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Comparative analysis of proteomic changes during cadmium stress metabolic responses by quantitative iTRAQ in maize (*Zea mays* L.) seedling roots

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ABSTRACT: Cadmium (Cd), a nonessential heavy metal, has become the most important metal pollutant in soils. Cd inhibits plant growth and development and causes substantial reductions in crop yield. However, limited information is available regarding the heavy metal stress-related proteome of maize. We aimed to analyze the proteomic alterations induced by Cd exposure during maize root development to identify potential genes or pathways related to Cd stress. Consequently, an isobaric tag for relative and absolute quantification (iTRAQ)-based proteomic approach was used to analyze the protein expression from seedling roots of the non-hyperaccumulator inbred line B73 and hyperaccumulator inbred line Mo17 at four developmental stages (0, 12, 24, and 48 h) under heavy metal cadmium pollution. The results showed that 209, 294, 422, and 384, 352, 489 proteins were screened as differentially expressed proteins (DEPs) between the control and Cd-treated roots for 12, 24, and 48 h in B73 and Mo17, respectively. Ten DEPs were identified as commonly expressed in both B73 and Mo17, including Ribosomal protein L15, L17c, and L36, Peroxidase 3, GST, ADP-ribosylation factor A1B, voltage-dependent anion channel protein 1b, among others. In B73, DEPs related to ribosomal protein synthesis were significantly upregulated, while they were downregulated in Mo17. Simultaneously, CRPs associated with antioxidant activity were identified, suggesting that B73 might enhance cadmium tolerance by improving protein synthesis and ROS scavenging capacity. These discoveries will offer valuable perspectives into the molecular mechanisms of maize responses to cadmium toxicity and detoxification at the protein level.

KEYWORDS: cadmium stress, iTRAQ, proteomics, maize, pollution

INTRODUCTION

Cadmium (Cd) uptake by crops has hazardous effects on human and animal health and food safety because Cd is transferred through the food chain [1]. Maize (*Zea mays* L.), one of the most important cereals in the world, is sensitive to cadmium stress; thus, it is important to elucidate the molecular mechanisms and detoxification processes during Cd exposure in maize.

Plants that are exposed to high Cd concentrations show symptoms of heavy metal toxicity such as reductions in both root and shoot length, leaf bleaching [2], necrosis, and wilting [3], which lead to yield reduction. Uncontaminated soils typically exhibit cadmium concentrations ranging from 0.04 to 0.32 μM, whereas moderately polluted soils contain Cd levels in the range of 0.32 to 1.00 μM. Low concentrations of Cd are not toxic to plants [4]. At high concentrations, Cd is toxic to maize plant growth and development because it affects various biochemical processes or pathways [5, 6], including photosynthetic activity [2], carbohydrate metabolism, nitrogen metabolism [7, 8], and basic mineral nutrient uptake [9, 10]. Numerous studies have demonstrated that both abiotic and biotic stresses influence plant root morphology and their capacity for heavy metal uptake [11, 12]. In addition, plants cultivated in heavy metal—contaminated soils have developed integrated homeostatic mechanisms to regulate the absorption, mobilization, and intracellular levels of toxic metal ions, thereby mitigating stress-induced damage [13]. These mechanisms include association with mycorrhiza, binding of metals to the cell wall, reduced uptake or active efflux across the plasma membrane, repair and protection of the plasma membrane, and chelation of Cd by phytochelatins (PCs).

Though many studies have enhanced our knowledge of Cd toxicity symptoms and how plants respond to Cd stress, alterations in protein levels offer a more accurate reflection of changes in metabolic activity that take part in heavy metal detoxification [14]. iTRAQ, which has arisen as a new proteomics approach, pro-

vides more reliable quantitative measurements and comparisons among samples at the protein level [15], and it has greatly improved proteomic analyses by adding gene ontology (GO) and pathway studies [16] to data analysis. Through iTRAQ technology, many differentially expressed proteins involved in different pathways were revealed in hemp [17], cultivated tobacco (*Nicotiana tabacum*) leaves [18], soybean [19], barley [20], Arabidopsis [21], and rice [22] under diverse abiotic and biotic stress conditions.

In recent years, several transcriptomic and post-transcriptional studies have explored the molecular responses of maize roots under Cd stress. Peng et al [5] performed a comparative RNA-Seq analysis in B73 and Mo17 and identified DEGs involved in stress response, transport, and transcriptional regulation under Cd exposure. In a complementary study, Gao et al [23] investigated Cd-responsive microRNAs (miRNAs) and their target genes in the same two genotypes, revealing genotype-specific expression of miRNAs such as Zma-miR156b, Zma-miR167f, and Zma-miR171b, which negatively regulate key transcription factors including SBP-box, ARF, and GRAS, respectively. These studies highlighted complex regulatory networks at the transcriptional and post-transcriptional levels. However, little is known about the proteomic changes that directly reflect functional responses under Cd stress, especially between maize genotypes with contrasting Cd tolerance. Therefore, this study employed iTRAQ-based proteomics to provide novel insights into genotype-specific protein expression patterns and regulatory pathways associated with Cd stress adaptation in maize roots.

