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Foliar spraying of melatonin confers cadmium tolerance in *Nicotiana tabacum* L

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ABSTRACT

Melatonin is a multifunctional signaling molecule that regulates broad aspects of responses to environmental stresses in plants. Cadmium (Cd) is a persistent soil contaminant that is toxic to all living organisms. Recent reports have uncovered the protective role of melatonin in alleviating Cd phytotoxicity, but little is known about its regulatory mechanisms in plants. In this study, we found that foliar application of melatonin (in particular 100 μmol L^{-1}) remarkably enhanced Cd tolerance of tobacco (*Nicotiana tabacum* L.) leaves, as evidenced by less Cd accumulation and alleviation of growth inhibition and photoinhibition, compared with nontreated Cd-stressed plants. The addition of melatonin also controlled oxidative damage of Cd on tobacco through direct scavenging and by enhancing the activities of antioxidative enzymes. Melatonin application promoted Cd sequestration in the cell wall and vacuoles based on the analysis of subcellular distribution of Cd in tobacco cells. Structural equation modeling (SEM) analysis revealed that melatonin-induced Cd tolerance in tobacco leaves was modulated by the expression of Cd-transport genes. Molecular evidence illustrated that modulation of *IRT1*, *Nramp1, HMA2*, *HMA4*, and *HMA3* genes caused by melatonin could be responsible for weakening Cd uptake, Cd transportation to xylem, and intensifying Cd sequestration into the root vacuoles.

1. Introduction

Tobacco is a nice model plant for scientific experiments and production of beneficial compounds (Regassa and Chandravanshi, 2016; Feng et al., 2018). Tobacco also produces a large amount of biomass and accumulates relatively more Cd (Rubio et al., 2015). More than 50% of total Cd that has been taken up by tobacco is concentrated in the leaves (Rosén et al., 2012; Liu et al., 2016). Cd accumulation in tobacco leaves could significantly degrade the yield and negatively affect its quality, because Cd disturbs the balance of nitrogen, nicotine, and carbohydrate contents, and makes the tobacco taste worse (Liu et al., 2015). Long-term intake of Cd could exacerbate serious, chronic health problems for smokers, such as kidney damage, bladder, and lung cancer; these problems could also be intensified in nonsmokers through secondhand smoke (Regassa and Chandravanshi, 2016; Zaprjanova et al., 2010; Wang et al., 2017). Therefore, increasing Cd tolerance or reducing Cd accumulation in tobacco leaves are ongoing endeavors in production of tobacco products.

Generally, the plasma membrane of plants is the first target of Cd toxicity stress, and Cd stress in plant cells is indicated by the following aspects: it induces reactive oxygen species (ROS) accumulation; alters the permeability of plasma membrane by inhibiting H^+-ATPase; changes the composition and fluidity of membrane lipids; alters plant cell cycle, division etc.; finally damages cell organelles (DalCorso et al., 2010; Rother et al., 2010; Janicka-Russak et al., 2012). Among these, the production of ROS is one of the most common and serious damage to plants exposed to Cd stress, as it causes oxidative damages to plant cells, disrupts both redox homeostasis in cells and ROS-derived DNA oxidation, and even breaks single- and double-stranded DNA (Ahammed et al., 2018). Fortunately these excess ROS can be effectively scavenged by various antioxidative enzymes or antioxidants (Chen et al., 2017). For example, a stable free radical of hydrogen peroxide (H_2O_2) can be scavenged by catalase and peroxidase (Wang et al., 2013). Thus, an effective antioxidative system (the antioxidant enzyme activities and the redox state of primary antioxidants) is critical for plant cells in order to defend against Cd stress. In addition, gene expression patterns change in the uptake, transportation, distribution, and detoxification of Cd (Fässler et al., 2011). For example, Cd uptake

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by the root is mediated by natural resistance-associated macrophage protein 1 (*Nramp1*) and iron-regulated transporter 1 (*IRT1*); the transport of Cd to xylem is regulated by heavy metal ATPase2 (*HMA2*) and ATPase4 (*HMA4*); in contrast, Cd up-regulates the ATPase3 (*HMA3*) gene locates at the tonoplast of root cells, which can sequestrate Cd into the root vacuoles. Therefore, the responsive expression of these genes is believed to be mostly attributed to Cd detoxification and/or transport in roots and shoots (Vert et al., 2002; Byeon et al., 2015; Wu et al., 2015).

Melatonin (N-acetyl-5-methoxytryptamine), an amphiphilic low-molecular weight indoleamine, has a broad spectrum of biological functions in plant cells, serving as a growth regulator, biostimulator, anti-aging agent, and antioxidant, especially in response to various stresses. Specifically, its antioxidant activity mainly functions via: (i) scavenging free radicals, due to its chemical characteristics of amphipathic behavior, which facilitates melatonin crossing cell barriers and melatonin availability to every subcellular organelle (Posmyk et al., 2008; García et al., 2014); ii) stimulating antioxidant enzymes that remove free radicals and their precursors; (iii) improving the efficiency of electron transport in mitochondria, thereby decreasing electron leakage, which inhibits the generation of free radicals (Posmyk et al., 2008). The combination of these beneficial properties means that melatonin has powerful antioxidant capabilities. More recent reports have shown that plants treated with melatonin displays strong tolerance to heavy metal stresses. Gu et al. (2017) found that pretreatment with exogenous melatonin of alfalfa seedling could significantly alleviate Cd-induced growth inhibition. The authors suggested that melatonin enhanced Cd tolerance via reestablishing the redox homeostasis mediated by microRNAs. In a similar study using melatonin on weak Cd stress in *Solanum lycopersicum*, Hasan et al. (2015) concluded that melatonin-induced increases in antioxidant activity, phytochelatin biosynthesis, and Cd sequestration played critical roles in enhancing the tolerance to Cd stress. Although melatonin is well known to promote Cd detoxification and enhance Cd stress tolerance in plants, information about melatonin-induced Cd-related gene expression in plant cells is lacking.

