Exogenous melatonin alleviates cadmium uptake and toxicity in apple rootstocks

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Abstract

To examine the potential roles of melatonin in Cd uptake, accumulation and detoxification in *Malus* plants, we exposed two different apple rootstocks varying greatly in Cd uptake and

accumulation to either 0 or 30 μM Cd together with 0 or 100 μM melatonin. Cd stress stimulated endogenous melatonin production to a greater extent in the Cd-tolerant *M. baccata* than in the Cd-susceptible *M. micromalus* 'qingzhoulinqin'. Melatonin application attenuated Cd-induced reductions in growth, photosynthesis, and enzyme activity, as well as ROS and MDA accumulation. Melatonin treatment more effectively restored photosynthesis, photosynthetic pigments, and biomass in Cd-challenged *M. micromalus* 'qingzhoulinqin' than in Cd-stressed *M. baccata*. Exogenous melatonin lowered root Cd^{2+} uptake, reduced leaf Cd accumulation, decreased Cd translocation factors (T_fs), and increased root, stem, and leaf melatonin contents in both Cd-exposed rootstocks. Melatonin application increased both antioxidant concentrations and enzyme activities to scavenge Cd-induced ROS. Exogenous melatonin treatment altered the mRNA levels of several genes regulating Cd uptake, transport, and detoxification including *HA7*, *NRAMP1*, *NRAMP3*, *HMA4*, *PCR2*, *NAS1*, *MT2*, *ABCC1*, and *MHX*. Taken together, these results suggest that exogenous melatonin reduced aerial parts Cd accumulation and mitigated Cd toxicity in *Malus* plants, probably due to the melatonin-mediated Cd allocation in tissues, and induction of antioxidant defense system and transcriptionally regulated key genes involved in detoxification.

Introduction

Cadmium (Cd) is a nonessential and highly phytotoxic heavy metal (HM). It accumulates in orchard soils via the application of metal-based pesticides, fungicides, and fertilizers and wastewater irrigation (Pizzol et al. 2014, Duan et al. 2016, Sungur 2016). According to a recent survey, the Cd levels in 55% and 66% of the soil samples from 91 orchards exceeded the Cd safety standard before flowering and after harvesting, respectively (Fang and Zhu 2014). Cd is highly mobile and readily absorbed by plants (Luo et al. 2016). It negatively affects plant metabolism, causes various morphological, physiological, biochemical, and

cellular changes, and hampers plant growth (Clemens et al. 2013). Apple trees may accumulate toxic Cd levels (Fang and Zhu 2014, Sungur 2016). Cd accumulation in edible plant parts poses a serious threat to human and animal health, as Cd toxicity may cause various human and animal diseases (Nawrot et al. 2006). Therefore, reliable methods are needed to reduce Cd accumulation in the aerial organs of apple trees and other crops.

Melatonin (*N*-acetyl-5-methoxytryptamine) is a ubiquitous signal molecule. It enhances plant tolerance to various abiotic stressors such as extreme temperatures, drought, salinity, and HM exposure (Zhang et al. 2015, Lee et al. 2017). Exogenous melatonin may reduce Cd concentrations in the aerial organs of tomato (Hasan et al. 2015) and wheat (Kaya et al. 2019). Upregulation of the genes governing melatonin biosynthesis and elevated endogenous melatonin levels were observed in herbaceous plants subjected to Cd (Byeon et al. 2015, Hasan et al. 2015). Thus, melatonin may be implicated in Cd accumulation and translocation. However, it is still unknown how exogenous melatonin influences rhizosphere net Cd^{2+} fluxes or whether it affects Cd translocation from the roots to the aerial organs of apple trees.

Exogenous melatonin enhances Cd detoxification in herbaceous plants via several mechanisms (Zhang et al. 2015). First, melatonin and its synthetic precursors chelate heavy metals (HMs) and induce chelate production (Limson et al. 1998). Second, melatonin and its metabolites directly scavenge reactive oxygen species (ROS) (Reiter et al. 2007). Third, it promotes antioxidant enzyme activity which scavenges excess ROS induced by Cd (Zhang et al. 2015). Melatonin is critical for Cd detoxification. Therefore, exogenous melatonin has been applied to Cd-stressed herbaceous plants such as tomato (Hasan et al. 2015, Li et al. 2016), rice (Byeon et al. 2015), wheat (Ni et al. 2018, Kaya et al. 2019), and alfalfa (Gu et al. 2017) in an attempt to enhance their Cd tolerance. To our knowledge, however, there are no studies on the efficacy of exogenous melatonin in enhancing Cd detoxification in woody fruit crops or the putative mechanisms involved.

Cd uptake, translocation, and detoxification are mediated by several genes (Luo et al. 2016). Plasma membrane (PM) H^+-ATPases furnish the proton motive force needed to ferry ions across the PM and play important roles in Cd uptake (Ma et al. 2014). The transcript levels of *HA7* encoding H^+-ATPase were higher in the Cd-susceptible *Malus micromalus* 'qingzhoulinqin' than in the Cd-tolerant *M. baccata*, thus contributing to higher Cd influxes into roots (Zhou et al. 2016). Natural resistance associated macrophage protein 1 (NRAMP1) located in the PM affects Cd entry into root cell cytosols (Gao et al. 2011, Lin and Aarts 2012). Cd cations in the root cells may complex with metal-binding chelators such as nicotianamine (NA), phytochelatin (PC), and metallothionein (MT) (Luo et al. 2016). Cytosolic Cd cations or PC–Cd complexes may be sequestered in vacuoles via tonoplast-localized magnesium proton exchangers (MHX) and ATP-binding cassette transporter C1 (ABCC1) (Berezin et al. 2008, Park et al. 2012). Vacuole-sequestered HMs, including Cd, may be remobilized via the tonoplast-localized metal efflux transporter NRAMP3 and transported into the apoplast by PM-localized transporters such as HM ATPase 4 (HMA4) and plant cadmium resistance protein 2 (PCR2), which are critical for Cd translocation from the roots to the shoots (Thomine et al. 2000, Verret et al. 2004, Song et al. 2010, Lin and Aarts 2012). Exogenous melatonin upregulated *ABC transporter* and *PCR2* in alfalfa, and *HMA4* in *Arabidopsis* (Gu et al. 2017). To the best of our knowledge, however, there is no information on the transcriptional regulation of the genes governing melatonin-mediated Cd uptake, translocation, and tolerance in apple trees.

Fruit tree rootstocks are used in vegetative propagation and control HM uptake and translocation to the aerial organs (Podazza et al. 2016). Previously, we evaluated Cd accumulation and tolerance among various apple rootstocks and established that *M. baccata* and *M. micromalus* 'qingzhoulinqin' differed greatly in terms of Cd uptake and accumulation (Zhou et al. 2017). To examine the potential roles of melatonin in Cd uptake, accumulation

and detoxification in *M. baccata* and *M. micromalus* ‘qingzhoulinqin’, we exposed the rootstocks to either 0 or 30 μM Cd together with 0 or 100 μM melatonin for 20 d. We measured their growth characteristics, net root Cd^{2+} flux, Cd accumulation, melatonin concentrations, ROS and antioxidants levels, and the key genes involved in Cd uptake, translocation, and detoxification. The results obtained from this study will provide a basis for the development of reliable methods for reducing Cd accumulation and increasing Cd tolerance in apple and other fruit trees.

Materials and Methods

Plant material and Cd exposure

Seeds of *Malus baccata* Borkh. (Mb) and *M. micromalus* ‘qingzhoulinqin’ (Mm) were stratified at 0–4 °C for 40 d. After germination, the seedlings were cultivated for 45 d in a greenhouse under natural light and temperature conditions (day/night temperature: 26/18 °C; relative humidity (RH): 50–60%) on seedling matrices in nursery plates. Seedlings of uniform height (~8 cm) were transferred to plastic pots (20 cm × 20 cm × 18 cm) containing clean sand. One seedling per pot was irrigated with half-strength Hoagland nutrient solution (pH 6.0) every second evening. After 6 weeks, plants from both species with similar growth performance were transferred to aerated half-strength Hoagland nutrient solution (pH 6.0) that was refreshed every 2 d. After cultivation in a hydroponic system for 1 week, 72 plants of both species similar in size and growth performance were equally divided among four groups (18 plants per group). The plants in each group were exposed to either 0 or 30 μM Cd together with 0 or 100 μM melatonin (M5250; Sigma-Aldrich Corp., St. Louis, MO, USA) by adding $CdCl_2 \cdot 2.5H_2O$ or melatonin to the half-strength Hoagland nutrient solution. The Cd and melatonin treatments continued for up to 20 d before harvest. Six plants were randomly selected per experimental group to measure net Cd^{2+} fluxes. The other 12 plants were

harvested after measuring their gas exchange rates.

Measurements of root net Cd^{2+} fluxes

Root net Cd^{2+} fluxes in both apple rootstocks were measured as previously described (Zhou et al. 2016) by a noninvasive micro-test technique (NMT system BIO-IM; Younger Corp., Amherst, MA, USA) at Xuyue Science & Technology Co. Ltd. (Beijing, China). An ion-selective microelectrode with an external tip (diameter range: ~2–4 μm) was fabricated and silanized with tributylchlorosilane and the tip was backfilled with an ion-selective cocktail (XY-SJ-Cd; Younger Corp., Amherst, MA, USA). Before the measurements were taken, the microelectrode was calibrated with 10 μM and 100 μM Cd^{2+}.

