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## Research paper

# Exogenous melatonin alleviates cadmium uptake and toxicity in apple rootstocks

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**To examine the potential roles of melatonin in cadmium (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  $\mu\text{M}$  Cd together with 0 or 100  $\mu\text{M}$  melatonin. Cadmium stress stimulated endogenous melatonin production to a greater extent in the Cd-tolerant *Malus baccata* Borkh. than in the Cd-susceptible *Malus micromalus* 'qingzhoulinqin'. Melatonin application attenuated Cd-induced reductions in growth, photosynthesis and enzyme activity, as well as reactive oxygen species (ROS) and malondialdehyde 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  $\text{Cd}^{2+}$  uptake, reduced leaf Cd accumulation, decreased Cd translocation factors 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.**

**Keywords:** heavy metals, *Malus* rootstock, net  $\text{Cd}^{2+}$  influxes, toxicity, transcriptional regulation.

## 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). Cadmium 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). Cadmium accumulation in edible plant parts poses a serious threat to human and animal health, as Cadmium 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<sup>2+</sup> 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 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.

Cadmium uptake, translocation and detoxification are mediated by several genes (Luo et al. 2016). Plasma membrane (PM) H<sup>+</sup>-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<sup>+</sup>-ATPase were higher in the Cd-susceptible *Malus micromalus* 'qingzhoulinqin' than in the Cd-tolerant *Malus 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). Cadmium 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 Cadmium 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 Cd 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 days. We measured their growth characteristics, net root Cd<sup>2+</sup> 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 days. After germination, the seedlings were cultivated for 45 days 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 × 20 × 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 days. 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<sub>2</sub>·2.5H<sub>2</sub>O or melatonin to the half-strength Hoagland nutrient solution. The Cd and melatonin treatments continued for up to 20 days before harvest. Six plants were randomly selected per experimental group to measure net Cd<sup>2+</sup> fluxes. The other 12 plants were harvested after measuring their gas exchange rates.

### Measurements of root net $\text{Cd}^{2+}$ fluxes

Root net  $\text{Cd}^{2+}$  fluxes in both apple rootstocks were measured as previously described (Zhou et al. 2016) by a noninvasive micro-test technique (non-invasive 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:  $\sim 2\text{--}4\text{ }\mu\text{m}$ ) 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 and 100  $\mu\text{M}$   $\text{Cd}^{2+}$ .

To determine the position of maximal  $\text{Cd}^{2+}$  fluxes along the root tip, six fine white roots (diameter  $\sim 1.5\text{ mm}$ ) were selected from each rootstock species treated with 30  $\mu\text{M}$   $\text{Cd}^{2+}$ . A preliminary experiment was conducted by taking measurements at 300, 600, 1200, 1500 and 3000  $\mu\text{m}$  from the root tips. The fine roots were cut from the plants and immediately transferred to 5 ml measuring solution (0.03 mM  $\text{CdCl}_2$ , 0.1 mM KCl, 0.5 mM NaCl, 0.3 mM MES and 0.2 mM  $\text{Na}_2\text{SO}_4$ ; pH 6.0). After 15 min equilibration, each fine root was transferred to fresh measuring solution and the net  $\text{Cd}^{2+}$  fluxes were measured for 5 min per position. The  $\text{Cd}^{2+}$  gradients near the root surface ( $\sim 2\text{--}5\text{ }\mu\text{m}$ ) were measured by moving the  $\text{Cd}^{2+}$ -selective microelectrode between two positions 30  $\mu\text{m}$  apart and perpendicular to the root surface. Net  $\text{Cd}^{2+}$  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  $\text{Cd}^{2+}$  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  $-80^\circ\text{C}$  until the subsequent biochemical and molecular analyses. To calculate the fresh-to-dry mass ratio, fresh root, stem and leaf powders ( $\sim 50\text{ mg}$  each) were dried at  $60^\circ\text{C}$  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 spectrophotom-

etry (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  $\text{H}_2\text{O}$  and exposed to a staining solution (30 mg diphenylthiocarbazone in 60 ml acetone, 20 ml  $\text{H}_2\text{O}$  and 100  $\mu\text{l}$  glacial acetic acid) for 1 h. The tissues were quickly rinsed in deionized  $\text{H}_2\text{O}$ . 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 charge coupled device (CCD) camera (DS-Fi1; Nikon, Tokyo, Japan) and connected to a computer.

### Determinations of Cd concentration, bioconcentration factor and translocation factor

Fine root, stem and leaf powders ( $\sim 100\text{ mg}$  each) were digested in a mixture of 7 ml concentrated  $\text{HNO}_3$  plus 1 ml concentrated  $\text{HClO}_4$  at  $170^\circ\text{C}$  as previously described (Zhou et al. 2016). Cadmium 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  $4^\circ\text{C}$  in the dark. After centrifugation at 10,000g and  $4^\circ\text{C}$  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 (pH 7.5). The melatonin standard and the extracted samples were subjected to plant melatonin ELISA kit (MM-084801; Jiangsu Meimian industrial Co. Ltd, Jiangsu, China) according to the manufacturer's instructions.

### Determination of $\text{O}_2^{\bullet-}$ , $\text{H}_2\text{O}_2$ and malondialdehyde

The concentrations of  $\text{O}_2^{\bullet-}$  in the roots, stems and leaves were measured by monitoring the nitrite formation from hydroxylamine spectrophotometrically at 530 nm as previously described (Eltner and Heupel 1976) with some modifications (Verma and Mishra 2005). The  $\text{H}_2\text{O}_2$  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). According to Chance and Maehly (1955), the activity levels of catalase (CAT) and peroxidase (POD) were analyzed. 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). According to the manufacturer's instructions, total root RNA was extracted and purified with a plant RNA extraction kit (R6827; Omega Bio-Tek, Norcross, GA, USA). 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 (DRRO37A; 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 available as Supplementary Data at Tree Physiology Online) in a CFX96 Real Time System (CFX96; Bio-Rad Laboratories, Hercules, CA, USA). *β-Actin* was the reference gene (Table S1 available as Supplementary Data at Tree Physiology Online). A melting curve programme was used to confirm polymerase chain reaction product homogeneity. Relative mRNA expression was calculated according to the  $2^{-\Delta\Delta C_t}$  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. As previously described by Luo et al. (2015), 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/>).

### 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<sup>2+</sup> 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

Cadmium exposure severely reduced foliar carbon assimilation rate (*A*), stomatal conductance (*g<sub>s</sub>*) and transpiration rate (*E*) in *M. baccata* and *M. micromalus* 'qingzhoulinqin' relative to their controls (Table S2 available as Supplementary Data at Tree Physiology Online). 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 available as Supplementary Data at Tree Physiology Online). 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 available as Supplementary Data at Tree Physiology Online).