Most studies investigating melatonin application into Cd detoxification or sequestration were carried out using *Solanum lycopersicum* (Byeon et al., 2015), *Oryza sativa* (Li et al., 2016), or *Brassica campestris* (Wu et al., 2015) in hydroponic or soil systems. There are only few studies on melatonin-induced tolerance to Cd stress in tobacco plants. We hypothesize that: 1) foliar application of melatonin increases Cd tolerance of tobacco leaves; and 2) melatonin-induced Cd tolerance involves in antioxidant capacities and Cd-related gene expression. Therefore, the objectives of this study were to evaluate the potential roles of foliar application of melatonin counteracting Cd stress in tobacco leaves; and to explore the evidence showing how melatonin confers Cd tolerance at both the chemical and genetic scales. A detailed analysis of melatonin application affecting on oxidative stress markers and antioxidant enzymes of tobacco leaves was performed. The expression of genes that are associated with Cd uptake, accumulation and distribution in tobacco plants was assessed. This study explores crucial mechanisms involved in applying melatonin as an antagonist of Cd toxicity to tobacco, which might have implications for developing promising strategies for safe tobacco products.

2. Materials and methods

2.1. Raw materials and experimental design

Tobacco seeds (variety Yun-87) were selected and supplied by Hunan Tobacco Science Institute (Changsha, China); Yun-87 is one of the most common varieties cultivated in Chinese tobacco planting areas. Tobacco seeds were first soaked in sodium hypochlorite solution (10%) for 10 min and then rinsed with deionized-water for several times to ensure surface sterility. Seeds were germinated in the sterilized seedling substrate containing vermiculite and peat (1:1, v/v), until the two true leaves emerged. Then, a group of ten uniform-sized seedlings were collected and exposed to the solutions as described below.

In preliminary experiments, To analyze the toxic threshold of Cd stress on growth performance, tobacco seedlings were exposed to 0, 50, 100, and 200 μM $CdCl_2$ for 3 d: the lowest root elongation and fresh weight were observed in 100 μM Cd treatment (about 50% decrease compared with 0 μM Cd treatment), and there was no significant difference in inhibiting root growth after treated by 100 and 200 μM Cd. Therefore, 100 μM Cd (in the form of $CdCl_2$) was applied into the following experiments. In order to illustrate the effect of Cd stress along with foliar application of melatonin on tobacco growth, seedlings were transferred into plastic chambers containing Hoagland's nutrient solution with or without Cd. Specifically, foliar portions of tobacco plants were sprayed with 0 (CK), 25, 50, 100, and 250 μM melatonin (denoted as CK, MT25, MT50, MT100 and MT250, respectively). The solutions in the pot were changed every day followed by foliar spray of 10 mL melatonin. Melatonin was purchased from Acros Organics, Inc. (Geel, Belgium) with a purity of 99%. The plants in CK were sprayed with water alone. The solutions were sprayed by a hand-pump sprayer and made sure each piece of leaf was evenly wet. All chambers were placed in an illuminated growth chamber (14 h light with an intensity of 200 μmol m^{-2} s^{-1} irradiation, 25 ± 1 °C; 10 h dark, 23 ± 1 °C) with 75% relative humidity. All chambers were arranged in randomized blocks with four replicates of each treatment. The experiment ended one week after initial treatment. Afterwards, the plant samples were harvested and the fresh weight and root length were measured immediately. Each plant was separated into roots and shoots with the aid of a small knife and were rinsed with deionized water. A portion of each sample was dried in an oven (60 °C) until the weight was constant, and the leaf weights from each pot were recorded. The plant samples were ground and passed through a 0.25 mm sieve for further analyses.

2.2. Analyses

2.2.1. Determination of photosynthetic characteristics

Total chlorophyll of fresh seedlings was calculated by the detection at the absorbance at 649 nm and 665 nm after the extraction using 95% (v/v) ethanol for 24 h in darkness (Ahammed et al., 2018). The net photosynthetic rate (Pn), stomatal conductance (Gs), intracellular CO_2 concentration (Ci), and transpiration rate (Tr) of tobacco seedlings were determined according to the procedure of Chen et al. (2017).

2.2.2. Quantitative detection of H_2O_2 and $O_2^{\cdot-}$

The H_2O_2 concentration in leaves was assessed spectrophotometrically at OD_{412} as described in Willekens et al. (1997) The concentration of $O_2^{\cdot-}$ was measured at the absorbance of 530 nm by nitrite formation from hydroxylamine hydrochloride as described in Chen et al. (2017).

2.2.3. Assay of antioxidant enzyme activity

Leaf tissue (0.3 g) was homogenized in 3 mL ice-cold HEPES buffer (pH 7.8, 25 mM) containing EDTA (0.2 mM) and polyvinylpyrrolidone (2%, w/v). According to the method of Nakano (Nakano and Asada, 1981), the activity of ascorbate peroxidase (APX) was determined by monitoring the decrease in absorbance at 290 nm after the extraction by 2 mM ascorbic acid. The activity of superoxide dismutase (SOD) was measured at 560 nm by monitoring the inhibition photochemical reduction (50%) in nitroblue tetrazolium. Catalase (CAT) activity was measured by the decrease in absorbance at 240 nm due to decomposition of H_2O_2. The assessment of H^+-ATPase activity was conducted as described previously (Ahmed et al., 2013).

2.2.4. Separation of tissue fractionations and Cd analysis

To determine the effect of foliar application of melatonin on the subcellular compartmentalization of Cd, the leaves were separated into four fractions: cell wall (CW), vacuole (V), organelle (O), and soluble

(S) fractions as previously described (Wu et al., 2005). The different separated cell fractions (0.2 g, dry weight) or homogenized dry powdered leaves (0.2 g) were digested with the diacid mixture consisting of HNO_3 and H_2O_2 in a 3:1 ratio using a microwave digestion apparatus (Milestone MLS 1200 Mega). The Cd concentrations of plants in the different fractions and tobacco leaves were determined by a PerkinElmer 1100B atomic absorption spectrometer.

2.2.5. RNA isolation and quantitative real- time PCR (qRT-PCR)

Total RNA was extracted from tobacco root tissue by an RNA extraction kit (Tiangen, Shanghai, China) following the manufacturer's instructions. DNA-free total RNA (5 μg) from different treatments was reverse-transcribed to generate cDNA by reverse transcriptase. qRT-PCR was performed by the method described by Li et al. (2016) PCR primers were designed to detect the expression level of target genes IRT1 (accession number AY087095.1), IRT2 (accession number BT025714.1), Nramp1 (accession number AF165125.1), HMA2 (accession number NM119157.3), HMA3 (accession number DQ446885.1) and HMA4 (accession number AY096796) and the control gene Actin (accession number AF111812) (Supplementary Table S1). All primers were synthesized by Lingen Biotechnology Ltd. Company (Shanghai, China). Relative gene expression was calculated basd on Livak and Schmittgen (Livak and Schmittgen, 2001). The qRT-PCR data were collected from four replicates, and each sample was prepared in triplicate.

