Overexpression of Nelumbo nucifera metallothioneins 2a and 3 enhances seed germination vigor in Arabidopsis

Zhou, Yuliang; Chu, Pu; Chen, Huhui; Li, Yin; Liu, Jun; Ding, Yu; Tsang, Edward W. T.; Jiang, Liwen; Wu, Keqiang; Huang, Shangzhi

Publisher's version / Version de l'éditeur:
https://doi.org/10.1007/s00425-011-1527-4
Planta, 235, 3, pp. 523-537, 2012-03

Overexpression of *Nelumbo nucifera* metallothioneins 2a and 3 enhances seed germination vigor in *Arabidopsis*

Running title:

MTs in seed germination vigor

Authors:

YULIANG ZHOU[1], PU CHU[1], HUHUI CHEN[1], YIN LI[1], JUN LIU[2], YU DING[3], EDWARD W.T. TSANG[4], LIWEN JIANG[3], KEQIANG WU[5], JIARUI FU[1] & SHANGZHI HUANG[1]

Institution address:

[1] School of Life Sciences, Guangdong Provincial Key Laboratory of Plant Resource, Sun Yat-Sen University, Guangzhou 510275, China

[2] Guangdong Academy of Agricultural Sciences, Guangzhou 510640, China

[3] School of Life Sciences, Centre for Cell and Developmental Biology, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China

[4] Plant Biotechnology Institute, National Research Council of Canada, 110 Gymnasium Place, Saskatoon, SK S7N 0W9, Canada

[5] Institute of Plant Biology, College of Life Science, National Taiwan University, Taipei 106, Taiwan

Corresponding author:

Name: Shangzhi Huang

Address: School of Life Sciences, Guangdong Provincial Key Laboratory of Plant Resource, Sun Yat-Sen University, Guangzhou 510275, China

E-mail: huangsz@mail.sysu.edu.cn

Tel: 8620-84110797

Fax: 8620-84036215

ABSTRACT

Sacred lotus (*Nelumbo nucifera* Gaertn.) seeds are known for their 1300-year-old viability and tolerance of high temperatures. The seeds can therefore be an excellent source for identification of genes controlling seed vigor. Based on the oxidative stress hypothesis of aging, we reasoned that metallothioneins (MTs) with potential roles in scavenging reactive oxygen species (ROS) could contribute to seed vigor. Here, we report the isolation and characterization of *MT2a*, *MT2b* and *MT3* from sacred lotus and their roles in seed germination vigor. The expression of these MT genes was highly detected in developing and germinating seeds and was dramatically increased after accelerated aging (AA) and oxidative stress treatments. *Arabidopsis* seeds overexpressing *MT2a* and *MT3* displayed improved resistance to AA treatment, indicating their significant roles in seed germination vigor. These transgenic seeds also exhibited lower lipid peroxidation levels and higher superoxide dismutase activity compared to wild-type seeds after AA treatment. In addition, we showed that MT2a and MT3 conferred improved germination ability to NaCl and methyl viologen on transgenic *Arabidopsis* seeds. Taken together, these data suggest that MT2a and MT3 may function to enhance seed germination vigor under adverse conditions by alleviating ROS-imposed oxidative stress.

Key-words: sacred lotus; metallothionein; seed germination vigor; ROS; abiotic stresses; *Arabidopsis*.

INTRODUCTION

Seed vigor is an important seed quality for the success of plant propagation and food production. High vigor seed lots can maintain high germination rates and vigorous seedling development under adverse environmental conditions (Dickson 1980). However, seeds gradually lose vigor and viability during storage, become more sensitive to stresses during germination and eventually lose germinability (McDonald 1999; Holdsworth, Bentsink & Soppe 2008). Although seed longevity and vigor has been the subject of a number of studies (reviewed in Rajjou & Debeaujon 2008), the molecular mechanisms remain poorly understood. Generally, the oxidative stress hypothesis of aging is widely accepted by scientific community (Orr & Sohal 1994; Finkel & Holbrook 2000). Therefore, metallothioneins (MTs) that are highly active in scavenging reactive oxygen species (ROS) may be an important contributor to seed longevity and vigor.

MTs are small, cysteine (Cys)-rich and metal-binding proteins that are widely distributed in plants, animals, fungi and cyanobacteria (reviewed in Cobbett & Goldsbrough 2002). Based on the arrangement of the Cys residues, MTs are categorized into three classes (Robinson *et al*. 1993; Klaassen, Liu & Choudhuri 1999). Class I includes only MTs from mammalians and vertebrates; Class II contains all other MTs from plants, fungi, and invertebrate animals; Class III is enzymatically derived polypeptides with a poly (gamma-Glu-Cys)-glycine structure. Plant MTs are further subdivided into several types (type 1, 2, 3 and 4) according to the distribution of Cys residues (Cobbett *et al*. 2002). In animals, MTs are involved in metal

homeostasis and scavenging of reactive oxygen species (Thornalley & Vasak 1985; Palmiter 1998; Suhy *et al*. 1999; Coyle *et al*. 2002). It is also worth noting that many studies have demonstrated that there is a tight correlation between the expression of MT genes and many important physiological processes, such as aging (Yang *et al*. 2006) and cell apoptosis (Raymond *et al*. 2010). For example, MT transgenic mice were more resistant to oxidative stress and showed a prolonged life-span compared to wild-type mice (Yang *et al*. 2006). In fungi, *Magnaporthe* MT1 was a powerful antioxidant and could release Zn^{2+} during oxidative stress (Tucker *et al*. 2004).

In plants, MTs are also involved in responding to heavy metal tolerance (reviewed in Cobbett *et al*. 2002) and other abiotic stress conditions, such as high salinity (Wu *et al*. 2005) and drought (Yang *et al*. 2009). Noticeably, many studies have reported that plant MTs acted as protectants against oxidative stresses imposed by adverse environmental conditions (Akashi *et al*. 2004; Mir *et al*. 2004; Wong *et al*. 2004; Xue *et al*. 2009; Samardzic *et al*. 2010). In addition, other studies have highlighted the importance of plant MTs in plant growth and development, such as seed development and germination (Brkljacic *et al*. 2004; Yuan *et al*. 2008), tissue senescence (Bhalerao *et al*. 2003; Guo, Bundithya & Goldsbrough 2003), fruit ripeness (Clendennen & May 1997) and root development (Yuan *et al*. 2008). For example, the expression of most *Arabidopsis* MT genes was dramatically increased in senescing siliques and leaves (Guo *et al*. 2003).

Sacred lotus (*Nelumbo nucifera* Gaertn.), a primitive angiosperm (Les, Garvin & Wimpee 1991), is one of the oldest plants that have survived from the last ice age.

Sacred lotus seeds can be used as an excellent plant genetic resource for identification of genes controlling seed viability and vigor, particularly for exceptional seed quality (Shen-Miller *et al*. 1995). Furthermore, the sacred lotus seeds also show great ability to survive under harsh environmental conditions, such as extremely high temperatures (Ding, Cheng & Song 2008) and γ-radiation (Shen-Miller *et al*. 2002). In this study, we report the isolation and characterization of three sacred lotus MT genes, *MT2a*, *MT2b* and *MT3*, and their expression patterns during seed development and germination and under various stress conditions. We show that overexpression of *MT2a* and *MT3* can enhance seed germination vigor in *Arabidopsis* after accelerated aging (AA) treatment. Additionally, we present evidence that MT2a and MT3 confer resistance in transgenic *Arabidopsis* seeds against high salinity and oxidative stress imposed by methyl viologen (MV). Taken together, our data provide new insights into the important functions of MT2a and MT3 in seed germination vigor under adverse conditions.

MATERIALS AND METHODS

Plant materials and growth conditions

Seeds of sacred lotus (*Nelumbo nucifera* Gaertn.) were collected from plants grown in ponds in Sanshui Lotus World, Guangdong Province, China. Sacred lotus flowers were tagged on the day of anthesis and developing seeds were removed from the pods at different days after pollination (DAP). Developing seed embryonic axes were used for gene cloning and RNA preparation in the study of gene expression during seed

development. Mature seeds were imbibed in distilled water at 25°C for 0.5, 1, 2, and 3 d in germination test. The 3d-imbibed mature seeds treated at 42°C for 2 h were used to construct cDNA library. Seed vigor was not affected after the treatment. All treated materials were immediately frozen in liquid nitrogen and stored at -80°C until use.

