Acta Physiol Plant (2012) 34:1819–1826
DOI 10.1007/s11738-012-0980-4

ORIGINAL PAPER

Cloning and characterization of a type 1 metallothionein gene from the copper-tolerant plant *Elsholtzia haichowensis*

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Received: 29 November 2011 / Revised: 3 March 2012 / Accepted: 6 March 2012 / Published online: 21 March 2012

Abstract *EhMT1*, a type 1 metallothionein (MT) gene, was cloned from *Elsholtzia haichowensis*, a Cu-tolerant plant. Typical of plant type 1 MTs, *EhMT1* encodes a putative peptide of 74 amino acid residues containing cysteine-rich domains. Sequence comparisons with various databases revealed strong similarities at the nucleotide and amino acid levels between EhMT1 and the type 1 MT of *Mimulus guttatus*. *EhMT1* transcription was greater in the roots than in the leaves, and was markedly increased by treatments with Cu, H_2O_2, and heat shock. EhMT1-GUS was localized to the cytoplasm of onion epidermal cells. *Escherichia coli* cells expressing pET-30a-EhMT1 were more tolerant to Cu and accumulated more Cu than control cells. Our results show that *EhMT1* is involved in Cu tolerance and accumulation in *E. haichowensis*.

Keywords Copper tolerance and accumulation · EhMT1 · *Elsholtzia haichowensis* · Gene expression · Metallothionein

Introduction

Soil contamination with heavy metals has become a serious environmental problem due to human practices such as mining, metallurgy, disposal of sewage sludge, and the agricultural application of fertilizers, pesticides, and wastes. Copper (Cu) is an essential micronutrient with catalytic and structural roles in plant growth and development. However, excess Cu can be toxic to plants, causing a wide range of deleterious effects, such as the inhibition of photosynthesis and pigment synthesis, plasma membrane damage, functional changes, and other metabolic disturbances (Marschner 1995). The normal leaf Cu concentration in plants ranges from 5 to 30 μg/g dry weight (DW) (Kabata-Pendias and Pendias 1992). It has been reported that a limited number of wild plants are able to grow normally on metal-contaminated sites and accumulate high concentrations of Cu in their tissues without suffering any damage (Baker and Brooks 1989). Depending on the species, plants have evolved several mechanisms for metal detoxification, including exclusion, compartmentalization, chelation, and binding to organic ligands such as organic acids, amino acids, phytochelations (PCs), and metallothioneins (MTs) (Cobbett and Goldbrough 2002; Hall 2002).

MTs are low-molecular weight cysteine (Cys)-rich proteins that can effectively bind metals via their Cys residues (Cobbett and Goldbrough 2002). As shown by the molecular structure of the Cu–MT complex, MTs have a high Cu-binding capacity (Callahan et al. 2006). Based on the arrangement of Cys residues, MTs have been divided into three classes. All plant MTs belong to Class II and typically contain two structural metal-binding Cys-rich domains at their N- and C-terminal regions. Many genes encoding MTs have been isolated and characterized in plants, but the precise functions of MTs are not completely understood, partly because it is difficult to isolate the proteins intact from plant tissues. Evidence suggests that plant MTs play a role in maintaining the homeostasis of essential metal ions, detoxifying heavy metals, and scavenging reactive oxygen species (ROS) (Cobbett and

Communicated by T. Moriguchi.

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Goldbrough 2002; Mir et al. 2004; Wong et al. 2004; Nishiuchi et al. 2007; Xue et al. 2009). In some plant species, Cu tolerance is associated with constitutively enhanced transcript levels of a type 2 MT (Murphy and Taiz 1995; van Hoof et al. 2001; Mengoni et al. 2003). Gene expression analyses and functional testing in yeast have revealed a role for MTs in Cu homeostasis and detoxification (Zhou and Goldsbrough 1994; Ma et al. 2003; Roosens et al. 2004; Hassinen et al. 2007). In addition, plant MTs are involved in important physiological processes, including fruit ripening (Moyle et al. 2005), root development, embryo germination (Yuan et al. 2008), and suberization (Mir et al. 2004).

