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Flavonoid metabolism plays an important role in response to lead stress in maize at seedling stage

Zanping Han[1*†], Yan Zheng[1†], Xiaoxiang Zhang[2], Bin Wang[1], Yiyang Guo[1] and Zhongrong Guan[3]

Abstract

Background Pb stress, a toxic abiotic stress, critically affects maize production and food security. Although some progress has been made in understanding the damage caused by Pb stress and plant response strategies, the regulatory mechanisms and resistance genes involved in the response to lead stress in crops are largely unknown.

Results In this study, to uncover the response mechanism of maize to Pb stress phenotype, physiological and biochemical indexes, the transcriptome, and the metabolome under different concentrations of Pb stress were combined for comprehensive analysis. As a result, the development of seedlings and antioxidant system were significantly inhibited under Pb stress, especially under relatively high Pb concentrations. Transcriptome analysis revealed 3559 co-differentially expressed genes(co-DEG) under the four Pb concentration treatments (500 mg/L, 1000 mg/L, 2000 mg/L, and 3000 mg/L $Pb(NO3)_2$), which were enriched mainly in the GO terms related to DNA-binding transcription factor activity, response to stress, response to reactive oxygen species, cell death, the plasma membrane and root epidermal cell differentiation. Metabolome analysis revealed 72 and 107 differentially expressed metabolites (DEMs) under T500 and T2000, respectively, and 36 co-DEMs. KEGG analysis of the DEMs and DEGs revealed a common metabolic pathway, namely, flavonoid biosynthesis. An association study between the flavonoid biosynthesis-related DEMs and DEGs revealed 20 genes associated with flavonoid-related metabolites, including 3 for genistin and 17 for calycosin.

Conclusion In summary, the study reveals that flavonoid metabolism plays an important role in response to Pb stress in maize, which not only provides genetic resources for the genetic improvement of maize Pb tolerance in the future but also enriches the theoretical basis of the maize Pb stress response.

Keywords Maize, Pb stress, Transcriptome, Metabolome, Flavonoid biosynthesis

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Introduction

Maize (*Zea mays* L.) is a crucial crop that can serve multiple purposes, such as food, fodder, feed, and as an industrial raw material. With the development of industry, corn yields are also increasing; however, new crises and challenges have emerged, such as environmental pollution and climate change [1, 2]. Maize is exposed to many pollutants, which are abundant in the environment and enter plants through soil [3] or the atmosphere [4], where they affect the production of crops. Among pollutants, Pb is one of the most toxic and the most abundant [5]. The productivity of ten different nonferrous metals in China was about 58 million tons in 2018, which included 5.11 million tons of Pb [6]. Pb contents tend to be higher in mining areas. In the Baiyinhua mining area, Inner Mongolia, China, the Pb content in the soil is 29.9 $mg{\cdot}kg^{-1}$ [7]. In the Dabaoshan mining area, Guangdong Province, China, the Pb content in the soil ranges from 56.43 $mg{\cdot}kg^{-1}$ to 138.03 $mg{\cdot}kg^{-1}$, while the Pb content in the control is about 6 $mg{\cdot}kg^{-1}$ [8]. Pb in agricultural soils can not only results in changes in soil microorganisms and soil fertility [9], but also cause oxidative stress in plants through the replacement of essential metals and nutrients, reducing plant growth, water balance, and photosynthesis, resulting reduced yield [10]. Additionally, Pb can be absorbed and enriched by plants and can pass through the food chain to humans [11], resulting in damage to nerves, kidneys, blood, bones, and immune and reproductive systems [2]. Therefore, deciphering the mechanisms of Pb absorption, enrichment, and regulation in maize is crucial.

Plants absorb Pb through at root surfaces in soil, and accumulate in different plant tissues [12], which depends on H^+/ATPase pump activity to maintain its gradient potential in rhizoderm cells [13]. Most plant species have adopted a mitigation strategy to accumulate approximately 95% of the Pb absorbed through roots, while only a minute proportion is translocated to shoots and leaves, as reported for *V. unguiculata* [14], *Nicotiana tabacum* [15], *Lathyrus sativus* [16], and *Zea mays* [17]. The accumulation of Pb inhibits the germination and growth of plants [18]. Pb stress inhibits seed germination has been reported in many plants, such as *Brassica juncea* [19], *Phaseolus vulgaris* [20], and *Zea mays* [21]. When plants are exposed to Pb stress, the development of roots and aerial parts is attenuated, especially in roots, probably due to increased Pb accumulation [22, 23]. Previous studies have shown that the reasons for the retardation of seed germination and plant growth under Pb are as follows: inhibited enzyme activity [24], disruption of plant water status [25], disruption of nutrient uptake [26], photosynthesis inhibition [27], disruption of cell division [28], oxidative stress [29], and lipid peroxidation [30]. To survive in soils, plants adopt several mechanisms to mitigate Pb stress, including cell wall adsorption and obstruction [31], excretion of metal ions into extracellular spaces [32], cellular sequestration [32], an increase in nonenzymatic antioxidants [33], and an increase in metal-binding ligands [34]. Although the Pb stress response has been studied extensively in plants, the mechanism and key genes involved in the Pb response in maize are largely unknown.

Plants tolerate abiotic stresses through a cascade of complex biological processes involving a series of molecular and physio-biochemical changes [35, 36]. Traditional phenotypic identification is macroscopic, a comprehensive reflection of various changes, and cannot accurately reflect the internal mechanism of tolerance to abiotic stresses; therefore, physio-biochemical analysis, transcriptome analysis, and metabolome analysis are widely used for identifying abiotic stress in plants [26, 37]. A multiomics approach involving differential profiling of the transcriptome and metabolome in *Vitis quinquangularis* in response to aluminum (Al) stress revealed that the phenylalanine metabolic pathway could play a crucial role in alleviating Al stress in *Vitis quinquangularis* [38]. Similarly, integrated *omics* analysis confirmed that rice leaves respond to high saline–alkali stress by engaging ABC transporters, dicarboxylate metabolism, glyoxylate, amino acid biosynthesis, and glutathione metabolism [39]. Similarly, salt stress could be tolerated by alleviating phenylpropanoid biosynthesis, starch and sucrose metabolism, plant hormone signal transduction and alpha-linolenic acid pathways in *Beta vulgaris* [40].

In this study, to reveal the response mechanism to Pb stress and identified the candidate genes related to Pb tolerance in maize, we performed phenotypic identification, physiological and biochemical analyses, transcriptome analysis, and nontarget metabolome analysis under a series of Pb concentration gradients in the elite inbred line HCL624. As a result, the antioxidant system was significantly inhibited under Pb stress, and the membrane system was destroyed. The results of GO analysis of DEGs were also consistent to the results of physiological and biochemical analysis. Additionally, KEGG analysis of the DEMs and DEGs revealed a common metabolic pathway, namely, flavonoid biosynthesis, which plays an important role in response to Pb stress. Our findings will not only provide genetic resources for the genetic improvement of maize Pb tolerance but also enriches the theoretical basis of the maize Pb stress response.

Materials and methods

Plant material and treatments

A maize inbred line, Henan university of science and technology corn line 624 (HCL624), which was cultivated by Henan University of Science and Technology, was used to exploit the Pb stress response in maize at

the seedling stage. Seeds of HCL624 were sown in quartz sand under optimum growth conditions (16 h light/8 h darkness) at a temperature of 25/22℃. Seedlings grown to the two-leaf stage were transplanted into Hoagland solution for 3 days for adaptable growth. Then, the plants were divided into 5 groups for different degrees of Pb stress, which were watered with solutions of different lead concentrations, namely, 0 mg/L (control), 500 mg/L, 1000 mg/L, 2000 mg/L, and 3000 mg/L $Pb(NO_3)_2$. Two days later, they were sampled for physiological characteristics, transcriptome analysis, and metabolome analysis.

