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Melatonin enhances cadmium tolerance in Tartary buckwheat by regulating phenylpropane biosynthesis and redox gene expression

Hanmei Du

duhanmei1027@163.com

Xichang University

Lin Dong

Xichang University

Cui Wen

Xichang University

Hongjun Liu

Xichang University

Changhe Wei

Xichang University

Yan Yi

Xichang University

Lu Tan

Xichang University

Qiuzhu Yu

Xichang University

Shengchun Li

Xichang University

Peter R. Ryan

Australian National University

Dandan Wu

Sichuan Agricultural University

Anhu Wang

Xichang University

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Abstract

Background

Melatonin (MT) is a multifunctional molecule capable of alleviating biotic and abiotic stresses in plants including cadmium (Cd) toxicity. While studies show that MT treatment can reduce the damage caused by Cd, it remains unclear how it mitigates Cd toxicity in Tartary buckwheat.

Results

This study investigated the physiochemical and molecular mechanisms by which MT protects Tartary buckwheat from Cd stress. We found that Cd treatments in hydroponics (50 μM $CdCl_2$) or added to soil (100 μM μM $CdCl_2$) reduced root and shoot growth, but that the combination of these treatments with 100 or 200 μM MT, respectively, significantly reversed that inhibition of growth. The protection afforded by MT was associated with reduced Cd uptake by plants as well as lower concentrations of malondialdehyde and the reactive oxygen species superoxide anion and hydrogen peroxide. Moreover, the concentrations of glutathione and ascorbic acid in roots and shoots and the activities of superoxide dismutase and peroxidase increased with the Cd + MT treatment compared with Cd alone. The expression of glutathione S-transferase and phytochelatin synthase genes, both of which combat Cd toxicity in other species, also increased. Expression of the heavy metal transporter gene *FtHMA2* was induced by Cd but unchanged in the Cd + MT treatment. A full transcriptomic analysis revealed that the Cd + MT treatment significantly altered the expression of genes involved with redox regulation, phenylpropanoid synthesis, heavy metal transport, as well as heat shock proteins (HSPs) and transcription factors from the WRKY, Dof, and AP/ERF families.

Conclusion

This study provides insights into the mechanisms by with MT ameliorates Cd toxicity in Tartary buckwheat. The findings could support the development of genetic or agronomic strategies for enhancing the production of this valuable crop on Cd-contaminated soils.

Highlights

1. MT application significantly reduces the toxic effects of Cd in Tartary buckwheat.

2. MT reduces Cd uptake, alleviates oxidative stress, and increases the expression of and .

3. MT modulates the expression of genes involved in phenylpropanoid synthesis and redox regulation.

4. MT mediates the expression of heat shock proteins and transcription factors to protect plants from Cd-induced damage.

Background

Certain industries and agricultural practices throughout the world have left soils contaminated with heavy metals [1]. Approximately 20% of the arable land in China is subjected to heavy metal pollution with cadmium (Cd) being a major pollutant [2, 3]. Cd has deleterious effects on crop production and human health [4–6]. It adversely affects

plant growth and development by damaging membranes [7, 8], inhibiting photosynthesis, altering enzyme activities, and limiting the absorption of water and nutrients [9–11].

Tartary buckwheat (*Fagopyrum tataricum*) is a valuable crop in the food and medicinal industries. It is capable of being cultivated in more hostile environments where soils are relatively infertile and temperatures extreme [12, 13]. However, Tartary buckwheat production is affected by Cd toxicity because in Cd-contaminated soils germination, nutrition, yields and grain quality are all compromised [14–17]. Therefore, mitigating the adverse effects of Cd toxicity on important crops like Tartary buckwheat has become an active research topic. Variation in Cd tolerance has been reported among different genotypes of Tartary buckwheat [14] and this variation can be used to identify the mechanisms involved. Previous studies that have investigated how plants respond to Cd stress have proposed beneficial roles for glutathione and phytochelatins [18, 19], as well as for the heavy metal transporters that reduce Cd uptake and compartmentalize the harmful ions to less sensitive organelles within the cytosol [20, 21]. The addition of substances such as sulfur [17], zinc oxide nanoparticles [22, 23], abscisic acid [24], and melatonin (MT) [25] has also been shown to protect some plants from heavy metals like Cd.

MT is a multifunctional molecule present in all cells which affects gene expression and protein synthesis [26, 27], especially in response to stresses [28–30]. The protection afforded by MT can be explained in part, from its ability to trigger pathways that scavenge potentially harmful reactive oxygen species (ROS) and maintain the redox balance in cells [30, 31]. Exogenously applied MT has been shown to alleviate metal-ion toxicities [32–34] including copper stress in melon [35], aluminum stress in maize [36] and Cd toxicity in several cereal and non-cereal crops [33, 37, 38]. However, the protection that MT provides from Cd stress involves more than just the management of oxidative stress because MT was also shown to reduce Cd uptake by plants and to compartmentalise the Cd to less sensitive organelles such as the vacuoles and cell walls [39, 40]. Other studies conclude that certain hormones, signaling pathways and secondary metabolites that are capable of chelating Cd (such as phytochelatins) also contribute to tolerance [25, 38, 41–43].

Few details are available on the effects of exogenously applied MT on Tartary buckwheat and the extent to which it can alleviate Cd toxicity. This study performed hydroponic and soil experiments to investigate the effects on Cd and MT on plant growth. The responses were compared with the molecular changes occurring in seedlings and mature plants in an effort to reveal the mechanisms involved.

Results

Melatonin treatment reduces the inhibition of growth by Cd toxicity

The growth of Tartary buckwheat seedlings was examined after treatment with a control treatment (CK), Cd alone, Cd + MT, or MT alone. Compared with the CK and MT treatments, Cd-treated plants had significantly shorter root lengths and reduced root and shoot dry weights (DW). When MT was added to the Cd treatment (Cd + MT), the roots were 19% longer roots than Cd alone and root and shoot DWs were ~ 30% greater (Fig. 1). Similar results were obtained in the soil. In those experiments Cd significantly inhibited plant height and 1000-grain weight compared with the CK or the MT treatment (Fig. 2A, Table 1) whereas the Cd + MT treatment increased plant height by 14%, grain weight per plant by 49%, and 1000-grain weight by 17% compared with Cd alone. The rutin and quercetin concentrations in most samples of roots (R), stems (ST), leaves (L), and seeds (S) of seedlings and mature plants were greater in the Cd, MT and Cd + MT treatments compared with Cd alone (Fig. 2B, C). The Cd

treatment increased Cd concentrations in the roots, stems, leaves and grain, but Cd + MT reduced this accumulation by between 25% and 75% in all these tissues except the grain (Fig. 2D). These results demonstrate that Cd stress adversely affected the growth, development and yield of Tartary buckwheat and that addition of exogenous MT reduced the accumulation of Cd and maintained greater growth.

Table 1
Effects of melatonin on agronomic characters of Tartary buckwheat under Cd stress

Parameters	CK	MT	Cd	Cd + MT
Plant height (cm)	58.88 ± 3.56a	56.75 ± 3.82a	44.38 ± 1.43b	50.75 ± 0.26a
Grain weight per plant (g)	1.87 ± 0.01b	2.07 ± 0.07a	1.02 ± 0.04d	1.52 ± 0.04c
1000-grain weight (g)	12.35 ± 0.46a	12.21 ± 0.25a	10.41 ± 0.11b	12.14 ± 0.63a
Note: Different letters are significantly different within each group at $p \leq 0.05$ (n = 15) according to Duncan's test.				

Melatonin protects from Cd-induced oxidative damage

Treatment with MT alone had little or no effect on the concentrations of H_2O_2 and superoxide anion ($O_2^{\ddot{Y}-}$) in roots or shoots and slightly reduced the concentrations of MDA (Fig. 3A-C). Exposure to Cd stress, by contrast, increased all these values by between 1.5 and 2.0-fold in roots and shoots except for $O_2^{\ddot{Y}-}$ concentrations in the shoots which remained unchanged. Compared with Cd alone, the Cd + MT treatment reduced MDA and ROS concentrations by between 29% to 44% in all tissues except for $O_2^{\ddot{Y}-}$ production in shoots (Fig. 3A-C).

Activities of the superoxide dismutase (SOD) and peroxidase (POD) enzymes in the roots and shoots of seedlings significantly increased after Cd treatment compared with either the CK or MT alone, while Cd + MT treatment increased the activity of SOD by an additional 10–20% and the activity of POD by an additional 30–50% (Fig. 3D, E). By contrast, activity of the catalase (CAT) enzymes decreased with Cd stress but returned to near CK levels with the Cd + MT treatment (Fig. 3F). Cd induced small but significant increases in the concentrations of glutathione (GSH) and ascorbic acid (AsA) in the roots and shoots compared with CK and Cd + MT increased these changes further, except for AsA in the shoots (Fig. 3G, H). When applied alone, Cd treatment more than doubled the concentration of MT in the roots and shoots whereas Cd + MT increased MT concentrations in these tissues more than 10-fold (Fig. 3I). These results provide evidence that exogenous MT enhanced the capacity of Tartary buckwheat to scavenge ROS and combat Cd-induced oxidative stress.

Effect of MT on the expression of several Cd-responsive genes

The expression of three genes known to be responsive to Cd stress was investigated with RT-PCR. Glutathione S-transferase (*FtGST1*), phytochelatin synthase (*FtPCS1*) and a heavy-metal ATPase transporter (*FtHMA2*) expression was measured after treatment with Cd alone or Cd + MT. *FtGST1* expression in roots was induced 40% by Cd compared with CK, and this increased by a further 30% in the Cd + MT treatment (Fig. 4A). By contrast, the expression of *FtPCS1* remained unchanged under Cd stress, but more than doubled in the Cd + MT treatment (Fig. 4B). Finally, *FtHMA2* expression was induced 4.3-fold by Cd stress but remained at control levels in the Cd + MT treatment (Fig. 4C). These results indicate that the combination of Cd + MT is reducing Cd stress by boosting

antioxidant defenses. The finding that *FtHMA2* expression increased in Cd treatment but remains similar to control levels in Cd + MT could indicate that the plants are less stressed in this treatment.

