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The Inhibition Analysis of Two Heavy Metal ATPase Genes (NtHMA3a and NtHMA3b) in *Nicotiana tabacum*

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ABSTRACT Previous studies suggested that plants detoxified mercury and cadmium through similar mechanisms. A heavy metal ATPase (adenosine triphosphatase) gene, HMA3, plays a key role in the plant's cadmium detoxification. To prove whether HMA3 also participates in mercury detoxification in plants, an experiment was designed to inhibit the expressions of HMA3 genes (NtHMA3a and NtHMA3b) in tobacco plants. Results showed that plants' tolerance to mercury ions had not changed after the expressions of NtHMA3a and NtHMA3b were inhibited. When mercury content was measured from the whole seedlings, no differences had been observed among wild-type, NtHMA3a-NtHMA3b-RNAi, and the empty-vector transgenic plants. HMA3 was not the key gene responsible for plants' mercury ion uptake from soil. Although the mercury content in the root was higher than that in the shoot for each seedling, in each treatment, neither in shoots nor in roots were statistical differences in mercury content found among NtHMA3a-NtHMA3b-RNAi, empty-vector transgenic, and wild-type plants. After the expressions of NtHMA3a and NtHMA3b were inhibited, the movement of mercury ions from root to shoot had not been affected. HMA3 was not the key gene responsible for mercury ion transportation from root to shoot. When mercury content was measured from the whole seedling, no significant difference had been found among wild-type, NtHMA3a-NtHMA3b-RNAi, and the empty-vector transgenic plants. For mercury ion translocation across tonoplast, the main pathway might not be HMA3, but ABC (ATP-binding cassette) transporters.

KEYWORDS HMA3, mercury, RNAi, tonoplast

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INTRODUCTION

With the development of industry and gold-mining activities, large quantities of wastewater containing mercury (Hg) was discharged into agricultural soil (Feng et al. 2002; Hassett et al. 2009; Mukherjee et al. 2004; Wu and Cao 2010; Wu et al. 2006). Mercury has become one of the primary heavy metals

that pollute soil (Cui and Zhang 2004). In some sites of Dengfeng, China, mercury concentration had passed the warning level (Yang et al. 2010). According to the criterion of soil quality made by the Department of Environment, China (GB15618-1995), 1.0 mg/kg is the mercury threshold value for protecting agricultural production and human health, and 1.5 mg/kg is the mercury threshold value for normal growth of plants. However, mercury contents of all of the 48 samples from agricultural soil in Qingzhen, Guizhou Province, China, exceeded 3 mg/kg (Matsuyama, Taniguchi, and Yasuda 2009).

When soil is contaminated, mercury can flow into food through crops and underwater channels (Cachada et al. 2009). Finding ways to remove mercury from soil is important for protecting human health. Current methods applied mainly include physical methods and chemical methods (Wang et al. 2012). However, such methods bring in the need for large funds and manpower (Gardea-Torresdey et al. 1990; Ritter and Bibler 1992). It is easy to bring in second pollutions (Gardea-Torresdey et al. 1990; Ritter and Bibler 1992). They are not fit for remediation for a large area of polluted soil. Comparatively, phytoremediation can not only decrease the expense, but also reduce the impacts on the environment (Von Castein, Li, and Wagner-Dobler 1999; Von Castein et al. 2001; Wagner-Dobler et al. 2000).

Studies on the phytoremediation of mercury are now mainly focused on the introduction of bacterial genes *mer*B and *mer*A into plants, which convert organic mercury into mercuric ion and into the volatile Hg(0) (Nagata et al, 2006, 2009; Shafiul et al. 2010). However, how plants assimilate mercury from soil, which genes participate in this process, which organelles participate in transporting and storing mercury are not clear. In spite of these factors, several evidences have suggested that plants detoxify mercury and cadmium (Cd) through similar mechanisms (Chen, Yang, and Wang 2009; Iglesia-Turino et al. 2006; Mendoza-Cozatl et al. 2011; Gong, Lee, and Schroeder 2003; Gueldry et al. 2003). Hg(II) and Cd(II) were both reported to be conjugated by chelator phytochelatins (PCs) (Chen, Yang, and Wang 2009; Iglesia-Turino et al. 2006; Mendoza-Cozatl et al. 2011). Cad1, a loss-of-function mutant of AtPCS1, exhibited a hypersensitive phenotype to both Hg(II) and Cd(II) (Gong, Lee, and Schroeder 2003). For both Hg and Cd detoxification, transporting of metal ions into the vacuole is thought to be important (Gueldry et al. 2003; Mendoza-Cozatl et al. 2011). These studies demonstrated that the main mechanism for cadmium detoxification in plant may also play a key role in mercury phytoremediation.

