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The Heterologous Expression of the *Iris lactea* var. *chinensis* Type 2 Metallothionein *IlMT2b* Gene Enhances Copper Tolerance in *Arabidopsis thaliana*

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Abstract *Iris lactea* var. *chinensis* (*I. lactea* var. *chinensis*) is a widely adapted perennial species with a high level of copper tolerance. To evaluate the role of metallothioneins (MTs) in copper tolerance in *I. lactea* var. *chinensis*, a full-length cDNA homologue of MT2, designated *IlMT2b* (GenBank accession No. AB907788), was cloned using the RACE-PCR method. The expression level of *IlMT2b* in the leaves and roots of *I. lactea* var. *chinensis* was induced in response to copper (Cu) treatment. Ectopic expression of *IlMT2b* in *Arabidopsis thaliana* increased the Cu concentration and reduced H_2O_2 production in the transgenic plants. After treatment with 50 and 100 μM Cu, the root length of two transgenic seedlings was respectively about 1.5- and 3-fold longer than that of the wild-type. Together, these results suggested that *IlMT2b* may represent a useful target gene for the phytoremediation of Cu-polluted soil.

Keywords *Iris. lactea* var. *chinensis* · Metallothionein · Cu stress · *Arabidopsis thaliana*

The metallothionein (MT) family is one of the most important classes to protect plants from heavy metal toxicity (Hall 2002). It has features of low molecular weight, rich cysteine (Cys) content and strong metal-binding ability (Samardzic et al. 2010). Based on distribution of cysteine residues in the amino- and carboxy-terminal regions, MTs can be divided into four categories in plants (Cobbett and Goldsbrough 2002). Type 1 contains two Cys-rich domains with metal binding motif Cys–X–Cys (X represents another amino acid), while type 2 is composed of Cys–Cys, Cys–X–X–Cys and Cys–X–Cys in the N-terminal region and Cys–X–Cys in the C-terminal region. Phytochelatins are described as type 3 which have the structure of $[\gamma\text{-Glu–Cys}]_n$–X polymers, whereas type 4 has three cysteine-rich domains.

Copper (Cu) is an essential micronutrient for various plant physiological processes via the form of Cu-dependent enzymes (Cobbett and Goldsbrough 2002), but excess copper accumulation always results in plant poisoning (Harris and Gitlin 1996). Plant MTs have important roles in seed germination (Zhou et al. 2012), salinity stress (Kumar et al. 2012), drought stress (Samardzic et al. 2010), and low temperature stress (Xue et al. 2009). The most important role of plants MTs is in tolerance to metals, such as Cu, cadmium (Cd) and zinc (Zn) (Lv et al. 2012; Ren et al. 2012; Xia et al. 2012a, b). However, few *MTs* genes have been isolated from Cu-tolerant plant species. *Iris lactea* var. *chinensis* is a widely adapted perennial species, which is also a known Cu hyperaccumulator (Zhang et al. 2007). However, little is known about the molecular mechanism of Cu tolerance and accumulation in this species. The purpose of our study was to experimentally test if *IlMT2b* can represent a useful target gene for the phytoremediation of Cu-polluted soil.

Materials and Methods

Plants of *Iris lactea* var. *chinensis* (Fisch.) Koidz. were collected from the *Iris* Resource Collection Garden of the

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Institute of Botany, Jiangsu Province and Chinese Academy of Science. Plants were grown in pots containing 1/2 Hoagland nutrient solution (Han et al. 2007). Seedlings with 10 cm height were selected to optimize uniformity of the experiment. For quantitative real-time PCR (qRT-PCR), 100 μM $CuSO_4$ stress treatment was given to plants. Roots and leaves were sampled at 0, 1, 3, 6, 12 and 24 h after treatment. All samples were collected immediately into liquid nitrogen and then stored at −80 °C until used to extract RNA.

