulated. After 5 d of treatment, 1902 DEGs were screened, with 958 being up-regulated and 944 being down-regulated. According to the GO functional enrichment analysis, DEGs were mainly enriched in oxidation-reduction process, cell wall organization and plasma membrane. KEGG enrichment analysis showed that DEGs were mainly enriched in glutathione metabolism, glycolysis / gluconeogenesis and phenylpropanoid biosynthesis and other pathways. The above study affords theoretical foundation for further revealing the molecular mechanism of endophytic infection enhancing the response of rice seedling to Pb stress.
Keywords: *bioinformatic, endophyte, Oryza sativa L., RNA-seq, lead stress*

Introduction

With the development of society and the improvement of industrialization, more and more soil has been polluted by heavy metals, among which Pb is a common heavy metal pollutant (Rodriguez et al., 2015). About 783,000 tons of Pb have been discharged into the environment globally in the previous 50 years (Singh et al., 2020). Many research studies have suggested that Pb can affect the growth of plants (Shah and Dubey, 1998; Yang et al., 2016), resulting in a large decline in crop yield (Li et al., 2016).

Endophyte involves bacteria and fungi that live in plant that have no adverse effect on the host plant, and they are mutualistic with their host (Gond et al., 2015). Endophyte can assist plants to obtain nitrogen, phosphorus, minerals, and other resources or regulate plant hormone levels to directly promote plant growth, or indirectly promote plant growth by decreasing the inhibitory effect of a range of pathogens on plant growth and development (Rifat et al., 2010; Ahemad and Kibret, 2014). Endophyte can also enhance plant resistance to abiotic stress (Himanshi and Wusirika, 2022). Endophytic organisms isolated from desert plants can improve the drought resistance of wheat by increasing soluble sugar and reducing the accumulation of malondialdehyde (Chen et al., 2017). Endophyte could alleviate the harm prompted by salt stress on Arabidopsis seedlings by adjusting osmotic pressure and antioxidant enzymes (Fan et al., 2020). Dark septate endophyte could reduce the accumulation of metals in the buds and roots of tomato seedlings and improved antioxidant enzymes, thus improving the tolerance of tomato seedlings to metals (Zhu et al., 2018). Endophyte can also increase the resistance of rice to Pb stress by enhancing the activity of antioxidant system, improving photosynthesis

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capacity, promoting organic acid accumulation, and increasing root absorption of mineral elements (Li et al., 2012, 2019).

Zhao et al. (2023) used high throughput sequencing technology (RNA-seq) to study root tissues of two rice varieties under Cd stress and identified many differentially expressed genes (DEGs) associated with metabolic pathways, biosynthesis of secondary metabolites and so on. Ren et al. (2022) used RNA-seq technology to reveal that endophyte could alleviate rice seedlings growth under Na_2CO_3 stress via regulating metabolic pathways such as enzyme activity, energy metabolism, biosynthesis, ROS-scavenging system, photosynthesis, and hormonal signaling. Based on transcriptome technology, our research group revealed that endophyte enhance the tolerance of rice to Pb stress by affecting oxidative detoxification, photosynthesis, signal transduction and hormone synthesis (Li et al., 2023).

Plant roots are the first barrier for plants to absorb or withstand heavy metal stress (Horton and Roberts, 2016), and the concentration of Pb accumulated in roots is greater than that in buds, leaves and fruits (Ahmed et al., 2021). Therefore, research on rice roots could better reveal the mechanism of endophyte enhancing rice response to Pb stress. Therefore, this study analyzed transcriptome data from endophyte infected rice root tissues under Pb stress, to furnish theoretical foundation for studying the mechanism of endophyte infected rice improving resistance to Pb stress at the molecular level.

Materials and methods

Preparation of rice seedlings and endophyte

In this study, the rice (*Oryza sativa* L.) seeds were sterilized by 1% sodium hypochlorite for 20 mins and washed with distilled water. Then these full seeds were dark soaking at 28℃ for 1 d, and germination at 30℃ for 1 d. After rice seeds exposed their radicle, the seeds were spread on yarn nets, which mounted on beaker fulled Hoagland solution. Seeds were cultured in a light incubator (light and dark 16 h/ 8 h, day and night temperature 28℃/26℃, light intensity 10000 lux, relative humidity 80%), and Hoagland solution was supplemented every day. When rice grew for 4 d, Pb stress and endophyte infection were treated.