HMA3, a heavy metal ATPase (adenosine triphosphatase), was found as the key protein controlling the process of cadmium detoxification in plants (Mendoza-Cozatl et al. 2011; Ueno et al. 2011). HMA3 was highly expressed in the Cd hyperaccumulator *Arabidopsis halleri* (Becher et al. 2004). Subcellular localization of TcHMA3 showed that this protein localized on the tonoplast (Ueno et al. 2011). Overexpression of HMA3 in *Arabidopsis* can enhance the plant's accumulation of cadmium (Morel et al. 2009). HMA3 was a determinant for Cd hyperaccumulation in Cd hyperaccumulator *Thlaspi caerulescens* (Ueno et al. 2011). Storage of cadmium in the vacuole is the main mechanism for cadmium detoxification in plants (Mendoza-Cozatl et al. 2011). HMA3 might be the major protein responsible for transporting Cd-PCs chelators into the vacuole.

Based on the studies described above, it can be deduced that HMA3 might also play critical roles for mercury detoxification in plant. If proven, engineered plants with high efficiency may be constructed for removing mercury from soil. To verify this deduction, the expressions of HMA3 genes (NtHMA3a and NtHMA3b) were excluded in this study. The responses of the transgenic plants to mercuric ions were reported. Mercury contents in different tissues or whole seedlings were measured. The mechanisms for mercury detoxification in plants were also discussed.

MATERIALS AND METHODS

Plants, Bacteria, Vectors, and Chemicals

The agrobacterium used was EHA105. Tobacco (*Nicotiana tabacum*; Zhongyan100) used was donated by the Center of Tobacco Improvement, China (Qingdao, China). The entrance vector pDONR221, RNAi initial vector pSGRNAi, and Top10 cells were purchased from SinoGene Scientific (Beijing, China). The initial vector pSGRNAi contained fragments of *att*B, which resulted that foreign fragment can be integrated into pSGRNAi directly, without considering enzyme sites (Karimi, Depicker, and Hilson 2007). Unless otherwise specified, all of the chemical reagents used were bought from Takara Biotechnology Company (Dalian, China).

Plant Growth Conditions

Tobacco seeds of transgenic lines and wild-type were surface-sterilized by shaking in 70% (*v*/*v*) ethanol for 30 s, followed by 10 % (*v*/*v*) hydrogen peroxide for 10 min, and washed five times with sterile double-distilled water. Surface-sterilized seeds were transferred to plates containing half-strength MS medium with 0.3 % (*w*/*v*) phytogel (pH5.7). Plates were incubated in the dark at 4°C for 3 days and then were moved to a growth chamber with controlled temperature (22–24°C), relative humidity (75–90%), light (750 μE m^{-2}), and photoperiod (16-h day/8-h night).

Evaluation of Mercury Accumulation in Plants

Mercury content in different tissues was measured according to the experiment mentioned above (Nagata et al. 2009). Fifteen-day-old seedlings were harvested and washed thoroughly in distilled water, then shoots and roots were separated and immersed directly in liquid nitrogen. The frozen plants were dried using a freezer-dryer, and the dry weight was determined. After being grounded to a fine powder using liquid nitrogen, samples (three replicates of each treatment) were acid-digested by stepwise additions of 70% (*v*/*v*) nitric acid, 30% (*v*/*v*) hydrogen peroxide, and concentrated HCl. For measuring mercury content of the whole seedling, the procedure was as the same as described above, except that shoots and roots were not separated.

Development of Tobacco Transgenic Plants

A 2-week-old leaf was cut into pieces to a new size of 4–6 mm and co-cultured with yeast extract broth (YEB)-grown *Agrobacteria* following the standard procedure (Thakur et al. 2014). The samples were put onto the sterile filter paper to remove the excess bacteria and cultured on the differentiation medium (MS + 2 mg/L benzylaminopurine [6-BA] + 0.5 mg/L indole-3-acetic acid [IAA]) in dark for 2–4 days. Then, the samples were transferred onto differentiation medium containing 300 mg/L cefotaxime (cef) sodium and 50 mg/L kanamycin. The samples were maintained in 25°C with 16-h light until amounts of clumps of seedlings appeared. The seedlings were cut from the base and transferred onto root medium (MS + 0.5 mg/L IAA + 300 mg/L cef + 50 mg/L kanamycin). After enough roots appeared, the seedlings were transferred to jars containing potting soil for further growth and development. After 4 weeks, plants were transferred to the soil in a greenhouse and cultured continually.

