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Root-specific expression of rice *OsHMA3* reduces shoot cadmium accumulation in transgenic tobacco

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Abstract Cadmium (Cd) is a toxic trace element released into the environment by anthropogenic activities. Its release threatens the growth of plants and contaminates the food chain. Tobacco (*Nicotiana tabacum* L.) is a Cd accumulator, and tobacco smoking is a major source of Cd exposure for smoking people. In the present study, we generated transgenic tobacco plants expressing the rice heavy metal P-type ATPase 3 gene (*OsHMA3*) in the roots under the control of a novel root-specific promoter from a tobacco root extensin-like protein-coding gene (*NtREL1*). Transgenic plants showed significantly reduced Cd accumulation in the shoots in both hydroponic and soil pot experiments. Analysis of Cd concentration in xylem sap showed that the transgenic plants had significantly decreased root-to-shoot translocation of Cd relative to that of wild-type plants. Moreover, a significantly lower oxidative stress level under Cd stress was observed in the shoots of transgenic plants than in wild-type shoots. These results suggest that *OsHMA3* can be expressed in tobacco plants and may be useful for developing tobacco varieties with a reduced capacity to accumulate Cd in the shoots, potentially reducing the risk of Cd exposure for smoking people.

Keywords Cadmium · *OsHMA3* · Root-specific expression · Tobacco · Heavy metals

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Introduction

Cadmium (Cd) is a well-known toxic heavy metal that has been widely released into the environment as a consequence of rapid industrialization. Once released into the environment, Cd enters the biogeochemical cycle and tends to accumulate in soils and sediments, which results in the contamination of agricultural soils and makes Cd available to crop plants. In the latest national general survey on soil contamination in China, Cd ranked the highest among heavy metals and metalloids (Zhao et al. 2015). Due to its high mobility and toxicity, Cd is one of the most toxic heavy metals in soil and can exhibit highly adverse effects on soil biological activity and biodiversity as well as plant metabolism. Most importantly, as a nonessential element, Cd can be enriched in plants and thereby threaten the health of humans through the food chain (Grant et al. 2008). It has been estimated that over 70% of the dietary intake of Cd is contributed through the food chain (Wagner 1993). Additionally, tobacco smoking is a major source of Cd exposure for smokers (Lugon-Moulin et al. 2004;

Willers et al. 2005; Jarup and Akesson 2009; Clemens et al. 2013). Tobacco (*Nicotiana tabacum* L.) is considered a type of Cd accumulator, being capable of accumulating high concentrations of Cd in its leaves (Lugon-Moulin et al. 2004; Tsadilas et al. 2005; Lugon-Moulin et al. 2006; Geiss and Kotzias 2007; Verma et al. 2010). Currently, tobacco continues to be an important commercial crop in China. Besides, China is also the world's largest consumer of tobacco. In some of the economically under-developed areas of China, tobacco remains a crucial cash crop for farmers. Therefore, it is necessary to reduce leaf Cd accumulation in tobacco plants to minimize the potential health risks to humans.

Numerous studies have revealed a number of genes involved in Cd uptake and translocation in plants. The P1B type ATPases, also known as heavy metal ATPases (HMAs), play crucial roles in metal transport and homeostasis in plants (Williams and Mills 2005). HMA3, a member of the HMA family, has been shown to perform important functions in Cd tolerance and accumulation in the shoots of *Arabidopsis thaliana* (Morel et al. 2009; Chao et al. 2012) and in Cd translocation from roots to shoots in rice (Ueno et al. 2010; Miyadate et al. 2011). This transporter is localized at the tonoplast, which mediates the transport of Cd from the cytoplasm into the vacuoles. In addition, AtHMA3 is involved in the storage of zinc (Zn), cobalt (Co), and lead (Pb) in root vacuoles (Morel et al. 2009). In rice, *OsHMA3* was mainly expressed in the roots and was identified as a causal gene in the major quantitative trait locus responsible for rice shoot Cd accumulation (Ueno et al. 2010). Weak or loss-of-function alleles of *OsHMA3*, which have been characterized in some rice cultivars, result in the accumulation of high concentrations of Cd in the shoots and grains (Ueno et al. 2010; Miyadate et al. 2011; Yan et al. 2016). In contrast, overexpression of the functional *OsHMA3* gene was demonstrated to increase Cd sequestration in the roots and decrease Cd accumulation in the aboveground tissues (Ueno et al. 2010; Sasaki et al. 2014). A recent study revealed that the expression of *OsHMA3* driven by the *OsHMA2* promoter strengthened the expression level and altered the tissue localization of OsHMA3 in rice, resulting in a pronounced reduction of Cd accumulation in rice grains through the enhanced sequestration of Cd into vacuoles in the roots and nodes (Shao et al. 2018). Members of the HMA family in tobacco have been investigated by Hermand et al. (2014). Orthologues for HMA1, HMA2, HMA4, HMA5, HMA6, HMA7, and HMA8 were all uncovered in tobacco plants, whereas no orthologue for HMA3 was identified in Nicotiana species (Hermand et al. 2014). Although a study named the two HMA homologous genes of *AtHMA3* in tobacco *NtHMA3a* and *NtHMA3b* (Chang and Shu 2015), these two homologous genes are actually *NtHMA4.1* and *NtHMA4.2*. No *HMA3* orthologue gene has been identified in tobacco (Hermand et al. 2014; Liedschulte et al. 2017).

