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Cloning and characterization of *HbMT2a*, a metallothionein gene from *Hevea brasiliensis* Muell. Arg differently responds to abiotic stress and heavy metals

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ABSTRACT

Metallothioneins (MTs) are of low molecular mass, cysteine-rich proteins. They play an important role in the detoxification of heavy metals and homeostasis of intracellular metal ions, and protecting against intracellular oxidative damages. In this study a full-length cDNA of type 2 plant metallothioneins, *HbMT2a*, was isolated from 25 mM Polyethyleneglycol (PEG) stressed leaves of *Hevea brasiliensis* by RACE. The *HbMT2a* was 372 bp in length and had a 237 bp open reading frame (ORF) encoding for a protein of 78 amino acid residues with molecular mass of 7.772 kDa. The expression of *HbMT2a* in the detached leaves of rubber tree clone RY7-33-97 was up-regulated by Me-JA, ABA, PEG, H_2O_2, Cu^{2+} and Zn^{2+}, but down-regulated by water. The role of HbMT2a protein in protecting against metal toxicity was demonstrated *in vitro*. PET-28a-HbMT2-beared *Escherichia coli*. Differential expression of *HbMT2a* upon treatment with 10 °C was observed in the detached leaves of rubber tree clone 93-114 which is cold-resistant and Reken501 which is cold-sensitive. The expression patterns of *HbMT2a* in the two rubber tree clones may be ascribed to a change in the level of endogenous H_2O_2.

1. Introduction

Metallothioneins (MTs) belong to a family of intracellular metal-binding proteins, which are evolutionally conserved, low molecular mass, cysteine-rich proteins and present in virtually all living organisms including mammals, plants, and fungi as well as some prokaryotes [1,2]. The first MT identified in plants is the wheat *EcMT* protein [3], and since then more and more MT sequences have been recorded from various plant species. Based on the structure and sequence analysis of MTs, plant MT proteins have been historically divided into four types [1,4]. Although type 1 and type 2 of plant MTs are characterized by two cysteine-rich domains separated by a central cysteine-free spacer, there are differences in the arrangement of cysteine residues in the N- and C-terminal domains [5]. The type 1 has exclusively C-X-C clusters while the type 2 has C-C, C-X-C, and C-X-X-C clusters in N-terminal domains with a highly conserved sequence (MSCCGGNCGCGS) , and three C-X-C motifs in C-terminal domains [4]. Type 3 of plant MTs contains only four Cys residues in the N-terminal domain and the consensus sequence is Cys-Gly-Asn-Cys-Asp-Cys [1]. Type 4 of plant MTs is constituted of phytochelatins, enzymatically synthesized peptides with a poly-glycine structure [6].

MTs are not only involved in maintaining homeostasis of essential metals and metal detoxification [7,28], but are implicated in a range of physiological processes, including scavenging reactive oxidant species (ROS) [8,9], regulating cell growth, proliferation, and activity of metalloenzymes and transcription factors [9,10]. Thus, it is likely that MTs do not play a single role but that, as a result of evolution, some have acquired specific functions. Therefore, MTs, to some extent, could confer the adaptability of plants to different environmental stresses [11].

Although natural rubber is synthesized in over 2000 plant species, involving about 300 genera from 7 families [9], the rubber tree (*Hevea brasiliensis*) is a key tropical cash crop due to the good yield and excellent physical properties of its products. With the extension of rubber plantation from traditional area to nontraditional area, rubber tree suffers inevitable abiotic stresses, such as cold and drought. In the present paper, an *HbMT2a* gene was cloned and its expression was analyzed under the condition of heavy metal ions, H_2O_2, drought and cold. Our results showed that drought up-

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regulated the expression of *HbMT2a* relative to water and the gene *HbMT2a* exhibited inverse trend of expression in a cold-sensitive rubber tree clone and in a cold-resistant rubber tree clone.

