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Assessing the Effects of Cadmium Stress on the Growth, Physiological Characteristics, and Metabolic Profiling of Rice (*Oryza sativa* L.) Using HPLC-QTOF/MS

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Abstract: Cadmium (Cd) pollution is an important environmental problem, as it is easily absorbed by plants and gradually accumulates in the human body through the food chain. This study aimed to elucidate the changes in the metabolic response of the rice cultivar "TanLiangYou215" under Cd stress. Rice was grown in soil culture at 0 (Control), 2 (Low group), and 10 (High group) mg/kg $CdCl_2$ for 90 days. The ultrastructural, Cd content, antioxidant activity, and metabolic changes to the rice in different tissues were analyzed. Phenotypic characterization and ultrastructure showed that the rice roots and leaves were significantly damaged and plant growth was inhibited in the High group, while plant growth was promoted in the Low group. Overall, Cd showed a regularity of "low promotion and high inhibition". Physiological indices revealed that rice was significantly affected by Cd stress. Compared to the Control, Cd stress resulted in higher antioxidant enzyme activities, and the Low group suffered less oxidative damage than the High group. Metabolomic studies revealed that Cd stress significantly altered the metabolic profiles of rice plants. Rice responded to Cd stress by upregulating amino acids and regulating related pathways, including alanine, aspartate and glutamate metabolism, and arginine and proline metabolism. The significant expression of flavonoids with antioxidant properties helped rice resist the oxidative damage caused by Cd accumulation in the root tissue; Cd stress significantly downregulated glycerophospholipid metabolism in the stem and leaf tissues, which affected the cellular activities in rice stem and leaf tissues. We investigated the effects of Cd stress on ultrastructure, antioxidant activity, and metabolic changes in different tissues of the rice variety TLY215. Moreover, the different tissues of TLY215 can regulate these metabolic pathways to resist Cd stress, which provides valuable insights into the response of TLY215 to different concentrations of Cd.

Keywords: cadmium (Cd); rice (*Oryza sativa* L.); phenotypic characterization; physiological activity; metabolomics

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1. Introduction

Toxic heavy metals (THMs) are persistent environmental pollutants that do not degrade and constantly accumulate in the food chain, posing a serious threat to the environment and human health [1]. With the rapid development of the economy and society, human activities, such as urban waste disposal, smelting, mining, metal manufacturing, and excessive use of fertilizers, have led to the heavy metal pollution of soils [2]. Among the various heavy metals, cadmium (Cd) is considered the most phytotoxic due to its high water solubility and relatively long biological half-life [3]. Cd exhibits high phytotoxicity to plants [4,5], resulting in various adverse physiological effects, such as imbalanced water distribution, inhibited photosynthesis, delayed growth, and reduced plant productivity [2,6,7].

The uptake and transport of nutrients by plants is inhibited in cadmium-contaminated soils, including nitrogen (N), phosphorus (P), potassium (K), iron (Fe), calcium (Ca), magnesium (Mg), and manganese (Mn) [8]. Additionally, Cd pollution in the soil results in the excessive accumulation of reactive oxygen species (ROS), leading to oxidative damage in plants [9]. Accumulation of Cd has been proven to pose a significant risk to human health [10,11], because it causes irreversible damage to various vital organs, including the liver, kidneys, bones, immune system, and reproductive system [12,13].

Metabolomics is a high-throughput technology used to monitor changes in the metabolism of small molecules in perturbed organisms [14]. The entire metabolome can be analyzed by identifying and semi-quantifying thousands of metabolites in plants. Metabolomics serves as a downstream discipline of both genomics and proteomics, closely linked to the phenotype [15]. Moreover, nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC-MS), and liquid chromatography-mass spectrometry (LC-MS/MS) are the most widely used methods in metabolomics research [16]. The analytical principle of pattern recognition in chemometrics is usually applied during metabolomics technology analysis. Appropriate chemometrics tools can expedite the analysis process and streamline rapid data processing [17]. Commonly used multivariate statistical analysis methods include principal component analysis (PCA), partial least-squares discriminant analysis (PLS-DA), and orthogonal partial least squares discriminant analysis (OPLS-DA). Through the construction of mathematical models, key biological information was extracted from the vast amount of data obtained via instrumental analysis, and bioinformatics techniques were employed to further extract and interpret this information.

Rice is widely recognized as one of the most crucial staple foods globally. Leading in global rice production are several Asian countries, such as China, Thailand, Japan, and Indonesia [18]. A survey revealed that approximately 82% of China's farmland is contaminated with inorganic pollutants, with Cd being the most prevalent among them [19]. Cd contamination in the soil might trigger Cd accumulation in the grain crops cultivated in China. Among various grains, rice has been found to possess a high capacity for Cd absorption [20]. Metabolomics technology has been widely used to study heavy metal stress in rice. Reig et al. [21] identified 97 differential metabolites in the above-ground parts of rice under both Cd and Cu stress based on metabolomics technology. They also indicated that the effect of Cd stress on rice was more pronounced compared to Cu stress. Liu et al. [19] investigated the alterations in the roots of various rice varieties under Cd stress using metabolomics technology. Low-Cd accumulated rice upregulated lipids and fatty acids, while high-Cd accumulated rice upregulated phenylethanoid glycosides to cope with Cd-induced oxidative stress. Combined with phenomics, it was shown that rice roots utilized multiple strategies to improve their tolerance to Cd-induced oxidative stress. TanLiangyou215 (TLY215) is a two-line early season hybrid rice known for its high yield and adaptability as a low-Cd rice variety, extensively cultivated in southern rice regions. Despite its wide usage, the change pattern of metabolic regulation under Cd stress in this rice variety has not been previously reported.

The purpose of this study was to investigate the effect of Cd stress on different tissues of TLY215. Rice plants were cultivated in soil with different Cd concentrations, including the CK group (0 mg kg^{-1}), Low group (2 mg kg^{-1}) and High group (10 mg kg^{-1}), for a duration of 90 days. Phenotypes, ultrastructure, antioxidant activity, changes in soil Cd concentration, and different rice tissues (roots, stems, and leaves) were analyzed. Untargeted metabolomics based on LC-QTOF/MS was employed to investigate the metabolic small molecules in different rice tissues. MSDIAL software (version 4) was used to analyze the metabolism of different rice tissues, and the differences among different groups were assessed via partial least-squares discriminant analysis (PLS-DA). MS peaks with significant changes were statistically selected, and characteristic MS peaks of MS/MS were identified for metabolic pathway analysis. By analyzing the characteristics of metabolites in different rice tissues and the physiological activities of plant tissues under Cd stress, the results allowed us to gain insights into the responses of different rice tissues to Cd stress.

2. Materials and Methods

2.1. Design of the Experiment

This experiment was conducted in the greenhouse of Guilin University of Technology (110°17′50 E, 25°3′5 N). Planting pots were filled with a mixture of nutrient soil, organic matter soil, and sand and gravel in a ratio of 1:1:1. The soil was then treated with $CdCl_2$ (Aladdin, Fukuoka, Japan, 99.99%) to create soil samples with concentrations of 0 mg kg^{-1} (CK), 2 mg kg^{-1} (Low group), and 10 mg kg^{-1} (High group). Additionally, a Blank group was established, where no $CdCl_2$ was added, and no rice was planted. To maintain soil moisture, 300 mL of deionized water was sprayed daily. After soil samples were stabilized for 10 days, follow-up experiments were conducted.