Transcriptome analysis and DEGs identification

In addition to targeting the known Cd-responsive genes above, we also performed a full transcriptomic analysis of Tartary buckwheat seedlings treated with a control, Cd (100 μM), Cd + MT (100 μM Cd + 200 μM MT), and MT alone (200 μM). A total of 103.43 G bases were obtained, with clean bases in each sample being distributed in the range of 8.33 to 9.31 Gb. The GC content was 45.4% 46.3%, and the Q30 was >96.2% (Table 2), indicating that the quality of sequencing was high and reliable. The differentially expressed genes (DEGs) were identified from three pairwise comparisons: (i) CK vs. Cd, (ii) CK vs. MT, and (iii) Cd vs. Cd + MT. For the CK vs. Cd comparison, 2570 DEGs were identified (1546 genes up-regulated by Cd and 1024 down-regulated by Cd), for the CK vs. MT comparison 3696 DEGs were identified (2174 upregulated by MT and 1522 downregulated by MT) and in the Cd vs. Cd + MT comparison 511 DEGs were identified (315 up-regulated by Cd + MT and 196 down-regulated by Cd + MT) (Fig. 5A, B). Among these DEGs were 39 transcription factors (TFs) from a range of families including WRKY, AP2/ERF, bHLH, MYB, DNA-binding with one zinc finger (Dof), and cysteine-rich polycomb-like protein (CPP) (**Table S2**). Interestingly, 107 DEGs (77 up-regulated and 30 down-regulated) overlapped across the three comparisons, suggesting that these genes responded to both Cd and MT treatments (Fig. 5A, **Table S3**).

Table 2
Summary of sequence data

Sample	Raw_reads	Raw_bases	Clean_reads	Clean_bases	Error_rate	Q20	Q30	GC_pct
CK1	61497436	9.22G	57195848	8.58G	0.01	98.90	96.63	46.35
CK2	55644852	8.35G	55598314	8.34G	0.01	98.76	96.32	45.96
CK3	61902818	9.29G	56760996	8.51G	0.01	98.83	96.50	46.18
Cd1	61592842	9.24G	56093696	8.41G	0.01	98.85	96.50	45.69
Cd2	64045420	9.61G	58825590	8.82G	0.01	98.72	96.25	45.69
Cd3	60080552	9.01G	55526740	8.33G	0.01	98.77	96.36	46.16
MT + Cd 1	60340634	9.05G	55613396	8.34G	0.01	98.86	96.51	45.44
MT + Cd 2	62276888	9.34G	57628662	8.64G	0.01	98.81	96.47	45.54
MT + Cd 3	63705222	9.56G	59375060	8.91G	0.01	98.78	96.4	45.91
MT1	58032000	8.7G	57987694	8.70G	0.01	98.76	96.35	45.94
MT2	61726716	9.26G	56915896	8.54G	0.01	98.88	96.66	45.61
MT3	62077520	9.31G	62035362	9.31G	0.01	98.88	96.61	45.72

GO and KEGG enrichment analyses

To understand the biological roles of the DEGs identified above, gene ontology (GO) enrichment analysis was performed. DEGs from each comparison were divided into three groups, each containing about 200 functional groups (**Table S4**). Among the significantly enriched GO items, the top 20 items were further divided into three categories: “cellular components” (CC), “molecular functions” (MF), and “biological processes” (BP). Most DEGs in cellular components were related to membranes, cell parts and organelles while the DEGs in biological processes were mainly related to transport, localization, and signal transduction. Finally, the DEGs in the molecular functions category were related to oxidoreductase activity and transporter activity (Fig. 5C-E). Notably, the following ontology groups were enriched in at least two of three comparisons: oxidoreductase activity (GO:0016491), membrane (GO:0016020), transmembrane transport (GO:0055085) and transporter activity (GO:0005215).

KEGG enrichment analysis of the three comparisons described above indicated that 221 DEGs from the CK vs. Cd comparison were assigned to 82 KEGG pathways, 292 DEGs from the CK vs. MT comparison were assigned to 88 KEGG pathways, and 164 DEGs from the Cd vs. Cd + MT comparison were assigned to 84 KEGG pathways (**Table S5**). As shown in Fig. 5F, many DEGs identified in the CK vs. Cd comparison were connected with the flavonoid biosynthesis pathway (ath00941), phenylpropanoid biosynthesis (ath00940), secondary metabolite synthesis (ath00999), glutathione metabolism (ath00480) and amino sugar and nucleotide sugar metabolism (ath00520). In the CK vs. MT comparison, the top four pathways were related to the biosynthesis of nucleotide sugars (ath01250), amino sugar and nucleotide sugar metabolism, circadian rhythms (ath04712) and flavonoid biosynthesis (Fig. 5G). Finally, the top four KEGG pathways highlighted from the Cd vs. Cd + MT comparison were associated with ubiquinone and terpenoid-quinone biosynthesis (ath00130), flavonoid biosynthesis, glutathione metabolism and phenylpropanoid biosynthesis (Fig. 5H). A total of 107 DEGs overlapped all three pairwise comparisons and the dominant KEGG enrichment pathways among these were related to flavonoid biosynthesis, phenylpropanoid biosynthesis and glutathione metabolism.

DEGs involved in heavy metal (HM) transport and heat shock proteins

Previous studies have indicated that the control of uptake and subcellular compartmentalization of HMs are important for reducing cellular damage. Metal-ion transporters play a key role in these steps. In addition, heat shock proteins (HSPs) respond to HM stress by protecting and repairing damaged proteins. We identified several DEGs involved in these processes including ATP-binding cassette (ABC) transporters, heavy metal transporters and HSPs (Fig. 6, **Table S6**). Cd treatment was associated with the upregulation of two heavy metal-associated isoprenylated plant protein (HIPP) genes (*FtPinG0005387700.01*, *novel.1899*), one copper transporter gene (*FtPinG0008617900.01*), three HSP genes (*FtPinG0003974500.01*, *novel.2843*, *novel.3137*) and four ABC transporter genes (*FtPinG0006389100.01*, *FtPinG0008013800.01*, *FtPinG0008973700.01*, *novel.1890*). Indeed, the HIPP genes, the copper transporter gene and one of the HSP genes were upregulated genes across all three comparisons. Interestingly, both metal-nicotianamine transporters and both metal tolerance proteins showed the opposite responses and had decreased expression in Cd treatment. Finally, one HSP gene (FtPinG0004132400.01) and one ABC transporter gene (FtPinG0007917200.01) were down-regulated in Cd compared with CK and down-regulated in MT compared with CK, but upregulated in Cd + MT compared with Cd alone.

DEGs related to redox

The GO annotations established that one of the highest enrichment subcategories was oxidoreductase activity (GO:0016491) which is consistent with our earlier measurements of SOD and POD activities (Fig. 3). A total of 38 redox-related genes were detected including glutathione S-transferase (GST), Cytochrome P450 (CYP450), superoxide dismutase (SOD), and bifunctional 3-dehydroquinate dehydratase/shikimate dehydrogenase (DHQ-SDH) (**Table S7**). In the pairwise comparison CK vs. Cd, a total of 22 of the 28 genes shown in Fig. 7 were upregulated. In the Cd vs. Cd + MT pairwise comparison, expression of *CYP450-2*, *CYP450-3*, *CYP450-6*, *NADPH* (Oxidoreductase NAD-binding), *DHQ-SDH-1*, *GST3*, *GSTF*, *GSTFD*, and *6PGD1* (6-phosphogluconate dehydrogenase) was down-regulated by Cd + MT. Interestingly, expression levels of *DHQ-SDH-2*, *BADH* (benzaldehyde dehydrogenase), *BBE* (berberine bridge enzyme), *GSTX4*, *GSTX5*, *GSTX6*, *PEX5* (peroxisome biogenesis protein) and *PEX9* were higher in MT treatment than Cd treatment (Fig. 7). These results further support the conclusion that the addition of MT can alter gene expression in ways that are likely to improve the capacity of Tartary buckwheat to combat Cd-induced oxidative stress.

DEGs involved in the phenylpropane pathway

KEGG enrichment analysis showed that phenylpropanoid biosynthesis and flavonoid biosynthesis pathways were enriched in all three pairwise comparisons indicating they are also likely to play a role in the MT-dependent responses to Cd toxicity. A total of 25 DEGs were involved in these two pathways, five of which are involved in the conversion of phenylalanine to *p*-coumaroyl-CoA via the activities of ammonia lyase (PAL), trans-cinnamate 4-monooxygenase (C4H) and 4-coumarate: CoA ligase (4CL) (Fig. 8, **Table S8**). The pathway then divides in towards flavonoid synthesis or lignin synthesis. Eleven of the DEGs are related to lignin synthesis and nine DEGs to flavonoid synthesis. The expression of two *PAL* genes and two *4CL* genes was significantly induced by Cd, but decreased in Cd + MT. Compared with CK conditions, the expression of *C4H* was significantly reduced by the Cd, Cd + MT, and MT treatments. Most DEGs associated with lignin synthesis, including *CCoAOMT* (Caffeoyl-CoA *O*-methyltransferase), *F5H* (ferulate 5-hydroxylase), *COMT* (caffeic acid *O*-methyltransferase), and *CAD* (cinnamyl alcohol dehydrogenase) were induced by Cd and Cd + MT compared with CK. Three *PRX* genes were downregulated by Cd alone and by MT alone and two were also down-regulated by Cd + MT (Fig. 8). In the flavonoid pathway, two chalcone synthase (CHS) genes and one chalcone isomerase (CHI) gene were induced by Cd and, to a lesser extent, by Cd + MT. Naringenin 3-dioxygenase (F3H) and flavonol synthase (FLS) catalyze steps in flavonol and quercetin synthesis. Two *FLS* and one *F3H* were upregulated by the Cd and Cd + MT treatments compared with CK. Genes encoding enzymes in the anthocyanin pathway were also significantly induced by Cd and MT and to a lesser extent by Cd + MT (Fig. 8). These results indicate that both Cd and MT treatments affected the phenylpropane pathway.

Confirmations of the RNA-seq results with qRT-PCR

The results of the RNA-seq experiments were confirmed by measuring the expression of 14 DEGs with qRT-PCR. The genes tested included a HSP gene (*FtPinG0003974500.01*, Fig. 9A) and HM genes (*FtPinG0005387700.01*, Fig. 9B, *FtPinG0008013800.01*, Fig. 9C, and *FtPinG0008617900.01*, Fig. 9D), key redox genes (*novel.3679*, Fig. 9E, *novel.1291*, Fig. 9F, and *novel.838*, Fig. 9G), genes involved in the phenylpropane pathway (*novel.3144*, Fig. 9; *novel.3182*, Fig. 9I; *novel.3572*, Fig. 9J; *novel.801*, Fig. 9K; and *novel.1598*, Fig. 9L), and two TFs (*FtPinG0007226700.01*, Fig. 9M; *FtPinG0002967800.01*, Fig. 9N). The results show a strong similarity in results between the RNA-seq results and the RT-PCR data with a positive correlation coefficient (average R^2 = 0.84) (Fig. 9O) indicating that the RNA-seq results were reliable.