Construction of RNAi Vector

Primers osgL528: 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGTGGAAAGTGA-3' (the sequence of *att*B1 was underlined) and osgL529: 5′- GGGGACCACTTTGTACAAGAAAGCTGGGTCTGCTATTAAAACA-3' (the sequence of *att*B2 was underlined) were designed to isolate the common sequence of NtHMA3a (HF675180.1) and NtHMA3b (HF937053.1) from the cDNA of tobacco. The amplified fragment was recovered using a gel extraction kit (CWBIO, Beijing, China). BP clonase reaction (pDONR221, 2 μl; polymerase chain reaction [PCR] product, 4 μl; TE buffer (10 mM Tris-Hcl, 1 mM EDTA pH = 8.0), 2 μl; BP clonase, 2 μl) was performed using BP Clonase Mix (Life Technology, catalog no. 11789-020; Beijing, China) according to the manual. The reaction product was named as pENTR-HMA3. Primers P2F: 5′-TCATTAAGCATTCTGCCGACATG-3' and P2R: 5′-CACTGGATATACCACCGTTGATA-3' were designed to identify the entrance vector pENTR-HMA3. pENTR-HMA3 was transformed into *Escherichia coli* Top10 competent cell (SinoGene Scientific) using the standard hot-shot method (Sambrook, Fritsch, and Maniatis 1989). The plasmid was extracted using Plasmid DNA Miniprep kit (RealTimes, Beijing, China). LR clonase reaction (pENTR-HMA3, 4 μl; pSGRNAi, 2 μl; TE buffer, 2 μl; LR clonase, 2 μl) was performed using an LR Clonase Mix (Life Technology, catalog no. 11791-020) according to the protocol provided by the manufacturer. The reaction product was transformed into *E. coli* Top10 competent cells according to the standard method (Sambrook, Fritsch, and Maniatis 1989). The plasmid was extracted and digested with the enzyme PvuII. Those plasmids were then digested into four fragments, whose molecular sizes were 607, 1066, 1236, and 9035 bp, respectively, and were sequenced. The plasmid with the correct sequence was named as pSGRNAi-HMA.

Transgene Expression Analysis

Plant total RNA was isolated by using an RNeasy plant mini kit (Qiagen, Shanghai, China) following the manufacture's protocol. Reverse transcriptase reaction was performed by converting 1 μg of RNA into cDNA by using the avian myeloblastosis virus (AMV) reverse transcriptase of the One-step RT-PCR kit (Qiagen). Specific primers P3F: 5′-AATGCTGCTCGTCTAA-GATGGCTTC-3' and P3R: 5′- CGTGTTCCTTGT-CCGAACAGCCATG-3' were designed to amplify the partial sequence of NtHMA3a (HF675180). Specific primers P4F: 5′-CAGTGACAAAAAATGTTGT-CAATCC-3' and P4R: 5′- ACTCTTATTATTTCCG-CATGTCATT-3′ were used to amplify the partial sequence of NtHMA3b (HF937053). Reverse transcriptase polymerase chain reaction (RT-PCR) conditions were denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, run for 30 cycles, and final extension was performed at 72°C for 7 min.

RESULTS

Construction of Transgenic Tobacco Lines in Which the Expressions of NtHMA3a and NtHMA3b Were Inhibited Completely

Using AtHMA3 (GenBank accession number AY055217.1) as the template, BLAST was performed in tobacco genome. Two sequences (GenBank accession numbers HF675180.1 and HF937053.1, respectively) were obtained. Their similarities with AtHMA3 were 61% and 60%, respectively. They were named as NtHMA3a (HF675180.1) and NtHMA3b (HF937053.1). The homology between NtHMA3a and NtHMA3b was up to 97%. Therefore, one RNAi vector can be constructed to inhibit the expressions of these two genes (Figure 1). As shown in Figure 2A, a 450-bp band was amplified from the wild-type tobacco cDNA. After the fragment was inserted into the entrance vector and transformed into *E. coli* DH10B, the plasmids were extracted from the positive clones and PCR was performed. The clones from which a 600-bp fragment can be amplified were sequenced. The NtHMA3 fragment was transferred into the plasmid pSGRNAi from the entrance vector by LR clonase reaction. Since the vector pSGRNAi contained two inserting sites with inverse direction, one LR clonase reaction can guarantee that the NtHMA3 fragment can be inserted into the two sites with inverse direction from the entrance vector pDONR221 (Figure 3; Karimi, Depicker, and Hilson 2007). The plasmids were extracted from positive clones and digested with PvuII. The plasmids that can be digested into four fragments (607, 1066, 1236, and 9035 bp) were sequenced (Figure 2B). The identified plasmid was transformed into wild-type tobacco as described in Materials and Methods. A total of 156 transgenic plants were obtained. mRNA was extracted from them and reverse-transcription reactions were performed. Specific primers were used to amplify the partial sequences of NtHMA3a and NtHMA3b from the cDNA of the transgenic plants (Figures 4 and 5). There were 10 plants from which neither NtHMA3a nor NtHMA3b