Phenotypic and physiological measurements and pb content of maize

7 days after different degrees of Pb treatment (0 mg/L, 500 mg/L, 1000 mg/L, 2000 mg/L, and 3000 mg/L), the performance of the seedlings was photographed, and the shoot length and root length were measured. Three biological replications were used for statistical analysis. Data processing and management were conducted using Microsoft Excel (Microsoft Office Excel 2019, Microsoft Corporation) and the R statistical language (version 4.3.2), and the comparisons between the groups were done by Dunn multiple comparisons.

2 days after different degrees of Pb treatment (0 mg/L, 500 mg/L, 1000 mg/L, 2000 mg/L, and 3000 mg/L), whole-plant-dried samples were collected and analyzed, and various biochemical parameters, including superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and hydrogen peroxide (H_2O_2), as well as malondialdehyde (MDA) content, were determined according to the instructions of the reagent kit, and the information of the kits is as follows: SOD activity (Nanjing Jiancheng Bioengineering Institute, Nanjing China, kit code: A001-4-1), CAT activity (Nanjing Jiancheng Bioengineering Institute, Nanjing China, kit code: A007-1-1), POD activity (Nanjing Jiancheng Bioengineering Institute, Nanjing China, kit code: A084-3-1), H_2O_2 content (Nanjing Jiancheng Bioengineering Institute, Nanjing China, kit code: E004-1-1), and MDA content (Nanjing Jiancheng Bioengineering Institute, Nanjing China, kit code: A003-1-1). For each trait analysis, three biological replicates were used, and each biological replicate included 3 technical replicates. Data processing and management were conducted using Microsoft Excel (Microsoft Office Excel 2019, Microsoft Corporation) and the R statistical language (version 4.3.2), and the comparisons between the groups were done by Dunn multiple comparisons.

To quantify Pb^{2+} uptake and absorption in plant tissues, the roots and shoots of plants under different Pb gradient stress treatments (500 mg/L, 1000 mg/L, 2000 mg/L, and 3000 mg/L) were to harvest (in triplicate, $n=3$), dried at 65℃ and then digested by treatment with HNO_3. Thereafter, Pb^{2+} uptake was quantified by ICP–OES 5110 VDV (Agilent Instruments Inc. State of California, USA). General settings are as follows: RF power: 1120 w; flush pump speed: 50 r/min; analytical pump speed: 50 r/min; Auxiliary gas flow: 0.50 L/min; integral time: 30 s; times of repetition: 3; analytical wavelength: 220.35 nm. Data processing and management were conducted using Microsoft Excel (Microsoft Office Excel 2019, Microsoft Corporation) and the R statistical language (version 4.3.2), and the comparisons between the groups were done by Dunn multiple comparisons.

Transcriptomic sequencing and data analysis

2 days after different degrees of Pb treatment (0 mg/L, 500 mg/L, 1000 mg/L, 2000 mg/L, and 3000 mg/L), total RNA was extracted from the roots of HCL624 plants using TRIzol reagent (Thermo Fisher Scientific, USA, kit code: 15596018CN) and then treated with an RNeasy Mini Kit (Tiangen Biochemical Technology (Beijing) Co., LTD, Beijing, China, kit code: 217004) to harvest higher-quality total RNA. Three biological replicates were performed for transcriptome analysis. The prepared RNA libraries were loaded on the Illumina NovaSeq TM 6000 platform by LC Biotechnology Co., Ltd. (Hangzhou, China). Adapter sequences were trimmed, and poor-quality reads were filtered out using FastQC (v0.10.1, https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and RSeQC Toolkit (v4.0.0, http://code.google.com/p/rseqc/) to obtain clean read data. The obtained clean reads were aligned to the maize B73 genome 26 (RefGen_v4, www.maizesequence.org) and the transcriptome using HISAT2 (v2.2.1, http://ccb.jhu.edu/software/hisat2/index.shtml). Gene count quantification and normalization were performed using TopHat2 and Cufflinks, and digital gene expression values were determined in terms of fragments per kilobase million (FPKM) values. Thereafter, the DESeq2 (http://bioconductor.org/packages/stats/bioc/DESeq2/) was used with filter fold change ($|log2FC| \geq 1$ and $q<0.05$) to determine the set of genes differentially regulated in Pb-treated maize seedlings.

To further explore the functional aspects of the identified set of differentially expressed genes (DEGs), Gene Ontology (GO) terms were determined, and enrichment analysis (GSEA) was subsequently performed according to a previous study [41]. The p value was determined based on false discovery rate (FDR) correction with 0.05 as the threshold. Finally, GO terms with a false discovery rate (FDR) ≤ 0.05 were selected for subsequent analysis. Pathway enrichment was performed through the KEGG (Kyoto Encyclopedia of Genes and Genomes) online resource as described in previous studies [42, 43]. The adjusted P value (*P.adj*) value was determined based on FDR correction (FDR ≤ 0.05).

Sample extraction and measurements for metabolomic analysis

2 days after different degrees of Pb treatment (0 mg/L, 500 mg/L, and 2000 mg/L), the roots of HCL624 plants were collected for metabolomic analysis. Collected profiling samples were homogenized and thawed on ice, and 20 μL of each sample was dissolved in 120 μL of precooled 50% methanol buffer. The prepared mixture was vortexed for 1 min and then incubated for 10 min to ensure maximum metabolite extraction. These mixtures were then kept at -20℃ overnight. To prepare for metabolite quantification, the mixtures were centrifuged at 4000 × g for 20 min, and the resulting supernatant was transferred to a semiskirted 96-well plate (with 5 technical repeats, $n=5$). Thereafter, the samples were subjected to LC-MS analysis. To ensure the quality of the procedures, a pooled quality control (QC) sample containing 10 μL of the reaction mixture was also prepared.

LC–MS analysis

A TripleTOF 5600 Plus mass spectrometer (SCIEX, Warrington, UK) was used to analyze all the control and treated samples. Metabolites in these samples were analyzed and separated via chromatography using an ultra-performance liquid chromatography (UPLC) system (SCIEX, UK). Metabolite separation was performed in reversed-phase using an ACQUITY UPLC T3 column (100 mm × 2.1 mm, 1.8 μm, Waters, UK). During metabolite separation, two different solvents were used for the mobile phase. Two solvents with specific formulations were used (Solvent A: water, 0.1% formic acid; Solvent B: acetonitrile, 0.1% formic acid). The gradient elution conditions (flow rate of 0.4 ml/min: 5% solvent B for 0–0.5 min; 5-100% solvent B for 0.5–7 min; 100% solvent B for 7–8 min; 100-5% solvent B for 8–8.1 min; and 5% solvent B for 8.1–10 min) maintained at 35℃ were used to promote maximum metabolite separation.

The different metabolites were eluted based on their physiochemical properties and detected through a specified column fitted in a TripleTOF 5600 Plus system under specific conditions (curtin gas pressure=30 psi, gas1 pressure=60 psi, gas2 pressure=60 psi, and interface heater=650℃). Since the TipleTOF 5600 Plus system was run in both ion modes, the spray floating voltage was adjusted accordingly (for positive ions=5 kV, for negative ions = -4.5 kV). TOF mass spectrometer data (60-1200 Da) were collected. Repeated scans of the built-in metabolite library were performed with customized settings (total cycle time=0.56 s, pulse frequency=11 kHz and a multichannel TDC detector at 40 GHz, dynamic exclusion=4 s). To maintain quality throughout the whole data acquisition duration, mass accuracy calibrations were performed after every 20 measurements, while a quality check (QC) sample was also analyzed every 10 samples to evaluate the stability of the LC–MS.