In tobacco plants, *NtHMAa/NtHMA4.1* and *NtHMAb/NtHMA4.2*, which are orthologues of *AtHMA2* and *AtHMA4*, respectively, have been identified as responsible for Zn and Cd translocation from roots to shoots (Hermand et al. 2014; Liedschulte et al. 2017). Disruption of these two genes significantly reduced leaf Cd and Zn accumulation in tobacco plants (Hermand et al. 2014; Liedschulte et al. 2017). There have been numerous attempts to reduce Cd accumulation in tobacco leaves through genetic engineering (Korenkov et al. 2009; Han et al. 2014; Siemianowski et al. 2014; Liedschulte et al. 2017; Nesler et al. 2017). It was reported that tobacco plants expressing the bacterial efflux transporters of the CzcCBA system, which exports metals from the cytoplasm or the periplasm across the outer membrane, accumulated less Cd in the leaves than did wild-type plants (Nesler et al. 2017). In addition, overexpression of a xyloglucan endotransglucosylase/hydrolase gene from *Populus euphratica* was reported to reduce Cd accumulation in root cells and increase Cd tolerance in tobacco through limiting root Cd uptake (Han et al. 2014). Root-specific expression of the vacuolar divalent cation/H^+ antiporters AtCAX2 and AtCAX4 from *Arabidopsis thaliana* led to an approximately 20% Cd reduction in transgenic tobacco leaves relative to that of wild-type (Korenkov et al. 2009). Furthermore, ectopic overexpression of *AtHMA4* in tobacco reduced Cd uptake and accumulation by enhancing the physical apoplastic barrier (Siemianowski et al. 2014).

Enhancing vacuolar storage/sequestration of Cd in the roots is an effective strategy for reducing shoot Cd accumulation. Recently, a novel root extension-like protein-coding gene (*NtREL1*) from tobacco was found to be expressed specifically in the root (Zhang et al. 2016a). The root-specific promoter of *NtREL1* was also isolated by characterizing six root-specific motifs associated with root expression (Zhang et al. 2016a). Due to lack of *HMA3* in tobacco, the principal aim of this study was to determine the effect of root-specific expression of *OsHMA3* on Cd accumulation in the leaves of transgenic tobacco. In the present study, *OsHMA3* was

introduced into the roots of tobacco plants driven by the root-specific promoter of *NtREL1*. We investigated Cd accumulation in the roots and shoots and the oxidative stress differences between transgenic lines and wild-type plants. Our findings demonstrated that root-specific expression of *OsHMA3* could reduce Cd accumulation in shoots by enhancing Cd sequestration in root vacuoles in transgenic tobacco plants.

Materials and methods

Plant materials and hydroponic and pot experiments

Experiments were performed on wild-type tobacco (*Nicotiana tabacum* cv. K326) and three homozygous lines (T-1, T-2, and T-3) of transgenic tobacco expressing rice *OsHMA3* specifically in the roots with the root-specific promoter of *NtREL1* (Zhang et al. 2016a). Seeds were surface sterilized in 8% sodium hypochlorite solution and germinated on one-fourth strength Murashige and Skoog (MS) medium solidified with 1.5% agar on Petri dishes and incubated at 22 °C in the dark for 3 days. After germinated, the tobacco seedlings were transferred to a growth chamber under 25/16 °C day/night temperature, 40–50% humidity, and a 16-h photoperiod with a light intensity 250 μmol m^{-2} s^{-1}. Once the seedling roots grew to 2–3 cm, the seedlings were transferred to a 1-L plastic vessel containing an aerated one-fourth strength Hoagland's nutrient solution (pH 5.7) and grown for 2 weeks. Three-week-old seedlings were then transferred to 5 l plastic vessels (four seedlings per box) containing one-half strength Hoagland's nutrient solution (pH 5.7) for 2 weeks. The nutrient solution was aerated continuously and renewed every 3 days. Then, plants of the equal size were transferred to one-half strength Hoagland's nutrient solution supplemented with 0.5 or 5 μM Cd (added as $CdCl_2$) for 3 days. Each line grew in four replicated vessels of Cd treatment. At the end of the experiment, the roots were rinsed twice with 0.5 mM $CaCl_2$ to remove apoplastic Cd and were then rinsed twice with deionized water. Then, the roots and shoots were separated and immediately frozen in liquid nitrogen for H_2O_2 and malondialdehyde (MDA) determination. For Cd content determination, the shoot and root samples were dried at 65 °C for 3 days.

Based on the results obtained from the hydroponic experiment, the transgenic lines and wild-type plants were selected for further soil pot experiments. Soil slightly contaminated with Cd (total Cd 0.62 mg kg^{-1}, pH 6.0) was collected from a tobacco field in Changsha, Hunan Province, China. Pots were filled with 10 kg of soil each. Basal fertilizers (120 mg N kg^{-1} soil as NH_4NO_3, 30 mg P kg^{-1} soil and 75.5 mg K kg^{-1} soil as K_2HPO_4) were added and mixed thoroughly into the soil. Four-week-old seedlings of each line were planted in the soil, with four replicates per line. The plants were grown in a greenhouse under natural sunlight. The growth conditions were 25–31 °C and a constant relative humidity of 60%. Plants were grown for 30 days. Leaves were harvested for the measurement of Cd concentration.

Construction of plant expression vectors

A fragment of 1574 bp upstream of the translational initiation site (ATG start codon) of the root-specific expression gene *NtREL1*, which contained the promoter of *NtREL1* (Zhang et al. 2016a), was isolated and inserted into the *Hind*III/*Kpn*I restriction sites of pMDC32 (GenBank accession no. FJ172534) expression vector using primers pNtREL1-F and pNtREL1-R (Table S1). The *OsHMA3* coding region sequence was amplified from the cDNA library of *Nipponbare* rice using primers OsHMA3-F with the *Asc*I restriction site and OsHMA3-R with the *Pac*I restriction site and sequenced (Table S1). The verified fragment was then inserted downstream of the p*NtREL1* promoter between *Asc*I/*Pac*I restriction sites of the pMDC32 plasmid vector, generating the transformation construct pMDC32–p*NtREL1–OsHMA3*. The binary construct was subsequently introduced into *Agrobacterium tumefaciens* strain LBA4404 for *Agrobacterium*-mediated gene transformation.