Rice seeds (TanLiangYou215, TLY215) were surface-sterilized using a 2% sodium hypochlorite solution (Xi Long Scientific, Guangzhou, China, available chlorine 8%) for 15 min and thoroughly rinsed with deionized water. The rice seeds were germinated in a light incubator (dark, 30 °C), and the seedlings were transplanted into Cd-contaminated soil. During the germination, at the leaf stage, a limited amount of deionized water was used for cultivation. Between the one-leaf stage and the three-leaf stage of rice, 25% nutrient solution was applied, and after the three-leaf stage, 50% nutrient solution was used. The water surface depth was maintained at 2–3 cm. After a growth period of 90 days, samples of soil, roots, stems, and leaves were collected for further analysis.

2.2. Cadmium Analysis

Samples of different rice tissues (roots, stems, and leaves) were dried in an oven at 80 °C until a constant weight was achieved. The dried samples were then ground and sieved through a 100-mesh sieve. We accurately weighed 0.10 g of rice tissue sample, added 5 mL of HNO_3, and 2 mL of 30% H_2O_2. Ultrasound was applied for 15 min, and then it was sealed and placed in autoclave, overnight. Next, the mixture was subjected to ultrasonic treatment for another 15 min. It was then sealed again and left overnight in a high-pressure vessel. Subsequently, the sample was dried on a hot plate at 120 °C and 3 mL of HNO_3 was added and sealed in the autoclave. The sample was then dried at 160 °C for 10 h. Afterward, the sample was dried again on a hot plate at 120 °C, and 5 mL of 2% HNO_3 was added and heated at 100 °C for 10 min. Finally, the 2% HNO_3 was dispersed in a 10 mL volumetric flask. The solution was measured via ICP-MS. The Cd concentrations in the sample were determined via ICP-MS (Agilent 7500 Series, Agilent Technologies, Santa Clara, CA, USA). Each treatment was replicated three times. The ICP-MS was operated with the following parameters: RF (radio frequency) power, 1390 W; nebulizer gas flow rate, 0.90 L min^{-1}; auxiliary gas, 0.14 L min^{-1}; cooling flow, 17 L min^{-1}; sampler cones, 1 mm (Ni); skimmer cones, 0.7 mm (Ni); ion lens voltage, −230~40 V; dwell time, 10 ms; data acquisition mode, peak hopping; number of repeated scans, 5; and impact gas flow rate (He), 3.5 mL min^{-1}.

2.3. Scanning Electron Microscope (SEM) Observation

After subjecting rice plants to 90 days of Cd stress, uniformly grown plants from the CK, Low, and High groups were carefully selected and washed with deionized water. Subsequently, the root and leaf tissues of rice were cut into 5 mm segments and fixed in 2.5% glutaraldehyde solution for 2 h. The fixed tissues were then rinsed three times for 15 min each with a 0.1 M phosphoric acid buffer solution (pH 7.2). After fixation, the sample was dehydrated using a graded series of acetone (30–100% v/v) for 15 min at each step. Afterward, the samples were immersed in tert-butanol and stored at 4 °C, and followed by freezing and lyophilization. Once the sample was trimmed to a size of 2.5 mm, gold coating was applied through ion sputtering. Finally, the sample was photographed and observed using a scanning electron microscope (SU5000, Hitachi, Tokyo, Japan).

2.4. Physiological Activities in Rice Tissues

After a 90-day growth period, the height (cm) of the plants from the soil surface to the top was measured using a meter stick. To analyze physiological activities in plant tissues, including total protein (TP, A045-2-2), enzyme activity including superoxide dismutase (SOD, A001-1-2) and peroxidase (POD, A084-3-1), chlorophyll (chlorophyll a, chlorophyll b and total chlorophyll, A147-1-1), and malondialdehyde (MDA, A003-1-2), test kits from Nanjing Jiancheng Bioengineering Institute Co., Ltd. (Nanjing, China) were employed. For the analysis, plant tissues (0.5 g for roots, stems and leaves, respectively) were mixed with normal saline at a ratio of 1:9 w/v. The samples were extracted at low temperatures and centrifuged at 3500 rpm min^{-1} for 10 min. The amount of TP, SOD, POD, and MDA in the supernatant was determined using the test kits according to the manufacturer's instructions [22]. The absorbance readings for TP, SOD, POD, and MDA were obtained at 595 nm, 550 nm, 420 nm, and 532 nm, respectively. The chlorophyll reagent kit instructions were followed to obtain measurements. A mixture of leaf tissue and extraction solution (acetone:ethanol, 1:2, v/v) was prepared and centrifuged at 3500 rpm for 10 min after mixing in the dark until the leaves were bleached. Absorbance was measured at 645 nm and 663 nm, and the concentrations of chlorophyll a, b, and total chlorophyll were calculated accordingly.

2.5. Untargeted Metabolomics Analysis of Rice Tissues Using HPLC-QTOF/MS

2.5.1. Sample Extraction

After a growth period of 90 days, the roots, stems, and leaves of the plant were carefully separated and harvested. The collected samples were immediately frozen in liquid nitrogen and subsequently dried using a freeze-dryer (FD-1B-80, Boyikang, Beijing, China). Then, the powder was prepared by grinding at a low temperature with an automatic grinder (JXFSTPRP-24L, Tissuelyser, Shanghai, China) and stored at −80 °C for further analysis. For extraction, the sample powder was shaken with 3 mL of methanol–water (80% v/v) solution for 10 min. The mixture was then centrifuged at 5000 rpm for 15 min. This extraction process was repeated twice, and the resulting supernatants were combined. The supernatant was filtered through a 0.22 μm membrane and collected in a sample vial for HPLC-QTOF/MS analysis. A QC sample was prepared by combining 20 μL of each sample for quality control. Each treatment group consisted of six replicated samples for statistical reliability.

2.5.2. Sample Detection

The metabolic profiles of rice root, stem, and leaf tissues were determined via HPLC-QTOF/MS (LC-20A-TripleTOF 5600+, AB Sciex, Framingham, MA, USA) based on Zhang's detection method [23]. XSelect® HSS T3 column (2.1 mm × 150 mm, 3.5 μm, Waters, Milford, MA, USA) was used for chromatographic separation. The mobile phase A was water (ESI^+: 0.1% formic acid; ESI^-: 5 mM ammonium acetate) and B was acetonitrile. Sample volume: 2.00 μL; column temperature: 40 °C; flow rate: 0.3 mL min^{-1}. Gradient procedures: 0–3.0 min, 10% B; 3.0–21.0 min, 10–95%; and 28.0–34.0 min, 10% B. Mass scan range was set from 50 to 1000 m/z. For positive and negative ionization modes, the ion source voltage was set to 5500 V and 4500 V, respectively, and the collision energy (CE) was set to 30 V and −30 V, respectively. Before sampling, QC samples were used to balance the mass spectral state, and then QC samples were tested once every 5 samples to monitor instrument stability. The total ion flow chromatogram for the QC sample is displayed in Figure S1.

2.6. Statistical Analysis

The original LC-QTOF/MS data files of all samples were analyzed using MS-DIAL software to obtain MS peak information, including retention time, mass number, MS/MS, and peak area. The characteristic MS peaks obtained via MS/MS were identified through comparison with the public database (MassBank). Detailed settings for processing MSDIAL data are presented in Figure S2. Simultaneously, the results of the matching database reveal

the four metabolites with the highest and lowest matching degrees to the references, which are depicted in Figure S3. Variable important in projection (VIP) in the PLS-DA model denotes the extent to which each metabolite contributes to the model. Metabolites that exhibit VIP values exceeding 1 are typically regarded as significant metabolites. Hence, a PLS-DA model (VIP > 1) was established, and a *t*-test ($p < 0.05$) was performed to identify the significantly differentiated metabolites. To further explore the metabolic pathway changes of the identified characteristic differential metabolites, the common MetaboAnalyst platform (https://www.metaboanalyst.ca, accessed on 6 May 2023) was utilized, along with reference to the rice database in the Kyoto Encyclopedia of Genes and Genomes (KEGG).