Discussion

MT decreases Cd accumulation and improves Cd tolerance in Tartary buckwheat

Cd contaminated soils affect plant growth and compromise product quality and safety [5, 14, 44]. In this study, we found that Cd decreased root and shoot growth and increased Cd accumulation in Tartary buckwheat. The combination of Cd and MT ameliorated those negative effects of Cd on growth and reduced the accumulation of Cd in tissues (Fig. 1, Fig. 2D). Previous reports similarly found that MT reduced the harmful effects of Cd toxicity in a range of other species. Among the mechanisms proposed in those studies to explain the protective effects of MT are changes to Cd transport and compartmentation, the management of oxidative stress and modifications to the cell walls [25, 33, 45, 46].

Biotic and abiotic stresses often result in oxidative damage to cells and plants can reduce this by increasing the activity of enzymic and non-enzymatic antioxidants to control the concentration of harmful superoxide and H_2O_2 molecules [47]. The present study confirmed that treatment with Cd induced oxidative stress in Tartary buckwheat. This is supported by the measured increases in the concentrations of superoxide and H_2O_2 as well as MDA, which is a bio-indicator of ROS damage. These changes were largely reversed by combining Cd with MT. Cd treatment also increased the activities of SOD and POD enzymes and the cellular concentrations of GSH, AsA, and MT. These changes were even greater in the Cd + MT treatment than Cd alone even though MT alone had no effect (Fig. 3). This indicates a complex interaction occurs between Cd and MT. Generally similar findings have been reported in other species [25, 38, 48, 49] such as peanut [49] and cucumber [25] where the expression of antioxidant-related genes *(e.g. SOD* and *POD*) were more upregulated in the Cd + MT treatment than in the control or Cd alone treatment. The present study identified several other genes with redox-related functions (*e.g. CYP450*, *NADP*$^+$, *NADH*, *CAT*, *SOD*, *GST*, *PEX* and *6PGD* and *FtGST1*) that were induced by Cd, MT and Cd + MT treatments (Figs. 4 & 7) which collectively support the conclusion that MT mitigates Cd toxicity by inducing the expression of redox-related genes to scavenge ROS and minimize damage to membranes and organelles.

Transporters and heat shock proteins affected by MT during Cd stress

Another mechanism by which plants mitigate heavy metal toxicity is through regulating the uptake and compartmentation of the toxic metal ions. Metal ion transporters play a crucial role transporting Cd into and out of cells as well as moving phytochelatin-Cd or GSH-Cd complexes from the cytosol to the vacuoles [50, 51]. The present study showed that Cd + MT treatment substantially reduced the expression level of metal-ion transporter *FtHMA2* compared with Cd alone, but induced the expression of the phytochelatin synthase gene *FtPCS1* (Fig. 4). We also found that Cd + MT altered the expression of genes from other transporter families such as *ABC* (ATP-binding cassette) and *HIPP* (heavy metal-associated isoprenylated plant protein) some of which are known to transport heavy metals like Cd across membranes (Fig. 6). These results support the model that MT treatment reduces the damage to cellular organelles incurred by Cd by regulating the expression of metal ion transporters and promoting the partitioning of Cd to the vacuole. Similar findings for MT treatment have been made in other species. For instance, MT treatment altered the relative expression of the *PCS1* gene in bread wheat which reduced Cd uptake accumulation [52]. MT treatment in radish (*Raphanus sativus*) suppressed the expression of *HMA* genes [53] and the expression of *OsNRAMP1*, *OsNRAMP5*, *OsCd1*, and *OsIRT1* in rice [54]. Qiu et al. [33] found that the reduction of Cd accumulation by MT in rice was associated with greater *OsPCS1* expression and reduced

expression of *OsNRAMP5* and *OsCd1*. Furthermore, MT enhanced the tolerance of tomato to Cd by promoting the biosynthesis of GSH and other compounds that complex Cd [55]. The protective responses of MT have also been associated with changes in the expression of *OsHIPP42* and *OsHIPP56* in rice [33] and in *ABC* genes in tomato [38].

Heat shock proteins (HSPs) act as molecular chaperones in protein folding and assembly and can help combat a range of stresses including heavy metal stress [56]. Their function can be complex and in radish, for example, some HSP genes were induced by Cd stress while others were inhibited [57]. Similarly in oat (*Avena sativa*), the *AsHSP90-1*, *AsHSP90-2*, and *AsHSP90-12* genes were induced by Cd stress while *AsHSP90-3*, *AsHSP90-10*, and *AsHSP90-16* expression was decreased by Cd [58]. The present study identified four *HSP* genes that changed expression during the Cd and MT treatments. One of these showed lower expression in Cd + MT compared with controls while the other three *HSP* genes tended to increase their expression (Fig. 6). These results indicate *HSP* genes may also be combating Cd stress but further research is required to clarify the complex mechanisms involved.

Transcription factors (TFs) participate in the MT-related alleviation of Cd stress

TFs act as activators and/or inhibitors to regulate gene expression, signal transduction, and various physiological and biochemical processes [59]. Among the 39 differentially expressed TFs identified in the present study, 16 TFs were highly upregulated in Cd + MT compared with Cd alone (including members of the AP2/ERF, bHLH, MYB, Dof, CPP, and WRKY families) and seven were downregulated (**Table S2**). TFs from the MYB and WRKY families regulate multiple physiological processes and have previously been implicated in Cd stress [60–63]. Three WRKY and one MYB TFs were upregulated in Cd + MT compared with Cd alone and two other MYBs were downregulated (**Table S2**). Dof and CPP TFs also play a crucial role in plant growth, development and responses to stress [64–67]. The present study identified one Dof TF gene that was significantly downregulated when treated with Cd or with MT but significantly induced in Cd + MT compared with Cd (**Table S2**). A CPP gene was also significantly induced in the Cd + MT treatment (**Table S2**).

MT induced changes to the phenylpropane pathway

The phenylpropanoid pathway controls the synthesis of flavonoids and lignin in plants [68, 69]. The activation of the phenylpropanoid pathway by Cd is thought to be a defense response [18, 70] because flavonoids (such as rutin and quercetin), lignin and other phenolic compounds can mitigate oxidative damage in the cell walls and cytosol [71, 72]. In this pathway phenylalanine is first converted to p-coumaroyl-CoA by reactions involving PAL and C4H and then towards either flavonoid synthesis or lignin synthesis. The present study found that the expression of *PAL* and *4CL* in Tartary buckwheat was induced by both the Cd and Cd + MT treatments compared with control conditions (Fig. 8). In the flavonoid branch, the expression of *CHI*, *F3H*, *FLS*, *ANS*, and three *CHS* genes was induced by Cd stress. The expression of all these genes, except for *CHS-2*, remained unchanged or was reduced in the Cd + MT treatment compared with Cd alone (Fig. 8). Interestingly, MT application did not lead to an accumulation of quercetin in Tartary buckwheat and rutin concentration in roots decreased in the Cd + MT treatment compared with Cd stress (Fig. 2B). We speculate that MT might either be effectively mitigating Cd toxicity by other mechanisms and therefore flavonoid accumulation was not induced, or the accumulation of flavonoids depends on the severity and duration of Cd exposure. These can be tested with further investigations.

The cell wall is considered a key site for immobilizing HMs like Cd and serves as the primary barrier to the uptake of harmful ions [73, 74]. Lignin is a component of the secondary cell wall which can immobilize HM ions [75] by binding them to the functional groups like phenols and hydroxyls [76, 77]. Previous studies have shown that MT can promote lignin biosynthesis and Cd tolerance in peanut (e.g. [49]).

We identified 11 DEGs related to lignin biosynthesis including *HCTs*, *CADs*, *CCoAOMTs*, *F5H*, *COMT*, and *PRXs* (Fig. 8). Peroxidase (PRX) is not only a member of the antioxidant system, but also involved in lignin synthesis and resistance to HM stresses [78, 79]. Three *PRX* genes (*PRX52*, *PRX44*, and *PRX2*) were downregulated by Cd stress compared with CK conditions while *PRX52* was upregulated under Cd + MT compared with Cd alone (Fig. 8). Finally, the COMT enzyme is involved in MT synthesis in plants [80] and plays an important role in lignin biosynthesis [81]. Previous studies confirmed that overexpression of the *COMT* gene could enhance tolerance to environmental stress by elevating lignin and MT levels in plants [82, 83]. The transcriptomic analysis demonstrates that *COMT1* was upregulated under Cd, Cd + MT, and MT treatments (Fig. 8). These results suggest that MT could help mitigate Cd stress by inducing the accumulation of lignin in cell walls.

Conclusion

Cd is a harmful element to plant growth and development. In this study, we systemically evaluated the physiological and molecular mechanisms of exogenous MT in alleviating Cd toxicity to Tartary buckwheat seedlings. We demonstrated that MT reduced Cd-induced oxidative stress in Tartary buckwheat by inducing the expression of redox genes, increasing the activity of antioxidant enzymes, increasing the concentration of antioxidants, and inducing the expression of genes in the phenylpropane pathway to stimulate flavonoid and lignin synthesis. MT increased the expression of PCS and GST and increased GSH accumulation in cells to chelate Cd ions and altered the expression of HM transporters that move Cd in and out of cells and between sub-cellular compartments (Fig. 10). Collectively, these physiological, biochemical, and transcriptomic investigations show that the MT can mitigate Cd toxicity by activating multiple mechanisms to counter Cd-induced damage.