For gene cloning, *I. lactea* var. *chinensis* seedlings were grown in 1/2 Hoagland nutrient solution and then 100 μM $CuSO_4$ treatment for 6 h. Total RNA was extracted from *I. lactea* var. *chinensis* leaves using the TRIzol reagent (TaKaRa, Japan) according to the manufacturer's instructions. The first cDNA strand was synthesized using Oligo $(dT)_{18}$ and SuperScript III reverse transcriptase (Invitrogen, USA), following the manufacturer's instructions, and used as a template for a 25-μL PCR. Three specific primers (GSP1, GSP2, GSP3) were designed for the 5′ rapid application of cDNA ends (RACE) reaction based on the sequence of an *Iris* EST (EX953691) (Tang et al. 2009). The 5′ RACE method was acquired following the procedures described by Liu et al. (2012). Finally, a pair primer (IlMT2b-S, IlMT2b-X) was designed to amplify the complete *IlMT2b* open reading frame (ORF), followed by confirmation of the amplicon by DNA sequencing. The sequences for all of the above primers are given in Table 1. The *IlMT2b* cDNA ORF was identified using the ORF finder program. Multiple peptide alignments and the phylogeny of the sequences were derived using the DNAman software package (version 5.2.2.0; Lynnon Biosoft, St Louis, QC, CA).

The RNA purity was assessed spectrophotometrically at 260/280 and 260/230 nm. The integrity of the purified RNA was checked by denatured agarose gel electrophoresis. qRT-PCR assays were conducted and assessed following the procedures described by Gu et al. (2011). The gene-specific pair IlMT2b-RT-S/-X (sequences given in Table 1) amplified a 151-bp *IlMT2b* fragment, while IlUBC-S/-X (sequences given in Table 1) amplified a 224-bp fragment of the reference gene (*Iris* ubc, GenBank accession EX953716) (Gu et al. 2014).

IlMT2b coding sequence was amplified using a forward primer (IlMT2bc-S) incorporating a *Sma* I restriction site, and a reverse primer (IlMT2bc-X) incorporating a *Xba* I restriction site. *Sma* I-*Xba* I digested amplicons were inserted into pCAMBIA1301-220 to generate a *35S: IlMT2b* construct, which was introduced into *Arabidopsis thaliana* Col-0 (ecotype Columbia) via the floral dip method (Liu et al. 2012). Transformed lines were selected by germination on a standard medium containing 20 mg/L hygromycin, and confirmed by subsequent RT-PCR and qRT-PCR (sequences given in Table 1). T_2 generation *A. thaliana* was used for identifying *IlMT2b* function.

Seeds of wild-type and two *IlMT2b* transgenic lines were germinated on Murashige and Skoog (MS) agar medium containing 0, 50 μM $CuSO_4$, or 100 μM $CuSO_4$ in 9-mm-diameter plates. The plate was maintained vertically in the culture room for 10 days to compare the root length. At least 10 seedlings of each line were measured. For Cu accumulation and H_2O_2 content, four-leaf stage wild type and transgenic *A. thaliana* seedlings grown on MS agar medium were potted into a 1:1:1 perlite: vermiculite: soilrite mixture and grown for 4 weeks, then plants were transferred to a growth regulator-free Hoagland and Arnon's (1950) culture medium. After 2 days, 50 μM $CuSO_4$, or 100 μM $CuSO_4$ was added to the medium and plants were sampled after 7 days growth. The Cu concentration

Table 1 The sequences of the primers used in this study

Primer	Sequence (5′–3′)
GSP1	CGCACTTGCAGCCGTTCTCG
GSP2	TTTGCAGTTGCAGGGGTCGC
GSP3	TCTCGGACTCGATCATTTGC
AAP	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG
AUAP	GGCCACGCGTCGACTAGTAC
IlMT2b-S	ATGTCTTGCTGCGGAGGA
IlMT2b-X	TCATTTGCAGTTGCAGGG
IlMT2bc-S	CGGGATCCATGTCTTGCTGCGGAGGA
IlMT2bc-X	CGAGCTCTCATTTGCAGTTGCAGGG
IlMT2b-RT-S	GGAGGAAACTGCGGCTGTGGGTCT
IlMT2b-RT-X	CAAATCCCTCCACCACGCCTCCTT
IlUBC-S	TCTCGCTTGTCCGGTTTGTG
IlUBC-X	ACCTTGGGTGGCTTGAATGG
AtUBQs	AGGACAAAGAGGGTATCCCA
AtUBQx	CAGACGCAAGACCAAGTGAA

was determined by atomic absorption spectrometry as followed by Lv et al. (2012). H_2O_2 content was determined according to Veljovic-Jovanovic et al. (2002).