3. Results

3.1. Cd and SEM Analysis

Rice root and leaf tissues were examined using scanning electron microscopy (SEM). As shown in Figure 1, the SEM images revealed distinct differences among the CK group, Low group and High group. In the CK group, the root tissues exhibited the densest distribution of root hairs on their surface, followed by the Low group. Conversely, in the High group, the root hairs were noticeably reduced and the intercellular tissues appeared significantly disrupted (Figure 1(A_1,B_1,C_1)). From the root section diagrams, it was observed that Cd stress led to an increase in the number of channels in the vascular bundle, cortical layer, and cortical parts within the root. In addition, the size and number of periductal phloem, companion cells, and sieve tubes, were reduced under Cd stress (Figure 1(A_3,B_3,C_3)). In the SEM images of leaf tissue, it was evident that the number of stomata on rice leaves decreased with the addition of Cd (Figure 1(A_2,B_2,C_2)).

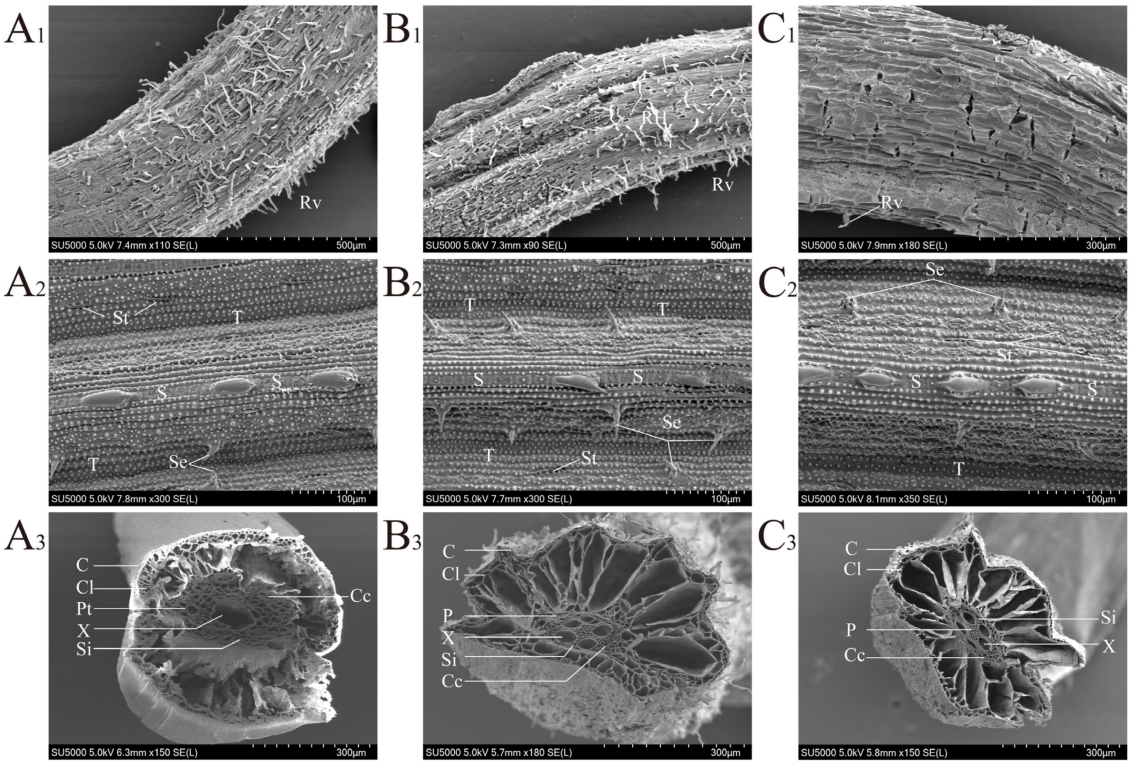

Figure 1. Scanning electron microscopy (SEM) micrographs of root surfaces (1), leaf surfaces (2), and root sections (3) of rice. (**A**–**C**) were CK, Low, and High groups, respectively. Rv: root velvet; S: silica-phellem; T: stomatal band; St: stomatal apparatus; Se: seta; P: phellem of vascular bundle; X: xylem; C: cortical; Cl: cork layer; Si: sieve tube; Cc: companion cell.

Moreover, we also conducted an analysis of the Cd content in various of tissues, the above-ground height of rice in different treatment groups, and the soil pH in each group

(Table 1). The Cd content was observed to follow the pattern: roots > stems > leaves. Regarding the above-ground height of rice in each treatment group, it was found that the Low group exhibited slightly higher values compared to the CK group, while the High group displayed a significantly lower height compared to other treatment groups. As for soil pH, there was a decreasing trend with the increase in Cd concentration in the soil, with the highest pH value observed in the Blank group and the lowest in the High group.

Table 1. Soil pH, Cd concentration, and growth height of rice (Mean ± SD, n = 3).

		Blank	CK	Low	High
Soil pH		7.33 ± 0.03	7.15 ± 0.03	6.95 ± 0.01	6.80 ± 0.01
Cd concentration in rice/mg kg^{-1}	Roots	/	1.45 ± 0.27	15.00 ± 1.68 **	58.34 ± 2.67
	Stems	/	0.22 ± 0.03	1.87 ± 0.18	3.72 ± 0.38 *
	Leaves	/	0.10 ± 0.01	1.20 ± 0.20 ***	2.97 ± 0.18 **
Above-ground height/cm	90 d	/	71.1± 5.93	86.98 ± 8.29 **	66.96 ± 6.08

*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ (compared to CK).

3.2. Physiological Indicators

The physiological parameters of rice were analyzed in the three groups (CK, Low, and High), including the total protein, enzyme activity (SOD, POD), chlorophyll (chlorophyll a, chlorophyll b, and total chlorophyll), and malondialdehyde (MDA) levels, as illustrated in Figure 2. Under Cd stress, the content of TP in the root and stem tissues of rice showed a negative correlation with increased stress concentration. However, TP levels in leaf tissue initially increased and later decreased (Figure 2A). SOD and POD activity in the root, stem, and leaf tissues of rice displayed a significant increase under Cd stress ($p < 0.05$). Furthermore, the content of chlorophyll a, chlorophyll b, and total chlorophyll in rice leaves increased significantly in the Low group ($p < 0.05$) but decreased in the High group (Figure 2D). Conversely, MDA levels decreased significantly in the Low group but increased in the High group (Figure 2E).

3.3. Metabolomics Analysis of Rice Root Tissue via HPLC-QTOF/MS

A total of 451 metabolites were detected in the analysis of root tissue using LC-QTOF/MS in both positive and negative ion modes. Detailed information about all the compounds is available in the Supplementary Materials (Table S1). To identify distinctions among the groups, we constructed a PLS-DA model for discriminant analysis. We ensured model stability and avoided overfitting concerns by analyzing 200 iterations (Figure 3A,B). Partial least squares-discriminant analysis (PLS-DA) served as the classification model with evaluation based on the goodness of fit (R^2) and predictive ability (Q^2). All sample R^2 values exceed their respective Q^2 values. This confirms the reliability of the model. Subsequently, we identified 103 significantly different metabolites (VIP > 1 and $p < 0.05$). These compounds were classified based on their differences using the PubChem database (https://pubchem.ncbi.nlm.nih.gov/, accessed on 1 May 2023). The identified compounds mainly include alkaloids, flavonoids, lipids, organic acids, amino acids, sugars and glycosides, terpenes, and various compounds (Figure 3C). Further classification of the 103 different metabolites was carried out using heat map analysis (Figure 4). Most flavonoids, alkaloids, lipids, sugars and glycosides, and terpenoids showed significant accumulation in the Low group but decreased in the High group. Additionally, Cd stress induced a decreasing trend in most amino acids compared to the CK group. Under the condition of fold change (FC) > 1.5, $p < 0.05$, 43 and 83 metabolites showed significant differences in the Low/CK and High/CK groups, respectively. In the Low/CK group, 16 metabolites were significantly downregulated and 27 metabolites were significantly upregulated. Conversely, 47 metabolites were significantly downregulated and 36 metabolites were significantly upregulated in the High/CK group (Figure S4A,B).

