

RESEARCH PAPER

Cotton metallothionein GhMT3a, a reactive oxygen species scavenger, increased tolerance against abiotic stress in transgenic tobacco and yeast

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Abstract

A cDNA clone encoding a 64-amino acid type 3 metallothionein protein, designated *GhMT3a*, was isolated from cotton (*Gossypium hirsutum*) by cDNA library screening. Northern blot analysis indicated that mRNA accumulation of *GhMT3a* was up-regulated not only by high salinity, drought, and low temperature stresses, but also by heavy metal ions, abscisic acid (ABA), ethylene, and reactive oxygen species (ROS) in cotton seedlings. Transgenic tobacco (*Nicotiana tabacum*) plants overexpressing *GhMT3a* showed increased tolerance against abiotic stresses compared with wild-type plants. Interestingly, the induced expression of *GhMT3a* by salt, drought, and low-temperature stresses could be inhibited in the presence of antioxidants. H₂O₂ levels in transgenic tobacco plants were only half of that in wild-type (WT) plants under such stress conditions. According to *in vitro* assay, recombinant GhMT3a protein showed an ability to bind metal ions and scavenge ROS. Transgenic yeast overexpressing *GhMT3a* also showed higher tolerance against ROS stresses. Taken together, these results indicated that GhMT3a could function as an effective ROS scavenger and its expression could be regulated by abiotic stresses through ROS signalling.

Key words: Abiotic stress, antioxidant, *GhMT3a*, ROS, transgenic tobacco, yeast.

Introduction

Drought, high salinity, and low temperature are three important abiotic stresses that are commonly encountered by plants growing in their native environments. To survive these challenges, plants have developed elaborate mechanisms to perceive external signals and to manifest adaptive responses with the proper physiological and morphological changes (Shinozaki and Yamaguchi-Shinozaki, 2000; Zhu, 2002). The three kinds of stresses are often interconnected and induce similar cellular damage, or often activate similar cell signalling pathways and cellular responses (Shinozaki and Yamaguchi-Shinozaki, 2000; Knight and Knight, 2001; Xiong *et al.*, 2002; Hu *et al.*, 2008). One of the most common and crucial consequences is the generation of ROS in plants, which can elicit a potentially damaging oxidative burden on cellular constituents and/or act as signals for

engaging mechanisms that ameliorate oxidative stress (Alvarez *et al.*, 1998; Foyer and Noctor, 2005; Mittler, 2002).

The oxidative burst, a transient increase of ROS production, predominantly superoxide (O₂⁻) and hydrogen peroxide (H₂O₂), is the first biochemical response of plants to abiotic stress and can cause extensive cell injury or death (Coelho *et al.*, 2002; Mittler, 2002; Joo *et al.*, 2005). On the other hand, ROS play a central role in many signalling pathways in plants involved in stress perception, photosynthesis regulation, pathogen response, programmed cell death, and plant growth and development (Apel and Hirt, 2004; Davletova *et al.*, 2005; Miller *et al.*, 2007). Although the deleterious effects of ROS have long been known, knowledge on the molecular mechanisms of ROS-mediated

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gene regulations is limited and whether they play different roles in plant stress response have remained largely unexplored (Kobayashi *et al.*, 2007).

Metallothioneins (MTs) are defined as a family of proteins with the characteristics of low molecular weight, high cysteine (Cys) residue content, and metal-binding ability. They have been widely found in animals, plants, fungi, and cyanobacteria, and are divided into three classes based on the arrangement of Cys residues. All MTs identified from plants so far belong to Class II and have been further grouped into four types according to Cys residue distribution (Robinson *et al.*, 1993; Palmiter, 1998; Cobbett and Goldsbrough, 2002).

In animals, MTs are ubiquitous proteins associated with numerous cellular functions, including the regulation of metal homeostasis in cells and the response to metal toxicity and oxidative stress (Mattie and Freedman, 2004; Zatta *et al.*, 2005; Stankovic *et al.*, 2007). In fungi, MTs have been proposed to be primarily involved in the response to metal toxicity or as general stress proteins (Lanfranco *et al.*, 2002; Tucker *et al.*, 2004). Recently, increasing numbers of reports have indicated that plant MTs may play important roles as they do in animals and fungi (Adams *et al.*, 2002; Cobbett and Goldsbrough, 2002; Chiang *et al.*, 2006; Zhigang *et al.*, 2006). In addition, plant MTs are involved in some important developmental processes, such as fruit ripeness, root development, and suberization (Chatthai *et al.*, 1997; Clendennen and May, 1997; Mir *et al.*, 2004; Moyle *et al.*, 2005; Yuan *et al.*, 2008). In *Arabidopsis*, it has been demonstrated that different types of MTs exhibit distinct and overlapping functions in maintaining the homeostasis of essential transition metals, detoxification of toxic metals, and protection against intercellular oxidative stress (Robinson *et al.*, 1996; Murphy *et al.*, 1997; Garcia-Hernandez *et al.*, 1998; Miller *et al.*, 1999; Kiddle *et al.*, 2003; Lee *et al.*, 2004).

Cotton is one of the most important fibre and oil crops, and its growth and yield are severely inhibited in high salinity soil, especially at the germination and emergence stages (Gouia *et al.*, 1994; Gossett *et al.*, 1996; He *et al.*, 2005). To identify genes whose expression is correlated with salinity stress in cotton, a cDNA library was constructed by using mRNA isolated from salt-induced seedlings of a salt-tolerant cotton cultivar, ZM3, and screened by differential hybridization cDNAs encoding specific proteins whose activity may contribute to salt tolerance. A cDNA clone, *GhMT3a*, which encodes a type 3 plant MT, was isolated and characterized. Northern blot analysis indicated that the expression of *GhMT3a* in cotton seedlings was induced by several abiotic stresses factors, including salinity, drought, and low temperature, and these induced expression patterns of *GhMT3a* could be inhibited in the presence of antioxidants. Recombinant GhMT3a protein showed an ability to bind metal ions and scavenge ROS *in vitro*. Transgenic tobacco and yeast that overexpress *GhMT3a* displayed increased tolerance to environmental stresses, indicating its role in response to abiotic stresses is by mediating the ROS balance as a ROS scavenger in plants.