Gene transformation of tobacco plants

Agrobacterium-mediated gene transformation of tobacco (*Nicotiana tabacum* cv. K326) was carried out following the standard protocol with some modifications (Gallois and Marinho 1995). A positive *Agrobacterium* colony harboring the recombinant plasmid vector was inoculated into LB (Luria–Bertani) liquid medium containing rifampicin (50 mg/L) and kanamycin (50 mg/L) overnight. The activated *Agrobacterium* strain was transferred to antibiotic-free LB liquid medium and cultivated for 4–5 h until the OD_{600} reached 0.5. Small pieces (2 cm × 2 cm) of sterilized young leaves of wild-type tobacco were immersed into the *Agrobacterium* suspension solution for 10 min and subsequently

transferred to antibiotic-free MS medium for co-cultivation in the dark for 2–3 days. Then, the pieces were transferred onto MS differentiation medium (MS + 2 mg/L 6-BA + 0.1 mg/L NAA + 50 mg/L kanamycin + 200 mg/L cefotaxime) for approximately 20 days. The regenerated buds were cut and transferred to a rooting medium (MS + 0.1 mg/L NAA + 50 mg/L kanamycin + 200 mg/L cefotaxime) for approximately 15 days. DNA was extracted from the rooted tobacco leaves and tested for positive transformation by PCR with the specific primer pair of the *OsHMA3* gene (Table S1). The PCR-positive plantlets were transplanted into soil for growing in a growth room. The progenies of transgenic lines were selected using antibiotics as well as PCR-based screening. The positive lines maintained growth to set seeds until T-2 generation with regular management. Five independent homozygous transgenic lines (named T-1, T-2, T-3, T-4, and T-5) were developed.

RNA isolation and RT–PCR analysis

Plants were pre-cultured in one-half strength Hoagland's nutrient solution for 2 weeks. Total RNA was extracted from the roots and shoots with the use of an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, followed by DNase I digestion (Takara). All RNA samples were quantified at A_{260} using a Nanodrop ND2000 spectrophotometer (Nanodrop, Wilimington, DE, USA). A 1-μg aliquot of total RNA was used to synthesize first-strand cDNAs by using the HiScript First-Strand cDNA Synthesis Kit (Vazyme) according to the manufacturer's protocol. The expression of the transgene in the transgenic lines was evaluated by RT-PCR with the gene-specific primers RTHMA3-F and RTHMA3-R (Table S1), which produced a 460-bp product. RT–PCR was performed on a BioRad CFX96 real-time system using a SYBRGreen Master Mix kit (Vazyme) following the manufacturer's instructions. The *NtActin* gene (GenBank accession no. X63603) was used as a reference gene. The primers used for RT-PCR analysis are listed in Table S1.

Measurement of the cadmium content in plant tissues

The dry plant tissues were ground to fine powder and digested with 5 mL of $HNO_3/HClO_4$ (4:1, *v*/*v*) in a heating block at 100 °C for 20 min. They were then heated 190 °C for 60 min until the liquid evaporated. A certified reference material (rice flour GBW10045, Institute of Geophysical and Geochemical Exploration, China) and blanks were included in the digestion for quality control. The residues were re-dissolved in 100 mL of deionized water for Cd content measurement by inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7500, Agilent Technologies Inc., USA). The shoot/root ratio of Cd was calculated as follows: Cd content in leaves/Cd content in roots. To analyze Cd concentration in the xylem sap, 4-week-old tobacco seedlings of wild-type and of transgenic lines with root-specific expression of *OsHMA3* were exposed to one-half strength Hoagland's solution supplemented with 10 μM Cd. After 30 min, stems were cut at 4 cm above the roots, and xylem exudates were collected by using clean absorbent cotton for 12 h. After centrifugation, 0.3–1.5 mL of xylem sap were collected from each seedling. The collected sap was filtered through a 0.45 μm membrane filter, and total Cd concentration was determined by ICP-MS (Agilent 7500, Agilent Technologies Inc., USA). The experiment was performed with three replicates per line, with each replicate containing four to six seedlings.

Determination of H_2O_2 and MDA contents

H_2O_2 concentration was determined colorimetrically as described by Jana and Choudhuri (1982) with some modifications. Fresh leaf material (0.5 g) was ground to a fine powder in liquid nitrogen and homogenized with 5 mL of 5% trichloroacetic acid (TCA) and 0.15 g of activated charcoal. The homogenate was centrifuged at 6000×*g* for 25 min. Three milliliters of extracted solution was mixed with 1 mL of 0.1% titanium chloride in 20% (*v*/*v*) H_2SO_4. The mixture was then centrifuged at 6000×*g* for 15 min. The absorbance of the colored supernatant was measured at 410 nm. H_2O_2 concentration was calculated using the extinction coefficient 0.28 $mmol^{-1}$ cm^{-1}.

Malondialdehyde (MDA), routinely used as an indicator of lipid peroxidation, was determined colorimetrically as described previously (Shah et al. 2001). Briefly, leaf tissues were grounded to a fine powder in liquid nitrogen and homogenized in 5% (*w*/*v*) trichloroacetic acid (TCA). The re-suspended solution was then centrifuged at 4000×*g* for 10 min. The supernatants were collected and reacted with an equal volume of 0.67% (*w*/*v*) thiobarbituric acid (TBA) in a boiling water bath for 30 min. After cooling, the mixture was centrifuged at 4000×*g* for 10 min. The absorbance of the colored

supernatant was measured at 532 nm, and the nonspecific turbidity was corrected by subtracting the absorbance at 600 and 450 nm.