Endophyte EF0801 isolated from *Suaeda salsa* is congeneric to *Sordariomycetes* sp. (99% similarity) and screened for Pb tolerance (Bu et al., 2012; Li et al., 2017). Endophyte EF0801 was transferred to potato liquid medium containing 125 mL PDA and cultured at 25±1℃, 125 r/min on a shaking table for 11 d.

Rice seedlings were divided into 2 treatments. Based on our previous studies (Li et al., 2012, 2023), we chose 100 μM$Pb(NO_3)_2$ concentration in this study. Pb stress treatment (Pb) was cultivated with Hoagland solution containing 100 μM$Pb(NO_3)_2$. The endophyte infection and Pb stress combined treatment (EPb) was cultivated with 5% EF0801 endophyte suspension and 100 μM$Pb(NO_3)_2$. Each treatment was repeated three times. Rice root tissues were frozen by liquid nitrogen on the 1st and 5th day of treatment, then root tissues stored at -80℃, until the RNA-seq and qRT-PCR verification.

Measurement of growth parameters

Ten rice seedlings were collected randomly in each treatment to measure plant height, maximum root length, fresh weight and dry weight of above-ground and under-ground

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parts. To measure the dry weight, the fresh weight samples were dried at 105℃ for 30 min and then at 80℃ until constant weight.

Determination of chlorophyll content and net photosynthetic rate

Refer to the method of Zhang and Qu (2004) to determine chlorophyll (Chl) content. The net photosynthetic rate (Pn) was measured by Li-6400 portable photosynthetic apparatus. The measurement time was 9:30-11:30 a.m. During the measurement, the airflow speed in the system was set to 500 $\mu mol \cdot s^{-1}$, and the light intensity was set to 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and indoor air circulation was maintained. The measurement was repeated 3 times for each treatment.

RNA extraction and sequencing

In this study, total RNA was extracted from root tissues using the RNA isolation Kit RN40 (Aidlab Bio Co Ltd, Beijing). NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE) was used to evaluate the concentration and purity of the extracted RNA, and RNA Nano 6000 Assay kit of Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA) was used to determine RNA integrity. After detecting, different libraries were clustered based on the quantity of target data and sequenced using the Illumina HiSeq^{TM} 2500 platform (Biomarker Technologies Co. Ltd, Beijing, China).

Bioinformatics analysis

Clean RNA-seq data was obtained after eliminating the low-quality reads and adapter sequences from the original data. DEGs was obtained by differential expression analysis of DESeq2 (version 1.6.3) among sample groups. The genes with fold changes (FC) ≥ 1.5 and false discovery rate (FDR) ≤ 0.01 were DEGs. Gene Ontology (GO) was applied to enrich DEGs, using GOseq R packages. Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to enrich DEGs by KOBAS software (http://www. genome.jp/kegg/). P-value ≤ 0.05.

qRT-PCR verification of DEGs

In order to prove that RNA-Seq was accurate, 8 DEGs (5 of them were treated for 1 d and the others were treated for 5 d) were selected for qRT-PCR verification. TubA is a ubiquitin extension protein homologous gene, and it has good stability (Li et al., 2016), so it was used as internal reference gene. Amplification was performed by LightCycler96 PCR system and LightCycler®480 SYBR Green I Master (Takara, Kyoto, Japan), and gene specific primers were designed by Biotechnology Information (NCBI) (*Table 1*). The relative gene expression was calculated by $2^{-\Delta\Delta Ct}$. The accuracy of RNA-Seq was assessed by analyzing whether the trend of qRT-PCR was coincident with that of RNA-Seq.

Statistical analysis

All data were the average of three replicates. One-way analysis of variance was used to calculate significant differences in growth parameters, Chl content and Pn. Data analysis was performed by SPSS 25.0, P-value ≤ 0.05.

