Integration of Transcriptome and Metabolome Analyses Reveals the Mechanistic Basis for Cadmium Accumulation in Maize

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Highlights
L63 showed normal vacuolar formation under Cd stress
Multiomics approach revealed the mechanistic basis for low GCA in maize
Eighty-four low-GCA-associated genes were identified
SAM cycle plays key roles in Cd accumulation

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Integration of Transcriptome and Metabolome Analyses Reveals the Mechanistic Basis for Cadmium Accumulation in Maize

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SUMMARY
Cadmium (Cd) pollution in soil has become a major environmental issue worldwide. However, the underlying molecular mechanism of low grain-Cd accumulation (GCA) in maize is still largely unknown. Herein, we report the mechanistic basis for low GCA in maize by a multiomics approach. The low GCA genotype L63 showed normal vacuolar formation and a lower capacity of xylem loading of Cd than the high-accumulator L42 under Cd stress. Transcriptomic sequencing identified 84 low-GCA-associated genes which are mainly involved in the S-adenosylmethionine (SAM) cycle, metal transport, and vacuolar sequestration. A metabolome analysis revealed that L63 plants had a more active SAM cycle and a greater capacity for terpenoid synthesis and phenylalanine metabolism than L42. Combining the analysis of transcriptome and metabolome characterized several genes as key genes involved in the determination of Cd accumulation. Our study identifies a mechanistic basis for low Cd accumulation in maize grains and provides candidate genes for genetic improvement of crops.

INTRODUCTION
Cadmium (Cd), a toxic heavy metal with no known biological function in plants, represents one of the most toxic substances released into the environment (Cao et al., 2020). Cd ranks first among the top six toxic metals (Cd, Cr, Cu, Hg, Ni, and Pb) released into ecosystems and has been recognized as a dire threat to human health (Han et al., 2002). As Cd shows a biological half-life of 10–30 years and is known to be stored in the human kidney, serious health threats through cumulative accumulation can arise even from low levels of chronic Cd exposure (Nordberg, 2009). Furthermore, when accumulated in plants, Cd is known to affect crop yield and cause various pathological symptoms such as chlorosis, oxidative stress, and cell death (Cao et al., 2015, 2020). Thus, there is a clear need to understand the mechanisms of Cd accumulation in food crops, to minimize human exposure.

Cd toxicity induces immediate responses in plants that are manifested in rapid changes at the transcriptional level with simultaneous changes at the physiological and metabolic levels. To minimize the detrimental effects of Cd toxicity, plants have evolved a range of detoxification mechanisms, such as Cd exclusion, chelation, and vacuolar and cell wall compartmentalization (Nakanishi et al., 2006; Jozefczak et al., 2012). Most of the Cd absorbed by roots is retained in roots, and only a small amount is transported to the aboveground tissues of plants. Studies have shown that the efficiency of Cd translocation from roots to shoots depends on the compartmentalization capacity of root vacuoles and cell walls for Cd and the loading capacity of the xylem (Xiong et al., 2009; Luo et al., 2019). Hundreds of genes are involved in these processes. Thus, identification of Cd-toxicity-induced genes is the basis for understanding the molecular mechanisms of Cd accumulation and developing transgenic crops with low Cd accumulation and high tolerance.

Many genes associated with Cd accumulation have been identified in a variety of plant species, most of which are related to ion transport. This includes various transporters such as low-affinity cation transporter 1, heavy metal ATPases 2 (HMA2), HMA3, natural resistance–associated macrophage proteins 1 (Nramp1), and Nramp5 (Ju et al., 2009; Sasaki et al., 2012; Zhu et al., 2016; Ma et al., 2021). For instance, Nramp5 is a major transporter for Cd uptake in different crops (Sasaki et al., 2012; Wu et al., 2016). OsHMA3 and

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ZmHMA3 have been found to affect root-to-shoot Cd translocation in rice and maize by mediating its efflux into vacuoles (Miyadate et al., 2011). Besides, ZIP, IRT, and ABC families were also found to be involved in the efflux and uptake of Cd (Mei et al., 2022; Tang et al., 2021). In addition, several transcription factors (TFs), such as MYBs and WRKYs, have also been found to be activated in plants under Cd stress (Opdenakker et al., 2012). Phytochelatin (PC) is synthesized in the cytoplasm, cell wall, and root surface and decreases free Cd$^{2+}$ levels to protect plants from the deleterious effects of Cd$^{2+}$ (Brunetti et al., 2015; Das et al., 2021). In addition, glutathione (GSH), serving as a precursor for PCs, contributes to increased Cd tolerance by regulating reactive oxygen species (ROS) homeostasis (Rodriguez-Serrano et al., 2006; Cui et al., 2014). However, despite the identification of such key genes, the issue of their regulation remains elusive. Little is known about transcriptional mechanisms regulating Cd transport, in particular, how crops perceive Cd stress signals and the molecular basis of Cd compartmentalization in vacuoles and cell walls.