2. Materials and methods

2.1. Plant materials and treatments

Adult trees of *H. brasiliensis* clone RY7-33-97 were planted at the experimental farm of Chinese Academy of Tropical Agricultural Sciences on Hainan Island, in China. Except for cold stress, leaf samples were collected from five trees and the detached compound leaves were kept in an illuminating incubator with a temperature of 28 °C and a relative humidity of 80%. The leaves were treated with 25 mM PEG8000, 50 μM H_2O_2, 100 μM ABA, 0.071% Me-JA, 50 μM $ZnSO_4$ and 50 μM $CuSO_4$ for 0 h, 1 h, 3 h, 5 h, and 7 h, respectively. For cold stress, detached leaves from cold-resistant rubber tree clone 93114 and cold-sensitive rubber tree clone Reken-501 were kept in an illuminating incubator with a temperature of 10 °C for 0 h, 2 h, 4 h, 6 h and 8 h, respectively. Samples of the whole middle leaflets were collected from control (treated with water) and treated leaves at the pointed intervals as above, immediately frozen in liquid nitrogen and stored at −70 °C for subsequent RNA isolation.

2.2. Isolation of RNA and DNA

Total RNA and DNA were extracted according to the method of Kim and Hamada [12]. The quality and concentration of the extracted RNA and DNA were checked by agarose gel electrophoresis and quantified by a spectrophotometer (Alphalmager2200, USA).

2.3. Internal conserved fragment cloning

The degenerate primers, P1 (5′-TCW TGY TGY GGW GGH AAY TGY GG -3′) and P2 (5′-CAR RTG CAW GGG TYR CAS STG C-3′), were designed according to the conserved region of MT members from other species in the GenBank. Total RNA (2 μg) was used in reverse transcription PCR (RevertAidMT H Minus First Strand cDNA Synthesis Kit, Fermentas, USA) in accordance with the instructions provided by the manufacturer. The resulting cDNAs were used as a template for PCR amplification with oligonucleotide primers (P1, P2) under the following conditions: 3 min denaturation at 94 °C, followed by 36 cycles of amplification (94 °C for 30 s, 62 °C for 30 s, 72 °C for 1 min). The amplified products were purified and cloned into the TaKaRa pMDTM-19T vector (D102A), followed by sequencing.

2.4. 3′-RACE (rapid amplification of cDNA ends)

The 3′-ready cDNA was synthesized by reversely transcribing 1 μg total RNA with oligo dT-3site adaptor primer (5′-CCA GTG AGC AGA GTG ACG AGG ACT CGA GCT CAA GCT TTT TTT TTT TTT TT-3′) supplied by 3′-RACE kit (Takara, Dalian, China). The specific primers for 3′-RACE were designed as primer 3P1 (5′- GCT GAG AGT GGG AAT GGC TGC -3′) and the nested primer 3P2 (5′- GCT GTG GCT CCG GAT GCA AG -3′) based on the cloned internal conserved fragment. The first round of 3′-RACE was performed using the universal primer (5′-CCA GTG AGC AGA GTG ACG-3′) and the specific primer 3P1 in a total volume of 25 μL under the following conditions: 3 min denaturation at 94 °C, followed by 31 cycles of amplification (94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min). The PCR product was diluted ten-fold as a template for the second round of 3′-RACE using the universal primer (5′-GAG GAC TCG AGC TCA AGC-3′) and the specific primer 3P2 under the same conditions as the first round. The products were purified and cloned into pMDTM-19T vector, followed by sequencing.

2.5. 5′-RACE (rapid amplification of cDNA ends)

The 5′ end of cDNA was amplified using 5′ RACE System for Rapid Amplification of cDNA Ends (invitrogen) following the manufacturer's instructions. cDNAs were synthesized from 2 μg total RNA with the oligo (dT)18 primer of RevertAidTM H Minus First Strand cDNA Synthesis Kit (Fermentas), and subsequently amplified by two successive steps. The first step consisted of 94 °C for 3 min and 30 cycles of amplification (94 °C for 30 s, 59 °C for 30 s, 72 °C for 1 min) by using the anchor AAP primer (5′-GGC CAC GCG TCG ACT AGT ACG GGG GGG GGG-3′) and antisense primer P1 (5′-CTC TCA GCA CTA AAG TTC ATC TCA G-3′). The PCR product was diluted ten-fold as a template for the second step, which consisted of 94 °C for 3 min, and then 36 cycles of amplification (94 °C for 30 s, 59 °C for 30 s, 72 °C for 1 min) by using the anchor AUAP primer (5′-GGC CAC GCG TCG ACT AGT AC -3′) and the specific primer 5P2 (5′-GGG TCT CAG TTC TGG TGT TCT CT AC-3′). The products were purified and cloned into pMDTM-19T vector, followed by sequencing.