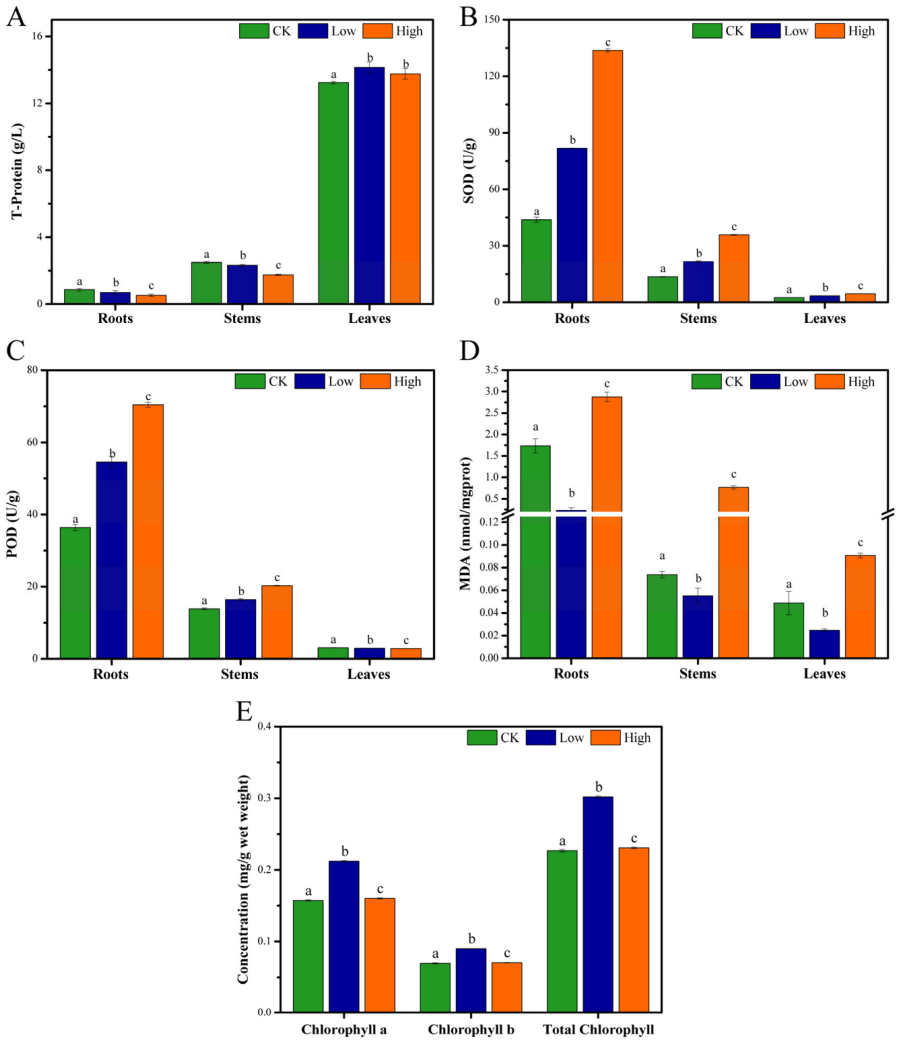

Figure 2. Effect of three concentrations of Cd stress on physiological activities in various tissues of rice. (**A**) Total protein (TP), (**B**) superoxide dismutase (SOD), (**C**) peroxidase (POD), (**D**) malondialdehyde (MDA), and (**E**) chlorophyll (for rice leaves only). CK: control; Low: 2 mg kg^{-1}; High: 10 mg kg^{-1}, respectively. Different letters above each bar denote significant differences ($p \leq 0.05$) between plant groups according to a one-way ANOVA. Each column bar represents the mean ± SD. Each treatment included six independent biological replicates.

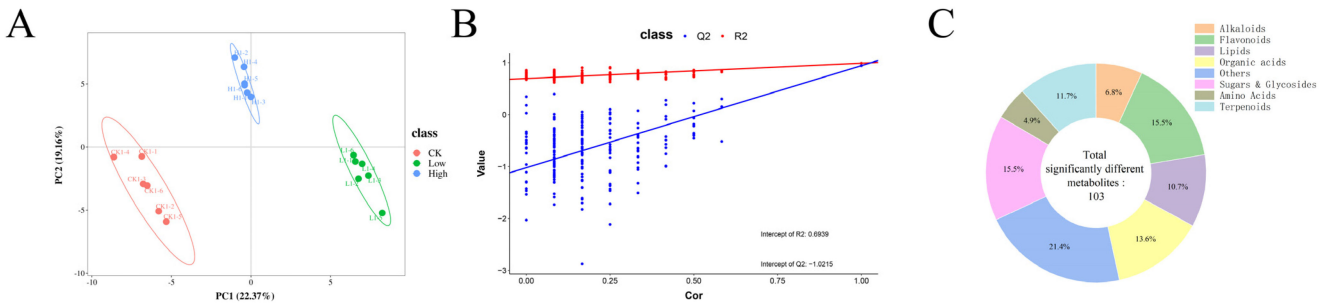

Figure 3. (**A**) Partial least squares (PLS-DA) scores plot; (**B**) permutations plot of PLS-DA; (**C**) the category percentages of significantly altered metabolites (VIP > 1 and $p < 0.05$) in rice roots. CK: control; Low: 2 mg kg^{-1}; High: 10 mg kg^{-1}, respectively. Each treatment included six independent biological replicates.