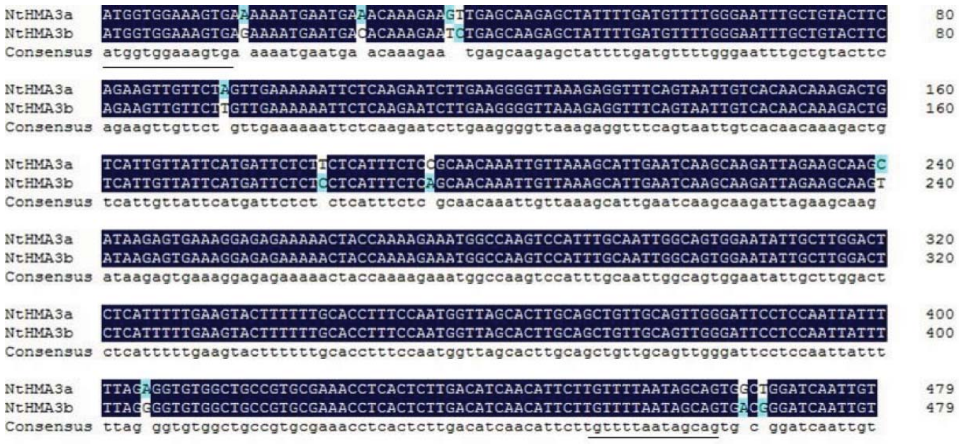

FIGURE 1 **Sequence used for constructing RNAi vector. Sequences used for designing primers were underlined.**

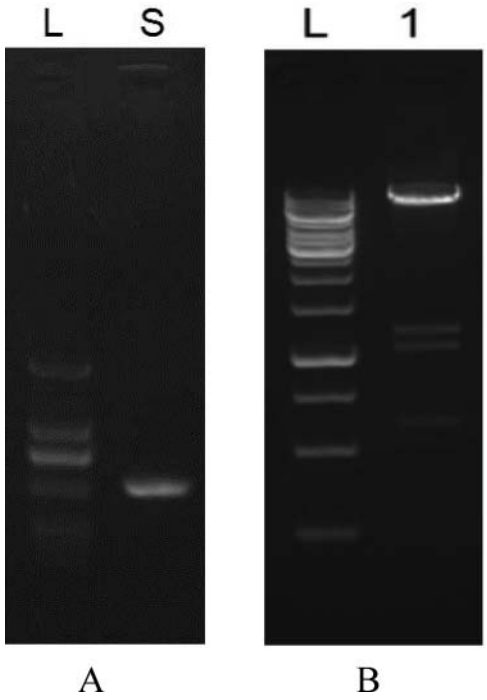

FIGURE 2 **The construction of RNAi vector pSGRNAi-NtHMA. Partial common sequences of NtHMA3a and NtHMA3b were amplified from tobacco cDNA (*A*). After RNAi vector pSGRNAi-NtHMA was constructed, the plasmid was digested with PvuII (*B*). The predicted result contained four fragments (607, 1066, 1236, and 9035 bp).**

could be amplified, indicating that the expressions of NtHMA3a and NtHMA3b had been inhibited completely in these lines (Figure 5). They were selected for getting seeds and used in the following experiments.

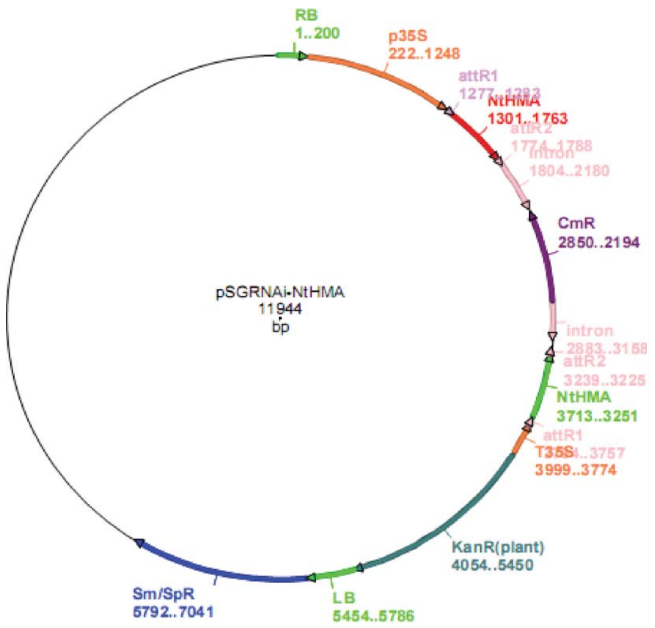

FIGURE 3 **The map of the constructed RNAi vector pSGRNAi-NtHMA.**

Mercury Tolerance of the NtHMA3a-NtHMA3b-RNAi Plants

Two methods were used to measure plants' tolerance to mercury. The surface-sterilized seeds of wild-type and transgenic tobacco were aligned in a horizontal array and grown vertically in MS agar medium containing mercuric chloride. After 2 weeks, the root length was measured for evaluating the plant's tolerance to mercury. The other method for evaluating mercury tolerance of plants is performed as Nagata et al. (2006) and modified. Plant seeds were germinated on MS agar medium for 3 weeks. Then the seedlings were transferred to MS liquid medium containing mercuric ions and cultured for 1 week. The seedlings that were transferred to MS liquid medium without mercury were selected as the control variable. Fresh wet weight of the seedling was measured.