2.6. Cloning of ORF and intron regions

After aligning and assembling, a full-length cDNA sequence was deduced, and subsequently confirmed by RT-PCR. The open reading frame (ORF) was amplified by using a pair of primers: HbMT2F (5′-CCA AGA GTT GAA GGA TTC AAT TAC C-3′) and HbMT2R (5′- TGA GAA CAT CGA GTA CAG CTT TGC -3′) under the following conditions: 3 min denaturation at 94 °C, followed by 32 cycles of amplification (94 °C for 30 s, 62 °C for 30 s, 72 °C for 1 min) and a 10 min final extension at 72 °C. The same primer pair was used to isolate the intron(s) within ORF by using genomic DNA as template under the following conditions: 3 min denaturation at 95 °C, followed by 32 cycles of amplification (94 °C for 30 s, 62 °C for 30 s, 72 °C for 1 min) and a 10 min final extension at 72 °C. The products were purified and cloned into pMDTM-19T vector (TaKaRa), and sequenced.

2.7. Multiple alignments and bioinformatic analysis

The full-length cDNA sequence was compared to the non-redundant peptide database at NCBI (http://www.ncbi.nlm.nih.gov) using BLAST version 2.2.17. The multiple alignment was performed by using Multiple Sequence Alignment of DNAMAN software 6.0. A phylogenetic tree was constructed using Multiple Sequence Alignment of DNAMAN software 6.0.

2.8. Quantitative real-time RT-PCR (qPCR)

Prior to cDNA synthesis, contaminating DNA was removed from RNA samples using Ambion DNA-free Dnase Treatment and Removal Reagents. First strand cDNA was synthesized from 2 μg of RNA with M-MuLV reverse transcriptase and random hexamer primers (RevertAidMT H Minus First Strand cDNA Synthesis Kit, Fermentas, USA) according to the manufacturer's instructions. The cDNA was diluted 1:20 with nuclease-free water. Aliquots of the same cDNA sample were used for quantitative real-time PCR with specific primers designed for the genes *HbMT2a* and *HbMT2* (GenBank accession number: FJ229481) (HbMT2af:5′AAGGATTCAATTACCAAGTAAAGGCC3′, HbMT2ar:5′ATGTCAGGGTACATACCACATCCG3′; HbMT2f: TGAAGGGTACATCTTGGAAAATT TG, HbMT2r: ATGTCAGGGTACATA

CCACATCCG) and for a housekeeping gene 18S (GeneBank accession number: AY435212) (Y18Sf: 5′ GCT CGA AGA CGA TCA GAT ACC 3′; Y18Sr5′ TTC AGC CTT GCG ACC ATA C 3′). Real-time RT-PCR was performed using the fluorescent dye SYBR-Green (Takara, Dalian, China) and the LightCycler 2.0 system (Roche Diagnostics, Germany). The reactions were carried out as follows: 30 s at 95 °C for denaturation, 5 s at 94 °C, 20 s at 60 °C, and 20 s at 72 °C for amplification. Each PCR reaction was done in triplicate and no-template controls were included. Accumulation of PCR products was detected in real time and the results were analyzed with Exor3 System Software (Applied Biosystem, Swiss) and presented as 2-dCt, where dCt was the difference between Ct values of *HbMT2a* and the reference gene.

Statistical analysis: Each of the experiments was performed 3 times, and values were presented as mean ± S.D. The statistical significance of the values was determined by the *T-test*. The P values <0.05 and <0.01 were considered to be significant and very significant, respectively.

2.9. Recombinant expression

The ORF of *HbMT2a* with introduced NdeI and SalI sites was inserted into PET-28a plasmid via the restriction sites. The resulting PET-HbMT2a construct was confirmed by sequencing and transferred into *Escherichia coli* strain BL21. The BL21 cells with PET-HbMT2a and PET-28a plasmids were grown at 37 °C in 20 mL of luria bertani (LB) medium containing 50 mg/L ampicillin. 1 mL of overnight-cultured transformants was inoculated into 40 mL of fresh LB medium containing 50 mg/L ampicillin. After the transformants in medium were grown to an OD600 value of 0.6, isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and then followed an incubation at 37 °C for 4–5 h. Total proteins were separated by 12% SDS-PAGE according to the standard method by Laemmli [13].