Results were expressed as means ± standard errors. SPSS v13.0 (SPSS Inc., Chicago, IL, USA) and Microsoft Excel 2003 (Microsoft, Redmond, WA, USA) were used for statistical analysis. To determine differences among treatments for each variable at each sampling time point, a one-way analysis of variance (ANOVA) was employed.

Results and Discussion

The full-length cDNA *IlMT2b* sequence (AB907788) was isolated by RT-PCR and RACE based on an EST created by Tang et al. (2009). It consisted of 422 nucleotides, of which 237 bp represented an ORF encoding 78 residues. The predicted gene product is a protein of molecular mass 7.64 kDa and a pI of 4.31, containing a conserved CC, CXC and CXXC domain in the N-terminal, and three CXC units in the C-terminal position (Fig. 1a). The alignment of the *IlMT2b* sequence with those of homologous MT2b proteins showed levels of similarity ranging between 49.38 % and 65.82 %, and suggested a close phylogenetic relationship with NpMT2 (Fig. 1b). To gain the expression pattern of *IlMT2b*, qRT-PCR was carried out to analyze samples from leaf and root of Cu stressed plants. From 0 to 3 h treatment, *IlMT2b* transcript abundance increased gradually in leaf and there was no significant difference in root (Fig. 2). The transcription accumulation of *IlMT2b* in leaf and root was dramatically increased in response to Cu treatment after 6 h, and then decreased. The four types of plant MTs differ in sequences (Cobbett and Goldsbrough 2002). Compared with homologues from other species, the positions of MT2 Cys residues are conserved (Fig. 1). So, we concluded that the *IlMT2b* gene encodes a type-2 MT protein. Differential expression of *MT2* genes varies among plant species (Ahn et al. 2012; Ren and Zhao 2009). In the present study, *IlMT2b* was induced by Cu stress. The same Cu inducible expression was found in the *AtMT2a* in leaves and *AtMT2b* in roots (Guo et al. 2003), while *BcMT2* and *TcMT2* were unaffected by Cu treatment (Lv et al. 2012; Roosens et al. 2005). These findings suggest that different plant MTs have distinct functions in Cu tolerance and homeostasis (Lv et al. 2012).

To further study the function of *IlMT2b*, 27 lines of hygromycin-resistant *A.thaliana* plants were selected as putative transgenic plantlets, 21 of which were positive in a glucuronidase (GUS) assay (data not shown). Based on the PCR and RT-PCR assays, two independent transgenic *A. thaliana* lines (*35S:IlMT2b-1*, *35S:IlMT2b-2*) which showed higher levels of transgene expression were selected for further experiments (Fig. 3a, b). For the Cu tolerance analysis of *IlMT2b*-overexpressing *A. thaliana,* two transgenic lines of T_2 progeny and wild-type plants were grown in normal and different Cu concentrations conditions. After 10 days of growth on the MS agar medium under normal conditions, no obvious difference in the root length between wild-type and transgenic seedlings was observed (Fig. 4a, b). After treatment with 50 and 100 μM Cu, the root length of two transgenic seedlings was respectively about 1.5- and 3-fold longer than that of the wild-type