Elsholtzia haichowensis Sun, in the Lamiaceae, is a Cu accumulator widely distributed on Cu-mining waste and Cu-contaminated soil along the middle and lower reaches of the Yangtze River in China (Tang et al. 1999; Lou et al. 2004; Qian et al. 2005). It has been suggested that the Cu tolerance of *E. haichowensis* is based on an exclusion mechanism, similar to that of *Silene vulgaris* (Song et al. 2004). The concentration of Cu in the roots of *E. haichowensis* is much higher than that in shoots, and most of this Cu is bound to root cell walls (more than 60 % of the total) (Lou et al. 2004). However, our and other studies have shown that *E. haichowensis* can accumulate Cu at levels exceeding 100 μg/g DW in the shoots (Lou et al. 2004; Song et al. 2004; Qian et al. 2005), which is much higher than the toxicity threshold of 20–30 μg/g DW reported in Cu non-tolerant crop species (Marschner 1995). Using synchrotron radiation X-ray fluorescence spectroscopy (SRXRF), Shi et al. (2008) demonstrated that the relative Cu levels were much higher in mesophyll tissue than in the leaf epidermis. These results imply that *E. haichowensis* employs an internal mechanism to tolerate the elevated levels of Cu in shoot tissues, in addition to its exclusion mechanism. Qian et al. (2005) reported that protein thiols might be involved in the adaptive tolerance mechanisms in response to Cu toxicity in this plant. However, the molecular mechanisms underlying the Cu tolerance of *E. haichowensis* are unclear. In this study, we isolated an *MT1*-type gene from *E. haichowensis*, and investigated its expression pattern in various organs. Moreover, the Cu tolerance and accumulation in *Escherichia coli* expressing EhMT1 were analyzed. Transient EhMT1-GUS expression was also analyzed in onion epidermal cells. The aim of this study was to increase our understanding of the Cu-tolerance mechanisms in Cu-accumulating plants.

Materials and methods

Plant materials and bacterial stains

Seeds of *E. haichowensis* were collected from Cu mine tailings near Tongling, Anhui Province, China. Seedlings were grown hydroponically in a greenhouse with a 12-h photoperiod at 25 °C and at 20 °C in darkness for 1 month before treatment. The plants were then exposed to 50 μM $CuSO_4$ for 48 h, heat shocked at 42 °C, and treated with 0.01 % H_2O_2 for 1 h. All treatments were repeated three times; the results represent the means of three independent experiments. Subsequently, the shoots and roots were collected separately, frozen in liquid nitrogen, and stored at −80 °C until use.

Escherichia coli strain JM109 was used for the cloning of all recombinant plasmid vectors, while *E. coli* strain BL21 (DE3) was used for His fusion expression. *Agrobacterium tumefaciens* strain EHA105 was used for the transformation of onion epidermal cells.

RNA isolation and first-strand cDNA synthesis

Total RNA was isolated from the collected samples using a Plant RNApure Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. The quality and quantity of the RNA were assessed by measuring the A260/A280 ratio and by 1 % agarose gel electrophoresis with ethidium bromide staining.

Single-stranded cDNA was synthesized using 1 μg of total RNA and Oligd$(T)_{18}$ primer with the Promega RT-PCR system (Madison, WI, USA) in a total volume of 20 μL. The RT reaction was performed at 70 °C for 10 min, 37 °C for 1 h, and 94 °C for 5 min. One microliter of 100 U/μl M-MLV reverse transcriptase was added for 1 h at 37 °C.

Cloning of the full-length cDNA

To amplify the coding region of *EhMT1*, the cDNA was cloned using a pair of degenerate primers, P1 and P2, synthesized by Sangon (Shanghai, China). The PCR products were purified from agarose gels using a Gel Extraction Kit (Huashun, Shanghai, China), cloned into pGEM-T (Promega), and sequenced.

SMART RACE cDNA Amplification Kits (Clontech, Mountain View, CA, USA) were used to amplify the 5′ and 3′ ends of the cDNA to obtain the full-length cDNA sequence. Gene-specific nested primers were designed on the basis of the obtained sequence information. The gene-specific and nested primers used for 5′ RACE were NGSP1 and NGSP2; the gene-specific primer for 3′ RACE was GSP. The PCR program was 29 cycles of 94 °C for 5 s, 68 °C for 10 s, and 72 °C for 2 min.