Materials and methods

Plant materials, growth conditions, and treatments

Seeds of cotton (*G. hirsutum* L.) ZM3 were provided by the Chinese Academy of Agricultural Sciences. Seedlings were grown in MS-liquid medium in a growth chamber for 9 d with $300 \mu\text{M m}^{-2} \text{s}^{-1}$ light intensity and day/night temperatures of 25 °C. For different stress treatments, uniformly developed seedlings were transferred into liquid medium containing the indicated concentrations of NaCl, PEG, CuSO₄, ZnCl₂, ABA, ethylene, H₂O₂ or PQ for 12 h. For the low temperature treatment, the seedlings were transferred to an incubator at 4 °C for 12 h. To study the effect of *N*-acetyl cysteine (NAC) on the induced expression of *GhMT3a* by stresses, the seedlings were transferred into liquid medium containing the indicated concentrations of NAC, together with the indicated concentrations of NaCl, PEG or a temperature of 4 °C. Then, cotyledons were harvested at 0, 1, 3, 6, and 12 h points directly into liquid nitrogen and stored at -80 °C for later use.

cDNA library construction and screening

Poly(A)⁺ RNA (0.5 μg) isolated from cotyledons of ZM3 seedlings treated with 300 mM NaCl for 24 h was used to synthesize first-strand cDNA, which was then amplified by long-distance PCR according to the manufacturer's protocol (SMART™ cDNA Library Construction Kit, Clontech, Mountain View, CA, USA). The double-stranded cDNA was digested by the *Sfi*I enzyme, and then fractionated by Chroma Spin-400. Fragments longer than 500 bp were cloned into *Sfi*I-digested dephosphorylated λTriplEx2 arms with T4 DNA ligase. The recombinants were packaged *in vitro* with Packagene (Promega, Madison, WI, USA). The cDNA library was screened by differential hybridization (once with an untreated cotyledon cDNA probe, once with a 300 mM NaCl-treated cotyledon cDNA probe). Plaques at a density of 10⁴ plaques/plate (15 cm diameter) were transferred onto the membrane. Prehybridization, hybridization, and washing were performed as described previously (Zheng *et al.*, 1998). Positive clones were plaque purified by two additional rounds of plaque hybridization with the same probes. Clones exclusively or preferentially hybridized by the NaCl-treated cotyledon cDNA probe were selected. Among these, one cDNA clone, *GhMT3a*, is described in this paper.

Northern blot analysis

Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Fremont, CA, USA). RNA samples for each experiment were analysed in at least two independent blots. Hybridization was performed in the same manner as the cDNA library screening. The specific *GhMT3a* cDNA fragment was labelled with [α -³²P]dCTP by the Prime-a-Gen labelling system from Promega, and used for the hybridization probe.

Analysis of transgenic tobacco plants under various stress conditions

Tobacco (*Nicotiana tabacum* cv. NC89) seedlings were grown on sterile MS medium and were used for leaf disc transformation. The *Agrobacterium* strain LBA4404 and the pBI121-based binary vector pHAGSK were used for transformation. The *GUS* gene of the vector was replaced with *GhMT3a* at the *Xba*I and *Sac*I restriction sites. The *Agrobacterium*-mediated transformation and regeneration procedures were as previously described by Kano-Murakami *et al.* (1993).

T₀ transgenic tobacco plants were identified by PCR to amplify the *nptII* gene with specific primers (5'-CGCAT-GATTGAACAAGATGG-3' and 5'-TCCCGCTCAGAA-GAACTCGTC-3'). The corresponding T₁ transgenic tobacco seedlings segregated at a ratio of ~3:1 (resistant:sensitive) were selected to propagate the T₂ generation, which was used for further analysis. Every 16 uniformly developed seedlings of transgenic and WT tobacco plants were treated with 200 and 300 mM NaCl for 20 d, 4 °C for 3 d, and 25% PEG for 15 d, respectively. The seedlings treated by NaCl and PEG were grown at 25 °C. These experiments were performed three times. The seedlings were photographed after recovery at 25 °C for 2 d.

Quantification of H₂O₂ levels

Cotyledons of cotton seedlings (1 g fresh weight) were homogenized in 5 ml cold acetone in a mortar with silica sand (Ferguson *et al.*, 1983). The extract and washings were centrifuged at 1250 g⁻¹ and the chlorophyll contents were adsorbed by activated carbon. Then 200 µl supernatant were added to 1 ml of reaction buffer [0.25 mM FeSO₄, 0.25 mM (NH₄)₂SO₄, 25 mM H₂SO₄, 1.25 mM xylene orange, and 1 mM sorbitol] at room temperature for 1 h. H₂O₂ levels were quantified at 560 nm absorbance, and H₂O₂ levels were calculated by reference to standards (He *et al.*, 2000; Suharsono *et al.*, 2002).