Materials and Methods

Plant materials, growth conditions, and treatment

The Tartary buckwheat cultivar Xiqiao No.2 was used in this study because it was previously shown to be more tolerant to Cd stress than other cultivars tested [15]. In the hydroponic experiments, seed were first surface sterilised with 75% ethanol for 60 s and 0.8% NaClO for eight minutes, and then rinsed more than eight times with sterile water. The seed were then germinated in petri dishes on moist dust-free paper for three to four days in darkness and room temperature. Seedlings were transplanted into 2.4 L containers with 1/2 Hoagland solution (pH = 5.80). They were held in holes of the container lids with soft plastic foam. The containers were kept in a greenhouse under controlled conditions: 14 h/25 ℃ and 10 h/22 ℃ day-night cycle, 70% relative humidity, and ~ 300 μM m^{-2} s^{-1} light intensity. Each container was aerated with one pipe attached to aquarium air pumps. After one week, the seedlings were transferred to new containers with 1/2 Hoagland solution and one of the following treatments: Control (CK), Cd alone (50 μM $CdCl_2$), Cd + MT (50 μM $CdCl_2$ + 100 μM MT), and MT alone (100 μM MT), all at pH = 5.80. The solutions were replaced after three days and pH checked daily and adjusted as required using 1 M HCl or 1 M NaOH. Plants were harvested after five days and stored for subsequent measurements.

The soil experiment included 36 pots (nine pots for each of the four treatments) with dimensions 19×24×24.5 cm and containing 8 kg of soil. The physicochemical properties of the soil were as follows: urea content (available nitrogen ≥ 46%), 400 mg/kg; phosphate content (P_2O_5, available phosphorus ≥ 16%), 200 mg/kg; and potassium sulfate content (K_2O, available potassium ≥ 52%), 100 mg/kg. Ten seeds were germinated in each pot and grown in a greenhouse as described above. After three weeks growth the seedlings were watered with 1.0 L of the following solutions: Control (CK, 1/2 Hoagland), Cd treatment (1/2 Hoagland + 100 μM $CdCl_2$), Cd + MT treatment (1/2 Hoagland + 100 μM $CdCl_2$ + 200 μM MT), or MT alone treatment (1/2 Hoagland + 200 μM MT). After seven days, whole plants from three of the pots in each treatment were harvested, frozen in liquid nitrogen, and stored at -80℃ for transcriptome sequencing. The roots, stems, leaves, and seeds were later collected for other measurements from seedlings (three pots per treatment) and mature plants (three pots per treatment). All measurements had three biological replicates. In summary, the agronomic traits, flavonoid polyphenol and Cd contents, and transcriptomic experiments all used plants grown in soil and the other measurements used hydroponically-grown plants.

Analysis of oxidative damage, enzyme activities, and antioxidant content

The concentrations of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) and the rate of superoxide anion ($O_2^{\cdot-}$) production were measured as described previously [49]. The activities of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) were measured using the nitro blue tetrazolium, guaiacol and spectrophotometric methods, respectively [84]. The content of glutathione (GSH) and ascorbic acid (AsA) was detected with commercial kits (PYHA3-C48 and PYHE8-C48; Nanjing Mofan Biotechnology Co., Ltd, Nanjing, China).

Determination of rutin and quercetin content

Concentrations of the flavonoid polyphenols rutin and quercetin were measured in the roots, stems, leaves, and seeds of soil-grown plants. High-performance liquid chromatography (HPLC; Agilent Series 1100 liquid chromatograph with C18, 150 × 4.6 mm, 5 μm) was used as follows: 0.2 g samples were placed in 50 mL centrifuge tubes with 30 mL of 70% methanol and shaken in the dark at 65℃. The extracts were then passed through a 0.45 μm filters, and 50 μL stored in new light-protected liquid chromatography vials for later testing. Measurement conditions included: 30℃ column temperature, mobile phases A (chromatographically pure methanol) and B (0.1% formic acid), 0.1 mL/min flow rate, 15 μL injection volume, elution gradient and detection at a wavelength of 350 nm. The elution gradient process was 20% A and 80% B, 100% A and 0% B, 20% A and 80% B at 0 ~ 15 min, 15 min, and 15 ~ 28 min, respectively.

Measurement of Cd content and endogenous MT

The roots, stems, leaves, and seeds from soil-grown plants were washed at least three times with distilled water, dried at 80℃ for two days before being weighed and digested with 68% concentrated HNO_3. The Cd content was detected by inductively coupled plasma mass spectrometry (ICP-MS, NexION, 2000) (PerkinElmer, Waltham, MA, USA).

To measure tissue MT concentrations, the roots and shoots of hydroponically grown plants were collected, rapidly sonicated with 5 mL methanol for 30 min at 4 ℃ in the dark conditions. The samples were then centrifuged at 5000 rpm for 10 min and the removed by drying. After being resuspended with 1.0 mL of methanol, the sample

was filtered with a 0.22 μm filter membrane and MT measured with HPLC-Tandem Mass Spectrometry (HPLC-MS/MS) (Vanquish UHPLC and TSQ Altis Plus, ThermoFisher, USA). The HPLC collection conditions included the chromatographic column: Syncronis aQ 100×2.1×1.7 μm, 40℃ column temperature, mobile phases A (ultrapure water added with 0.1% formic acid) and B (methanol with 0.1% formic acid), 5 μL injection volume, 0.3 mL/min flow rate. Mobile phase A/B was 97:3 (v/v), 97:3, 10:90, 97:3, and 97:3 at 0 min, 1.5 min, 4 min, 4.01 min, and 6 min, respectively. The MS/MS collection conditions included Electron Spray Ionization (ESI+), multiplex reaction monitoring (MRM) detection mode, mass spectrum voltage (4000 V under positive ion mode and − 3500 V under negative ion mode), 45 Arb sheath gas flow, 10 Arb auxiliary airflow velocity, 325℃ ion transfer tube temperature, and 450℃ nebulizer temperature.

RNA extraction and transcriptome sequencing

The HiPure Plant RNA Kits (Guangzhou Magen Biotechnology Co., Ltd, Guangzhou, China) were used to extract the total RNA from 12 frozen plant samples comprised of four treatments (CK, Cd, Cd + MT and MT) and with three replicates per treatment. The quantity and integrity of the RNA were evaluated with a NanoDrop 8000 spectrophotometer (NanoDrop, Wilmington, DE) and the 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively. The complementary deoxyribonucleic acid (cDNA) libraries were constructed using the NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, Ipswich, MA, USA). The cDNA library from each sample was sequenced on an Illumina Hiseq™ 6000 system by Beijing Novogene Technology Co., Ltd (Beijing, China), and paired-end reads were generated.

After filtering the raw reads, clean reads were obtained by removing low-quality reads as well as reads containing adapters and ploy-N regions. The Q20, Q30, and GC contents of the clean data were calculated. All the downstream analyses were based on high-quality clean data that were mapped on the *Fagopyrum tataricum* genome (https://mbkbase.org/Pinku1/) [85] using Hisat2 v2.0.5 [86] and assembled by StringTie v1.3.3b [87]. Gene expression levels were estimated as fragments per kilobase of transcript per million fragments mapped (FPKM).

Transcriptomic data analysis

Differential expression genes (DEGs) were determined in three comparisons: CK vs. Cd, CK vs. Cd + MT, and CK vs. MT. These were performed using the DESeq2 R package (1.20.0) [88]. Resulting *p*-values were adjusted using Benjamini and Hochberg's method. The DEGs were screened using adjusted *p*-value ≤ 0.05 and |log2foldchange| ≥ 1. In addition, both Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were conducted using the clusterProfiler R package. GO terms with adjusted *p*-value < 0.05 were considered significantly enriched by DEGs.

Quantitative real-time PCR (qRT-PCR) validation

The qRT-PCR procedures used a CFX96 Touch Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA) using TB Green® Premix Ex Taq™ （Takara, Co. Ltd, Dalian, China). Primers were designed using Primer Premier 5 (Premier Biosoft, Palo Alto, CA, USA) and listed in **Table S1**. All reactions were repeated three times, and quantification was calculated with the $2^{-\Delta\Delta CT}$ method [89].

Statistical analysis

Data obtained from this study were analyzed using SPSS 21.0 software (Chicago, IL, USA). The statistical significance was determined using one-way analysis of variance (ANOVA), performed by Duncan's multiple range test. Images were drawn and merged using Origin 2021 (OriginLab Corporation, Northampton, MA, USA) and Adobe Photoshop CC 2018 software (San Jose, California, USA), respectively.

Declarations

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals. The collection materials of the plants comply the relevant institutional, national, and international guidelines and legislation.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author Contribution

D.H. participated in experimental design, methods and provided the findings. D.H, W.C.H., and D.L. wrote the main manuscript text. W.C., D.L., L.H., Y.Y., Y.Q., P.R., and W.C.H. analyzed the data. W.A. provided the plant resource used in this study. All authors reviewed the manuscript.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The datasets generated or analyzed during the current study are available in the CNGB repository with accession number CNP0009606 (https://db.cngb.org/cnsa/project/page/sub087008/view/).