2.10. Tolerance of HbMT2a-beared E. coli to heavy metal ions

To investigate the tolerance of *HbMT2a-beared E. coli* to heavy metal ions, PET-HbMT2a- and PET-28a-beared *E. coli* cells were separately inoculated into 40 mL of LB medium with appropriate antibiotics, 1 mM IPTG, and either 3.5 mM $CuSO_4$ or 1.75 mM $ZnSO_4$ at 35 °C, and shaken at 200 rpm shaker. The OD600 value was measured at 1 h intervals.

3. Results

3.1. Isolation and characterization of HbMT2a

A 227-bp conserved fragment was amplified with a pair of degenerate primers designed according to the conserved region of MT members from other plant species. 3′-RACE and 5′-RACE generated a 292-bp fragment and a 172-bp fragment, respectively. By alignment and assembly of these three fragments, a full-length cDNA was deduced, and its open reading frame (ORF) was amplified by PCR and confirmed by sequencing. The cDNA, referred to as *HbMT2a* (GenBank accession number: HQ687666), was 372-bp in length containing a 237-bp ORF, flanked by a 55-bp 5′-UTR (untranslated region) upstream of the start codon and a 80-bp 3′-UTR including a poly (A) tail of 12 bp downstream of the stop codon.

The putative HbMT2a protein was composed of 78 amino acid residues and contained two cysteine-rich domains which were separately located at N-terminal and C-terminal regions (Fig. 1A). The N-terminal domain had eight Cys residues with patterns of C-C, C-X-C and C-X-X-C, and also had a highly conserved domain, MSCCGGNCGCGS while there were three C-X-C motifs in the C-terminal domain (Fig. 1A). There characters were shared with other members of type 2 MTs (Fig. 1A). The HbMT2a protein was clustered into type 2 MTs by the analysis of phylogenetic tree (Fig. 1B), sharing 85.9%, 75%, 71.6%, 64.43%, 60.49%, 59.76%, 59.26% identity with HbMT2, PtMT2b, PtMT2a, OsMT2, NcMT2a, SnMT2, AtMT2a. To test whether *HbMT2a* contained intron (s), the primers used to amplify the ORF of *HbMT2a* were used to amplify the corresponding genomic fragment from genomic DNA. The genomic fragment contained three exons of 64 bp, 76 bp and 97 bp in length, which were separated by two introns of 99 bp and 173 bp in length, respectively (Fig. 2A).

3.2. Expression profiles of HbMT2a upon drought, heavy metal iron and oxidative stresses

The specific primers were designed to amplify 120-130 bp fragments of the genes *HbMT2a* for quantitative real-time PCR (Fig. 2B). The expression of *HbMT2a* upon drought, heavy metal ions and oxidative stresses was analyzed by quantitative real-time RT-PCR (qPCR). Water could markedly down-regulate the expression of *HbMT2a* in detached leaves up one to seven hours of treatment (Fig. 3A–F). The level of *HbMT2a* expression upon treatment with PEG, H_2O_2, Me-JA, ABA, $CuSO_4$ and $ZnSO_4$ in water was significantly higher than that upon treatment with water, among which, the effect of PEG was the strongest (Fig. 3A–F).

3.3. Differential expression patterns of HbMT2a and HbMT2 in rubber tree clones with different tolerance to cold

It has been demonstrated that rubber tree clone 93-114 is cold-resistant while the rubber tree clone Reken501 is cold-sensitive. DNA sequence analysis showed that *HbMT2a* and *HbMT2* had high homology (80.1%). Specific primers were designed to amplify 120-130 bp fragments of the genes *HbMT2a* and *HbMT2* for quantitative real-time PCR (Fig. 2B). In detached leaves of the two rubber tree clones upon treatment with 10 °C, the two genes exhibited similar trend of transcript accumulations with different relative abundances (Fig. 3G and H). In rubber tree clone 93-114, the expression of *HbMT2a* was high at 0 h, down-regulated remarkably and remained constant at 2 h, 4 h and 6 h of treatment, and then up-regulated at 8 h after treatment with 10 °C. By contrast, in rubber tree clone Reken-501, the expression of *HbMT2a* was low at 0 h and 2 h, increased at 4 h and 6 h, and then down-regulated at 8 h. The gene *HbMT2a* exhibited inverse trend of expression in cold-sensitive rubber tree clone and in cold-resistant rubber tree clone (Fig. 3H).