MATERIALS AND METHODS

Plant materials and experimental design

The inbreeding corn line B73 has been shown to be more resistant to stress from heavy metals than Mo17 [24], particularly in the setting of Cd stress. Seeds from B73 and Mo17 were treated with 10% H_2O_2 for 1 h, rinsed for 24 h in distilled water, and sprouted at 25 °C for 3 days in this investigation. The seedlings were grown in modified Hoagland solution [25] under conditions of a 14-h light and 10-h dark cycle at temperatures ranging from 24 to 26 °C. The seedlings were separated into 2 groups, the control group and the treatment group, at random after 20 days. The treated and control seedlings were treated with 20 μM $CdCl_2 \cdot 2.5H_2O$ solution and Cd-free Hoagland solution, respectively. Subsequently, we gathered maize seedlings in the control and treatment groups following 0, 12, 24, 48, 72, and 96 h of immersion with 20 μM $CdCl_2 \cdot 2.5H_2O$. Superoxide dismutase (SOD) and peroxidase (POD) activities were evaluated following the protocols outlined by Beyer et al [26] and Kim et al [27], respectively. Catalase (CAT) activities and malondialdehyde (MDA) activities were determined following the method by Hussain et al [28]. Three biological replicates were utilized, and each measurement was performed 3 times. Utilizing the assay results of enzyme activity, the seedling roots from 2 groups were individually gathered at 0, 12, 24, and 48 h and then subjected to iTRAQ analysis for the proteomics experiments with two biological replicates.

Protein preparation and digestion

The root tissue, with a fresh weight of approximately 0.5 g, was promptly pulverized into a powder in liquid nitrogen. The resulting powder was then suspended in a lysis buffer (PH 8.0) consisting of 8 M urea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS), and 40 mM Tris-HCl to achieve the final concentration. Subsequently, 1 mM PMSF and 2 mM ethylenediaminetetraacetic acid (EDTA) were added. After vigorous vortexing for 5 min, dithiothreitol (DTT) was introduced to reach a final concentration of 10 mM. The cells were then disrupted by sonication for 15 min. The supernatant was collected after centrifugation at $25{,}000 \times g$ for 20 min and mixed thoroughly with 10 mM DTT at 57 °C for 1 h. Following this, iodoacetamide (IAM) was added to a final concentration of 55 mM and incubated in the dark for 45 min. The solution was then mixed with prechilled acetone (1:4, v/v) and incubated at −20 °C for 2 h. After centrifugation at $25{,}000 \times g$ for 20 min, the supernatant was discarded, and the resulting precipitate was resuspended in 200 μl of 0.5 M triethylammonium bicarbonate (TEAB) and subjected to sonication for 15 min. Finally, the supernatant was collected after a further centrifugation at $25{,}000 \times g$ for 20 min and quantified using the Bradford method. A total of 100 μg of protein from each sample was accurately weighed and digested with trypsin at 37 °C for 12 h (Sigma-Aldrich, St. Louis, MO, USA; added at a 1:20 w/w ratio at 0 and 4 h).

iTRAQ labeling and LC-MS/MS proteomic analysis

By the manufacturer's operating procedures, samples from the control group and the Cd-treated group were selected. Peptide samples were labeled using the iTRAQ reagent 8-plex kit (Applied Biosystems, Foster City, USA). For Cd-treated samples in B73, samples taken at 0, 12, 24, and 48 h were labeled with iTRAQ reagents, corresponding to molecular masses of C1_113, C2_114, C3_115, and C4_116 Da, respectively. Cd-treated samples for Mo17 were labeled T1_117 (0 h), T2_118 (12 h), T3_119 (24 h), and T4_121 (48 h) Da iTRAQ tags. A robust SCX column was used in Agilent 1200 HPLC (Agilent, Santa Clara, California, USA) for the purification of all pooled samples following labeling. The use of an Eksigent nanoLC-Ultra 2D system (AB SCIEX, Massachusetts, USA) allowed for the conduct of a sophisticated liquid

chromatography (LC) separation. After that, the acquisition of mass spectrometry data was accomplished utilizing the Triple TOF 5600 system (AB SCIEX), which was equipped with the Nanospray III source (AB SCIEX). Data were acquired as follows: nebulizer gas at 15 psi, curtain gas at 30 psi, interface heater temperature of 150 °C, and ion spray voltage of 2.5 kV. Survey scans were obtained at 250 ms for information-dependent acquisition (IDA), and if a product ion scan exceeded 150 counts per s (counts/s) with a charge-state of 2+ to 5+, up to 35 product ions were collected. The overall time for cycles was set to 2.5 s. The collision-induced dissociation (CID) for all precursor ions utilized the same rolling collision energy setting. Dynamic exclusion was applied to the Kof peak width (18 s), and then the precursor was refreshed off the exclusion list.