Metabolomic data processing

The platform-generated metabolite profiling data from LC–MS were preanalyzed with XCMS software (https://sciex.com/products/software/xcms-plus-software) to generate the raw data points. These data points were first transferred to the mzXML format. Further data processing involved R environment-compatible packages such as XCMS, CAMERA and metaX (http://metax.genomics.cn/). Each metabolite was identified based on critical data attributes relevant to retention time, peak area and m/z. The intensity of each peak was determined by analyzing a three-dimensional matrix in which peak attributes (retention time-m/z pairs) were arbitrarily assigned. This information was processed for all samples, and variable names were retrieved. The information generated was cross-matched, and annotation profiles were generated for all detected metabolites using public databases such as KEGG (http://www.genome.jp/kegg/) MetaCyc (https://metacyc.org/smarttables) and HMDB (http://hmdb.ca). To further improve the peak quality attributes, the metaX tool was used to retrieve missing peaks based on the k-nearest neighbor algorithm.

Irrelevant differences observed in metabolomics datasets could lead to false detection and thus deterioration of the overall analysis quality. Orthogonal least squares-discriminant analysis (OPLS-DA) can aid in determining variance while removing irrelevant differences. Through the OPLS-DA model, variable importance in projection (VIP) can be identified from different sets of treatments. QC data based on the order of injection and signal intensity over a specific time drift were used to fit the QC-based robust LOESS signal. To ensure quality and data stability, QC samples with a standard deviation≥30% were removed. Data normalization was performed using QC-robust spline batch correction for the QC samples, while for the remaining samples, normalization was performed using a probabilistic quotient algorithm. The significance of pairwise comparisons was determined using Student's t-test, and the P value calculated was adjusted using FDR (Benjamini–Hochberg) correction. Differentially regulated metabolites in response to Pb stress were identified using fold change (FC≥1.5 or FC≤1/1.5) along with a VIP≥1. In addition, a supervised PLS-DA was performed (VIP cutoff=1.0) to detect group-specific differentially regulated metabolites.

Spearman's correlation coefficient for correlation analysis between gene expression and metabolic abundance

To reveal the association between key genes and target metabolites, we used Spearman's correlation coefficient for correlation analysis between gene expression and

metabolic abundance. Finally, hub genes were further filtered based on absolute correlation (>0.8) and adjusted P value (<0.01).

Results

Phenotypic and physiological characteristics of maize under pb stress

To uncover the Pb stress on the development of the maize, about 2-leaf stage seedlings were treated with lead solutions with different concentrations (CK, 500 mg/L, 1000 mg/L, 2000 mg/L, 3000 mg/L). One weeks after treatment, the first leaf of the seedlings under different concentrations of lead showed withered, while the CK showed still green (Fig. 1A). Additionally, the length of shoot and root was also measured. The results showed that, the length of shoot was significantly inhibited when the lead content was above 2000 mg/L (Fig. 1B), while the root length was significantly inhibited when the lead content was above 1000 mg/L. Additionally, the seedlings under 3000 mg/L Pb stress showed the weakest performance and wilted (Fig. 1A-C).

Abiotic stress disturbed the overall metabolic balance of ROS scavengers, leading to oxidative stress. Therefore, physiological measurements of maize under Pb stress were performed. As a result, the activity of SOD was significantly down-regulated when the lead concentration of solution is greater than or equal to 1000 mg/L (Fig. 2A). The activity of CAT was significantly down-regulated when the lead concentration of solution is greater than or equal to 2000 mg/L (Fig. 2B). The activity of POD was significantly down-regulated under the Pb treatments, however, the activity of POD under 2000 mg/L Pb treatment showed no difference to the control (Fig. 2C). Moreover, the net accumulation of H_2O_2 and MDA was significantly increased under Pb stress (Fig. 2D-E), while there were no differences between the different Pb contents. The Pb content in roots and shoots under different degrees of Pb stress was also detected. The results showed that, the plants under 2000 mg/L Pb treatment had the height Pb contents (93.38 g/kg) in the root, while the plants under 500 mg/L and 1000 mg/L Pb treatments have the lowest Pb contents (58.21 g/kg, 61.71 g/kg) in the root (Fig. 2F); the plants under 3000 mg/L Pb treatment had the height Pb contents (5.36 g/kg) in the shoot, while the plants under 500 mg/L and 1000 mg/L Pb treatments have the lowest Pb contents (0.21 g/kg, 0.29 g/kg) in the shoot (Fig. 2G). Additionally, 15-fold (3000 mg/L) to 280-fold (500 mg/L) greater Pb uptake in roots than in shoots (Fig. 2F-G).

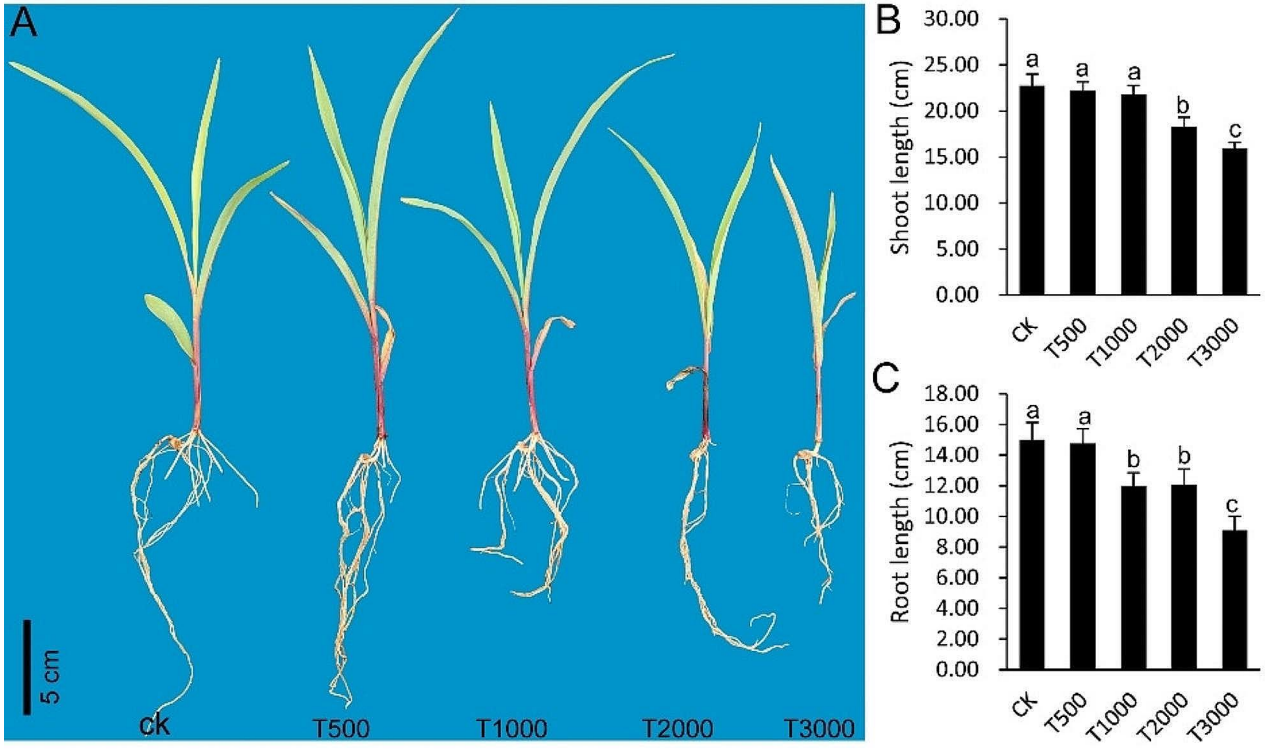

Fig. 1 The phenotype of HCL624 under different contents of Pb stress. (**A**) The performance of HCL624 after one week of different contents of Pb treatment. (**B**) The shoot length of HCL624 after one week of different contents of Pb treatment. (**C**) The root length of HCL624 after one week of different contents of Pb treatment