2.2.6. TEM observations and energy-dispersive spectroscopy (EDS) analysis

To achieve direct evidence of the mechanism of alleviated Cd phytotoxicity induced by foliar application of melatonin, tobacco roots under different treatments were examined by transmission electron microscopy (TEM). The procedure for sample preparation was described in Shi et al. (2014) The samples were observed under Ni grids using TEM (JEOL JEM-2011, Japan) equipped with X-ray EDS during observations.

2.3. Data presentation and statistical analysis

Structural equation modeling (SEM) was applied to explore the interrelationships among the changes in tobacco leaf parameters, melatonin application, and Cd accumulation. In this study, the SEM framework was applied to investigate the direct and indirect effects of melatonin on Cd accumulation. The model fit was assessed by means of a Chi-square test; a non-significant Chi-square test ($0.05 \leq P \leq 1.00$) indicates a good fit of the model to the data. The strength of the modelled relationships between variables was quantified using the estimated coefficients and significance was assessed using z statistics (Rao et al., 2018). SEM analyses were performed using AMOS 21.0 (Amos Development Corporation, Crawfordville, FL, USA). Values are shown as the means ± SE of the independent experiments with four replicates each. SPSS v20.0 (SPSS Inc., USA) was used to perform one-way analysis of variance (ANOVA) with post hoc Tukey's honest significant difference (HSD) tests and to calculate Spearman's rank correlations.

3. Results

3.1. Tobacco growth

Biomass weight usually reflects the response of plants to adverse environments; seedling root growth is a rapid and broadly used acute phytotoxicity indicator taking the benefits of sensitivity, simplicity, low cost, and suitability. In this study, melatonin-treated plants had better growth and stronger root development with and without Cd-stress (Fig. 1C). Melatonin alone did not have a significant effect on tobacco fresh weight or root length (compared to the CK) when the concentration was low (25 or 50 μmol/L). Application of higher concentrations of melatonin significantly increased tobacco growth (Fig. 1). Melatonin application even at lower concentrations significantly increase tobacco fresh weight and root length under Cd stress, which suggested that the toxic effect of Cd on tobacco growth was mitigated by melatonin application. The growth of tobacco seedlings was significantly inhibited by $CdCl_2$ as evaluated by a significant decrease in both fresh weight and root length by 39.1% and 39.9%, respectively (Fig. 1). It should be noted that higher concentrations of melatonin application is not always desirable; in this study, it seems that a moderate concentration, 100 μM of melatonin, was best for ameliorating Cd-induced inhibition and promoting tobacco growth.

3.2. Photosynthetic characteristics

The effect of foliar application of melatonin on photosynthetic parameters of tobacco leaves with or without Cd stress is illustrated in Table 1. All photosynthetic parameters were significantly reduced after exposure to Cd compared with their corresponding no-Cd-stress control. Foliar spraying of melatonin on tobacco seedlings under Cd stress could effectively alleviate these adverse effects. For example, exogenous melatonin led to statistical recovery of some chlorophyll content. Again, a moderate concentration of melatonin displayed the most effective ameliorative effect (100 vs. 25, 50, or 250 μM) compared with Cd treatment alone. In addition, as a growth-promoting molecule, melatonin application increased the concentrations of all photosynthetic parameters under no Cd stress, indicating that melatonin might play a role in regulating plant growth and development.

3.3. ROS accumulation

After Cd exposure, ROS ($O_2^{\cdot-}$and H_2O_2) concentrations in leaves of tobacco seedlings were significantly elevated when compared with CK (Cd0) treatment (Fig. 2). Application of melatonin significantly inhibited the oxidative damage indicated by decreased $O_2^{\cdot-}$and H_2O_2 contents. Again, application of 100 μM melatonin resulted in the stronger reduction in ROS accumulation indicated by up to 28.3% and 29.1% reduction of $O_2^{\cdot-}$and H_2O_2 levels. These results suggest that foliar application of melatonin could be effective to minimize Cd-induced oxidative stress in the tobacco leaves. In addition, melatonin application alone also significantly reduced $O_2^{\cdot-}$and H_2O_2 levels when compared with their corresponding no-Cd-stress controls.

3.4. Activities of antioxidant enzymes and H^+-ATPase

Modification of antioxidant enzymes (APX, SOD, and CAT) and H^+-ATPase of tobacco leaves under various treatments with Cd and melatonin alone or in combination is shown in Fig. 3. All test enzymes displayed similar trends: toxic Cd had strong inhibitory effects on all enzymes, while foliar application of melatonin significantly recovered their activities. For example, exogenous melatonin treatment combined with Cd stress enhanced SOD activity by 37.5% compared with just Cd stress. Similarly, an approximately 30.0% increase in the activity of H^+-ATPase was induced by 100 μM melatonin addition, compared with CK under Cd stress. These results imply that exogenous melatonin-induced modulation of the activities of antioxidant enzymes and H^+-ATPase conferred tolerance to oxidative stress caused by Cd in tobacco leaves.

3.5. Cd accumulation and distribution

Excess Cd exposure for 1 week substantially elevated endogenous Cd contents in tobacco leaves (Fig. 4A), while foliar application of melatonin significantly inhibited Cd uptake by leaves compared to Cd stress alone; up to 30.1% reduction in Cd was observed in treatment after adding 100 μM melatonin. To explore the effect of melatonin on the cellular distribution of Cd in tobacco leaf cells, the Cd content in cellular compartments of leaf tissues was quantified. The subcellular distribution of Cd was mainly dominated by the soluble and organelle