All wild-type and transgenic *Arabidopsis thaliana* (ecotype Columbia-0) plants were grown routinely in a greenhouse (22°C with a 16-h photoperiod). For aseptic growth, *Arabidopsis* seeds were surface sterilized in 70% ethanol for 2 min, followed by soaking in 10% bleach for 20 min, and then rinsed extensively in sterile water for at least five times. Sterilized seeds, stratified at 4°C for 2 d , were sown on Petri dishes containing half-strength Murashige & Skoog (MS) medium (Duchefa) with 3% (w/v) sucrose and 0.6% (w/v) agar (pH=5.8) under the same growth parameters as above. In all experiments, seeds of wild-type and transgenic lines were produced in the same culture cycle.

cDNA library construction

Total RNA was isolated from embryonic axes of late-maturation (22 DAP) sacred lotus seeds and embryonic axes of 3d-imbibed and 2h-heat-treated mature sacred lotus seeds using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. cDNA library construction was performed using a SMART cDNA library construction kit (Clontech).

Phylogenetic analysis

Protein sequences from species other than sacred lotus were retrieved from GenBank and SwissProt. Protein sequences of MT2a, MT2b, MT3 and their corresponding homologs from other species were initially aligned by DNAMAN software with default gap penalties. The Phylogenetic tree was constructed by MEGA4.1 (Tamura *et al*. 2007) with the neighbor-joining algorithm using default settings. A bootstrap analysis of 1000 replicates was performed.

Accelerated aging

Accelerated aging (AA) was carried out by exposing seeds to a temperature of 41 or 43°C for 72 h in tightly closed boxes with 100% relative humidity (RH) (Byrd & Delouche 1971; Sattler *et al*. 2004). The RH was obtained by introducing sterile distilled water into the boxes. Before the treatments, the AA boxes were thoroughly washed with a 10% bleach solution, rinsed, and dried to prevent fungal contamination.

Stress treatments of sacred lotus seeds

After AA treatment (43°C for 72 h at 100% RH), the treated and untreated seeds were simultaneously imbibed in distilled water at 25°C in a greenhouse with a 16-h photoperiod. When the plumule protruded 0 cm, 0.2 cm and 0.5 cm, respectively, the embryonic axes were collected. For other abiotic stress treatments, 3d-imbibed seeds were transferred to fresh distilled water with or without 150 mM NaCl, 0.5% H_2O_2 and 20 μM MV. These embryonic axes were sampled at 2, 6, 12 and 24 h. After the treatments, the embryonic axes were immediately frozen in liquid nitrogen and stored

at -80°C until used for RNA extraction and real-time PCR analysis.

Transient expression and confocal imaging

For transient expression, fragments of *MT2a* and *MT3* coding sequences were introduced into *Xho*I and *Sma*I sites, which were underlined in the following primers, and amplified by PCR. Primers (forward and reverse, respectively) for *MT2a* were 5′-CCGCTCGAGATGTCTTGCTGCGGAGGAA-3′ and 5′-TCCCCCGGGTTTGCAGTTGCAAGGGTTAC-3′; for *MT3*, 5′-CCGCTCGAGATGTCGACCTGCGGCAACT-3′ and 5′-TCCCCCGGGGTGACCACCACAGGTGCAA-3′. The PCR products were digested with *Xho*I and *Sma*I, and cloned into pA7-YFP to produce constructs MT2a-YFP and MT3-YFP for protein transient expression analysis. The recombinant constructs were electroporated into protoplasts of *Arabidopsis* suspension cultured cells and empty pA7-YFP was used as a control. Transient expression was carried out essentially as previously described (Miao & Jiang 2007).

Construction of transforming vectors and plant transformation

To construct the plant transforming vectors, fragments of *MT2a* and *MT3* coding sequences were introduced into *Sma*I and *Sac*I sites, which were underlined in the following primers, and amplified by PCR. Primers (forward and reverse, respectively) for *MT2a* were 5′-TCCCCCGGGAATGTCTTGCTGCGGAGGA-3′ and 5′-CTGGAGCTCGCCCTCCTCTCATTTGCAG-3′; for *MT3*, 5′-TCCCCCGGGATGTCGACCTGCGGCAACTG-3′ and 5′-CTGGAGCTCTTAGTGACCACCACAGGTGC-3′.

PCR was performed using Ex Taq polymerase (Takara) at 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s for 30 cycles. The PCR products were first cloned into pGEMT-Easy vector (Promega) to form the intermediate vectors pGEMT-MT2a and pGEMT-MT3 and sequenced. Then, the intermediate vectors were digested by *Sma*I and *Sac*I, and then subcloned into the corresponding sites of the binary plasmid pBI121 by replacing the beta-glucuronidase (*GUS*) gene to generate the transforming vectors pBI121-MT2a and pBI121-MT3. *MTs* were expressed under the control of the cauliflower mosaic virus 35S promoter in the plant transforming vectors, which were electroporated into *Agrobacterium tumefaciens* strain EHA105. Transformation of *Arabidopsis* plants was conducted by the floral dip method (Clough & Bent 1998). T0 seeds were selected on half-strength MS agar medium containing 50 mg/L kanamycin (Sigma-Aldrich). Leaves from kanamycin-resistant seedlings were collected and verified by PCR using a cauliflower mosaic virus 35S promoter-specific forward primer 5′-CCTTCGCAAGACCCTTCCTC-3′ and gene-specific reverse primers: 5′-GCTCATCTCGGACCCTTC-3′ (for *MT2a*), and 5′-CCGCTGGAACCTCACTCA CA-3′ (for *MT3*). The seeds of the positive lines were further characterized for a 3:1 segregation. T3 homozygous seeds were obtained in the subsequent generation and were confirmed by the lack of segregation of antibiotic resistance.

RNA extraction, RT-PCR and real-time PCR

For RT-PCR analysis, total RNA was isolated from dry mature seeds and developing green siliques of transgenic and wild-type *Arabidopsis* plants using the Universal

Plant RNA Extraction Kit (BioTeke). For real-time PCR analysis, total RNA was extracted from embryonic axes of developing and germinating seeds, roots, stems, leaves and flowers of sacred lotus using RNAprep pure Plant Kit (TianGen) according to the manufacturer's instructions. After the RNA was digested with RNase-free DNase I (Takara), RNA quality and quantity were determined by electrophoresis and spectrophotometry. First-strand cDNA was synthesized from the total RNA with the PrimeScript 1st Strand cDNA Synthesis Kit (Takara).

Primers (forward and reverse, respectively) used in RT-PCR for *MT2a* were 5′-ACCCGGACTTCAGCTTCT-3′ and 5′-GCTCATCTCGGACCCTTC-3′; for *MT3*, 5′-CAAGAGCCAGTGCGTGAAGA-3′ and 5′-GACCACCACAGGTGCAATCC-3′; for *Arabidopsis actin2*, used as reference for standardization of cDNA amounts, 5′-ATTACCCGATGGGCAAGTCA-3′ and 5′-TGCTCATACGGTCAGCGATA-3′. PCR was performed at 94°C for 15 s, 58°C for 15 s, and 72°C for 20 s for different cycles (for dry seeds, 30 cycles for *MT2a* and 32 cycles for *Act2*; 32 cycles for *MT3* and *Act2*; for green siliques, 28 cycles for *MT2a* and 30 cycles for *Act2*; 30 cycles for *MT3* and *Act2*). Each reaction was performed at least three times.

Real-time PCR was performed on a LightCycler 480 (Roche) in the presence of SYBR Green Mix (Takara). Gene-specific primers for *MT2a* and *MT3* were the same as above. Primers (forward and reverse, respectively) for *MT2b* were 5′-ACTGTGGCTGTGGGTCTG-3′ and 5′-CTCCGCTCCAAAGTTCAT-3′. The sacred lotus 18S rRNA was used as an internal control, which was amplified using the following primers: 5′-CCATAAACGATGCCGAC-3′ (forward) and 5′-CACCACCC

ATAGAATCAAGA-3′ (reverse). The PCR program was 95°C denaturation for 30 s, 40 cycles of 95°C for 5 s, 60°C for 20 s. The specificity of the PCR amplification was checked with fusion curves (60°C-95°C) following the final cycle of the PCR. The amount of cDNA was calculated using LightCycler 480 Software. Data represent two biological replicates and three technical replicates.

