Transcriptome analysis Cladophora rupestris absorption and response to Pb stress

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Abstract

This study aimed to analyze the transcriptome of C. rupestris under Pb stress by using high-throughput sequencing technology, observe the changes of gene expression and metabolic pathway after 3 and 5 days under 1.0 and 5.0 mg/L Pb2+ treatment, and analyze the differentially expressed genes (DEGs) and related functional genes. Metabolic pathways were revealed through Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Results show that DEGs increased significantly with the increase of Pb2+ concentration and stress time. A total of 32 genes were closely related to Pb stress response. GO analysis identified two major transporter proteins, namely, ATP-binding transport protein-related (ABC transporters) and zinc finger CCHC domain containing protein (Zfp) in C. rupestris. Pthr19248, pthr19211, Zfp pthr23002, Zfp p48znf pthr12681, Zfp 294 pthr12389, and Zfp pthr23067 played important roles against Pb infection and its absorption in C. rupestris. KEGG pathway analysis indicate that ABCA1, ATM, and ABCD3 were closely related to Pb absorption. Pb stress was mainly involved in metallothionein, plant hormone signal transduction, ABC transporters, and glutathione metabolism.

1. Introduction

Lead (Pb) is one of the most toxic pollutants and is a persistent environmental contaminant (Wang et al., 2020). Some plants such as *Rhus chinensis* Mill (*Anacardiaceae*) and *Conyza canadensis*. (Huang et al., 2017; Li et al., 2016) could over-accumulate heavy metals (HM), tolerance and detoxification HMs. *Cladophora rupestris* has a strong ability to absorb and tolerate Pb stress and alter the distribution of Pb at both the tissue and the sub-cellular levels to reduce toxicity (Cao et al., 2015a; Wang et al., 2021). However, the tolerance and detoxification of hyperaccumulate have complex mechanisms (Cao et al., 2015a; Liu et al., 2017; Chen et al., 2021a b). The overexpression of ATPase 3 (SpHMA3) increases the accumulation of Cd in non-hyper-accumulating ecotype of *Sedum alfredii* (Liu et al., 2017), and HM transporter plays an important role in detoxification of plants by sequestering toxic or excess HMs into the vacuoles (Sharma et al., 2016). The defense mechanism of *Cassia alata* is based on Cd accumulation in roots, coupled with increase in GSH (Silva et al., 2018). SpHMA1 functions as a chloroplast Cd exporter and prevents Cd accumulation; the hyperexpression of SpHMA1 contributes to Cd hypertolerance in *S. plumbizincicola* (Zhao et al., 2019). MT could enhance Pb accumulation in *Cladophora* (Cao et al., 2015a). The expression of MT, ATP-binding cassette (ABC) transporter, and zinc and manganese transporter genes depend on both the concentration of cadmium and exposure time (Cao et al., 2020). ABC transporters and HM ATPases (HMA) are involved in Cd transportation and sequestration in radish (Xu et al.,2020).

High-throughput RNA sequencing (RNA-seq) is rapidly emerging as a major quantitative transcriptome profiling platform (Wang et al., 2010; Zheng.et al. (2020) identified salt-responsive genes by using transcriptome sequencing in *Dunaliella viridis*. Li Y.et al. (2016) sequenced and analyzed the transcriptome of Tree Penoy Petals by using RNA-seq and investigated the molecular response of tree peony petals for grafting with two different rootstocks. Comparative transcriptome analysis was used to provide new insights into the mechanism of biodegradation and biosorption of SMZ/Cu^{2+} (Chen et al., 2020). Transcriptomic and physiological analyses were performed to enhance the understanding of cadmium accumulation and detoxification in *P. canescens* (He et al., 2013).

C. rupestris has some adsorptive ability and tolerance to Pb^{2+} (Cong et.al., 2014). Based on our previous research, *Cladophora* could accumulate Pb but not to Zn and Cu (Cao et al., 2015a, b). The mechanism underlying Pb detoxification by *C. rupestris* is interesting, but the mechanism remains unclear.

In the present study, we analyzed the transcriptome of *C. rupestris* under Pb stress by using high-throughput sequencing technology. Comparative transcriptome analysis of cells was conducted under different concentrations of Pb treatment and stress time. DEGs were analyzed, and the related functional genes and metabolic pathways were revealed through GO and KEGG analysis. These processes were conducted to clarify the difference in transcriptional responses of Pb. Furthermore, understanding on the genetic basis underlying these physiological processes would help reveal the mechanism of Pb biosorption and tolerance.

2. Materials And Methods

2.1. *C. rupestris* cultivation and preparation

C. rupestris was collected from surface water of a pond in Hefei in Anhui Province, China (31°50' N, 117°11'E). The configuration of the culture medium of *C. rupestris* was BBM, and the samples were grown in a sterile incubator at 25 °C (SPX-250B-G) with light intensity ranging from 3,000 to 4,000 lx (light and dark cycle of 12:12 h) and cultured at a pH value of approximately 7.5± 0.5 (Cao et al., 2015a,b).

2.2. Experimental design

2.0g samples were grown in 1000 ml BBM and contained various concentrations of $Pb(NO_3)_2$.The control group was treated without lead polluted and other experimental groups were treated $Pb(NO_3)_2$, and grown without antibiotics. Pb concentrations were set as 0, 1, and 5.0 mg/L, while Pb stress time was set as 3 and 5 days. The samples were numbered as Pb1_3 and Pb1_5 for the 1.0 mg/L treatment group on the third and fifth day, and Pb5_3 and Pb5_5 for the 5 mg/L treatment group on the third and fifth day, respectively. Three biological replicates were set up for each group,the culture solution was added every 2 days to prevent the losses from evaporation.