Production of recombinant GhMT3a

A fragment containing the entire open reading frame of GhMT3a was cloned by PCR into the *Bam*HI site of the *E. coli* expression vector pGEX4T-1 (Amersham Pharmacia Biotech, Hong Kong, China). To overexpress GST-GhMT3a and the control GST proteins, the pGEX and pGEX-GhMT3a plasmids were transformed into BL21 *E. coli* cells. Transformed cells were grown to A₆₀₀ 0.8 at 37 °C before expression of the recombinant proteins was induced by the addition of 1 mM isopropyl β-D-thiogalactoside, followed by growth at 25 °C for 4 h. The cells were harvested by centrifugation and lysed by sonication, as described previously (Valls *et al.*, 2001). The GST and GST-GhMT3a proteins in the recovered supernatant were purified by batch affinity chromatography with glutathione-Sepharose 4B (Amersham Pharmacia Biotech, Hong Kong, China) according to the manufacturer's instructions. The purified proteins were dialysed with three changes

against 500 vols of phosphate-buffered saline overnight at 4 °C and concentrated by Centriprep Concentrators. The tag of concentrated GST-GhMT3a was digested by Thrombin Cleavage Capture Kit (Novagen, San Diego, CA, USA). To prevent protein oxidation, the buffer solutions were bubbled with pure nitrogen gas in all the purification steps.

Hydroxyl radical scavenging assays

For hydroxyl radical scavenging assays, antioxidant-mediated competitive inhibition of the salicylate hydroxylation by hydroxyl radicals was performed as described previously (Smirnov and Cumbe, 1989).

Functional analysis of GhMT3a in yeast

Saccharomyces cerevisiae strain W303 was used as the wild type. Yeast strains were routinely cultured in YPD (1% yeast extract, 2% peptone, and 2% dextrose) or synthetic dropout (SD) media with appropriate supplements at 30 °C. A *GhMT3a* expression vector was made by subcloning the *GhMT3a* gene by PCR into a pYES2 shuttle vector (Invitrogen, San Diego, CA, USA), which contains the Ura3 selection marker and is driven by a GAL1 promoter. Yeast transformation was carried out using the standard lithium acetate method (Madeo *et al.*, 1999). Growth assays were performed according to a method described previously by Nass and Rao (1999) by inoculating 2 µl of saturated seed culture into a tube with 3 ml of selective medium (2% galactose, 0.67% yeast nitrogen base without amino acid, and 0.077% Ura DO Supplement, pH 4.0), containing 2 mM H₂O₂ or 2 mM PQ and the absorbance at 600 nm was measured. Growth on selective plates was performed as described previously (Yokoi *et al.*, 2002).

Results

Characterization of a NaCl-induced MT cDNA clone in cotton

A cDNA clone, *GhMT3a* (AY857933), was isolated from a NaCl-induced *G. hirsutum* cotyledon cDNA library by differential hybridization screening to identify genes involved in salt stress. The full sequence of the *GhMT3a* cDNA consisted of 499 nucleotides, encoding a polypeptide approximately 6.6 kDa of 63 amino acids. The N-terminal and C-terminal domains contain 4 and 6 Cys residues, respectively, separated by a central Cys-free spacer. In agreement with other higher plant MTs, all the Cys residues are located in the N- and C-terminal domains of GhMT3a. Multiple alignments also showed that GhMT3a shared high homology with many MTs from other plant species (Fig. 1).

To obtain clues about the evolutionary history of GhMT3a, a phylogenetic tree was constructed based on the similarities of deduced amino acid sequences of 67 available MT genes from various plant species. Consulting Cobbett and Goldsbrough's classification of plant MT, we also divided plant MTs into four types (Fig. 2), and *GhMT3a*



Fig. 1. Comparison of the deduced amino acid sequences of *Gossypium hirsutum* MT3a with its homologues from other plant species. Sequence alignment is optimized by inducing gaps using DNAMAN software. Conserved cysteine residues are indicated by the letter C. Identical or conserved amino acids are shaded in dark or grey, respectively. The accession numbers in GenBank of other plant type 3 MTs are as follows: *Gossypium hirsutum* (GhMT3a, AY857933), *Hordeum vulgare* subsp. *vulgare* (CAD88266), *Oryza sativa* (AF001396), *Oryza coarctata* (AAF68995), *Actinidia chinensis* (P43389), *Carica papaya* (CAA69624), *Citrus unshiu* (AAK08209), *Metroxylon sagu* (ABA43635), *Vitis vinifera* (CAB85630), and *Populus alba* × *Populus tremula* var. *glandulosa* (BAD95608). The amino acid numbers of these MTs are indicated to the right of the sequences.

falls into the type 3 category. Although MTs in types 1 and 2 share identical Cys distribution, respectively, the low bootstrap values in the internal nodes of type 1 or 2 MTs indicate that the orthologous relationships among type 1 or 2 MTs from different plant species are far from each other. By contrast, the bootstrap values of type 3 or 4 are relatively high, suggesting that MT members in type 3 or 4 might have common origins and are most probably derived from gene duplications.

The expression of GhMT3a is up-regulated by abiotic stresses and phytohormones

To determine which kind of stress could induce *GhMT3a* expression, Northern blot analysis was performed using total RNA from 9-d-old cotton seedlings treated with 300 mM NaCl, 4 °C, and 25% PEG, respectively. The results showed that the expression of *GhMT3a* was induced not only under the condition of salt stress, but also by drought and low temperature (Fig. 3A). In addition, the *GhMT3a* transcripts were detected in all organs of cotton seedlings under the induced conditions (data not shown). Since ABA and ethylene are known to play important roles in response to multiple environmental stimuli (Bleecker and Kende, 2000; Leung and Giraudat, 1998), their effect on *GhMT3a* transcription was examined. The results showed that the expression of *GhMT3a* in cotton seedlings was rapidly induced to high levels by the application of 10 μM ABA and 0.3% ethylene (Fig. 3B). Overall, the induced expression of *GhMT3a* revealed the involvement of GhMT3a in adaptation against various environmental stresses.

In agreement with other reports from animals (Palmiter, 1998; Saydam et al., 2002), the transcriptional level of *GhMT3a* in cotton seedlings was also up-regulated by the application of Cu²⁺ and Zn²⁺ (Fig. 3C).