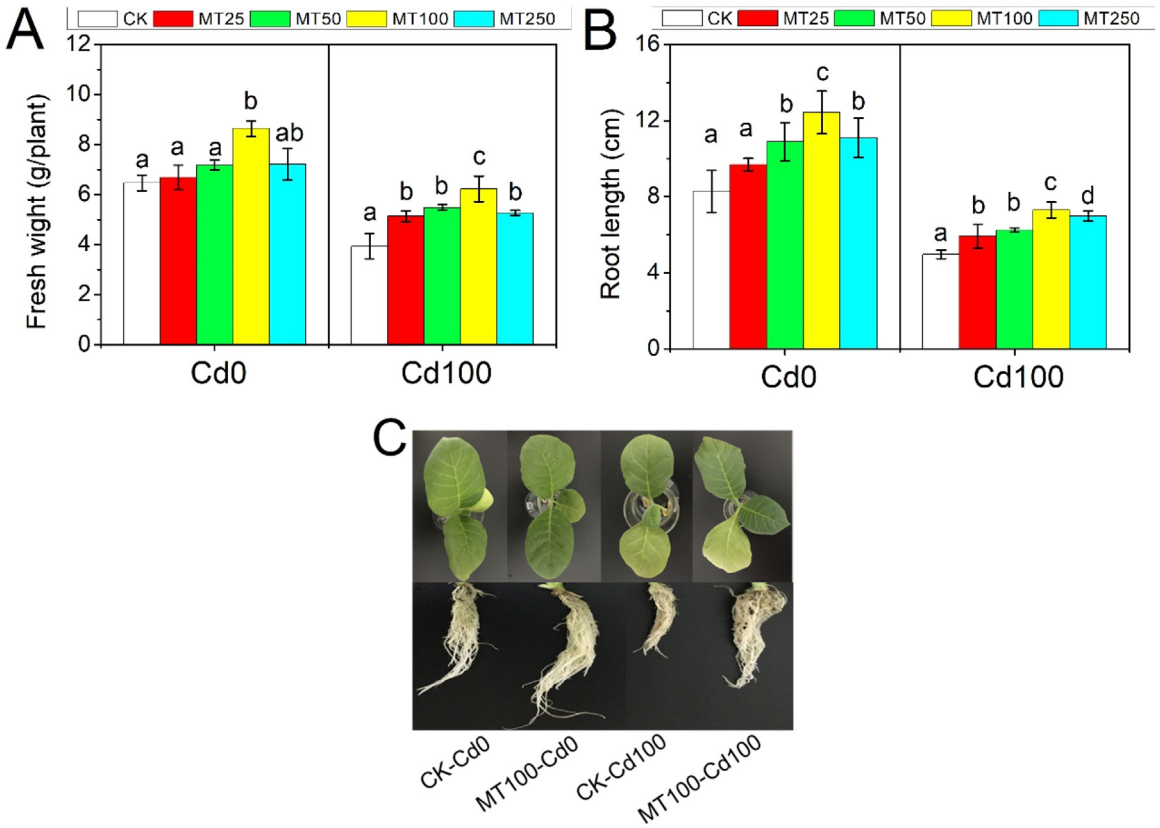

Fig. 1. Foliar application of melatonin alleviates Cd-induced inhibition of tobacco growth. The roots of tobacco seedlings at the two-leaf stage were treated with exogenous MT with/without Cd for 1 week. Afterwards, fresh weight of tobacco seedlings (A) and root elongation (B) were measured, and the corresponding pictures were taken (C). The values are expressed as mean ± SD (standard deviation) of triplicate samples with 10 seedlings each. Columns with different letters denote significant differences at $P < 0.05$ according to Duncan's multiple range test.

Table 1
Exogenous melatonin changes photosynthetic parameters (photosynthetic pigment (Chl), net photosynthetic rate (Pn), stomatal conductance (Gs), intracellular CO_2 concentration (Ci), and transpiration rate (Tr)) in tobacco leaves whether under Cd stress or not. Values are expressed as means ± SD. Significant differences are indicated by different lowercase letters ($p < 0.05$) (these comparisons were done separately for the parameters in each column).

Treatment	Chl (mg g^{-1} fresh weight)	Pn (μmol CO_2 m^{-2} s^{-1})	Gs (mmol H_2O m^{-2} s^{-1}	Ci (μmol CO_2 mol^{-1}	Tr (mmol H_2O m^{-2} s^{-1}
CK-without Cd	2.79 ± 0.15 a	19.5 ± 2.06 a	0.51 ± 0.03 a	320 ± 10.2 a	6.18 ± 0.22 a
CK-Cd100	1.96 ± 0.06 a	11.9 ± 0.62 a	0.37 ± 0.04 a	235 ± 12.2 a	4.86 ± 0.41 a
MT25-without Cd	3.10 ± 0.09 b	20.0 ± 1.66 a	0.59 ± 0.03 b	319 ± 22.1 a	6.25 ± 0.32 b
MT25-Cd100	2.28 ± 0.18 b	13.9 ± 0.98 b	0.49 ± 0.03 b	262 ± 14.5 b	5.92 ± 0.23 b
MT50-without Cd	3.34 ± 0.11c	24.1 ± 1.05 b	0.61 ± 0.05 b	348 ± 25.6 ab	6.89 ± 0.25c
MT50-Cd100	2.59 ± 0.08c	17.8 ± 0.46 d	0.57 ± 0.01c	303 ± 20.1c	7.35 ± 0.29c
MT100-without Cd	3.89 ± 0.07 d	29.3 ± 2.65c	0.69 ± 0.01c	369 ± 14.2 b	7.48 ± 0.36 d
MT100-Cd100	2.59 ± 0.08c	17.8 ± 0.46 d	0.57 ± 0.01c	303 ± 20.1c	7.35 ± 0.29c
MT250-without Cd	3.26 ± 0.09 e	24.4 ± 1.36 b	0.62 ± 0.02 b	342 ± 15.6 a	6.94 ± 0.21c
MT250-Cd100	2.29 ± 0.12 b	15.1 ± 0.66c	0.51 ± 0.01 b	294 ± 11.5c	6.79 ± 0.15 d

Significant differences are indicated by different lowercase letters.

fractions, which accounted for more than 65% of total Cd content under all treatments (Fig. 4B). Importantly, foliar application of melatonin changed subcellular distribution of Cd in tobacco leaves. In particular, the proportions of Cd in vacuole and cell wall fractions increased significantly under melatonin treatment compared with CK, but the Cd levels in the soluble and organelle fractions decreased. Melatonin is beneficial for promoting Cd immobilization and minimizing its cellular toxicity, because Cd in the soluble and organelle fractions is mostly bioavailable and highly toxic.

3.6. Gene expression

Toxic and biologically non-essential metals like Cd can enter plants by the same transporters used for uptake of essential nutrients. Once the toxic metals replace or interfere with the function of essential nutrients, organisms need to maintain specific levels of these nutrients through biochemical methods or changes in gene expression (Mendoza-Cózatl et al., 2011). In this study, the expression of Cd-related genes in tobacco root tissue was investigated. As shown in Fig. 4C, exogenous melatonin dramatically changed the transcript levels of Cd transport genes in tobacco leaves under Cd stress. For instance, the transcript levels of *IRT1*, *Nramp1*, *HMA2*, and *HMA4* were significantly increased in plants treated with melatonin compared to Cd stress alone, while the responses observed in *IRT2* and *HMA3* genes were opposite.