Semi-quantitative RT-PCR

The primers used for semi-quantitative RT-PCR were P1 and P2 (for primer sequences, see Table 1). *18S rRNA* was

Table 1 Primers used to clone the full-length *EhMT1* cDNA, examine its expression, and assess expression of the fusion protein in *E. coli*

Primer name	Sequence
P1	5′-GAAAATGTCTTGCTGTGG(T/A)GG-3′
P2	5′-GTGCAAGGGTTGCAGGTGCAG-3′
NGSP1	5′-ACCCCTTCAACGGAGATAAC-3′
NGSP2	5′-GCGATCTTCTCCACATCTGC-3′
GSP	5′-TGCACGGTGAAGGATCTGAG-3′
18S	5′ -TGGGATACCTGCCAGTAGTCAT-3′
18A	5′ -CTGGATCCAATTACCAGACTCAA-3′
EcoP	5′-GGATATCAAGAAAATGTCGAGTGGA-3′
BamP	5′-GGATCCTCAGCAGGGCCTAGTTAA-3′

used as an internal control; the primers were 18S and 18A. The *EhMT1* and *18S rRNA* products were 225 and 500 bp in length, respectively. All PCRs were replicated at least three times.

Expression of the EhMT1 fusion protein and analysis of Cu concentration in *E. coli*

EhMT1 was overexpressed in *E. coli* as a His fusion protein using the plasmid vector pET-30a. The coding region of *EhMT1* was amplified by PCR using Taq Plus DNA polymerase with two primers (forward: EcoP, reverse: BamP) containing *Eco*RI and *Bam*HI restriction sites, respectively. After digestion with *Eco*RI/*Bam*HI, the *EhMT1* coding region fragment was inserted into pET-30a using the same restriction sites. Sequence analysis of the coding regions of *EhMT1* did not reveal any mutations, and the recombinant vector was named pET-30a-EhMT1. Empty pET-30a and pET-30a-EhMT1 were transformed into *E. coli* BL21 (DE3) cells.

Escherichia coli cells transformed with pET-30a-EhMT1 or pET-30a (control) were cultured in Luria–Bertani (LB) medium supplemented with 50 μg/ml kanamycin and shaking at 200 rpm at 37 °C. At an OD_{600} of 0.5–1.0, the cells were induced with 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG), grown for an additional 3 h, and harvested by centrifugation at 4,000*g* for 10 min. After boiling for 5 min, 15 μl of the samples was analyzed by Coomassie blue staining using 15 % SDS–polyacrylamide gels.

To test the effect of EhMT1 expression in *E. coli*, the growth of *E. coli* cells carrying empty pET-30a was compared with cells carrying pET-30a-EhMT1. Each strain was inoculated separately ($OD_{600} = 0.4$) into 50 mL of LB medium with 1.0 mM IPTG, with or without 1.0 mM $CuSO_4$. The cultures were shaken at 200 rpm at 37 °C, and the OD_{600} was measured at 2-h intervals.

The aliquots cultured in LB medium with 1.0 mM $CuSO_4$ for 4 h were centrifuged at 5,000*g* for 10 min. Cells were washed three times in LB medium, then dry weights were measured. The dried samples were completely digested with extra pure grade HNO_3 and $HClO_4$ (87:13, v/v). The concentration of Cu was determined by flame atomic absorption spectrometer (novAA® 400; Analytik Jena, Jena, Germany). Blanks were also used for background correction and other sources of error.

Analysis of transient EhMT1-GUS expression

To generate the construct EhMT1-GUS (β-glucuronidase) for the transformation of onion epidermal cells, a *Xba*I-*Bam*HI fragment containing the coding region of the EhMT1 fusion GUS cassette was inserted into the *Xba*I-*Bam*HI site in pBI121 (Clontech).

GUS staining was conducted according to Jefferson et al. (1987). Onion epidermal samples were vacuum infiltrated and then incubated in X-gluc solution [1 mM X-gluc, 50 mM sodium phosphate buffer (pH 7.2), and 5 mM potassium ferricyanide] for 8 h at 37 °C. Next, the samples were washed with 70 % (V/V) ethanol at 60 °C for 2 h and photographed under a stereomicroscope (SZX21; Olympus). More than six individual samples were stained and analyzed.