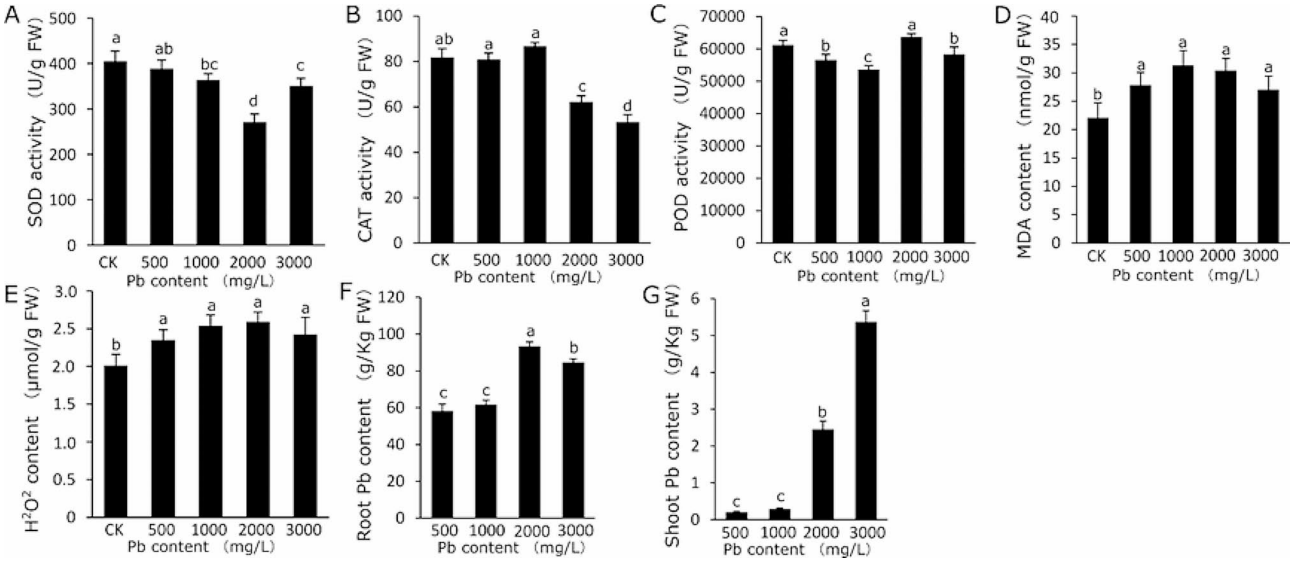

Fig. 2 Physiological response of maize plants under Pb stress. (**A-C**) SOD, CAT and POD activities under different concentrations of Pb stress in maize. (**D-E**) MDA and H_2O_2 contents of maize under Pb stress. (**F-G**) Pb content under Pb stress in maize. Mean comparisons denoted using similar letters were not found to be significant according to *Duncan's* multiple range test ($P < 0.05$)

Transcriptomic analysis of maize roots under different degrees of pb stress

To elucidate the response mechanism to Pb stress, RNA-seq analysis of roots at 2 days of 4 degrees of Pb stress (500 mg/L Pd $(NO_3)_2$, 1000 mg/L Pd $(NO_3)_2$, 2000 mg/L Pd $(NO_3)_2$, 3000 mg/L Pd $(NO_3)_2$ and the control (0 mg/L Pd $(NO_3)_2$)) was performed. The raw reads were obtained and trimmed to remove adapter sites, followed by quality control filtering to obtain high-quality clean read data. The average percentage of clean reads was 94.9%, the average Q20 (percentage of bases with mass values greater than or equal to 20) was 99.0%, and the average Q30 (percentage of bases with mass values greater than or equal to 30) was 95.5%. The average GC content of these clean read data was 50.5% (Table S1), which suggested that the cleaned read data could be used for further analysis. Principal component analysis (PCA) represents the degree of variability among the samples and treatments. The PCA plot showed that variability among the control and treatment groups could be related to Principal component 2(PC2), while PC1 corroborated the degree of variability among the treatment groups (Fig. 3A). Different sets of DEGs were identified under 4 levels of Pb stress in maize. In total, 5836, 8484, 19,074 and 9038 DEGs were identified in maize under 500 mg/L, 1000 mg/L, 2000 mg/L, and 3000 mg/L Pd$(NO_3)_2$, respectively. Among them, 3625, 3951, 13,778, and 5115 genes were downregulated, and 2211, 4533, 5296 and 3923 genes were upregulated (Fig. 3B). Additionally, to identify genes that were co-upregulated or downregulated under 4 degrees of Pb stress, Venn diagram analysis was performed; 1762 genes were assigned as co-downregulated genes (Fig. 3C), and 1797 genes were assigned as co-upregulated genes (Fig. 3D).

To further explore the functional aspects of DEGs involved in the genetic regulation of the Pb stress response in maize, GO enrichment analysis of the co-upregulated or downregulated genes was performed. Under Pb stress, biological processes (such as response to stress, transmembrane transport, response to water deficit stress, response to salicylic acid, metal ion transport, and cell death) and molecular functions (such as transporter activity, DNA-binding transcription factor activity, metal cluster binding, ubiquitin-like protein transferase activity) were significantly enriched in the upregulated genes (Fig. 4A). Biological processes (such as cellular component organization or biogenesis, root system development, plant type cell wall organization, cell division and so on), cellular components (such as plasma membrane, cell wall, vacuole, cytoskeleton, ribosomal subunit, and Golgi cisterna membrane), and molecular functions (such as peroxidase activity, 2-alkenal reductase [$NADP^+$] activity, transmembrane receptor cellulose synthase (UDP-forming) activity, and miRNA binding) were significantly enriched in the downregulated genes (Fig. 4B).

Metabolic analysis of maize roots under different degrees of pb stress

To further reveal the mechanism underlying the metabolic response of maize to Pb stress, metabolome-based profiling was used to identify the differentially regulated metabolites. To predict a representative model, OPLS-DA was used to create a relationship interaction model