Transcriptome and metabolome analyses are powerful tools for analyzing the genome-wide changes in genes and metabolites related to biotic and abiotic stress in plants. Maize (Zea mays), as one of main cereals in the world, is a major source of Cd intakes for humans. Compared with other crops, the molecular mechanisms of Cd accumulation in maize are still poorly understood. Our previous study has identified two maize genotypes, L42 and L63, which are high- and low-grain-Cd accumulation varieties, respectively (Lin et al., 2022). This difference was partially attributed to a lower root-to-shoot Cd translocation rate in L63. However, the mechanistic basis of this process has not been revealed. The aim of this study was to reveal the mechanistic basis for low grain-Cd accumulation (LGCA) in maize through ionomic, transcriptomic, metabolomic, and genetic approaches.

RESULTS
Cd Caused Damage to Cell Ultrastructure in Root Tips and ROS Scavenging and Energy Consumption in Roots

To investigate the effect of Cd on the cell ultrastructure of root tips, transmission electron microscopy (TEM) was performed. TEM showed more-severe Cd-induced damage to the organelles of L42 plants than to those of L63 plants (Figure 1). Cd-stressed L42 seedlings had a high number of distorted vacuoles than controls, while Cd stress did not increase the number of distorted vacuoles in L63. GSH acts indirectly as a substrate for some enzymes or PC to alleviate Cd toxicity. GSH content in roots was much higher in L63 than in L42.
RNA-seq Analysis Revealed LGCA-Related Genes in the LGCA Genotype L63

To understand the molecular mechanisms of LGCA and identify potential candidate genes, we performed RNA-seq of L42 and L63 in the presence and absence of Cd. Gene expression profiles of the two genotypes were significantly changed by 24-h Cd treatment compared with those of the control (Figure 2, Table S2). Based on Log2FC being >1 (upregulated) or < -1 (downregulated) and p < 0.05, a total of 680 genes were characterized as differentially expressed genes (DEGs) in the two genotypes. Among them, 267 (299) and 88 (26) genes were upregulated (downregulated) in L42 and L63, respectively (Figures 2A–2D). To verify the
RNA-seq dataset, 5 genes were selected for qRT-PCR (Figure S2). The expression patterns of the selected genes were consistent with RNA-seq. For the DEGs differing between L42 and L63, we mainly focused on genes that were upregulated in L63 but remained unchanged or downregulated in L42 (Table S3). The DEGs in this group are considered more likely to play critical roles in Cd accumulation as key genes for low GCA would be highly expressed in L63 while downregulated in L42. There were 84 DEGs matching this requirement (Figure 2E). Twenty-one genes (25%) are involved in stress and defense responses, 21 genes (25%) are involved in transport processes, and 6 genes are involved in transcription processes, such as heat shock proteins, ABC transporter, and RING zinc finger protein 5. Other functional categories are signal transduction, transcription, cell division and growth, and protein synthesis and degradation (Figure S3). Among 297 genes that were unchanged in L63 and downregulated in L42, 29 are involved in stress and defense responses, which accounted for 9.8% in this group (Table S4).

The Involvement of S-Adenosylmethionine Cycle in Cd Accumulation

The expression of two key genes (TRIBOA-glucoside O-methyltransferase BX7-like and homocysteine S-methyltransferase 3) involved in the S-adenosylmethionine (SAM) cycle was significantly upregulated in L63 and unchanged in L42 after Cd treatment compared with the control (Figure 3A). Meanwhile, one gene encoding 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase (ACO), an important enzyme in ethylene biosynthesis, was also upregulated only in L63. We then measured the ethylene emission in the two genotypes under Cd stress. Cd stress induced a significant increase in ethylene emission in both genotypes, with L42 showing a greater increase (Figure 4). We further found that the ACC content, the precursor of ethylene biosynthesis, was unchanged in L42 under stress and significantly downregulated in L63, compared with control (Figure 4). The results demonstrated that ethylene might play an important role in LGCA in L63. To further explore whether the contents of SAM cycle–related metabolites were affected by Cd, we analyzed the relative contents of L-homocysteine (Hcy), L-methionine (L-Met), SAM, and S-adenosyl-homocysteine (SAH) based on the metabolome (Figure 3B). The results showed that the contents of Hcy, L-Met, and SAH were all significantly elevated in L63 after 24 h of Cd treatment compared with those in the control, while no change was found in L42 (Figure 3B). However, the SAM content was significantly increased in both genotypes, indicating that the higher expression of HMT3 and TGMT induced higher accumulation of SAM cycle–related metabolites.