Germination assays

For sacred lotus seeds, four replicates of 20 AA treated or untreated seeds were germinated in 15-cm Petri dishes containing distilled water. To eliminate the effect of AA treatment on seed water content, the control seeds were balanced with 100% RH at 25°C for 72 h. In another case, four replicates of 20 dry seeds were germinated in 15-cm Petri dishes containing distilled water with or without 150 mM NaCl, 0.5% H_2O_2 and 20 μM MV. Seeds with plumule emerged 2 mm were scored as germinated.

For *Arabidopsis* seeds, four replicates of 100 seeds of each genotype were germinated in 9-cm Petri dishes containing half-strength MS medium to study seed germination performances after AA treatment. In another case, four replicates of 50 seeds for each genotype were germinated in 9-cm Petri dishes containing half-strength MS medium supplemented with various concentrations of NaCl and MV, as indicated in the Figure legends. Petri dishes were sealed with Parafilm (American National Can). Germination was scored daily after the radicles protruded the seed envelope.

Tetrazolium assay

Seeds were scarified and soaked in a 1% (w/v) aqueous solution of 2,3,5-triphenyltetrazolium chloride (Alfa) at 30°C in darkness for 2 d according to the procedure described by Wharton (1955). Tetrazolium salts were metabolically reduced to highly colored end products called formazans by NADH-dependent reductases of the endoplasmic reticulum.

Lipid peroxidation assay

The thiobarbituric acid (TBA) test was used to analyze lipid peroxidation (Bailly *et al*. 1996). Briefly, 0.1g seeds were ground in 5 ml distilled water and homogenized with an equal volume of 0.5% (w/v) 2-TBA in 20% (w/v) trichloroacetic acid. The homogenate was incubated in a boiling water bath for 30 min and then quickly cooled in an ice bath. After centrifugation at 12,000g for 15 min, the absorbance of the supernatant was measured at 532 and 600 nm. Results are expressed as nmol g^{-1} fresh weight.

SOD enzymatic assay

0.1 grams of seeds were ground in an ice-chilled mortar and homogenized with 0.1g polyvinylpyrrolidone (PVP) in 5 ml of 0.1 M potassium phosphate buffer (pH 7.8) containing 2 mM dithiothreitol, 0.1 mM EDTA and 1.25 mM PEG-4000. After centrifugation at 4°C, 16 000 g for 15 min, supernatants were collected and used to determine superoxide dismutase (SOD) activity. SOD (EC1.15.11) activity was assayed by measuring its ability to inhibit the photochemical reduction of nitro blue

tetrazolium (NBT). One unit of the enzyme activity was defined as the amount of enzyme required to achieve a 50% inhibition of the rate of NBT reduction at 560 nm as previously described (Bailly *et al*. 1996). Results were expressed as SOD (Units mg prot.$^{-1}$).

RESULTS

Isolation and characterization of MT genes from sacred lotus

To investigate the molecular events of sacred lotus seed development and germination, two cDNA libraries were constructed using mRNAs isolated from embryonic axes of late-maturation (22 days after pollination, DAP) sacred lotus seeds and 3d-imbibed and 2h-heat-treated mature sacred lotus seeds. A large number of ESTs isolated from these two libraries showed high sequence similarities to those MT-like proteins from other plant species. Phylogenetic analysis revealed that they were classified into type 2 and 3, designated as *MT2a*, *MT2b* and *MT3* (Supporting Information Fig. S1). Analysis of ESTs showed that *MT2a* comprised approximately 1.17% of the total transcripts in embryonic axes of late-maturation seeds and *MT2b* 0.05%, whereas *MT3* was not detected (Fig. 1a). On the other hand, *MT2a* and *MT3* accounted for 1.94% and 0.28% of the transcripts in embryonic axes of 3d-imbibed and 2h-heat-treated seeds, respectively, whereas *MT2b* was not detected (Fig. 1a).

The full length cDNA of *MT2a* contains an open reading frame of 243 nucleotides, flanked by 99 bp 5′-UTR and 357 bp 3′-UTR, encoding a putative protein of 80 amino acids with a predicted molecular mass of 8.1 kDa and an isoelectric point of 5.2. The

full length cDNA of *MT2b* contains an open reading frame of 231 nucleotides, flanked by 88 bp 5′-UTR and 312 bp 3′-UTR, encoding a putative protein of 76 amino acids with a predicted molecular mass of 7.6 kDa and an isoelectric point of 4.6. The full length cDNA of *MT3* contains an open reading frame of 201 nucleotides, flanked by 97 bp 5′-UTR and 470 bp 3′-UTR, encoding a putative protein of 66 amino acids with a predicted molecular mass of 7.0 kDa and an isoelectric point of 5.5. Multiple amino acid sequence alignments of the deduced MT2a, MT2b and MT3 with their corresponding homologues from other plant species were conducted (Fig. 1b,c). MT2a and MT2b showed high sequence identities with other homologues ranging from 54% to 78%, and MT3 from 50% to 71%. All MTs contain two Cys-rich domains separated by Cys-free linker regions of approximately 40 amino acids containing aromatic amino acids. The large spacer in the plant MTs is the most distinctive feature that separates them from those of animals. In the animal MTs, Cys-rich domains are separated by a short spacer of less than 10 amino acids that do not include aromatic residues (Cobbett *et al*. 2002). The sequence of the N-terminal domain of MT2a, MT2b and MT3 is variable. By contrast, the distribution of Cys residues within the C-terminal of MT2a, MT2b and MT3 shares the highly conserved motif CxCxxxCxCxxCxC (x denoting any amino acid other than Cys).

Temporal and spatial expression profiles of *MT2a*, *MT2b* and *MT3*

Sacred lotus seed development consists of three stages: tissue differentiation (0-6 DAP), cell expansion (maturation, 7-22 DAP) and maturation drying stage (23-30

DAP) (Huang *et al*. 2003). Although considerable research of MTs has been conducted, the expression of plant MTs has not been studied thoroughly during seed development and seed germination. To characterize the expression patterns of *MT2a*, *MT2b* and *MT3*, transcript levels were analyzed in embryonic axes of sacred lotus at various stages of seed development and seed germination and in other organs by real-time RT-PCR (Fig. 2). Analysis of *MT2a* revealed that high levels of expression were detected in the embryonic axes of developing and germinating seeds, and much lower levels in the roots, stems, leaves and flowers (Fig. 2a). In the developing seeds, the expression of *MT2a* was low initially at 15 DAP and peaked at 20 DAP followed by a sharp decrease during subsequent development. In the germinating seeds, the transcript levels of *MT2a* increased significantly within 24 h after imbibition and subsequently decreased to the level found in embryonic axes of the dry seed. The expression data of *MT2b* showed that it was predominantly expressed in the roots and was also detected in the seeds, but was weakly expressed in other organs (Fig. 2b). In the developing seeds, the expression of *MT2b* was detected from 15 to 40 DAP with the highest levels observed at 30 DAP. In the germinating seeds, the expression of *MT2b* was increased slightly at 12 h after imbibition and decreased during the following germination process. Unlike *MT2a* and *MT2b*, *MT3* was strongly expressed in the roots, stems, and leaves with the highest transcript level in the leaves, but was weakly expressed in the flowers and seeds (Fig. 2c). Although *MT3* was normally expressed at low levels during seed development and germination, relatively high levels of expression were observed in the embryonic axes at early developmental

stage (15 DAP) and at 2 d after germination.

To get more information about the expression of MT genes, the expression of *Arabidopsis MT2a*, *MT2b* and *MT3* was also analyzed in seeds and other organs by gene expression tool *Arabidopsis* eFP Browser at BAR (Bio-Array Resource for Plant Biology, http://bar.utoronto.ca). The bioinformatics analysis showed that the expression of *Arabidopsis MT2a* was similar with that of sacred lotus *MT2a*. The expression of *Arabidopsis MT2a* was strongly detected in the developing seeds from stage 8 to 10, but was lower in the roots, stems, leaves and flowers (Supporting Information Fig. S2a). During the germination process, the expression of *Arabidopsis MT2a* was significantly elevated at 1 and 3 h after imbibition and then declined at 6, 12 and 24 h. In contrast to sacred lotus *MT2b* whose expression was found predominantly in the roots, the expression of *Arabidopsis MT2b* was found extensively in the developing seeds from stage 3 to 10 and in the roots, stems, leaves and flowers with the highest level in the leaves (Supporting Information Fig. S2b). In the germinating seeds, the expression of *Arabidopsis MT2b* was much lower than in the developing seeds, and a slightly increased expression was observed at 1 h after imbibition. In addition, the expression of *Arabidopsis MT3* was high in the developing seeds from early stage 3 to 5 and in the stems and leaves but was lack in the dry seeds, which was consistent with sacred lotus *MT3* (Supporting Information Fig. S2c). However, the expression of *Arabidopsis MT3* was barely founded in the germinating seeds, while that of sacred lotus was increase at 48 h and 72 h after imbibition. Based on these comparisons, we can find that there are differences as well as similarities in

the expression for MT genes between sacred lotus and *Arabidopsis*. However, the results indicate that both sacred lotus and *Arabidopsis* MTs are highly expressed in the seeds.