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Table 1. *List of specific primers for qRT-PCR*

Treatment time	Gene	Forward primer（5'-3'）	Reverse primer（5'-3'）
	TubA	TCGCAGCATCAACCCAATC	GCAACCAGTCCTCACCTCAT
1 d	Os04g0679400	CCGTCTTTATCCCGTTCGCT	ACGGGATTTAGGATGGCACG
	Os10g0463800	TTGCTGAACGCCTGGTACTC	TTGTCCGCATTCCCTACCTC
	Os03g0103100	ACTACTGCCACAAGACCTGC	AAGTACGTGCGAAAGGGTTT
	Os03g0103200	ATCAACATCAACGTCCCCGT	CTGCAACACACAGACTAAGCC
	Os01g0294500	TCCACTCTCTGTACCGTCGT	ATGATAGACACCCAGCCGTG
5 d	Os06g0347700	GAGGCAAAGTTCTGGCAAGC	GCAAGCTCGTCTCCAGTTGA
	Os12g0571000	AACCCCTGCAACTGCTAAGA	CATTACACAGGGCACACTGG
	Os12g0568500	CAAGGCAGGTGAGTCTGGTG	TTTCCAGCCACGGACTTTTG

Results

Effects of endophyte on growth

Endophyte can alleviate the harm of Pb stress to rice seedlings and make the leaves more green (*Fig. 1*). As shown in *Table 2*, endophyte infection under Pb stress increased plant height by 0.33 times, Chl content by 0.51 times, and Pn by 0.41 times, and the differences reached extremely significant levels. The above-ground dry weight was significantly increased by 0.1 times. However, the root length was decreased by 0.49 times, and the difference reached extremely significant level.

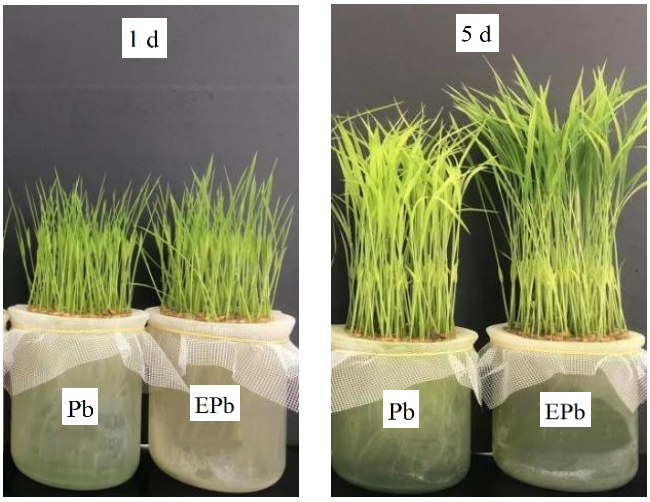

Figure 1. *Growth status of rice seedlings*

Table 2. *Changes of growth parameters, Chl content and Pn of endophyte infected rice seedlings under Pb stress, * and ** denote $p<0.05$ and $p<0.01$*

Treatment	Plant height (cm)	Root length (cm)	Above-ground dry weight (g)	Under-ground dry weight (g)	Chl content (mg/g·FW)	Pn (μmol/m²·s)
Pb	16.75±0.17	12.40±0.53	0.071±0.003	0.029±0.001	2.26±0.078	6.67±0.926
EPb	21.11±0.34	8.85±0.22	0.076±0.004	0.026±0.003	3.22±0.208	8.89±0.945
Fold Change	0.33**	-0.49**	0.10*	-0.16	0.51**	0.41**

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Quality control analysis of sequencing data

Transcriptome sequencing was performed to study endophyte infected rice root tissues response to Pb stress. The cDNA library was sequenced by Illumina high-throughput sequencing platform, and the obtained transcriptome sequencing data were analyzed for quality control (*Table 3*). The percentage of Q20 base and Q30 base reached 97.32% and 92.51%. It could be seen that the transcriptome of this sample had good sequencing quality, and the data would be utilized for subsequent bioinformatics analysis.

Table 3. *Statistics of sequencing data*

Treatment time	Sample	Clean reads	Clean bases	GC(%)	Q20(%)	Q30(%)
1 d	Pb1	24760784	7394944990	51.95	97.96	94.29
	Pb2	26685523	7971360240	51.77	97.96	94.26
	Pb3	25358479	7568160924	51.83	97.76	93.8
	EPb1	23476795	7024946986	51.86	97.98	94.33
	EPb2	22831112	6823509566	51.22	97.75	93.81
	EPb3	24206217	7237161278	51.26	97.85	94.04
5 d	Pb1	19669411	5874242278	51.19	97.32	92.51
	Pb2	25012148	7470503290	51.43	97.88	94.03
	Pb3	23979962	7160845018	51.03	98.05	94.47
	EPb1	20092475	6003773604	48.95	98.04	94.41
	EPb2	22574407	6749441364	50.53	97.93	94.21
	EPb3	27103733	8082701936	50.53	97.99	94.24

Analysis of DEGs

The screening criteria for DEGs were FC≥1.5 and FDR<0.05. After 1 d of treatment, 9022 DEGs were screened, with 4641 being up-regulated and 4381 being down-regulated (*Fig. 2A*). After 5 d of treatment, 1902 DEGs were screened, with 958 being up-regulated and 944 being down-regulated (*Fig. 2B*). It is observed that, the number of DEGs of 5 d treatment was significantly less than that of 1 d treatment.