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Figures

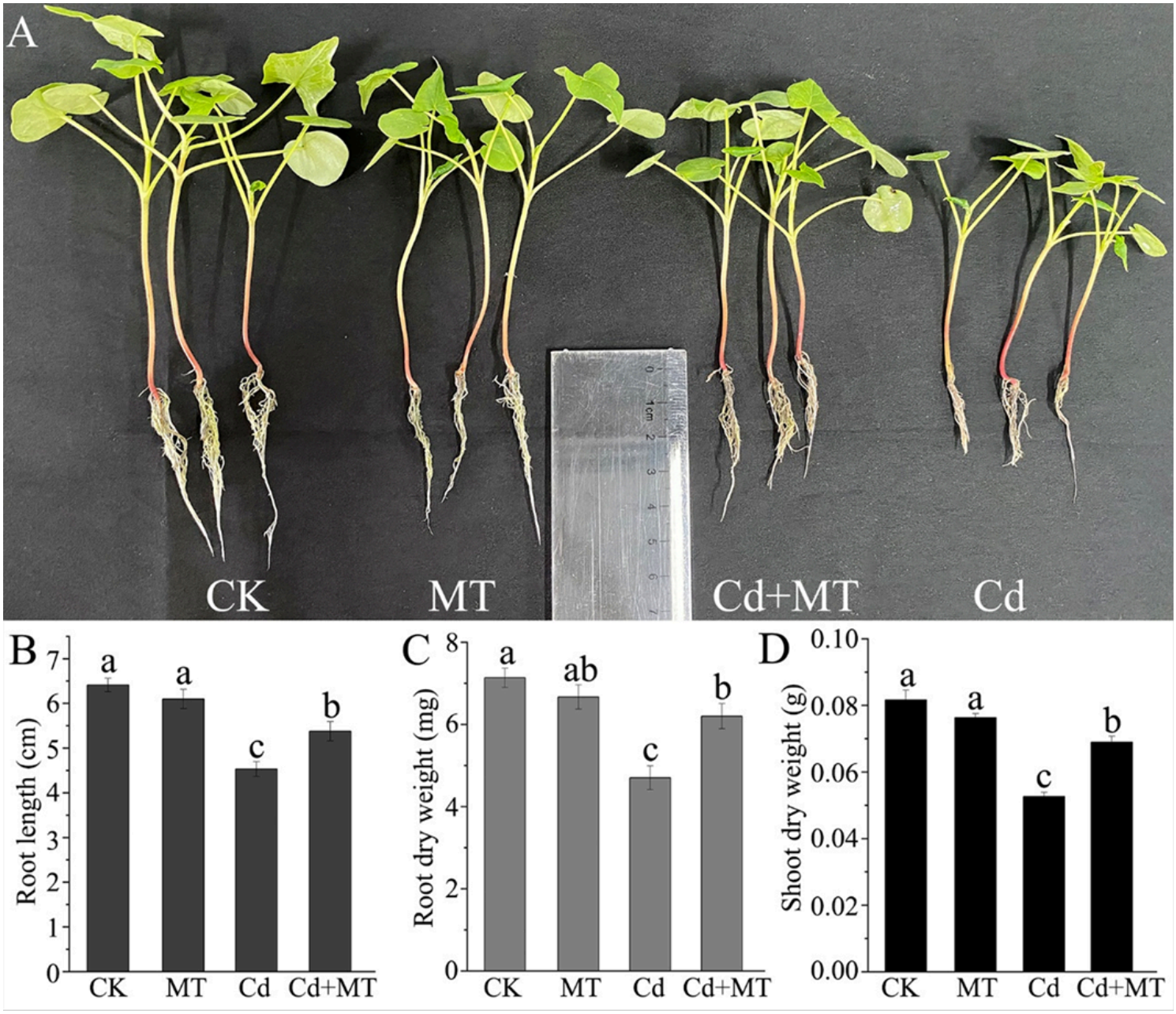


Figure 1

Effect of MT treatment on the morphological indices of Tartary buckwheat seedlings under Cd stress

(A) The phenotype, **(B)** root length, **(C)** root dry weight, and **(D)** shoot dry weight in Tartary buckwheat seedlings under Cd and MT treatment conditions. CK, 1/2 Hoagland; MT, 1/2 Hoagland + 100 μM MT; Cd+MT, 1/2 Hoagland + 50 μM $CdCl_2$ + 100 μM MT; Cd, 1/2 Hoagland + 50 μM $CdCl_2$. Plants were cultivated for five days after treatment. Values represent means ± SD (n = 14). Different letters indicate significant differences at $P < 0.05$ by Tukey's test.

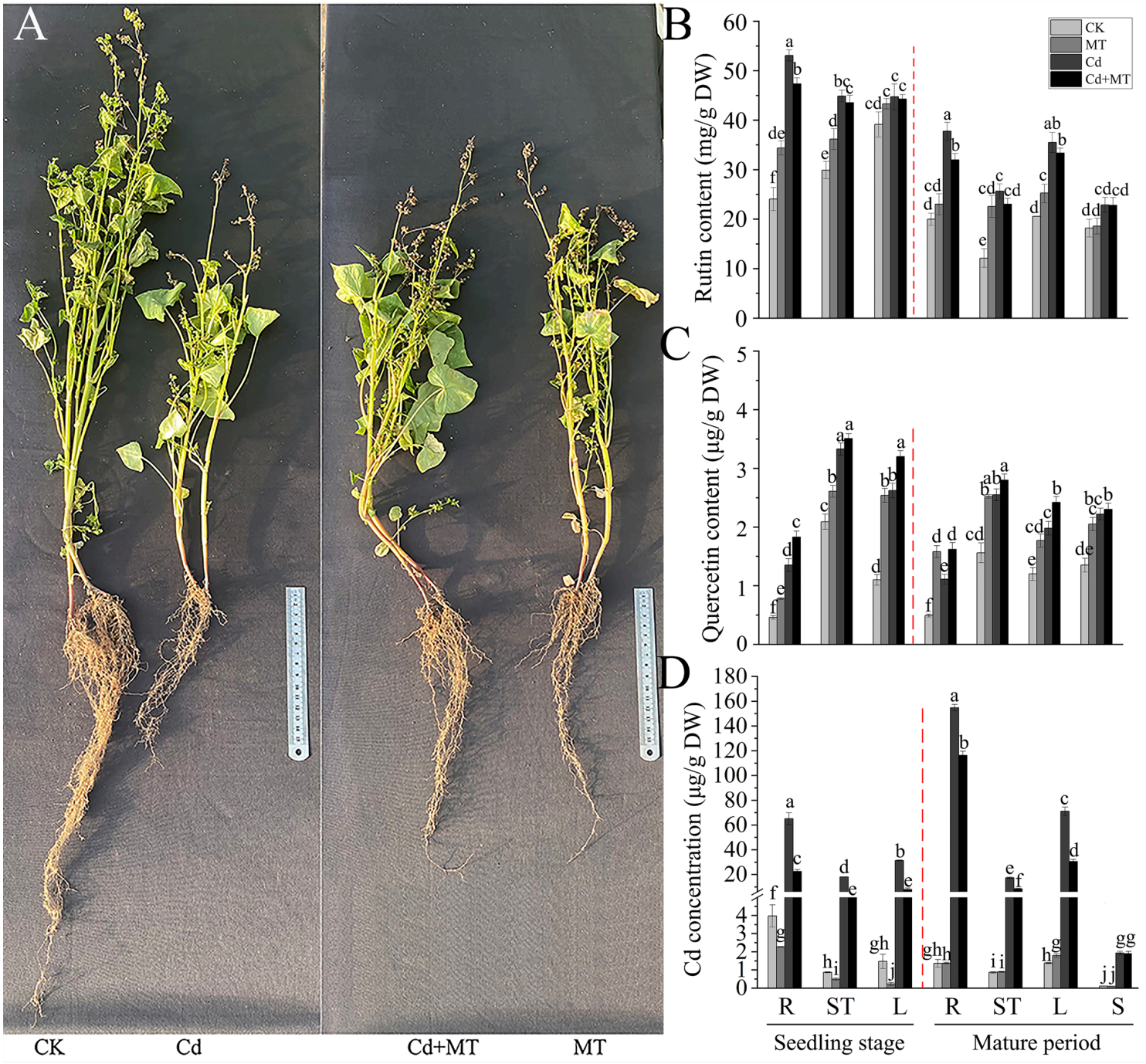


Figure 2

Tartary buckwheat phenotype, quality, and Cd content after MT and Cd stress treatment

(A) The whole plant of Tartary buckwheat at the mature stage. The concentration of **(B)** rutin, **(C)** quercetin, and **(D)** Cd in different tissues at the seedling and mature stage after exposure to Cd and MT treatment. CK, control; MT, 200 μM MT; Cd+MT, 100 μM $CdCl_2$ + 200 μM MT; Cd, 100 μM $CdCl_2$. Values represent means ± SD (n = 15). Different letters indicate significant differences between different treatments at $P < 0.05$ by Tukey's test.

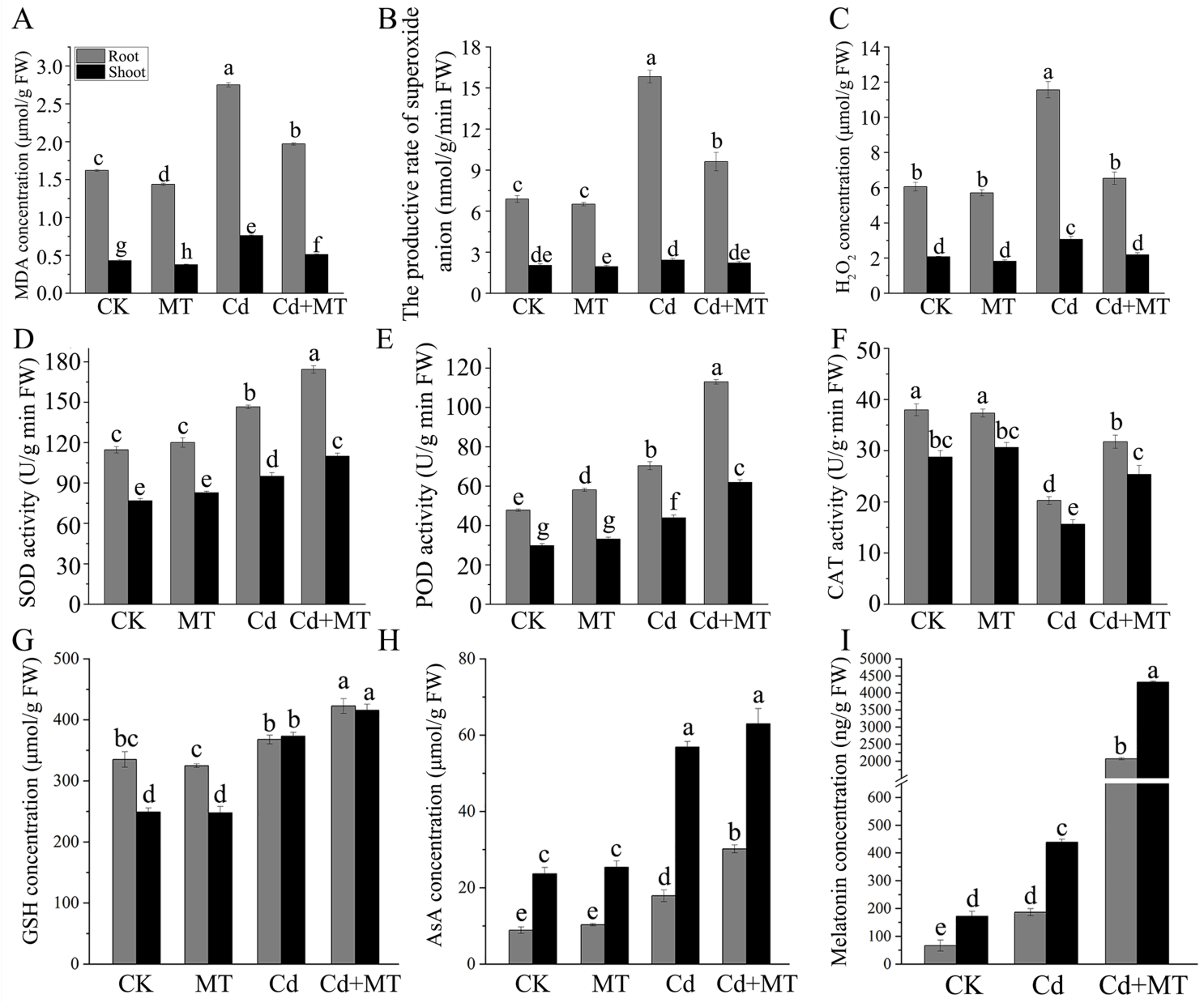


Figure 3

Effect of MT treatment on antioxidant defense capacity of Tartary buckwheat seedlings under Cd and MT treatment

(A) The concentration of malondialdehyde (MDA). **(B)** The productive rate of superoxide anion. **(C)** hydrogen peroxide (H_2O_2) concentration. The activity of **(D)** superoxide dismutase (SOD), **(E)** peroxidase (POD), and **(F)** catalase (CAT). The concentration of **(G)** glutathione (GSH), **(H)** ascorbic acid (AsA), and **(I)** melatonin. Treatment and sample acquisition refer to Fig. 1. Values represent means ± SD (n = 14). Different letters indicate significant differences at $P < 0.05$ by Tukey's test.