Expression of MT genes is increased after AA and oxidative stress treatments

The EST analysis showed that MT genes were one of the most abundant gene families in sacred lotus seeds, which are known for their astonishing seed viability (Shen-Miller *et al*. 1995). To investigate the relationship between MTs and seed vigor, dry mature sacred lotus seeds were subjected to AA treatment (43°C for 72 h at 100% RH) which is used by seed technologists to assess seed vigor by a short exposure of the seeds to elevated temperatures and 100% relative humidity. AA treatment caused damage to sacred lotus seeds and reduced their germination rates to 81% after germination at 25°C for 5 d, while those of control seeds were nearly 100% (Fig. 3a). Also, the relative length and fresh weight of 10-d-old AA treated seedlings were only 77% and 85%, respectively, when compared to those of control seedlings (Fig. 3b,c). After AA treatment, the embryonic axes of treated and control seeds with the similar growth status (plumule protruded 0 cm, 0.2 cm and 0.5 cm, respectively) were collected for gene expression analysis (Fig. 3d). In response to AA treatment, the expression of *MT2a* and *MT2b* in embryonic axes of the AA treated seeds was up-regulated when the plumule had protruded 0.2 cm (Fig 3e,f). Similarly, significant up-regulation of *MT3* was observed when the plumule protrusion was 0 cm (Fig 3g).

To further study the sacred lotus seed germination behaviors, dry mature seeds were

germinated under various abiotic stress conditions, such as high salinity and oxidative stress induced by H_2O_2 and MV, which delayed germination by different hours. For example, the germination rates under the conditions of control, NaCl (150 mM), H_2O_2 (0.5%) and MV (20 μM) at 25°C after 72 h were 92%, 0%, 0% and 75%, respectively (Fig. 4a). Also, the relative length of 10-d-old seedlings treated with NaCl, H_2O_2 and MV were only 28%, 42% and 46%, respectively, when compared to those of control seedlings (Fig. 4b). Similar results were also obtained from the relative seedling fresh weight (Fig. 4c). In addition, the expression of MT genes under these stressful conditions was examined in 3d-imbibed sacred lotus seeds which were fully rehydrated, and the seed system can therefore respond effectively to environmental factors. The results of quantitative real-time PCR showed that the transcript levels of *MT2a*, *MT2b* and *MT3* were up-regulated in response to various stresses. The transcripts of *MT2a*, *MT2b* and *MT3* in the embryonic axes were elevated between 2 and 6 h, and down-regulated thereafter when treated with 150 mM NaCl (Fig. 4d,e,f). Significant up-regulation of *MT2a*, *MT2b* and *MT3* was detected at 6 h and subsequently at 24 h for *MT2b,* and 12 and 24 h for *MT3* after treatment with H_2O_2 (Fig. 4d,e,f). With MV treatment, up-regulation of all transcripts was detected between 6 and 12 h, with the most significantly up-regulation at 24 h (Fig. 4d,e,f). In addition, the results of RT-PCR showed that the expression of *MT2a*, *MT2b* and *MT3* was also up-regulated in the presence of heavy metals (Supporting Information Fig. S3). Taken together, the above observations suggest that MT genes can respond to AA treatment and oxidative stresses by up-regulation of their expression.

Since *MT2a* and *MT2b* belong to the same subfamily and *MT2a* was expressed predominantly in sacred lotus seeds, *MT2a* was chosen as the representative of type 2 MTs for further analysis with *MT3*.

MT2a and MT3 are localized in both cytoplasm and nucleoplasm

Since plant MTs do not contain signal peptides, it is predicted that these proteins are localized in the cytoplasm. To verify the location of the proteins in plant cells, we generated constructs containing full-length cDNA of MT2a and MT3 fused to a yellow fluorescent protein (YFP) at their C-terminal (MT2a-YFP and MT3-YFP, respectively). These vectors were electroporated into protoplasts prepared from *Arabidopsis* suspension cultured cells. After electroporation 12-16 h, the subcellular locations of each fusion protein were examined by confocal microscopy. As shown in Fig. 5, the free YFP was found distributed in both the cytoplasm and nucleus, including the nucleolus. In contrast, in protoplasts electroporated with MT2a-YFP and MT3-YFP, the YFP signal of both vectors was found in cytoplasm and nucleoplasm but was excluded from the nucleolus. Recent evidence from other plant species also shows similar localization patterns (Wong *et al*. 2004; Zhigang *et al*. 2006). Collectively, the results demonstrate the cytosolic localization of MT2a and MT3, indicating that these two proteins are located predominantly in the cytoplasm and nucleoplasm of the plant cells.

Enhanced seed germination vigor in *MT2a* and *MT3* overexpressing *Arabidopsis*

plants

In an attempt to reveal the *in vivo* functions of MT2a and MT3 in seeds, transgenic *Arabidopsis* plants expressing *MT2a* and *MT3* under the control of the cauliflower mosaic virus 35S promoter were generated. Homozygous transgenic lines containing a single copy of the transgene were used for further analysis. Three independent transgenic lines MT2a-O1, MT2a-O2 and MT2a-O3 overexpressing *MT2a* and three independent transgenic lines MT3-O1, MT3-O2, MT3-O3 overexpressing *MT3* were selected. Under normal growth conditions, plants expressing *MT2a* and *MT3* transgenes were indistinguishable from wild-type plants with respect to growth, development, seed size and seed morphology (Supporting Information Fig. S4 & S5). However, RT-PCR analysis showed that both *MT2a* and *MT3* were overexpressed in dry mature seeds (DS) and green developing siliques (GS) of transgenic plants, whereas no expression was detected from the wild-type seeds or siliques (Fig. 6a & 7a).

To further explore the roles of MT2a and MT3 in seed vigor, dry mature seeds from various transgenic lines and wild-type plants were subjected to AA treatment (43°C for 72 h at 100% RH). All untreated seeds displayed nearly 100% germination by 2 d at 22°C (Fig. 6b & 7b), indicating a high viability of the untreated seeds. In response to AA treatment, the rates of seed germination (measured in percentages) from both the transgenic lines and wild type decreased, but the germination rates of transgenic lines were dramatically higher than those of wild type (Fig. 6c & 7c). For example, germination rates in the transgenic lines overexpressing *MT2a* at 22°C ranged from 45

to 51% after 4 d, 66 to 69% after 6 d, 72 to 77% after 8d and 75 to 81% after 10d. In the wild type, the germination rates at 22°C were 6% after 4 d, 14% after 6 d, 20% after 8 day and 22% after 10 d (Fig. 6c). Similar results were also obtained from transgenic lines overexpressing *MT3* (Fig. 7c).

Among the seeds that survived the AA treatment, transgenic seeds germinated faster and finished germination earlier than their corresponding wild-type seeds, indicating a faster repair and recovery mechanism of the transgenic seeds after the AA treatment. After 10 d of germination, the final germination rates of transgenic seeds overexpressing *MT2a* and *MT3* were approximately four-fold greater than those of wild-type seeds. Strikingly, better seedling vigor was observed in the seedlings of transgenic lines after AA treatment, as indicated by the bigger plants and higher percentages of seedlings with cotyledon expansion (Fig. 6d,e & 7d,e). Without AA treatment, it is difficult to make a distinction between the wild-type and transgenic seedlings on the basis of these characteristics (Supporting Information Fig. S6).

Vital stain with tetrazolium salt can be used to demonstrate the differences in seed viability. After AA treatment (41°C for 72 h at 100% RH), the wild-type line displayed a higher number of dead seeds than those of the transgenic lines, as indicated by the numbers of unstained seeds (Fig. 6f & 7f). The differences in seed viability correlated well with the germination behavior after AA treatment. Collectively, these results provide direct evidence that MT2a and MT3 play a key role in the maintenance of seed vigor and viability during AA treatment.