Cd Toxicity Induced Large-Scale Changes in Metabolite Levels

Besides the involvement of ethylene biosynthesis, SAM cycle affects the metabolism of many metabolites. To better understand the mechanistic basis of low GCA in maize, root metabolite profiling was conducted by ultra-high performance liquid chromatography-quadrupole electrospray field orbital trap mass spectrometry (UHPLC-QE-MS). A total of 18,705 metabolites were detected. Among them, 8,885 and 9,820 metabolites were detected in negative and positive ion modes, respectively, (two modes of mass detection). Principal component analysis demonstrated that PC1 explained 44.6% and 51.2% of the total variation under positive and negative ion modes, respectively, (two modes of mass detection). Principal component analysis demonstrated that PC1 explained 44.6% and 51.2% of the total variation under positive and negative ion modes, respectively, and the four groups were well-separated (Figures S4A and S4B). The results indicated that the changes in metabolites induced by Cd had significant genotypic differences.

Based on the threshold of fold change being >1.5 (upregulated) or <0.67 (downregulated) and p < 0.05, a total of 659 differentially accumulated metabolites (DAMs) between two genotypes were found up on Cd treatment. Among these DAMs, 111 were upregulated in L42 but unchanged or downregulated in L63 and 49 were downregulated in L42 but upregulated or unchanged in L63 after Cd treatment. Correspondingly, 126 were upregulated in L63 but unchanged or downregulated in L42, and 34 were downregulated in L63 but upregulated or unchanged in L42, respectively (Figures S4C–S4E; Tables S5 and S6). According to these DAMs, key metabolites were selected to produce major metabolic pathways using the KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis (Figure 5, Table S7). These crucial metabolites are related to fatty acid synthesis, terpenoid synthesis, phenylalanine, the TCA (Tricarboxylic acid) cycle, and GSH metabolism. Most of these metabolites were upregulated in L63 and downregulated or unchanged in L42 after Cd stress compared with control. For instance, malic acid (1.80-fold), citric acid (2.15-fold), L-linolenic acid (1.52-fold), and octadecadienoic acid (1.88-fold) involved in the TCA cycle and fatty acid synthesis; naringenin (3.54-fold) and plathymenin (1.66-fold) involved in phenylalanine synthesis; and costunolide (1.92-fold) and retinol (4.67-fold) in terpenoid synthesis were all significantly upregulated in L63. Even though some of DAMs, such as α-D-glucose, cis-aconitic acid, and octadecenoic acid, showed similar changes in the two genotypes when exposed to Cd treatment, the majority of them were upregulated only in L63 after Cd stress. Additionally, the contents of some sugars, such as α, β-trehalose (2.00-fold) and maltotriose
Expression and Haplotype Analysis of ZmHMT3

To verify the aforementioned conclusions of SAM cycle as a determinant of differential Cd accumulation in maize, we selected ZmHMT3, a key gene involved in the SAM cycle, for gene expression and haplotype analysis. ZmHMT3 was significantly downregulated in L42 and upregulated in L63 after 24-h Cd treatment (Figure 6A). The expression of ZmHMT3 was markedly induced over Cd-exposure time and reached the maximum value of about 50-fold greater expression at 6 h, which then gradually reduced within 24 h (Figure 6B). Besides Cd, ZmHMT3 was also significantly induced by Fe deficiency (Figure 6C). To further investigate whether ZmHMT3 is involved in the regulation of grain-Cd accumulation in maize, we cloned and compared the coding sequence (CDS) sequence from 30 maize genotypes with diverse genetic backgrounds and grain Cd concentrations. The CDS of ZmHMT3 in L42 and B73 (a variety of reference genome) were identical, while CDS of L63 contained three SNPs (Figure S7). We then sequenced the CDS from the left 28 maize genotypes. The 3 SNPs resulted in 2 haplotypes (Hap1 and Hap2), which showed significant differences in grain Cd concentration (Figure 6). The results demonstrated that ZmHMT3 might play important roles in grain-Cd accumulation in maize.
DISCUSSION

Comparative Transcriptome and Metabolome Analysis Revealed Key Genes Associated With Low Cd Accumulation in Maize Grain