2.3. Total RNA extraction

After 3 and 5 days, *C. rupestris* samples were collected and rinsed with deionized water. Approximately 80 mg *C. rupestris* cells were with liquid nitrogen into powder,Total RNA was isolated from *C. rupestris*,Ethanol precipitation protocol and CTAB-PBIOZOL reagent were used for the purification of total RNA according to the manual instructions(Meng et al., 2018.)

2.4 mRNA Library Construction

mRNA was purified from total RNA by using Oligo(dT)-attached magnetic beads. Purified mRNA was fragmented into small pieces with fragment buffer at high temperature. First- and second-strand cDNA synthesis was generated using Shah's method (Shah et al., 2020.)

Microarray analysis was carried out using the Agilent Technologies 2100 Bioanalyzer. Microarray hybridization, washing, staining, scanning, and data processing were conducted by Beijing Genomics Institute (BGI, BGI-Shenzhen, China).

2.5 Differential expression analysis

The original sequencing data contained reads with low quality, joint contamination, and high unknown base nitrogen content. These reads were removed before data analysis to ensure the reliability of the results. For samples lacking reference genomes, clean reads were assembled to obtain a reference sequence (Shah. M et al.., 2020). Tgicl was used to cluster the transcript to remove redundancy. The DEG screening method is based on Poisson distribution, and all the data from Pb^{2+} treatments were quantified as fold change (FC) compared with the control (Wang et al. 2010). Genes were considered screened as DEGs when log2 FC $\geq$2 and Q-value $\leq$ 0.001.

2.6 Data analysis

The DEGs were classified and grouped by GO functional (http://geneontology.org) and KEGG pathway (http://www.genome.jp/kegg). Then, FDR correction was carried out for P value, and significant enrichment was considered at *Q* value $\leq$ 0.05.

The data were analyzed using Excel 2019 to average the sum of three parallel samples and expressed as mean ± standard deviation (SD). The DEGs was statistically analyzed with one-way ANOVA ($P < 0.05$). Duncan test was conducted with SPSS18.0, were tested using Pearson's coefficient to determine the presence of positive or negative correlations at *$P < 0.05$ or **$P < 0.01$.Originpro8.5 software was used to analyze data and create diagrams.

3. Results

3.1.Quality evaluation of sequencing reads

The quality assessment of sequencing reads was evaluated before the subsequent bioinformatics analysis (Li et al. 2020).As shown in Table 1, the filtered reads for each group were approximately 43 M, and the percentage of clean reads for each group was over 93%. Q20 and Q30 refer to the proportion of base with Phred scores >20 or 30 in the total bases, and these values were calculated to determine the quality of clean data (Meng et al., 2018; Wu et al., 2019). A high score ensures the accuracy and data quality. The average readings of the filtered Q20 and Q30 were approximately 97% and 90% of the original readings, respectively (Table 1). These findings indicate low quality base ratios and good sequencing quality for further Denovo assembly (Wu et al., 2019). Trinity assembly results show that each group had an N50 of over 1,200 and GC content of approximately 46%; no significant difference was observed between the control and treatment group, indicating high assembly quality and can be used for subsequent analysis (Cao et al., 2020; Wang et al., 2021).

3.2 Identification of DEGs responding to Pb^{2+}

3.2.1 Number of DEGs

DEGs with a difference multiple of more than two and a *Q* value≤0.001 were selected as significantly differential expressed genes (Wang et al., 2010). As shown in Fig. 1A, differential analysis showed that 21,893 genes were differentially expressed in *C. rupestris* under Pb^{2+} treatment.

According to Fig. 1A, DEG analysis showed that compared with the control group, the expression levels of 1400 unigenes in *C. rupestris* exposed to 1.0mg/L Pb at day 3 were up-regulated, whereas the expression levels of 6,815 unigenes were downregulated, and while the expression levels of 3,783 were upregulated and 4,312 were downregulated when exposed to 5.0 mg/L Pb for 3 days,this time *Cladophora* could accumulate Pb (Cao et al., 2015a), and the Pb concentration in the cell wall was 558.9 and 1661.2 mg/kg, organelle was 231.5 and 286.3 mg/kg ,soluble fraction was 103.2 and 258.6 mg/kg when Pb level was 1.0 mg/L and 5.0 mg/L(Wang et al., 2021).The number of upregulated gene increased significantly with the increase of Pb^{2+} concentration, reaching 3,783, and these genes may play important roles in tolerance to Pb stress (Meng et al., 2018). Therefore, Pb stress at 5.0 mg/L concentration had a more obvious effect than that at 1.0 mg/L concentration on *C. rupestris* (Meng et al., 2018). These DEGs may play a key role in the stress process (Cao et al., 2020; Zheng et al., 2020). The comparison results imply the differences in gene expression in *C. rupestris* exposed to Pb^{2+}, and the number of DEGs increased significantly with the increase of Pb^{2+} concentration, and this result was consistent with the findings of Cao et al. (2015a) and Chen et al. (2021ab).

Furthermore, Pb^{2+} exposure time had a considerably effect (Fig. 1A). A total of 11,169 upregulated and 7,670 downregulated genes were differentially expressed in 1.0 mg/L Pb after 3 and 5 days, and 8,393 upregulated and 10,732 downregulated genes were differentially expressed in 5.0 mg/L pb comparisons 3d and 5d. DEGs increased significantly with the increase of Pb^{2+} stress time, indicating significant difference in stress time of Pb to *C. rupestris*, and 1,869 common DEGs was found in Pb1_3vsCK, Pb1_5vsCK, Pb5_3vsCK and Pb5_5vsCK groups (fig.1.B), this DEGs play a key role in the stress process (Cao et al., 2020).