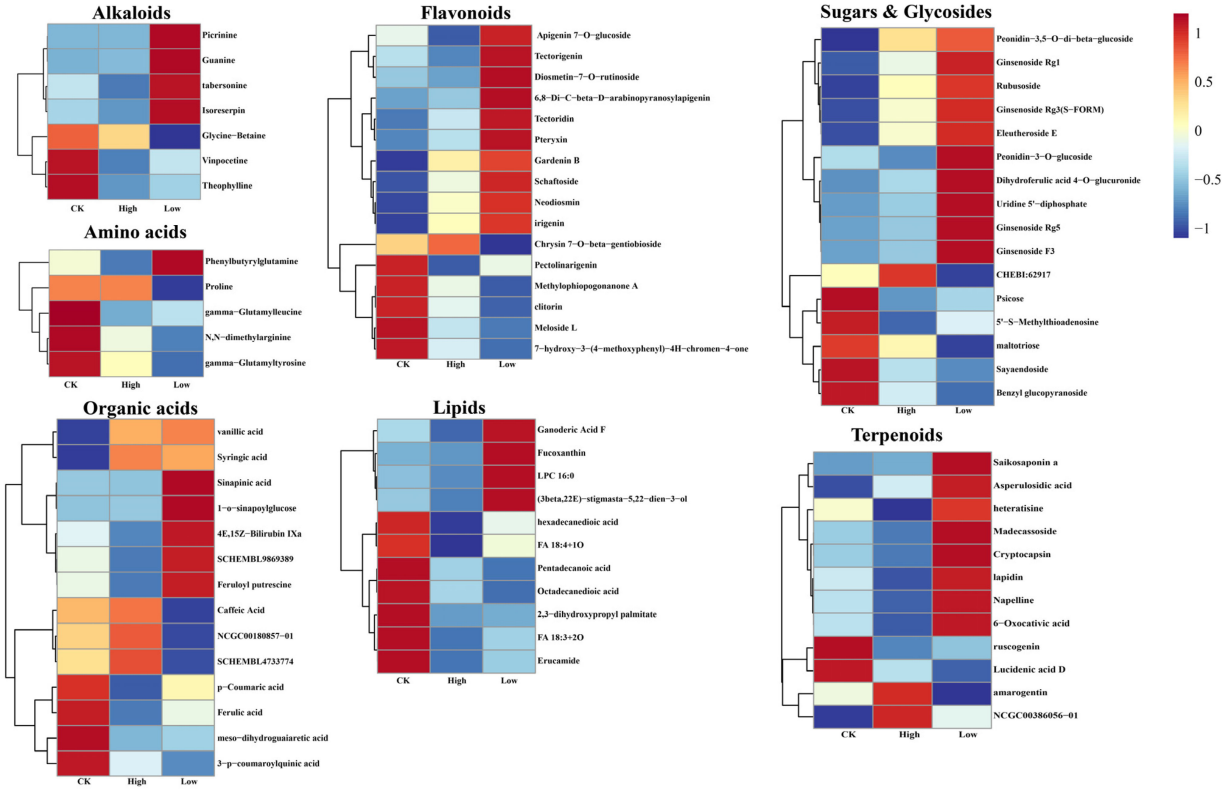

Figure 4. Heat map analysis of metabolites identified in rice root tissues with significant differences by substance ($p < 0.05$). CK: control; Low: 2 mg kg^{-1}; High: 10 mg kg^{-1}, respectively. The red and blue blocks indicate significant upregulation and downregulation, respectively.

As shown in Figure 5, the MetaboAnalyst web tool (https://www.metaboanalyst.ca, accessed on 6 May 2023) was used to examine the metabolites with significant differences to gain insights into changes in their metabolic pathways. It was found that metabolites with significant differences in the Low/CK treatment group could not undergo pathway enrichment analysis. On the other hand, the metabolic pathways in the High/CK group were identified, including flavonoid biosynthesis; phenylpropanoid biosynthesis; purine metabolism; nitrogen metabolism; flavone and flavonol biosynthesis; arginine biosynthesis; and alanine, aspartate, and glutamate metabolism. The results indicated that the application of low-concentration Cd had no significant effect on the metabolic pathways of rice root tissues. However, multiple metabolic pathways in rice were significantly affected in the High group, suggesting that the high-concentration Cd treatment imposed a more pronounced stress effect on rice roots than the low-concentration Cd treatment.

3.4. Metabolomics Analysis of Rice Stem and Leaf Tissue via HPLC-QTOF/MS

A total of 191 and 201 metabolites were found in the stem and leaf tissues, respectively. Detailed information about all the compounds is available in the Supplementary Materials (Tables S2 and S3). PLS-DA methods were employed to analyze the metabolic profiles of the stem (Figure 6(A_1)) and leaf (Figure 6(A_2)) tissues. The results demonstrated significant differences in the stem and leaf tissues among the CK, Low, and High groups, with clear separation of all three treatment groups. Subsequently, we identified 51 and 50 significantly different metabolites (VIP > 1, $p < 0.05$) in the stems and leaves, respectively. These metabolites mainly included flavonoids, lipids, organic acids, amino acids, sugars and glycosides, alkaloids, and others (Figure 6(C_1,C_2)). Heatmap analysis (Figure 7) further illustrated significant changes in the content of metabolites in stem and leaf tissues under Cd stress. In stem tissue, most alkaloids, organic acids, amino acids, sugars and glycosides, and flavonoids were obviously accumulated, while some lipids were significantly

decreased. However, in leaf tissue, the pattern of change was not as pronounced as in stem tissue, but a significant increase in many flavonoids was observed under high Cd treatment. Furthermore, in stem tissue, 33 and 77 different metabolites were screened for the Low/CK and High/CK groups, respectively (FC > 1.5, $p < 0.05$). In the Low/CK group, 10 metabolites were downregulated, and 23 metabolites were upregulated. In the High/CK group, 9 metabolites were downregulated and 68 metabolites were upregulated (Figure S2(A1,B1)). In leaf tissue, 12 and 16 different metabolites were screened for the Low/CK and High/CK groups, respectively. In the Low/CK group, 10 metabolites were downregulated and 2 metabolites were upregulated. In the High/CK group, 8 metabolites were downregulated and 8 metabolites were upregulated (Figure S5(A2,B2)). Overall, these results strongly suggested that Cd stress had a significant effect on plant metabolism.

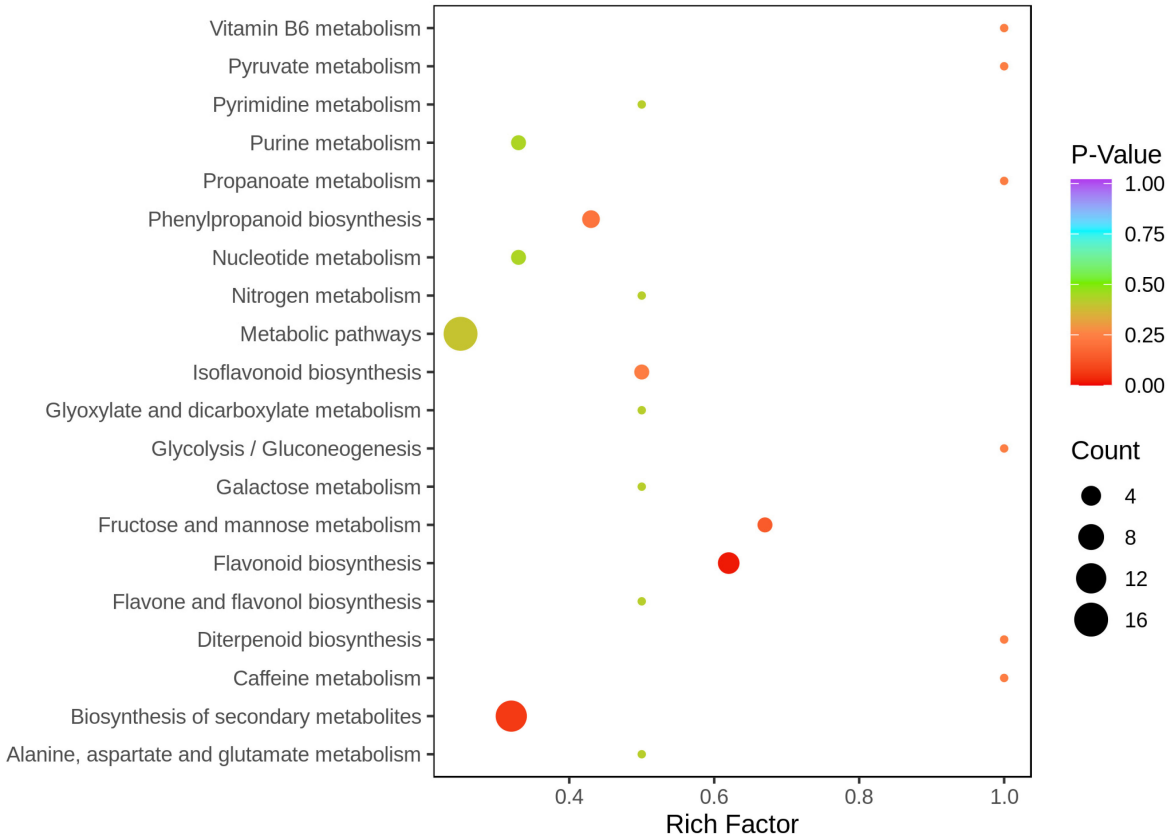

Figure 5. Pathway analysis of significantly different metabolites in root tissue treated with High/CK. The count is the number of compounds matched from our data and p values with the p-value calculated during pathway analysis. A larger Rich factor indicates a greater degree of enrichment.