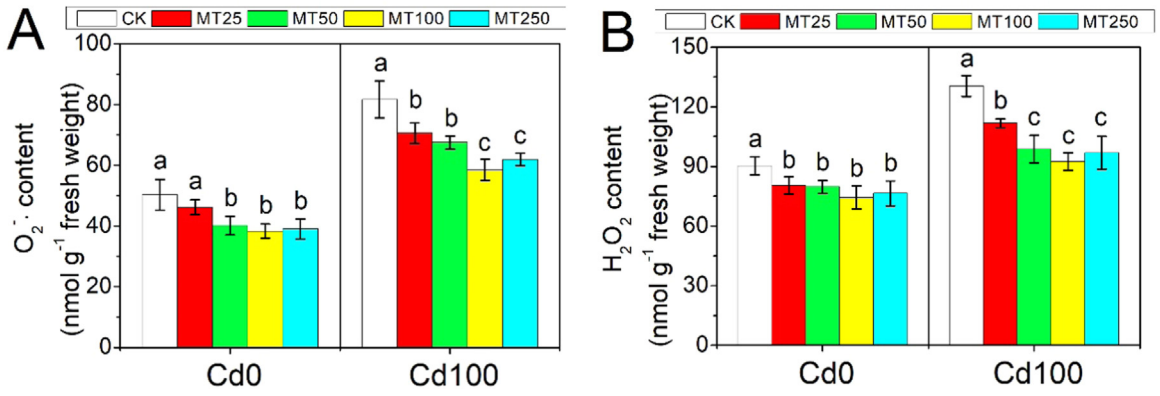

Fig. 2. Exogenous melatonin influences oxidative stress-related parameters whether under Cd stress or not. The roots of tobacco seedlings at the two-leaf stage were treated with exogenous MT with/without Cd for 1 week. Afterwards, quantitative contents of O_2·-(A) and H_2O_2 (B) in tobacco leaves were analyzed.

3.7. TEM-EDS

To explore the visible evidence of decreased Cd phytotoxicity induced by foliar application of melatonin, TEM micrographs were made of root samples taken from no treatment, Cd stress, and 100 μM melatonin under Cd stress after 1-week of exposure. The cross sections of the root after Cd exposure exhibited considerable morphological and physiological differences when compared with the control (no treatment). Roots exhibited degradation of plasma membranes and partial cell wall, cytoplasmic disruptions, and root surface clutter (Fig. 5B). However, full cell structure without disintegration in the root was observed after spraying melatonin (Fig. 5C). The results indicated that melatonin application could be effective in alleviating Cd stress on tobacco root cells. The EDS spot-scanning technique was applied to identify Cd composition of the electron-dense deposits in the intercellular spaces of the root cells. The presence of Cd in the deposits was significant (0.38%) in the treatment of Cd stress (Fig. 5E), while the Cd peak was lower (0.19%) following melatonin addition (Fig. 5F; Table S2).

3.8. SEM analysis

To better integrate the complex interrelationships among changes in tobacco leaf parameters, melatonin application and Cd accumulation, structural equation modeling was used. The latent variables (denoted in dashed rectangles in Fig. 6) were successfully represented as photosynthetic parameters, ROS, enzyme activities and gene expression levels. Results showed that foliar application of melatonin had significant direct effects on improving tobacco leaf quality and inhibiting Cd phytotoxicity, and melatonin influenced Cd availability indirectly through ROS, antioxidative enzyme activities, and the expression levels

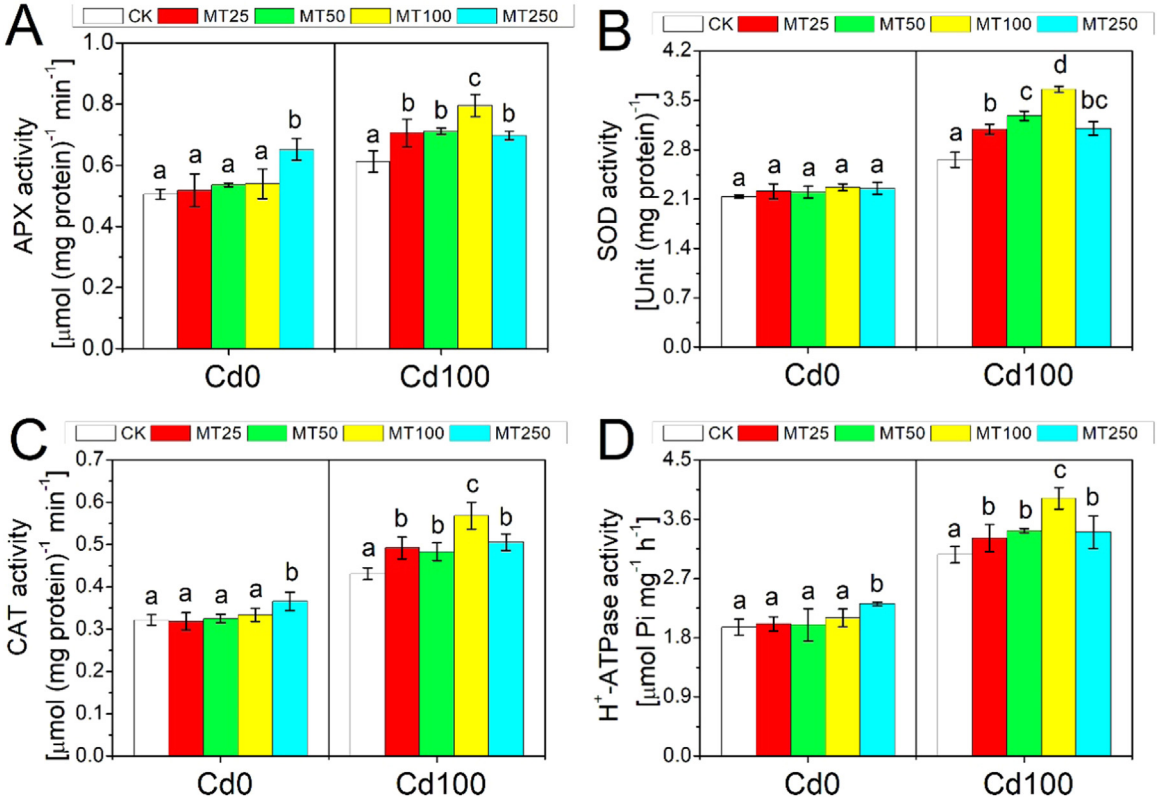

Fig. 3. Foliar application of melatonin alters the activities of enzymatic antioxidants particularly under Cd stress. The roots of tobacco seedlings at the two-leaf stage were treated with exogenous melatonin with/without Cd for 1 week. The data shown are the average of three replicates, with the standard errors indicated by the vertical bars. The means denoted by the same letter do not significantly differ at $P < 0.05$ (Tukey's test).