As shown in Figure 6, with mercuric content increasing, the plant roots became shorter. However, in each treatment there was no significant difference between the root length of the NtHMA3a-NtHMA3b-RNAi plants and that of the wild-type. The fresh wet weight of the wild-type plants cultured in higher content of mercury was significantly lighter than that of those cultured in lower content of mercury (Figure 7). Similar results had also been found in NtHMA3a-NtHMA3b-RNAi plants and empty-vector transgenic line. However, in each treatment, no statistical differences were found among the fresh wet weight of the wild-type, the NtHMA3a-NtHMA3b-RNAi plants, and the empty-vector transgenic line (Figure 7).

Mercury Content in NtHMA3a-NtHMA3b-RNAi Plants

As shown in Figure 8, the growth of wild-type and NtHMA3a-NtHMA3b-RNAi plants were both inhibited when they were transferred into MS medium containing mercury ions. To understand the changes during this process, mercury content was measured from whole seedlings. It was found that with the mercury concentration in the medium increasing, the mercury content in the NtHMA3a-NtHMA3b-RNAi seedlings increased (Figure 8). For example, when mercury treatment concentration was 0.5 μmol/L, the mercury content in the NtHMA3a-NtHMA3b-RNAi plants was 4 μg/g. Yet when the mercury concentration in MS liquid medium increased up to 10 μmol/L, the

FIGURE 4 **Sequences of NtHMA3a and NtHMA3b used for transgenic plants identification. Specific sequences used for designing primers were underlined.**

mercury content in the NtHMA3a-NtHMA3b-RNAi plants was 60 μg/g. Similar results were also found in the wild-type plants and empty-vector transgenic line (Figure 8). Nevertheless, for each treatment, no significant difference had been found among the mercury content of the wild-type, that of the NtHMA3a-NtHMA3b-RNAi plants, and that of the empty-vector transgenic line (Figure 8).

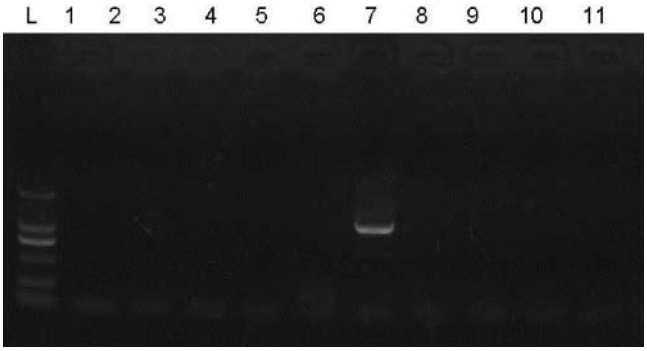

FIGURE 5 **The identification of transgenic plants. L was the DNA ladder. 1–11 represented different transgenic seedlings. Total RNA was extracted from the leaves of transgenic seedlings. cDNA was obtained from mRNA by reverse-transcriptase reaction. PCR was performed using NtHMA3a- and NtHMA3b-specific primers, respectively. PCR products from the same seedling were combined and electrophoresed in 0.8% agrose gel. Results showed that except seedling 7, the expressions of NtHMA3a and NtHMA3b in another 10 seedlings examined had been inhibited completely.**

Mercury Contents in the Shoots and Roots of the Transgenic Plant

To clarify whether more mercury was transferred into the shoots after the expressions of NtHMA3a and NtHMA3b were inhibited, mercury contents in shoots and roots were measured, respectively. It was found that in each treatment, the mercury content in roots was much higher than that in the shoots (Figures 9, 10). For example, the mercury content in the shoots of the NtHMA3a-NtHMA3b-RNAi seedling cultured in medium containing 1.25 μmol/L of mercuric chloride was 3 μg/g, whereas the value in the roots of the NtHMA3a-NtHMA3b-RNAi seedling cultured in the same medium was about 10 μg/g (Figures 9, 10). The mercury content in roots was about 2 times higher than that in the shoots (Figures 9, 10). Similar results were also found in wild-type and empty-vector transgenic line (Figures 9, 10). But for each treatment, neither in shoots nor in roots were any statistical differences in mercury content found among the NtHMA3a-NtHMA3b-RNAi line, empty-vector transgenic line, and wild-type (Figure 9, 10). These results demonstrated that after the expressions of NtHMA3a and NtHMA3b were knocked down in tobacco, the root-to-shoot ratio of mercury had not changed.