Changes in lipid peroxidation and SOD activity in seeds after AA treatment

It has been proposed that lipid peroxidation and changes in free radical scavenging enzyme activities are involved in seed deterioration induced by AA treatment (Kumar & Knowles 1993; Bailly *et al*. 1996). Therefore, the levels of thiobarbituric acid-reactive-substances (TBARS) were chosen as a marker to quantify the extent of lipid peroxidation. No difference in the TBARS content was observed between wild-type and transgenic lines without AA treatment (Fig. 8a,b), whereas the TBARS levels of *MT2a* and *MT3* overexpressing lines were lower than those of wild-type lines after 3 d AA treatment (43°C at 100% RH). Similarly, although there was no difference in superoxide dismutase (SOD) activity in untreated seeds, AA treatment resulted in a significant decrease in SOD activity in wild-type seeds in comparison with that (only a slight decline) of the transgenic seeds (Fig. 8c,d). These results show that the expression of *MT2a* and *MT3* in transgenic *Arabidopsis* seeds is correlated with a low TBARS, high SOD activity and enhanced seed germination vigor after AA treatment.

Overexpression of *MT2a* and *MT3* enhances seed tolerance to NaCl and MV

To investigate the effects of altered expression of *MT2a* and *MT3* on seed germination ability, the germination tests of wild-type and transgenic lines were performed in the presence of various concentrations of NaCl and MV. In the absence of NaCl or MV, germination between wild-type and transgenic lines were similar (Fig. 6b & 7b). In contrast, germination was delayed for 1 d on medium supplemented with various

concentrations of NaCl and MV. However, seeds overexpressing *MT2a* and *MT3* were substantially more tolerant than the wild-type seeds to NaCl and MV (Fig. 9). For examples, after germination at 22°C for 4 d, seeds overexpressing *MT2a* showed germination rates ranged from 71 to 95% and 49 to 69% in the presence of 150 mM NaCl and 175 mM NaCl, respectively (Fig. 9a,b). In contrast, only 53% and 39% of the wild-type seeds germinated under the same conditions. Similar results were also observed from transgenic lines overexpressing *MT3* (Fig. 9c,d). With 10 μm MV for 4 d, germination of seeds overexpressing *MT2a* and *MT3* ranged from 68 to 81% and 68 to 89%, respectively, in comparison with 57% from the wild-type seeds (Fig. 9e,f). Taken together, the results from the germination test support the idea that MT2a and MT3 play a role in overcoming high salinity and oxidative stress in *Arabidopsis* seeds.

DISCUSSION

MTs are small, Cys-rich and metal-binding proteins which are found ubiquitously in all living systems. Many reports have documented that plant MTs function similarly as their mammalian counterparts in metal tolerance and homeostasis and controlling ROS balance (reviewed in Cobbett *et al*. 2002; Akashi *et al*. 2004; Mir *et al*. 2004; Xue *et al*. 2009). It has also been shown that plant MTs are involved in plant stress tolerance and plant growth and development processes (Bhalerao *et al*. 2003; Guo *et al*. 2003; Yuan *et al*. 2008; Xue *et al*. 2009; Yang *et al*. 2009). However, the functions of MTs in seed vigor are unclear. In this study, we focus on the functions of MTs in

seeds by gene expression analysis and transgenic approach. We have used sacred lotus seeds and *Arabidopsis* seeds overexpressing *MT2a* and *MT3* to study their unique roles in seed germination vigor under sub-optimal conditions. Further detailed analysis of the transgenic plants grown under stressful conditions will provide more evidence for the roles of MTs.

_MT2a_, _MT2b_ and _MT3_ are expressed during sacred lotus seed development and germination

Although the expression of plant MTs has been investigated in a variety of plant species, their expression in seeds has not been well elucidated. Previous studies indicate that type 2 MTs are expressed mainly in the leaves, while type 3 MTs are preferentially expressed in the fleshy fruits (reviewed in Cobbett *et al*. 2002) and in the leaves of *Arabidopsis* that do not produce fleshy fruits (Guo *et al*. 2003). However, our real-time RT-PCR results revealed that the transcripts of *MT2a* were predominantly detected in the embryonic axes of developing and germinating sacred lotus seeds (Fig. 2a). The similar results were also obtained from bioinformatics analysis of *Arabidopsis MT2a* (Supporting Information Fig. S2a). Interestingly, the expression of *MT2b* in the roots was much higher than in other organs of sacred lotus, but the expression of both sacred lotus and *Arabidopsis MT2b* was also found during seed development and early germination processes (Fig. 2b & Supporting Information Fig. S2b). In agreement with this, the GUS staining of rice MT2b was strong in the germinating embryos and could also be detected at the developing embryos (Yuan *et*

al. 2008). These data demonstrate that type 2 MT genes are expressed during seed development and seed germination, indicating that type 2 MTs may play an important role in the seeds. Unlike type 2 MTs, *MT3* was strongly expressed in the vegetative organs of sacred lotus with the highest level in the leaves (Fig. 2c), which is consistent with the observations in *Arabidopsis* (Guo *et al*. 2003; Supporting Information Fig. S2c) and buckwheat (Brkljacic *et al*. 2004; Samardzic *et al*. 2010). However, the transcripts of *MT3* were also found in the embryonic axes of sacred lotus at mid-maturation stage (15 DAP) and late-germination process, suggesting that *MT3* may function in the reproductive process and germination process. In agreement with this, *Arabidopsis MT3* was expressed at high levels at early stages (3 to 5) of seed development (Supporting Information Fig. S2c). This result is in contrast with the previous report that *Arabidopsis MT3* cannot be detected in seeds or green siliques (Guo *et al*. 2003), but was supported by other studies from buckwheat (Brkljacic *et al*. 2004; Samardzic *et al*. 2010). However, these data suggest that both sacred lotus and *Arabidopsis* MTs may play an important role in seed development and seed germination.

Both MT2a and MT3 are important for seed germination vigor

Seed vigor is crucial for plant success and crop production. However, the molecular mechanism of seed vigor is unclear. During the past decades, considerable efforts have been made to disclose the mechanisms of the underlying characteristics of seed storability and seed vigor (Debeaujon, Leon-Kloosterziel & Koornneef 2000; Clerkx

et al. 2003; Rosnoblet *et al*. 2007; Oge *et al*. 2008; Shin, Kim & An 2009; Waterworth *et al*. 2010). In this study, we show that the transcripts of *MT2a*, *MT2b* and *MT3* in embryonic axes of AA treated sacred lotus seeds were increased during germination process (Fig. 3e,f,g), suggesting that MT genes may be one of the genetic factors associated with seed germination vigor of sacred lotus. This hypothesis was further supported by the enhanced seed germination vigor of transgenic *Arabidopsis* seeds overexpressing *MT2a* and *MT3* after AA treatment. The germination rates and seedling vigor of the transgenic *Arabidopsis* lines were significantly higher than those of the wild-type seeds after AA treatment (Fig. 6c,d,e & 7c,d,e). Furthermore, vital stain showed the substantially high seed viability of transgenic *Arabidopsis* lines after AA treatment (Fig. 6f & 7f). Based on these results, it is reasonable to speculate that both MT2a and MT3 are important for seed germination vigor. The enhanced germination ability in transgenic *Arabidopsis* seeds (Fig. 9) in the presence of NaCl and MV lend further support to the idea that MT2a and MT3 are correlated with seed germination vigor. In attempting to get more evidence about the roles of MTs in seed vigor, we examined the behavior of seeds from mutants of the *Arabidopsis MT2a* (SALK_021037C & SALK_059712C) and *MT2b* (SALK_037601) by AA test, but no obvious effect was observed. The reason may be the function redundancy of MT genes in *Arabidopsis* and any single MT gene mutation could not cause obvious phenotype.