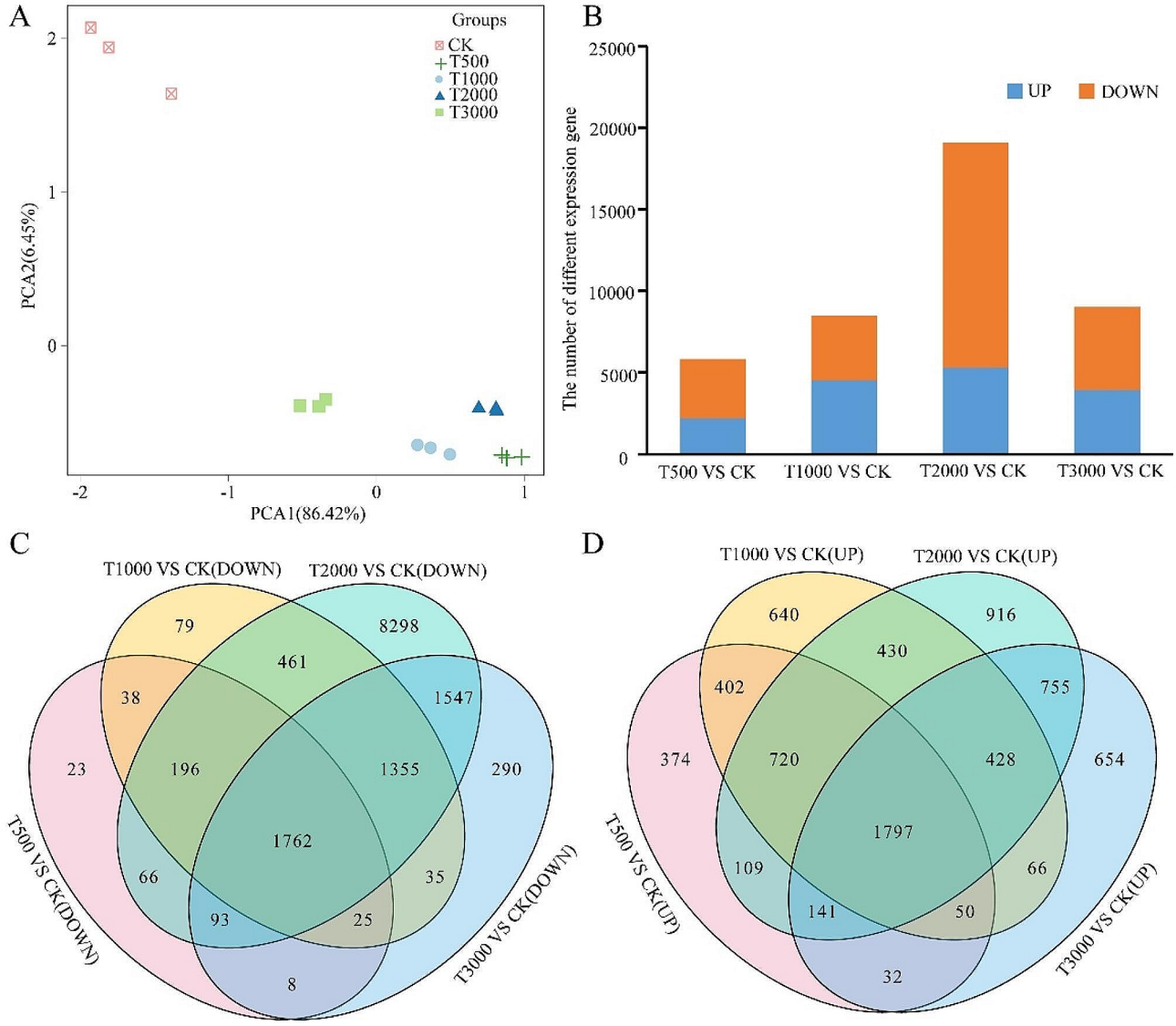

Fig. 3 PCA and analysis of differentially expressed genes (DEGs) in the transcriptome of plants subjected to Pb stress along different gradients. (**A**) PCA of RNA-seq samples. (**B**) Graphical representation of DEGs; 'up' denotes upregulated genes, and 'down' denotes downregulated genes. Venn diagram of downregulated (**C**) and upregulated genes (**D**)

among the metabolite regulation and the sample category (CK, T500, T2000). The metabolite peak regions from the Pb-treated and control groups were adjusted, and OPLS-DA was subsequently performed (Fig. 5A). As a result, the samples from the same treatment were clearly clustered, which suggested that there was good repeatability between biological replicates and strong specificity between treatments.

Based on the VIP score (VIP≥1), Student's t-test statistic ($P<0.05$) and degree of shift in abundance upon Pb exposure (ratio≥1.5 or ≤1.5/1), a group of metabolites were identified for their contribution to sample segregation between the CK and Pb treatments. As a result, 72 differentially expressed metabolites (DEMs) and 107 DEMs were identified from T-500 and T-2000, respectively. Among the DEMs, 36 were detected in both groups (Fig. 5B). There were 11 upregulated DEMs and 23 downregulated DEMs (Fig. 5C). According to the taxonomical characterization of the metabolites, the 36 metabolites were divided into 8 groups: benzenoids (4 metabolites), lipids and lipid-like molecules (7 metabolites), nucleosides (2 metabolites), organic oxygen compounds (3 metabolites), organic acids and derivatives (4 metabolites), organoheterocyclic compounds (6 metabolites), phenylpropanoids and polyketides (2 metabolites), and unknown metabolites (6 metabolites) (Fig. 5D). Among them, the metabolites of nucleosides, organic acids and derivatives were downregulated, while the metabolites of phenylpropanoids and polyketides were upregulated. Six of the metabolites of lipids and lipid-like

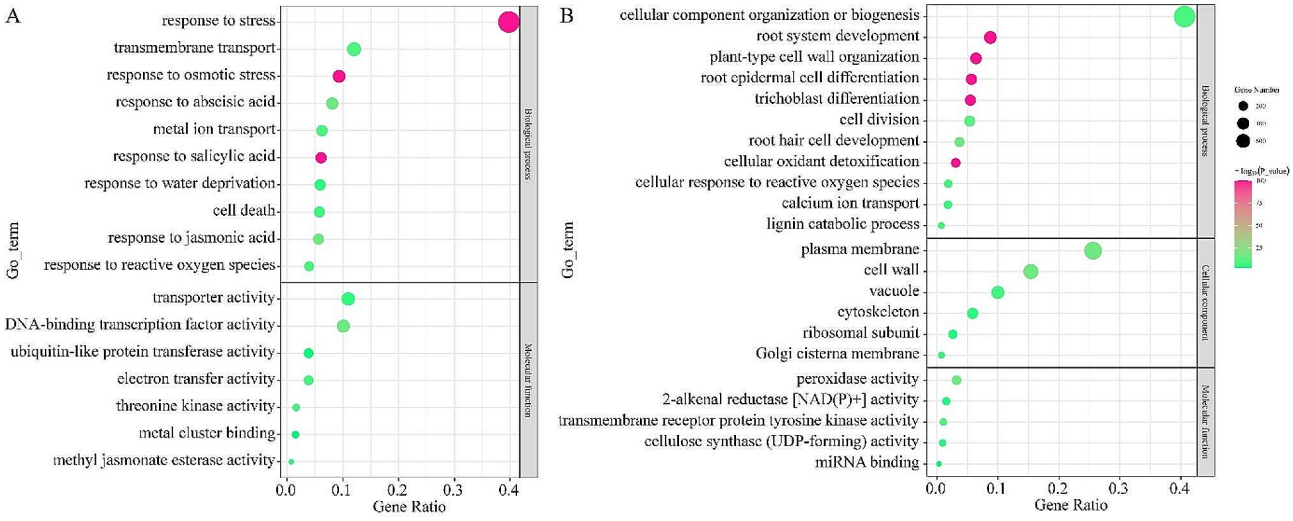

Fig. 4 GO enrichment analysis of co-DEGs under 4 degrees of Pb stress in maize. (**A**) GO enrichment analysis of co-upregulated genes. (**B**) GO enrichment analysis of co-downregulated genes