To determine the position of maximal Cd^{2+} fluxes along the root tip, six fine white roots (diameter ~1.5 mm) were selected from each rootstock species treated with 30 μM Cd^{2+}. A preliminary experiment was conducted by taking measurements at 300 μm, 600 μm, 1,200 μm, 1,500 μm, and 3,000 μm from the root tips. The fine roots were cut from the plants and immediately transferred to 5 mL measuring solution (0.03 mM $CdCl_2$, 0.1 mM KCl, 0.5 mM NaCl, 0.3 mM MES, and 0.2 mM Na_2SO_4; pH 6.0). After 15 min equilibration, each fine root was transferred to fresh measuring solution and the net Cd^{2+} fluxes were measured for 5 min per position. Cd^{2+} gradients near the root surface (~2–5 μm) were measured by moving the Cd^{2+}-selective microelectrode between two positions 30 μm apart and perpendicular to the root surface. Net Cd^{2+} fluxes data were processed in imFlux coupled to the NMT system.

Gas exchange measurements and plant harvest

Before harvest, gas exchange was measured for three mature leaves (leaf plastochron index range: 7–9) per plant in a CIRAS-2 photosynthesis system (PP Systems, Amesbury, MA, USA) as recommended by Zhou et al. (2017).

Root, stem, and leaf tissues were then excised from each plant. The roots were rinsed

with 20 mM EDTA disodium for 5 min and washed with deionized water for 5 min to remove any Cd^{2+} adhering to their surfaces (Shi et al. 2019). The fresh weights of the excised root, stem, and leaf tissues were recorded. The materials were immediately frozen in liquid nitrogen, ground to a fine powder in a ball mill (MM400; Retsch, Haan, Germany) and stored at -80 °C until the subsequent biochemical and molecular analyses. To calculate the fresh-to-dry mass ratio, fresh root, stem, and leaf powders (~50 mg each) were dried at 60 °C for 72 h. The biomass of each tissue type was calculated according to its fresh-to-dry mass ratio and fresh weights. Fine root and stem subsamples were harvested for histochemical analysis.

Chlorophyll content determination

To measure the chlorophyll concentrations, fine leaf powder was extracted in 5 mL of 80% (v/v) acetone in the dark for 24 h. Absorbances of the extract were measured by spectrophotometry (UV-3802, Unico Instruments Co. Ltd, Shanghai, China) at 663, 646 and 470 nm, respectively (Wellburn 1994).

Histochemical Cd staining

Root and stem Cd localization was investigated by histochemical staining method as suggested by He et al. (2013). Intact tissues or hand sections of fresh fine root and stem samples were rinsed in deionized H_2O and exposed to a staining solution (30 mg diphenylthiocarbazone in 60 mL acetone, 20 mL H_2O, and 100 μL glacial acetic acid) for 1 h. The tissues were quickly rinsed in deionized H_2O. Samples containing abundant red-black Cd-dithizone precipitate were immediately observed and photographed under an Eclipse E200 light microscope (Nikon, Tokyo, Japan) fitted with a CCD camera (DS-Fi1; Nikon, Tokyo, Japan) and connected to a computer.

Determinations of Cd concentration, BCF, and T_f

Fine root, stem, and leaf powders (~100 mg each) were digested in a mixture of 7 mL concentrated HNO_3 plus 1 mL concentrated $HClO_4$ at 170 °C as previously described (Zhou et al. 2016). Cd concentrations were determined by flame atomic absorbance spectrometry (Hitachi 180-80; Hitachi Ltd., Tokyo, Japan).

The bioconcentration factor (BCF) is the ratio of the root and shoot Cd content to the Cd concentration of the solution (He et al. 2015). The translocation factor (T_f) is the ratio of the shoot to root Cd concentrations multiplied by 100% (He et al. 2015).

Melatonin quantitation

Melatonin concentrations in the roots, stems and leaves were measured by enzyme-linked immunosorbent assay (ELISA) according to Pape and Lüning (2006) with some modifications. Frozen tissue samples were extracted with 80% (v/v) methanol for 12 h at 4 °C in the dark. After centrifugation at 10,000 × *g* and 4 °C for 15 min, the supernatant was purified on a C18 solid phase extraction column (Waters, Milford, MA, USA). After drying, the eluent was dissolved in phosphate-buffered saline (PBS; pH 7.5). The melatonin standard and the extracted samples were subjected to plant melatonin ELISA kit (MM-0848O1; Jiangsu Meimian industrial Co. Ltd, Jiangsu, China) according to the manufacturer's instructions.

Determination of $O_2^{\bullet-}$, H_2O_2, and MDA

The concentrations of $O_2^{\bullet-}$ in the roots, stems, and leaves were measured by monitoring the nitrite formation from hydroxylamine spectrophotometrically at 530 nm as previously described (Elstner and Heupel 1976) with some modifications (Verma and Mishra 2005). The H_2O_2 concentrations were measured in a spectrophotometer according to the method of Patterson et al. (1984). The malondialdehyde (MDA) concentrations were measured spectrophotometrically at 450, 532 and 600 nm as suggested by Hodges et al. (1999).

Antioxidant levels and antioxidant enzyme activity

The concentrations of free proline were determined according to the method of Bates et al. (1973), whereas soluble phenolics were measured as described previously by Swain and Goldstein (1964), total thiols (T-SH) according to Sedlak and Lindsay (1968), ascorbate (ASC) according to Kampfenkel et al. (1995), and reduced glutathione (GSH) according to Griffith (1980) with minor modifications.

Soluble proteins were extracted from the roots, stems, and leaves and quantified according to a previously described method (Bradford 1976). The activity levels of catalase (CAT) and peroxidase (POD) were analyzed according to Chance and Maehly (1955). The ascorbate peroxidase (APX) activity was measured according to the method of Nakano and Asada (1981). The activity of glutathione reductase (GR) was measured according to the method of Connell and Mullet (1986).

Gene transcript measurement

The gene transcript levels were measured according to a previously described method (Zhou et al. 2016). Total root RNA was extracted and purified with a plant RNA extraction kit (R6827; Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's instructions. RNA concentration and quality in each sample were determined by spectrophotometry (NanoDrop 2000; Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis, respectively. One-microgram aliquots of total RNA were used to generate first-strand cDNA with a PrimeScript RT Reagent Kit with gDNA Eraser (DRR037A; Takara, Dalian, China) in a total volume of 20 μL. Quantitative reverse-transcription polymerase chain reaction was run with 10 μL of 2×SYBR Green Premix Ex Taq II (DRR820A; Takara, Dalian, China), 0.5 μL cDNA, and 0.2 μM primer per gene (Table S1) in a CFX96 Real Time System (CFX96; Bio-Rad Laboratories, Hercules, CA, USA). *β-Actin* was the reference gene (Table S1). A melting curve program was used to confirm PCR product homogeneity.

Relative mRNA expression was calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The expression level was set to 1 for each gene in the roots of *M. baccata* (Mb) treated with 0 μM Cd and 0 μM melatonin (-Cd-MEL). The corresponding fold changes relative to this control were calculated for the other treatments. A gene expression heatmap was plotted according to the average log base 2 expression fold changes in heatmap.2 of the "gplots" package in R (http://www.r-project.org/) as previously described (Luo et al. 2015).

Statistical analysis

Data were processed in Statgraphics (Statgraphics Technologies, Inc., The Plains, VA, USA) after checking for normality. To test for significant changes in root average net Cd^{2+} fluxes, the main effects of species (S) and melatonin (M) were analyzed by two-way ANOVA. For the other data, three-way ANOVA was used with Cd treatment (T), species (S), and melatonin (M) as factors. All *P* values obtained from the multiple comparisons were corrected by the Tukey-HSD method to reduce the chance of type I errors. Differences between means were considered significant at $P < 0.05$. For the principal component analysis (PCA), the data were standardized and computed with the prcomp package in R (http://www.r-project.org/) as previously recommended (He et al. 2015).

Results

Growth characteristics

Cd exposure severely reduced foliar carbon assimilation rate (*A*), stomatal conductance (g_s), and transpiration rate (*E*) in *M. baccata* and *M. micromalus* 'qingzhoulinqin' relative to their controls (Table S2). However, under Cd exposure conditions, *A* and *E* were 1.3- and 1.6-fold higher, respectively, in *M. micromalus* 'qingzhoulinqin' treated with melatonin than those without exogenous melatonin treatment (Table S2). The Cd treatment also lowered foliar photosynthetic pigment concentrations in both apple rootstocks exposed to Cd, but to a

relatively greater extent in the Cd-susceptible *M. micromalus* 'qingzhoulinqin'. Exogenous melatonin alleviated Cd-induced adverse effects. These were more pronounced in *M. micromalus* 'qingzhoulinqin' (Cd-susceptible) than in *M. baccata* (Cd-tolerant) (Table S2).

Consistent with Cd-induced photosynthetic inhibition, the root, stem and leaf dry weights in both species were substantially reduced by Cd exposure (Table 1). However, the melatonin treatment markedly alleviated Cd-induced decreases in stem and leaf dry biomass for *M. micromalus* 'qingzhoulinqin' but not *M. baccata* (Table 1).

Root Net Cd^{2+} fluxes

For all Cd exposures and melatonin treatments, the maximum net Cd^{2+} influxes in *M. baccata* and *M. micromalus* 'qingzhoulinqin' occurred 300–600 μm from the root tips (Figure 1a). In both rootstocks, the net Cd^{2+} fluxes dramatically decreased with increasing distance from the root tips. In response to Cd exposure without melatonin, *M. baccata* even presented with a net Cd^{2+} efflux of 3,000 μm from the root tips. For all Cd exposures and melatonin treatments, the net Cd^{2+} fluxes were always greater in the roots of *M. micromalus* 'qingzhoulinqin' than in those of *M. baccata* (Figure 1a). Exogenous melatonin reduced the average net Cd^{2+} fluxes by 65.4 % and 10.4% at 300 μm and 600 μm from the root tips of Cd-exposed *M. baccata* and *M. micromalus* 'qingzhoulinqin', respectively (Figure 1b).