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<sup>2+</sup> fluxes

For all Cd exposures and melatonin treatments, the maximum net Cd<sup>2+</sup> influxes in *M. baccata* and *M. micromalus* 'qingzhoulinqin' occurred 300–600 µm from the root tips (Figure 1a). In both rootstocks, the net Cd<sup>2+</sup> 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<sup>2+</sup> efflux of 3000 µm from the root tips. For all Cd exposures and melatonin treatments, the net Cd<sup>2+</sup> 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<sup>2+</sup> fluxes by 65.4 and 10.4% at 300 and 600 µm from the root tips of Cd-exposed

Table 1. The dry mass (g) of root, stem, leaf and whole plant of *M. baccata* (Mb) and *M. micromalus* 'qingzhoulinqin' (Mm) exposed to 0 or 30  $\mu\text{M}$   $\text{CdCl}_2$  combined with either 0 or 100  $\mu\text{M}$  melatonin for 20 days. Data indicate means  $\pm$  SE ( $n = 6$ ). Different letters after 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  $\times$  S: TS; T  $\times$  M: TM; S  $\times$  M: SM; and T  $\times$  S  $\times$  M: TSM) are indicated.

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

\* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ ; \*\*\*\* $P \leq 0.0001$ ; ns: not significant.

*M. baccata* and *M. micromalus* 'qingzhoulinqin', respectively (Figure 1b).

#### Cadmium 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).

Cadmium 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 (Figure 3a and c). 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). The 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  $\sim 5.8$  and  $\sim 6.1\%$  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 (Figure 5a and c). Cadmium 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 (Figure 5a and c). 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 (Figure 5). 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 (Figure 5).

#### Reactive oxygen species and antioxidants

Compared with the untreated controls of both species, the plants exposed to Cd alone presented with significantly elevated root,

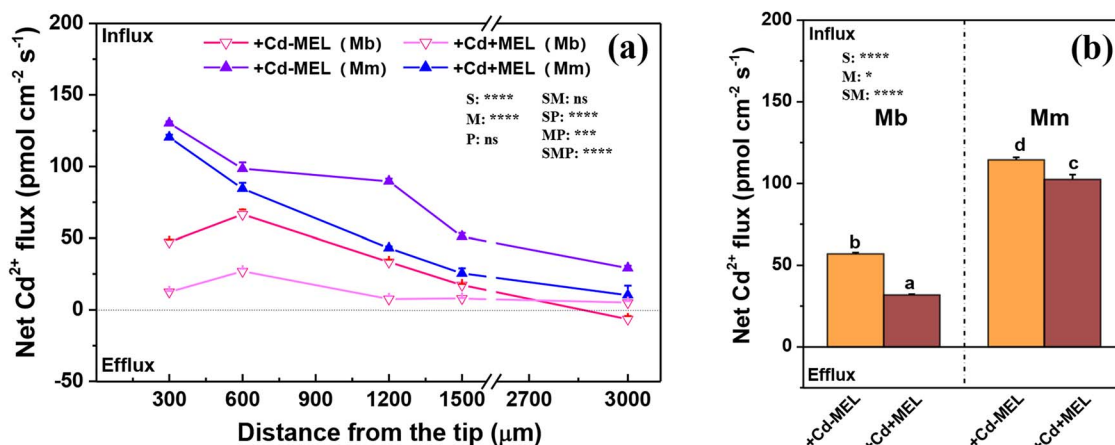


Figure 1. Net Cd<sup>2+</sup> fluxes along root tips (a) and the average of net Cd<sup>2+</sup> fluxes at 300 and 600 μm (b) from the root tips of *M. baccata* (Mb) and *M. micromalus* 'qingzhoulinqin' (Mm) exposed to 30 μM CdCl<sub>2</sub> (+Cd) combined with either 0 (–MEL) or 100 μM melatonin (+MEL) 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* ≤ 0.05; \*\*\**P* ≤ 0.001; \*\*\*\**P* ≤ 0.0001; ns: not significant.

stem and leaf O<sub>2</sub><sup>•−</sup> concentrations (Figure 6). Nevertheless, these increments were greater for *M. micromalus* 'qingzhoulinqin' than for *M. baccata* (Figure 6). Melatonin application significantly reduced O<sub>2</sub><sup>•−</sup> 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<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> production in all tissues of both species (Figure 6). Cadmium 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 (Figures S1 and S2 available as Supplementary Data at Tree Physiology Online). 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 (Figure S1 available as Supplementary Data at Tree Physiology Online). Melatonin application sometimes enhanced the Cd-induced increases in the levels of the aforementioned antioxidants (Figure S1 available as Supplementary Data at Tree Physiology Online). 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 (Figure S2 available as Supplementary Data at Tree Physiology Online). Exogenous melatonin enhanced the Cd-induced antioxidant enzymatic activity in all tissues except for GR in *M. micromalus* 'qingzhoulinqin' stems (Figure S2 available as Supplementary Data at Tree Physiology Online).

Principal component analysis 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, Tables S3–S4 available as Supplementary Data at Tree Physiology Online). 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 (Table S3 available as Supplementary Data at Tree Physiology Online). 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 (Table S3 available as Supplementary Data at Tree Physiology Online). 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