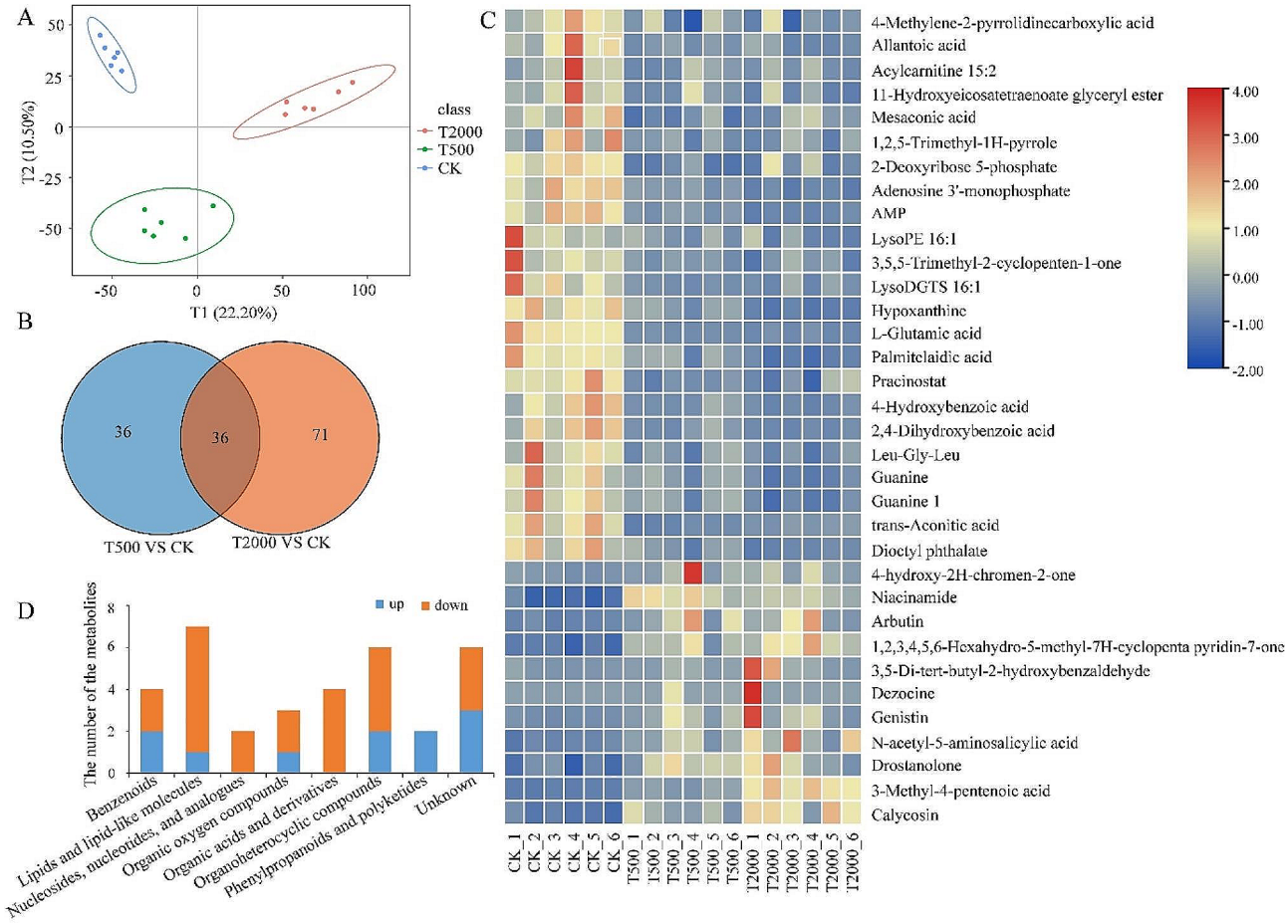

Fig. 5 Metabolic profiling in response to Pb stress in maize. (**A**) PLSDA analysis of treatment samples. (**B**) Venn diagram showing the DEMs shared between T500 and T2000. (**C**) Heatmap of shared DEMs in the roots of maize under different Pb stresses. (**D**) Statistical analysis of the shared DEMs. 'up' represents the upregulated metabolites, while 'down' represents the downregulated metabolites

molecules were downregulated, while only one was upregulated. The number of downregulated metabolites of benzenoids and unknown compounds was the same as that of upregulated metabolites, while the number of downregulated metabolites of organic oxygen compounds and organoheterocyclic compounds was twice that of upregulated metabolites (Fig. 5D).

Association analysis of transcriptomic and metabolomic expression levels and biological pathways under Pb stress in maize

Furthermore, to explore the response pathway to Pb stress, KEGG enrichment analysis of common DEGs and DEMs was performed. The common DEGs among the different Pb concentrations were significantly mapped to 19 distinct biological pathways, including those involved in the biosynthesis of secondary metabolites, phenylpropanoid biosynthesis, brassinosteroid biosynthesis, pentose and glucuronate interconversions, terpenoid and polyketide metabolism, flavonoid biosynthesis, and starch and sucrose metabolism (Fig. 6, Table S2).

As the number of common DEMs was low, the KEGG analyses of the DEMs were performed separately for T500 and T2000, and the results showed that 19 distinct pathways were enriched in both treatments, including biosynthesis of plant secondary metabolites, glyoxylate and dicarboxylate metabolism, arginine biosynthesis, flavonoid biosynthesis, beta-alanine metabolism, nitrogen metabolism, glycolysis/gluconeogenesis, biosynthesis of plant hormones, alanine, aspartate and glutamate metabolism, taurine and hypotaurine metabolism, and biosynthesis of alkaloids derived from histidine and purine (Fig. 7, Table S3, S4). Finally, both DEGs and DEMs were enriched in the flavonoid biosynthesis KEGG pathway (Figs. 6 and 7). There were 2 shared candidate metabolites of flavonoid biosynthesis in T500 and T2000, namely, calycosin and genistin, and 27 shared DEGs.

To better understand the contributions of calycosin and genistin to the Pb stress response, the abundances of these two metabolites were determined from the metabolome, and the results showed that both metabolomes significantly increased under Pb stress (Fig. 8A). To further identify interactions between the DEGs and DEMs that exhibited the same KEGG pathways, association analysis was subsequently performed between the DEGs and the DEMs. There were 18 genes associated with calycosin (12 positive, 6 negative), 3 genes associated with genistin (1 positive, 2 negative), and one gene negatively associated with both metabolites. The correlation coefficient ranged from 0.80 to 0.95 (Fig. 8B Table S5). Additionally, the expression of the associated genes was analyzed, among which 7 genes were downregulated and 13 genes were upregulated under Pb stress (Fig. 8C). In summary, the 20 candidate genes related to the flavonoid synthesis were identified, which played key roles in response to Pb stress.

Discussion

Pb stress inhibited maize root development by inhibiting the root cell wall, cell membrane and DNA synthesis

Plants are rooted in the soil and can absorb the nutrients and water they need directly from the soil via their roots. Soil is not only the source of nutrients for roots but also the living environment of roots, which can cause stress to plant growth and development, including drought stress, salt stress, waterlogging stress, and heavy metal stress [44–47]. As roots are in the soil, they are always the first part to be exposed and respond to stress from the soil [48]. Pb stress is one of the most common heavy metal stresses in soil and is increasing with the development of industry [49]. Previous studies have shown that root development, including lateral root formation and root morphological parameters, including length, surface area, volume, bushiness, and biomass, is inhibited by Pb [50, 51]. Root cell viability was attenuated, leading to induced cell death under Pb stress [52]. In our study, the Pb uptake in the roots and shoots tended to increase in response to the different Pb treatments, while the Pb uptake in the roots was relatively greater than that in the shoots, which is consistent with previous studies. The transcriptome analysis revealed that the downregulated genes were enriched in the root development-, plasma membrane-, cell division-, and cell wall-related pathways, and the upregulated genes were enriched in the cell death- and response to stress-related pathways. Additionally, among the 36 metabolites shared between T500 and T2000, six of the lipid and lipid-like molecule metabolites, two of the nucleoside metabolites, and two of the benzenoid metabolites were downregulated, which is consistent with the transcriptome results. According to the results, root development was strongly inhibited by Pb stress, which was mainly due to the inhibition of the synthesis of the cell wall, plasma membrane, and nucleic acid and the acceleration of cell death.