Data analysis

Data were analyzed by analysis of variance (ANOVA) followed by comparisons between means using the Tukey's HSD test at a probability of $P < 0.05$. Statistical analyses were performed using SPSS18.0.

Results

Root-specific expression of *OsHMA3* cDNA in transgenic tobacco

To enable the root-specific expression of the *OsHMA3* gene in tobacco plants, a fragment of 1574 bp upstream of *NtREL1*, which contained the promoter of root-specific expression gene *NtREL1* (Zhang et al. 2016a), was isolated and inserted into the *Hind*III/*Kpn*I restriction sites of pMDC32 expression vector (GenBank: FJ172534.1). Then, the full-length cDNA of *OsHMA3* was isolated and linked to the *NtREL1* promoter for root-specific expression, thereby generating the construct pMDC32-p*NtREL1-OsHMA3* (Fig. 1a). The construct was introduced into tobacco using *Agrobacterium tumefaciens*-mediated transformation, which yielded 18 independent positive transgenic lines. Among the T1 lines, five lines segregated at an approximate ratio of 3:1 based on hygromycin resistance, and their seeds were harvested. Moreover, seedlings from all the transgenic T-2 lines grew well on MS medium containing 25 mg L^{-1} hygromycin. Three independent T-2 homozygous lines of p*NtREL1*:*OsHMA3* plants, named T-1, T-2 and T-3, were selected for further analysis.

The transcript levels of *OsHMA3* in the transgenic plants were determined by RT-PCR analysis. For this purpose, total RNA was extracted from the leaf and root tissues of 14-day-old seedlings grown under normal growth conditions. *OsHMA3* expression was clearly detectable in the roots of all the transgenic lines but not in the wild-type control (Fig. 1b). The transgene was expressed at high levels in the roots of the p*NtREL1*:*OsHMA3* plants but not in the shoots, thus verifying the root-specific expression of *OsHMA3* in the transgenic tobacco plants (Fig. 1b).

Root-specific expression of *OsHMA3* in tobacco reduced shoot Cd accumulation

To investigate the effect of root-specific expression of *OsHMA3* on Cd accumulation in transgenic tobacco plants, 4-week-old tobacco seedlings of T-1, T-2, T-3, and the wild-type plants were exposed to 0.5 and 5 μM Cd for 3 days. There were no significant differences between the transgenic lines and the wild-type plants in the shoot and root biomass at any level of Cd (Fig. 2a, c). The Cd concentrations in the shoots of T-1, T-2, and T-3 lines were significantly ($P < 0.01$) lower than those in the shoots of wild-type plants, being lower by 37–47% and 33–36% at 0.5 and 5 μM Cd, respectively (Fig. 2b). Although the transgenic lines showed increases in root Cd concentration relative to wild-type plants at 0.5 μM Cd, the differences were not statistically significant except for line T-1 (Fig. 2d). In contrast, at 5 μM Cd, the Cd concentrations in the roots of the transgenic lines were significantly ($P < 0.05$) higher, by 17–24%, than those of wild-type plants (Fig. 2d).

Significant differences were found in the shoot/root ratio of Cd concentration between the transgenic lines and wild-type plants. The shoot/root ratio of Cd concentration was 0.57 and 0.50 in wild-type plants under the 0.5 and 5 μM Cd conditions, respectively, whereas that in transgenic lines was 0.27–0.29 and 0.23–0.26 in the 0.5 and 5 μM Cd treatments, respectively (Fig. 3).

Root-specific expression of *OsHMA3* in tobacco decreased Cd concentration in xylem sap

The phenotype of decreased Cd accumulation in the shoots of transgenic tobacco lines suggests that root-specific expression of *OsHMA3* reduces Cd translocation from the roots to the shoots. To investigate whether root-specific expression of *OsHMA3* affects the root-to-shoot translocation of Cd, xylem exudates from transgenic lines and wild-type plants grown in hydroponics and subjected to 10 μM Cd treatment were collected and analyzed. The total Cd concentrations of the xylem sap were significantly lower, by 32–37% in the transgenic lines than in the wild-type plants (Fig. 4). These results indicate that root-specific expression of *OsHMA3*