Proteomic data analysis

MASCOT Engine (Matrix Science, London, UK; Version 2.2) was integrated into Proteome Discoverer 1.4 (Thermo Electron, San Jose, USA) and then through the corn protein database search (http://ftp.maizegdb.org/MaizeGDB/FTP/maize_proteome/proteome.fasta) for MS/MS. Subsequently, the following parameters were employed for protein identification: fragment mass tolerance of 0.1 Da, trypsin enzyme, peptide mass tolerance of ±0.05 Da, maximum missed cleavage of 1, missed values of monoisotopic, fixed modifications including carbamidomethyl (C), iTRAQ 8-plex (N-term), and iTRAQ 8-plex (K), and variable modification as iTRAQ 8-plex (Y) [29].

Differentially expressed proteins (proteins showing significant downregulation or upregulation) were calculated with ProteinPilot software. Fold changes in Cd-treated vs control plants were determined by calculating the ratio of protein abundances. Duncan's multiple range test was employed to scrutinize statistically meaningful differences ($p < 0.05$) in means between plants subjected to Cd treatment and their corresponding control counterparts. Furthermore, confidence values greater than 1.5 or less than 0.66-fold, along with a peptide confidence level of 95%, were used for the qualification criterion [30]. Proteins that differed from their matched controls by at least 1.0-fold were considered differentially expressed [31].

GO annotation and KEGG pathway enrichment of DEPs related to Cd stress

We utilized the agriGO tool, which can be available at http://bioinfo.cau.edu.cn/agriGO/, to assign DEPs associated with Cd stress related to GO functional categories. The statistical enrichment of DEPs in KEGG pathways related to pathway enrichment analysis was tested by KOBAS software [32].

RESULTS

The impact of Cd stress on maize root system development

Here, the enzymatic activities of SOD, POD, MDA, and CAT were conducted in B73 and Mo17 in a series of treatment times (0, 12, 24, 48, 72, and 96 h). To ascertain the suitable duration for validating candidate Cd-regulated DEPs, we analyzed maize roots treated with Cd over 0 to 96 h. The results showed that significant increases in SOD, POD, and MDA activities were observed in both Mo17 and B73 within 12 h after Cd treatment and decreased after 24 h. Interestingly, SOD and POD activities were higher in B73 than in Mo17, while MDA showed the opposite trend. These results indicate that B73 has a stronger defense against oxidative stress. In addition, CAT activity decreased rapidly after the 12 h increase, suggesting that this enzyme is involved in the response at an early stage (Fig. 1). We demonstrated that shortly after the Cd treatment, certain Cd-tolerant mechanisms were activated to maintain normal physiological and metabolic activities. Following that, B73 and Mo17 treatment and control (CK) were used for protein isolation to provide a protein basis for further study.

Summary of quantitative proteomics analysis

Subsequently, to identify DEPs, using the iTRAQ method, proteome profiles were generated quantitatively for seedling roots exposed to Cd stress at 4 development stages (0, 12, 24, and 48 h). A comparison of the 2 biological replicates revealed that more than 80% of the proteins were reproducible with a delta error under 0.5 (Fig. 2A, Table S1). A total of 2,573 and 2,440 proteins were obtained from 6,479 and 6,029 unique peptides that matched 26,083 and 24,151 unique spectra, respectively, with high scores from the 2 replicates of iTRAQ LC-MS/MS proteomic analysis (Fig. 2B). In terms of protein mass distribution, a wide molecular weight (MW) range for proteins larger than 10 kDa was obtained, with good coverage of 30 to 45% of the total proteins in each MW group (Fig. 2C). Among these proteins, 46% were identified with a sequence coverage of more than 10%, and 20% exhibited a sequence coverage exceeding 20% (Fig. 2D). The above results indicated that the protein molecular weight range was broad, with good coverage.

Identification of differentially expressed proteins (DEPs) in response to Cd treatment

To additionally pinpoint the DEPs in maize roots under Cd stress, DEPs were identified in a series of treatment times under Cd stress in B73 and Mo17 (Table S2). Proteins exhibiting a fold change of over 1.0 with a p-value below 0.05 were deemed significantly altered in comparison with the control group (CK). Of the identified DEPs, 105, 148, 252, and 181, 207, 229 upregulated DEPs were obtained from the 3 dynamic