The possible explanation accounting for the link between MTs and seed germination vigor may be the antioxidant properties via their sulfhydryl groups in

proteins. The production of ROS, such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($HO^{\bullet}$) and singlet oxygen ($^1O_2$), is an unavoidable consequence of aerobic metabolism and occurs in a wide range of places in living cells (Moller, Jensen & Hansson 2007). In seed community, ROS are often considered to be a main cause of seed deterioration which results in a loss of seed vigor and viability (McDonald 1999; Bailly, El-Maarouf-Bouteau & Corbineau 2008). To cope with seed deterioration, plants have developed antioxidant defense systems, including non-enzymatic antioxidants and enzymatic antioxidants, which are often involved in seed storability and vigor (Kumar *et al*. 1993; Bailly *et al*. 1996; Lee *et al*. 2010). However, plants are equipped with specific enzymes or compounds to scavenge the above mentioned ROS except $HO^{\bullet}$, which can cause the most detrimental damages to cells (Moller *et al*. 2007). Noticeably, MT2 in wild watermelon (*Citrullus lanatus* sp.) was firstly proven to have an extraordinary high activity for detoxifying $HO^{\bullet}$ (Akashi *et al*. 2004). Thereafter, a number of studies further demonstrated that many plant MTs, especially type 2 and type 3, can function as ROS scavengers (Mir *et al*. 2004; Xue *et al*. 2009; Samardzic *et al*. 2010). Besides the known antioxidant compounds and enzymes, plant MTs may represent a new and effective antioxidant system in plant cells. In concert with this, the expression of *MT2a*, *MT2b* and *MT3* in embryonic axes of sacred lotus seeds was remarkably up-regulated under oxidative stresses (Fig. 4d,e,f). Furthermore, we observed lower levels of lipid peroxidation and higher SOD activity in transgenic *Arabidopsis* seeds than those of wild-type seeds after AA treatment (Fig. 8), suggesting that MTs are involved in oxidative stress tolerance. The

better germination behaviors of transgenic *Arabidopsis* seeds under oxidative stress condition also support our hypothesis (Fig. 9e,f). Taken together, these data suggest that MT2a and MT3 may participate in antioxidant response and scavenge ROS in response to AA treatment and adverse germination conditions, and hence contribute to seed germination vigor.

In summary, our results provide evidence that MT2a and MT3 contribute significantly to seed germination vigor under stress conditions. The biological roles of MT2a and MT3 in seed vigor are of significantly theoretical and practical values, which may be applicable to agricultural crops such as rice, wheat, maize and soybean for the success of germplasm conservation and crop yield.

ACKNOWLEDGMENTS

This study was supported by Natural Science Foundation of China (30370912) and Natural Science Foundation of Guangdong Province (2006B20101010 and 9151027501000075) to S. Huang and Guangdong Provincial Science and Technology Program (2010D020301003) to J Liu.

REFERENCES

Akashi K., Nishimura N., Ishida Y. & Yokota A. (2004) Potent hydroxyl radical-scavenging activity of drought-induced type-2 metallothionein in wild watermelon. *Biochemical and Biophysical Research Communications* **323,** 72-78.

Bailly C., Benamar A., Corbineau F. & Come D. (1996) Changes in malondialdehyde

content and in superoxide dismutase, catalase and glutathione reductase activities in sunflower seeds as related to deterioration during accelerated aging. *Physiologia Plantarum* **97,** 104-110.

Bailly C., El-Maarouf-Bouteau H. & Corbineau F. (2008) From intracellular signaling networks to cell death: the dual role of reactive oxygen species in seed physiology. *Comptes Rendus Biologies* **331,** 806-814.

Bhalerao R., Keskitalo J., Sterky F., *et al*. (2003) Gene expression in autumn leaves. *Plant Physiology* **131,** 430-442.

Brkljacic J.M., Samardzic J.T., Timotijevic G.S. & Maksimovic V.R. (2004) Expression analysis of buckwheat (*Fagopyrum esculentum* Moench) metallothionein-like gene (*MT3*) under different stress and physiological conditions. *Journal of Plant Physiology* **161,** 741-746.

Byrd H.W. & Delouche J.C. (1971) Deterioration of soybean seed in storage. *Proceedings of the Association of Official Seed Analysts* **61,** 41-57.

Clendennen S.K. & May G.D. (1997) Differential gene expression in ripening banana fruit. *Plant physiology* **115,** 463-469.

Clerkx E.J., Vries H.B., Ruys G.J., Groot S.P. & Koornneef M. (2003) Characterization of *green seed*, an enhancer of *abi3-1* in *Arabidopsis* that affects seed longevity. *Plant Physiology* **132,** 1077-1084.

Clough S.J. & Bent A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant Journal* **16,** 735-743.

Cobbett C. & Goldsbrough P. (2002) Phytochelatins and metallothioneins: Roles in heavy metal detoxification and homeostasis. *Annual Review of Plant Biology* **53,** 159-182.

Coyle P., Philcox J.C., Carey L.C. & Rofe A.M. (2002) Metallothionein: the multipurpose protein. *Cellular and Molecular Life Sciences* **59,** 627-647.

Debeaujon I., Leon-Kloosterziel K.M. & Koornneef M. (2000) Influence of the testa on seed dormancy, germination, and longevity in *Arabidopsis*. *Plant Physiology* **122,** 403-414.

Dickson M.H. (1980) Genetic aspects of seed quality. *HortScience* **15,** 771-774.

Ding Y., Cheng H. & Song S. (2008) Changes in extreme high-temperature tolerance and activities of antioxidant enzymes of sacred lotus seeds. *Science in China Series C: Life Sciences* **51,** 842-853.

Finkel T. & Holbrook N.J. (2000) Oxidants, oxidative stress and the biology of ageing. *Nature* **408,** 239-247.Guo W.J., Bundithya W. & Goldsbrough P.B. (2003) Characterization of the *Arabidopsis* metallothionein gene family: tissue-specific expression and induction during senescence and in response to copper. *New Phytologist* **159,** 369-381.

Holdsworth M.J., Bentsink L. & Soppe W. (2008) Molecular networks regulating *Arabidopsis* seed maturation, after-ripening, dormancy and germination. *New Phytologist* **179,** 33-54.

Huang S., Tang X., Zhang L. & Fu J. (2003). Thermotolerance and activity of antioxidative enzymes in lotus seeds. *Journal of Plant Physiology and Molecular*

Biology **29,** 421-424 (in Chinese).

Kawasaki S., Borchert C., Deyholos M., Wang H., Brazille S., Kawai K., Galbraith D. & Bohnert H.J. (2001) Gene expression profiles during the initial phase of salt stress in rice. *The Plant Cell* **13,** 889-905.

Klaassen C.D., Liu J. & Choudhuri S. (1999) Metallothionein: an intracellular protein to protect against cadmium toxicity. *Annual Review of Pharmacology and Toxicology* **39,** 267-294.

Kumar G. & Knowles N.R. (1993) Changes in lipid peroxidation and lipolytic and free-radical scavenging enzyme activities during aging and sprouting of potato (*Solanum tuberosum*) seed-tubers. *Plant Physiology* **102,** 115-124.

Lee Y.P., Baek K.H., Lee H.S., Kwak S.S., Bang J.W. & Kwon S.Y. (2010) Tobacco seeds simultaneously over-expressing Cu/Zn-superoxide dismutase and ascorbate peroxidase display enhanced seed longevity and germination rates under stress conditions. *Journal of Experimental Botany* **61,** 2499-2506.

Les D.H., Garvin D.K. & Wimpee C.F. (1991) Molecular evolutionary history of ancient aquatic angiosperms. *Proceedings of the National Academy of Sciences of the United States of America* **88,** 10119-10123.

McDonald M.B. (1999) Seed deterioration: physiology repair and assessment. *Seed Science and Technology* **27,** 177-238.

Miao Y. & Jiang L. (2007) Transient expression of fluorescent fusion proteins in protoplasts of suspension cultured cells. *Nature Protocols* **2,** 2348-2353.

Mir G., Domenech J., Huguet G., Guo W.J., Goldsbrough P., Atrian S. & Molinas M.

(2004) A plant type 2 metallothionein (MT) from cork tissue responds to oxidative stress. *Journal of Experimental Botany* **55,** 2483-2493.

Moller I.M., Jensen P.E. & Hansson A. (2007) Oxidative modifications to cellular components in plants. *Annual Review of Plant Biology* **58,** 459-481.

Oge L., Bourdais G., Bove J., *et al*. (2008) Protein repair l-isoaspartyl methyltransferase1 is involved in both seed longevity and germination vigor in *Arabidopsis*. *The Plant Cell* **20,** 3022-3037.

Orr W.C. & Sohal R.S. (1994) Extension of life-span by overexpression of superoxide dismutase and catalase in *Drosophila melanogaster*. *Science* **263,** 1128-1130.