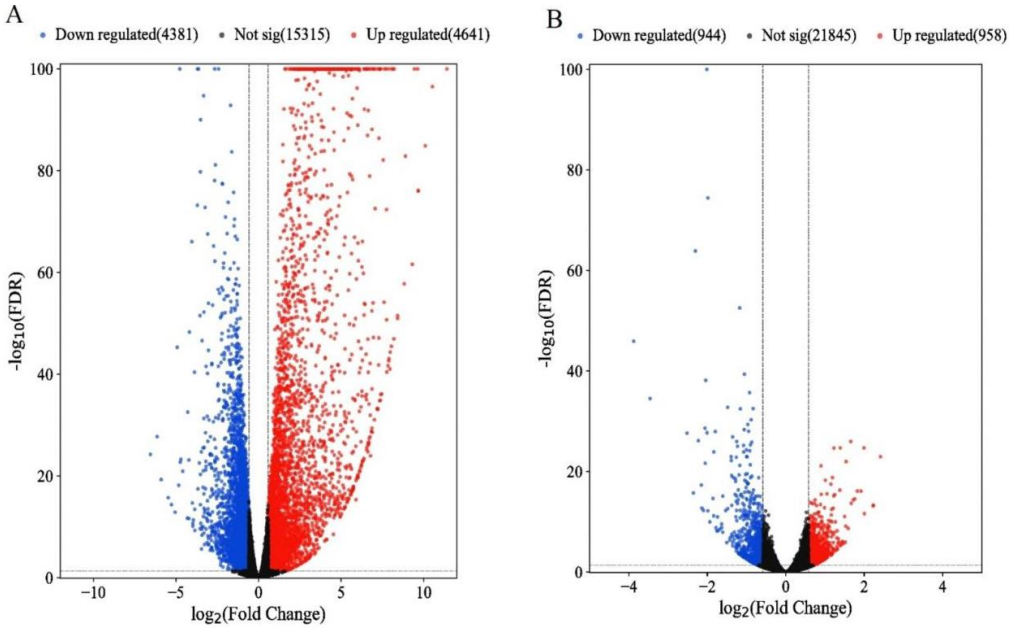

Figure 2. *Volcano map of DEGs*

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As shown in Venn diagram, among all up-regulated DEGs, 188 DEGs were identical after 1 d and 5 d of treatment, and 4453 DEGs were specific of 1 d and 770 were specific of 5 d (*Fig. 3*). Among all down-regulated DEGs, there were 222 identical DEGs, and the specific DEGs were 4159 and 722, respectively.

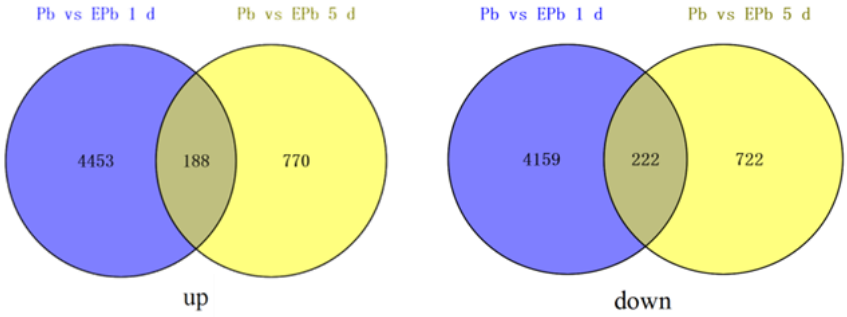

Figure 3. *Venn diagram of DEGs*

GO enrichment analysis of DEGs

To determine endophyte infected rice root tissues response to Pb stress, GO enrichment analysis was performed for DEGs. The GO annotation divides DEGs into biological process (BP), cellular component (CC) and molecular function (MF), and the DEGs induced by endophytic infection after 1 d and 5 d of treatment were shown respectively (*Fig. 4*).