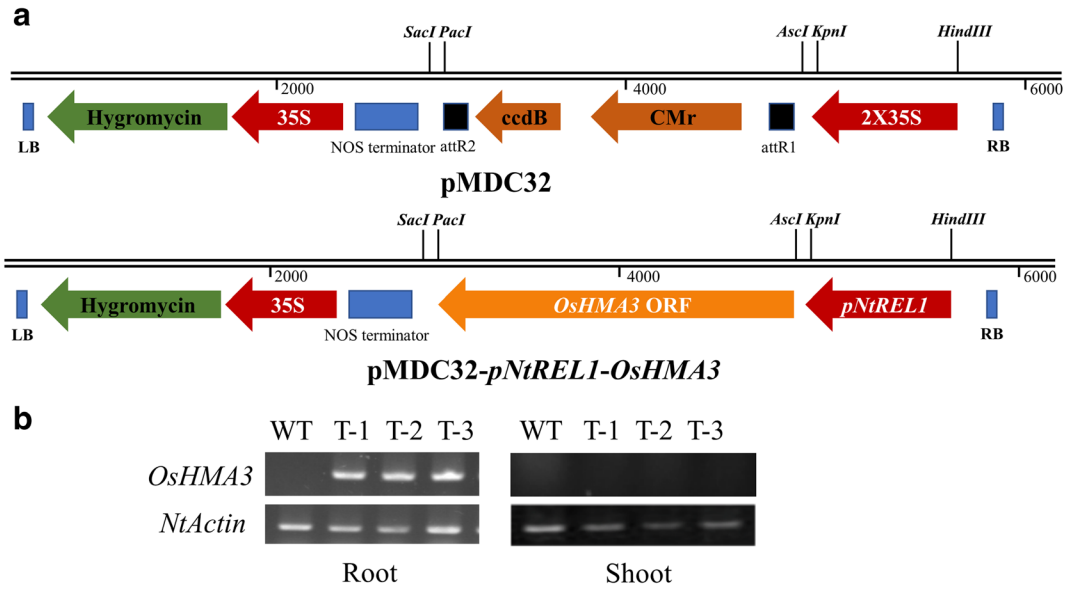

Fig. 1 Expression of *OsHMA3* in tobacco roots is controlled by the *NtREL1* promoter. **a** Schematic diagram of root-specific expression construction. *pNtREL1* root specific promoter from tobacco root extensin-like protein (NtREL1) gene, *OsHMA3* heavy metal ATPase 3 gene from rice, NOS nopaline synthase transcriptional terminator, 35S cauliflower mosaic virus 35S promoter, Hygromycin hygromycin resistance gene, LB left border, RB right border. **b** PCR products of the *OsHMA3* gene from the root and shoot of transgenic tobacco plants. WT wild-type tobacco plant, T-1, T-2, and T-3 individual transgenic tobacco lines

reduces shoot Cd concentration by limiting Cd loading into the xylem.

Root-specific expression of *OsHMA3* in tobacco decreased H_2O_2 and MDA contents in shoots

After exposing the plants to Cd stress, the degree of oxidative stress caused by Cd accumulation in transgenic plants was estimated from the H_2O_2 and MDA contents, which indicate ROS level and the extent of lipid peroxidation, respectively. H_2O_2 and MDA contents in both leaf tissues and roots were measured. There were no significant differences in root H_2O_2 concentration between the transgenic lines and wild-type plants at either 0.5 or 5 μM Cd (Fig. 5a). However, root-specific expression of *OsHMA3* resulted in a significant decrease in H_2O_2 concentration in the shoots of transgenic lines relative to the level in wild-type shoots in both the 0.5 and 5 μM Cd treatments (Fig. 5b). At 0.5 and 5 μM Cd, the shoot H_2O_2 contents of transgenic lines were 21–22% and 32–36%, respectively, lower than those of wild-type plants (Fig. 5b).

Consistent with the variation in H_2O_2 content, no significant differences in root MDA concentration were observed between the transgenic lines and the wild-type plants in either the 0.5 or 5 μM Cd treatment (Fig. 5c). Further analysis showed that the MDA concentrations in the shoots of transgenic lines following 0.5 μM Cd treatment were significantly reduced from the corresponding wild-type levels by 20–26% (Fig. 5d). At the Cd concentration of 5 μM, the shoot MDA contents of transgenic lines were decreased by 14–17% relative to the shoot content of wild-type plants (Fig. 5d).

Root-specific expression of *OsHMA3* in tobacco reduced leaf Cd accumulation in the soil pot experiment

To confirm the effects of root-specific expression of *OsHMA3* on Cd accumulation in tobacco leaves, the transgenic lines with root-specific expression of *OsHMA3* were further tested in a pot experiment with Cd contaminated soil. After 30 days of growth in the pots, Cd accumulation in the leaves of the transgenic lines was 30–34% lower than that in the leaves of wild-type plants (Fig. 6). The Cd bioconcentration factor, i.e., the ratio of leaf Cd concentration to soil Cd concentration, was 18.2 and 12.0–12.7 for wild-type plants and transgenic lines, respectively, indicating generally high Cd accumulation in tobacco plants.