GhMT3a involved in oxidative stress in cotton seedlings

Most environmental stresses are known to induce the accumulation of ROS such as superoxide, hydrogen peroxide, and hydroxyl radicals, which, in turn, results in cell damage in plants (Alvarez et al., 1998; Apel and Hirt, 2004). To elucidate if the regulation of *GhMT3a* expression under

conditions of drought, salt, and cold stress is under the control of ROS, the mRNA levels of *GhMT3a* in cotton seedlings treated with solutions of 10 mM H₂O₂ and 100 μM paraquat (PQ) as sources for the generation of ROS, were examined first. The results showed that just as in the cases of drought, salt, and cold stresses, both H₂O₂ and PQ significantly enhanced the accumulation of *GhMT3a* transcripts (Fig. 4A). Thereafter, the cotton seedlings were treated with 300 mM NaCl, 25% PEG, 4 °C, 10 mM H₂O₂, and 100 μM PQ together with the antioxidant *N*-acetyl cysteine (NAC) and the levels of *GhMT3a* transcripts and H₂O₂, respectively, were examined. The results indicated that NAC, as a kind of antioxidant, could effectively reduce not only ROS accumulation (Fig. 4B) but also the *GhMT3a* transcript level induced by salinity, drought, and cold stresses (Fig. 4C), suggesting that *GhMT3a* might be regulated by ROS production under such abiotic stress conditions.

Overexpression of GhMT3a in tobacco plants improves tolerance to abiotic stress

To confirm the *in vivo* functions of the *GhMT3a* gene during abiotic stress in plants, ectopic expression of the *GhMT3a* gene was carried out in tobacco. A total of 17 transgenic tobacco plants were obtained. Northern blot analysis showed that, although the transcriptional levels of the MT gene from individual tobacco plants varied, the signal intensity in transgenic plants was much stronger than that in wild-type (WT) plants (Fig. 5A). Sixty-four kanamycin-resistant T₂ plantlets (from eight lines) were selected for the stress tolerance assay. Sixteen 4-week-old uniformly developed seedlings of transgenic and WT tobacco plants were treated with 4 °C, 25% PEG, 200 mM and 300 mM NaCl, respectively. As shown in Fig. 5C, the transgenic plants exhibited enhanced tolerance against high salinity, low temperature, and drought compared with WT plants. Although all plants showed wilting and dehydration of young leaves with a concomitant loss of chlorophyll, the damaging levels in transgenic lines were lower than those of WT lines. All transgenic tobacco plants were able to survive following recovery, whereas 82% of WT plants died.

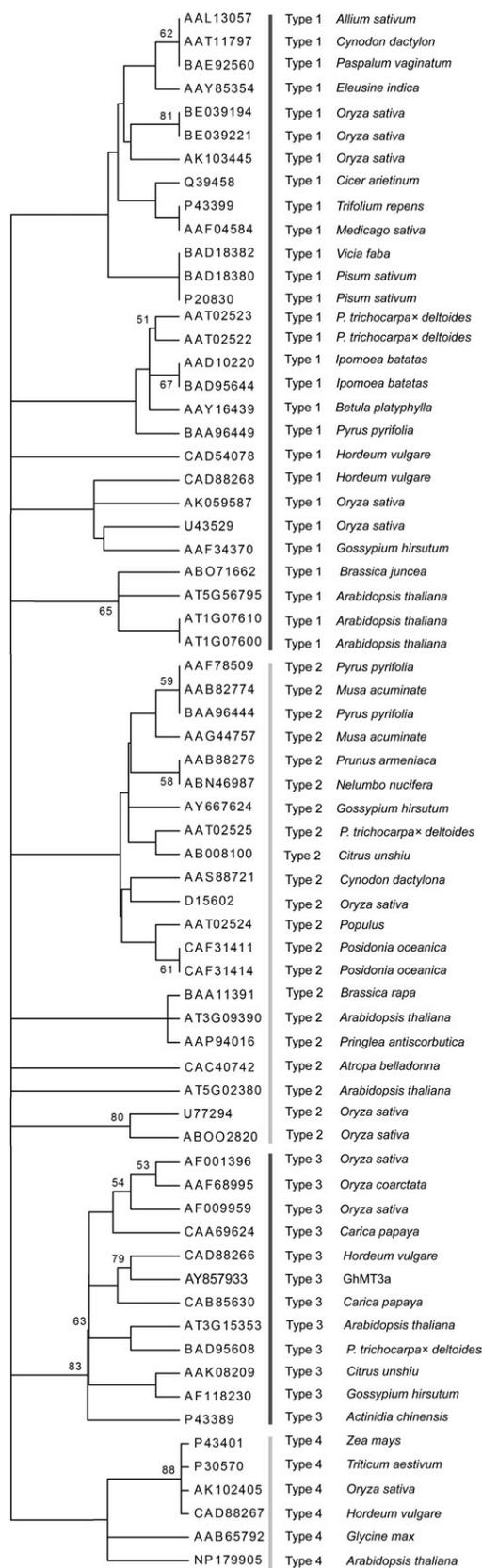


Fig. 2. Phylogenetic tree constructed with plant MT sequences retrieved by BLAST searches in the NCBI database, using MTs from *Gossypium hirsutum*, *Arabidopsis thaliana*, *Oryza sativa* as queries. Alignment was performed using Clustal X and the

Moreover, H_2O_2 levels in transgenic tobacco plants were only half of those in WT plants under such stress conditions (Fig. 5B), indicating that the improved stress tolerance might be due to the change of ROS balance in tobacco by overexpressing *GhMT3a*.