Results

EhMT1 encodes a type 1 MT

RT-PCR and RACE were used to obtain full-length cDNA fragments of interest. The sequences of the clones were identified using a BLAST search (http:\\www.ncbi.nlm.nih.gov/BLAST). The cDNA, which showed strong similarity to the cDNA of *MT1* (CAA36249), was named EhMT1 and submitted to GenBank under accession number DQ059081. The cDNA contained a 225-bp open reading frame (data not shown) encoding a 74 amino acid polypeptide with a theoretical molecular mass of 7.14 kDa and a *p*I of 5.13. Similar to other plant MTs, there was no Cys in the spacer region between the terminal Cys-rich domains (Fig. 1). A sequence alignment of the *EhMT1* cDNA with genomic DNA revealed that *EhMT1* contains two introns starting with the typical 5′-GT and ending with AG-3′ in the genomic DNA (data not shown). The intron sizes were 197 and 90 bp, respectively.

A multiple sequence alignment was conducted with EhMT1 and other proteins, including MgMT1 (CAA36249) from *Mimulus guttatus*, PmMT1 (CAH59439) from *Plantago major*, and IbMT1 (BAD95644) from *Ipomoea batatas* (Fig. 1a). Type 1 MTs contain two Cys-rich domains separated by a spacer of approximately 40 amino acids. The N-terminal sequence in this type of MT is highly conserved,

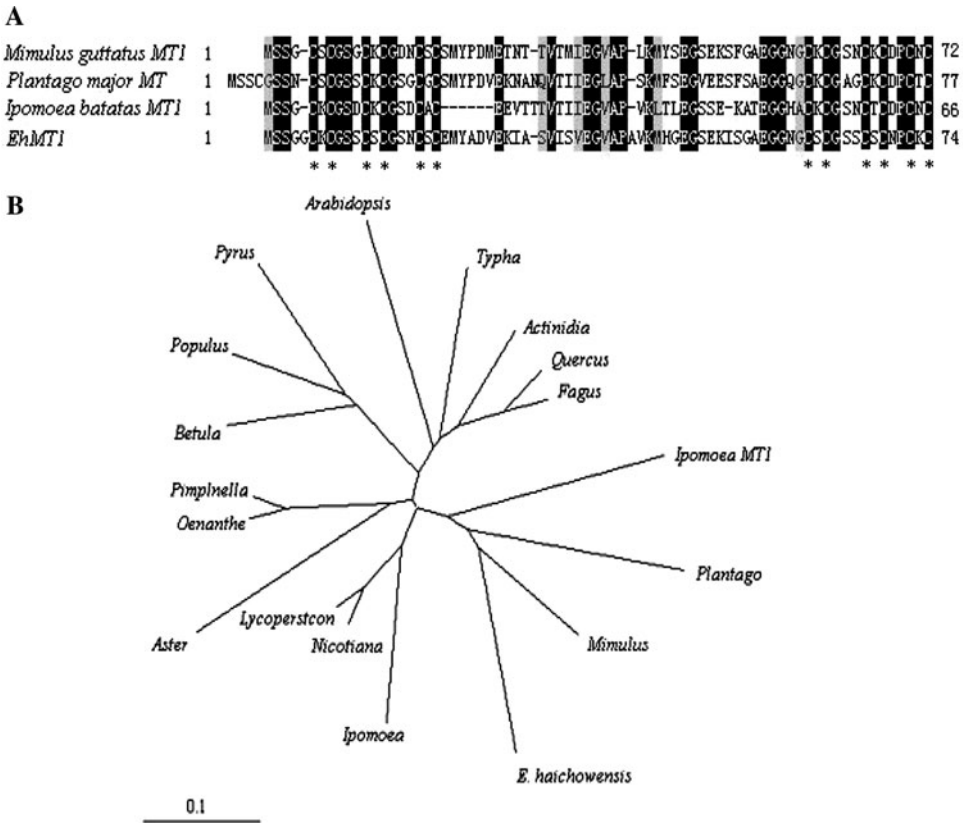

Fig. 1 Comparison of the deduced amino acid sequence for *EhMT1* with that for other MTs (**a**), phylogenetic tree analysis (**b**)