Cd localization and accumulation

Dithizone histochemical staining revealed that Cd was deposited in the roots and stems of Cd-exposed *M. baccata* and *M. micromalus* 'qingzhoulinqin' (Figure 2). The Cd localization had a similar pattern in both species (Figure 2). In the roots, the Cd was localized mainly to the cell walls and intercellular spaces. In the stems, dark brown deposits were detected in phloem parenchyma cells. There was more Cd-dithizone staining in the roots and stems of *M. micromalus* 'qingzhoulinqin' than in those of *M. baccata* (Figure 2). In both species,

exogenous melatonin application had no obvious effect on root Cd deposition but significantly decreased Cd accumulation in the stems relative to the Cd treatment alone (Figure 2).

Cd accumulated in the root, stem, and leaf tissues of both *Malus* species (Figure 3). However, the Cd concentrations were lower in all tissues of *M. baccata* than in those of *M. micromalus* 'qingzhoulinqin'. Melatonin application did not alter root Cd accumulation but significantly reduced foliar Cd accumulation in both species relative to the Cd treatment alone (Figures 3a and 3c). Nevertheless, the melatonin treatment decreased the relative foliar Cd accumulation in *M. micromalus* 'qingzhoulinqin' by 51.4% whereas for *M. baccata* it lowered it by only 38.2% (Figure 3c). BCF and T_f were calculated for both species to compare their Cd accumulation and translocation capacities (Figure 4). Root and aerial organs BCF were consistently higher for *M. micromalus* 'qingzhoulinqin' than for *M. baccata* (Figure 4a). Exogenous melatonin had no influence on root or aerial organs BCF in Cd-exposed *M. baccata*. Relative to *M. micromalus* 'qingzhoulinqin' exposed to Cd alone, however, the melatonin treatment significantly decreased root and aerial organs BCF by 10.9% and 14.4%, respectively (Figure 4a). Melatonin application significantly reduced T_f by ~5.8% and ~6.1% in Cd-exposed *M. baccata* and *M. micromalus* 'qingzhoulinqin', respectively, relative to the Cd treatment alone (Figure 4b).

Endogenous melatonin concentration

Endogenous melatonin concentrations were determined for the roots, stems, and leaves of both *Malus* species under study (Figure 5). In Cd-exposed plants, endogenous melatonin concentrations increased in the roots and leaves of *M. baccata* and *M. micromalus* 'qingzhoulinqin' relative to that in plants not exposed to Cd (Figures 5a and 5c). Cd exposure had no significant effects on endogenous melatonin concentration in the stems of either species (Figure 5b). Endogenous melatonin concentrations were always higher in the roots

and leaves of *M. baccata* than in those of *M. micromalus* 'qingzhoulinqin' without exogenous melatonin addition (Figures 5a and 5c). Irrespective of Cd exposure, increased endogenous melatonin concentrations were found in the roots, stems, and leaves of both apple rootstocks treated with exogenous melatonin, except for leaves of *M. baccata* treated with exogenous melatonin under no Cd exposure conditions (Figures 5a-c). Further, the increase in endogenous melatonin was more pronounced in *M. micromalus* 'qingzhoulinqin' than in *M. baccata* after the addition of exogenous melatonin, irrespective of Cd exposure (Figures 5a-c).

ROS and antioxidants

Compared with the untreated controls of both species, the plants exposed to Cd alone presented with significantly elevated root, stem, and leaf $O_2^{\bullet-}$ concentrations (Figure 6). Nevertheless, these increments were greater for *M. micromalus* 'qingzhoulinqin' than for *M. baccata* (Figure 6). Melatonin application significantly reduced $O_2^{\bullet-}$ accumulation by 19.4%, 24.7%, and 34.8%, respectively, in the roots, stems, and leaves of Cd-challenged *M. baccata* and by 29.4%, 29.1%, and 8.7%, respectively, in the same organs of Cd-stressed *M. micromalus* 'qingzhoulinqin' (Figure 6). Relative to the untreated controls, the Cd-treated *M. baccata* presented with H_2O_2 concentrations that were 30.3%, 30.5%, and 59.0% higher in the roots, stems, and leaves, respectively. For Cd-treated *M. micromalus* 'qingzhoulinqin', the root, stem, and leaf H_2O_2 concentrations were 68.5%, 57.8%, and 60.4% higher, respectively, than those of the untreated controls (Figure 6). Exogenous melatonin alleviated Cd-induced H_2O_2 production in all tissues of both species (Figure 6). Cd exposure also promoted MDA accumulation in all tissues of *M. baccata* and *M. micromalus* 'qingzhoulinqin' (Figure 6). Melatonin treatment inhibited Cd-induced MDA accumulation in all tissues of both apple rootstocks except for *M. micromalus* 'qingzhoulinqin' roots (Figure 6).

The Cd-induced burst in ROS production stimulated antioxidant responses in all tissues

of both tree species (Supplementary Figures S1 and S2). The concentrations of non-enzymatic antioxidants such as free proline, soluble phenolics, T-SH, and ASC were significantly higher in all tissues of Cd-treated plants than in those of untreated ones (Supplementary Figure S1). Melatonin application sometimes enhanced the Cd-induced increases in the levels of the aforementioned antioxidants (Supplementary Figure S1). The activities of the antioxidant enzymes such as CAT (roots and leaves), POD (stems and leaves), APX (roots), and GR (roots and leaves) were consistently higher in *M. baccata* than in *M. micromalus* 'qingzhoulinqin' for all Cd and melatonin treatments (Supplementary Figure S2). Exogenous melatonin enhanced the Cd-induced antioxidant enzymatic activity in all tissues except for GR in *M. micromalus* 'qingzhoulinqin' stems (Supplementary Figure S2).

PCA was conducted on non-enzymatic and enzymatic antioxidants to evaluate Cd detoxification patterns in the apple rootstock tissues and to establish whether exogenous melatonin mediated these trends (Figure 7, Supplementary Tables S3-S4). There were clear and distinct response patterns to Cd stress and exogenous melatonin between two apple rootstocks (Figure 7). For *M. baccata*, PC1 separated the effects of Cd treatment and accounted for 66% of the observed variance (Figure 7a). The concentrations of proline in the roots, stems and leaves, the T-SH and ASC concentrations in the leaves, and the POD activity in the roots , stems and leaves, the GR activity in the stems and leaves were the main positive contributors to PC1 (Supplementary Table S3). PC2 reflected the effects of melatonin and covered 21% of the total variance (Figure 7a). The concentrations of soluble phenolics in the roots, the GSH concentrations in the roots and leaves, and the CAT and GR activities in the roots, the APX activity in the roots and leaves were the major positive contributors to PC2 (Supplementary Table S3). For *M. micromalus* 'qingzhoulinqin', however, the opposite results were returned. PC1 separated the effects of melatonin treatment while PC2 disclosed variations in the effects of Cd treatment (Figure 7b). PC1 and PC2 accounted for 58% and 24%

of the observed variation, respectively (Figure 7b). The key positive contributors to PC1 were the concentrations of free proline in the roots, stems and leaves, the T-SH concentrations in the stems and leaves, the ASC concentrations in the leaves, the POD and GR activities in the roots, stems, and leaves (Supplementary Table S4). In contrast, the concentrations of GSH, ASC, and the soluble phenolics in the roots, the APX activity in the roots were the important positive factors for PC2 (Supplementary Table S4).

Notably, treatments of +Cd-MEL and +Cd+MEL located at different quadrants of PCA in both apple rootstocks (Figures 7a and 7b), indicating the significant effects of melatonin on the detoxification system of both apple rootstock to Cd stress. In *M. baccata*, the concentrations of ASC in the stems, the soluble phenolics concentrations in the leaves, and the activities of CAT, GR and APX in the roots positively contributed to the effects of melatonin (Figure 7a). In *M. micromalus* 'qingzhoulinqin', however, the concentrations of proline in the roots, the T-SH concentrations in the stems, the GSH concentrations in the leaves, and the POD activity in the stems and leaves, the CAT activity in the roots and stems, the GR activity in the roots and leaves, the APX activity in the roots were the dominant positive contributors to the effects of melatonin (Figure 7b). Melatonin treatment more strongly induced the antioxidant system of *M. micromalus* 'qingzhoulinqin' than that of *M. baccata* (Figures 7a and 7b).

Regulation of the transcription of the genes involved in Cd uptake, translocation, and detoxification

The transcript levels of the genes involved in Cd uptake, transport, and detoxification were assessed for the roots of *M. baccata* and *M. micromalus* 'qingzhoulinqin' (Figure 8). *HA7* and *NRAMP1* probably participate in root Cd uptake and transport. Relative to the control, the abundances of *HA7* in the roots of *M. baccata* and *NRAMP1* in the roots of both species markedly decreased after Cd treatment, irrespective of melatonin application (Figure 8).

Under Cd exposure, the mRNA levels of *HA7* and *NRAMP1* decreased by 2.3- and 49.1-fold, respectively, in the roots of *M. baccata* due to the application of exogenous melatonin. Whereas exogenous melatonin raised *HA7* transcript level in the Cd-treated roots of *M. micromalus* 'qingzhoulinqin' (Figure 8). The transcript levels of *HA7* were 8.4-fold higher in the roots of *M. micromalus* 'qingzhoulinqin' than in those of *M. baccata* supplied with exogenous melatonin under Cd exposure (Figure 8). Regardless of melatonin treatment, reduced transcript levels of *NRAMP1* were found in the roots of Cd-challenged *M. baccata* compared with Cd-exposed *M. micromalus* 'qingzhoulinqin (Figure 8).