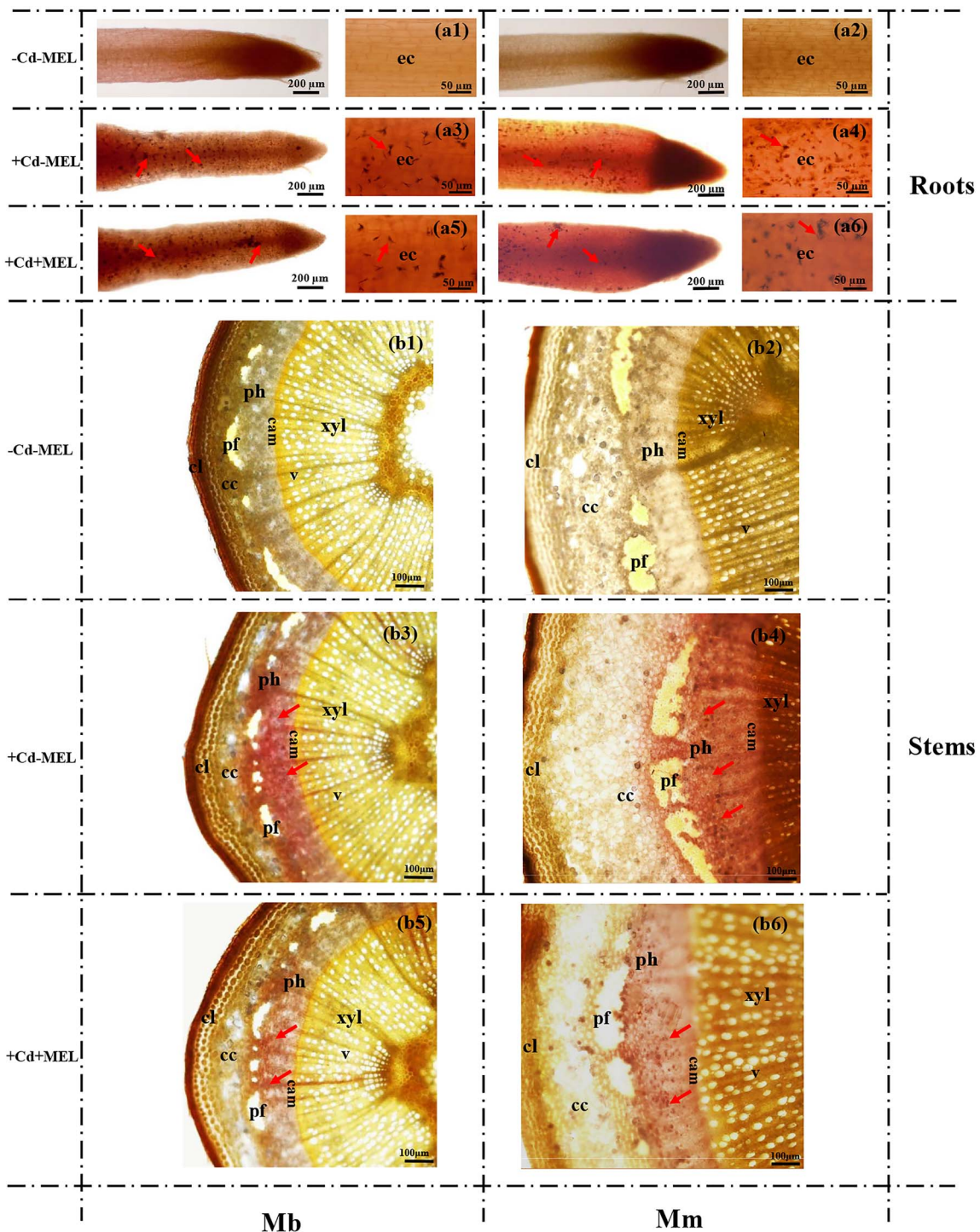


Figure 2. Cadmium localization in the roots (a1–a6) and stems (b1–b6) of *M. baccata* (Mb) and *M. micromalus* 'qingzhoulinnqin' (mm) exposed to 0 (–Cd) or 30  $\mu\text{M}$   $\text{CdCl}_2$  (+Cd) combined with either 0 (–MEL) or 100  $\mu\text{M}$  melatonin (+MEL) for 20 days. Arrows point to precipitates of Cd-dithizone. The histochemical detection of Cd in the roots and stems of both species treated with 0  $\mu\text{M}$  Cd and 100  $\mu\text{M}$  melatonin (–Cd+MEL) was similar to that in the roots treated with 0  $\mu\text{M}$  Cd and 0  $\mu\text{M}$  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.

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 (Table S4 available as Supplementary Data at Tree Physiology Online). In contrast, the concentrations of

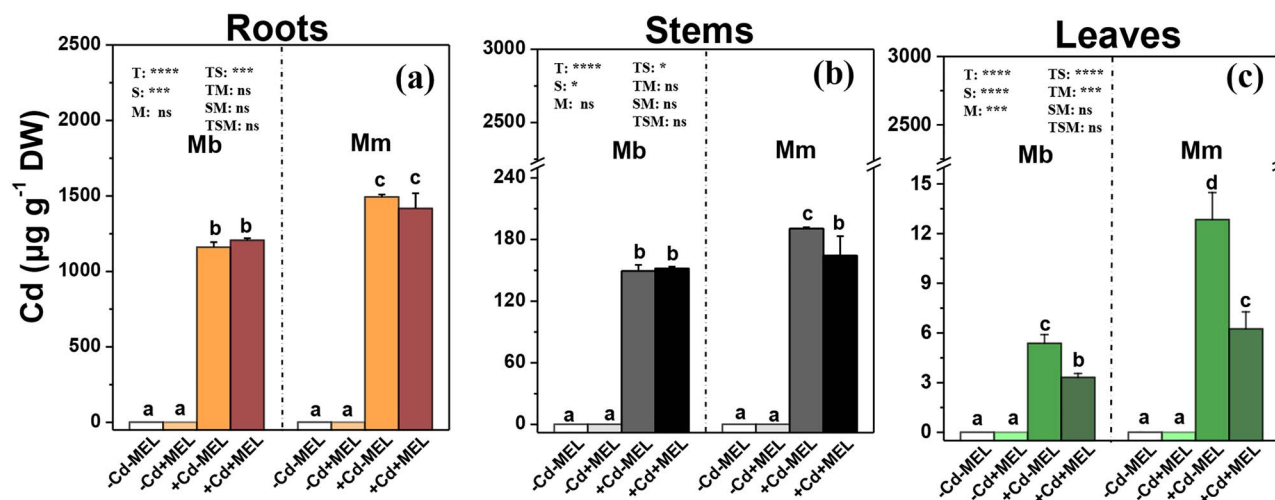


Figure 3. Cadmium concentration in the roots, stems and leaves of *M. baccata* (Mb) and *M. micromalus* 'qingzhoulinnqin' (Mm) exposed to 0 (–Cd) or 30  $\mu\text{M}$   $\text{CdCl}_2$  (+Cd) combined with either 0 (–MEL) or 100  $\mu\text{M}$  melatonin (+MEL) for 20 days. Data indicate means  $\pm$  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  $\times$  S: TS; T  $\times$  M: TM; S  $\times$  M: SM; and T  $\times$  S  $\times$  M: TSM) are indicated. \* $P \leq 0.05$ ; \*\*\* $P \leq 0.001$ ; \*\*\*\* $P \leq 0.0001$ ; ns: not significant.

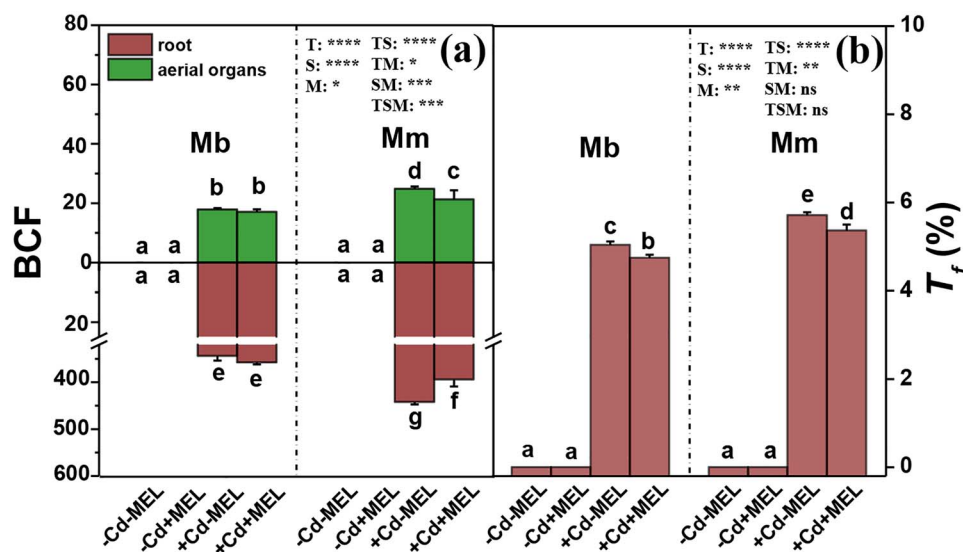


Figure 4. Bioconcentration factor (BCF) (a) in the roots and aerial organs, and translocation factor ( $T_f$ , b) of *M. baccata* (Mb) and *M. micromalus* 'qingzhoulinnqin' (Mm) exposed to 0 (–Cd) or 30  $\mu\text{M}$   $\text{CdCl}_2$  (+Cd) combined with either 0 (–MEL) or 100  $\mu\text{M}$  melatonin (+MEL) for 20 days. Data indicate means  $\pm$  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  $\times$  S: TS; T  $\times$  M: TM; S  $\times$  M: SM; and T  $\times$  S  $\times$  M: TSM) are indicated. \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ ; \*\*\*\* $P \leq 0.0001$ ; ns: not significant.