3.4. Function analysis of HbMT2a in vivo

Recombinant HbMT2a protein which was an expected molecular weight of 10.064 kDa was expressed in PET-28a-*HbMT2a*-beared *E. coli* (Fig. 4A). In the absence of Cu^{2+} and Zn^{2+}, the growth kinetics of PET-28a-beared *E. coli* cells was identical with that of PET-28a-*HbMT2a*-beared *E. coli* cells (Fig. 4B). Addition of either 3.5 mM $CuSO_4$ or 1.75 mM $ZnSO_4$ inhibited the growth of the both cell strains. The effect of $CuSO_4$ and $ZnSO_4$ on inhibiting the growth of PET-28a-beared *E. coli* cells was much stronger than that of PET-28a-*HbMT2a*-beared *E. coli* cells (Fig. 4C and D), suggesting that production of recombinant HbMT2a protein gave *E. coli* cell's more tolerance to Cu^{2+} and Zn^{2+}.

4. Discussion

MT genes have been isolated from rice, poplar, watermelon, olive, Jatropha and other plant species [6,8,14,29,30]. Type 2 of

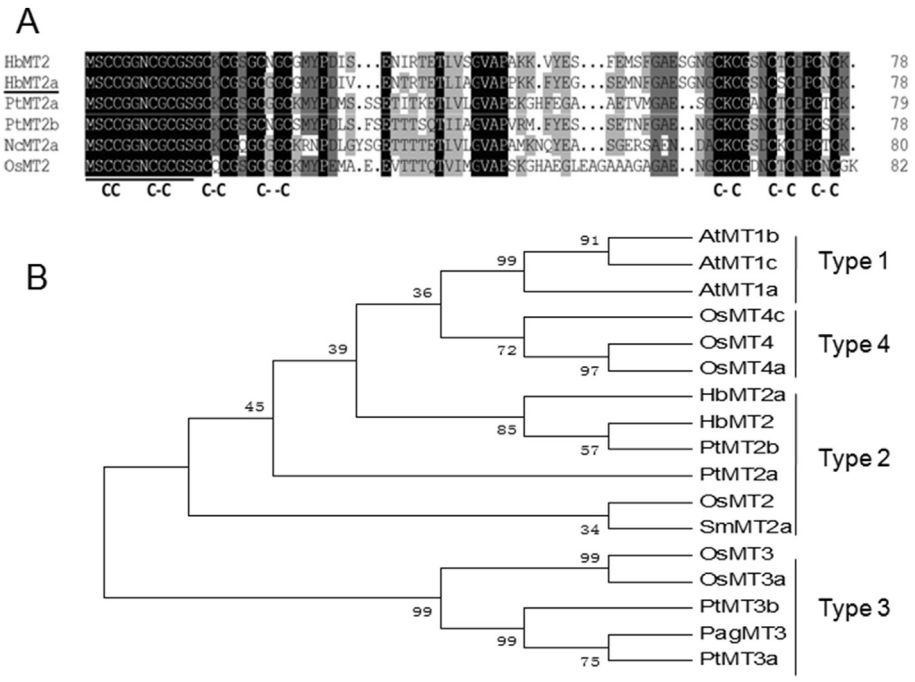

Fig. 1. Amino acid alignment and Phylogenetic tree of HbMT2a and other MT2 proteins. (A)The identical amino acids were shaded in dark and the well-conserved residues were shaded in gray. The highly conserved domain (MSCCGGNCGCGS) was underlined. The conserved cysteine residues were indicated by letter C. (B) Phylogenetic tree of deduced amino acid sequences from MTs was constructed by neighbor-joining method with 1000 bootstrap replication using MEGA 5.0 software. The amino acid HbMT2a: HQ687666 and HbMT2: ACI42984 were from *Hevea brasiliensis*, NcMT2a: ACR46970 was from *Noccaea caerulescens*, PtMT2a: AAT02524, PtMT2b: AAT02525, PtMT3a: AAT02526 and PtMT3b: AAT02527 were from *Populus trichocarpa* × *Populus deltoides*, PagMT3: BAD95608 from *Populus alba* × *Populus tremula*, OsMT2: P94029, OsMT4c: Q2QNC3, OsMT4a: Q0IMG5, OsMT3: ABL74404, OsMT4: NP_001067081 and OsMT3a: A2WLS0 from rice, AtMT1c: Q38804, AtMT1b: Q38803 and AtMT1a: NP-172239 from *Arabidopsis thaliana*, and SmMT2a ABR92329 from *Salvia miltiorrhiza*.