Function of *GhMT3a* as a ROS scavenger both in vitro and in yeast

To explore the biochemical properties of *GhMT3a* further, a recombinant GST-*GhMT3a* fusion protein was constructed and expressed in *E. coli*. The purified GST, *GhMT3a*, and GST-*GhMT3a* fusion proteins were determined *in vitro* for their ability to bind metal ions and function as a ROS scavenger. Because zinc is not a Fenton-active metal and

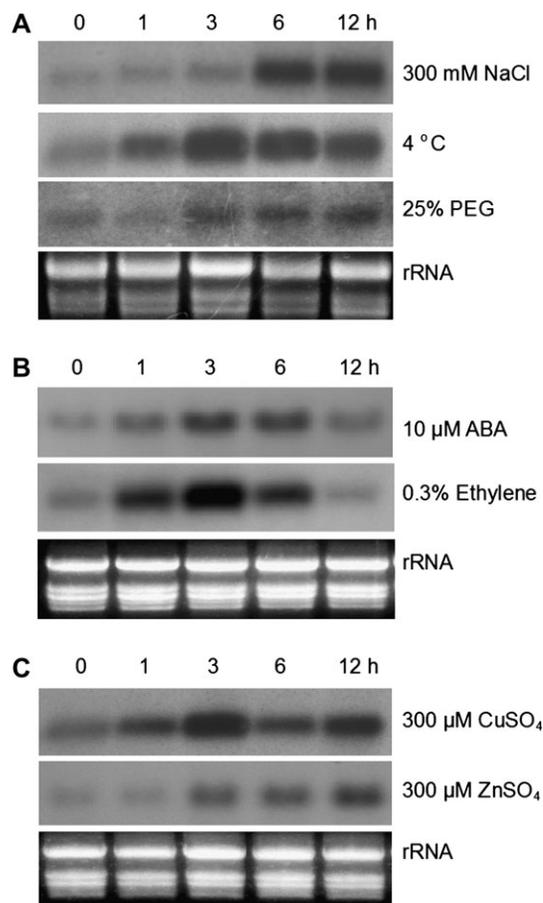


Fig. 3. Northern blot analysis of *GhMT3a* expression induced by stresses and hormone signals in cotton. Total RNA was extracted from cultivar ZM 3. About 20 μ g of total RNA was analysed by RNA gel blotting. The blot was hybridized with total cDNA fragment of *GhMT3a*. The ethidium bromide-stained rRNA is shown as a loading control.

phylogenetic tree was constructed using MEGA 3.1. The numbers on the branches indicate the percentage of 1000 bootstrap replicates that support the adjacent node; low bootstrap support (<50%) was not reported. The accession numbers, types and species of MTs from different plants were given.

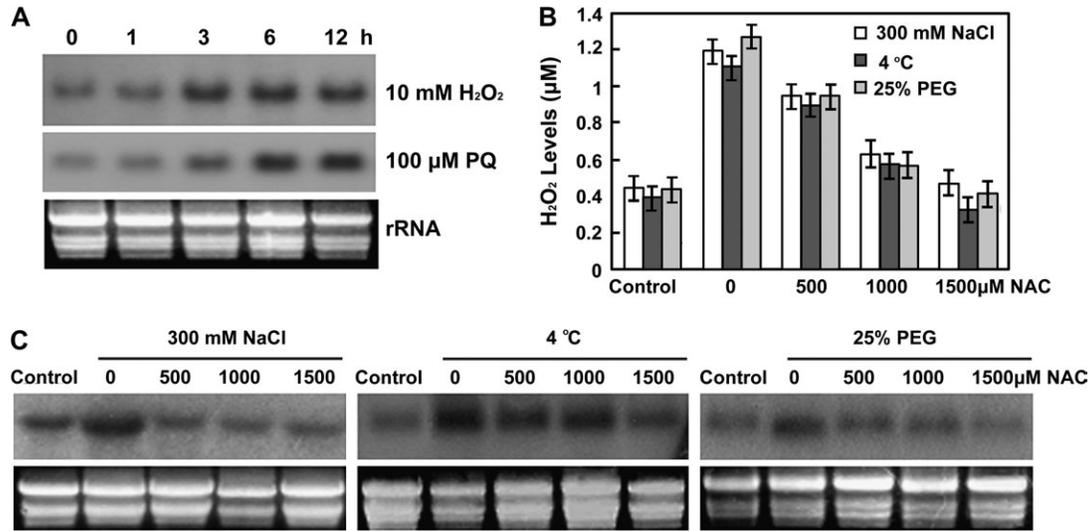


Fig. 4. *GhMT3a* expression and H_2O_2 accumulation in cotton seedlings inhibited by NAC under stress conditions. (A) RNA-gel blot analysis of total RNA isolated from cotton seedlings with treatments of 10 mM H_2O_2 and 100 μ M PQ for the indicated hours. (B) H_2O_2 production in cotton seedlings with treatments of 300 mM NaCl, 4 $^{\circ}$ C, 25% PEG, together with NAC in different concentrations for 6 h, respectively. Values are the mean obtained from three experiments, and error bars indicate SEM. (C) RNA-gel blot analysis of total RNA isolated from cotton seedlings with treatments of 300 mM NaCl, 4 $^{\circ}$ C, 25% PEG₆₀₀₀, together with NAC in different concentrations for 6 h, respectively. The ethidium bromide-stained rRNA is shown as a loading control.