This study used physiological analysis, large-scale transcript, and metabolite profiling to examine cellular responses affected by Cd stress in roots of low-GCA genotype L63 and high-GCA genotype L42. A number of key LGCA-associated genes were identified under Cd stress. Based on these identified DEGs and DAMs, we proposed DEG- or DAM-based integrated schematic models of the mechanisms involved in LGCA in L63 under Cd stress (Figures 3, 5, and 7). Figure 7 shows a tentative model of transport and signaling network involved in Cd accumulation based on the identified DEGs in the two groups. These genes included six cell wall synthesis–related genes (e.g., CCR1, XET23, Expansin-B4), three antioxidant enzymes (e.g., GLP2, POD52, POD72), three plasma membrane–localized cation transporter genes (PMC, ZIP5, ZIP1), four vascular (ABCC8, VPE, AP8d, and XCP1) and three mitochondria (NADHOOR, HSP, and AACP) localized genes. There were also functional genes encoding a diverse set of TFs (e.g., MYB, WRKY, ERF, HSF, bZIP, RINGZF families), protein synthesis–related ribosomal proteins (e.g., 30S ribosomal protein), and protein secretion and vesicle-mediated transport-related proteins (STDGCR and clathrin). Meanwhile, the expression level of GPDH which is involved in the pentose-phosphate pathway was significantly increased in L63 after Cd treatment, but no change was found in L42 (Figure 7). These results indicated that L63 could rapidly induce defense responses in roots, thus decreasing Cd translocation to aboveground tissues. The low-GCA genotype and several candidate genes can be effectively used in breeding programs in maize.

SAM Cycle May Induce Multiple Cellular Pathways Through Regulating Ethylene Production, Methionine Metabolism and Methylation, and Controlling Accumulation of Many Key Metabolites Against Cd Stress in L63

SAM cycle plays important roles in many key biological processes, such as methylation, Met metabolism, and ethylene and secondary metabolites biosynthesis (Heidari et al., 2020). Transcriptome sequencing revealed that two important genes involved in the SAM cycle, HMT3 and TGMT, were significantly upregulated only in L63 after Cd treatment (Figure 3). Metabolome profiling showed that the relative content of four important metabolites (Met, SAM, SAH, and Hcy) was significantly elevated in L63 and that Cd-induced elevation in SAM level was significantly higher in L63 than that in L42. The results suggested that SAM cycle plays an important role in decreasing Cd accumulation in maize. High production of Cd-induced endogenous ethylene negatively affects plant growth and development, but within an optimized level, it shows positive effects on plants against Cd stress (Thao et al., 2015). Ethylene is also involved in sulfur (S)-mediated increase in GSH, which plays key roles in Cd detoxification (Khan et al., 2015; Masood et al., 2015). In this study, ethylene emission rate was drastically increased in L42 but only moderately in L63 (Figure 4). The expression of ACO was upregulated in L63 and unchanged in L42 (Figure 3A). Moreover, compared with control, ACC content was reduced in L63 under Cd stress but unchanged in L42 (Figure 4). The results suggested a mechanism for fine-tuning of ethylene biosynthesis in L63, thus controlling the emission of ethylene at an optimized level and finally activating the expression of downstream Cd-responsive genes.
The transportation and assimilation of sulfate, as well as the synthesis of a series of sulfur-containing metabolites with important biological functions, are not only closely related to plant growth and development but also affect biotic and abiotic stress tolerance of crops (Nazar et al., 2011). Met, an S-containing essential amino acid in all plants, acts as the cornerstone of protein synthesis. SAM, as an important methyl donor for nucleic acid and many proteins and a sulfonium metabolite, controls many key physiological pathways, such as Met metabolism, ethylene and polyamine biosynthesis, and transmethylation and transsulfuration (Heidari et al., 2020). SAM is widely known as a precursor of GSH, which is an important ROS scavenger and a precursor of PC (Noriega et al., 2007). Although the relative content of GSH was significantly downregulated in L63, it was still more than 10 times higher than that of L42 upon Cd stress (Figure S1A). The results suggested that L63 has a more active sulfur metabolism which contributes to decreased GCA. Together, these results demonstrated that Cd can promote SAM cycle in L63 which in turn activates the expression of a series of downstream GCA-related genes and the accumulation of key metabolites. HMT family plays central roles in the SAM cycle. Genotypic differences were found in the expression level of ZmHMT3 under Cd stress between L42 and L63. Meanwhile, the 2 haplotypes significantly correlated with grain Cd concentration. The results suggested that ZmHMT3 might be a key regulator of grain-Cd accumulation in maize. Further studies will be performed to investigate the function of ZmHMT3 in Cd uptake, translocation, and accumulation.