3.2.2 Functional annotation of MT related genes

As shown in Table 2, DEGs were annotated through Swissprot (https://www.uniprot.org/) and Pfam (http://pfam.xfam.org) databases, and eight MT-related genes were annotated. Three MT genes were upregulated, including PEC family and two MTs, indicating that CL7373.Contig1_All (FC was 1.64), Unigene37386_All (FC was 3.5), and Unigene2683_All (FC was 1.39) might play a key role in *C. rupestris* response to Pb stress, and these genes can be used as key candidate genes (Cao et al., 2020).

3.2.3 GO enrichment analysis of functional DEGs

The GO enrichment analysis results of DEGs in Pb1_5 vs Pb5_5 were classified according to molecular functions (MF), biological processes (BP), and cell components (CC). To identify the candidate genes for Pb^{2+} stress resistance in *C. rupestris*, the GO items with the most significant (filtering with *Q* value ≤ 0.05) enrichment were selected from each GO classification for analysis (Fig. 2a).

The terms of ribosome (GO:0005840) were dominant in cell components, those of structural constituent of ribosome (GO:0003735) and peptidase activity (GO:0008233) were mainly involved in molecular function, while those of translation (GO:0006412) and transmembrane transport (GO:0055085) were mainly involved in biological processes.

As shown in Fig. 2, the ribosome and structural constituents of ribosome were dominant in CC and MF respectively, was indicate that the gene about protein synthesis and regulation was significantly affected by pb^{2+} stress (Xie et al., 2020). In addition, peptidase activity, protein-containing complex, cAMP-dependent protein kinase complex, intein-mediated protein splicing, glutamine biosynthetic process, protein stabilization, protein heterodimerization activity, glutamate-ammonia ligase activity, and cAMP-dependent protein kinase regulator activity and the like polypeptide associated genes was significantly affected by Pb^{2+} stress. MT concentration in *Cladophora* increased as the Pb concentration increased (Cao et al., 2015a). Non-protein thiols (NPT), GSH, and phytochelatins (PCs) increased significantly with increasing Pb stress in the cell wall and soluble fraction (Chen et al., 2021a), suggesting that MT, NPT, GSH, and PCs are important candidate genes for Pb^{2+} stress resistance in *C. rupestris* (Cao et al., 2015a; Chen et al., 2021a). Genes related to GSH (GSH1 and GSH2) and PCs (PCS1 and PCS2) were also increased in apx1-3 plants subjected to Pb stress (Jiang et al., 2017).

Genes related to photosynthesis, such as chloroplast thylakoid membrane (GO:0009535), photosystem I (GO:0009522), photosystem (GO:0009523) in CC, photosynthesis, light harvesting in photosystem I (GO:0009768), and response to light stimulus (GO:0009416) in BP (Fig. 2), were significantly affected by Pb^{2+} stress, indicating that the photosynthesis of *C. rupestris* was influenced by Pb^{2+} stress, and the controlling genes were GO:0009535, GO:0009522, GO:0009523, GO:0009768, and GO:0009416. Cao et al. (2015a) reported that photosynthesis is highly sensitive to Pb, and chlorophyll content is negatively correlated with Pb^{2+} concentration, supporting the experimental results in this paper.

Transfer-related genes, such as transmembrane transport, structural constituent of ribosome, copper ion import, ferric iron import across cell outer membrane, transferase activity, and transferring phosphorus-containing groups were significantly affected by Pb^{2+} stress (Fig.2). We identified two ABC transporter genes, including ATP-binding transport protein-related pthr19248 (EUGRSUZ_A00112; EUGRSUZ_A00046;A0A2I2YPV7; ABC transporter E family member 2; Kcr_0070;103639168; 101778969; UMAG_03351; LOC100184673; BRADI_3g33470v3; LOC104606445; 100217065; GSPATT00016840001; EUGRSUZ_A00294; CL6EHI_156200; CL6EHI_150540; EHI_150530; W5MR47; ATRLI2; TVAG_249850; A0A287NTT5;A0A287JRE8 Q93YY0 M1A0G7 THAPSDRAFT_bd1652; POPTR_004G235900; 100804088;SELMODRAFT_413898; PRUPE_1G366500;PRUPE_3G017900; SORBI_3004G128100; SS1G_06617;AMTR_s00066p00025940 and so on),and atp-binding transport protein-related pthr19211(UMAG_04152;CND02420 and the like), which was the largest subfamily and the dominant ABC transporter gene subfamily in *C. rupestris*. The ABC-B1 subfamily group proteins from systems involved in the uptake of organic and inorganic ions (Saurin et al., 1999) enhanced Pb tolerance mainly by activating the expression of the ABC-type transporters (Jiang et al., 2017). Most flax ABC transporters participate in

ATP binding, transport, ATPase activity, and metal ion binding (Khan et al., 2020). These genes are the most likely candidates for Pb accumulation in *C. rupestris*.