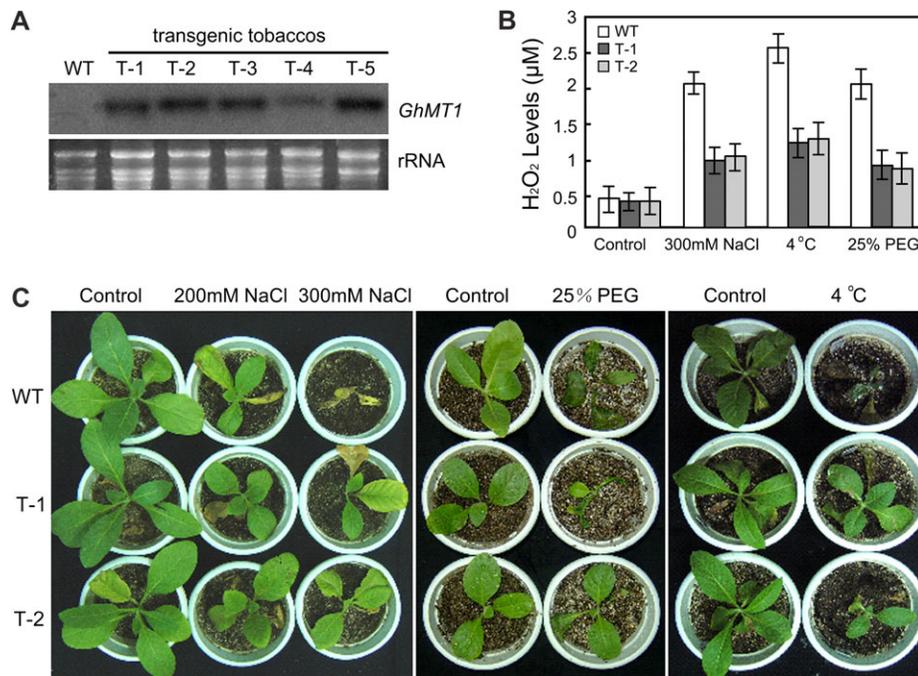


Fig. 5. Overexpression of *GhMT3a* in tobacco and stress tolerance of wild-type (WT) and transgenic tobacco plants. (A) Northern blot analysis of *GhMT3a* gene expression in wild-type (WT) and transgenic tobacco plants. T-1 to T-5 represent five independent T_2 transgenic lines. (B) The H_2O_2 levels in leaves of WT and transgenic tobacco lines under those stress conditions. Values are the mean obtained from three experiments, and error bars indicate SEM. (C) Plants were treated with or without salt solution every 2 d for 30 d; plants were grown at 25 $^{\circ}$ C as a control or at 4 $^{\circ}$ C for the low temperature for 3 d. Plants were treated with or without 25% PEG as a drought condition every 3 d for 20 d.

would not have deleterious effects on the purified proteins, metal binding experiments were performed using zinc (Tucker *et al.*, 2004; Hao and Maret, 2006; Qiao *et al.*, 2006). The results showed that with increasing concentra-

tions of Zn^{2+} , oxidation of the Cys residues in GhMT3a by 5,5'-dithiobis-2-nitrobenzoic acid (DTNB, a thiol-specific oxidizing agent) occurred more slowly, indicating that binding of Zn^{2+} to Cys residues inhibited the oxidation

reaction (Fig. 6A). In the control experiment using Ca^{2+} to replace Zn^{2+} , the presence of Ca^{2+} could not prevent GhMT3a from oxidation by DTNB (Fig. 6A). Moreover, when GhMT3a was incubated with 5-fold molar excesses of Zn^{2+} , the DTNB oxidation was not retarded further, suggesting that the binding capacity per GhMT3a molecule would be no more than five Zn^{2+} .

To determine the efficiency of GhMT3a as a ROS scavenger, its ability to inhibit superoxide- and hydroxyl radical-mediated oxidation *in vitro* compared with other antioxidants was measured. As shown in Fig. 6B, GST-GhMT3a displayed higher antioxidant activity against superoxide and hydroxyl radicals than the known antioxidants, including reduced glutathione (GSH) and thiourea at the same concentrations. Interestingly, GhMT3a protein without a GST tag also showed higher antioxidant activity than GSH, revealing the novel role of GhMT3a as an efficient antioxidant.

To confirm the function of GhMT3a as a ROS scavenger *in vivo* further, *GhMT3a* was transformed into *Saccharomy-*

ces cerevisiae strain W303. The results indicated that the GhMT3a-overexpressing yeast cells were less sensitive to oxidants such as PQ and H_2O_2 than the control cells (Fig. 7), suggesting that GhMT3a could scavenge ROS effectively in eukaryotic cells.

Discussion

High salinity, low temperature and drought are critical environmental factors that limit agricultural production worldwide, mainly by affecting plant growth and development. The cellular and molecular responses of plants to these stresses have been studied intensively (Hasegawa *et al.*, 2000; Thomashow, 1999; Xiong *et al.*, 2002). Oxidative stress occurs as an essential response when plants are challenged with abiotic stresses. Oxidative stress results from the disturbance in balance between ROS production and scavenging such as hydrogen peroxide, superoxide anions, and hydroxyl radicals that damage or kill cells by destroying lipids, nucleic acids, and proteins (Apel and Hirt, 2004;