Cell Wall and Vacuolar Sequestration and Chelation of Small Molecules in Roots Decrease Shoot-to-Root Cd Translocation in L63 Under Cd Stress

Cell wall in roots is the primary structure directly exposed to Cd and is also a major region of heavy metal accumulation in roots (Fernandez et al., 2014). A higher binding capacity of the cell wall to Cd in roots may, therefore, result in lower Cd translocation to the shoot (Lin et al., 2022) as was observed in L63. Cd exposure has been reported to induce endodermal changes such as thickening of inner tangential walls and formation of...
Casparian strips (Schreiber et al., 1999; Schreiber, 2010). Moreover, the composition of the cell wall characterized by lignin, proteins, polysaccharides, and other phenolic compounds confers the ability to bind Cd via functional groups (Parrotta et al., 2015). These responses contribute to reduced xylem Cd loading, thereby decreasing its translocation to the shoot. Meanwhile, our previous study found that most of Cd in L63 was accumulated in epidermal cells, while Cd was distributed at the entire cross-section of root tips in L42 (Lin et al., 2022), confirming that L63 has different mechanisms for preventing Cd uptake and xylem loading. Several DEGs were found to be involved in cell wall extension and modification (Figure 7). Among them, cinnamoyl CoA reductase (CCR) catalyzes the first committed step of the lignin-specific branch of monolignol synthesis (Lacombe et al., 1997; Lauvergeat et al., 2001). Lignification provides an effective barrier against the entry of Cd, resulting in a reduced cell wall penetration (Loix et al., 2018). CCR expression was significantly upregulated in L63, while no change was found in L42 (Figure 7; Table S3), which was further confirmed by a metabolome analysis. Lignin is synthesized through the phenylpropanoid pathway responsible for the synthesis of secondary metabolites such as polyphenols and flavonoids (Sonbol et al., 2009). As shown in Figure 5, some metabolites that are assigned to the phenylpropanoid synthesis pathway, such as trans-ferulic acid and 5-coumaroylquinic acid, participate in the biosynthesis of lignin directly, while other flavonoids are indirectly related to lignin metabolism. The results suggested that lignin plays an important function in decreasing grain-Cd accumulation. XET23 cleaves and relegates xyloglucan polymers, which is an essential constituent of the primary cell wall (Rose et al., 2002). UDP-glycosyltransferase plays a role in callose deposit in the cell wall as a defense response to heavy metals (Clay et al., 2009; Pastorczyk and Bednarek, 2016); thus, L63 can form a thicker corpus callosum to reduce Cd toxicity. Meanwhile, fasciclin-like arabinogalactan protein 7 and
expansin-B4 are known to be involved in cell wall biogenesis and degradation (Lee et al., 2001), which leads to a greater ability of L63 to maintain cell wall formation and expansion under Cd toxicity. Therefore, the Cd-induced transcriptional and metabolic changes that involve cell wall extension and modification contribute to decreasing grain-Cd accumulation in maize.

TEM analysis found that more severe destruction occurred in the root vacuole of L42, while L63 was almost not affected (Figure 1). In response to Cd stress, plant cells synthesize various components such as PCs, which are involved in binding and detoxification of Cd, to be transported into vacuoles via ABC transporters (Heiss et al., 2003; Emamverdian et al., 2015). Apart from this, other intracellular ligands such as GSH and a range of organic compounds contribute to the detoxification of Cd in plants.

**Figure 7. A Predicted DEG-Based Low-Cd-Accumulation–Associated Model in L63 in Response to Cd Stress**

AACP, ADP and ATP carrier protein 2; ABBCC8, ABC transporter C family member 8-like; ATL1Q, RING-H2 finger protein ATL1Q; AP8d, autophagy-related 8 days; bZIP, bZIP transcription factor superfamily protein; Cd, cadmium; CCR, cinnamoyl CoA reductase 1; CCRZF, CHY-type/CTCHY-type/RING-type Zinc finger protein; FLAP, fasciclin-like arabinogalactan protein 7; G-6-P, glucose-6-phosphate; GAP, glyceraldehyde-3-phosphate; Gn-6-P, glucono-6-phosphate; GLP2, germin-like protein 2; GPDH, glucose-6-phosphate dehydrogenase; HSP, heat shock protein; MT, mitochondria; MYB, MYB-like DNA-binding protein myb-1; NADHOOR, NADH-ubiquinone oxidoreductase 10.5 kDa subunit; NTF2, nuclear transport factor 2 family protein; POD, peroxidase; Pr, protein; PMC, plasma membrane–associated cation-binding protein 1; R, ribosome; RINGZF, RING zinc finger domain superfamily protein; ROS, reactive oxygen species; RP, ribosomal protein; STDGCR, Ser/Thr-rich protein T10 in DGCR region; TF, transcription factor; TPR, tetratricopeptide repeat-like superfamily protein; UDPGT, UDP-glycosyltransferase; VPE, vacuolar-processing enzyme gamma-isozyme; WRKY, WRKY DNA-binding domain–containing protein; XET23, xyloglucan endotransglucosylase/hydrolase protein 23; XCP1, cysteine protease XCP1; ZIP, zinc transporter.
acids are involved in binding Cd (Emamverdian et al., 2015). For instance, the carboxylic acids citrate, malate, and oxalate are known to be involved in xylem transport and vacuolar sequestration. According to the transcriptome analysis, several vacuolar localized genes, such as VPE, were significantly upregulated in L63 but downregulated in L42 or unchanged in L63 but downregulated in L42 (Figure 7). As a precursor for PCs, the content of GSH in L63 was ten times higher than that in L42 (Figure S1A). Besides GSH, malic acid and citric acid were significantly increased only in L63 after Cd stress compared with control (Figure 5). Taken together, L63 is more capable to produce Cd chelates and sequester more Cd into vacuoles.