The results of the pathway analysis are presented in Figure 8. In stem tissues (Figure 8A,B), the Low/CK group exhibited unique pathways, including alanine, aspartate and glutamate metabolism; arginine biosynthesis; nitrogen metabolism; pyrimidine metabolism; ABC transporters, and so on. On the other hand, the High/CK group had distinctive pathways, such as flavone and flavonol biosynthesis; amino sugar and nucleotide sugar metabolism; tyrosine metabolism; linoleic acid metabolism; phenylpropanoid biosynthesis; anthocyanin biosynthesis; betalain biosynthesis, and more. Both the Low/CK and High/CK groups displayed significant changes in several pathways due to Cd stress in rice stem tissues. These pathways included glycerophospholipid metabolism; flavonoid biosynthesis; glutathione metabolism; valine, leucine, and isoleucine metabolism; and arginine and proline metabolism in rice stem tissues. These findings suggest that Cd stress significantly affected these metabolic pathways in the stem tissues of rice.

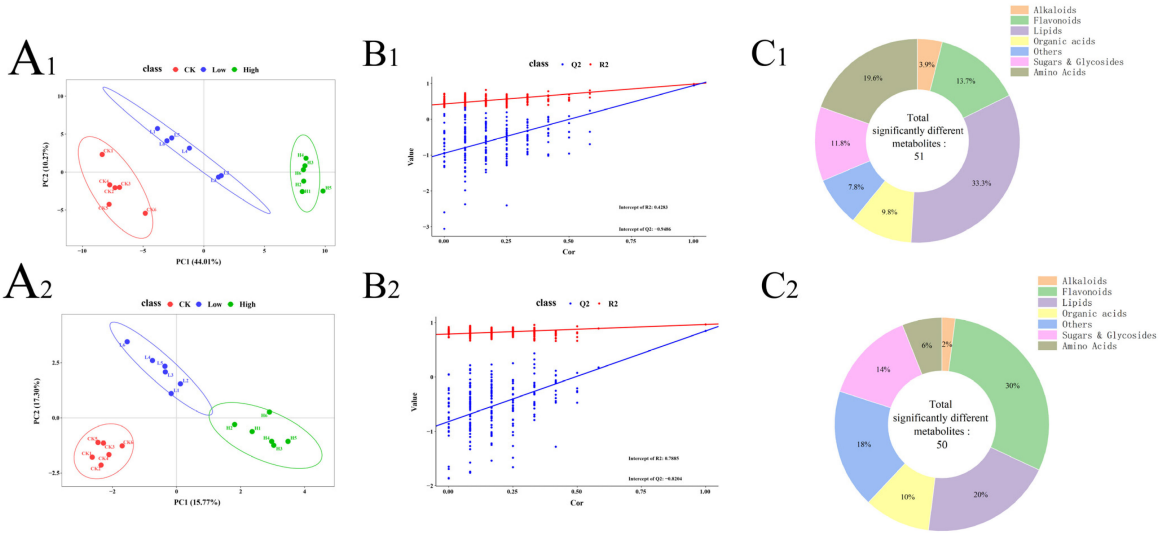

Figure 6. (**A**) Partial least square method (PLS-DA) scores plot; (**B**) permutations plot of PLS-DA; (**C**) proportions of significantly altered metabolites (VIP > 1 and $p < 0.05$) in different categories in rice roots. CK: control; Low: 2 mg kg^{-1}; High: 10 mg kg^{-1}, 1 and 2 represent stems and leaves, respectively. Each treatment included six independent biological replicates.

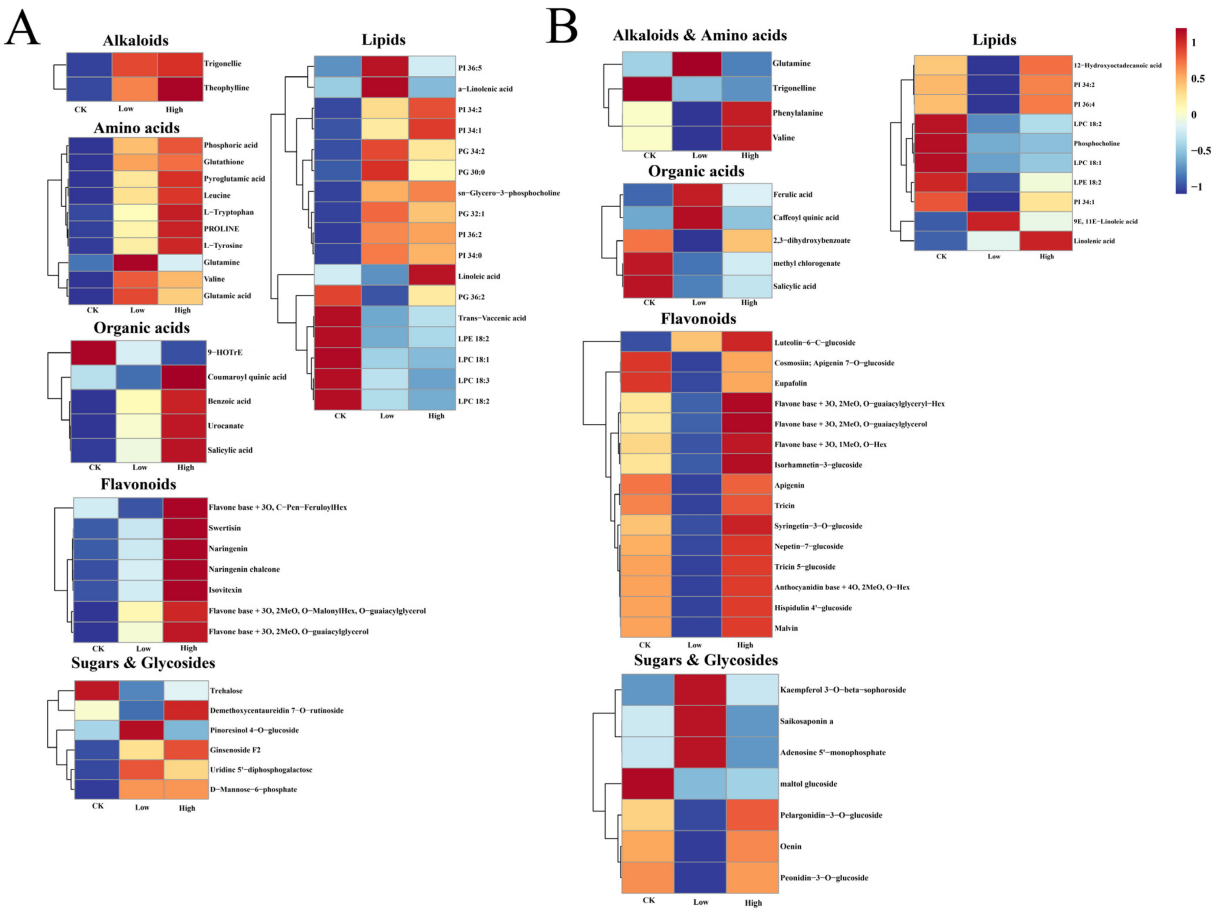

Figure 7. Heat map analysis of metabolites identified in rice stem (**A**) and leaf (**B**) tissues with significant differences by substance ($p < 0.05$). CK represents the control group, and Low, and High represent low and high cadmium treatment groups, respectively. The red and blue blocks indicate significant upregulation and downregulation, respectively.