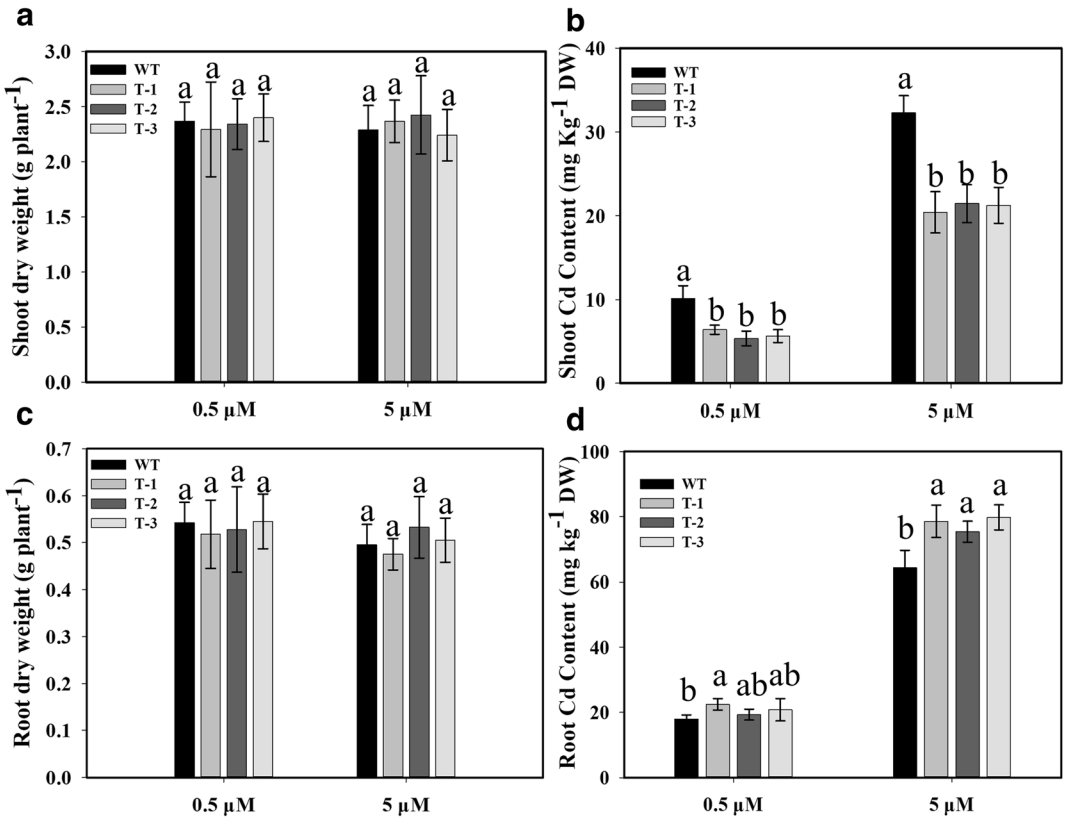

Fig. 2 Differences in biomass and Cd accumulation between wild-type tobacco plants and transgenic tobacco lines grown in hydroponic culture with 0.5 or 5 μM Cd for 3 days. **a**, **c** Shoot and root dry weight of wild-type plants and transgenic lines exposed to 0.5 or 5 μM Cd. **b**, **d** Cd accumulation in shoots and roots of wild-type plants and transgenic lines exposed to 0.5 or 5 μM Cd. Data are means ± SD ($n = 4$ replicates). Significant differences between the wild-type plants and transgenic lines are indicated by different letters ($P < 0.05$)

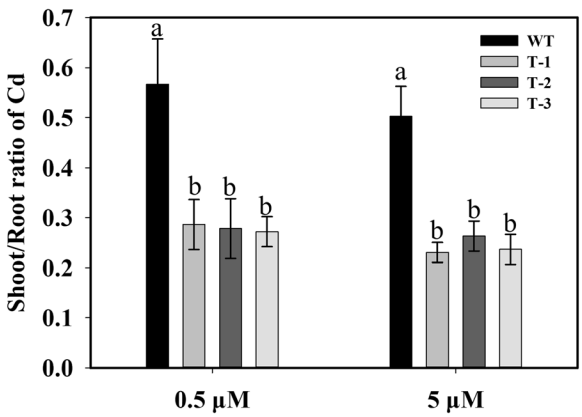

Fig. 3 The shoot to root Cd concentration ratio in wild-type plants and transgenic lines with root-specific expression of *OsHMA3* exposed to 0.5 or 5 μM Cd for 3 days. Data are means ± SE ($n = 4$ replicates). Significant differences between the wild-type plants and transgenic lines are indicated by different letters ($P < 0.05$)

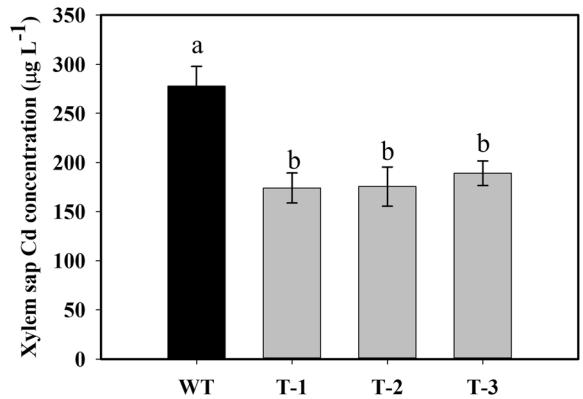

Fig. 4 Cd concentrations in the xylem sap of wild-type plants and transgenic tobacco lines with root-specific expression of *OsHMA3*. Four-week-old tobacco seedlings of wild-type plants and transgenic lines were grown in one-half strength Hoagland's solution containing 5 μM Cd. After 30 min, the rice plants were cut 4 cm above the roots, and xylem exudates were collected for 12 h. Data are the means ± SD ($n = 4$). Significant differences between the wild-type plants and transgenic lines are indicated by different letters ($P < 0.05$)