**TFs Are Largely Responsible for LGCA in L63**

TFs regulate gene expression by conveying messages under biotic or abiotic stresses, including Cd stress. In the present study, a number of TFs, such as MYB, WRKY, and bZIP, were upregulated in L63 but downregulated in L42 under Cd stress. These TF families play important roles in response to Cd stress. For instance, WRKY13 was reported to target PDR8 directly to positively regulate Cd tolerance in Arabidopsis (Sheng et al., 2019). MYB7 can positively regulate the phenylpropanes pathway to enhance flavonoid production, which can control lignin biosynthesis to decrease Cd accumulation (Gharari et al., 2020). Many TFs belonging to the bZIP family are involved in various abiotic stresses, with some being engineered to improve Cd stress tolerance in plants (Huang et al., 2016). Zinc finger proteins were also found to be involved in response to abiotic stress (Davletova et al., 2005). The results demonstrated that TFs play a vital role in regulating signals to reduce shoot Cd concentration.

**Strong Capacities for Energy Supply and ROS Scavenging Enable Cells to Maintain Normal Homeostasis Under Cd Stress in L63**

The expression levels of NADHOOR, AACP, and HSP, and GPDH, which are involved in the glycolysis cycle, were upregulated or unchanged in L63 but downregulated in L42 upon Cd stress (Figure 7). The TCA and glycolysis cycles are a series of reactions ultimately leading to the production of ATP. He et al. (2019) revealed that a decrease in ATP content and reduction in TCA activity resulted in increased Cd accumulation in Arabidopsis plants. Increases in aconitic acid, citric acid, and malic acid metabolism were congruent with the transcriptome results (Figure 5) and were higher in L63 than those in L42. Interestingly, the ATP content in roots of L63 after 24 h of Cd treatment decreased significantly, while the content in L42 showed a slight increase (Figure S1B). This may imply that more ATP produced in L63 is consumed to activate a series of pathways against Cd stress, such as providing energy to sequestrate more Cd in vacuoles via the ABC transporter family (Brunetti et al., 2015; Wang et al., 2019). The results suggested that L63 can accelerate glycolysis and TCA cycle via upregulating key genes and metabolites under Cd stress, and the energy generated from this is used to drive various metabolic pathways against Cd stress.

A plant’s ability for ROS scavenging is another hallmark of Cd tolerance aimed to protect the membrane system from being destroyed (Marques et al., 2019). In the present study, genes encoding peroxidase, POD52 and POD72 and GLP2 which possess oxalate oxidase or superoxide dismutase activity, displayed high expressions in L63. In addition, GSH has been confirmed to have a potential role in decreasing Cd toxicity either through reduced ROS or sequestration (Hernandez et al., 2015; Chen et al., 2016). Although L42 had a higher ratio (relative to control) of GSH, the content of GSH was 10-folds higher in L63 than that in L42. Interestingly, the ATP content in roots of L63 after 24 h of Cd treatment decreased significantly, while the content in L42 showed a slight increase (Figure S1B). These results imply that L63 has a stronger ability to scavenge Cd-induced ROS overaccumulation, thus maintaining the normal function of the membrane system. Cd also affects lipid biochemistry by inhibiting the biosynthetic pathway, leading to the reduction of unsaturated fatty acids (UFAs) (Mohamed et al., 2012). The increase in root linoleic acid content (an UFA) in rice is negatively correlated with malonaldehyde content and thus can alleviate Fe toxicity stress (Turhadi et al., 2019). The content of elaidic acid, another UFA, has been found to be high in a Cd-tolerant variety of rice (Turhadi et al., 2019). These results suggest that UFAs may play an important role in regulating ROS homeostasis under Cd toxicity. In our study, the contents of several UFAs, such as linoleic acid, octadecadienoic acid, decanoic acid, pinolenic acid, and dehydroabietic acid, were significantly increased only in L63, suggesting less lipid peroxidation (Figure 5). As a result, L63 was more capable of scavenging Cd-induced ROS by increasing the activity of antioxidant enzymes, GSH and UFA contents.