Transcription factors of Zfp are differentially expressed during Pb stress to *C. rupestris*, and four kinds of transcription factors might be associated with Pb.Zfp pthr23002(A0A2I2YNE6;UMAG_11314; v1g120512; ZCCHC13; SNOG_11691; UMAG_11258; BRADI_4g18203v3; POPTR_010G092600; SORBI_3009G088550; SORBI_3009G098801; SORBI_3009G080150; SORBI_3009G085250; SORBI_3009G133201; SORBI_3009G072366; SORBI_3009G099450; SORBI_3009G109775; SORBI_3009G092850; SORBI_3009G085051; SORBI_3009G071401; SORBI_3009G080266; SORBI_3009G064250; CNBP; NCU05800; LOC107769619; W5N9C9; ZCCHC13; ANIA_05111; LOC107796219; Tb11.01.1270; PRUPE_1G269000; PGTG_10593;SORBI_3001G275950 100823164 SORBI_3001G384900 H3GI81 LSAT_4X8860 CL6EHI_055640 CNE02090 SORBI_3009G091980; SORBI_3009G095480; SORBI_3009G095560;DAPPUDRAFT_112160;A0A2I2YYV6;101784076; CNBP; ZEAMMB73_Zm00001d011718; GSPATT00016774001; GLYMA_18G125100 ;SELMODRAFT_450823; F7EJV2;SS1G_04861 and so on),Zfp p48znf pthr12681(F2E763; EUGRSUZ_A01194; YALI0_D07018g; LOC107791523; ANIA_06922; HannXRQ_Chr17g0559121; LSAT_8X20961 CNK01650 W4Z699 ZC3H15;GSPATT00004747001;POPTR_005G167200;100783113;F6W619;SORBI_3001G353600; SORBI_3003G346700; THAPSDRAFT_4543 ;BRAFLDRAFT_288490;PF3D7_1244800; PRUPE_1G511400 and so on),Zfp 294 pthr12389(G3QDL6;ltn1 W5MRG2 CL6EHI_190430 CNE03250 LOC104597233;G3QDL6; 8030755; ANIA_06404; TVAG_343770; TCM_042247; A0A3B6N2V3; AMTR_s00007p00201600; 29273;BATDEDRAFT_33847 100775576 ;SS1G_07566 and so on), and Zfp pthr23067(A0A2I2ZRX7). An HM transporter cDNA, ZNT1(Zfp), was cloned from *Thlaspi caerulescens* through functional complementation in yeast and mediated high-affinity Zn and low-affinity Cd uptake (Pence et al., 2000). Our results were consistent with previous reports on annotated sequences matching with Zfp proteins of *C. rupestris*, indicating that the protein regulate dthe absorption and migration of Pb in *C. rupestris* (Pence et al., 2000). The genes may play important roles in the Pb absorption of *C. rupestris* (Cao et al. 2015a).

3.3 KEGG analysis of DEGs in *C. rupestris* under Pb stress

KEGG analysis was performed to characterize the pathway enrichment of the identified DEGs, amd these genes were classified into including cellular processes, organismal systems, environmental information processing, genetic information processing, and metabolism. A total of 7,845 differential genes were observed in Pb5_5vsPb1_5, and 2,919 differential genes were annotated to 121 KEGG pathways, among which 30 metabolic pathways were significantly enriched (Fig. 3A).

3.3.1 DEGs in ABC transporters

A total of 31 DEGs of ABC transporters (ko02010) were detected from *C. rupestris* treated with 1.0 and 5.0 mg/L Pb, 13 genes were upregulated, and 18 genes were downregulated (Fig. 3B), including two ABCA1 genes, one ABCA3 gene, two ABCB1genes, one ABCB8 gene, 15 ABCC1 genes, two ABCC2 genes, one ABCD3 gene, six ABCG2 genes, and one ATM (mitochondrial ABC transporter) gene. Among them, 17 DEGs include CL2046. Contig1_All, CL2046.Contig2_All, Unigene15678_All, and Unigene28062_All were up-

regulated (|log2FC| ≥ 2, Table 3). These genes were associated with absorption of Pb in *C. rupestris* (Zhang.et al 2019).

ABC transporter proteins were present in almost all prokaryotic and eukaryotic cells (Saurin et al., 1999), and many ABC genes were involved in HM tolerance (Wang et al.2020). In the present study, the induction of ABCC1 and ABCG2 transporter proteins was more pronounced during Pb stress in *C. rupestris*, ABCA1, ATM, and ABCD3 were upregulated when the Pb concentration was increased (Fig. 3C), while ABCC2, ABCB1, ABCB8, and ABCB10 were all downregulated. Besides, ABCA3, ABCC1, and ABCG2 were up- and down-regulated. All 31 ABC transporter proteins belong to six subfamilies, namely ABCA, ABCB, ABCC, ABCD, ABCG, and ATM; ABCB, ABCC, and ABCG played important roles in HM detoxification (Shi et al., 2020). The differential expression of ABC transporters in *C. rupestris* under Pb stress suggests that it needs to regulate its tolerance through up- or down-regulation of transporter protein activity (Wang et al., 2020). Hence, ABCC1 and ABCG2 transporter proteins were more pronounced during Pb stress in *C. rupestris*, and ABCA1, ATM, and ABCD3 were also closely related to Pb absorption.

3.3.2 Identification of genes in GSH metabolism

Based on KEGG pathway analysis (Fig. 3B), 34 DEGs were identified and were involved in several processes in the GSH metabolism, and 17 DEGs had log2Fold change≥ 2 . These genes encoded different types of enzymes, such as isocitrate dehydrogenase, glucose-6-phosphate 1-dehydrogenase, 6-phosphogluconate dehydrogenase, glutathione S-transferase, and ribonucleoside-diphosphate reductase subunit M2. CL168.Contig2_All, CL3367.Contig4_All, Unigene17078_All, Unigene28066_All, Unigene53785_All, Unigene54130_All, Unigene55336_All, and Unigene55337_All were upregulated. These genes were associated with plant defense response to Pb stress in *C. rupestris* (table 3).