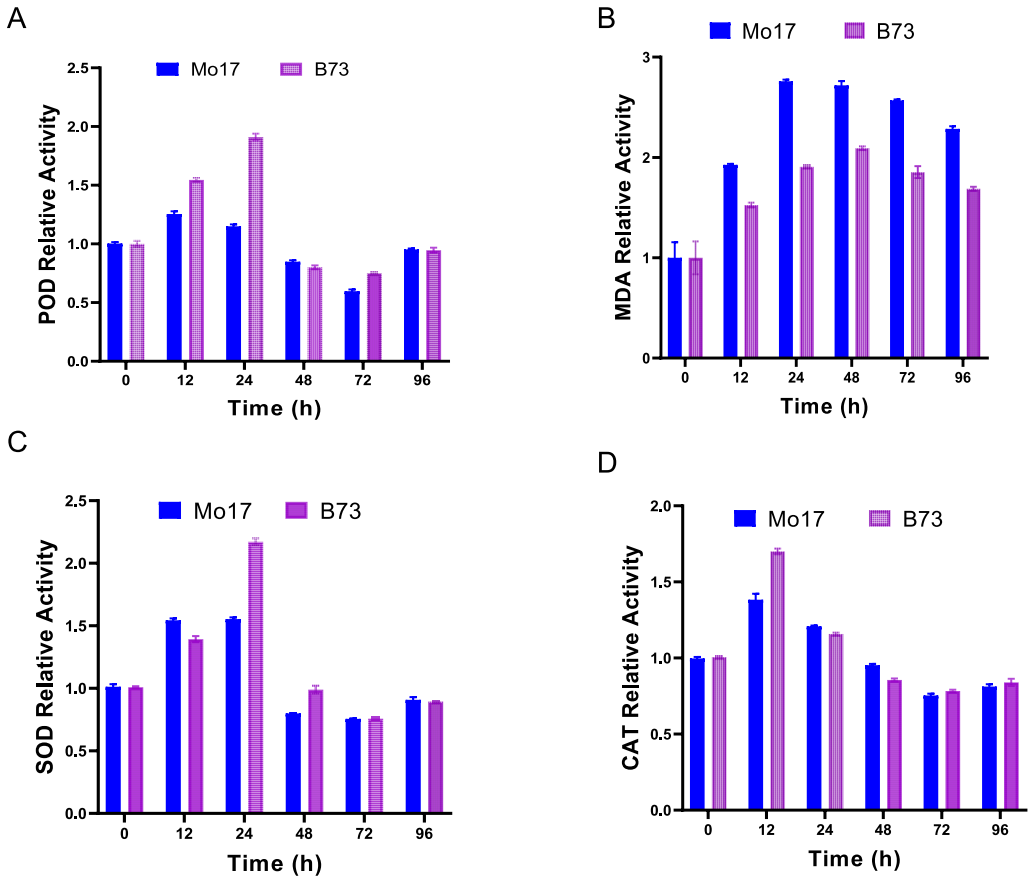

Fig. 1 SOD, POD, MDA, and CAT conducted in B73 and Mo17 in a series of treatment times. All data were normalized to the 0 h value and expressed as relative values.

development stages (12, 24, and 48 h) in B73 and Mo17, respectively, as well as 104, 146, 170, and 203, 145, 260 downregulated DEPs (Fig. 3A). Mo17 has a higher number of DEPs than B73 in the early response, suggesting its more sensitive but possibly more chaotic regulation. Subsequent Venn analysis showed that a total of 10 proteins were changed in both B73 and Mo17 in the treatment groups at different time points (Fig. 3B,C). Compared with the CK group, Zm00001d034356, Zm00001d031345, and Zm00001d031611, which were upregulated at 12, 24, and 48 h in both B73 and Mo17, encode glutathione transferase 5, beta-glucosidase aggregating factor-like protein, and ribosomal protein L17c, respectively. Zm00001d001d008295 (ADP ribosylation factor A1B), Zm00001d01205 (RNA binding protein), Zm00001d014467 (Peroxidase 3), Zm00001d018903 (Ribosomal protein L15), and Zm00001d020219 (voltage-dependent anion channel protein 1b) were downregulated in both lines. However, we found that Zm00001d038374, encoding 60S ribosomal protein L36, was upregulated at 12, 24, and 48 h in B73, but downregulated in Mo17. In addition, Zm00001d029969 (npdk1) was upregulated in B73 at 48 h and downregulated in Mo17. To further dissect the specificity of protein expression under Cd stress, candidate regulated proteins (CRPs) and super candidate regulated proteins (SCRPs) were identified as the regulated proteins that were observed in two or more different time stages, in both B73 and Mo17 maize inbred lines. The results showed that 176 CRPs and 43 SCRPs were identified in B73, and 234 CRPs and 52 SCRPs were identified in Mo17, respectively (Fig. S1A,B).

GO annotation of proteins differentially expressed under Cd stress

For a more in-depth investigation into the function of DEPs associated with the Cd stress response, these 410 CRPs and 95 SCRPs after Cd pollution were used for GO analysis.