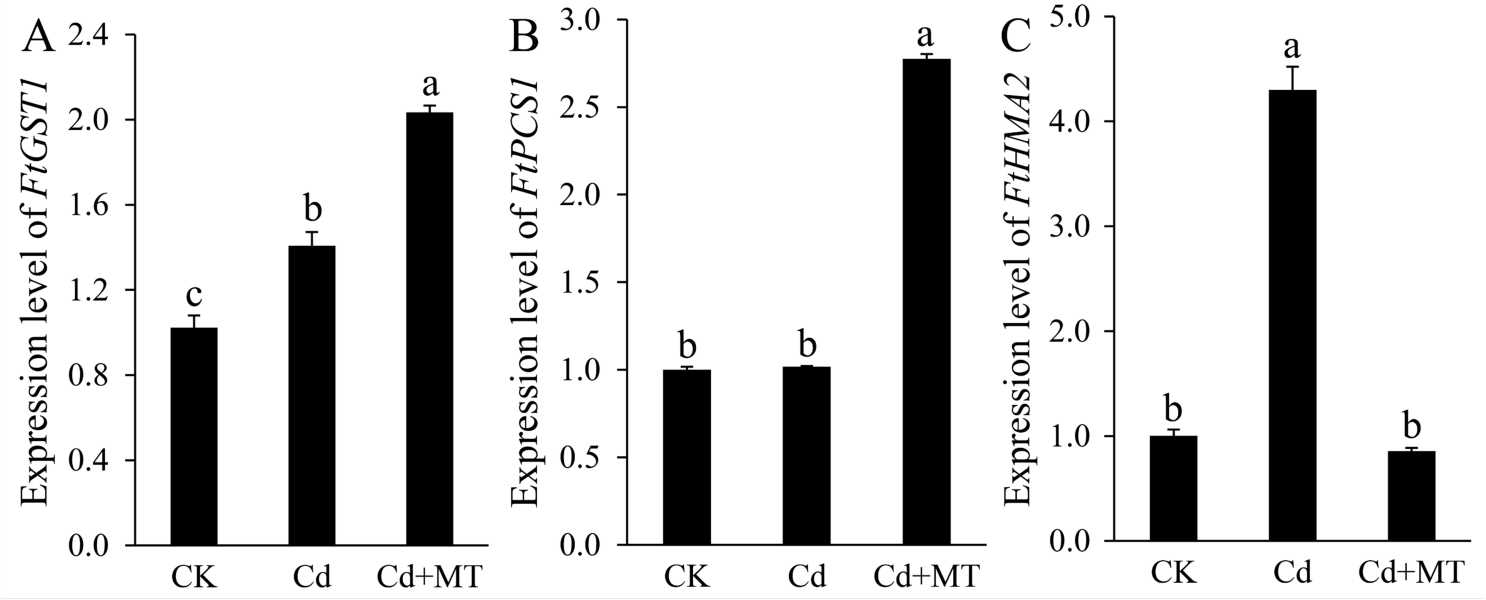


Figure 4

Effect of MT on the expression level of *FtGST1*, *FtPCS*, and *FtHMA*in Tartary buckwheat roots under Cd stress

RT-PCR analysis of (A) *FtGST1*, (B) *FtPCS*, and (C) *FtHMA* with the reference of *FtH3*. Treatment and root samples acquisition refer to Fig. 1. Values represent means ±SD (n = 15). Different letters indicate significant differences at $P < 0.05$ by Tukey's test.

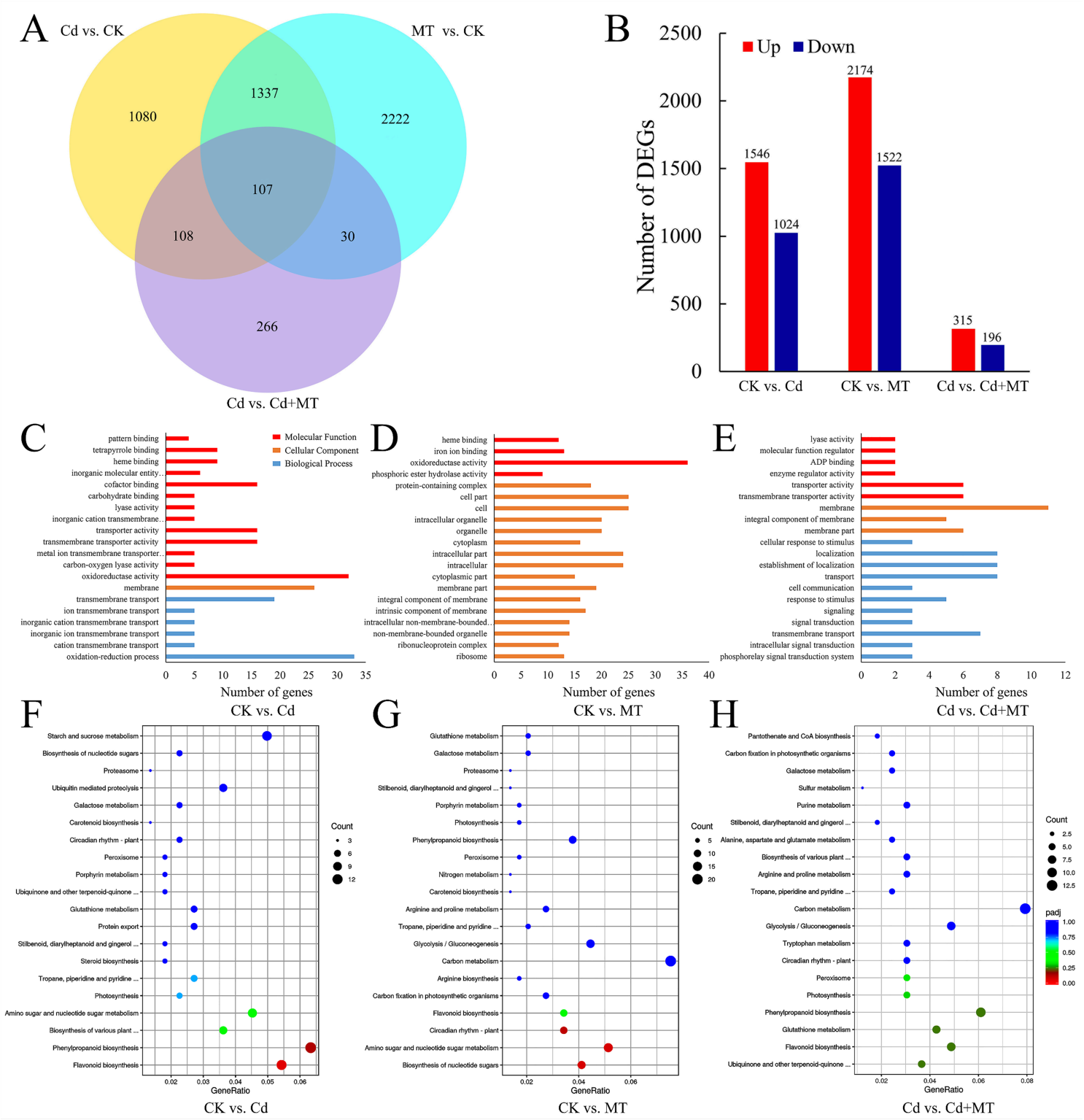


Figure 5

The DEGs analysis of RNA-Seq after exposure to different treatments

(A) Venn diagrams of DEGs in the three comparisons: CK vs. Cd, CK vs. MT, and Cd vs. MT+Cd. **(B)** The numbers of DEGs in three different comparisons, red and blue, represent upregulated expression and downregulated expression DEGs, respectively. The top 20 GO enrichment of the DEGs in **(C)** CK vs. Cd,**(D)** CK vs. MT, and **(E)** Cd

vs. MT+Cd comparison. The top 20 KEGG pathway enrichment of the DEGs in **(F)** CK vs. Cd, **(G)** CK vs. MT, and **(H)** Cd vs. MT+Cd comparison.