and the C-terminal domain contains three Cys-Xaa-Cys motifs (Fig. 1a). Plant MTs have been divided into different types based on the arrangement of the Cys residues at their N- and C-termini (Robinson et al. 1993; Rauser 1999). The 18 proteins were divided into several small subgroups by phylogenetic analysis (Fig. 1b); EhMT1 was clustered with MgMT1, PmMT1, and IbMT1. Therefore, we classified *EhMT1* as a type 1 MT. Our analysis indicates that homologous MT1 genes exist ubiquitously in planta, suggesting that this gene family plays important roles in higher plants; thus, we chose EhMT1 for further study.

Analysis of *EhMT1* expression

EhMT1 expression was investigated using semi-quantitative RT-PCR. The *EhMT1* transcript level was higher in roots than in leaves, and it was weak in stems (Fig. 2a). To investigate whether metal ions, heat, or oxidative stress are involved in the regulation of *EhMT1*, we harvested *E. haichowensis* seedlings treated with Cu, heat, or oxidative stress and assessed the transcriptional level of *EhMT1*. *EhMT1* expression was markedly increased in both roots and leaves treated with Cu and H_2O_2; in contrast, heat shock did not increase root *EhMT1* expression (Fig. 2b).

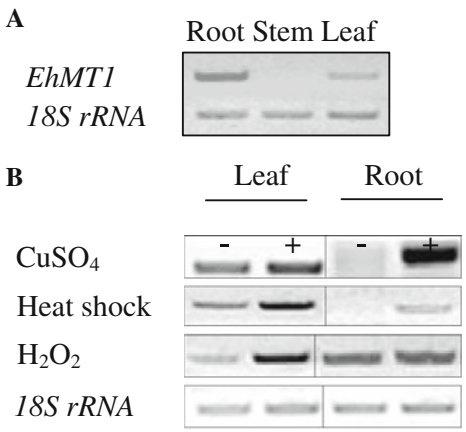

Fig. 2 Expression analysis of *EhMT1* as determined by semi-quantitative RT-PCR. *EhMT1* mRNA levels in the roots, stems, and leaves of *E. haichowensis* (**a**); *EhMT1* mRNA levels in leaves and roots of plants exposed to 50 μM $CuSO_4$ for 48 h, heat shocked at 42 °C for 1 h, and treated with 0.01 % H_2O_2 for 1 h (**b**). *18S rRNA* was used as an internal control

Efficient expression of functional EhMT1 as a fusion protein in *E. coli*

Figure 3 shows the expression of the 18-kDa fusion protein in BL21 (DE3) cells transformed with pET-30a-EhMT1

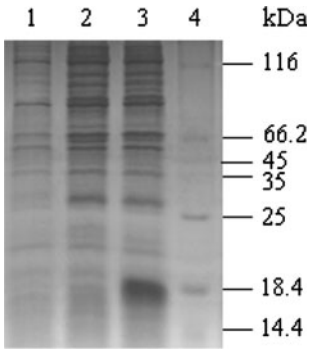

Fig. 3 Expression of EhMT1 in *E. coli*. Bacterial lysates from *E. coli* cells transformed with: (*1*) empty vector (pET-30a) without IPTG induction, (*2*) pET-30a with IPTG induction, and (*3*) recombinant plasmid (pET-30a-EhMT1) with IPTG induction, (*4*) protein markers, including β-galactosidase (116 kDa), BSA (66.2 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35 kDa), endonuclease *Bsp*98I (25 kDa), β-lactoglobulin (18.4 kDa), and lysozyme (14.4 kDa)

and induced with IPTG. To confirm the function of *EhMT1* in vivo, bacterial growth in the presence of Cu was compared using empty vector (pET-30a)- and pET-30a-EhMT1-expressing cells. The pET-30a-EhMT1 transformed cells showed significantly improved growth in LB media supplemented with 1.0 mM $CuSO_4$, whereas the growth of control cells carrying the empty vector was significantly inhibited. In the absence of Cu, the two strains exhibited identical growth kinetics (Fig. 4). The concentration of Cu in *E. coli* cells expressing EhMT1 was approximately 1.6-fold greater than that in expressing empty vector cells when exposed to 1 mM $CuSO_4$ for 4 h (Fig. 5).