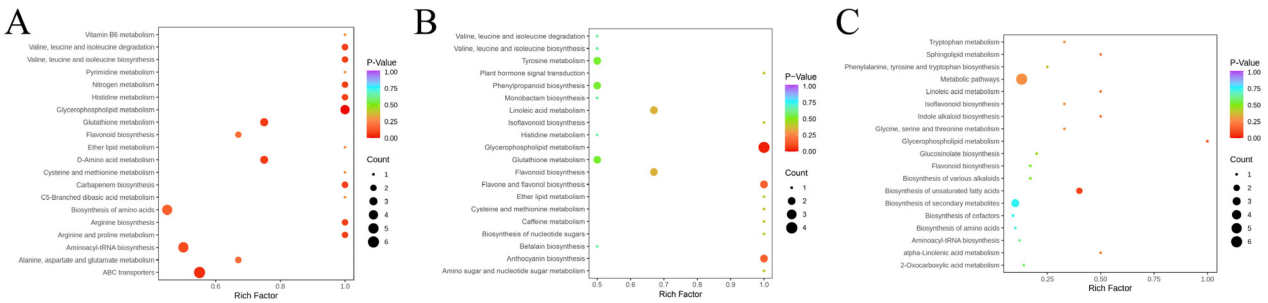

Figure 8. Pathway analysis of metabolites with significant differences in stem tissue in Low/CK group (**A**) and High/CK group (**B**), and leaf tissue in the High/CK group (**C**). The count is the number of compounds matched from our data and *p* values with the *p*-value calculated from pathway analysis. A larger Rich factor indicates a greater degree of enrichment.

In leaf tissue, we observed that the Low/CK group did not work in the effective enrichment analysis. However, the High/CK group revealed various enriched pathways, such as unsaturated fatty acid biosynthesis; tryptophan metabolism; phenylalanine, tyrosine, and tryptophan biosynthesis; alpha-linolenic acid metabolism; flavonoid biosynthesis; sphingolipid metabolism; aminoacyl-tRNA biosynthesis, and more (Figure 8C). Our results indicated that metabolic pathways in rice stem and leaf tissues were significantly affected by different Cd treatments. Notably, the pathways in stem tissues appeared to be more complex, indicating that stem tissues were more susceptible to Cd stress than leaf tissues.

4. Discussion

Cadmium(Cd) is a non-essential and toxic element known for its high level of toxicity to plants, which hinders their growth [4]. Generally, plants respond to environmental stress by adapting their metabolism and morphology. To investigate the effects of Cd-induced stress on rice, we analyzed plant morphology, physiological activities, and tissue metabolism in roots, stems, and leaves.

Under Cd stress, rice regulates the absorption and transport of external substances by altering the number and length of root hairs and multiple stomata on the leaves in order to adapt to environmental pressures [24]. As displayed via SEM, the root hair was inconspicuous, and the cell tissue space was considerably enlarged in the High group, which would affect the uptake of soil nutrients by the rice because of the reduced contact area between the rice roots and soil nutrients [25]. The structural changes in root tissue might lead to a decrease in plant biomass in the High group. In addition, the Cd addition resulted in a decrease in the number of stomata in the leaf tissue of rice leaves. Our results were consistent with what was found by Wang [26]. The aboveground height measurements of rice revealed that the High group exhibited a significantly lower height compared to the other groups. This could be attributed to the damage on the roots and leaves of the High group. The analysis of Cd concentration in different tissue types revealed that rice roots had significantly higher levels of Cd in all Cd-contaminated soils compared to the stems and leaves. This result suggested that rice roots were more susceptible to Cd absorption and accumulation. Additionally, it was easier to transfer Cd from roots to stems, but relatively difficult to transfer Cd from stems to leaves. We found that soil pH was decreased with increasing Cd concentration and the highest soil pH was in the Blank group. Studies have shown that soil pH in the rhizosphere contaminated with Cd was typically lower than in unpolluted soil. Our results were consistent with that discovered by Xie et al. [27]. At the same time, Cd activation and absorption were accelerated as the soil pH decreased, which promoted the accumulation of Cd in rice plants [28]. This finding was consistent with the results of increased Cd accumulation in rice plants in the High group.

Proteins can bind to heavy metal ions to reduce the toxicity of free heavy metal ions to plants [29]. Therefore, the TP content of rice decreased with the increased Cd concentration in this study. SOD and POD were considered protective enzymes due to their ability to

scavenge active free radicals [30]. SOD and POD levels in the root, stem, and leaf tissues of rice significantly increased under Cd stress ($p < 0.05$). The observed alterations in SOD and POD levels suggest that Cd stress induced damage to the rice cells. This outcome could also be confirmed by the damage to the cell wall surface of rice roots in the High group (Figure 1(A_1–C_1,A_3–C_3)). We observed that rice incurred oxidative damage in the following order: root > stem > leaf. Furthermore, we found a positive correlation between the accumulated Cd content in the various tissues and the degree of oxidative damage inflicted on the plant. The Cd content was greater in the roots compared to the stems and leaves. The results demonstrated that the higher the accumulated Cd content in each tissue, the more severe the oxidative damage to the plant. Chlorophyll is a crucial factor in plant photosynthesis and growth, which makes it a significant determinate of plant abiotic tolerance [31]. Chlorophyll a, b, and the total chlorophyll content in rice leaves exhibited an initial increase followed by a decrease as the Cd stress concentration increased. In comparison to the control group (CK), there was a significant increase ($p < 0.05$) in the Low group and a decrease in the High group. The protective adaptability of the plant probably stimulated chlorophyll synthesis in the Low group. Cd accumulation in rice plants might disrupt the plant's defense system, resulting in chlorophyll degradation and chloroplast structure damage [32]. MDA could reflect the degree of lipid peroxidation in rice and, indirectly, the degree of cell damage [33]. Our study found that MDA decreased significantly in the Low group and increased significantly in the High group. The reason for such a result might be that Cd might activate antioxidant enzymes to inhibit MDA synthesis in the Low group, while high concentrations might promote increased reactive oxygen species (ROS) and consequently MDA synthesis [34]. The SEM analysis also yielded similar results. Moreover, the study indicated that the root surface damage in the Low group was less than that in the High group.

In this study, an untargeted metabolomics analysis of plant metabolites was performed based on LC-QTOF/MS. The goal was to identify the metabolites differentially abundant in root, stem, and leaf tissues. A total of 103, 51, and 50 significantly different metabolites were identified in root, stem, and leaf tissues, respectively. These included alkaloids, flavonoids, glycosides, lipids, organic acids, amino acids, sugars and glycosides, terpenoids, and other compounds. Amino acid metabolism is one of the most important sources of energy in cells [35]. It is also a precursor of various essential metabolites that regulate gene expression, post-translation protein modification, cell fate, and so on [36]. In root tissue, five amino acids were significantly altered. Arginine is a vital medium for nitrogen transport and storage [37]. The concentration of Cd in the roots was found to have an inverse correlation with the level of N,N-dimethylarginine, thereby disrupting arginine biosynthesis and nitrogen metabolism in rice roots. Glutamic acid serves as an essential precursor for the metabolism of amino acids in plants [38]. Our study found that Cd stress significantly downregulated gamma-glutamyltyrosine and gamma-glutamylleucine in root tissues, which in turn affected root alanine, aspartate, and glutamate metabolism. There were 10 and 3 significant variations in the amino acids in stems and leaves, respectively. Isoleucine, valine, and proline are crucial amino acids, with proline performing various protective functions, such as osmotic protection, scavenging reactive oxygen species (ROS), and maintaining redox balance under adverse conditions [39]. In stems, leucine, valine, and proline expression levels were upregulated by 1.71-, 1.54-, and 2.60-fold in the Low/CK group, respectively, and they were upregulated by 2.07-, 1.45-, and 3.85-fold in the High/CK group, respectively. Additionally, Cd stress significantly affected the metabolic pathways related to valine, leucine, and isoleucine degradation and biosynthesis, and arginine and proline metabolism. The results suggest that precursor regulated genes and related pathway enzymes associated with the expression of isoleucine, valine, and proline were altered in response to the effects of Cd stress in rice. Glutathione is an important antioxidant that can protect cells from oxidative stress and aging, and it plays an important role in living organisms [40]. In stem tissues, glutathione was upregulated 7.76-fold in the Low/CK and 8.55-fold in the High/CK group. Plants might respond to external stress by removing excess