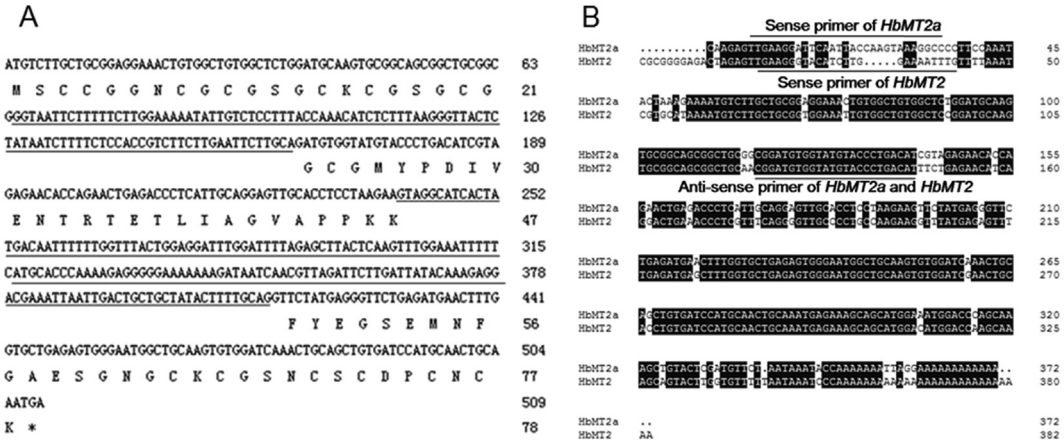

Fig. 2. The positions and sequences of two introns and specific primers in the HbMT2a gene. (A) nucleotide and deduced amino acid sequences of *HbMT2a*, indicating the positions and sequences of two introns (underlined). (B) specific primers were designed to amplify 120-130 bp fragments of the genes *HbMT2a* and *HbMT2* for quantitative real-time PCR.

plant MTs (MT2) is characterized by two cysteine-rich domains located separately at N-terminal and C-terminal, one C-C, two C-X-C and one C-X-X-C motifs in order as well as a highly conserved sequence, MSCCGGNCGCGS in the N-terminal domain and three C-X-C motifs in the C-terminal domain [1,4]. In this study, the putative HbMT2a protein possesses all the characteristics mentioned above. Besides, it is homologous with MT2 members from different plant species. Taken all these data together, HbMT2a is a member of type 2 of plant MTs.

Plants produce JA under drought stress, which subsequently stimulates the production of ABA [15,16]. The transcript expression of a metallothionein gene, *PpMT2* from *Physcomitrella patens* can be induced by ABA [17]. Drought stress also up-regulates *MT2* expression in barley [18], and it was suggested that CLMT2 was implicated in improving tolerance of watermelon to drought stress [8]. In this study, the expression of *HbMT2a* was up-regulated upon drought stress-related factors, Me-JA, ABA and PEG, but down-regulated by water, implying that *HbMT2a* may positively mediate the response of rubber tree to drought stress.

The high percentage of cysteine residues confers the role of MTs in heavy metal detoxification [19,20,31–34] as well as in scavenging ROS (reactive oxygen species) [1,8,21,22]. Plant MT2s have been shown to increase Cd^{2+} and Cu^{2+} tolerance in plants when overexpressed [23], implying that they are important in metal