Flavonoid metabolism plays an important role in the plant response to abiotic stress

Flavonoids are a group of secondary metabolites that are mainly composed of compounds with a C6-C3-C6 framework and are common in plants [53]. To date, more than 8000 flavonoids have been identified in plants [54]. Flavonoids play critical roles in the stress response by maintaining redox homeostasis through several mechanisms, including (1) attenuation of singlet oxygen; (2) attenuation of enzyme activity involved in ROS production; (3) chelation of transition metal ions; (4) quenching of free radical cascades produced during lipid peroxidation metabolism; and (5) recycling of other antioxidants

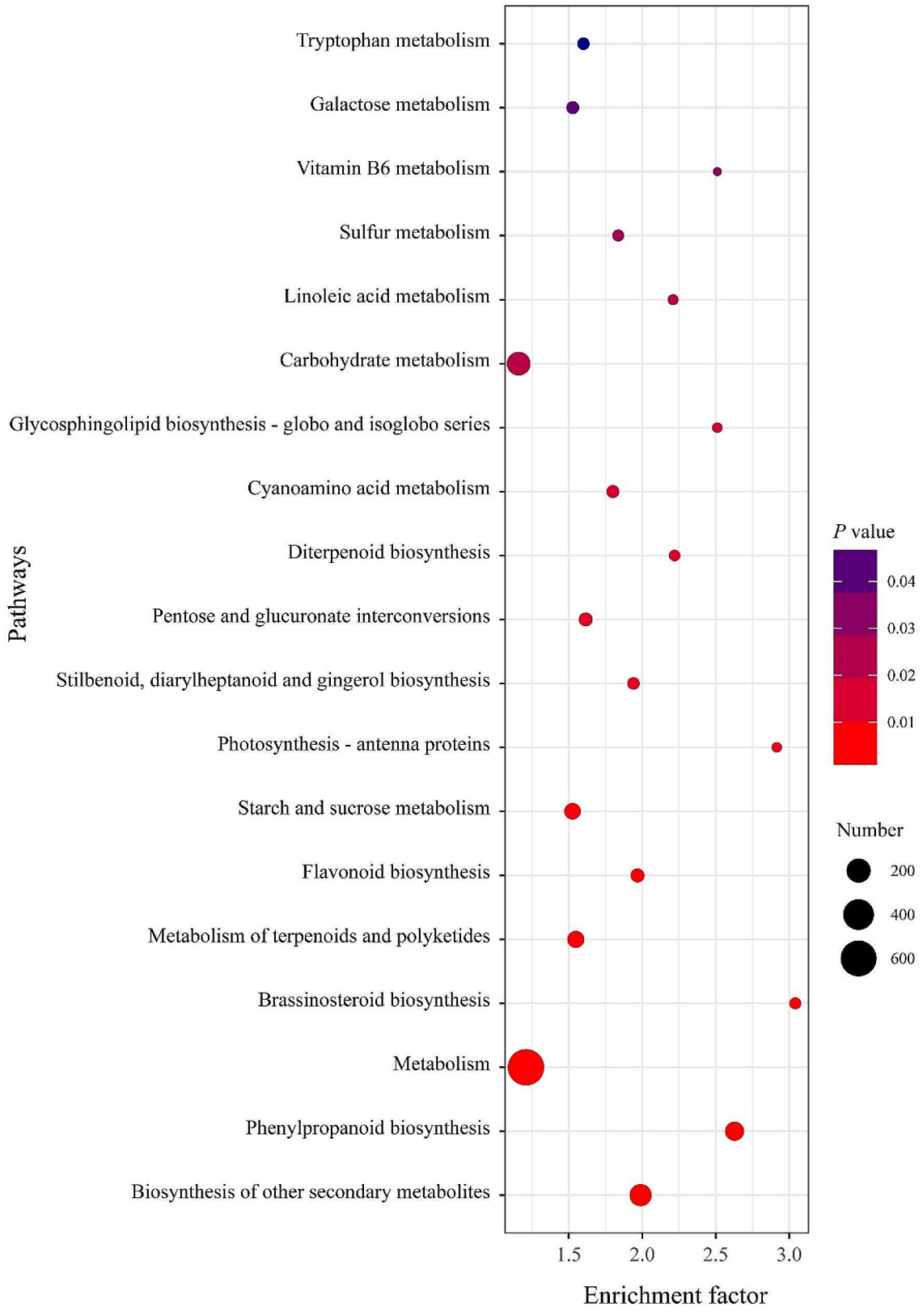

Fig. 6 KEGG enrichment analysis of common DEGs under four Pb treatments

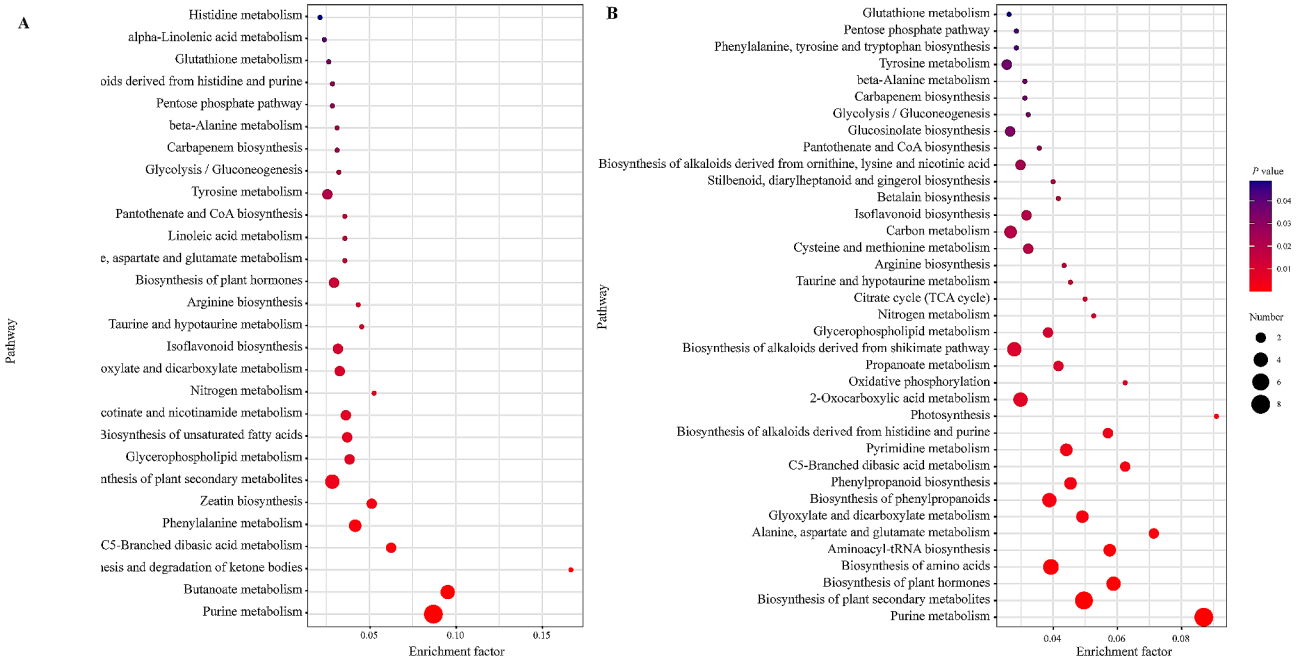

Fig. 7 KEGG analysis of the shared DEMs. A and B: KEGG analysis of DEMs under T500 (**A**) and T2000 (**B**)