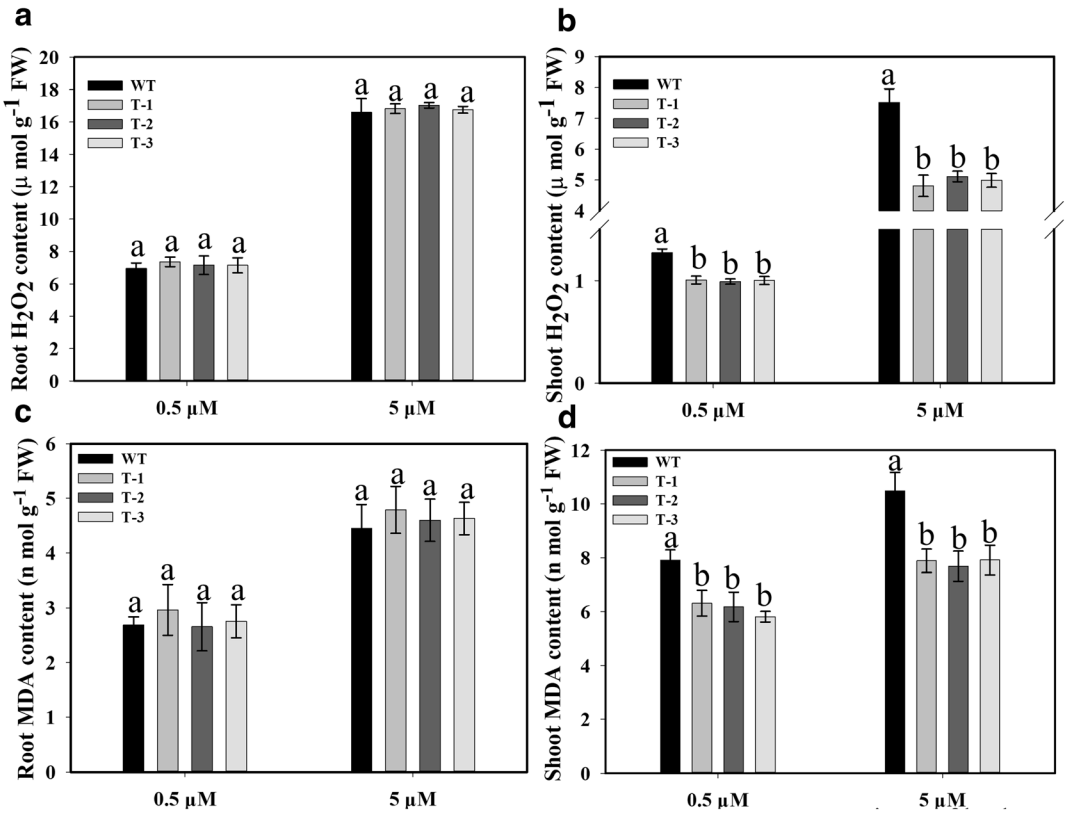

Fig. 5 Effects of root-specific expression of *OsHMA3* on the concentrations of hydrogen peroxide in roots (**a**) and shoots (**b**) and on malondialdehyde (MDA) in roots (**c**) and shoots (**d**) in transgenic tobacco plants. Plants were grown hydroponically under different Cd concentrations for 3 days. Data are means ± SD ($n = 4$). Significant differences between the wild-type plants and transgenic lines are indicated by different letters ($P < 0.05$)

Discussion

Nicotiana tabacum (common tobacco) has a capacity to accumulate high concentrations of Cd in the shoots. This capacity was evident from the high Cd bioconcentration factor observed in the pot experiment as well as previous reports (Lugon-Moulin et al. 2004; Lugon-Moulin et al. 2006; Verma et al. 2010; Liu et al. 2011). In tobacco plants, roots control the amount of Cd translocated to the shoots and xylem loading is the limiting step in this process (Lugon-Moulin et al. 2004). The strategy to minimize Cd accumulation in tobacco leaves is to reduce Cd uptake or xylem loading in the roots. As the entry of Cd into plant root cells is mainly mediated by transporters for other essential metals, disruption of these transporters can be expected to negatively affect the uptake of essential elements for plant growth while reducing Cd uptake. Therefore, limiting xylem loading of Cd, which can be achieved by disrupting the transporters for xylem loading of Cd or sequestering Cd in root vacuoles, is a better way to reduce Cd accumulation in tobacco leaves.

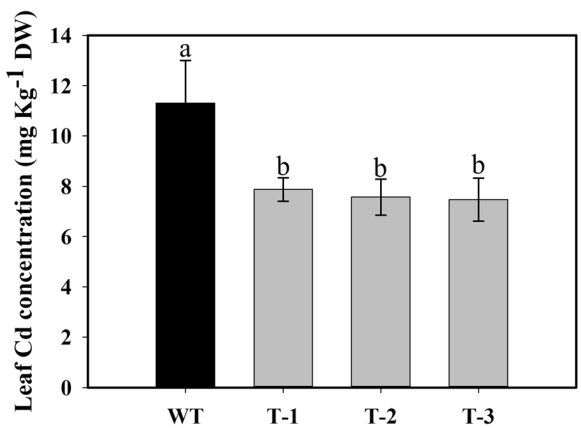

Fig. 6 Cd concentrations in the leaves of wild-type plants and transgenic lines in a pot experiment with Cd-contaminated soil. Data are means ± SD ($n = 4$ biological replicates). Significant differences between the wild-type plants and transgenic lines are indicated by different letters ($P < 0.05$)

In tobacco plants, mutation of the *NtHMA4.1/4.2* genes, which are mainly expressed in root vascular cells and leaf veins, lead to 50–90% reduction of Cd in leaves (Hermand et al. 2014; Liedschulte et al. 2017), suggesting that such mutation may be an effective way to reduce Cd accumulation in tobacco leaves. However, the *NtHMA4.1/4.2* genes seem to be essential for the transport of Zn into the pollen grain and for its germination, furthermore, mutation of these genes have negative effects on plant growth (Hermand et al. 2014). Thus, increasing vacuolar storage/sequestration of Cd in the roots may represent a better way to minimize xylem loading of Cd.

In recent years, the physiological roles of *HMA3* in plants have been extensively studied, such as those of *AtHMA3* in *A. thaliana* and *OsHMA3* in rice. Overexpression of *AtHMA3* in *A. thaliana* or of *OsHMA3* in rice greatly enhances tolerance to Cd toxicity (Morel et al. 2009; Sasaki et al. 2014). Functional *AtHMA3* or *OsHMA3* is expressed in root cells and transports most Cd into the root vacuoles, causing high Cd retention in the roots. This activity restricts Cd translocation from roots to shoots. Therefore, in plants, *HMA3* not only plays an important role in Cd tolerance but also contributes to the regulation of Cd accumulation in shoots. The lack of the *HMA3* gene in tobacco may partially explain the high Cd accumulation in the shoots of this species. A previous study reported that overexpression of *OsHMA3* increased the accumulation of both Cd and Zn in rice roots, suggesting that OsHMA3 is able to transport both Cd and Zn into the root vacuoles (Sasaki et al. 2014). However, previous studies have also demonstrated that *OsHMA3* mainly affects the vacuolar sequestration and subsequent root-to-shoot translocation of Cd, thus, silencing or overexpressing *OsHMA3* has little effect on Zn accumulation in rice shoots or grains (Ueno et al. 2010; Sasaki et al. 2014; Yan et al. 2016). These studies suggest that *OsHMA3* may be a good candidate gene for vacuolar sequestration of Cd in plant root cells.