DISCUSSION

Cadmium and mercury are both toxic in biology. The process that plants transport and detoxify cadmium ions had been well studied (Lux et al. 2011; Mendoza-Cozatl et al. 2011). However, the

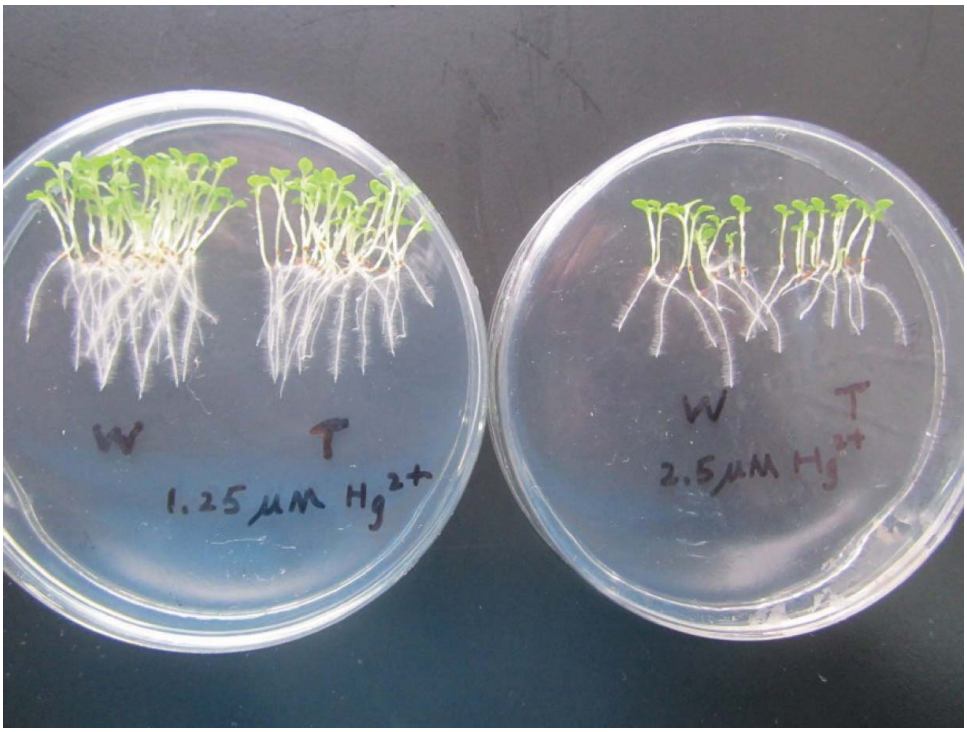

FIGURE 6 **Plants' tolerance to mercury. W referred to the wild-type tobacco. T was the NtHMA3a-NtHMA3b-RNAi tobacco.**

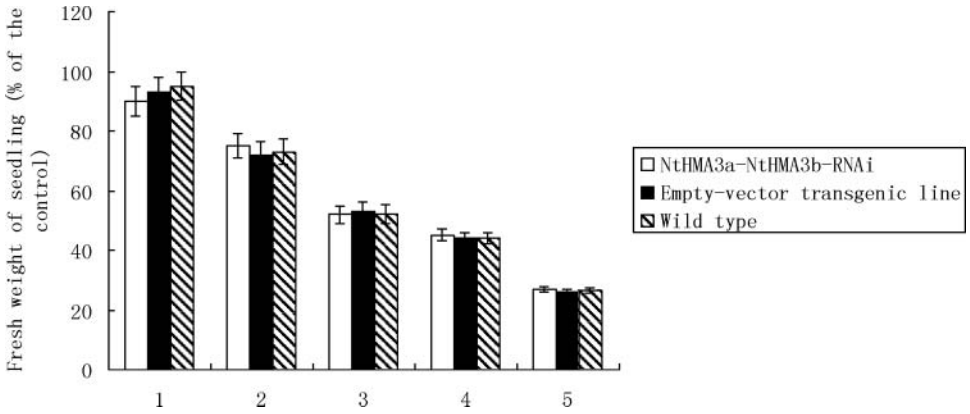

FIGURE 7 **Plants' tolerance to mercury. 1–5 represented the mercury concentrations in MS liquid medium in which the seedlings were cultured: 0.5, 1.25, 2.5, 5, and 10 μmol/L of mercuric chloride, respectively.**

mechanism that plants use to resist mercury ions is still unclear. Some researches suggest that plants detoxify mercury and cadmium through similar ways (Chen, Yang, and Wang 2009; Iglesia-Turino et al. 2006; Mendoza-Cozatl et al. 2011; Gong, Lee, and Schroeder 2003; Gueldry et al. 2003). If this is true, genes participating in cadmium transport in plants can be used for constructing transgenic plants with higher mercury accumulation ability.