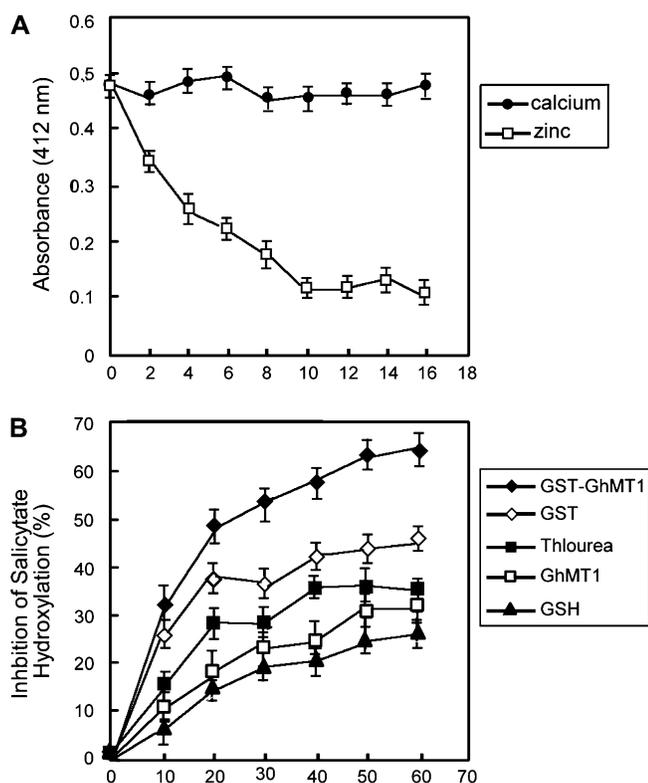


Fig. 6. The ability of GhMT3a to bind metal ions and its function as a ROS scavenger *in vitro*. (A) GhMT3a (2 mM) was incubated with DTNB (100 mM) in HEPES buffer (nitrogen purged, 25 °C) and thiol oxidation monitored spectrophotometrically at 412 nm. As the concentration of metal ions in the solution increased, the rate at which this oxidation occurred slowed, which was indicative of metal binding to GhMT3a, which interferes with thiol oxidation. (B) Comparison of ROS scavenging activity between GST-GhMT3a, GhMT3a, and other known antioxidants. Inhibition of hydroxyl radical-mediated salicylate hydroxylation by antioxidants was shown. Values are the mean obtained from three experiments, and the error bars indicate SEM.

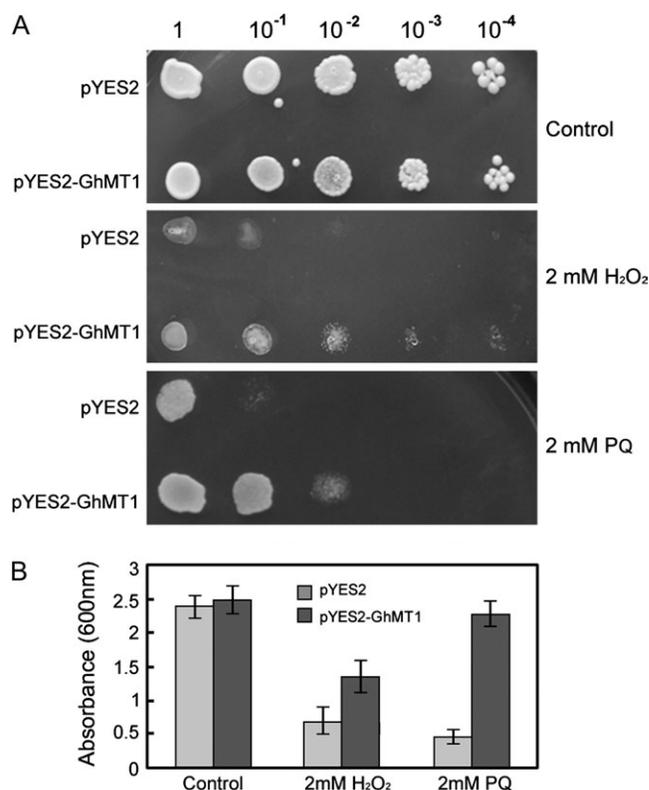


Fig. 7. Function of GhMT3a as a ROS scavenger based on overexpression in yeast. The pYES2 empty vector and pYES2-GhMT3a construct were transformed into wild-type strain W303. (A) Yeast strains in a concentration grade grown on selective plate with 2 mM H_2O_2 or 2 mM PQ; photos were taken after 72 h at 30 °C. (B) Growth of two transgenic lines (pYES2 empty, white squares; and pYES2-GhMT3a, black sequences) in liquid medium. Growth was detected by measuring the absorbance at 600 nm after culturing for 48 h at 30 °C in liquid medium containing 2 mM H_2O_2 or 2 mM PQ. Values are the mean obtained from three experiments, and the error bars indicate SEM.

Knight and Knight, 2001). To cope with different internal and external stresses, plants have developed a variety of adaptive mechanisms for survival by activating cascades or network events starting with stress perception and ending with the expression of many effector genes (Mittler, 2002; Xiong *et al.*, 2002). It has been accepted that antioxidant defence systems, including non-enzymatic antioxidants such as ascorbate, reduced glutathione, and tocopherol, and enzymatic antioxidants such as SOD and CAT, play a crucial role in plants against various stresses. Previous studies demonstrated that the regulation of the concentrations of antioxidants and of the activities of antioxidant enzymes is an important mechanism for combating oxidative stress (Alscher *et al.*, 2002; Blokhina *et al.*, 2003; Heiber *et al.*, 2007). However, because of the complexity and diversity of cell metabolism, other unknown antioxidant systems may exist in plant cells and need to be clarified.

MTs are cysteine-rich, low molecular weight intracellular proteins that were initially shown to regulate the metabolism of metals such as zinc, copper, and cadmium, and play a role in heavy metal tolerance (Lanfranco *et al.*, 2002; Palmiter, 1998). Recently, a number of investigations have demonstrated MTs as being efficient scavengers of ROS production in animals (Li *et al.*, 2006; Dong *et al.*, 2007; Peng *et al.*, 2007). During oxidative stress, MTs protect against ROS-induced DNA degradation with higher molar efficiency than glutathione (Jourdan *et al.*, 2004). Plants also contain a multiple MT gene family in which different types may play distinct and overlapping biological roles by the regulation of gene expression or signalling networks. In *Arabidopsis*, all four types of MTs provided similar levels of Cu tolerance and accumulation to the yeast mutant $\Delta cup1$ (Lee *et al.*, 2004; Guo *et al.*, 2008). Cu^{2+} , Ag^+ , Cd^{2+} , Zn^{2+} , and Ni^{2+} all induced significant levels of *Arabidopsis* MT2 gene expression; however, MT1 in *Arabidopsis* could not be induced by these ions except for Cu^{2+} in excised leaves (Zhou and Goldsbrough, 1994; Murphy and Taiz, 1995). Recently, expression of *LSC54*, a rape MT1 gene, was proven to be induced by ROS production and related to the misbalance of ROS during leaf senescence (Navabpour *et al.*, 2003), and transgenic *Arabidopsis* plants overexpressing *cgMT1* from beefwood (*Casuarina glauca*) reduced the accumulation of H_2O_2 (Obertello *et al.*, 2007). In addition, OsMT2b may also function as a ROS scavenger involved in the response to bacterial blight and blast fungus infections in rice (Wong *et al.*, 2004).