After 1 d of treatment, DEGs associated with oxidation-reduction process (GO:0055114), carbohydrate metabolic process (GO:0005975), cell wall organization (GO:0071555), defense response (GO:0006952), plasma membrane (GO:0005886) and sequence-specific DNA binding (GO:0043565) had high enrichment frequency. After 5 d of treatment, DEGs associated with the oxidation-reduction process, translation (GO:0006412), cell wall organization, plasma membrane, heme binding (GO:0020037) and structural constituent of ribosome (GO:0003735) had high enrichment frequency. Oxidation-reduction process, cell wall organization and plasma membrane were mainly enriched in 1 d and 5 d treatment. Carbohydrate metabolic process, defense response was enriched in 1 d treatment, and structural constituent of ribosome was enriched in 5 d treatment. Most of these Go terms were associated with antioxidant and metabolism. The infection of endophyte resulted in higher enrichment of these processes, suggesting that endophyte improved the resistance of rice to Pb by influencing these processes.

KEGG enrichment analysis of DEGs

In 1 d and 5 d treatment, the top 20 of the most significant enrichment metabolic pathways were screened respectively (*Fig. 5*). After 1 d of treatment, 1589 DEGs were annotated to 128 metabolic pathways. DEGs associated with carbon metabolism (ko01200), glutathione metabolism (ko00480), beta−Alanine metabolism (ko00410), pyruvate metabolism (ko00620), glycolysis / gluconeogenesis (ko00010) and phenylpropanoid biosynthesis (ko00940) had high enrichment frequency. After 5 d of treatment, 365 DEGs were annotated to 112 metabolic pathways. DEGs associated with glutathione metabolism, glycolysis / gluconeogenesis, phenylpropanoid biosynthesis, biosynthesis of various secondary metabolites − part 2 (ko00998), nitrogen metabolism

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(ko00910) and ribosome (ko03010) had high enrichment frequency. In 1 d and 5 d treatment, the top 6 of the mainest enrichment metabolic pathways were revealed in *Table 4*. Glutathione metabolism, glycolysis / gluconeogenesis and phenylpropanoid biosynthesis were mainly enriched in 1 d and 5 d treatment. Carbon metabolism, beta−Alanine metabolism and pyruvate metabolism were mainly enriched in 1 d treatment. Biosynthesis of various secondary metabolites − part 2 pathways, nitrogen metabolism and ribosome were mainly enriched in 5 d treatment. Although these enrichment pathways were different, most of they were related to metabolism.