GSH plays a central role in the plant tolerance against metals, can bind and help Pb^{2+} enter the cell (Chen et al., 2021b), and was a key antioxidant that acts as a cofactor for glutathione S-transferase (Rojas et al., 2014). GSH was the most important non-enzymatic intracellular defense mechanism against reactive oxygen species (ROS) and controls the plant response to toxic elements (Chen et al., 2021b). In addition, it acts as a substrate for divalent cations such as Pb^{2+}, where GSH acts as a precursor for PCs and as a substrate for GSH metabolizing enzymes to protect the -SH group in the enzyme (Anjum et al .2012). In the present study, 34 DEGs were annotated into the GSH metabolic pathway and were mainly divided into dehydrogenase and GSH S-transferase. The formation of GSH-Pb conjugate(GS-Pb) is dependent on GSTs and plays an important role in Pb detoxification (Sousa et al .2015; Chen et al., 2021b). The upregulation of GSH transferase helped in promoting the formation of GSH-Pb, which enhances Pb detoxification (GS-Pb). In addition, ABCC proteins transport substrates act as GSH conjugates (Langmead al., 2009). Therefore, up-regulated changes in ABCC transporter proteins contribute to the transport of GSH conjugates. In summary, Pb binding to GSH was catalyzed by glutathione transferase, followed by GS-Pb binding to the vesicle via the ABCC transporter (Sousa et al .2015).

3.3.3 Identification of peroxidase-related genes

As shown in Fig. 3B, 72 DEGs were annotated into the peroxisomal pathway through KEGG enrichment analysis, 35 genes were up-regulated, 37 genes were down-regulated, and 10 of these genes are involved in the encoding of SOD, CAT, epoxide hydrolase, and a class of peroxidases, which may play a key role in oxidative stress of *C. rupestris* under Pb stress(Cao.et al,2015a).

Under Pb stress, SOD and POD enzyme activities in *Pogonatherum crinitum* leaves were increased, which could reduce the cell membrane damage (Hou et al., 2018). SOD and CAT in *Datura stramonium Linn* under Cd stress showed an increasing trend at the beginning of the treatment and gradually decreased with time. Excessive Cu stress increased SOD and ROS activity in *Closterium ehrenbergii* cells (Wang et al., 2020). In the present study, 72 DEGs were annotated to the peroxisomal pathway, and 10 DEGs were identified as related to SOD, CAT, and other enzymes, which may be related to the oxidative stress response in *C. rupestris* response to Pb stress (Cao et al.,2015a).

3.3.4 Identification of phenylpropanol biosynthesis gene

A total of 116 DEGs were annotated into phenylpropanol synthesis pathway (Fig. 3B), and 47 DEGs were upregulated and 69 were downregulated. Phenylpropanol is a natural plant compound commonly extracted from phenylalanine (Michal G,2012), which plays an important role in the response of plants to a variety of biotic and abiotic stresses (Vogt. T,2010). Cinnamyl alcohol dehydrogenase and cinnamoyl coenzyme a reductase were identified from DEGs (Table 2). cinnamyl alcohol dehydrogenase included Unigene55182_All, Unigene54923_All, and Unigene53862_All; and cinnamoyl coenzyme a reductase included Unigene53942_All, Unigene13418_All, and Unigene10106_All.In addition, the branching biochemical reactions of phenylpropanoid biosynthesis, and protect plants from Pb-induced oxidative stress by scavenging H_2O_2 and reactive free radicals (Casta et al., 2009).

3.3.5 Hormone signaling and Mitogen-activated protein kinase（MAPK）signaling genes

As shown in Table 3, two genes were identified which were related to growth hormone among plant hormones (Unigene34841_All and CL263.Contig32_All), while many genes were involved in MPAK transduction, amd two representative genes (Unigene3664_All and Unigene55189_All) were selected with FC≥2. Environmental stressors are delivered to target transcription factors (TFs) via the hormone signalling pathway and MAPK signaling pathway (Lin et al., 2012). Heavy metal-related hormones include abscisic acid, growth hormone, jasmonic acid, and salicylic acid (Yan et al., 2013). Pb-organic acids to resist the toxicity of Pb^{2+}(Chen et al., 2021b). The expression of MAPKs and GSH metabolism genes was up-regulated under HM stress (Agrawal.et al,2003). The MAPKs signaling pathway plays a key role in regulate crosstalk between plant signalling systems as a means of facilitating adaptation, and coordination of plant responses to various stressors (Smekalova V et al,2014).

3.4 Pb absorb and response pathways in *C. rupestris*

As shown in the Figure 4, combined with the above analysis, when Pb^{2+} entered the cell via a transporter such as ABC transporters and zinc finger protein (Zheng et al., 2020), Pb^{2+} was sent to the vesicles storage and isolation by MT, GSH, PCs, and Pb-organic acids to resist the toxicity of Pb^{2+} in *C. rupestris*, SOD and

CAT were increased through the expression of peroxidase genes. On the other hand, Pb^{2+} affects the phenylpropanoid biosynthesis and hormone cellular transmission processes in *C. rupestris*, which involve the synthesis of anthocyanins, carotenoids and flavonoids, Pb-induced oxidative stress by scavenging H_2O_2 and reactive free radicals (Casta et al., 2009). At the same time, Pb^{2+} enter cell causes changes in phytohormone concentrations, induced of GSH,via hormone signaling,MAPK signalling, and ROS interacts with phytohormones , protection against and mitigation of Pb stress (Wang.et al,2020).

4. Conclusion

The filtered reads for each group were approximately 43 M, and the percentage of clean reads for each group was over 93%. Q20and Q30 were approximately 97% and 90% of the original readings respectively,this indicates high assembly quality and can be used for subsequent analysis.

Pb stress caused changes in DEGs increased significantly with the increase of Pb^{2+} concentration and stress time. Moreover, 35 genes closely related to Pb stress response were screened. Three metallothionein-related genes were identified as candidate genes in Swissprot and Pfam databases, namely CL7373.Contig1_All, Unigene37386_All, and Unigene2683_All. GO analysis identified two major transporter proteins, namely, ABC transporters and Zfp in *C. rupestris*. Pthr19248, pthr19211, Zfp pthr23002, Zfp p48znf pthr12681, Zfp 294 pthr12389, and Zfp pthr23067 played important roles against Pb infection and its absorption in *C. rupestris*.