Transient expression of EhMT1 in onion epidermal cells

To identify the cellular compartment in which EhMT1 functions, we assessed the location of EhMT1 in onion epidermal cells. When EhMT1 fused with GUS under the control of the cauliflower mosaic virus *35S* promoter was introduced into onion epidermal cells (Fig. 6a), GUS staining was localized into the cytoplasm. Cytoplasmic localization was also observed in onion epidermal cells expressing 35S-GUS (Fig. 6b).

Discussion

According to the classification system proposed by Cobbett and Goldbrough (2002), plant MTs are subdivided into four types based on their primary sequences, and specifically on the positions of their Cys residues. Type 1 MTs possess CXCXXXCXCXXXCXXC and CXCXXXCXCXXCXC (X corresponds to amino acids other than Cys) motifs at their N- and C-termini, respectively (Cobbett and Goldbrough 2002). As shown in this study, the *MT* cDNA isolated from *E. haichowensis* encodes a protein with two Cys-rich regions. These regions show significant identity with the N- and C-terminal domains of different type 1 MTs from other plants; therefore, the MT was designated EhMT1. EhMT1 exhibits a high degree of similarity to the type 1 MT from *M. guttatus* (de Miranda et al. 1990), a Cu-tolerant plant, indicating that EhMT1 may perform functions similar to the *M. guttatus* MT. To verify the identity of the isolated cDNA, genomic fragments covering the coding region were isolated by PCR and sequenced. Our results indicate that the genomic fragments were divided into three exons by two introns (data not shown). *MT* genes with two introns have been reported in rice and cotton (Hudspeth et al. 1996; Chen et al. 1998).

It has been reported that different members of the plant *MT* gene family show distinct spatial and temporal expression patterns, probably correlated with distinct roles in plant tissues (Guo et al. 2003, 2008). In general, type 1 *MTs* are predominantly expressed in roots, whereas type 2 and 3 *MT* genes are mainly expressed in shoots (Zhou and Goldsbrough 1994, 1995; Hsieh et al. 1995, 1996; Guo

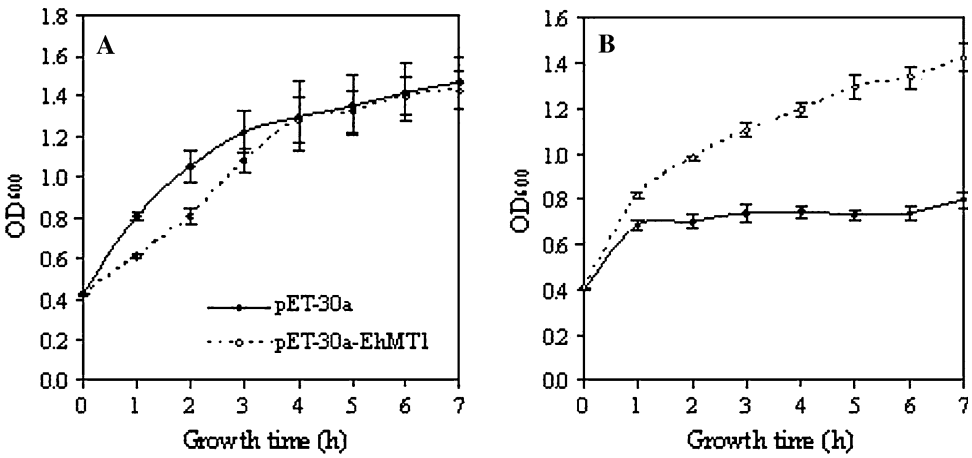

Fig. 4 Growth curves of *E. coli* BL21 (DE) carrying pET-30a-EhMT1 or empty vector in the absence (**a**) or presence (**b**) of 1.0 mM $CuSO_4$. The results are the mean ± SE of three independent experiments