GSH, ASC and the soluble phenolics in the roots, the APX activity in the roots were the important positive factors for PC2 (Table S4 available as Supplementary Data at Tree Physiology Online).

Notably, treatments of +Cd–MEL and +Cd+MEL located at different quadrants of PCA in both apple rootstocks (Figure 7), 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* 'qingzhoulinnqin', 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



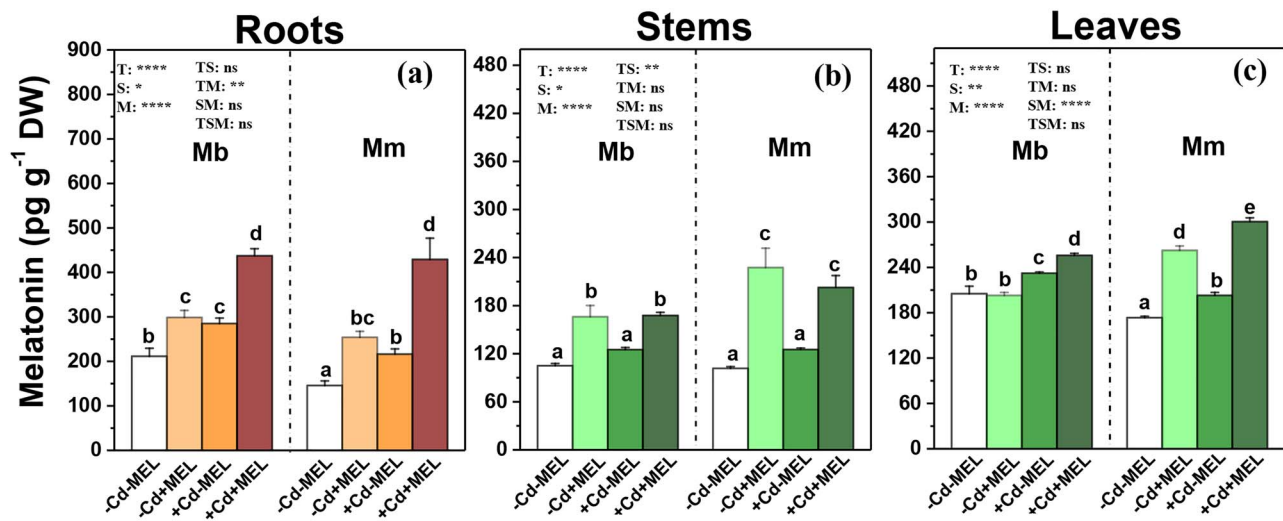


Figure 5. Melatonin concentration in the roots, stems and leaves of *M. baccata* (Mb) and *M. micromalus* 'qingzhoulinqin' (Mm) exposed to 0 (–Cd) or 30  $\mu$ M CdCl<sub>2</sub> (+Cd) combined with either 0 (–MEL) or 100  $\mu$ M melatonin (+MEL) for 20 days. Data indicate means  $\pm$  SE ( $n = 6$ ). 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  $\times$  S: TS; T  $\times$  M: TM; S  $\times$  M: SM; and T  $\times$  S  $\times$  M: TSM) are indicated. \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.0001$ ; ns: not significant.

more strongly induced the antioxidant system of *M. micromalus* 'qingzhoulinqin' than that of *M. baccata* (Figure 7).