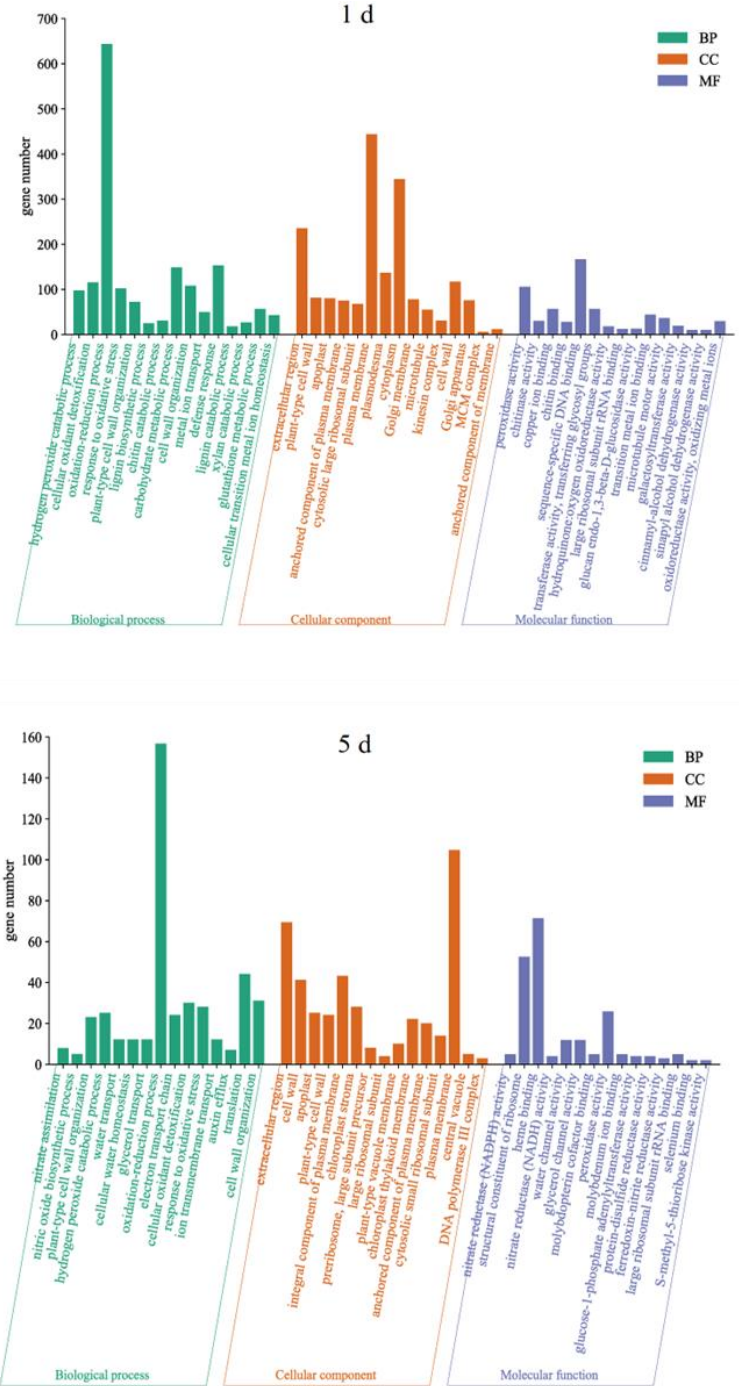

Figure 4. *Gene ontology (GO) enrichment of DEGs*

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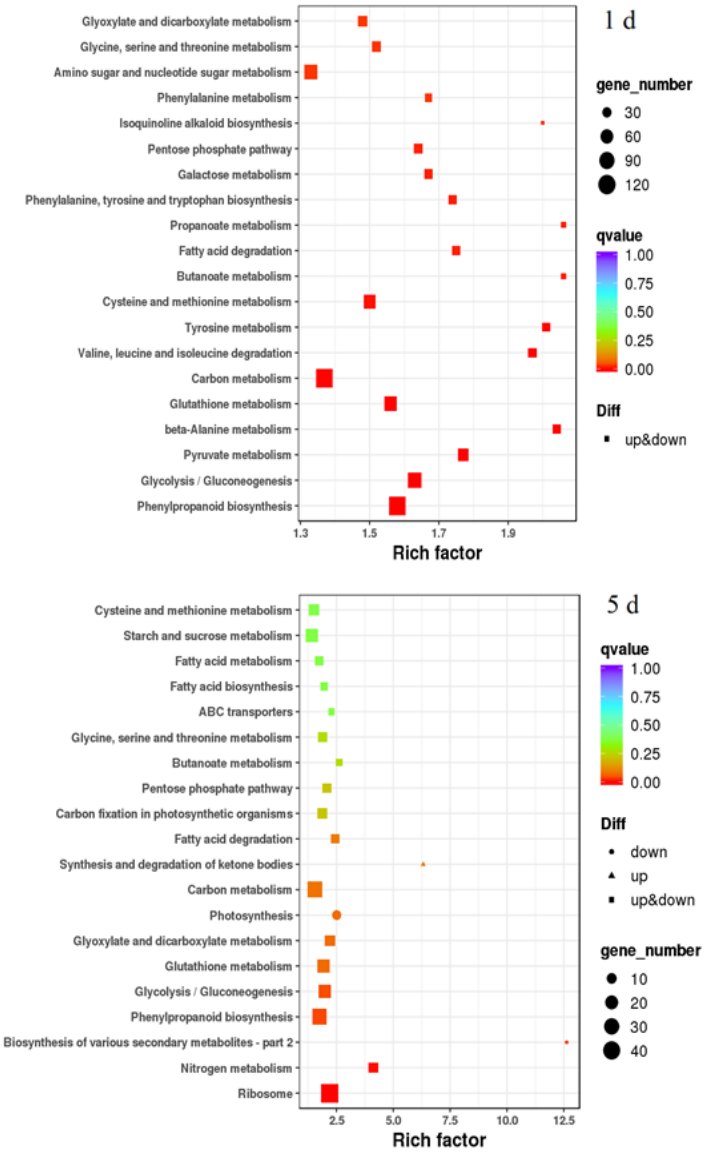