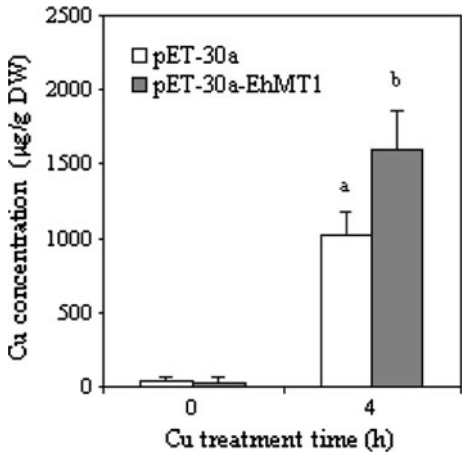

Fig. 5 The Cu concentration in *E.coli* cultured in LB medium with 1.0 mM $CuSO_4$ for 4 h. The results are the mean ± SE of three independent experiments

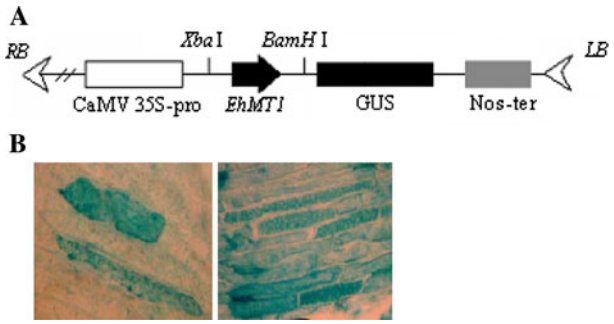

Fig. 6 Subcellular localization of EhMT1 overexpressed in onion epidermal cells. **a** Schematic diagram of the pBI121 expression vector. **b** Colocalization of 35S-GUS (*left*, control) and 35S-EhMT1-GUS (*right*) in the cytoplasm of onion epidermal cells

et al. 2003; Castiglione et al. 2007). Guo et al. (2003) further found that *MT1a* and *MT2b* were expressed in the phloem of *Arabidopsis* leaves and roots, whereas *MT2a* and *MT3* were mainly expressed in root tips and young leaves. In the present study, *EhMT1* RNA was detected in the leaves of *E. haichowensis* although the level was lower than that in the roots. It is possible that MT2, MT3, and MT4 homologs are also present in *E. haichowensis*. Further studies, analyzing other *MT* family members are needed to confirm the effect of Cu on *MT* expression in *E. haichowensis*.

Plant *MT* gene expression has been shown to be regulated by many factors, including metal ions, stresses such as salt, heat shock, wounding, and H_2O_2, and developmental stage. Our data indicate that *EhMT1* is induced by treatments with Cu, H_2O_2, and heat shock. The expression of plant type 1 *MT* genes is induced by Cu in a number of plant species (Zhou and Goldsbrough 1994; Hsieh et al. 1995; Snowden et al. 1995; Choi et al. 1996; García-Hernández et al. 1998; Morris et al. 1999; Giordani et al. 2002; Ma et al. 2003; Guo et al. 2003; Roosens et al. 2004). In this study, Cu treatment increased *EhMT1* expression more strongly in roots than in leaves. This suggests a particular role for EhMT1 in Cu tolerance in *E. haichowensis* roots. Guo et al. (2008) observed that MT1a but not MT2b deficiency led to a 30 % decrease in Cu accumulation in *Arabidopsis* roots, suggesting that MT1a functions in the sequestration of excess Cu in root cells. It has been shown that the Cu tolerance of *E. haichowensis* involves the accumulation of Cu in roots and the restricted translocation of Cu toward shoots, thereby excluding this metal from regions of active growth and metabolism (Lou et al. 2004; Song et al. 2004). Ni et al. (2005) confirmed that the isolation of Cu in vacuoles may be involved in the detoxification process in roots of *E. haichowensis*.

Our data support the hypothesis that *MT1* genes are involved in Cu tolerance and accumulation in plants. *E. coli* cells expressing pET-30a-EhMT1 were more tolerant to Cu exposure and accumulated more Cu than control cells. Similar results were reported in *E. coli* expressing $PsMT_A$ and *rgMT* (Evans et al. 1992; Jin et al. 2006). *AtMT1* from *Arabidopsis* enhances Cu resistance in yeast cells (Zhou and Goldsbrough 1994). Functional complementation studies have shown that *mcMT1* from the metal-tolerant grass *Festuca rubra* functions to sequester Cu and other metal ions (Ma et al. 2003).