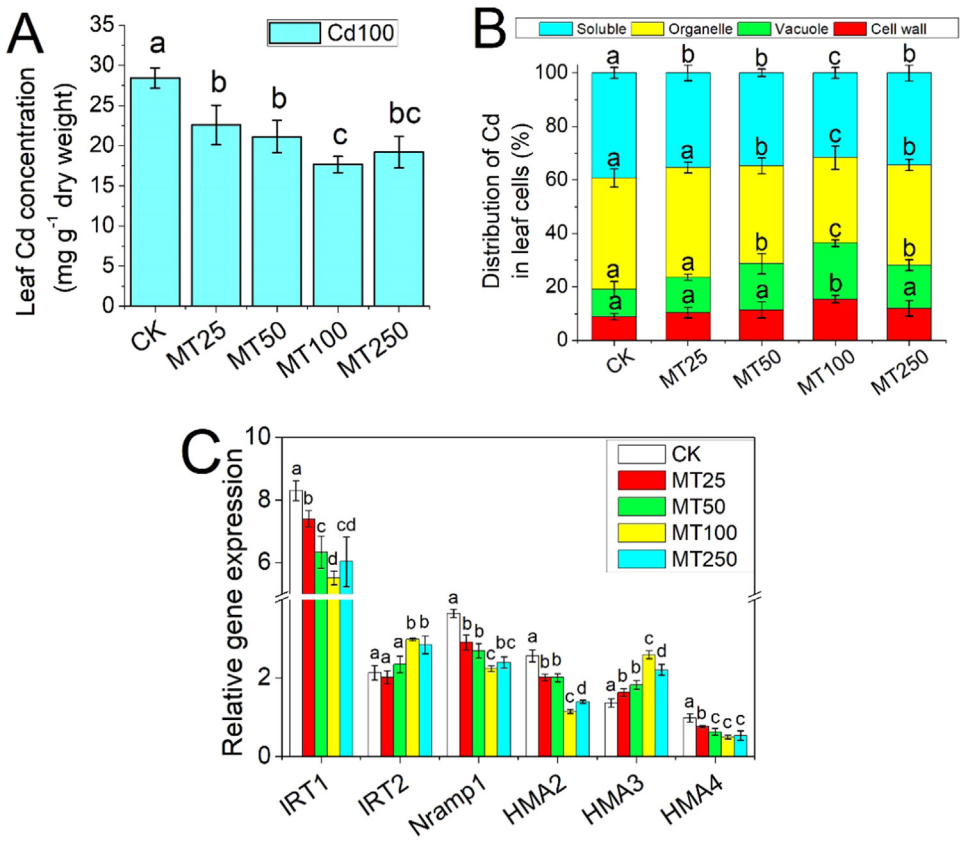

Fig. 4. Melatonin decreases Cd accumulation (A) and changes its subcellular distribution in tobacco leaves (B). Melatonin differentially regulates the expression of Cd transport genes in tobacco root (C). Expression levels of corresponding genes are presented relative to the control samples. The roots of tobacco seedlings at the two-leaf stage were treated with exogenous melatonin with/without Cd for 1 week.

of Cd-related genes.

4. Discussion

Cd is a non-essential and very toxic heavy metal that can enter plant or animal cells by competitively binding to membrane proteins, because these proteins are exclusively used to transport essential divalent metals (iron, zinc, or calcium) (Hasan et al., 2015; Cao et al., 2017). Once taken up by the cells, Cd causes visible symptoms, oxidative stress, lipid peroxidation, and hence malfunctions of cellular organelles and further cell damage (Valko et al., 2005). Melatonin is an animal hormone and plays key roles in modulating circadian rhythms and antioxidant activity (Tan et al., 2012; Manchester et al., 2015). Although in recent years, the protective roles of melatonin in the response of plants to biotic and abiotic stresses due to its ability to scavenge free radicals have been broadly characterized (Posmyk et al., 2008; Li et al., 2016), the corresponding mechanism involved in Cd stress tolerance in tobacco leaves has not been fully explored. In this study, foliar application of melatonin in tobacco leaves significantly improved Cd tolerance as evidenced by promoting plant growth, enhancing antioxidant capacities, and restricting Cd root-to-shoot transportation, ultimately compartmentalizing Cd in cell walls and vacuoles. Melatonin also suppressed the expression of Cd-related genes and minimized Cd uptake or Cd toxicity in roots, which might be responsible for conferring Cd tolerance in tobacco leaves.

4.1. Melatonin counteracts Cd-induced inhibition of tobacco growth

Excess Cd exposure significantly increased Cd concentrations in tobacco leaves to toxic levels (Fig. 4A), which further negatively affected tobacco growth and development. For example, Cd altered basic cell metabolic processes, inhibited photosynthetic abilities, decreased chlorophyll concentration and reduced the weight of tobacco biomass (Table 1; Fig. 1). In this study, foliar application of melatonin dramatically alleviated Cd phytotoxicity in tobacco, as indicated by the larger root length (up to 47.0% increase) and heavier fresh weight of tobacco seedlings (up to 58.4% increase) (Fig. 1). Melatonin addition was also effective in counteracting the degrading effects of Cd on photosynthesis and chlorophyll accumulation. It is worth noting that melatonin application significantly reduced the leaf Cd content, and increased Cd immobilization in the cell wall and vacuoles. The results indicate that melatonin could not only induce a barrier for preventing root-to-shoot translocation of Cd but also promote cell wall binding and vacuole sequestration of Cd, thus minimizing Cd toxicity. It is believed that melatonin-treated plants tend to accumulate higher levels of thiol-peptide in roots, which might act as a sink for thiol-reactive metal and thus play a critical role in sequestering higher levels of Cd (Hasan et al., 2015), Similar to what was found by Li et al. (2016) and Gu et al. (2017) In addition, our data showed that different doses of melatonin (25, 50, 100, and 250 μM) relieved Cd-induced inhibition of tobacco seedling growth at different levels. Moderate concentrations, particularly 100 μM, of melatonin displayed the maximal protective roles. Previous results suggested that as a potential modulator of plant growth, the most effective concentration of melatonin to alleviate stress might depend on the stress itself (Bajwa et al., 2014; Zhang et al., 2015). For example, 10 and 30 μM melatonin played a specific, important role for *Arabidopsis thaliana* challenged with cold stress at 4 °C for 72 and 120 h, respectively (Bajwa et al., 2014). Posmyk et al. (2008) reported that 1 or 10 μM melatonin eliminated the inhibitory effect of 0.5 mM toxic copper ion concentration on seed germination of *Brassica oleracea rubrum*, while 100 μM melatonin had a negative effect. In addition, this study showed that melatonin application without Cd stress also improved tobacco growth to some extent. Melatonin is a kind of indoleamine, it shares same initial biosynthesis compound with auxin, and has similar functions as hormones (H.J. Zhang et al., 2015; N. Zhang et al., 2015; Arnao and Hernández-Ruiz, 2014; Fan et al., 2018).