Vacuolar Cd cations may be exported to the cytoplasm by tonoplast-localized transporters such as NRAMP3 which also initiates Cd translocation to the shoot (Thomine et al. 2000). In the absence of exogenous melatonin, the *NRAMP3* transcript levels were higher in the roots of both tree species exposed to Cd than in those of the untreated controls (Figure 8). However, the *NRAMP3* transcript levels decreased in the roots of Cd-stressed *M. baccata* and *M. micromalus* 'qingzhoulinqin' when exogenous melatonin was applied (Figure 8). In root cell cytosols, HMs such as Cd are transported to the xylem vessels and thence to the shoots by the PM-bound transporters HMA4 and PCR2 (Song et al. 2010, Siemianowski et al. 2011). Relative to the untreated, Cd exposure decreased the *HMA4* and *PCR2* transcript levels in the roots of both tree species irrespective of exogenous melatonin (Figure 8). Under Cd exposure conditions, *HMA4* and *PCR2* were downregulated in the roots of Cd-challenged *M. baccata* and *M. micromalus* 'qingzhoulinqin' after the addition of exogenous melatonin. This effect was especially pronounced for *PCR2* in the roots of *M. micromalus* 'qingzhoulinqin' (Figure 8). Under Cd treatment, the *HMA4* and *PCR2* transcript levels were always higher in the roots of *M. micromalus* 'qingzhoulinqin' than in those of *M. baccata* regardless of exogenous melatonin (Figure 8).

NAS1 and MT2 may detoxify Cd in plants (Luo et al. 2016). Transcriptional *NAS1*

upregulation occurred in the roots of both apple rootstocks subjected to Cd and melatonin together but not in those exposed to melatonin alone (Figure 8). Under Cd treatment, the root *NAS1* transcript levels in *M. baccata* were 1.6- and 2.6-fold higher than those of *M. micromalus* 'qingzhoulinqin' without and with melatonin treatment, respectively (Figure 8). In contrast, Cd treatment significantly downregulated *MT2* in the roots of both *Malus* species relative to the control rootstocks (Figure 8). However, melatonin application upregulated root *MT2* mRNA levels in the roots of both Cd-treated apple rootstocks (Figure 8).

Cd and Cd-containing complexes in the cytosol may be transported to the vacuoles by MHX and ABCC1, respectively, in the tonoplast (Berezin et al. 2008, Park et al. 2012). In the absence of melatonin, Cd exposure significantly downregulated *MHX* in the roots of both species (Figure 8). However, exogenous melatonin attenuated Cd-induced root *MHX* downregulation in both species (Figure 8). Cd stress upregulated root *ABCC1* in *M. baccata*, whereas 100 μM exogenous melatonin further increased Cd-stressed root *ABCC1* transcript levels by ~1.3-fold in this species (Figure 8). In contrast, melatonin application upregulated root *ABCC1* in *M. micromalus* 'qingzhoulinqin' regardless of Cd exposure (Figure 8). The *MHX* and *ABCC1* mRNA levels were higher in the roots of Cd-exposed *M. baccata* than in those of Cd-treated *M. micromalus* 'qingzhoulinqin' in the absence of melatonin (Figure 8).

Discussion

Exogenous melatonin reduced root Cd uptake and translocation to apple rootstock aerial organs

Cd is not essential element for plant growth. However, it is readily absorbed by the roots and is partially transported to the shoots (Salt et al. 1995). Plants tolerate Cd by excluding and detoxifying it and by mobilizing antioxidant defense mechanisms (Baker 1987). Exclusion comprises the inhibition of root Cd uptake and the restriction of Cd translocation to the

shoots (Hasan et al. 2009). Previous studies reported that exogenous melatonin influences metal exclusion in herbaceous plants. Gu et al. (2017) found that melatonin pretreatment significantly inhibited Cd accumulation in alfalfa seedling roots but not in their shoots. In contrast, exogenous melatonin had no effect on tomato root Cd accumulation but substantially lowered tomato leaf Cd content (Hasan et al. 2015). These discrepancies suggest that exogenous melatonin may have different effects on various plant species in terms of Cd uptake and accumulation. Only limited information is available regarding the mechanisms by which melatonin mediates Cd uptake and translocation from the roots to the aerial organs in *Malus* species.

To clarify the influences of exogenous melatonin on Cd uptake, net Cd^{2+} fluxes along the root tips of two apple rootstocks differing in Cd accumulation were monitored by NMT fitted with a Cd-selective microelectrode (Figure 1). The relatively low Cd uptake rates in the roots of *M. baccata* and *M. micromalus* 'qingzhoulinqin' after exogenous melatonin application showed that this treatment inhibited root Cd uptake. A previous study showed that melatonin and its synthetic precursors combine with HMs such as Cd^{2+}, thereby reducing their absorption by plants (Limson et al. 1998). Earlier studies showed that reducing Cd translocation from the roots limits Cd accumulation in the shoots (Akhter et al. 2012, 2014). Here, decreasing net Cd^{2+} influx did not lower root Cd accumulation. Nevertheless, it significantly reduced Cd accumulation and T_fs in the aerial organs of both species treated with melatonin (Figures 3 and 4). Thus, melatonin application altered Cd allocation to various apple rootstock organs. Melatonin treatment may promote the formation of a barrier against Cd translocation to the shoots and protect the photosynthetic apparatus from Cd-induced damage (Jakovljevic et al. 2014). There was far less Cd accumulation in the aerial organs of Cd-stressed *M. micromalus* 'qingzhoulinqin' than in those of Cd-treated *M. baccata* due to the addition of exogenous melatonin (Figure 3). Therefore, melatonin

application had a comparatively stronger impact on Cd-susceptible apple species. This conclusion aligns with previous findings reported for *Malus* species subjected to drought stress (Li et al. 2015).

Melatonin-suppressed root Cd uptake and translocation to the aerial organs in both apple rootstocks is probably associated with melatonin-modulated transcription of genes regulating Cd uptake and accumulation. PM-bound H^+-ATPases, NRAMPs, HMA4, and PCR2 regulate divalent cation uptake and transport in plants (Luo et al. 2016, Zhou et al. 2016, Shi et al. 2019). *HA7* encodes PM-bound H^+-ATPases. Its downregulation lowered net Cd^{2+} influxes into the roots of *M. baccata* more than those of other apple rootstocks (Zhou et al. 2016). In the present study, exogenous melatonin decreased *HA7* and *NRAMP1* mRNA levels (Figure 8). This finding was consistent with the observed decreases in root Cd influxes (Figure 1). The localized PM transporters *HMA4* and *PCR2* exclude cytosolic Cd^{2+} to the apoplast. This process is crucial for loading HMs into the root stele xylem (Song et al. 2010, Nouet et al. 2015). Melatonin-regulated genes participating in Cd^{2+} transport have been reported for herbaceous plants. Melatonin application to alfalfa seedling roots increased their *PCR2* mRNA levels and decreased their Cd content (Gu et al. 2017). Here, however, we found that melatonin downregulated root *HMA4* and *PCR2* in both *Malus* species subjected to Cd (Figure 8). These inconsistencies may be explained by the relative differences between herbaceous and woody plants in terms of Cd uptake and accumulation. In general, fruit trees have strong abilities to accumulate Cd in their roots and block its translocation to their aerial organs (Nada et al. 2007, Zhou et al. 2017). In the present study, melatonin-promoted *HMA4* and *PCR2* downregulation further restricted Cd translocation from the roots to the aerial organs of the apple trees. These observations were confirmed by the measured decreases in stem and leaf Cd content and T_f (Figures 3 and 4). Regardless of exogenous melatonin, the roots of Cd-exposed *M. micromalus* 'qingzhoulinqin' had relatively higher *HA7*, *NRAMP1*,

HMA4, and *PCR2* mRNA levels than those of Cd-stressed *M. baccata* (Figure 8). These results were consistent with the comparatively higher root Cd influxes and aerial organ Cd translocation and accumulation rates (Figures 1, 3 and 4).

Exogenous melatonin alleviates Cd toxicity in apple rootstocks

Cd has no known biological function and is toxic to most organisms (Luo et al. 2016). Here, the observed reductions in photosynthetic rate, chlorophyll, content, and overall biomass and increases in ROS and MDA in the apple rootstocks subjected to Cd suggest that they were reacting to Cd exposure and toxicity. A previous study reported that melatonin might regulate plant growth and development (Tan et al. 2012). Our results indicated that exogenous melatonin enhanced the photosynthetic rates, increased the chlorophyll levels and biomass, and reduced the ROS and MDA levels in various tissues of both apple rootstocks subjected to Cd stress. However, these beneficial effects were particularly strong in *M. micromalus* 'qingzhoulinqin'. Thus, exogenous melatonin alleviated the toxic effects of Cd in these plants, which aligns with previous reports (Li et al. 2016, Ni et al. 2018). Under Cd exposure conditions, more endogenous melatonin was found in the roots and leaves of *M. baccata* than in those of *M. micromalus* 'qingzhoulinqin' (Figure 5). Therefore, the former produced sufficient melatonin to contend with Cd-induced oxidative stress. This response is consistent with its relatively high Cd tolerance and comparatively low loss of photosynthetic pigments and biomass in response to Cd stress. However, exogenous melatonin enhanced endogenous melatonin concentrations and alleviated Cd-induced toxicity in all tissues of both apple rootstocks but especially in *M. micromalus* 'qingzhoulinqin'. Thus, exogenous melatonin may have antioxidant efficacy in addition to its other regulatory functions in several Cd tolerance pathways.