GO enrichment analysis indicates that these CRPs occurred in diverse biological processes (BP) and cellular components (CC), spanning single-organism processes, cellular processes, metabolic processes, cells, cell organelles, and macromolecular complexes. In addition, those CRPs in categories associated with bind-

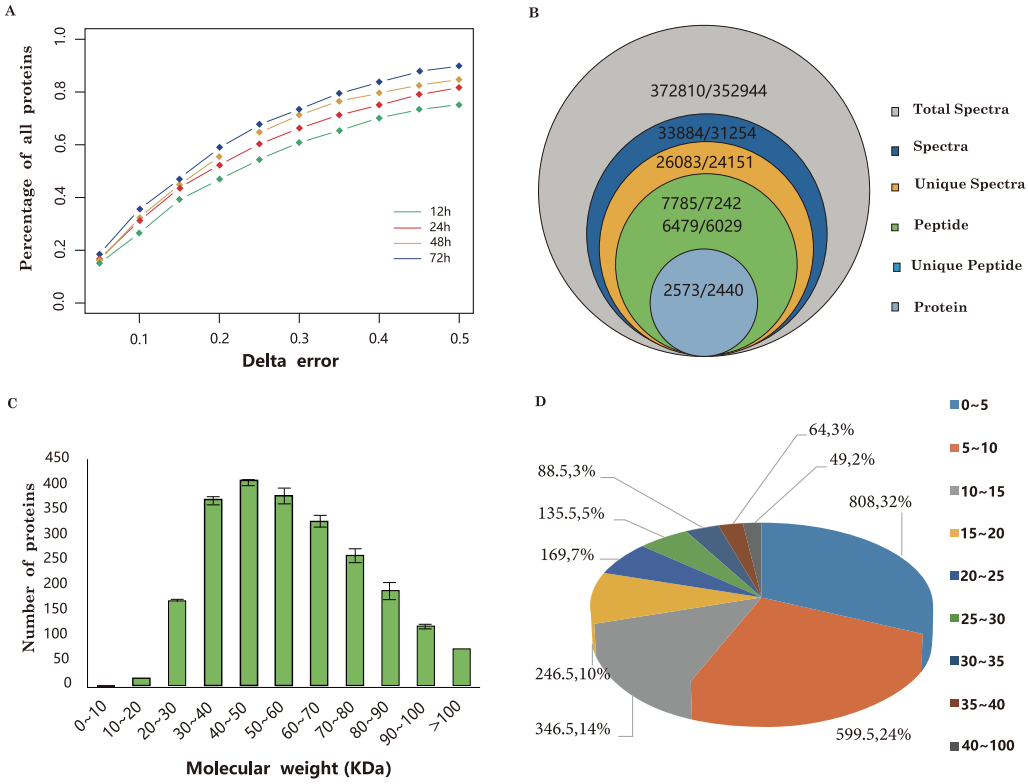

Fig. 2 Overview of quantitative proteomics analysis at different times in the Cd treatment assays.

ing, antioxidant activity, and catalytic activity were identified in MF (Fig. 4).

KEGG pathway analysis of proteins differentially expressed under Cd stress

To gain insights into the functions of CRPs with common responses to Cd stress, KOBAS software was used to identify their functions. Here, more than 50 DEPs involved in different KEGG pathways were selected (Fig. S2). Of those, metabolic pathways, biosynthesis of secondary metabolites, and ribosome were identified as significant pathways, followed by phenylpropanoid biosynthesis, protein processing in endoplasmic reticulum, methane metabolism, and spliceosome. In addition, glycolysis/gluconeogenesis, oxidative phosphorylation, as well as RNA transport were also identified (Fig. S2).

DISCUSSION

Identifying DEPs and their associated pathways in response to Cd exposure is crucial for understanding the molecular mechanisms of Cd stress responses in plants. This study identified some DEPs with various functions that showed large alterations in response to Cd stress. Of these, most DEPs related to protein degradation and ribosomal protein synthesis increased dramatically, while those involved in signaling, binding, antioxidant, energy, and transport pathways decreased significantly. Previous studies reported that the methylation status of DNA-associated proteins is crucial for the gene regulation response to Cd stress in maize seedling roots [33]. Here, proteins related to DNA synthesis or the chromatin structure of histones among upregulated CRPs were detected in our study. Our previous study revealed differential expression of miRNAs in Cd-stressed maize seedling roots but lacked results on protein expression levels [23]. Therefore, in this study, we analyzed the differences in protein expression in the roots of maize seedlings under Cd stress.

Studies have shown that defense proteins that facilitate effective ROS scavenging and molecular chaperones aiding in the restoration of normal protein conformation contribute to maintaining redox homeostasis in heavy metal (HM)-stressed plants [14]. In this study, we detected many upregulated genes involved in defense and stress, such as Zm00001d024750 (annotated as peroxidases), involved in scavenging H_2O_2 and protecting the cell membrane from lipid peroxidation induced by hydroxyl radicals. Additionally, Zm00001d034356, characterized as a glutathione S-transferase (GST)-like protein, was found to engage in multifunctional activities including methylglyoxal detoxification, ROS scavenging, and phytochelatin (PC) synthesis through the ascorbate-glutathione (GSH) cycle [10, 14]. Moreover, 6 CRPs