Figure 5. *KEGG enrichment of DEGs*

Table 4. *KEGG enrichment pathways*

Treatment time	KEGG ID	Annotated	Total DEGs	P value
1 d	ko01200	Carbon metabolism	122	3.55682E-05
	ko00480	Glutathione metabolism	67	2.42663E-05
	ko00410	beta−Alanine metabolism	30	1.32942E-05
	ko00620	Pyruvate metabolism	48	7.32202E-06
	ko00010	Glycolysis / Gluconeogenesis	75	1.17665E-06
	ko00940	Phenylpropanoid biosynthesis	121	5.15973E-09
5 d	ko00480	Glutathione metabolism	19	4.23E-03
	ko00010	Glycolysis / Gluconeogenesis	21	1.85E-03
	ko00940	Phenylpropanoid biosynthesis	31	1.29E-03
	ko00998	Biosynthesis of various secondary metabolites - part 2	3	7.94E-04
	ko00910	Nitrogen metabolism	11	4.53E-05
	ko03010	Ribosome	47	1.42E-07

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Upregulated DEGs in the phenylpropanoid biosynthetic pathway encodes seven enzymes, including: phenylalanine ammoniumlyase (PAL), cinnamic acid 4-hydroxylase (C4H), 4-coumarate-CoA ligase (4CL), ferulic acid 5-hydroxylase (F5H), cinnamoyl-CoA reductase (CCR), cinnamyl-alcohol dehydrogenase (CAD) and peroxidase (EC:1.11.1.7) (*Fig. 6*). In 1 d and 5 d treatment, the genes encoded phenylalanine ammonlyase (Os04g0518400) and peroxidase (Os07g0677100, Os12g0112000 and Os07g0638400) were up-regulated.

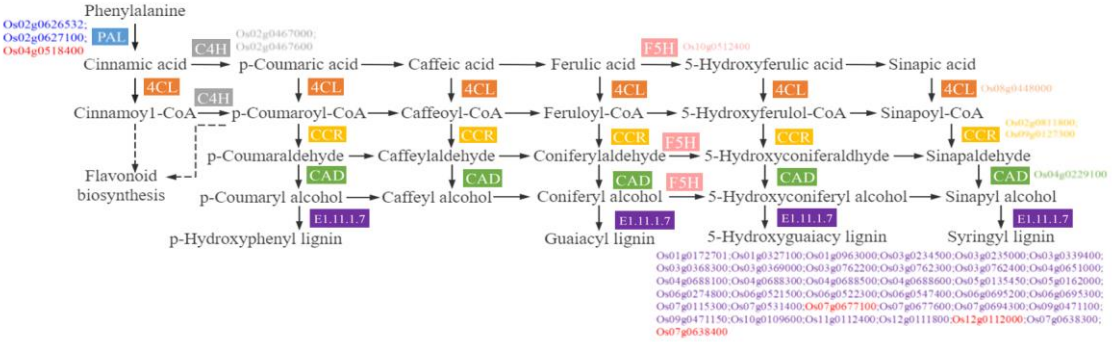

Figure 6. *Phenylpropanoid biosynthesis pathway. The annotated DEGs are up-regulated, and the color of the DEGs is the same as that of their encoded enzymes. Red DEGs are up-regulated genes after 1 d and 5 d treatment*

qRT-PCR verification

Eight DEGs were randomly selected for qRT-PCR verification, including three up-expressed genes (Os04g0679400, Os10g0463800 and Os06g0347700). Five down-regulated genes (Os03g0103100, Os03g0103200, Os01g0294500, Os12g0571000 and Os12g0568500) were detected. As shown in *Figure 7*, the expression changes of 8 selected DEGs were the same as the expression changes of genes in RNA-Seq (R^2=0.922), indicating high reliability of RNA-Seq.

Figure 7. *Validation of transcriptome sequencing data by qRT-PCR*

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Discussion

Pb stress inhibits plant growth (Shah and Dubey, 1998; Yang et al., 2016), while endophyte can accelerate plant growth and enhance plant resistance (Wu et al., 2021; Elshahawy et al., 2022). Similarly, this study demonstrated that plant height and above-ground dry weight of rice infected with endophyte increased significantly under Pb stress. While, with no significant difference of the under-ground dry weight, the root length decreased significantly. Endophyte can promote adventitious roots and lateral roots growth (Ban et al., 2017), so plants infected with endophyte do not need too long roots to absorb enough water. Endophyte could increase the photosynthetic capacity of plants under high NaCl concentration (Wang et al., 2019). In present study, Pn and Chl content of rice seedlings infected with endophyte increased significantly under Pb stress.