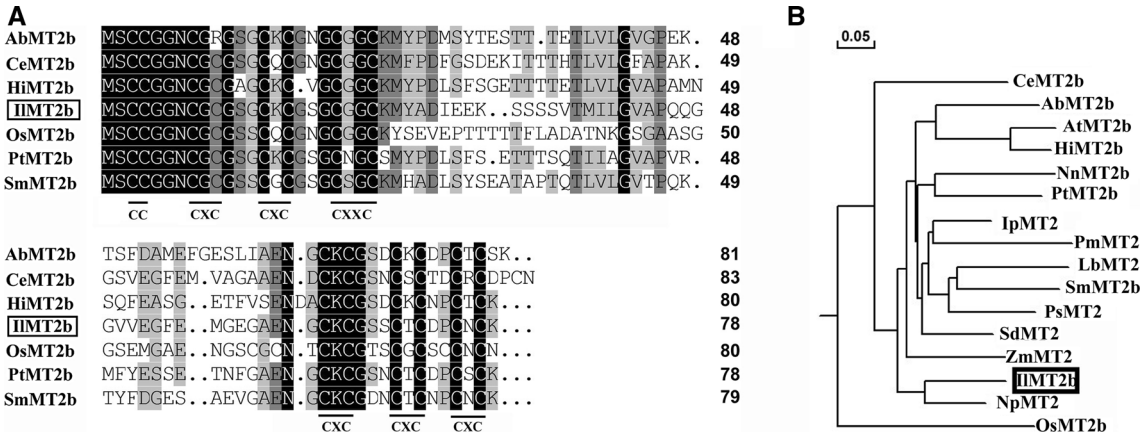

Fig. 1 The deduced peptide sequence of *IlMT2b* (marked in *box*) and related MT2. **a** Peptide alignment. Conserved sequences are shown underlined. **b** The phylogeny of *IlMT2b* and related MT2s. Bootstrap values of each branch of the derived tree are given. The genes encoding the amino acid sequences and their GenBank accession numbers are: *CeMT2b* (*Colocasia esculenta;* Q19LA2), *AbMT2b* (*Atropa belladonna*; CAC40757), *AtMT2b* (*Arabidopsis thaliana*; AED90465), *HiMT2b* (*Hirschfeldia incana*; AGQ45633), *NnMT2b* (*Nelumbo nucifera*; ABN46988), *PtMT2b* (*Populus trichocarpa*; AAT02525), *IpMT2* (*Ilex paraguariensis*; AFP93964), *PmMT2* (*Plantago major*; CAH59436), *LbMT2* (*Limonium bicolor*; ABL10086), *SmMT2b* (*Salvia miltiorrhiza*; ABR92330), *PsMT2* (*Pisum sativum*; BAD18383), *SdMT2* (*Sesbania drummondii*; ABQ44281), *ZmMT2* (*Zea mays*; ACG42263), *IlMT2b* (*Iris. lactea* var. *chinensis*; AB907788), *NpMT2* (*Narcissus pseudonarcissus*; AAL16908), *OsMT2b* (*Oryza sativa*; Q5JM82). *IlMT2b* is in box

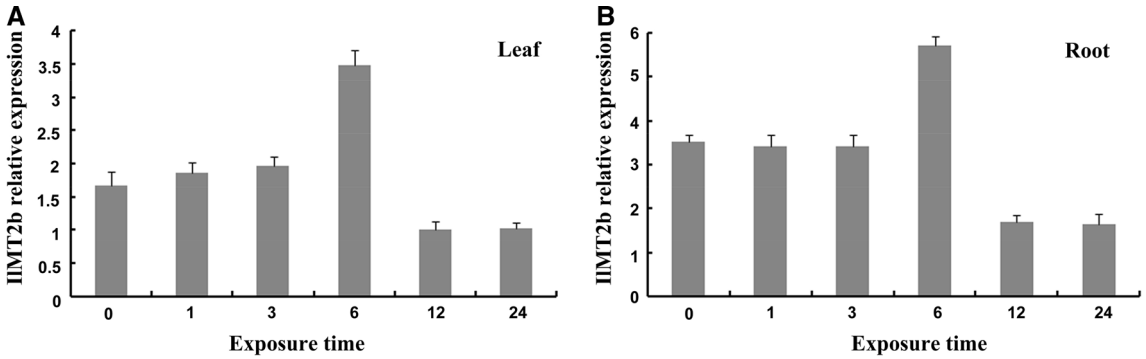

Fig. 2 The induced transcription accumulation of *IlMT2b* in leaves (**a**) and roots (**b**) of plants exposed to 100 μM Cu following a period of Cu starvation, as determined by triplicate qRT-PCR assays