#### 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).

Cadmium 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

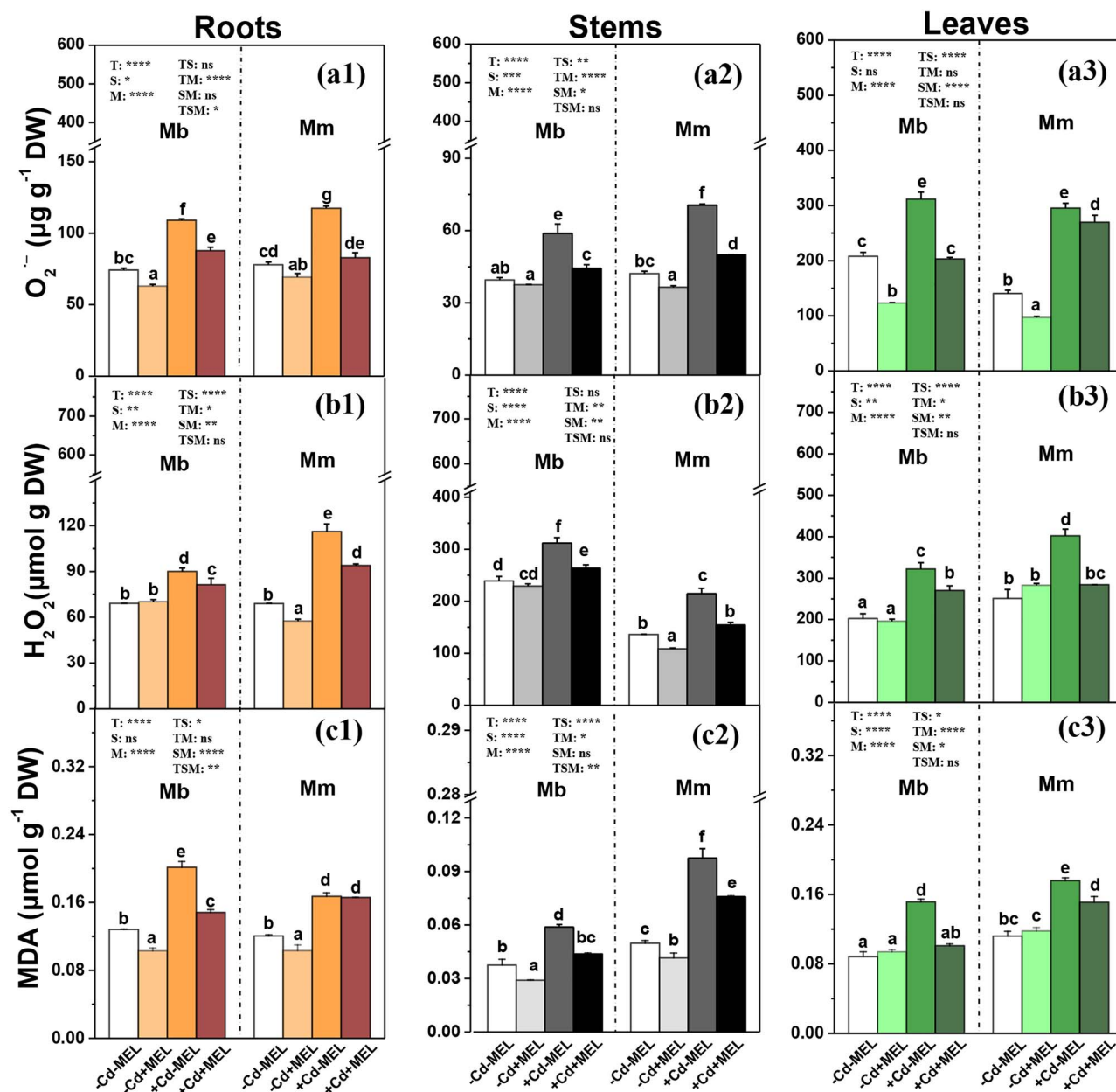


Figure 6.  $O_2^{\bullet-}$ ,  $H_2O_2$  and malonaldehyde (MDA) in the roots, stems and leaves of *M. baccata* (Mb) and *M. micromalus* 'qingzhoulinqin' (Mm) exposed to 0 (–Cd) or 30  $\mu M$   $CdCl_2$  (+Cd) combined with either 0 (–MEL) or 100  $\mu M$  melatonin (+MEL) for 20 days. Data indicate means  $\pm$  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  $\times$  S: TS; T  $\times$  M: TM; S  $\times$  M: SM; and T  $\times$  S  $\times$  M: TSM) are indicated. \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ ; \*\*\*\* $P \leq 0.0001$ ; ns: not significant.

absence of melatonin, Cadmium exposure significantly down-regulated *MHX* in the roots of both species (Figure 8). However, exogenous melatonin attenuated Cd-induced root *MHX* down-regulation in both species (Figure 8). Cadmium stress up-regulated root *ABCC1* in *M. baccata*, whereas 100  $\mu M$  exogenous melatonin further increased Cd-stressed root *ABCC1* transcript levels by  $\sim 1.3$ -fold in this species (Figure 8). In contrast, melatonin application up-regulated 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

Cadmium 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

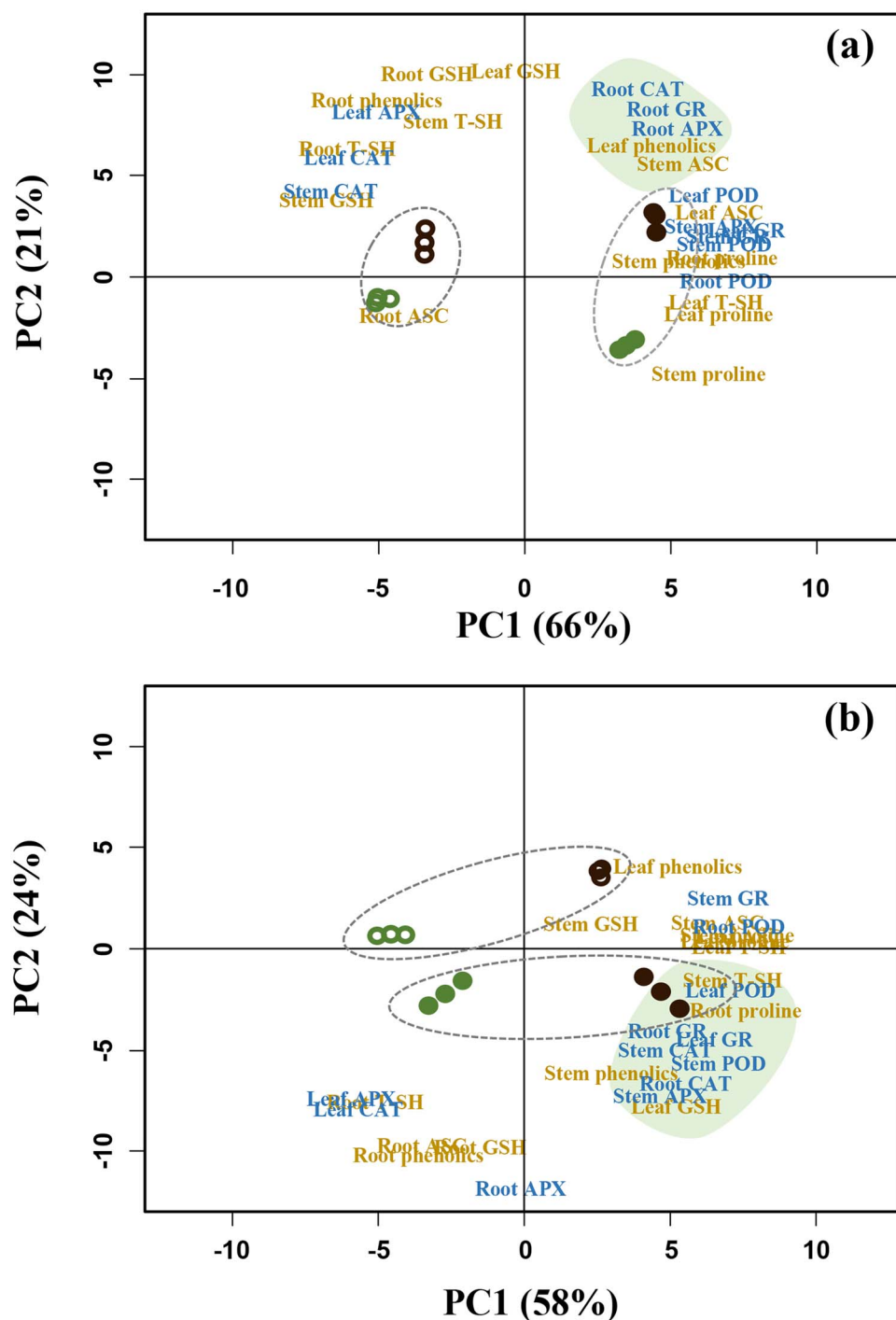


Figure 7. The plots of PCA of non-enzymatic and enzymatic antioxidants involved in Cd detoxification in the roots, stems and leaves of *M. baccata* (a) and *M. micromalus* 'qingzhoulinqin' (b) exposed to 0 (–Cd) or 30  $\mu\text{M}$   $\text{CdCl}_2$  (+Cd) combined with either 0 (–MEL) or 100  $\mu\text{M}$  melatonin (+MEL) for 20 days. The hollow and solid symbols indicate 0 and 30  $\mu\text{M}$  Cd, respectively. The orange and black colors indicate 0 and 100  $\mu\text{M}$  melatonin, respectively. The PCA loadings are presented in Tables S3 and S4 available as Supplementary Data at Tree Physiology Online.