The main mechanism that plants detoxify cadmium is that cadmium ions are transported into a vacuole and stored there (see review in Lux et al. 2011 and Mendoza-Cozatl et al. 2011). The metabolism activity in the vacuole is much lower than that in the cytoplasm. The injuries on the vacuole caused by heavy metal ions are much lower than those on the cytoplasm. Cadmium ions can be transported into vacuoles by H^+/Cd^+ antiporters (such as orthologues of AtCAX2 and AtCAX4) (Korenkov et al. 2007, 2009), by heavy metal P1B-ATPases (such as orthologues of AtHMA3) (Morel et al. 2009), and as Cd chelates by ABC transporters (such as orthologues of AtMRP3) (Tommashini et al. 1998; Cobbett 2000; DalCorso et al. 2008; Verbruggen, Hermans, and Schat 2009). Considering the phenotypes of the HMA3-RNAi plants, that cadmium ions were translocated into vacuoles through HMA3 was considered as the main way for plant to detoxify cadmium (DalCorso et al. 2008; Ueno et al. 2011, 2010; Miyadate et al. 2011). In the vacuole, cadmium ions were bonding with S-containing ligands (such as glutathione, metallothioneins, and phytochelatins), and then the toxicity of cadmium was reduced (DalCorso et al. 2008; Ueno et al. 2011, 2010; Miyadate et al. 2011).

HMA3 is located in tonoplast (Ueno et al. 2011). If plants use similar pathways with detoxifying mercury ions and cadmium ions, HMA3 may also play a key role in translocating mercury ions across tonoplast. To identify whether this deduction was true or not, the expressions of HMA3 genes were knocked down in

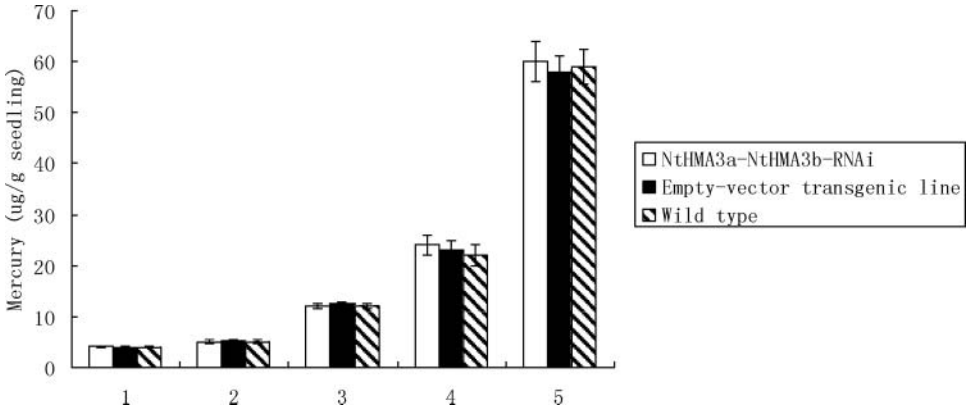

FIGURE 8 **Mercury content in whole tobacco seedlings. 1–5 represented the mercury concentrations in MS liquid medium in which the seedlings were cultured: 0.5, 1.25, 2.5, 5, and 10 μmol/L of mercuric chloride, respectively.**

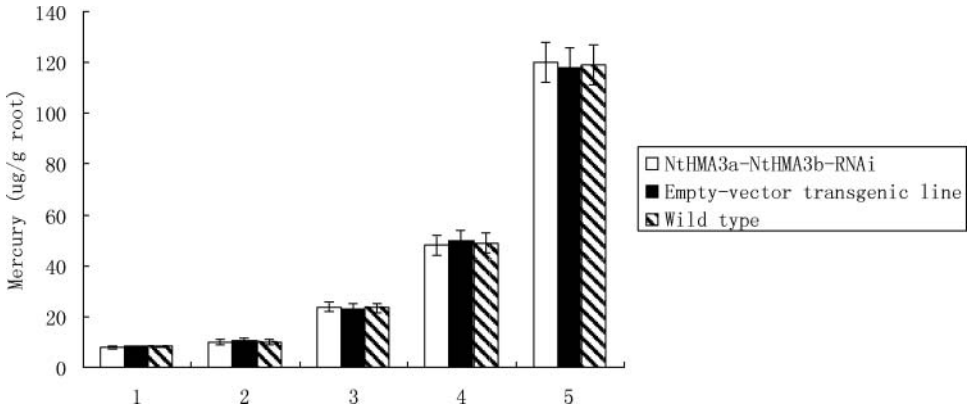

FIGURE 9 **Mercury content in roots. 1–5 represented the mercury concentrations in MS liquid medium in which the seedlings were cultured: 0.5, 1.25, 2.5, 5, and 10 μmol/L of mercuric chloride, respectively.**