In this study, a type 3 MT encoding cDNA, *GhMT3a*, was isolated from an NaCl-induced cotton cotyledon cDNA library. The up-regulation of *GhMT3a* expression was observed in cotton seedlings treated not only with high salinity but also with drought and low temperature (Fig. 3A, B, C). Interestingly, the levels of *GhMT3a* in cotton seedlings were also markedly increased by H_2O_2 and PQ treatment (Fig. 4A). The induced expression of *GhMT3a* by these abiotic stresses could be completely inhibited in the presence of 1500 μM NAC, an antioxidant (Fig. 4B). Just as in the case of *GhMT3a*, NAC also decreased the levels of H_2O_2 in cotton seedlings (Fig. 4C), indicating that there is

a high correlation between the expression of *GhMT3a* and the misbalance of ROS production in cotton and *GhMT3a* may act as an antioxidant to minimize ROS toxicity, which was further confirmed by overexpressing *GhMT3a* in tobaccos and yeast. As shown in Figs 5 and 6, transgenic tobaccos displayed high tolerance against salt, drought, and low temperature stresses, and their H_2O_2 levels were only half of that in WT plants. Transgenic yeast overexpressing *GhMT3a* showed more tolerance to ROS toxicity than the control. The purified GhMT1 protein from *E. coli* exhibited antioxidative capacity *in vitro* when no other metals and other antioxidants were applied. A number of studies have proved that the cysteine ligands in proteins are remarkably reactive towards oxidizing agents (Chae *et al.*, 1994; Haslekas *et al.*, 2003; Maret, 2004; Hao and Maret, 2006), including MTs (Zhou *et al.*, 2002; Maret, 2004; Hao and Maret, 2006). Therefore, it could be concluded that GhMT1 acts as an endogenous antioxidant to respond to ROS stress in a direct manner.

It has been accepted that high levels of ROS lead to phytotoxicity, while relatively low levels can be signals inducing ROS scavengers and other protective mechanisms in plants (Couee *et al.*, 2006; Gadjev *et al.*, 2006; Miller *et al.*, 2007). These results strongly support the idea that ROS signalling is indispensable for the regulation of *GhMT3a* expression during environmental stresses in plants. The fact that GhMT3a had antioxidant ability *in vitro* indicated the function of GhMT3a as a ROS scavenger (Fig. 6), revealing that plant metallothioneins play important roles as do their animal counterparts (Mattie and Freedman, 2004; Hao and Maret, 2006). Based on evidence that a number of transgenic plants or mutants with higher ROS scavenging ability showed increased tolerance to environmental stresses (Avsian-Kretschmer *et al.*, 2004; Moradi and Ismail, 2007), it is proposed that the higher tolerance against abiotic stresses in transgenic tobaccos might be due to the scavenging of ROS production by the overexpression of *GhMT3a*. In addition, previous studies demonstrated that ROS may act as second messengers in redox signal transduction and are implicated in hormonal mediated events (Guan *et al.*, 2000; Zhang *et al.*, 2001). Thus, the ROS signal may also be the intermediate for the induced expression of *GhMT3a* by ABA and ethylene in our study.

Most previous research on plant MTs focus on heavy metal ions. The effects of metal ions on the expression of plant MT genes vary with plant species, tissues, and types of MT genes (Foley *et al.*, 1997; Chang *et al.*, 2004; Bellion *et al.*, 2007). However, very little is known about the mechanism for the regulation of plant MT gene expression by metal ions. In this study, GhMT3a showed a high affinity to Zn^{2+} *in vitro*. The cysteine ligands in proteins are reactive towards oxidizing agents and release zinc (Maret, 2004; Hao and Maret, 2006). When released from MT, zinc may become available for the synthesis of antioxidant metal-binding proteins, such as Cu, Zn-superoxide dismutase, and at the same time be part of a mechanism that conducts spatial regulation of the oxidoreductive environment in the cell (Liochev and Fridovich, 2004). There is evidence that

MTs release bound metals during oxidative stress and trigger a Zn-mediated antioxidant response in mammals and fungi (Maret, 1994; Tucker *et al.*, 2004). The zinc-released MT can function as a reducing agent because of its high content of cysteines or rebind zinc under reducing conditions. Therefore, MTs may interact with other metal-proteins by releasing zinc within cells in response to spatial or temporary changes in the redox environment, which might be another function of MT in plant.

Taken together, the results indicate that the rapid accumulation of ROS in cotton plants after abiotic stresses (high salinity, drought, and low temperature) and the application of ABA or ethylene will induce the expression of *GhMT3a*. As a ROS scavenger, accumulation of GhMT3a during defence signalling would diminish ROS damage and then increase the tolerance of plants against abiotic stresses. Future studies are required to determine the relationship between GhMT3a and other antioxidant metalloproteins and whether the release of zinc ions from GhMT3a could be beneficial to the synthesis of other antioxidant metalloproteins or facilitate the activation of these proteins.

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