Plants subjected to Cd stress present with impaired redox homeostasis, accumulate ROS, and undergo PM lipid peroxidation (Podazza et al. 2012). Plants have several non-enzymatic

and enzymatic antioxidant mechanisms to counteract the destructive effects of Cd-induced ROS (Guo et al. 2019). Non-enzymatic antioxidants such as free proline, soluble phenolics, T-SH, GSH, and ASC, while enzymatic antioxidants including CAT, POD, APX, and GR, can scavenge Cd-induced ROS (He et al. 2013, Shi et al. 2019). Melatonin induces the antioxidant system to scavenge stress-induced ROS, prevent lipid peroxidation, and protect cells from oxidative damage (Romero et al. 2014, Zhang et al. 2015). In wheat, melatonin application mitigated Cd-induced ROS by upregulating SOD, POD, and APX (Ni et al. 2018). Here, exogenous melatonin increased the free proline, soluble phenolic, T-SH, GSH, and ASC concentrations (Supplementary Figure S1) and activated CAT, POD, APX, and GR (Supplementary Figure S2). All of these might have contributed to Cd-induced ROS scavenging. Overall, melatonin may modulate Cd chelator levels, initiate antioxidant defense systems, thereby attenuate Cd-induced oxidative stress in apple rootstocks.

Cd-stressed plants modulate the transcription of genes participating in Cd detoxification (Ding et al. 2017). Our previous study showed that the mRNA levels of the *AtNAS1* ortholog in the roots of *M. baccata* were higher than those of three other apple rootstocks (Zhou et al. 2016). *Arabidopsis thaliana* overexpressing the *Brassica juncea* gene encoding MT2 shows elevated Cd tolerance relative to the WT seedlings (An et al. 2006). Previous studies have shown that *MHX* and *ABCC1* transport Cd ions or PC–Cd into the vacuoles, thereby detoxifying Cd (Gaash et al. 2013, Song et al. 2014). There is little empirical evidence that melatonin mediates transcriptional regulation of the genes involved in Cd detoxification. Here, elevated endogenous melatonin was detected in the roots of apple rootstocks treated with exogenous melatonin. This response might have upregulated Cd-detoxifying genes such as *NAS1*, *MT2*, *MHX*, and *ABCC1* (Figure 8). However, further study is required to elucidate the mechanisms underlying the melatonin-mediated transcription of genes involved in Cd detoxification.

As summarized in Figure 9, Cd was absorbed by the roots and partially translocated to the aerial organs of apple rootstocks. In response, photosynthesis, pigment content, and biomass accumulation were all reduced. Cd stress alone more strongly decreased photosynthesis and biomass and perturbed redox homeostasis and enzyme activity in *M. micromalus* 'qingzhoulinqin' than in *M. baccata*. A plausible explanation is that the former had lower endogenous melatonin levels than the latter. Cd treatment increased root endogenous melatonin levels in both species and while exogenous melatonin further stimulated endogenous melatonin production. External melatonin was more efficacious at mitigating Cd toxicity in *M. micromalus* 'qingzhoulinqin' than in *M. baccata*. Exogenous melatonin reduced net root Cd^{2+} influxes and aerial organ Cd contents. It also promoted ROS scavenging by the antioxidant system. Furthermore, exogenous melatonin downregulated several genes involved in Cd uptake and translocation, including *HA7*, *NRAMP1*, *NRAMP3*, *HMA*4, and *PCR2*, but upregulated those participating in Cd detoxification, including *NAS1*, *MT2*, *ABCC1*, and *MHX*.

Supplementary Data

Fig. S1 Free proline, soluble phenolics, T-SH, GSH and ASC.

Fig. S2 Activities of CAT, POD, APX and GR.

Table S1 Primers used for qRT-PCR.

Table S2 Photosynthetic capacity and pigments.

Table S3 Principal component analysis (PCA) of *Malus baccata*.

Table S4 Principal component analysis (PCA) of *M. micromalus* 'qingzhoulinnqin'.

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Conflict of Interest

None declared

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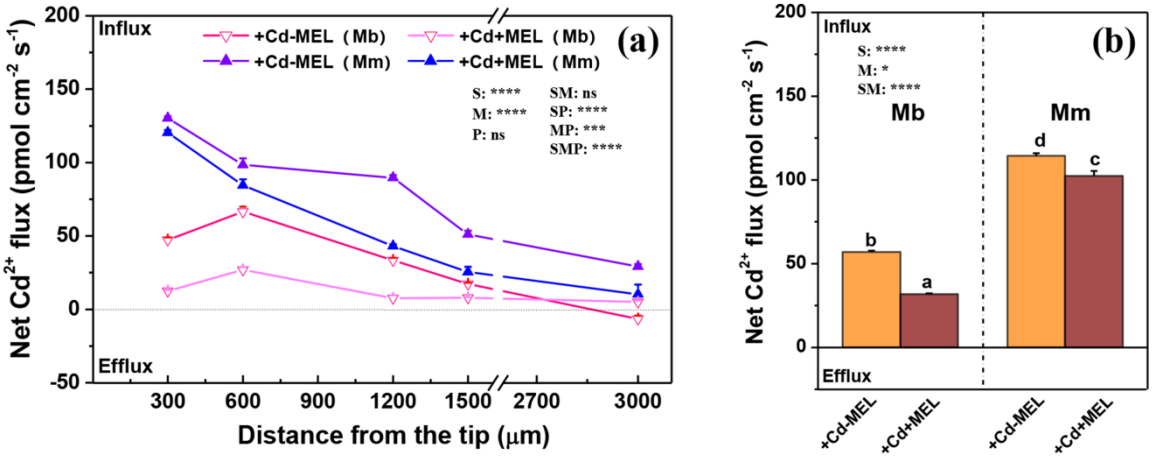

Figure 1 Net Cd^{2+} fluxes along root tips (a) and the average of net Cd^{2+} fluxes at 300 and 600 μm (b) from the root tips of *Malus baccata* (Mb) and *M. micromalus* 'qingzhoulinnqin' (Mm) exposed to 30 μM $CdCl_2$ (+Cd) combined with either 0 (-MEL) or 100 μM melatonin (+MEL) for 20 days. Data indicate means ± SE (n = 6). Different letters on the bars indicate significant difference between the treatments. *P*-values of the ANOVAs of species (S), melatonin (M), position (P) and their interaction (S × M: SM; S × P: SP; M × P: MP and S × M × P: SMP) for panel (a), and species (S), melatonin (M) and their interaction (S × M: SM) for panel (b) are indicated. *: $P \leq 0.05$; ***: $P \leq 0.001$; ****: $P \leq 0.0001$; ns: not significant.