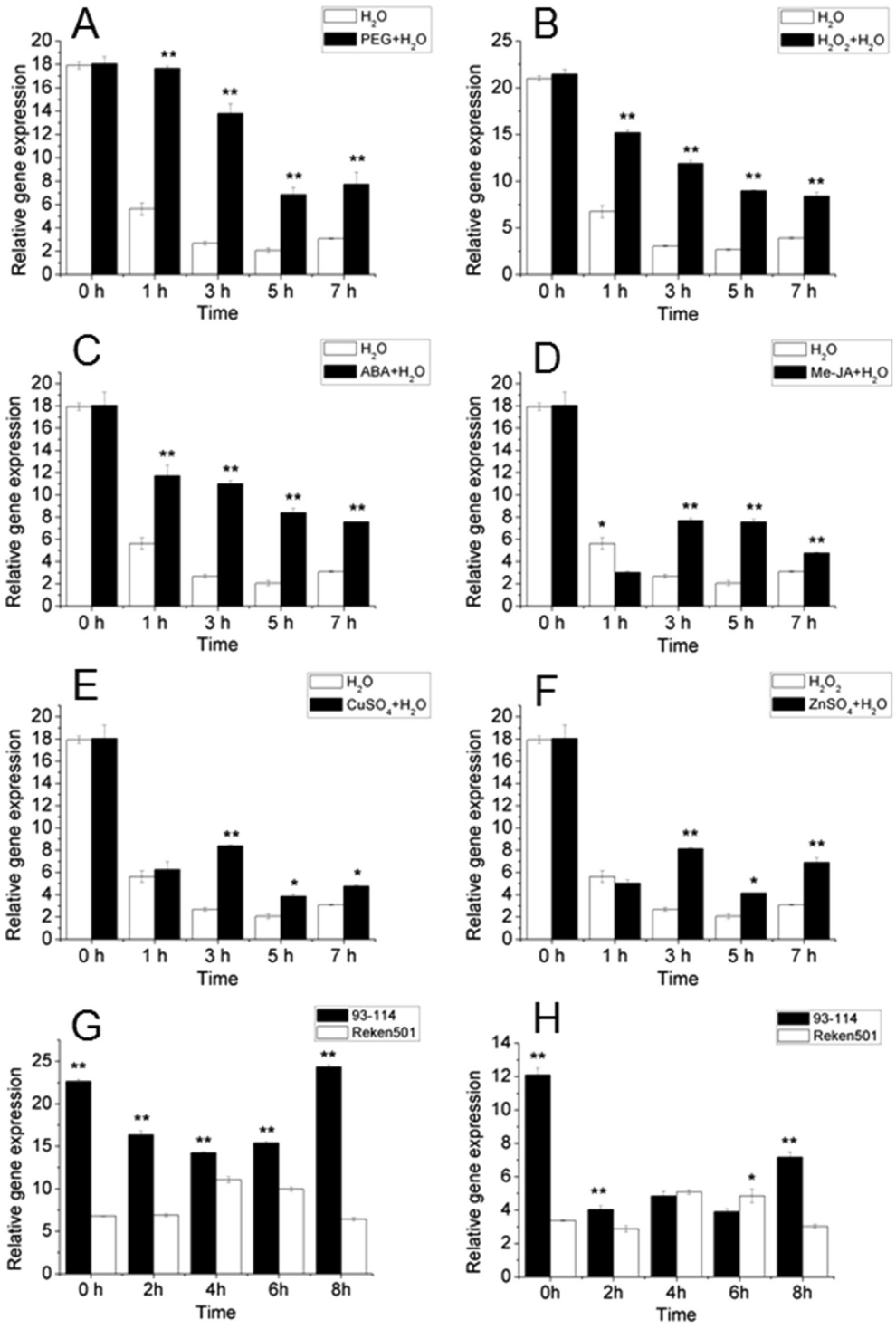

Fig. 3. Expression patterns of *HbMT2a*. expression patterns of *HbMT2a* in leaves of rubber tree clone RY7-33-97 treated with PEG (A), H_2O_2 (B), ABA (C), Me-JA (D), $CuSO_4$ (E), and $ZnSO_4$ (F), respectively. And differential expression of *HbMT2* (G) and *HbMT2a* (H) in leaves of rubber tree clones 93-114 and Reken501 under low temperature (10 °C). One asterisk indicates significant differences ($P < 0.05$), two asterisks indicate very significant differences ($P < 0.01$) between treatments.