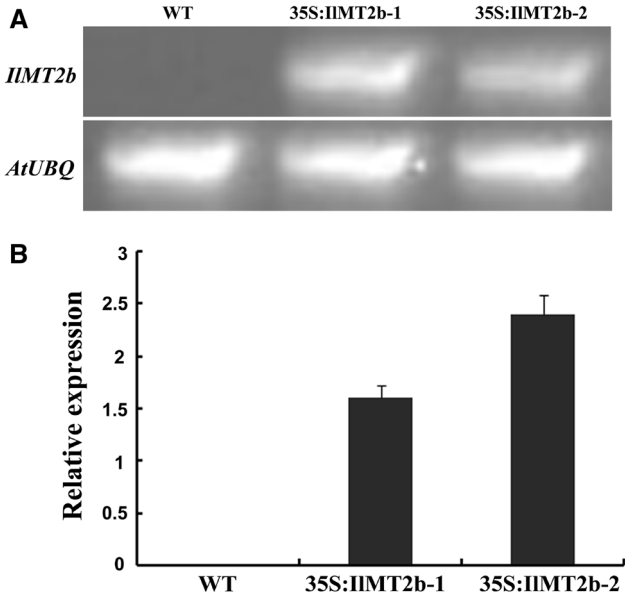

Fig. 3 PCR and qRT-PCR validation of transgenic. **a** RT-PCR demonstrating the heterologous expression of *IlMT2b* in *A. thaliana*. The *AtUBQ* sequence was used as an internal control; **b** qRT-PCR based on mRNA. Transcript abundance was normalized against the expression of the constitutively expressed *AtUBQ*. The PCR for each sample was replicated three times, and the data efficiency of each reaction was $2^{-\Delta\Delta Ct}$. *WT* wild type *A. thaliana*; 35S: IlMT2b-1 and 35S:IlMT2b-2, two presumptive *IlMT2b*-carrying transgenic lines

(Fig. 4c–f). Although wild-type and transgenic seedlings grew normally, their root lengths were inhibited under the two Cu concentrations compared to control. There was no significant root length difference between the two transgenic lines. As root length has been considered as a reliable standard for measuring heavy metal tolerance (Gasic and Korban 2007), our work demonstrated that heterologous *IlMT2b* expression has provided evidence for Cu tolerance in *A. thaliana.* Interestingly, conflicting results have been reported on *BjMT2*. Ectopic expression of *BjMT2* reduced root growth in the absence of Cu exposure, whereas in the presence of Cu stress, root growth in wild-type and *BjMT2* transgenic lines was identical (Zhigang et al. 2006). However, the root length of *BcMT2* transgenic lines was longer under Cu stress (Lv et al. 2012). It is possible that the MT2-mediated root elongation reduction is related to interference of excess MT2 with the cellular redox balance or signalling processes (Mir et al. 2004; Thomas et al. 2005). Thus, more studies are under way to elucidate the MT2-mediated root growth mechanism.

The concentration of Cu within the plants reflects their ability to cope with Cu stress. Copper concentrations in wild-type and transgenic seedlings were higher in plants exposed to 100 μM Cu than in those exposed to 50 μM Cu (Fig. 5). However, Cu concentrations in the two transgenic seedlings were higher than in the wild-type exposed to the two copper concentrations (Fig. 5). It is likely that high levels of *IlMT2b* expression may be required for Cu homeostasis (Roosens et al. 2004). Some observations support the hypothesis. Thomas et al. (2003) observed that overexpression of the yeast metallothionein (CUP 1) in tobacco plants results in two to three times the copper content as that of the control plants. Furthermore, a predominant Cu-homeostasis function has also been found in *TcMT3* (Roosens et al. 2004). In *A. thaliana*, constitutive expression of *AtMT2b* increased Cu accumulation in the yeast *Dcup1* mutant (Guo et al. 2003, 2008). Also, a pea class-2 MT increased Cu tolerance and accumulation, when overexpressed in *Escherichia coli* and *A. thaliana* (Evans et al. 1992).