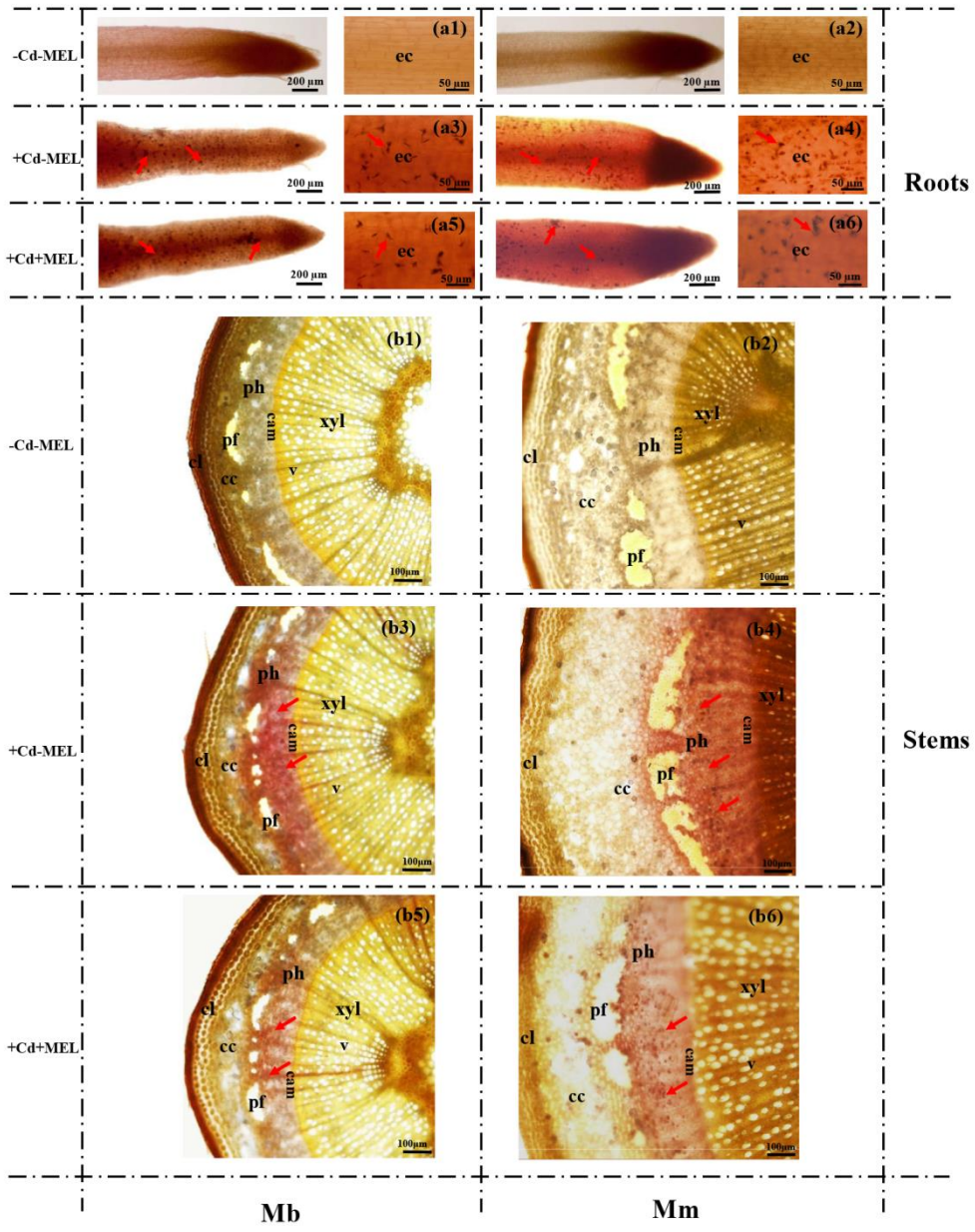

Figure 2 Cadmium (Cd) localization in the roots (a1-a6) and stems (b1-b6) of *Malus baccata* (Mb) and *M. micromalus* 'qingzhoulinnqin' (Mm) exposed to 0 (-Cd) or 30 μM $CdCl_2$ (+Cd) combined with either 0 (-MEL) or 100 μM melatonin (+MEL) for 20 days. Arrows point to precipitates of Cd-dithizone. The histochemical detection of Cd in the roots and stems of both species treated with 0 μM Cd and 100 μM melatonin (-Cd+MEL) was similar to that in the roots treated with 0 μM Cd and 0 μM melatonin (-Cd-MEL), and the data were not shown. ec:

epidermal cells; cl: collenchyma; cc: cortical cells; pf: phloem fiber; ph: phloem; cam: cambium; v: vessel; xyl: xylem.

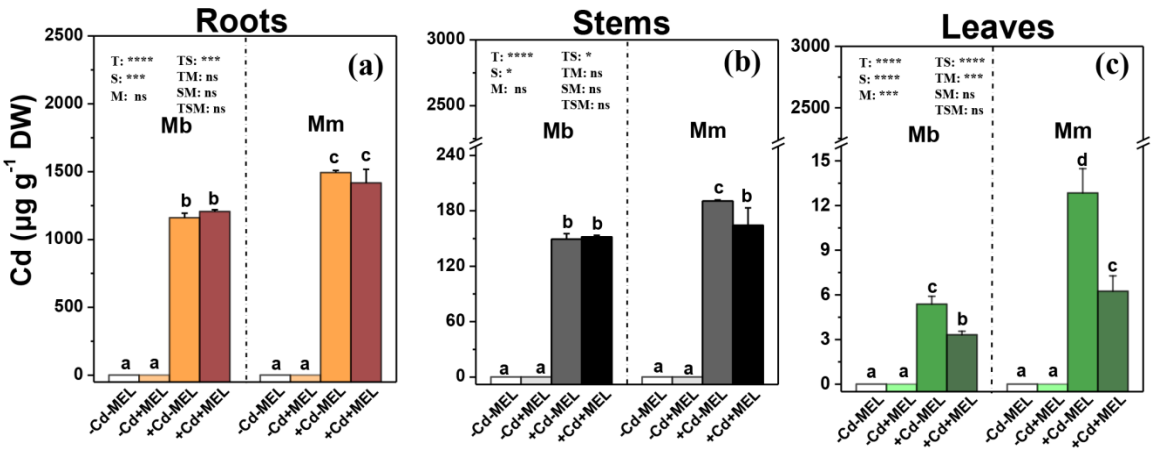

Figure 3 Cd concentration in the roots, stems and leaves of *Malus baccata* (Mb) and *M. micromalus* 'qingzhoulinnqin' (Mm) exposed to 0 (-Cd) or 30 μM $CdCl_2$ (+Cd) combined with either 0 (-MEL) or 100 μM melatonin (+MEL) for 20 days. Data indicate means ± SE (n = 6). Different letters on the bars indicate significant difference between the treatments. *P*-values of the ANOVAs of Cd treatment (T), species (S), melatonin (M) and their interaction (T × S: TS; T × M: TM; S × M: SM and T × S × M: TSM) are indicated. *: $P \leq 0.05$; ***: $P \leq 0.001$; ****: $P \leq 0.0001$; ns: not significant.

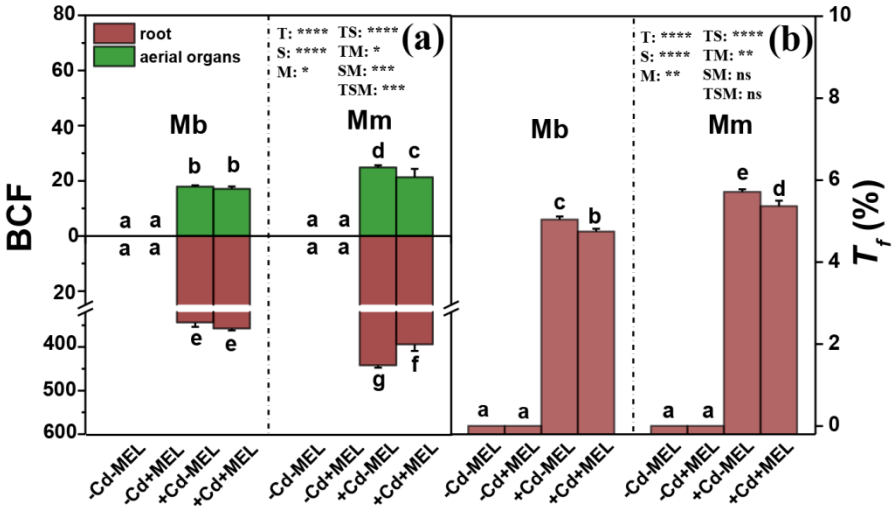

Figure 4 Bio-concentration factor (BCF, a) in the roots and aerial organs, and translocation factor (T_f, b) of *Malus baccata* (Mb) and *M. micromalus* 'qingzhoulinnqin' (Mm) exposed to 0 (-Cd) or 30 μM $CdCl_2$ (+Cd) combined with either 0 (-MEL) or 100 μM melatonin (+MEL) for 20 days. Data indicate means ± SE (n = 6). Different letters on the bars indicate significant difference between the treatments. *P*-values of the ANOVAs of Cd treatment (T), species (S), melatonin (M) and their interaction (T × S: TS; T × M: TM; S × M: SM and T × S × M: TSM) are indicated. *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$; ****: $P \leq 0.0001$; ns: not significant.

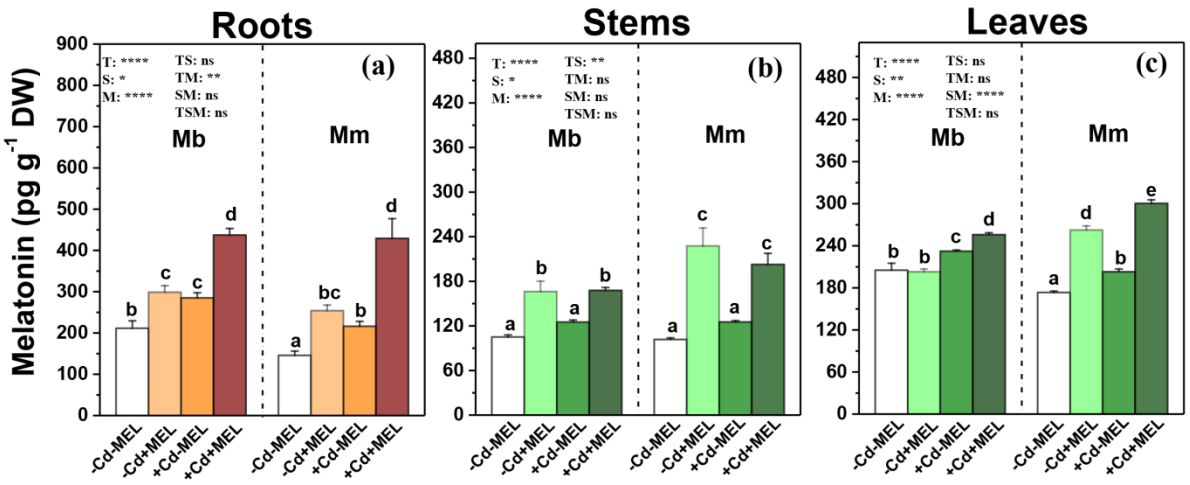

Figure 5 Melatonin concentration in the roots, stems and leaves of *Malus baccata* (Mb) and *M. micromalus* 'qingzhoulinnqin' (Mm) exposed to 0 (-Cd) or 30 μM $CdCl_2$ (+Cd) combined with either 0 (-MEL) or 100 μM melatonin (+MEL) for 20 days. Data indicate means ± SE (n = 6). Different letters on the bars indicate significant difference between the treatments. *P*-values of the ANOVAs of Cd treatment (T), species (S), melatonin (M) and their interaction (T × S: TS; T × M: TM; S × M: SM and T × S × M: TSM) are indicated. *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$; ****: $P \leq 0.0001$; ns: not significant.