By analyzing the transcriptome of 1 d and 5 d samples, large amounts of the DEGs were detected, and the amount of DEGs in 1d treatment was greater than that in 5 d treatment. Ren et al. (2022) also demonstrated that endophyte infection under salt-alkali stress caused DEGs in rice root tissues, and DEGs of 1 d was significantly more than that of 5 d. This indicates that when rice root tissue is subjected to abiotic stress, endophytes can induce rice seedlings to rapidly alleviate the harm caused by abiotic stress in a short time.

Reactive oxygen species (ROS) is a group of chemicals that interact with multiple metabolites and cellular molecules, leading to irreversible metabolic abnormalities and cell necrosis (Ogbe et al., 2020). The antioxidant system of plants can resist oxidative stress, thereby temporarily clearing ROS (De Palma et al., 2021), and endophytic fungi can further improve the antioxidant mechanism of plants (Zhang et al., 2019). Plants infected with endophytic fungi had increased catalase, peroxidase, and polyphenol oxidase activities (Dastogeer et al., 2018). De Palma et al. (2021) revealed that tomato inoculated with endophyte could activate various antioxidant enzymes under water stress. Ren et al. (2021) showed that endophyte infection improved the activity of glutathione reductase, enhanced the ability to clear ROS, and thus improved the resistance of rice seedlings to Na_2CO_3 stress. Wang et al. (2021) demonstrated that phenylpropanoid can also remove ROS by adjusting related enzyme genes. In this study, glutathione metabolism and phenylpropanoid biosynthesis were enriched in KEGG enrichment analysis after 1 d and 5 d treatment, suggesting that endophyte could promote glutathione metabolism and phenylpropanoid biosynthesis and contributed to ROS clearance in rice. In addition, ROS can damage the membrane system of plants. In this study, plasma membrane was enriched in GO enrichment after 1 d and 5 d of treatment, suggesting that endophyte helped rice repair the damaged plasma membrane caused by ROS, by regulating plasma membrane related genes.

Cell wall is the outermost barrier of plant cells, which is an important tissue to protect cells from heavy metal poisoning. Phenylpropanoids can affect the stability of cell wall by affecting lignin synthesis (Deng and Lu, 2017). Shen et al. (2020) demonstrated that endophyte significantly increased the activity of some enzymes in maize under Cd stress, affected the expression and regulation of related genes in synthetic cell wall, and enhanced Cd compartmentation, thus reduced the toxicity of the Cd for maize. In this study, cell wall organization was enriched in GO enrichment after 1 d and 5 d treatment, and phenylpropanoid biosynthetic pathway was enriched in KEGG enrichment after 1 d and 5 d treatment, indicating that endophyte could reduce Pb stress influence on rice seedlings and improve their tolerance to Pb stress by regulating cell wall related genes.

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Metabolism is very important for plants, and endophytic infection also affects plant metabolism. Chen et al. (2020) demonstrated that infection by endophytic fungi improved the metabolic activity and survival of ryegrass. Cui et al. (2020) showed that endophyte *Phialocephala fortinii* promotes carbohydrate metabolism, lipid metabolism and secondary metabolite synthesis of *Rhodiola crenulata* at low altitudes. In GO enrichment analysis, carbohydrate metabolism process was enriched after 1 d treatment, structural constituent of ribosome was enriched after 5 d treatment. In the KEGG enrichment analysis, after 1 d and 5 d treatment, glycolytic / gluconeogenic mainly enriched. Carbon metabolism, beta−Alanine metabolism and pyruvate metabolism were enriched after 1 d treatment. Biosynthesis of various secondary metabolites − part 2, nitrogen metabolism and ribosome were enriched after 5 d treatment. They were closely related to metabolism and biosynthesis. All these indicated that endophyte accelerated and promoted the metabolism of rice root tissue at molecular level. Therefore, we hypothesized that endophyte infection coordinates the expression of various metabolism related genes, thus enhancing the tolerance of rice seedlings to Pb stress.

Conclusions

Endophyte infection could enhance the resistance of rice to Pb stress, and there were significantly more DEGs after 1 d treatment than after 5 d treatment (9022 and 1902). GO enrichment showed that DEGs were mainly enriched in cell wall organization, oxidation-reduction process and plasma membrane. KEGG enrichment showed that glutathione metabolism, glycolysis / gluconeogenesis and phenylpropanoid biosynthesis and other pathways were involved in the response to Pb stress in rice root tissue infected by endophyte.

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