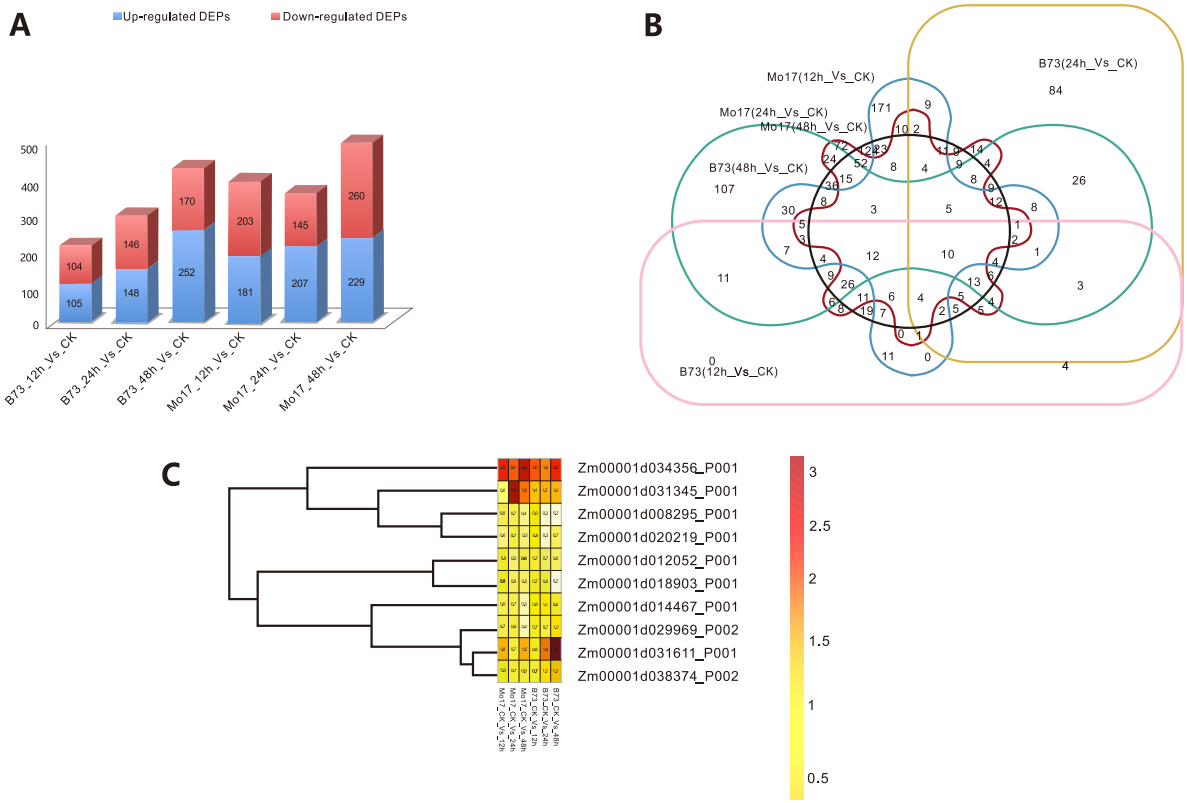

Fig. 3 Proteomic variations at different times in the Cd treatment assays. A: Upregulated and downregulated DEPs identified in B73 and Mo17 at different treatment times under Cd stress. B: Venn diagram of the differentially expressed proteins at 12, 24, and 48 h, compared with CK (0 h). C: Heatmap of Co-modulated DEPs association with Cd stress (Common DEPs identified in both B73 and Mo17).

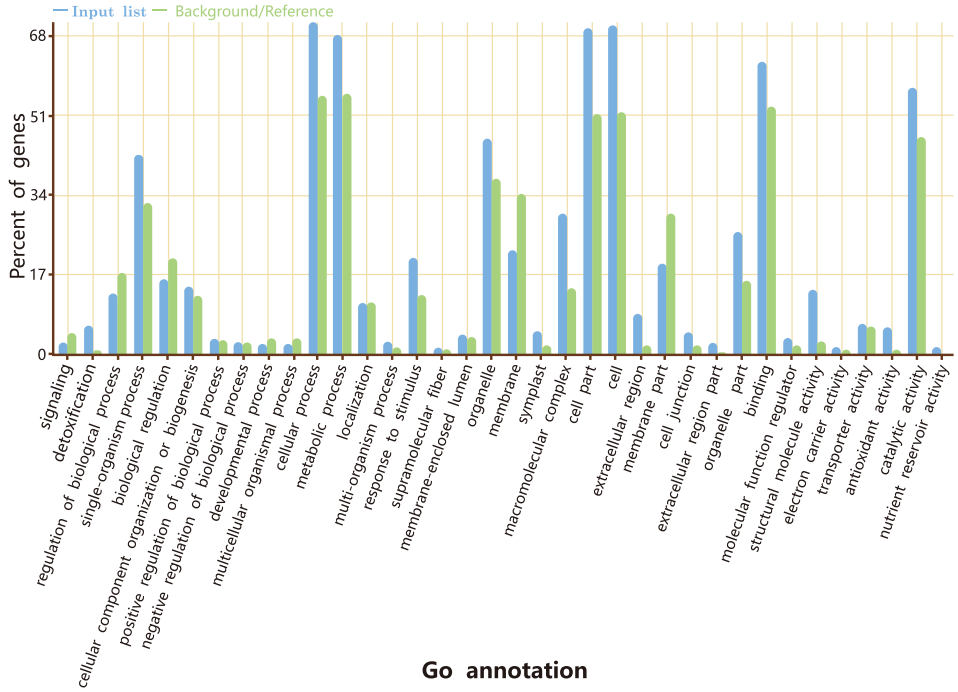

Fig. 4 Gene ontology categories for CRPs identified from both B73 and Mo17 samples.