Palmiter R.D. (1998) The elusive function of metallothioneins. *Proceedings of the National Academy of Sciences of the United States of America* **95,** 8428-8430.

Rabbani M.A., Maruyama K., Abe H., *et al*. (2003) Monitoring expression profiles of rice genes under cold, drought, and high-salinity stresses and abscisic acid application using cDNA microarray and RNA gel-blot analyses. *Plant Physiology* **133,** 1755-1767.

Rajjou L. & Debeaujon I. (2008) Seed longevity: survival and maintenance of high germination ability of dry seeds. *Comptes Rendus Biologies* **331,** 796-805.

Raymond A.D., Gekonge B., Giri M.S., *et al*. (2010) Increased metallothionein gene expression, zinc, and zinc-dependent resistance to apoptosis in circulating monocytes during HIV viremia. *Journal of Leukocyte Biology* **88,** 589-596.

Robinson N.J., Tommey A.M., Kuske C. & Jackson P.J. (1993) Plant metallothioneins. *Biochemical Journal* **295,** 1-10.

Rosnoblet C., Aubry C., Leprince O., Vu B.L., Rogniaux H. & Buitink J. (2007) The regulatory gamma subunit SNF4b of the sucrose non-fermenting-related kinase complex is involved in longevity and stachyose accumulation during maturation of *Medicago truncatula* seeds. *Plant Journal* **51,** 47-59.

Samardzic J.T., Nikolic D.B., Timotijevic G.S., Jovanovic Z.S., Milisavljevic M.D. & Maksimovic V.R. (2010) Tissue expression analysis of *FeMT3*, a drought and oxidative stress related metallothionein gene from buckwheat (*Fagopyrum esculentum*). *Journal of Plant Physiology* **167,** 1407-1411.

Sattler S.E., Gilliland L.U., Magallanes-Lundback M., Pollard M. & DellaPenna D. (2004) Vitamin E is essential for seed longevity and for preventing lipid peroxidation during germination. *The Plant Cell* **16,** 1419-1432.

Shen-Miller J., Mudgett M.B., Schopf J.W., Clarke S. & Berger R. (1995) Exceptional seed longevity and robust growth: ancient sacred lotus from China. *American Journal of Botany* **82,** 1367-1380.

Shen-Miller J., Schopf J.W., Harbottle G., Cao R., Ouyang S., Zhou K., Southon J.R. & Liu G. (2002) Long-living lotus: germination and soil γ-irradiation of centuries-old fruits, and cultivation, growth, and phenotypic abnormalities of offspring. *American Journal of Botany* **89,** 236-247.

Shin J.H., Kim S.R. & An G. (2009) Rice aldehyde dehydrogenase7 is needed for seed maturation and viability. *Plant Physiology* **149,** 905-915.

Suhy D.A., Simon K.D., Linzer D. & O Halloran T.V. (1999) Metallothionein is part of a zinc-scavenging mechanism for cell survival under conditions of extreme zinc

deprivation. *Journal of Biological Chemistry* **274,** 9183-9192.

Tamura K., Dudley J., Nei M. & Kumar S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24,** 1596-1599.

Thornalley P.J. & Vasak M. (1985) Possible role for metallothionein in protection against radiation-induced oxidative stress. Kinetics and mechanism of its reaction with superoxide and hydroxyl radicals. *Biochimica et Biophysica Acta* **827,** 36-44.

Tucker S.L., Thornton C.R., Tasker K., Jacob C., Giles G., Egan M. & Talbot N.J. (2004) A fungal metallothionein is required for pathogenicity of *Magnaporthe grisea*. *The Plant Cell* **16,** 1575-1588.

Waterworth W.M., Masnavi G., Bhardwaj R.M., Jiang Q., Bray C.M. & West C.E. (2010) A higher plant DNA ligase is an important determinant of seed longevity. *Plant Journal* **63,** 848-860.

Wharton M.J. (1955) The use of tetrazolium test for determining the viability of seeds of the genus Brassica. *Proceedings of the International Seed Testing Association* **20,** 81-88.

Wong H.L., Sakamoto T., Kawasaki T., Umemura K. & Shimamoto K. (2004) Down-regulation of metallothionein, a reactive oxygen scavenger, by the small GTPase OsRac1 in rice. *Plant Physiology* **135,** 1447-1456.

Wu Y., Wang Q., Ma Y. & Chu C. (2005) Isolation and expression analysis of salt up-regulated ESTs in upland rice using PCR-based subtractive suppression hybridization method. *Plant science* **168,** 847-853.

Xue T., Li X., Zhu W., Wu C., Yang G. & Zheng C. (2009) Cotton metallothionein GhMT3a, a reactive oxygen species scavenger, increased tolerance against abiotic stress in transgenic tobacco and yeast. *Journal of Experimental Botany* **60,** 339-349.

Yang Z., Wu Y., Li Y., Ling H.Q. & Chu C. (2009) OsMT1a, a type 1 metallothionein, plays the pivotal role in zinc homeostasis and drought tolerance in rice. *Plant Molecular Biology* **70,** 219-229.

Yang X., Doser T.A., Fang C.X., Nunn J.M., Janardhanan R., Zhu M., Sreejayan N., Quinn M.T. & Ren J. (2006) Metallothionein prolongs survival and antagonizes senescence-associated cardiomyocyte diastolic dysfunction: role of oxidative stress. *The FASEB Journal* **20,** 1024-1026.

Yuan J., Chen D., Ren Y., Zhang X. & Zhao J. (2008) Characteristic and expression analysis of a metallothionein gene, *OsMT2b*, down-regulated by cytokinin suggests functions in root development and seed embryo germination of rice. *Plant Physiology* **146,** 1637-1650.

Zhigang A., Cuijie L., Yuangang Z., Yejie D., Wachter A., Gromes R. & Rausch T. (2006) Expression of BjMT2, a metallothionein 2 from *Brassica juncea*, increases copper and cadmium tolerance in *Escherichia coli* and *Arabidopsis thaliana*, but inhibits root elongation in *Arabidopsis thaliana* seedlings. *Journal of Experimental Botany* **57,** 3575-3582.

FIGURE LEGENDS

Figure 1. Isolation and characterization of *MT2a*, *MT2b* and *MT3* from sacred lotus. (a) ESTs of *MTs* derived from embryonic axes of sacred lotus seeds. ESTs, expressed sequence tags; LM, late-maturation sacred lotus seeds; HT, 3d-imbibed and 2h-heat-treated mature sacred lotus seeds. (b) Protein sequence multiple alignment of MT2a and MT2b. (c) Protein sequence multiple alignment of MT3. Conserved Cys residues are indicated by letter C. Identical amino acids are shaded in black and conserved changes in gray. Sequence data used in the multiple alignment analysis can be found in GenBank/EMBL data libraries under the following accession numbers: *Arachis hypogaea* MT2b (DQ178617), *Cicer arietinum* MT2a (Q39459), *fagus sylvatica* MT2 (CAA10232), *Limonium bicolor* MT2 (EF103574), *Nelumbo nucifera* MT2a (EF421200), *Nelumbo nucifera* MT2b (EF421201), *Salvia miltiorrhiza* MT2a (EF666996) and *Vigna angularis* MT2 (AB176561); *Brassica juncea* MT3a (AB057413), *Brassica juncea* MT3b (AB057414), *Elaeis guineensis* MT3a (EU499363), *Musa acuminata* MT3 (Q40256), *Noccaea caerulescens* MT3 (FJ439651), *Nelumbo nucifera* MT3 (EF421199), *Prosopis juliflora* MT3 (EU447159) and *Typha angustifolia* MT3 (GQ426488).

Figure 2. Temporal and spatial expression profiles of *MT2A* (a), *MT2b* (b) and *MT3* (c). Transcript levels of developing and germinating sacred lotus seeds and other different organs were shown. All transcript levels were assessed by quantitative real-time PCR using 18s rRNA as an internal control.

Figure 3. Up-regulation of MT genes in AA treated embryonic axes of sacred lotus. (a) Germination performance of control and AA treated sacred lotus seeds. The values are from four replicates of 20 seeds (4×20) (mean ± SD). C, control; AA, accelerated aging (43°C for 72 h at 100% RH). (b) to (c) Relative length (b) and fresh weight (c) of 10-d-old seedlings after AA treatment. The values are from four replicates of 20 seeds (4×20) (mean ± SE). Fresh weight of seedlings did not include cotyledons. FW, fresh weight. (d) The phenotype of sacred lotus seeds at different growth stages. 0 cm, 0.2 cm and 0.5 cm represent the length of the protrudent plumule. The scale bars in mm were indicated on the left. (e) to (g) Quantitative real-time PCR analysis of MT genes transcript levels in AA treated embryonic axes of sacred lotus. 18s rRNA was used as an internal control. The expression levels of control samples with plumule emerged 0 cm were normalized to 1.0.