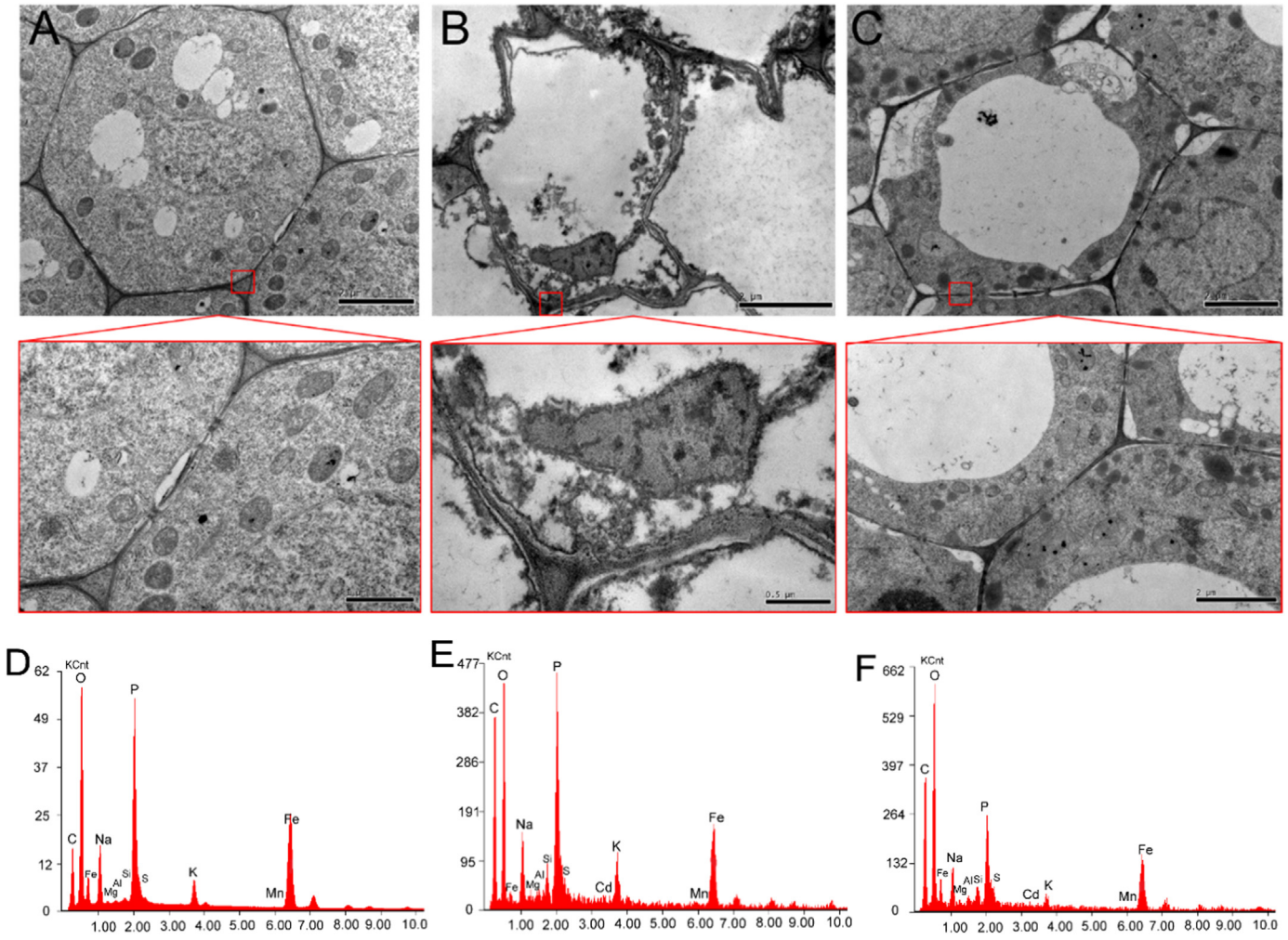

Fig. 5. Transmission electron micrographs show the changes in root cross-sections of tobacco grown in Cd solution with and without melatonin application. (A) no treatment (CK-Cd0); (B) Cd stress (CK-Cd100); (C) treated with 100 μM melatonin under Cd stress (MT100-Cd100). The corresponding EDS spectra of dark dots for A, B, & C in the square area are presented in D, E & F, respectively.

4.2. Melatonin confers Cd tolerance by enhancing antioxidant defense capacities

Numerous studies have shown that exposure to Cd could cause oxidative damage on plants by inducing increased production of the outburst of ROS (Rodríguezserrano et al., 2009). Next, overproduction of ROS could cause the cellular redox perturbation in different cellular compartments, which further leads to damage to proteins, nucleic acids