To elucidate the function of a protein, it is important to know its location. It has been difficult for MT proteins in planta, since cell fractionation destroys MT proteins that are unstable in the presence of oxygen (Lee et al. 2004). We showed that EhMT1 fused to GUS was localized in the cytosol of onion epidermal cells, indicating that EhMT1 may bind Cu in the cytoplasm, thereby decreasing the activity of free Cu^{2+} ion and blocking Cu^{2+} from interacting with cytoplasmic components in *E. haichowensis*. It has already been shown that the AtMT2a and AtMT3, and BjMT2 were localized in the cytoplasm of the *Vicia faba* guard cells and tobacco leaf cells, respectively, and that this localization did not change after Cd or Cu treatment (Lee et al. 2004; An et al. 2006). On the other hand, MT protein itself might act as an endogenous antioxidant since the cys ligands in proteins are remarkably reactive toward oxidizing agents (Chae et al. 1994).

Oxidative burst is a common response to abiotic and biotic stresses. To avoid the deleterious effect of ROS, plant cells have evolved antioxidant defense mechanisms, which include enzymatic and non-enzymatic components (Mittler 2002). MTs themselves are considered to be antioxidants in plant systems (Cobbett and Goldbrough 2002). In this study, enhanced *EhMT1* transcription in the roots and leaves of *E. haichowensis* was found in response to H_2O_2 exposure. This observation is consistent with studies of the oxidative stress-induced expression of *MTs* in other plant species, including *Chloris virgata* (Nishiuchi

et al. 2007), *Gossypium hirsutum* (Xue et al. 2009), and *Arabidopsis* (Zhu et al. 2009). The treatment of *Arabidopsis* cotyledon and leaf tissues with a catalase inhibitor resulted in enhanced expression of *LSC54*, a rape *MT1* gene (Navabpour et al. 2003). Combined treatment with quenchers of ROS indicated that this induced expression was due to increased levels of ROS. Transgenic *Arabidopsis* plants overexpressing *BrMT1* from *Brassica rapa* (Kim et al. 2007) or *cgMT1* from *Casuarina glauca* (Obertello et al. 2007) show reduced accumulation of H_2O_2. Under conditions of high salinity, drought, and low temperature, the H_2O_2 levels in tobacco seedlings overexpressing *GhMt3a* from *G. hirsutum* were only half that in control plants (Xue et al. 2009). Transgenic yeast overexpressing *GhMT3a* showed high levels of tolerance to ROS stress. Purified OsMT2b demonstrated greater protection against hydroxyl radical-mediated salicylate hydroxylation compared to the vector (GST) alone (Wong et al. 2004). We previously demonstrated that excess Cu treatment induced oxidative stress and increased the accumulation of superoxide anion ($O_2^{\bullet-}$) and H_2O_2 in *E. haichowensis* roots and leaves (Zhang et al. 2008, 2010). The increased need for MT caused by Cu and H_2O_2 treatment suggests a role for EhMT1 in ROS scavenging. In this study, however, the role of oxidative signaling in the expression of *MT* genes was not clarified. Other oxidative stressors and signaling molecules will be investigated to define the regulation of *MT* genes in this plant species.

Heat shock had obvious effects on the expression of *EhMT1* in roots and leaves. Thus, EhMT1 may be involved in heat shock responses. Similarly, it has been shown that heat shock induces the expression of *OsMT-1* and *-2* in rice (Hsieh et al. 1995, 1996).

Conclusions

The present study showed that *EhMT1* expression in *E. haichowensis* was induced by Cu, H_2O_2, and heat shock. The tolerance of *E. coli* expressing pET-30a-EhMT1 to Cu stress was improved compared with control cells. Our results suggest that EhMT1 functions to moderate the toxicity of Cu ions; however, the detailed mechanism remains to be elucidated.

Author contribution Zhenguo Shen designed and instructed the research work. Yan Xia, Yanyan Lv and Yuxiang Yuan performed the experiments. Guiping Wang and Yahua Chen contributed the data analysis. Yan Xia, Zhenguo Shen, and Hongsheng Zhang wrote the manuscript.

Acknowledgments This study was supported by Doctoral Program Foundation of Institutions of Higher Education of China (20070307010).

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