Figure 4. Expression patterns of MT genes in embryonic axes of sacred lotus during abiotic stress treatments. (a) Germination performance of control and stress treated sacred lotus seeds. Dry seeds were germinated with or without 150 mM NaCl, 0.5% H_2O_2 and 20 μM methyl viologen (MV). The values are from four replicates of 20 seeds (4×20) (mean ± SD). C, control. (b) to (c) Relative length (b) and fresh weight (c) of 10-d-old seedlings after stress treatments. The values are from four replicates of 20 seeds (4×20) (mean ± SE). Fresh weight of seedlings did not include cotyledons. FW, fresh weight. (d) to (f) *MT2a* (d), *MT2b* (e) and *MT3* (f) expression patterns.

3d-imbibed seeds were treated with or without 150 mM NaCl, 0.5% H_2O_2 and 20 μM MV. The expression levels of control samples at 2 h stage of imbibition were normalized to 1.0. 18s rRNA was used as an internal control.

Figure 5. Subcellular localization of YFP-fused MT2a and MT3. (a) to (c) Bright field images. (d) Yellow fluorescent image of free YFP in the protoplasts shown in (a). (e) Yellow fluorescent image of MT2a-YFP in the protoplasts shown in (b). (f) Yellow fluorescent image of MT3-YFP in the protoplasts shown in (c). DIC, differential interference contrast. Bar=25 μm.

Figure 6. Characterization of *MT2a* overexpressing lines. (a) RT-PCR analysis of *MT2a* expression levels. *Arabidopsis Actin2* was shown as a loading control. DS, dry mature seeds; GS, green developing siliques. (b) Germination performance of untreated dry mature seeds. (c) Germination performance of AA (43°C for 72 h at 100% RH) treated dry mature seeds. wild type, black squares; MT2a-O1, gray circles; MT2a-O2, gray triangles; MT2a-O3, gray diamonds. The values are from four replicates of 100 seeds (4×100) (mean ± SD). (d) Phenotypes of 10-d-old seedlings grown on half-strength MS media after AA treatment (43°C for 72 h at 100% RH). (e) Percentages of expanded cotyledons after AA treatment (43°C for 72 h at 100% RH). The numbers of expanded cotyledons were scored after 7 d (in parentheses). The values are from four replicates of 100 seeds (4×100) (mean ± SD). (f) Viability test of seeds before (upper panel) and after (lower panel) AA treatment (41°C for 72 h at 100%

RH). Dark-red staining indicates viable seeds; light-pink staining indicates reduced seed viability. Bar = 1 mm.

Figure 7. Characterization of *MT3* overexpressing lines. (a) RT-PCR analysis of *MT3* expression levels. DS, dry mature seeds; GS, green developing siliques. *Arabidopsis Actin2* was shown as a loading control. (b) Germination performance of untreated dry mature seeds. (c) Germination performance of AA (43°C for 72 h at 100% RH) treated dry mature seeds. wild type, black squares; MT3-O1, gray circles; MT3-O2, gray triangles; MT3-O3, gray diamonds. The values are from four replicates of 100 seeds (4×100) (mean ± SD). (d) Phenotypes of 10-d-old seedlings grown on half-strength MS media after AA treatment (43°C for 72 h at 100% RH). (e) Percentages of expanded cotyledons after AA treatment (43°C for 72 h at 100% RH). The numbers of expanded cotyledons were scored after 7 d (in parentheses). The values are from four replicates of 100 seeds (4×100) (mean ± SD). (f) Viability test of seeds before (upper panel) and after (lower panel) AA treatment (41°C for 72 h at 100% RH). Dark-red staining indicates viable seeds; light-pink staining indicates reduced seed viability. Bar = 1 mm.

Figure 8. Effects of AA treatment on lipid peroxidation and SOD activity. AA treatment, 43°C for 72 h at 100% RH. The significance of differences was calculated using Student's *t* test. One asterisk represents $P < 0.05$, and two asterisks represent $P < 0.01$. Data are the mean ± SD of three independent experiments. TBARS,

thiobarbituric acid-reactive-substances; FW, fresh weight; C, control. (a) to (b) TBARS content of *MT2a* (a) and *MT3* (b) overexpressing seeds. (c) to (d) SOD activity of *MT2a* (c) and *MT3* (d) overexpressing seeds.

Figure 9. Enhanced germination of transgenic seeds to NaCl and MV. wild type, black squares; MT2a-O1 and MT3-O1, gray circles; MT2a-O2 and MT3-O2, gray triangles; MT2a-O3 and MT3-O3, gray diamonds. The values are from four replicates of 50 seeds (4×50) (mean ± SD). MV, methyl viologen. (a) to (b) Sensitivity of wild-type and *MT2a* overexpressing seeds to NaCl. (c) to (d) Sensitivity of wild-type and *MT3* overexpressing seeds to NaCl. (e) Sensitivity of wild-type and *MT2a* overexpressing seeds to MV. (f) Sensitivity of wild-type and *MT3* overexpressing seeds to MV.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Phylogenetic analysis of putative sacred lotus MT2a, MT2b and MT3. The phylogenetic tree was constructed from an alignment of the amino acid sequences of MT2a, MT2b, MT3 and other members of the plant MT superfamily. Their sequence data can be found in GenBank/EMBL data libraries under the following accession numbers: *Glycine max* MT1 (AB176558), *Medicago sativa* MT1 (AF189766), *Pisum sativum* MT1 (AB176564), *Vigna angularis* MT1 (AB176560), and *Vicia faba* MT1 (AB176562); *Pisum sativum* MT2 (AB176565); *Plantago major* MT3 (AJ843995) and *Thlaspi caerulescens* MT3 (AY531114); *Arabidopsis thaliana* MT4a (At2g42000), *Glycine max* MT4 (AF010186), *Oryza sativa* MT4 (AY572960), *Triticum aestivum* MT4 (P30570), and *Zea mays* MT4 (NM_001112029). Other accession numbers of type 2 and 3 MTs can be found in the legend to Fig. 1.

Figure S2. Temporal and spatial expression profiles of *Arabidopsis MT2A* (a), *MT2b* (b) and *MT3* (c). All expression data were from *Arabidopsis* eFP Browser (http://bar.utoronto.ca/). Data were normalized by the GCOS method, TGT value of 100. *Arabidopsis* MTs sequence data can be found in GenBank/EMBL data libraries under the following accession numbers: *MT2A* (AT3g09390), *MT2b* (AT5g02380.1) and *MT3* (AT3g15353). root, young root; stem, the second internode; leaf, rosette leaf 4; flower, stage 9 flower.

Figure S3. The expression of *MT2a*, *MT2b* and *MT3* in embryonic axes of sacred lotus in response to heavy metals. The 3d-imbibed sacred lotus seeds were exposed to

1000 μM Zn^{2+}, 1000 μM Cu^{2+}, 250 μM Pb^{2+} and 250 μM Cd^{2+} for another 1 d. 18s rRNA from sacred lotus was used as an internal control.

Figure S4. Phenotypic comparison between wild-type and three independent *MT2a* overexpressing lines. (a) dry mature seeds. Bar=0.1 mm. (b) 2-w-old seedlings cultivated on half-strength MS medium. (c) 4-w-old seedlings cultivated in soil. (d) 12-d-old seedling roots cultivated on half-strength MS medium. (e) 6-w-old plants cultivated in soil.

Figure S5. Phenotypic comparison between wild-type and three independent *MT3* overexpressing lines. (a) dry mature seeds. Bar=0.1 mm. (b) 2-w-old seedlings cultivated on half-strength MS medium. (c) 4-w-old seedlings cultivated in soil. (d) 12-d-old seedling roots cultivated on half-strength MS medium. (e) 6-w-old plants cultivated in soil.

Figure S6. Phenotypes of 10-d-old untreated WT and transgenic seedlings grown in half-strength MS media. (a) Wild-type and *MT2a* overexpressing lines. (b) Wild-type and *MT3* overexpressing lines.