KEGG pathway analysis showed that ABC transporter proteins belong to six subfamilies, namely ABCA, ABCB, ABCC, ABCD, ABCG, and ATM, with some members of the subfamilies ABCB, ABCC, and ABCG playing important roles in Pb detoxification in different species. Moreover, ABCA1, ATM, and ABCD3 were closely related to lead absorption. Pb binding to GSH was catalyzed by glutathione transferase, followed by GS-Pb binding to the vesicle via the ABCC transporter. In addition, the analysis of the KEGG metabolic pathway indicate that glutathione metabolism, phenylpropanoid synthesis, hormone signalling and MAPK signalling pathways are important in the defense mechanism of *C. rupestris* under lead stress. A total of 32 key genes were also screened. This research will contribute to the development and study of HM remediation techniques for aquatic plants in the future.

Declarations

Ethical Approval

Not applicable.

Consent to Participate

We consent to participate this manuscript.

Consent to Publish

We consent to publish this manuscript.

Authors Contributions

Lei Liu: Investigation, Resources, Data curation, Writing - original draft, Lu-sheng Zhang have designed experimental scheme, Liu Yang Conceptualization, Methodology, did experiments,Writing - review & editing, Investigation, Resources,Qiu-yu Chen : Methodology, Writing - review & editing, Qian Zhang, Zhao-wen Liu, De-ju Cao: have collated and analyzed experimental data.

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Competing Interests

The authors declare that they have no conflict of interest.

Data accessibility

Data are deposited in the Mendeley Data Translated with www.DeepL.com/Translator (free version)

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Tables

Table 1

The reads and the quality index of Unigenes

Sample	Filtered reads(Mb)	Q20(%)	Q30(%)	Filtered reads radio(%)	N50	GC(%)
CK	42.5±0.2a	97.3±0.1a	90.1±0.3a	93.4±0.440a	1534±75ab	46.3±0.4a
Pb1_3	42.5±0.2a	97.2±0.1a	89.7±0.3a	93.341±0.4a	1584±28 b	46.1±0.5a
Pb1_5	43.2±0.1b	97.2±0.2a	89.8±0.5a	94.7±0.3b	1346±156a	46.7±0.9a
Pb5_3	43.3±0.1b	97.2±0.0a	89.8±0.1a	95.0±0.3b	1533±132ab	46.0±0.700a
Pb5_5	43.3±0.2b	97.4±0.4a	90.3±0.9a	95.1±0.4b	1380±131ab	46.101±0.3a

Note:"±" indicates mean ± standard deviation, and different letters in each column indicate differences between treatment groups (same letters indicate non-significant differences $p > 0.05$, different letters indicate significant differences $p < 0.05$, Duncan);Q30,Q20 refers to the proportion of base with Phred scores >30 or 20 in the total bases.A high score ensures the accuracy and data quality.

Table 2

Metallothionein-related genes

gene	Fold change	Subject Annotation
CL7373.Contig1_All	1.64	Metallothionein
Unigene11338_All	-1.2	Yeast metallothionein
Unigene23676_All	—	Metallothionein
Unigene2683_All	1.39	Plant PEC family metallothionein
Unigene37386_All	3.5	Metallothionein
Unigene8627_All	-1.0	Metallothionein
Unigene36067_All	—	Metallothionein-like protein
Unigene47760_All	—	Metallothionein-like protein

Note: The differential multiple is positive, which means the expression level is up-regulated; the differential multiple is negative, which means the expression level is down-regulated; the differential multiple is —, which means there is no change before and after

Table 3

Candidate gene of KEGG analysis

Pathway name	Pathway Id	Up genes	Note
ABC transporters	ko02010	CL189.Contig3_All(ABCA1) CL189.Contig9_All(ABCA1) Unigene3596_All(ABC.ATM) Unigene42923_All(ABCG2)	ABCA1:ATP-binding cassette, subfamily A (ABC1), member 1 ABC.ATM:mitochondrial ABC transporter ATM ABCG2:ATP-binding cassette, subfamily G (WHITE), member 2
GSH metabolism.	ko00480	CL168.Contig2_All(IDH1, IDH2, icd) CL3367.Contig4_All(G6PD, zwf) Unigene17078_All(PGD, gnd, gntZ) Unigene28066_All(GST, gst) Unigene53785_All(PGD, gnd, gntZ) Unigene54130_All(RRM2) Unigene55336_All(RRM2) Unigene55337_All(RRM2)	IDH1,IDH2,icd: isocitrate dehydrogenase [EC:1.1.1.42] G6PD,zwf:glucose-6-phosphate 1-dehydrogenase [EC:1.1.1.49 1.1.1.363] PGD,gnd,gntZ:6-phosphogluconate dehydrogenase [EC:1.1.1.44 1.1.1.343] GST, gst:glutathione S-transferase [EC:2.5.1.18] RRM2:ribonucleoside-diphosphate reductase subunit M2 [EC:1.17.4.1]
Peroxisome	ko04146	CL2228.Contig2_All SOD CL3513.Contig1_All CAT Unigene16634_Al EPHX4) Unigene1686_All PRDX Unigene28141_All PRDX Unigene2866_All SOD Unigene37952_All CAT Unigene54054_All SOD Unigene682_All CAT Unigene8435_All SOD	SOD:superoxide,dismutase CAT:catalase EPHX4:epoxide hydrolase 4 PRDX peroxiredoxin
Phenylpropanoid biosynthesis	Ko00940	Unigene53942_All CCR Unigene55182_All E1.1.1.195	CCR cinnamoyl-CoA reductase E1.1.1.195:cinnamyl-alcohol dehydrogenase