Copper stress can lead to the generation of reactive oxygen species (ROS), such as H_2O_2 (Kumar et al. 2012). In our study, H_2O_2 accumulation in wild-type and transgenic seedlings was significantly increased, as seen by measuring H_2O_2 content during Cu stress (Fig. 6). However under 50 μM Cu stress, the leaves of two *IlMT2b* transgenic lines showed remarkably less accumulation of H_2O_2 content compared to wild-type (Fig. 6). This contrast in H_2O_2 levels between wild-type and transgenic lines was noticed even under 100 μM Cu stress (Fig. 6). In animals,

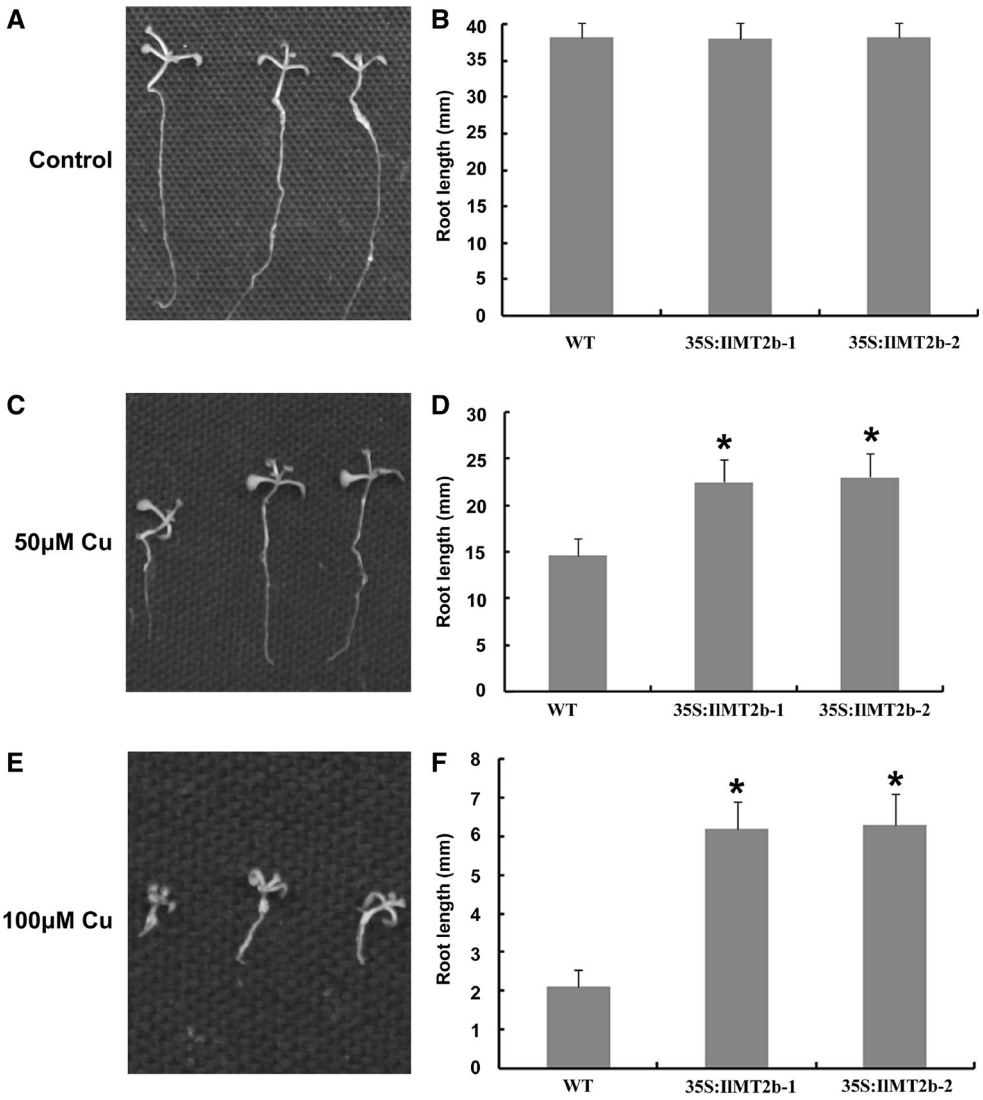

Fig. 4 Tolerance to Cu in wild-type and two *IlMTb* transgenic lines. Seeds were germinated on MS agar medium containing 0 μM (**a**, **b**), 50 μM (**c**, **d**), or 100 μM (**e**, **f**) $CuSO_4$, and petri dishes were placed in a vertical orientation upon onset of growth. After 10 days of growth, root lengths of two *IlMT2b* lines and WT were measured. An *asterisk* indicates a significant difference (by the *t* test at $p < 0.05$) from WT of the same treatment. Values correspond to mean ± SE of 10 plants