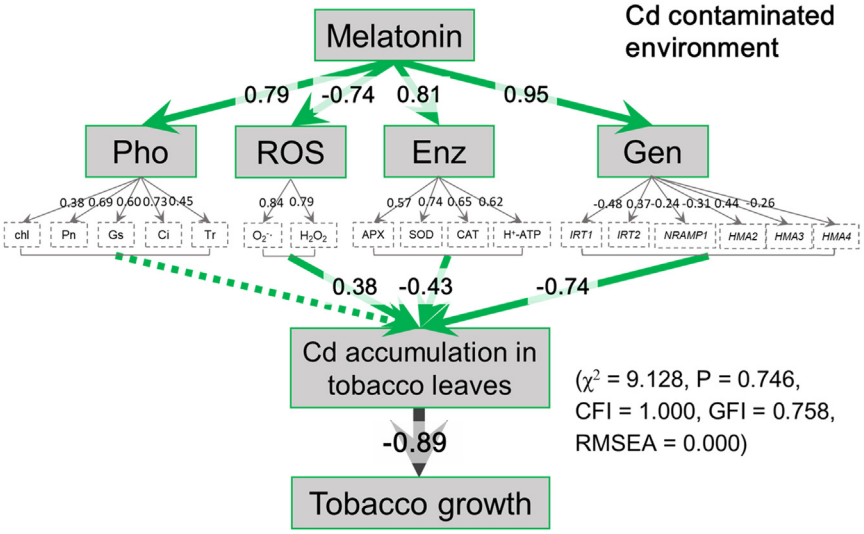

Fig. 6. Structural equation model (SEM) shows the relationship of different parameters. The loadings for five photosynthetic parameters, $O_2^{\cdot-}$ and H_2O_2 contents, activities of four enzymes, and expression levels of six genes that create the latent variables are shown in dashed rectangles. A significant positive correlation is represented by a solid arrow, and a non-significant path is indicated by a dashed arrow. The numbers adjacent to arrows are standardized path coefficients. Pho: photosynthetic parameters; Enz: activities of enzymatic antioxidants; Gen: expression levels of genes.

and metabolic processes in plant cells, which might be related to Cd-dependent phytotoxicity symptoms (López et al., 2006; Ruelland et al., 2009). In the current study, significantly increased concentrations of $O_2^{\bullet-}$ and H_2O_2 were exhibited on tobacco leaves after one week of Cd exposure. It is also known that plants have evolved a complex antioxidative system. Antioxidant enzymes, for example SOD, can catalyze the dismutation of superoxide union to H_2O_2, and then H_2O_2 is further metabolized to H_2O and O_2. SOD is the only enzyme able to scavenge $O_2^{\cdot-}$; APX belongs to the ascorbate-glutathione cycle and plays a critical role in eliminating H_2O_2, similar with CAT (Chen et al., 2017; Lin and Aarts, 2012). Thus, the change in the concentrations of these antioxidant enzymes represents the status of the antioxidative defense in plant cells. Serving as a signal, melatonin plays key roles in scavenging stress-induced ROS and activating the antioxidative system in plants exposed to various stresses like drought, cold, age, salinity, or heavy metals (Arnao and Hernández-Ruiz, 2014; Fan et al., 2018). In the present study, melatonin application significantly inhibited the oxidative damage of Cd stress on tobacco seedlings indicated by decreased $O_2^{\cdot-}$ and H_2O_2 contents. This could be due to the enhanced capacity of the ROS scavenging system, including significant upregulation in the activities of SOD, CAT, and APX.

Lipid peroxidation, induced by ROS injury to biological membranes, is another important stress marker (García et al., 2014). The function of H^+-ATPase is transporting ions and organic compounds through the plasma membrane (Morsomme and Boutry, 2000). In agreement with previous finding (Oner et al., 2002), the current study found that melatonin application increased H^+-ATPase activity in tobacco leaves under Cd stress, indicating that melatonin plays an important role in maintaining plasma membrane stability. This effect might be explained by the fact that under stress exposure, melatonin can be transformed into 5-methoxytryptamine, which can stimulate H^+-ATPase activity in plants (Masson-Pévet et al., 2010). Therefore, we inferred that melatonin might operate by activating antioxidative enzymes and stabilizing the plasma membrane to reconstruct the ROS-balance and to confer tolerance of Cd-induced oxidative stress.

4.3. Melatonin alleviates Cd accumulation by modulating genes involved into Cd uptake and transport

To survive under Cd stress, plants activate a set of genes related to Cd detoxification that may help plants to extrude, sequester, or detoxify Cd ions (Cai et al., 2017). In this study, we found that Cd accumulation in tobacco leaves depended on the genes that regulated Cd sequestration, uptake, and transport (Fig. 6). *HMA* genes are members of the P_{1B}-type ATPase superfamily and they regulate metal homeostasis in plant cells (Lin and Aarts, 2012). Specifically, *HMA2* and *HMA4* are Zn^{2+}-ATPases and have the ability to drive the efflux of metals out of the cell (Barabasz et al., 2013). The downregulation of *HMA2* and *HMA4* was found to play a major role in preventing Cd root-to-shoot transport in the plants *A. halleri* and *N. caerulescens* (Hammond et al., 2006). In the current study, we found that decreased transcript levels of *HMA2* and *HMA4* in melatonin-treated seedlings beneficially reduced Cd root-to-xylem translocation efficiency. In contrast, *HMA3* is a promising candidate for Cd sequestration: *HMA3* usually locates at the tonoplast of root cells and limits Cd root-to-shoot translocation by effective sequestration of Cd into the root vacuoles (Ueno et al., 2009). Therefore, overexpressing *HMA3* due to foliar application of melatonin increased Cd tolerance in tobacco leaves (Fig. 4C). Moreover, *IRT1* and *Nramp1* play critical roles in the passive transport of Cd through channel proteins into plants (Lin and Aarts, 2012). Melatonin application in this study significantly inhibited the up-regulation of the transcript levels of *IRT1* and *Nramp1* induced by Cd (Fig. 4C), thus leading to less accumulation of Cd in leaves (Fig. 4A). Although both *IRT1* and *IRT2* are the members of the ZIP gene family and have similar functions of Fe transport, they had inconsistent expression patterns in melatonin treatments with Cd exposure. A similar phenomenon was observed in a previous study (Wu et al., 2015) and the authors explained that the suppression of *IRT1* might block Fe uptake from the medium, and overexpressing *IRT2* might play a compensation effect to reestablish Fe^{2+} homeostasis. Taken together, the results suggest that sequestrating Cd and decreasing Cd uptake by modulating the Cd-related gene expression might explain how foliar application of melatonin conferred Cd tolerance in tobacco.

In summary, this study provides intriguing evidence that supports our assumption that foliar application of melatonin induces Cd tolerance of tobacco leaves by enhancing antioxidative defense capacities, modulating the expression of Cd-related genes, and promoting cell wall or vacuolar sequestration of Cd. Taken together, our findings provide compelling support for the use of melatonin as an antagonist for Cd toxicity, with major benefits for sustainable tobacco production worldwide.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2018.11.127.

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