		Unigene54923_All E1.1.1.195 Unigene13418_All CCR Unigene53862_All E1.1.1.195 Unigene10106_All CCR	
Plant hormone signal transduction	ko04075	Unigene34841_All ARF CL263.Contig32_All ARF	ARF auxin response factor
MAPK signaling pathway - plant	ko04016	Unigene3664_All PAF1 Unigene55189_All MPK4	PAF1 RNA polymerase II-associated factor 1 MPK4 mitogen-activated protein kinase 4

Figures

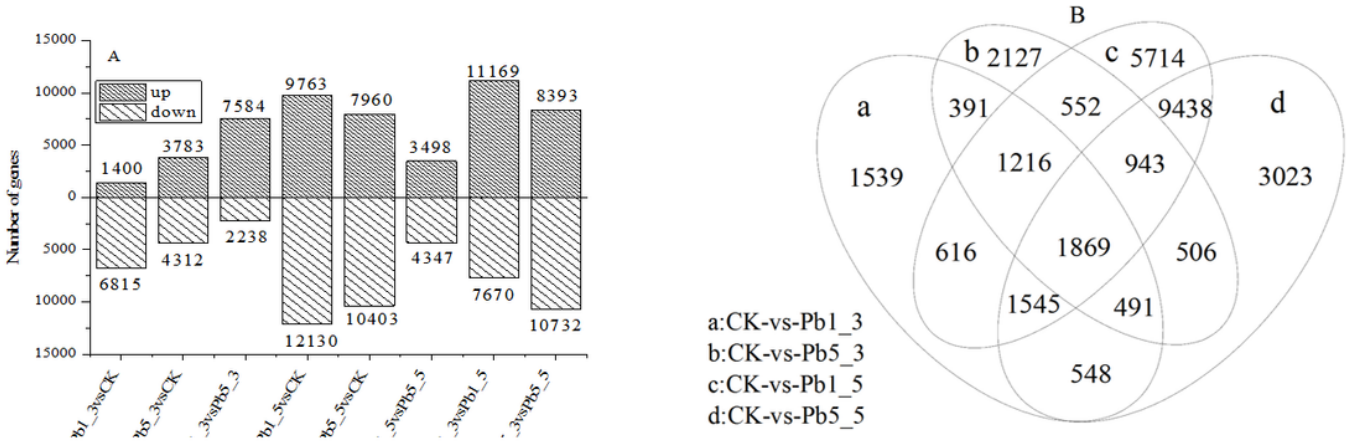

Figure 1

DEGs of *C. rupestris* under Pb

Note:(A) under different concentration of Pb; (B) Venn diagram Common DEGs

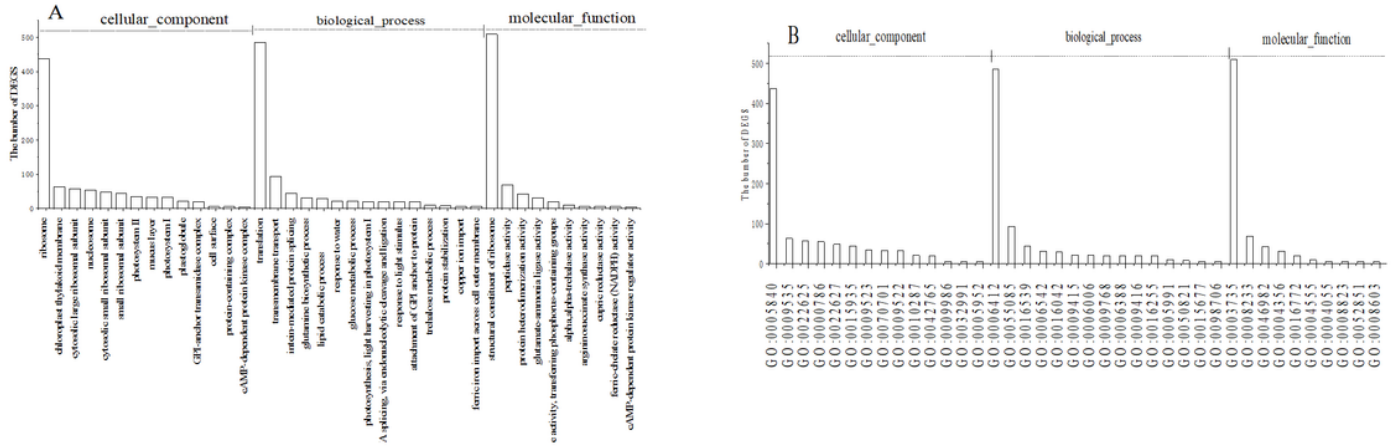

Figure 2

GO annotation of the DEGs. (A, GO classification of the DEGs.B, GO term ID of the DEGs)