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



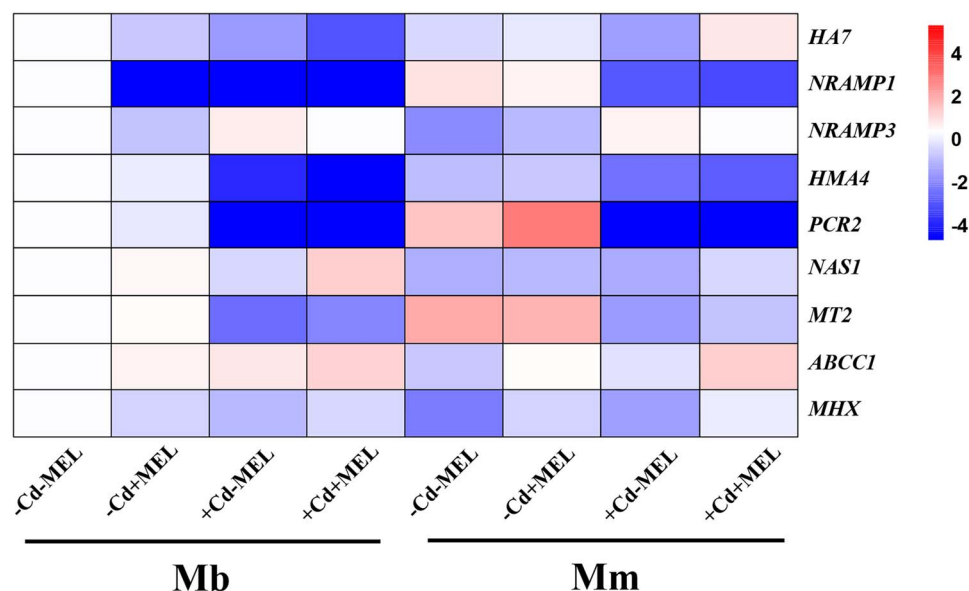


Figure 8. Heatmap of genes encoding proteins involved in Cd uptake, transport and detoxification in the roots of *M. baccata* (Mb) and *M. micromalus* 'qingzhoulinqin' (Mm) exposed to 0 (–Cd) or 30  $\mu$ M CdCl<sub>2</sub> (+Cd) combined with either 0 (–MEL) or 100  $\mu$ M melatonin (+MEL) for 20 days. For each gene, the expression level was set to 1 in the roots of *M. baccata* (Mb) treated with 0  $\mu$ M Cd and 0  $\mu$ M 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.

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<sup>2+</sup> 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<sup>2+</sup>, 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<sup>2+</sup> influx did not lower root Cd accumulation. Nevertheless, it significantly reduced Cd accumulation and *T<sub>f</sub>*s 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. Plasma membrane-bound H<sup>+</sup>-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<sup>+</sup>-ATPases. Its downregulation lowered net Cd<sup>2+</sup> 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<sup>2+</sup> 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<sup>2+</sup> 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

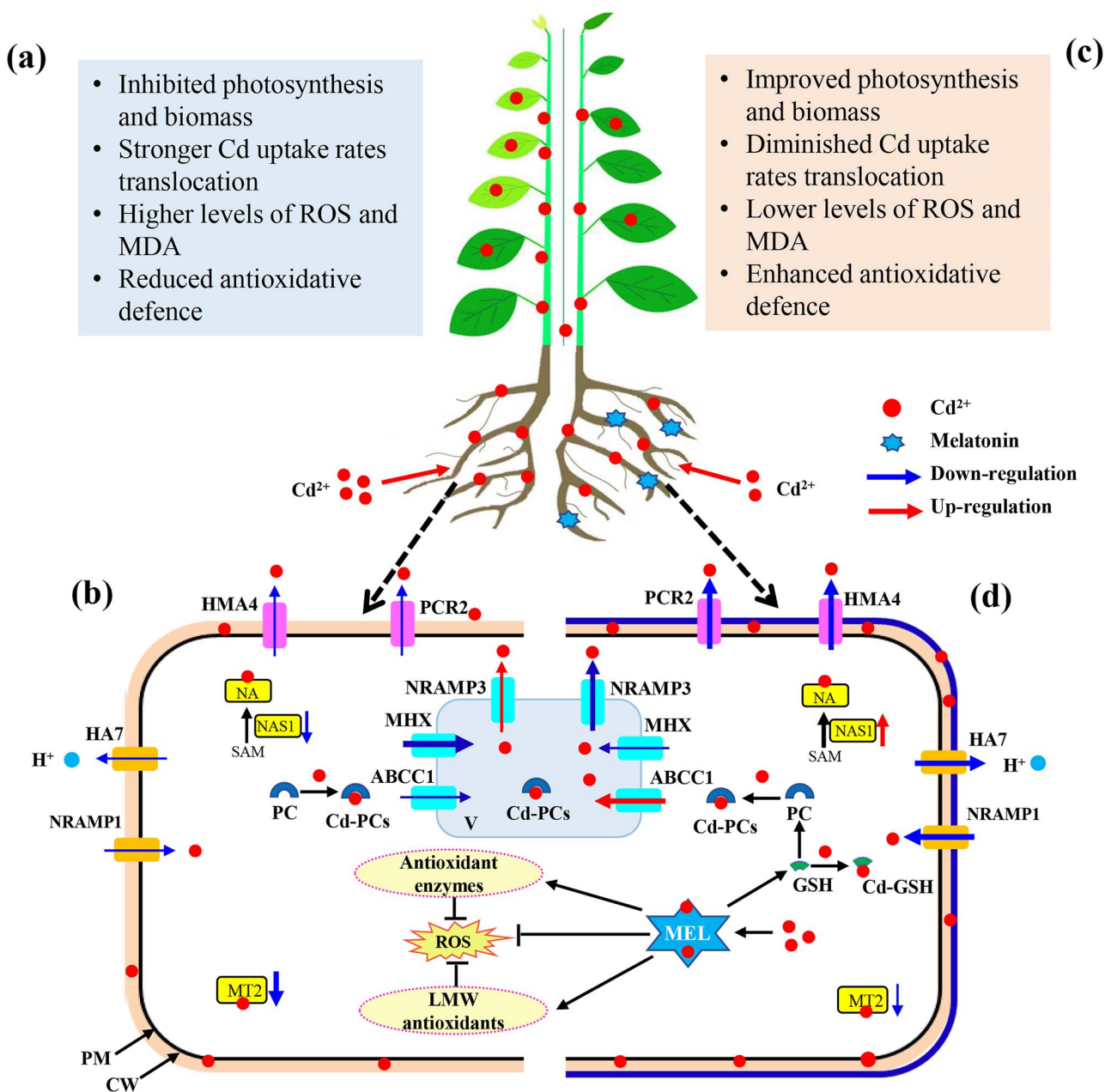


Figure 9. A schematic model illustrating the effects of melatonin on Cd uptake, translocation and toxicity in apple rootstocks exposed to Cd. The left chart represents apple rootstocks exposed to Cd alone (a–b), and the right chart depicts apple rootstocks treated with Cd and exogenous melatonin (c–d). 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, melatonin; SAM, S-adenosyl-L-methionine; HA7, PM H<sup>+</sup>-ATPases 7; NRAMP1 and NRAMP3, natural resistance associated macrophage protein 1 and 3; HMA4, P-type HM ATPase 4; PCR2, plant Cd resistance protein 2; NAS1, nicotianamine synthase 1; MT2, metallothionein 2; ABCC1, ATP-binding cassette transporter C1; MHX, magnesium proton exchanger protein.

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

Cadmium 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 (Figure S1 available as Supplementary Data at Tree Physiology Online) and activated CAT, POD, APX and GR (Figure S2 available as Supplementary Data at Tree Physiology Online). 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.

Cadmium-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 wild-type 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. Cadmium 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. Cadmium 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<sup>2+</sup> 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*, *HMA4* and *PCR2*, but upregulated those participating in Cd detoxification, including *NAS1*, *MT2*, *ABCC1* and *MHX*.