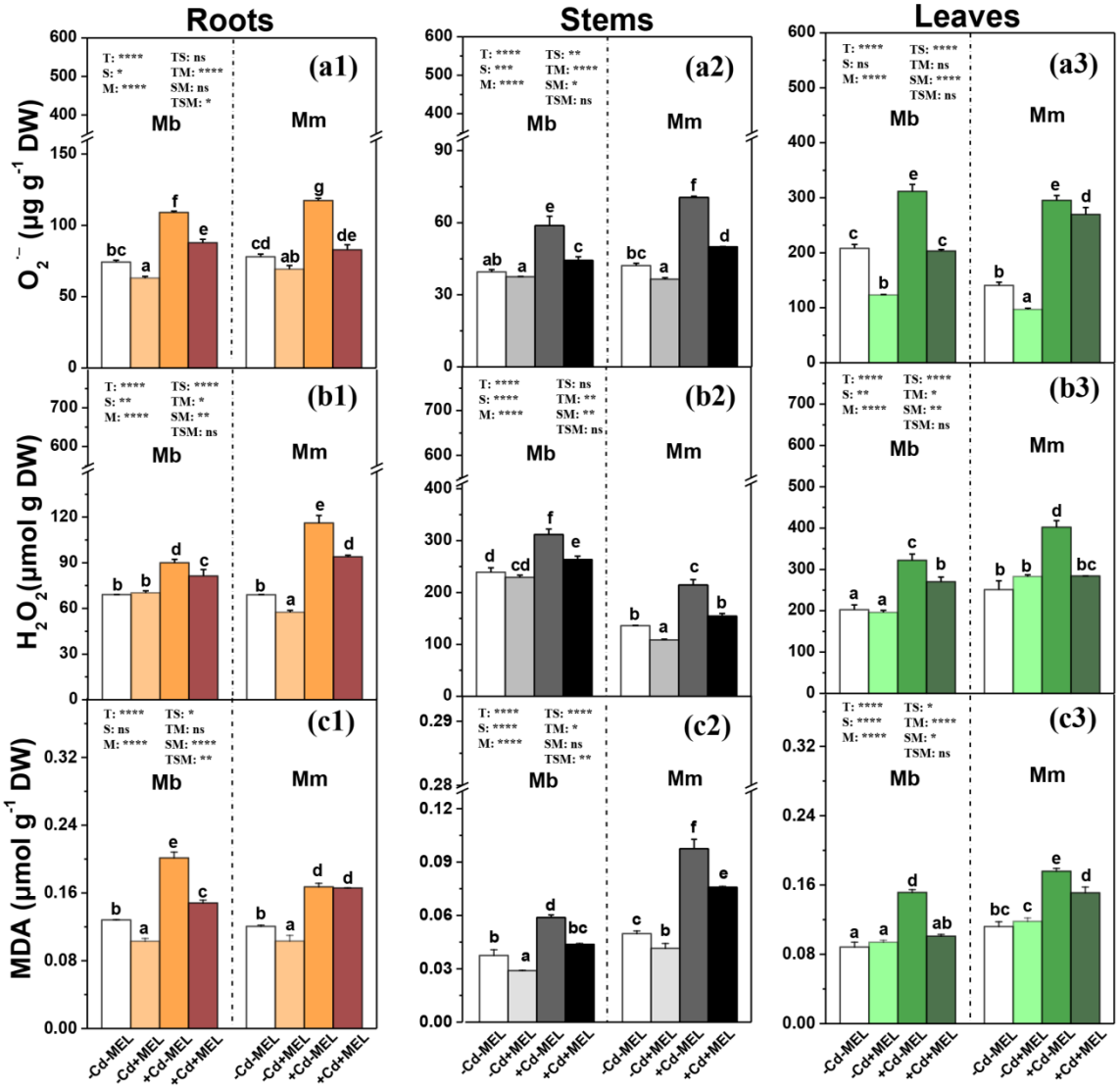

Figure 6 $O_2^{\bullet-}$, H_2O_2 and malonaldehyde (MDA) in the roots, stems and leaves of *Malus baccata* (Mb) and *M. micromalus* 'qingzhoulinnqin' (Mm) exposed to 0 (-Cd) or 30 μM $CdCl_2$ (+Cd) combined with either 0 (-MEL) or 100 μM melatonin (+MEL) for 20 days. Data indicate means ± SE (n = 6). Different letters on the bars indicate significant difference between the treatments. *P*-values of the ANOVAs of Cd treatment (T), species (S), melatonin (M) and their interaction (T × S: TS; T × M: TM; S × M: SM; and T × S × M: TSM) are indicated. *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$; ****: $P \leq 0.0001$; ns: not significant.

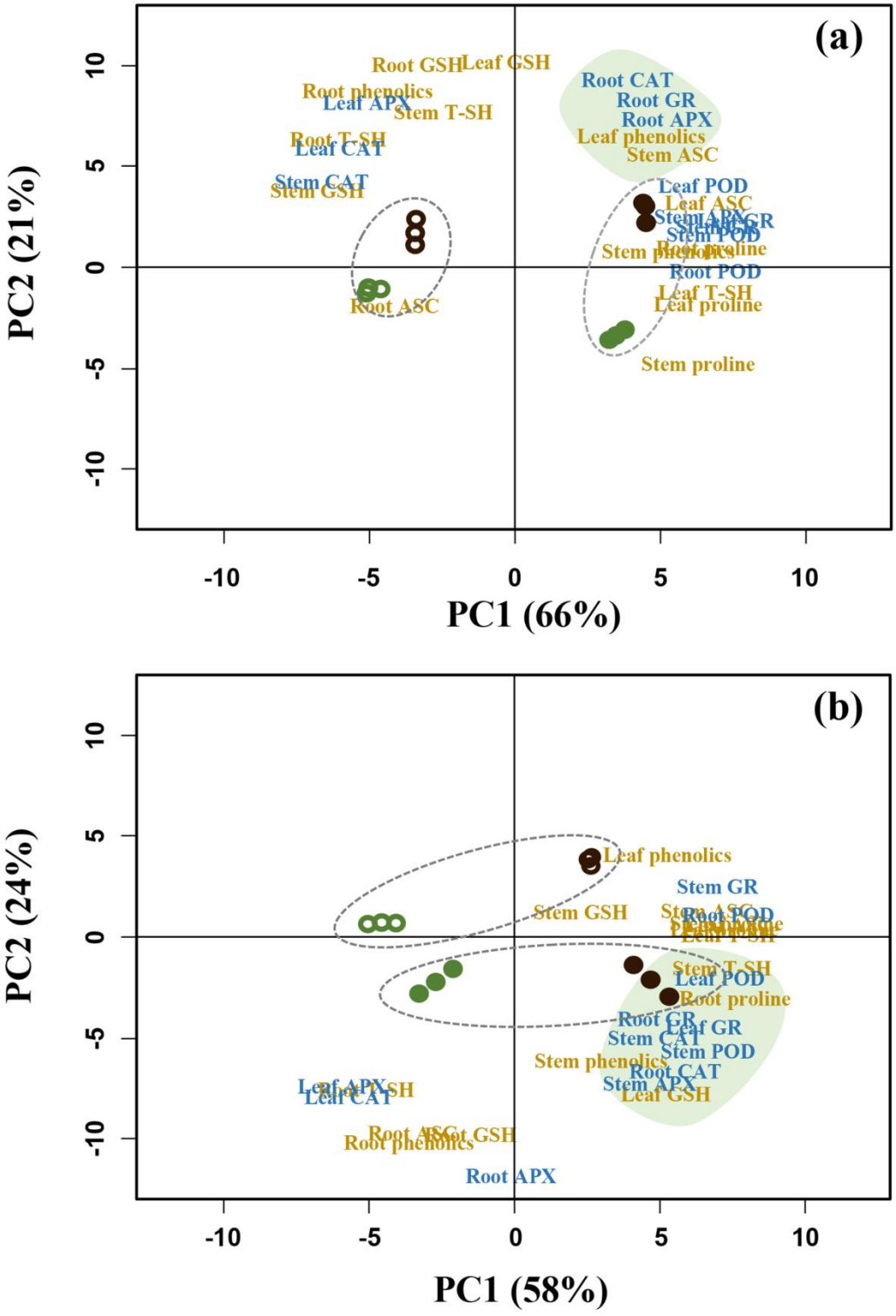

Figure 7 The plots of principal component analysis (PCA) of non-enzymatic and enzymatic antioxidants involved in Cd detoxification in the roots, stems and leaves of *Malus baccata* (a) and *M. micromalus* 'qingzhoulinnqin' (b) exposed to 0 (-Cd) or 30 μM $CdCl_2$ (+Cd)

combined with either 0 (-MEL) or 100 μM melatonin (+MEL) for 20 days. The hollow and solid symbols indicate 0 and 30 μM Cd, respectively. The orange and black colors indicate 0 and 100 μM melatonin, respectively. PCA loadings are presented in Tables S3–S4.