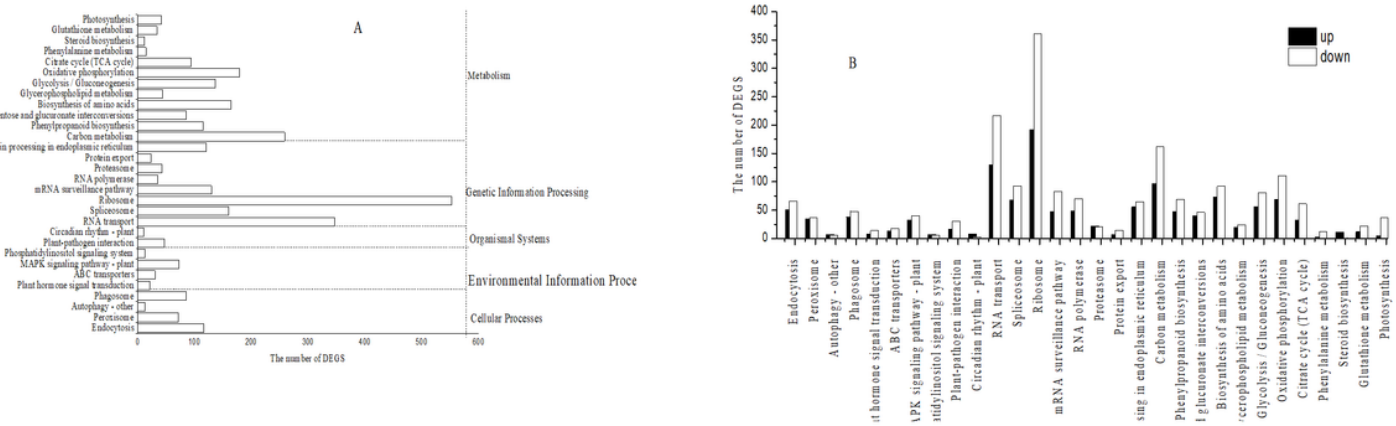

Figure 3

KEGG analysis of the identified DEGs

Note A was KEGG classification of DEGs; B was Up-regulated and Down-regulated DEGs of every pathway

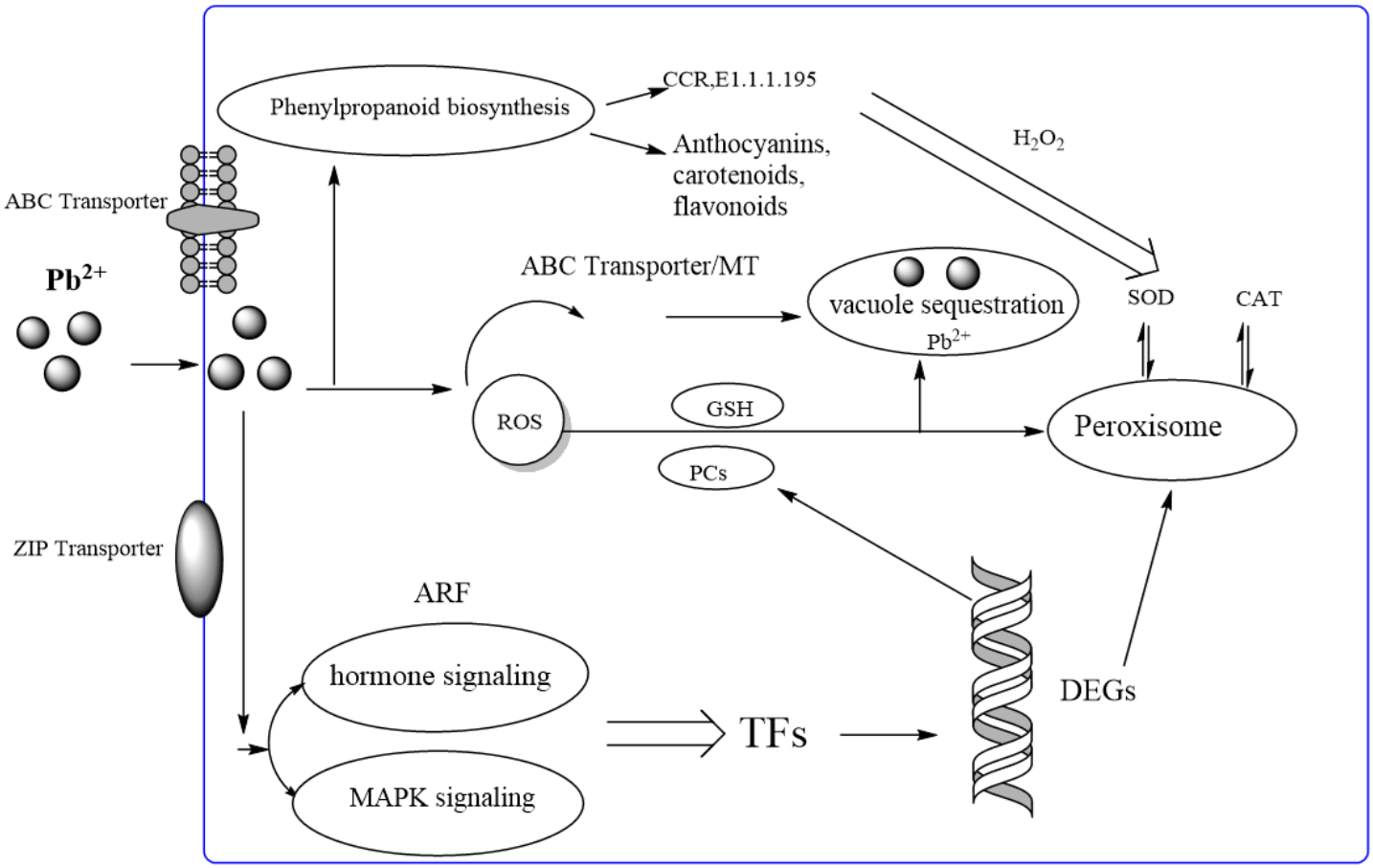

Figure 4

Pb absorb and emergency response of *C. rupestris*

Note:CCR cinnamoyl-CoA reductase E1.1.1.195:cinnamyl-alcohol dehydrogenase; GSH, glutathione; TF, transcription factors; ROS, reactive oxygen species;ARF auxin response factor; DEGs, differentially expressed genes;MT:Metallothionein;PCs:phytochelatins.