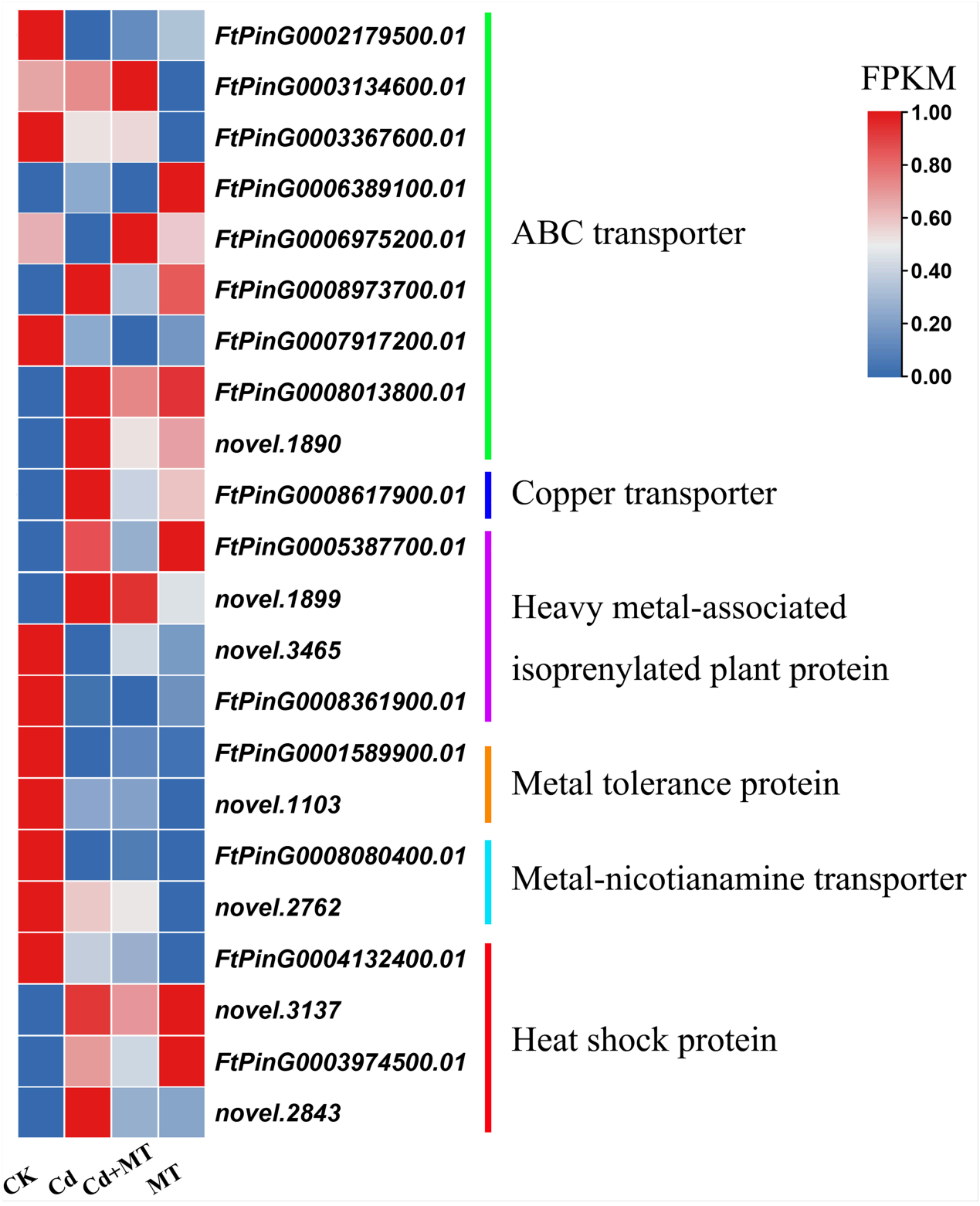


Figure 6

Expression of DEGs involved in heavy metal detoxification

Red means upregulation, and blue means downregulation. Color bars: FPKM value.

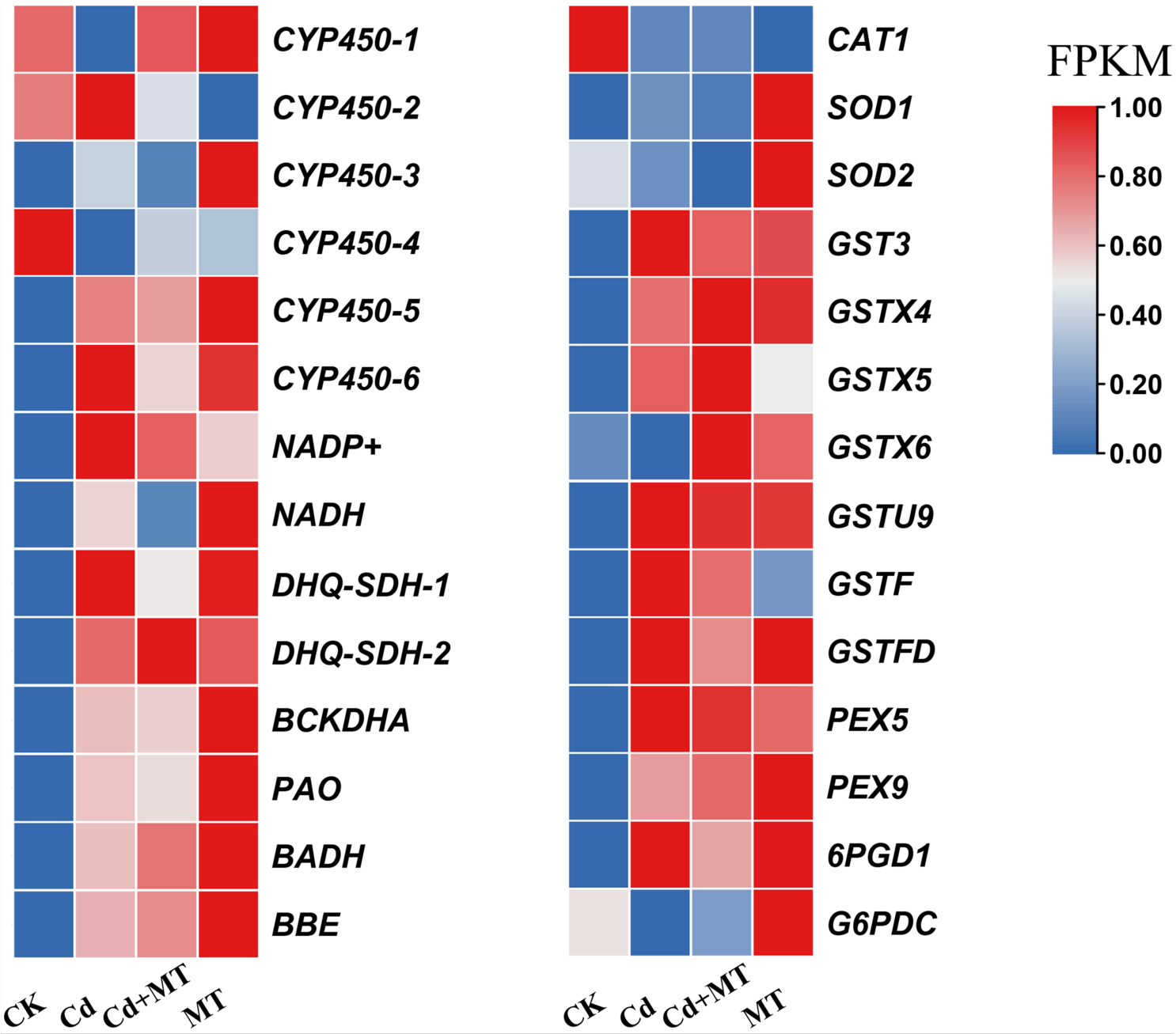


Figure 7

Heat map of DEGs associated with redox genes

The color and related explanation in this figure legend is referred to Fig.5. CYP450, Cytochrome P450; $NADP^+$, 2-alkenal reductase (NADP(+)-dependent); NADH, NADH--cytochrome b5 reductase 1; DHQ-SDH, Bifunctional 3-dehydroquinate dehydratase/shikimate dehydrogenase; BCKDHA, 2-oxoisovalerate dehydrogenase subunit alpha 1; PAO, Polyamine oxidase 1; BADH, Benzaldehyde dehydrogenase; BBE, Berberine bridge enzyme; CAT, Catalase isozyme; SOD, Superoxide dismutase; GST, Glutathione S-transferase; PEX, Peroxisome biogenesis protein; 6PGD1, 6-phosphogluconate dehydrogenase; G6PDC, Glucose-6-phosphate 1-dehydrogenase.

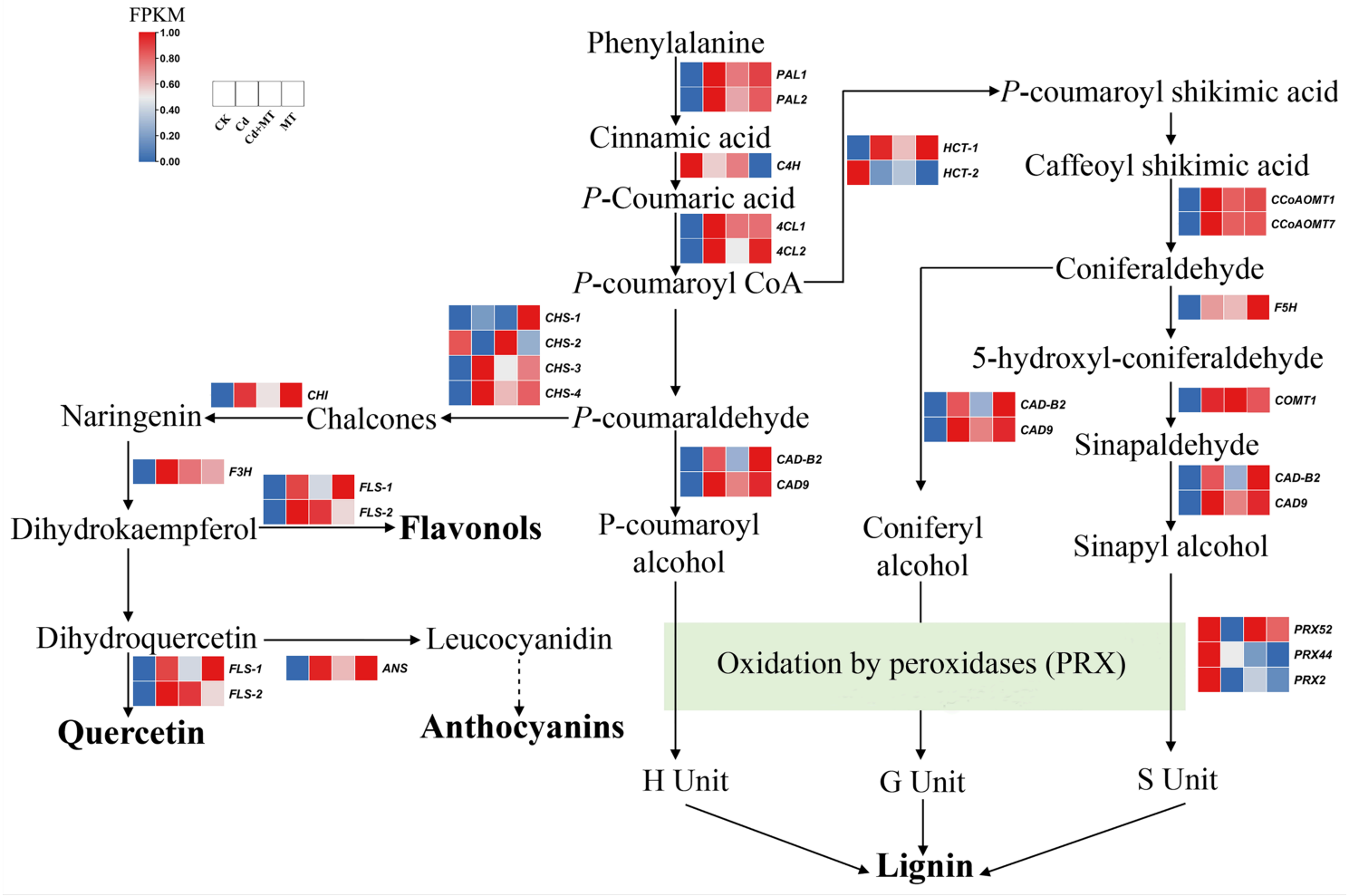


Figure 8

Effect of CK, Cd, Cd+MT, and MT treatments on the expression patterns of DEGs involved in the phenylpropane pathway related to flavonoid and lignin biosynthesis