free radicals, thereby protecting the plant from damage caused by Cd stress [41]. The KEGG of stem tissue also confirmed that rice protects itself from Cd stress through the glutathione pathway. Organic acids have been associated not only with energy production and the formation of precursors for amino acid biosynthesis but also with the tolerance of the plant to environmental stress, which could regulate its ability to adapt to the environment at the physiological level [42]. We found a total of 14 organic acids with significant changes in the root tissue. Caffeic acid is a natural phenolic acid belonging to the category of organic acids known for its strong antioxidant properties. Ferulic acid is one of its derivatives and acts as a free radical scavenger [43]. Caffeic acid was downregulated 0.61-fold in the Low/CK but slightly accumulated in the High/CK group in root tissue. Ferulic acid was downregulated 0.76- and 0.61-fold in the Low/CK and High/CK groups, respectively. The results showed that organic acid metabolites with antioxidant activity were significantly affected with the increase in the concentration of Cd stress, which might be caused by the consumption of these substances in response to Cd stress in rice.

Flavonoids are naturally occurring polyphenolic compounds that act as secondary metabolites in plants, with diverse functions such as the regulation of cell growth and providing resistance to biotic and abiotic stress. It is well-known that abiotic stress triggers an increase in the synthesis and accumulation of flavonoids in plants [44]. Heatmap data from root tissues showed that most flavonoids were significantly upregulated in the Low/CK group and slightly upregulated in the High/CK group. In contrast to the studies conducted by Chang et al. [45] on Cd-stressed NH199 and NH224 rice varieties, TLY215 exhibited a significant regulation of flavonoid-related pathways, including flavonoid biosynthesis, isoflavonoid biosynthesis, and flavone and flavonol biosynthesis. Apigenin has been shown to promote cell cycle arrest and induce apoptosis by regulating the cellular responses to oxidative stress and DNA damage [46]. For example, apigenin-7-O-glucoside and 6,8-Di-C-beta-D-arabinopyranosylapigenin were upregulated 1.27- and 2.77-fold, respectively, in the Low/CK group. The results demonstrated that rice root tissues exhibit resilience to Cd stress by secreting a large amount of antioxidant flavonoids and enhancing their antioxidant defense mechanisms under low concentrations of Cd, which helped us to understand the role of flavonoids in plant tissue defense after Cd stress. In stem tissues, apigenin levels were upregulated 1.13- and 1.84-fold in the Low/CK and High/CK groups, respectively, while in leaf tissues they were downregulated 0.76- and 0.95-fold, respectively. Honeysuckle flavonoids provided cellular protection against oxidative damage by scavenging free radicals, enhancing non-enzymatic antioxidant activity and regulating redox balance [47]. In leaf tissues, several types of honeysuckle flavonoids (including luteolin-6-C-glucoside and eupafolin) were significantly upregulated 1.36-fold and 1.04-fold in the High group, respectively, which might have an impact on the growth of the leaf and thus affect the energy absorption and conversion and photosynthesis of rice. Metabolic pathway enrichment analysis indicated that both flavonoid and flavonol biosynthesis and flavonoid biosynthetic pathways were significantly affected, suggesting that flavonoid conversion in rice stem and leaf tissues was also influenced.

Lipids have been implicated in various biological functions, including signal transduction, the formation of biological membranes, carbon storage, the regulation of cellular signaling pathways, and stress responses [48]. Heatmap analysis showed that some lipids were significantly upregulated in the Low/CK group, but most lipids were significantly downregulated in the High/CK group. Research showed that fucoxanthin exhibits effective resistance to external stress, ensuring normal cell growth and development, and it exerts a physiological effect on optimizing plant growth, development, and immunity [49]. In root tissues, fucoxanthin was upregulated 4.37-fold in the Low/CK group and downregulated 0.82-fold in the High/CK group. Lipid was significantly decreased in stems and leaves under the influence of Cd stress. Phosphorylcholine is a vital component of the outer layer structure of cell membranes composed of phospholipids [50]. LPC 18:1, LPC 18:2, LPC 18:3, and other similar components were significantly downregulated in the stems and leaves. Metabolic pathway analysis revealed that the glycerophospholipid pathway was similarly

affected. These results indicate that the plants might have experienced oxidative damage and disruption to lipid metabolism, subsequently affecting cellular activities. Sugars and glycosides are crucial components of plant growth, responsible for nutrient storage and forming the plant's structural framework. In addition, they interacted with phytohormones to regulate and control plant growth and development [51,52]. Sugars and glycosides were significantly increased in stem tissues under Cd stress, which might contribute to promoting cell wall synthesis and providing an energy source for cellular responses to specific stresses.

5. Conclusions

In this study, untargeted metabolomics technology was employed to evaluate the metabolic effects of Cd on the root, stem, and leaf tissues of the rice cultivar "TanLiangYou215" at different concentrations. The results indicated that Cd had a significant effect on the growth and development of rice. The growth height and ultrastructure of the rice was significantly different from the CK group. The growth of rice plants was promoted under low Cd stress, while high Cd stress inhibited their development, leading to an overall trend of "low promotion and high inhibition". Physiological indicators such as total protein (TP), enzyme activities (SOD, POD), chlorophylls (including chlorophyll a, chlorophyll b, and total chlorophyll) and malondialdehyde (MDA) showed the significant effects of Cd stress on the physiology of rice tissues. The Low group exhibited lower levels of oxidative damage compared to the High group. The untargeted metabolomics analyses demonstrated that the physiological metabolism of all rice tissues was influenced by Cd stress. A total of 103, 51, and 50 metabolites with significant differences were detected in root, stem, and leaf tissues, respectively. Most of the alkaloids, flavonoids, organic acids, amino acids, lipids, terpenoids, sugars and glycosides were significantly altered by Cd stress. To resist Cd stress, rice upregulated amino acids and controlled related pathways, including alanine, aspartate and glutamate metabolism, and arginine and proline metabolism, among others. Rice was able to resist the oxidative damage caused by Cd accumulation in root tissue thanks to the significant expression of flavonoids with antioxidant properties. However, Cd stress had an impact on the cellular activities in rice stem and leaf tissues by significantly downregulating glycerophospholipid metabolism. These results indicated the adaptability of TLY215 to environmental changes and its ability to exhibit proactive metabolic regulation. These findings contributed significantly to our understanding of the physiological and metabolic effects of Cd on rice. In addition, these findings provide valuable insights for further exploring the potential of TLY215 to resist stress under Cd stress conditions and promote its growth and development even in the presence of Cd contamination.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/chemosensors11110558/s1, Figure S1: total ion flow chromatogram of QC samples. Figure S2: MSDIAL data processing parameter settings for positive (A) and negative (B) ions. Figure S3: identification information for four compounds. The red and blue colors represent references and measurements, respectively. Figure S4: rice root tissue differential metabolites' volcano plots in the Low group (A) and High group (B) compared with the CK group, CK: control; Low: 2 mg kg^{-1}; High: 10 mg kg^{-1}, respectively. Figure S5: differential metabolites' volcano plots in the Low group (A) and High group (B) compared with the CK group, CK: control; Low: 2 mg kg^{-1}; High: 10 mg kg^{-1}; 1 and 2 represent stems and leaves, respectively. Tables S1–S3: information about MS peaks identified from the roots, stems, and leaves of rice.

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