**Limitations of the Study**

This work revealed the mechanistic basis of Cd accumulation in maize and the processes controlling Cd exclusion from the shoot. The following features explain the superior ability of a low-Cd accumulating line to deal with Cd load: (1) higher capacity to scavenge Cd-induced ROS; (2) ability to maintain ion homeostasis and
sequester more Cd into the vacuoles and cell wall; (3) providing more energy for Cd detoxification in maize; and (4) a more efficient SAM cycle. Although ZmHMT3 has been identified as a candidate gene for LGCA in maize, the functional verification and a detailed mechanism needs to be explored.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105484.

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**AUTHOR CONTRIBUTIONS**

M.Z., and F.C. planned and designed the research. K.L., M.Z., D.V.W., S.S., and F.C. carried out the experiments. K.L., D.V.W., W.H., and F.C. analyzed data. K.L., D.V.W., S.S., M.Z., and F.C. drafted the manuscript. All authors have read and agreed to the published version of the manuscript.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

**INCLUSION AND DIVERSITY**

We support inclusive, diverse, and equitable conduct of research.

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STAR METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Biological samples** | | |
| Maize genotypes: L42, L63, B73 | This paper | |
| **Critical commercial assays** | | |
| MiniBEST Plant RNA Extraction Kit | Takara | 9769 |
| PrimeScriptTM RT reagent Kit | Takara | 6210A |
| **Chemicals, peptides, and recombinant proteins** | | |
| K2SO4 | AR | CAS 7778-80-5 |
| MgSO4.7H2O | AR | CAS 10034-99-8 |
| KCl | AR | CAS 3811-04-9 |
| KH2PO4 | AR | CAS 7778-77-0 |
| Ca(NO3)2.4H2O | Sigma-Aldrich | CAS 13477-34-4 |
| Fe(III)-EDTA-Na | Sigma-Aldrich | CAS 15275-07-7 |
| MnSO4.H2O | AR | CAS 10034-96-5 |
| ZnSO4.7H2O | AR | CAS 7446-20-0 |
| CuSO4.5H2O | AR | CAS 7758-99-8 |
| (NH4)6Mo7O24.4H2O | AR | CAS 13106-76-8 |
| H3BO3 | AR | CAS 10043-35-3 |
| **Oligonucleotides** | | |
| Primers for qPCR see Table S1 | This paper | N/A |
| Primer for Haplotype analysis of ZmHMT3 Forward: ATGGTGGG GACCGCGGAAAGG | This paper | N/A |
| Primer for Haplotype analysis of ZmHMT3 Reverse: TGCTACAG GTAACTGATGCTTGTGGCAG | This paper | N/A |
| **Software and algorithms** | | |
| DESeq2 | Love et al. (2014) | v. 1.18.1 |
| R | https://cran.r-project.org/ | 3.3.1 |
| Adobe Acrobat DC origin | | 2019b |
| TB tools | https://github.com/CJ-Chen/TBtools/releases | |
| **Other** | | |
| LC-MS | Agilent Technologies | |
| Dionex Ultimate 3000 series UHPLC | Thermo Scientific | https://www.thermofisher.com/de/en/home.html |

RESOURCE AVAILABILITY

**Lead contact**

Further information and requests for resources should be directed to and will be fulfilled by the lead contact Fangbin Cao (caofangbin@zju.edu.cn).
Materials availability
This study did not generate new unique reagents.

Data and code availability
The raw sequence data reported in this study have been deposited in the Genome Sequence Archive in National Genomics Data Center, Beijing Institute of Genomics (China National Center for Bioinformation), Chinese Academy of Sciences, under accession number CRA008372 that are publicly accessible at https://bigd.big.ac.cn/gsa.

This study does not report original code. We have provided all detailed information to reanalyze this study in STAR Methods to the best of our knowledge.