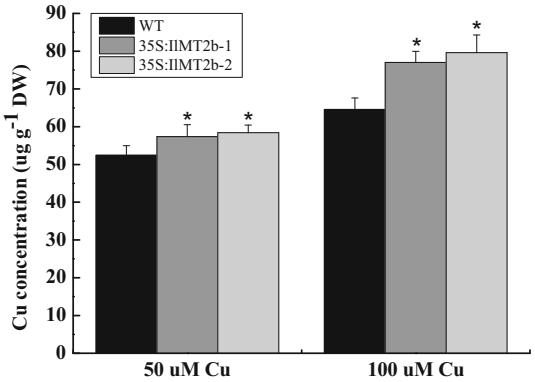

Fig. 5 Cu accumulation in leaves of wild-type and two *IlMTb* transgenic lines exposed to 50 or 100 μM Cu stess for 7 days. WT: wild type *A. thaliana*; *35S:IlMT2b-1* and *35S:IlMT2b-2*: *IlMT2b* transgenic lines. An *asterisk* indicates a significant difference (by the *t* test at $p < 0.05$) from WT of the same treatment. Values correspond to mean ± SE of of 10 plants

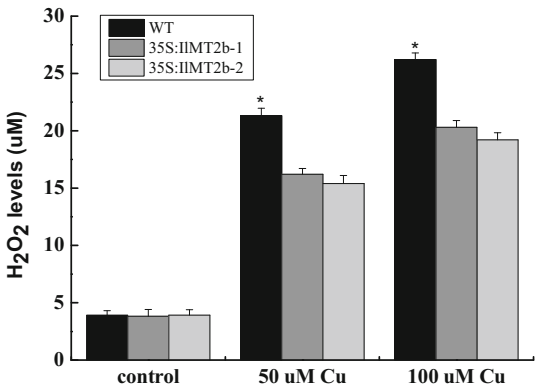

Fig. 6 H_2O_2 level in leaves of wild-type and two *IlMTb* transgenic lines exposed to 50 or 100 μM Cu stress for 7 days. An *asterisk* indicates a significant difference (by the *t* test at $p < 0.05$) from WT of the same treatment. Values correspond to mean ± SE of 5 plants

MTs act as antioxidants against ROS (Palmiter 1998). However, plant MTs may also provide protection against ROS during abiotic stress. In our study, injured leaves were shown to contain lower concentrations of H_2O_2 in two *IlMT2b* transgenic lines under Cu stress (Fig. 6). It is possible that the ROS scavenging in plants might have been affected by overexpression of *IlMT2b*, and this may have resulted in the observed Cu tolerance in transgenic lines. Similar results have been reported previously. For example, *AtMT2a* mediates ROS balance during oxidative stress (Zhu et al. 2009). *BcMT1* and *BcMT2* decreased production of Cu induced ROS (Lv et al. 2012), while *OsMT1e-P* conferred multiple abiotic stress tolerance in tobacco via ROS scavenging (Kumar et al. 2012). Based on these studies, it was proposed that higher ROS scavenging ability resulted in increased tolerance to abiotic stress, and that ROS may act as secondary messengers in redox signal transduction (Xue et al. 2009).

In the present study, we described the isolation of a *MT2b* cDNA from *I. lactea* var. *chinensis* and showed that its transcription accumulation is induced by an exogenous supply of Cu. Its heterologous expression in *A. thaliana* demonstrated that the *IlMT2b* transgenic lines enhanced the tolerance and accumulation of Cu. Further, we also showed that *IlMT2b* overexpressing plants accumulated lower amounts of hydrogen peroxide (H_2O_2) under Cu stress. Based on these results, *IlMT2b* may serve as an important target gene for phytoremediation of Cu-contaminated soils.

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