In the present study, we generated transgenic tobacco lines expressing *OsHMA3,* with *OsHMA3* expression driven by the root-specific *NtREL1* promoter (Fig. 1). It has been reported that *NtREL1* is specifically expressed in the roots of tobacco and that the promoter of *NtREL1* is a novel root-specific promoter with high transcriptional activity (Zhang et al. 2016a). The presence of heterologous *OsHMA3* mRNA specifically detected in the roots of transgenic lines indicated that the rice gene can be functionally transcribed in tobacco root cells (Fig. 1). In rice plants, expression of *OsHMA3* under the control of the *OsHMA2* promoter does not significantly affect growth at the vegetative stage (Shao et al. 2018). Furthermore, overexpression of *OsHMA3* in rice does not significantly affect growth under low Cd exposure (Sasaki et al. 2014). In the current study, there were no significant differences in biomass between any of the transgenic lines and the wild-type plants exposed to either 0.5 or 5 μM Cd (Fig. 2a, c), suggesting that root-specific expression of *OsHMA3* in tobacco has no significant effects on growth.

After Cd treatment in the hydroponic experiment, the transgenic lines had significantly decreased Cd accumulation in the shoots relative to that in wild-type plants under both the 0.5 and 5 μM Cd conditions (Fig. 2b). The decreased Cd concentrations in the shoots of the transgenic lines were caused by the enhanced sequestration of Cd into vacuoles in the roots, consistent with the function of *OsHMA3* in sequestering Cd into vacuoles (Ueno et al. 2010; Sasaki et al. 2014). Consistent with the hydroponic experiment, in the pot experiment, the transgenic lines showed significantly lower Cd accumulation in the leaves than did the wild-type plants (Fig. 6). In rice, the overexpression of functional *OsHMA3* enhances the vacuolar sequestration of Cd in roots, resulting in high accumulation of Cd in the roots and low accumulation of Cd in the shoots (Sasaki et al. 2014). In the present study, the expression of *OsHMA3* under the control of the *NtREL1* promoter in tobacco successfully achieved the overexpression of functional *OsHMA3* specifically in the roots of transgenic tobacco plants, suggesting that root-specific expression of *OsHMA3* can effectively reduce Cd accumulation in tobacco leaves.

In contrast, the transgenic lines showed higher Cd accumulation in the roots than did the wild-type plants when exposed to 0.5 or 5 μM Cd, although the difference was not significant for the T-2 and T-3 lines at 0.5 μM Cd (Fig. 2d). The enhanced Cd accumulation in the roots of the transgenic lines, caused by root-specific expression of *OsHMA3*, suggests that *OsHMA3* functions in Cd sequestration into vacuoles for detoxification in tobacco plants. The root-to-shoot translocation efficiency of Cd, measured as the ratio of shoot Cd concentration to root Cd concentration, was significantly lower in the transgenic plants than in the wild-type plants under both Cd levels (Fig. 3). This finding is consistent with results regarding *SaHMA3h* overexpression in tobacco (Zhang

et al. 2016b). These results suggest that root-specific expression of *OsHMA3* in tobacco is involved in Cd translocation from roots to shoots. However, there was no significant difference in total Cd content between wild-type plants and the transgenic lines (Supplemental Fig. S1). It is possible that preferential capture of Cd by the root vacuoles of the transgenic plants decreased the root-to-shoot translocation of Cd (Ueno et al. 2010). Cd translocation via xylem loading is a key process in Cd accumulation in the aerial parts of plants (Clemens et al. 2002; Uraguchi et al. 2009). The significantly reduced Cd concentrations in the xylem sap of transgenic lines relative to the corresponding concentrations in wild-type plants further demonstrate that the root-specific expression of *OsHMA3* reduced xylem loading of Cd in the tobacco plants.

Many studies have demonstrated that oxidative stress is a primary effects of Cd exposure (Smeets et al. 2009; He et al. 2011; Gallego et al. 2012; Tamas et al. 2017; Ahmadi et al. 2018). In the present study, we used H_2O_2 levels as an indicator of oxidative stress. Decreased levels of H_2O_2 in the shoots were observed in the transgenic lines exposed to either 0.5 or 5 μM Cd (Fig. 5). A consequence of oxidative stress is ROS-induced lipid peroxidation of membranes, which can be indirectly measured by measuring the production of MDA (Mittler 2002). Consistent with the H_2O_2 contents in shoots, the MDA contents in shoots were significantly lower in the transgenic lines than in the wild-type plants (Fig. 5). These results are in accordance with the decreased Cd accumulation in the shoots of the transgenic lines, suggesting that the root-specific expression of *OsHMA3* relieved the oxidative stress in the shoots of transgenic plants through reducing shoot Cd accumulation. Although root accumulation of Cd was higher in the transgenic lines than in the wild-type plants, no significant difference in H_2O_2 or MDA level in the roots was observed between the transgenic lines and wild-type plants (Fig. 5). This result may have been obtained because most of the accumulated Cd was stored in the vacuoles through the activity of OsHMA3, resulting in low oxidative stress to the root cells.

In conclusion, the results of the present study showed that the expression of *OsHMA3* in tobacco roots driven by the root-specific *NtREL1* promoter increased the vacuolar sequestration of Cd in the roots, resulting in reduced Cd loading into the xylem and decreasing Cd accumulation in leaves. This strategy may be useful for developing tobacco varieties with a reduced capacity to accumulate Cd in the shoots, potentially reducing the risk of Cd exposure for smoking people.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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