detoxification. The *MT2* expression in plants has been shown to be inducible by several metals, including Cu^{2+} [24] and Zn^{2+} [25–27] in Arabidopsis seedlings. The overproduction of recombinant *HbMT2* improves the tolerance on Cu^{2+} and Zn^{2+} in the *Escherichia coli* cells, and scavenges the reactive oxidant species (ROS) *in vitro*, implying that *HbMT2* increases the tolerance of rubber trees to heavy metal ions [9]. The *HbMT2a* and *HbMT2* are clustered into type 2 MTs, respectively share 80.1% and 85.9% identity in nucleotide and amino acid, In the present study, the role of HbMT2a protein in protecting against metal toxicity was demonstrated in PET-28a-HbMT2-beared *E. coli*, and the expression of *HbMT2a* is induced by H_2O_2, suggesting that HbMT2a protein could scavenge ROS. In cold-resistant rubber tree clone 93-114 and cold-sensitive rubber tree clone Reken501 upon cold treatment, The *HbMT2a* and *HbMT2* exhibit similar trend of transcript accumulations with different relative abundances. The inverse trend of *HbMT2a*

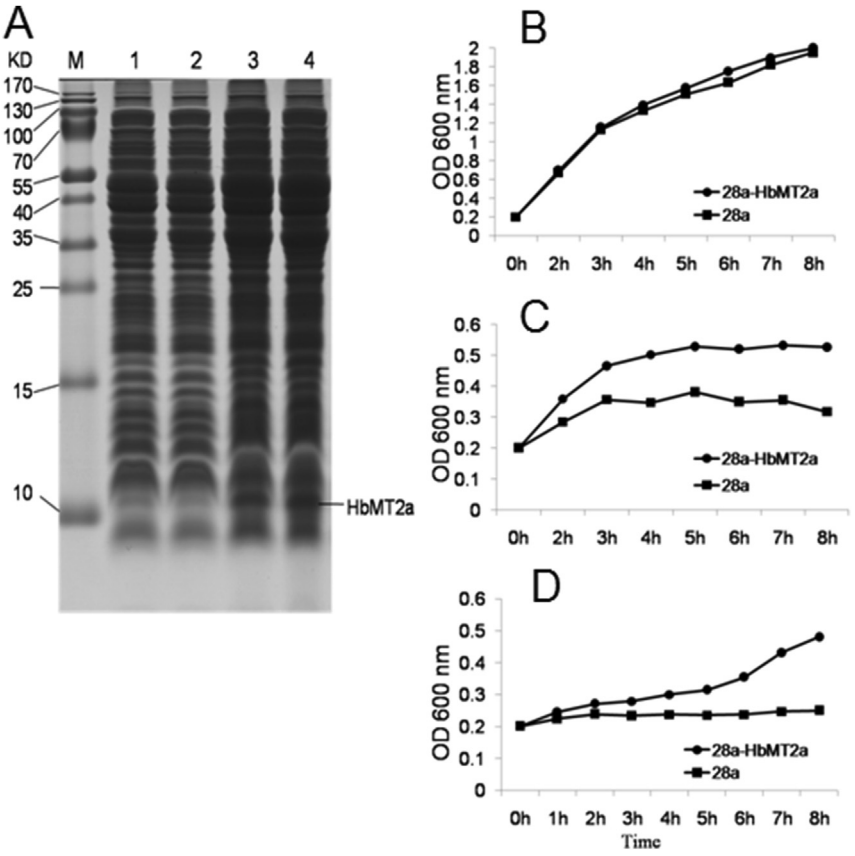

Fig. 4. Function analysis of *HbMT2a in vivo*. (A) SDS-PAGE analysis of recombinant HbMT2a in *E. coli*. Protein samples were from PET-28a-beared *E. coli* cultivated in medium without IPTG for 4 h (1) and PET-28a-HbMT2a-beared *E. coli* cultivated in medium without IPTG for 4 h (2) and with IPTG for 4 h (3) and 5 h (4). Growth kinetics of *E. coli* cells with different plasmids in LB (B) and LB containing 3.5 mM $CuSO_4$ (C) and 1.75 mM $ZnSO_4$ (D). The PET-28a- and PET-28a-HbMT2a-beared bacterial cells were indicated as PET-28a and PET-28a-HbMT2a, respectively.

expression in the two rubber tree clones may be ascribed to a change in the level of endogenous H_2O_2.

Conflict of interest

The paper has no conflict of interest.

Acknowledgments

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