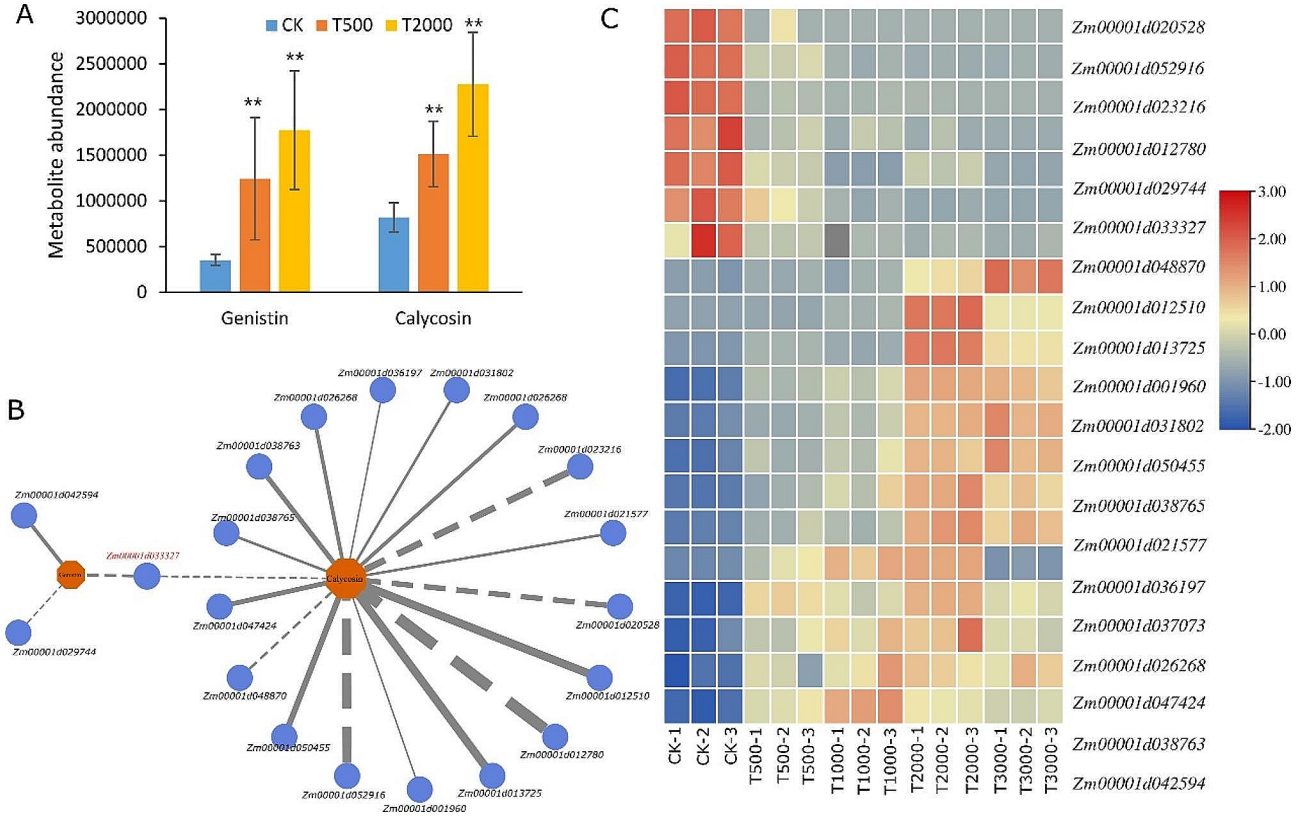

Fig. 8 Analysis of flavonoid biosynthesis-related metabolites and genes. (**A**) The abundance of calycosin and genistin under different Pb treatments. (**B**) Correlation analysis between DEMs and DEGs related to flavonoid biosynthesis. The solid line denotes a significant positive correlation, the dashed line denotes a significant negative correlation, and the thickness of the line denotes the magnitude of the correlation. (**C**) Expression heatmap of the DEGs associated with calycosin and genistin

[54–56]. In our study, Pb stress caused an increase in ROS and destruction of the cell membrane of maize plants. To survive, plants initiate a series of physiological and biochemical responses, including the accumulation of flavonoids.

This study provides genetic resources for innovation of Pb-tolerant maize germplasm

Crop Pb tolerance is a complex quantitative trait that is regulated through multiple processes, such as selective metal uptake, metal binding to the root surface, binding to the cell wall, and the induction of antioxidants [5]. In our study, we identified 20 candidate genes related to the Pb stress response and accumulation of flavonoids by combining transcriptomic and metabolomic data. Among the candidate genes, *Zm00001d038763* and *Zm00001d021577* encode UDP-glycosyltransferase proteins. Previous studies reported that UDP-glycosyltransferases are involved in mitigating abiotic stresses in plants through ROS scavenging [57, 58]. In the study, both the UDP-glycosyltransferase related genes were upregulated under Pb stress, which may be also involved in ROS scavenging under Pb stress. *Zm00001d047424* encodes a flavonoid 3'-monooxygenase that has been reported to be involved in regulating oxidative and salt stress tolerance [59]. In the study, *Zm00001d047424* was upregulated under Pb stress, which could increase the expression of reactive oxygen species (ROS) scavengers to regulate reactive oxygen balance. *Zm00001d031802* encodes a NADPH HC toxin reductase. In rice, overexpression of a NADPH HC toxin reductase gene, *YK1*, increased NAD(H) and NADP(H) levels by causing an increase in NAD synthetase and NAD kinase activities, which conferred the prevention of induced cell death in planta [60]. In the study, *Zm00001d031802* was up-regulated by Pb, which could increase NAD(H) and NADP(H) levels to alleviate superoxide stress caused by Pb. *Zm00001d037073* encodes a hydroxycinnamoyltransferase3. In maize, homologs of Hydroxycinnamoyltransferase are involved in lignin biosynthesis to modulate the defense response [61]. In the study, the cell wall related genes were down regulated by Pb stress, while the increased expression of Hydroxycinnamoyltransferase related gene could alleviate the effect of Pb on cell wall synthesis. According to the results, we believe that the identified genes in the study are probably involved in the Pb stress with different mechanisms. However, the function of these genes needs to be verified by genetic transformation in the future study.

Conclusion

In summary, Pb can accumulate in maize, especially in roots, where it suppresses the antioxidant system, root development, cell division, cell wall synthesis, and plasma membrane synthesis-related pathways and promotes responses to stress, cell death, and ABA response-related pathways. More importantly, flavonoids (genistin and calycosin) were shown to be involved in the response of maize to Pb stress, and 20 related genes were identified. This study not only provides genetic resources for the genetic improvement of maize Pb tolerance but also enriches the theoretical basis of the maize Pb stress response.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-024-05455-0.

Supplementary Material 1

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Author contributions

Z. H., and Y. Z. designed the overall experiment. Z. H., Y. Z., X. Z., B. W., Y. G., and Z. G. conducted the data processing for transcriptomics and metabolomics. Z. H., and Y. Z. wrote the main manuscript, while X. Z. and Z. H. reviewed and revised the paper. All authors read and approved the final manuscript.

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Data availability

The raw RNA-seq data have been uploaded to NCBI Sequence Read Archive (SRA) data-base (BioProject ID: PRJNA1113349, named from B1-B15).

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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