Any additional information is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS
Plant materials and Cd treatment
Hydroponic experiments were performed in a glasshouse at the Zijingang Campus, Zhejiang University, Hangzhou, China. Two inbred maize lines, L42 and L63, high- and low-grain Cd accumulation (GCA) genotypes were used (Lin et al., 2022). The seeds of L42 and L63 were germinated in vermiculite and 2-leaf-stage seedlings were transplanted into 5 L black plastic bins filled with nutrient solution. The components of the nutrient solution were (mg L\(^{-1}\)): K\(_2\)SO\(_4\), 130.7; MgSO\(_4\).7H\(_2\)O, 160.2; KCl, 7.5; KH\(_2\)PO\(_4\), 13.6; Ca(NO\(_3\))\(_2\).4H\(_2\)O, 472.3; Fe-EDTA-Na, 36.7; MnSO\(_4\).H\(_2\)O, 0.006; ZnSO\(_4\).7H\(_2\)O, 0.287; CuSO\(_4\).5H\(_2\)O, 0.025; (NH\(_4\))\(_6\)Mo\(_7\)O\(_24\).4H\(_2\)O, 0.006; H\(_3\)BO\(_3\), 0.062 (Lin et al., 2022). The pH of the nutrient solution was adjusted to 5.8 \pm 0.1 with NaOH or HCl.

Three days after transplanting, seedlings of both genotypes were treated in 5 mM CdCl\(_2\) solution for 24 h. Roots were then sampled for the Cd staining, and transcriptomic, metabolomic and physiological analysis. Observation of root ultrastructure was conducted after six days of Cd treatment.

METHOD DETAILS
Transmission electron microscopy and ATP determination
Transmission electron microscopy (TEM; JEM-1230, Japan) was performed on fresh root fragments as previously described (Lin et al., 2022). ATP content was measured using an assay kit (SS026, Beyotime, China) following the manufacturer’s instructions. Briefly, fresh root tissue was homogenized in lysis solution on ice and centrifuged at 12,000 g for 10 min at 4°C. The supernatant was used for ATP determination. ATP standard solution was diluted to various concentration to generate a calibration curve. The detection reagent was mixed with the supernatant, and the luminescence was detected by a microplate reader (FLUOstar Omega, Germany) and ATP content was calculated based on the standard curve.

Transcriptome analysis
Root RNA extraction, RNA-seq library construction and Illumina sequencing was conducted according to our previous study (He et al., 2015). The clean reads obtained from SOAPnuke were mapped to the maize reference genome using HISAT2. Clean reads were aligned with the reference sequence using Bowtie2. Gene expression level was calculated using RSEM. Pearson correlation was calculated using the cor function, while hierarchical clustering was performed using CLUSTER software. Differentially expressed genes (DE-Gs) between Cd and the control were detected using DEseq2. DE-Gs were expected to have \( \lvert \log_2(\text{fold change}) \rvert > 1.00 \) and the p value was <0.05.

To confirm the expression pattern of RNA-seq, L42 and L63 plants were grown as described above under control conditions or in the presence of 5 mM CdCl\(_2\) for 24 h. Total RNA was isolated from root tissues. Synthesis of the first strand cDNA and manipulation of qRT-PCR were performed according to Cao et al. (2014). The maize tubulin was used as a reference gene. Three biological and three technical replications were conducted. The specific primers for six genes are listed in Table S1.

Metabolomic analysis
Metabolites from fresh root tissues were extracted according to Hao et al. (2020). Briefly, 20 mg powder of the samples were weighed and placed in tubes. Then, 1000 \( \mu \)L extraction solution
(methanol: acetonitrile: water, 2: 2: 1) was added, triturated at 40 Hz for 4 min, and sonicated for 5 min. Samples were centrifuged at 12,000 rpm for 15 min at 4°C. The supernatants were used for LC-MS/MS analysis. Chromatographic separation was carried out to detect metabolites using a UHPLC system with a UPLC HSS T3 column (2.1 mm x 100 mm, 1.8 μm) coupled to Q Exactive (Orbitrap MS, Thermo). The raw data were first converted to mzXML files using ProteoWizard and processed by MAPS software v1.0. The preprocessing data matrix included the retention time (RT), Mass-to-charge ratio (m/z) values, and peak intensity. The MS2 database was used to identify metabolites. The principal component analysis (PCA) was conducted to analyze the variability among treatments. Metabolites with fold change (Cd vs. control) > 1.50 or <0.67, p < 0.05, were identified as differently accumulated metabolites.

**Gene cloning, haplotype analysis and expression analysis**

The full-length cDNA of homocysteine S-methyltransferase 3 (ZmHMT3) were amplified from 30 maize genotypes, which were then sequenced and compared to find the difference. The expression levels of ZmHMT3 were analyzed by qRT-PCR. The primers are shown in Table S1.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

All data are the averages of three replicates. Statistical analysis was carried out using DPS software (Tang and Feng, 2010). The graphs in the manuscript were prepared using Origin Pro v8.0 (Origin lab corporation, Wellesley Hills, Wellesley, MA, USA). ANOVA followed by a Duncan’s multiple range test (DMRT) was used to evaluate the differences among different treatments at a significance level of p < 0.05.