tobacco in this study. Results showed that the biomass of plants cultured in the medium containing mercury was lower than that of plants cultured in the control medium. With mercuric content increasing, the plant roots became shorter. However, in each treatment there was no significant difference among the NtHMA3a-NtHMA3b-RNAi plants, empty-vector transgenic line, and wild-type. The plants' tolerance to mercury ions had not changed after the expressions of NtHMA3a and NtHMA3b were inhibited. When the mercury content was measured from the whole seedlings, no significant difference was found among the wild-type, NtHMA3a-NtHMA3b-RNAi line, and the empty-vector transgenic plants. This demonstrated that the plant's mercury accumulation capacity had not changed after the expressions of NtHMA3a and NtHMA3b were knocked down. HMA3 might not be the key gene responsible for plants' mercury ion detoxifying.

Ueno et al. found that the expression of TcHMA3 in *T. caerulescens* was constitutive in shoots and roots (Ueno et al. 2011). After the expression of OsHMA3 was knocked down in rice, the cadmium concentration in the shoot was increased by 2.1–2.5 times in the RNAi lines compared with the empty-vector control plants and the root cadmium concentration was decreased by 74–60% in the RNAi line (Ueno et al. 2010). Moreover, if OsHMA3 was overexpressed in rice, the cadmium accumulation in the shoots decreased, but cadmium accumulation in the roots increased (Ueno et al. 2010). It seemed that HMA3 play roles mainly in roots, but not in shoots. If HMA3 worked in detoxifying mercury ions in similar ways with its translocating cadmium ions in plants, after the expression of HMA3 was inhibited, more mercury ions would be transferred into shoots from roots. The mercury content in NtHMA3a-NtHMA3b-RNAi shoots would be higher than that in wild-type shoots. However, although the mercury content in the root was higher than that in the shoot, for each treatment, neither in shoots nor in roots were differences found among NtHMA3a-NtHMA3b-RNAi plants, empty-vector transgenic line, and wild-type. After the

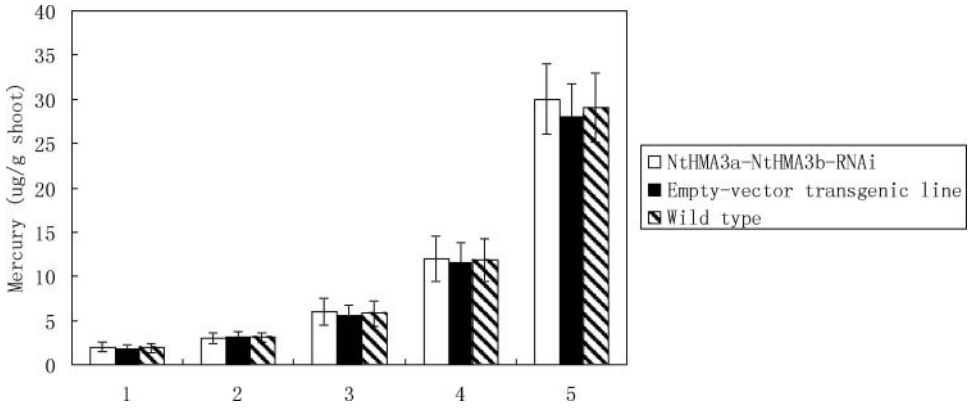

FIGURE 10 **Mercury content in shoots. 1–5 represented the mercury concentrations in MS liquid medium in which the seedlings were cultured: 0.5, 1.25, 2.5, 5, and 10 μmol/L of mercuric chloride, respectively.**

expressions of NtHMA3a and NtHMA3b were inhibited, the movement of mercury ions in plants had not been affected. HMA3 was not the main gene responsible for mercury ion translocation from root to shoot. If HMA3 controlled the process that mercury ion translocation into the vacuole, the mercury content in the root would be decreased significantly after the expression of HMA3 was knocked down. However, when mercury content was measured from the whole seedling, no significant difference had been found among wild-type, NtHMA3a-NtHMA3b-RNAi plants, and the empty-vector transgenic plants. Therefore, for mercury ion translocation across tonoplast, the main pathway might not be HMA3, but ABC transporters. Mercury ions might be chelated with phytochelatins in the cytoplasm. That mercury ions bond with phytochelatins might happen in the cytoplasm, but not in the vacuole.

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