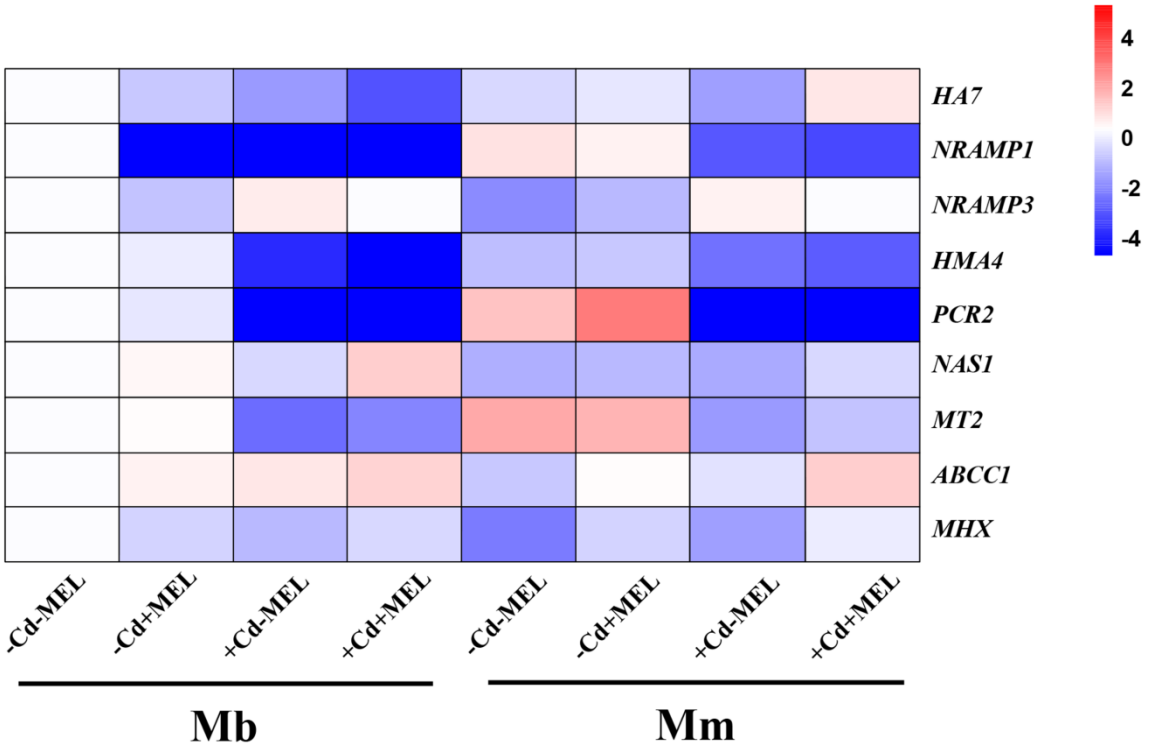

Figure 8 Heatmap of genes encoding proteins involved in Cd uptake, transport and detoxification in the roots of *Malus baccata* (Mb) and *M. micromalus* 'qingzhoulinnqin' (Mm) exposed to 0 (-Cd) or 30 μM $CdCl_2$ (+Cd) combined with either 0 (-MEL) or 100 μM melatonin (+MEL) for 20 days. For each gene, the expression level was set to 1 in the roots of *M. baccata* (Mb) treated with 0 μM Cd and 0 μM melatonin (-Cd-MEL), and the corresponding fold changes were calculated under other treatments. The gene expression heatmap was generated on the log base 2 average expression fold values.

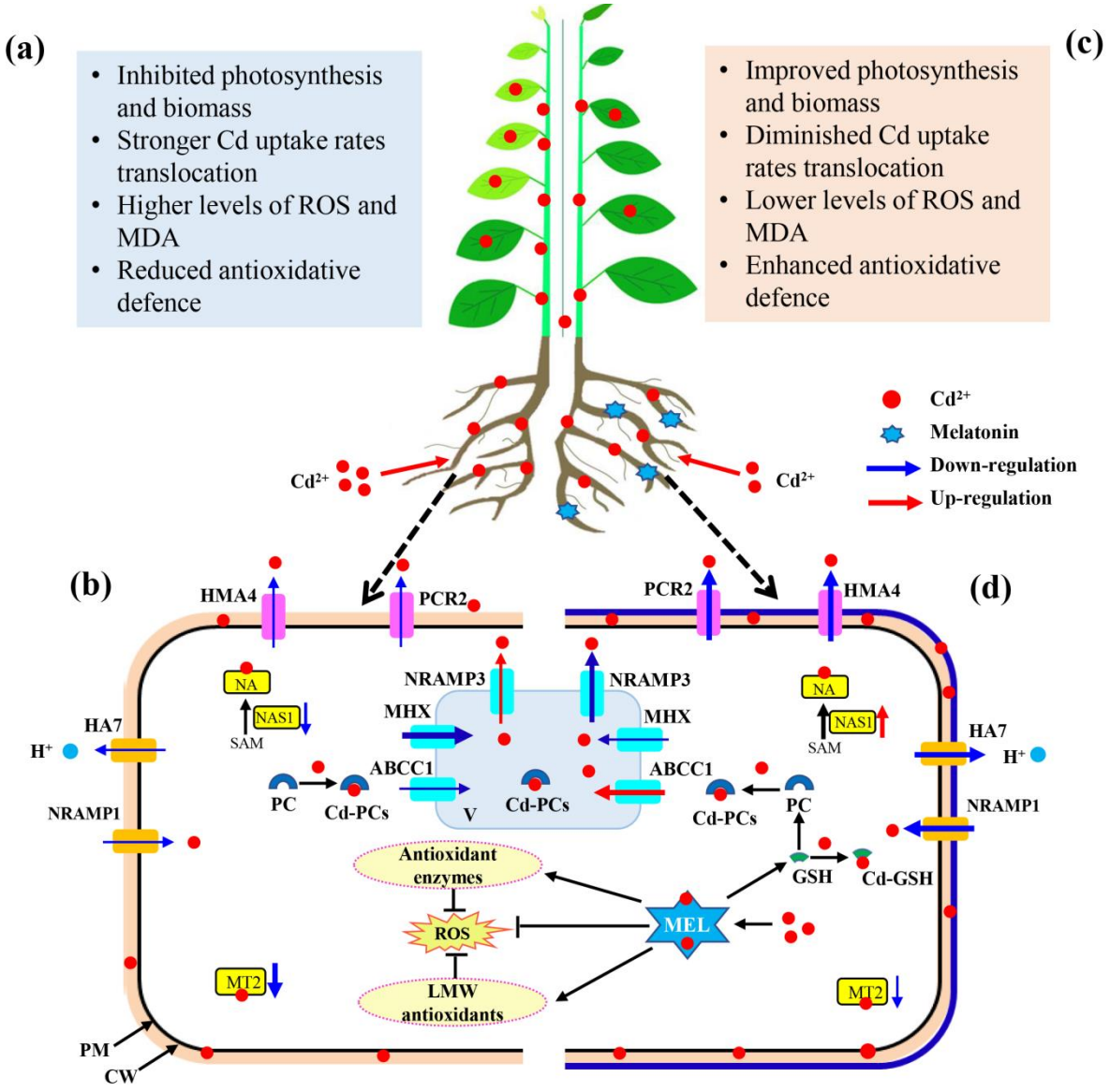

Figure 9 A schematic model illustrating the effects of melatonin on Cd uptake, translocation and toxicity in apple rootstocks exposed to Cd. The left chart represents apple rootstocks exposed to Cd alone (a–b), and the right chart depicts apple rootstocks treated with Cd and exogenous melatonin (c–d). Exogenous melatonin decreased Cd uptake rates in the roots and translocation to the aerial organs in the apple rootstocks. Moreover, exogenous melatonin relieved Cd-induced oxidative stress via improving antioxidant defense system. CW, cell wall; PM, plasma membrane; V, vacuole; MEL, metolanin. SAM, S-adenosyl-l-methionine; HA7, PM H^+-ATPases 7; NRAMP1 and NRAMP3, natural resistance associated macrophage

protein 1 and 3; HMA4, P-type heavy metal ATPase 4; PCR2, plant cadmium resistance protein 2; NAS1, nicotianamine synthase 1; MT2, metallothionein 2; ABCC1, ATP-binding cassette transporter C1; MHX, magnesium proton exchanger protein.

Table 1 The dry mass (g) of root, stem, leaf and whole plant of *Malus baccata* (Mb) and *M. micromalus* 'qingzhoulinnqin' (Mm) exposed to 0 or 30 μM $CdCl_2$ combined with either 0 or 100 μM melatonin for 20 days. Data indicate means ± SE (n = 6). Different letters behind the values in the same column indicate significant difference between the treatments. *P*-values of the ANOVAs of Cd treatment (T), species (S), melatonin (M) and their interaction (T × S: TS; T × M: TM; S × M: SM; and T × S × M: TSM) are indicated. *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$; ****: $P \leq 0.0001$; ns: not significant.

Rootstocks	Cd (μM)	Melatonin (μM)	Root	Stem	Leaf	Whole plant
Mb	0	0	2.30 ± 0.03 cd	1.64 ± 0.02 b	3.80 ± 0.01 c	8.08 ± 0.50 c
	0	100	2.91 ± 0.23 de	1.72 ± 0.00 b	3.79 ± 0.03 c	7.90 ± 0.32 c
	30	0	1.23 ± 0.04 a	1.27 ± 0.02 a	2.30 ± 0.05 a	4.93 ± 0.12 a
	30	100	1.37 ± 0.08 ab	1.36 ± 0.00 a	2.33 ± 0.03 a	4.89 ± 0.15 a
Mm	0	0	2.98 ± 0.06 ef	2.23 ± 0.04 c	8.69 ± 0.44 d	14.39 ± 0.32 d
	0	100	3.32 ± 0.02 f	2.65 ± 0.21 d	8.15 ± 0.03 d	13.77 ± 0.44 d
	30	0	1.78 ± 0.06 bc	1.63 ± 0.03 b	2.45 ± 0.08 a	6.20 ± 0.36 b
	30	100	2.06 ± 0.07 cd	2.10 ± 0.06 c	3.30 ± 0.21 b	7.56 ± 0.31 c
		T	****	****	****	****
		S	****	****	****	****
		M	*	***	ns	ns
P-values		TS	ns	ns	****	****
		TM	ns	ns	**	*
		SM	ns	**	ns	ns
		TSM	ns	ns	**	ns