The phenylpropane pathway produces p-coumarate CoA, a common precursor of flavonoids and lignin biosynthesis. The color scale from blue to red for the heatmap is based on the FPKM value. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate-CoA ligases; HCT, shikimate O-hydroxycinnamoyltransferase; CCoAOMT, caffeoyl CoA O-methyltransferase; F5H, ferulate 5-hydroxylase; COMT, caffeic acid O-methyltransferase; CAD, cinnamyl alcohol dehydrogenase; PRX, peroxidase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, naringenin 3-dioxygenase; FLS, flavonol synthase; ANS, anthocyanidin synthase.

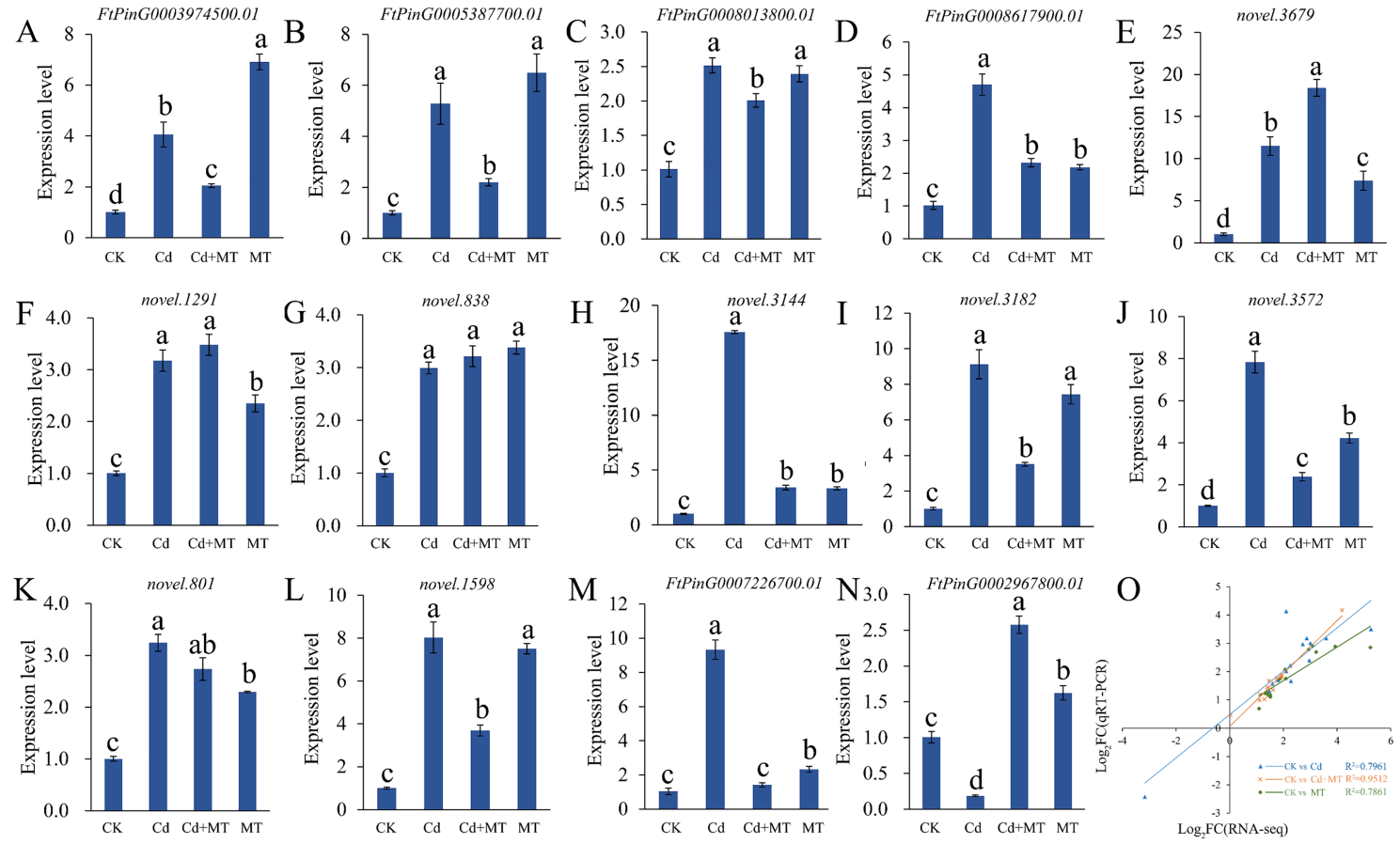


Figure 9

Real-time quantitative PCR verification and correlation analysis with RNA-seq

(**A-N**) The qRT-PCR analysis of 14 selected DEGs. (**N**) Relationship between qRT-PCR (log_2FC) and RNA-seq expression data (log_2FC).

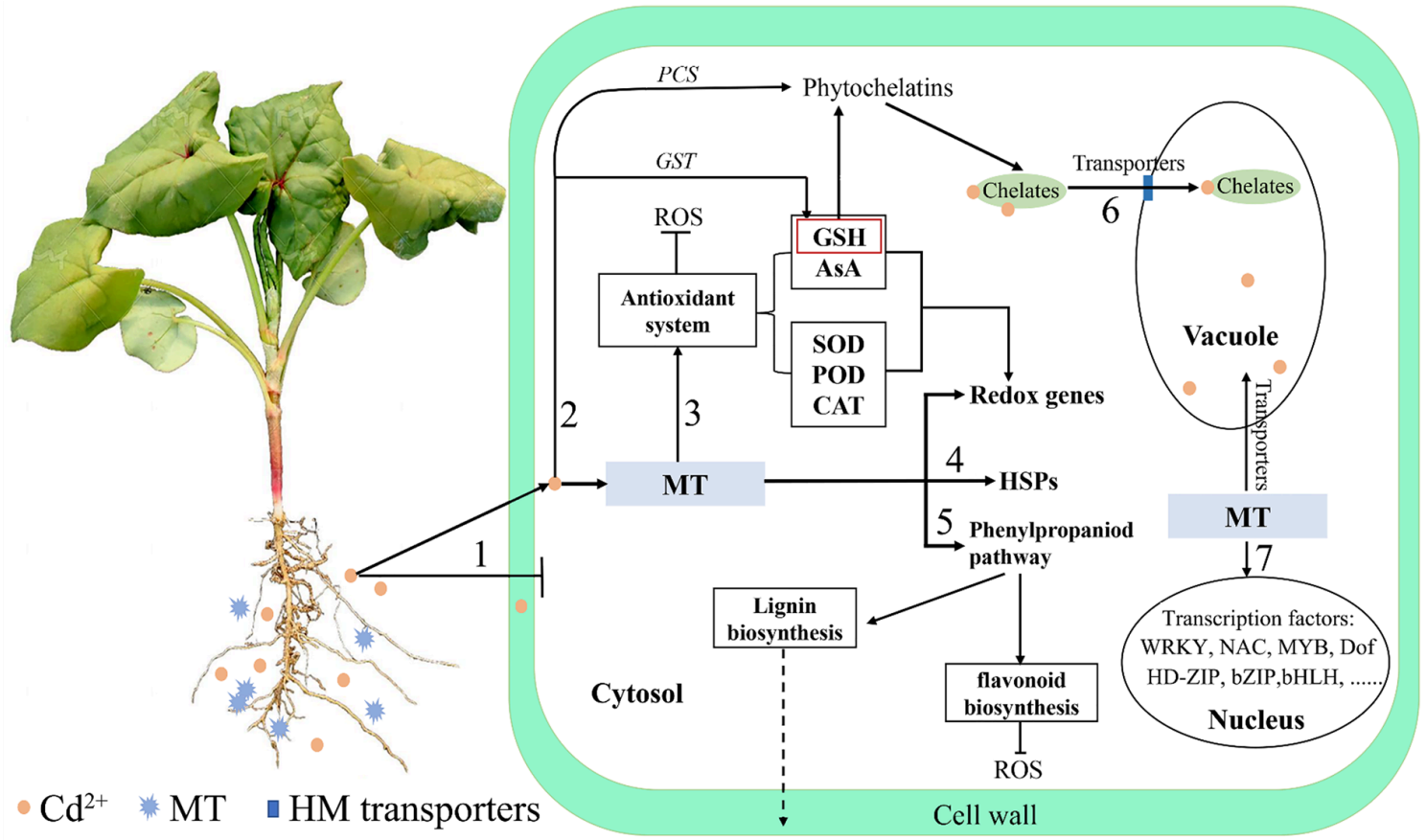


Figure 10

Schematic diagram showing melatonin (MT) alleviates Cd toxicity in Tartary buckwheat

ROS, reactive oxygen species; SOD, superoxide dismutase; POD, peroxidase; CAT, catalase; GSH, glutathione; AsA, ascorbic acid; PCS, phytochelatin synthase gene; GST, Glutathione S-transferases gene; HSPs, heat shock proteins; HM transporters, heavy metal transporters. 1. MT may mitigate the entry of Cd into Tartary buckwheat. 2. MT induces the expression of PCS and GST and the accumulation of glutathione to chelate metals in the cytosol; 3. MT stimulates the antioxidant system to remove reactive oxygen species. 4. MT induces the expression of HSPs. 5. MT induces flavonoid and lignin synthesis-related gene expression in the phenylpropane pathway. 6. MT induces heavy metal transporters to transfer metal-ligand complexes into the vacuole. 7. MT induces the expression of transcription factors.

Supplementary Files

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