involved in abiotic and biotic stress were identified, such as heat shock protein (a kind of molecular chaperone) Zm00001d006008; proteins from the universal stress protein (USP) family, Zm00001d043261 and Zm00001d012610; and pathogenesis-related (PR) genes. Moreover, some pathways related to glutathione metabolism and the synthesis of some amino acids were also found in this study, suggesting that these pathways were important for Cd detoxification through various functional genes. Interestingly, this study also identified CRPs related to ribosomal pathways and ribosome protein synthesis. The upregulation of ribosomal proteins (such as L15 and L36) in B73 suggests that enhanced protein synthesis capacity may be a key mechanism in the tolerant line. In rice, ribosome-related genes have different expression levels between sensitive and tolerant rice cultivars after treatment with Al^{3+} [34], suggesting that ribosomes may play a very important role in HM detoxification in plants. Moreover, the ribosome and proteasome pathways were identified as 2 significant pathways in the KEGG enrichment study, and this result was consistent with the gene annotations. Previous studies have demonstrated that plant—plant interactions or the application of soil amendments can alleviate the toxic effects of heavy metals on plants [35, 36]. Building on this, the differential proteomic analysis of maize under Cd stress conducted in the present study provides a molecular basis for the development of novel strategies to remediate heavy metal contamination in maize.

The biosynthesis of diverse cellular biomolecules is the primary way to tolerate or neutralize metal toxicity [37]. Moreover, ATPase and proton pumps are activated to protect cells against excess metals [38]. In our study, a vacuolar-type H^+-pumping pyrophosphatase 1 and a pyrophosphate-energized vacuolar membrane proton pump gene were induced. However, cell wall- and cell organization-related DEPs were downregulated in this study, and we speculated that Cd stress may lead to cell damage by degrading cell wall proteins such as AGPs (fasciclin-like protein) as well as LRRs (leucine-rich repeat family proteins). These results were following aluminum stress responses in rice roots [22]. In summary, some DEPs exhibiting significant alterations in response to Cd stress were detected in maize seedling roots by iTRAQ technology. Significant increases were observed in DEPs involved in protein degradation and ribosomal protein synthesis, while proteins functioning in signaling, binding, antioxidant, energy, and transport pathways showed marked decreases. Furthermore, KEGG pathway analysis revealed that proteins in the ribosome, proteasome, and glutathione metabolism pathways were upregulated. In contrast, proteins involved in phenylpropanoid biosynthesis, phenylalanine metabolism, and endocytosis pathways showed decreases in abundance. Moreover, many upregulated DEPs responsive to Cd involved in the ribosome pathway were first identified in this study, and their effects on Cd detoxification require further study. Notably, the DEPs identified at the protein level in this study more directly reflect physiological response mechanisms, complementing previously unreported expression patterns of Cd detoxification-related proteins in earlier work, thereby providing deeper molecular insights into Cd stress tolerance.

CONCLUSION

This study represents the first application of iTRAQ proteomics to investigate the dynamic responses to Cd stress in roots during development between Cd-tolerant B73 and Cd-sensitive Mo17 maize lines. Furthermore, KEGG pathway analysis confirmed that ribosomal, proteasomal, and glutathione metabolic pathways are key mechanisms in coping with Cd toxicity. The findings not only enrich the proteomic database of maize responses to Cd stress but also provide valuable molecular insights into Cd toxicity and detoxification mechanisms at the protein level.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at https://dx.doi.org/10.2306/scienceasia1513-1874.2025.072. Table S1. Fundamental proteomics data derived from maize roots subjected to Cd exposure, as revealed through iTRAQ with 2 replicates. Table S2. DEPs identified in a series of treatment times under Cd stress in B73 and Mo17. The full data for Tables S1 and S2 can be requested from authors.