### Supplementary Data

Supplementary Data for this article are available at *Tree Physiology* Online.

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### Conflict of interest

None declared.



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## References

- Akhter MF, McGarvey B, Macfie SM (2012) Reduced translocation of cadmium from roots is associated with increased production of phytochelatin and their precursors. *J Plant Physiol* 169:1821–1829.
- Akhter MF, Omelon CR, Gordon RA, Moser D, Macfie SM (2014) Localization and chemical speciation of cadmium in the roots of barley and lettuce. *Environ Exp Bot* 100:10–19.
- An ZG, Li CJ, Zu YG, Du YJ, Wachter A, Gromes R, Rausch T (2006) Expression of BjMT2, a metallothionein 2 from *Brassica juncea*, increases copper and cadmium tolerance in *Escherichia coli* and *Arabidopsis thaliana*, but inhibits root elongation in *Arabidopsis thaliana* seedlings. *J Exp Bot* 57:3575–3582.
- Baker A (1987) Metal tolerance. *New Phytol* 106:93–111.
- Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline in water stress studies. *Plant Soil* 39:205–207.
- Berezin I, Mizrahy-Dagry T, Brook E, Mizrahi K, Elazar M, Zhuo S, Saul-Tcherkas V, Shaul O (2008) Overexpression of *AtMHX* in tobacco causes increased sensitivity to  $Mg^{2+}$ ,  $Zn^{2+}$ , and  $Cd^{2+}$  ions, induction of V-ATPase expression, and a reduction in plant size. *Plant Cell Rep* 27:939–949.
- Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254.
- Byeon Y, Lee HY, Hwang OJ, Lee HJ, Lee K, Back K (2015) Coordinated regulation of melatonin synthesis and degradation genes in rice leaves in response to cadmium treatment. *J Pineal Res* 58:470–478.
- Chance B, Maehly AC (1955) Assay of catalases and peroxidases. *Methods Enzymol* 2:764–775.
- Clemens S, Aarts MGM, Thomine S, Verbruggen N (2013) Plant science: the key to preventing slow cadmium poisoning. *Trends Plant Sci* 18:92–99.
- Connell JP, Mullet JE (1986) Pea chloroplast glutathione reductase: purification and characterization. *Plant Physiol* 82:351–356.
- Ding S, Ma CF, Shi WG, Liu W, Lu Y, Liu QF, Luo ZB (2017) Exogenous glutathione enhances cadmium accumulation and alleviates its toxicity in *Populus × canescens*. *Tree Physiol* 37:1697–1712.
- Duan Q, Lee J, Liu Y, Chen H, Hu H (2016) Distribution of heavy metal pollution in surface soil samples in China: a graphical review. *B Environ Contam Tox* 97:303–309.
- Elstner EF, Heupel A (1976) Inhibition of nitrite formation from hydroxylammonium chloride: a simple assay for superoxide dismutase. *Anal Biochem* 70:616–620.
- Fang B, Zhu X (2014) High content of five heavy metals in four fruits: evidence from a case study of Pujiang County, Zhejiang Province, China. *Food Control* 39:62–67.
- Gaash R, Elazar M, Mizrahi K, Avramov-Mor M, Berezin I, Shaul O (2013) Phylogeny and a structural model of plant MHX transporters. *BMC Plant Biol* 13:75.
- Gao C, Wang Y, Xiao DS, Qiu CP, Han DG, Zhang XZ, Wu T, Han ZH (2011) Comparison of cadmium-induced iron-deficiency responses and genuine iron-deficiency responses in *Malus xiaojinensis*. *Plant Sci* 181:269–274.
- Griffith OW (1980) Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal Biochem* 106:207–212.
- Gu Q, Chen Z, Yu X, Cui W, Pan J, Zhao G, Xu S, Wang R, Shen W (2017) Melatonin confers plant tolerance against cadmium stress via the decrease of cadmium accumulation and reestablishment of microRNA-mediated redox homeostasis. *Plant Sci* 261:28–37.
- Guo J, Qin S, Rengel Z, Gao W, Nie Z, Liu H, Li C, Zhao P (2019) Cadmium stress increases antioxidant enzyme activities and decreases endogenous hormone concentrations more in Cd-tolerant than Cd-sensitive wheat varieties. *Ecotox Environ Safe* 172:380–387.
- Hasan MK, Ahammed GJ, Yin L, Shi K, Xia X, Zhou Y, Yu J, Zhou J (2015) Melatonin mitigates cadmium phytotoxicity through modulation of phytochelatin biosynthesis, vacuolar sequestration, and antioxidant potential in *Solanum lycopersicum* L. *Front Plant Sci* 6:601.
- Hasan SA, Fariduddin Q, Ali B, Hayat S, Ahmad A (2009) Cadmium: toxicity and tolerance in plants. *J Environ Biol* 30:165–174.
- He JL, Li H, Luo J et al. (2013) A transcriptomic network underlies microstructural and physiological responses to cadmium in *Populus × canescens*. *Plant Physiol* 162:424–439.
- He JL, Li H, Ma CF, Zhang YL, Polle A, Rennenberg H, Cheng XQ, Luo ZB (2015) Overexpression of bacterial gamma-glutamylcysteine synthetase mediates changes in cadmium influx, allocation and detoxification in poplar. *New Phytol* 205:240–254.
- Hodges DM, DeLong JM, Prange FRK (1999) Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. *Planta* 207:604–611.
- Jakovljevic T, Bubalo MC, Orlovic S, Sedak M, Bilandzic N, Brozincevic I, Redovnikovic IR (2014) Adaptive response of poplar (*Populus nigra* L.) after prolonged Cd exposure period. *Environ Sci Pollut R* 21:3792–3802.
- Kampfenkel K, Vanmontagu M, Inze D (1995) Extraction and determination of ascorbate and dehydroascorbate from plant tissue. *Anal Biochem* 225:165–167.
- Kaya C, Okant M, Ugurlar F, Alyemeni MN, Ashraf M, Ahmad P (2019) Melatonin-mediated nitric oxide improves tolerance to cadmium toxicity by reducing oxidative stress in wheat plants. *Chemosphere* 225:627–638.
- Limson J, Nyokong T, Oaya S (1998) The interaction of melatonin and its precursors with aluminium, cadmium, copper, iron, lead, and zinc: an adsorptive voltammetric study. *J Pineal Res* 24:15–21.
- Lee K, Choi G-H, Back K (2017) Cadmium-induced melatonin synthesis in rice requires light, hydrogen peroxide, and nitric oxide: key regulatory roles for tryptophan decarboxylase and caffeic acid O-methyltransferase. *J Pineal Res* 63:e12441.
- Li C, Tan DX, Liang D, Chang C, Jia D, Ma F (2015) Melatonin mediates the regulation of ABA metabolism, free-radical scavenging, and stomatal behaviour in two *Malus* species under drought stress. *J Exp Bot* 66:669–680.
- Li MQ, Hasan MK, Li CX et al. (2016) Melatonin mediates selenium-induced tolerance to cadmium stress in tomato plants. *J Pineal Res* 61:291–302.
- Lin YF, Aarts MGM (2012) The molecular mechanism of zinc and cadmium stress response in plants. *Cell Mol Life Sci* 69:3187–3206.

- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the  $2^{-\Delta\Delta CT}$  method. *Methods* 25:402–408.
- Luo J, Zhou J, Li H, Shi W, Polle A, Lu M, Sun X, Luo ZB (2015) Global poplar root and leaf transcriptomes reveal links between growth and stress responses under nitrogen starvation and excess. *Tree Physiol* 35:1283–1302.
- Luo ZB, He J, Polle A, Rennenberg H (2016) Heavy metal accumulation and signal transduction in herbaceous and woody plants: paving the way for enhancing phytoremediation efficiency. *Biotechnol Adv* 34:1131–1148.
- Ma Y, He J, Ma C, Luo J, Li H, Liu T, Polle A, Peng C, Luo ZB (2014) Ectomycorrhizas with *Paxillus involutus* enhance cadmium uptake and tolerance in *Populus × canescens*. *Plant Cell Environ* 37:627–642.
- Nada E, Ferjani BA, Ali R, Bechir BR, Imed M, Makki B (2007) Cadmium-induced growth inhibition and alteration of biochemical parameters in almond seedlings grown in solution culture. *Acta Physiol Plant* 29:57–62.
- Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant Cell Physiol* 22:867–880.
- Nawrot T, Plusquin M, Hogervorst J, Roels HA, Celis H, Thijs L, Vangronsveld J, Van Hecke E, Staessen JA (2006) Environmental exposure to cadmium and risk of cancer: a prospective population-based study. *Lancet Oncol* 7:119–126.
- Ni J, Wang Q, Shah FA, Liu W, Wang D, Huang S, Fu S, Wu L (2018) Exogenous melatonin confers cadmium tolerance by counterbalancing the hydrogen peroxide homeostasis in wheat seedlings. *Molecules* 23:799.
- Nouet C, Charlier J-B, Carnol M, Bosman B, Farnir F, Motte P, Hanikenne M (2015) Functional analysis of the three *HMA4* copies of the metal hyperaccumulator *Arabidopsis halleri*. *J Exp Bot* 66:5783–5795.
- Pape C, Lüning K (2006) Quantification of melatonin in phototrophic organisms. *J Pineal Res* 41:157–165.
- Park J, Song WY, Ko D, Eom Y, Hansen TH, Schiller M, Lee TG, Martinoia E, Lee Y (2012) The phytochelatin transporters *AtABCC1* and *AtABCC2* mediate tolerance to cadmium and mercury. *Plant J* 69:278–288.
- Patterson BD, Macrae EA, Ferguson IB (1984) Estimation of hydrogen peroxide in plant extracts using titanium (IV). *Anal Biochem* 139:487–492.
- Pizzol M, Smart JCR, Thomsen M (2014) External costs of cadmium emissions to soil: a drawback of phosphorus fertilizers. *J Clean Prod* 84:475–483.
- Podazza G, Arias M, Prado FE (2012) Cadmium accumulation and strategies to avoid its toxicity in roots of the citrus rootstock *Citrumelo*. *J Hazard Mater* 215–216:83–89.
- Podazza G, Arias M, Prado FE (2016) Early interconnectivity between metabolic and defense events against oxidative stress induced by cadmium in roots of four citrus rootstocks. *Funct Plant Biol* 43:973.
- Reiter RJ, Tan DX, Terron MP, Flores LJ, Czarnocki Z (2007) Melatonin and its metabolites: new findings regarding their production and their radical scavenging actions. *Acta Biochim Pol* 54:1–9.
- Romero A, Ramos E, de Los Ríos C, Egea J, Pino JD, Reiter RJ (2014) A review of metal-catalyzed molecular damage: protection by melatonin. *J Pineal Res* 56:343–370.
- Salt DE, Prince RC, Pickering IJ, Raskin I (1995) Mechanisms of cadmium mobility and accumulation in Indian mustard. *Plant Physiol* 109:1427–1433.
- Sedlak J, Lindsay RH (1968) Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Anal Biochem* 25:192–205.
- Shi WG, Liu W, Yu W, Zhang Y, Ding S, Li H, Mrak T, Kraigher H, Luo ZB (2019) Abscisic acid enhances lead translocation from the roots to the leaves and alleviates its toxicity in *Populus × canescens*. *J Hazard Mater* 362:275–285.
- Siemianowski O, Mills RF, Williams LE, Antosiewicz DM (2011) Expression of the P1B-type ATPase *AtHMA4* in tobacco modifies Zn and Cd root to shoot partitioning and metal tolerance. *Plant Biotechnol J* 9:64–74.
- Song WY, Choi KS, Kim DY et al. (2010) *Arabidopsis* *PCR2* is a zinc exporter involved in both zinc extrusion and long-distance zinc transport. *Plant Cell* 22:2237–2252.
- Song WY, Yamaki T, Yamaji N et al. (2014) A rice ABC transporter, OsABCC1, reduces arsenic accumulation in the grain. *Proc Natl Acad Sci USA* 111:15699–15704.
- Sungur A (2016) Heavy metals mobility, sources, and risk assessment in soils and uptake by apple (*Malus domestica* Borkh.) leaves in urban apple orchards. *Arch Agron Soil Sci* 62:1051–1065.
- Swain T, Goldstein JL (1964) The quantitative analysis of phenolic compounds. In: Pridham JB (ed) *Methods in polyphenol chemistry*. Macmillan, New York, NY, pp 138–146.
- Tan DX, Hardeland R, Manchester LC, Korkmaz A, Ma S, Rosales-Corral S, Reiter RJ (2012) Functional roles of melatonin in plants, and perspectives in nutritional and agricultural science. *J Exp Bot* 63:577–597.
- Thomine S, Wang RC, Ward JM, Crawford NM, Schroeder JI (2000) Cadmium and iron transport by members of a plant metal transporter family in *Arabidopsis* with homology to Nramp genes. *Proc Natl Acad Sci USA* 97:4991–4996.
- Verma S, Mishra N (2005) Putrescine alleviation of growth in salt stressed *Brassica juncea* by inducing antioxidative defense system. *J Plant Physiol* 162:669–677.
- Verret F, Gravot A, Auroy P, Leonhardt N, David P, Nussaume L, Vavasseur A and Richaud P (2004) Overexpression of *AtHMA4* enhances root-to-shoot translocation of zinc and cadmium and plant metal tolerance. *FEBS Lett* 576:0–312.
- Wellburn AR (1994) The spectral determination of chlorophyll-a and chlorophyll-b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. *J Plant Physiol* 144:307–313.
- Zhang N, Sun Q, Zhang H, Cao Y, Weeda S, Ren S, Guo YD (2015) Roles of melatonin in abiotic stress resistance in plants. *J Exp Bot* 66:647–656.
- Zhou J, Wan H, Qin S, He J, Lyu D, Li H (2016) Net cadmium flux and gene expression in relation to differences in cadmium accumulation and translocation in four apple rootstocks. *Environ Exp Bot* 130:95–105.
- Zhou J, Wan H, He J, Lyu D, Li H (2017) Integration of cadmium accumulation, subcellular distribution, and physiological responses to understand cadmium tolerance in apple rootstocks. *Front Plant Sci* 8:966.