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Appendix A. Supplementary data

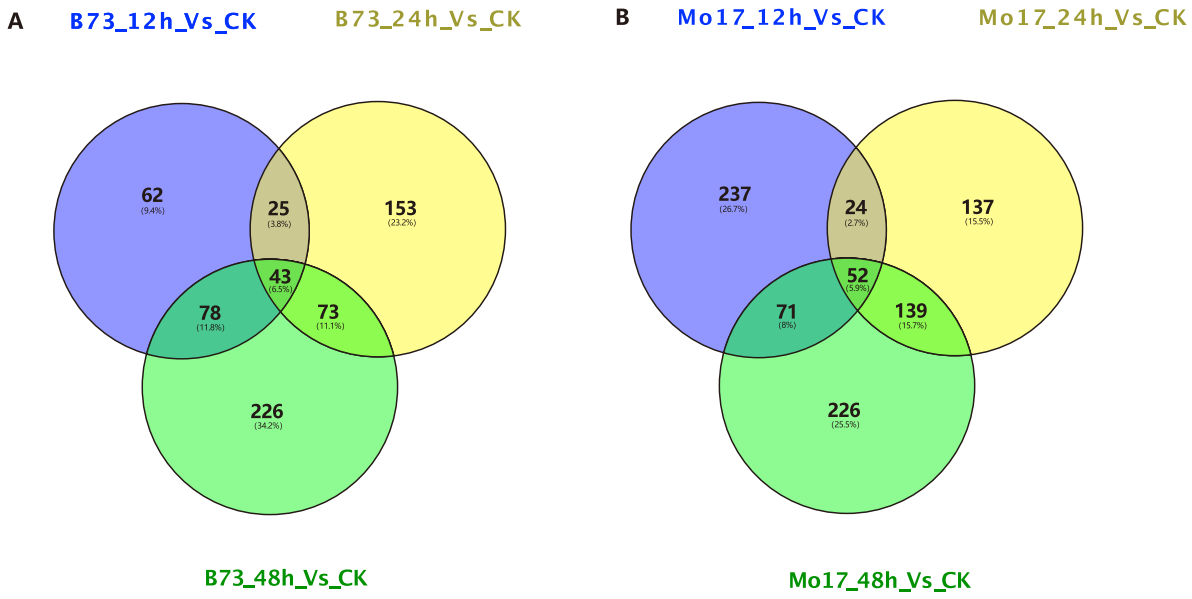

Fig. S1 CRPs and SCRPs related to Cd stress identified in B73 and Mo17 samples.

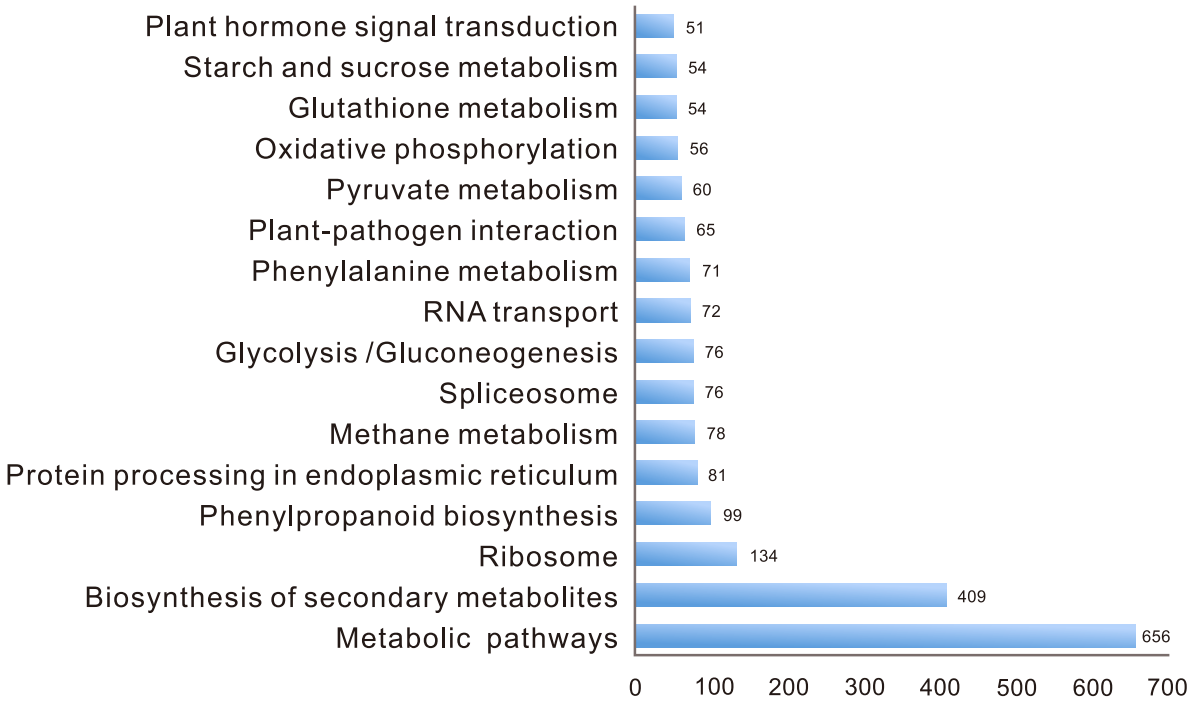

Fig. S2 KEGG pathway analysis of CRPs and SCRPs that are differentially expressed under Cd stress.