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ORIGINAL RESEARCH

Arabidopsis thaliana Metallothionein, AtMT2a, Mediates ROS Balance during Oxidative Stress

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Abstract Cold stress has been shown to induce the production of reactive oxygen species (ROS), which can elicit a potentially damaging oxidative burden on cellular metabolism. Here, the expression of a metallothionein gene (*AtMT2a*) was upregulated under low temperature and hydrogen peroxide (H_2O_2) stresses. The *Arabidopsis* T-DNA insertion mutant, *mt2a*, exhibited more sensitivity to cold stress compared to WT plants during the seed germination, and H_2O_2 levels in *mt2a* mutant were higher than that in WT plants during the cold stress. Synthetic GFP fused to AtMT2a was observed to be localized in cytosol. These results indicated that AtMT2a functions in tolerance against cold stress by mediating the ROS balance in the cytosol. Interestingly, mRNA level of *AtMT2a* was increased in seedlings of *Arabidopsis cat2* mutant after cold treatment compared to WT seedlings, and overexpression of *AtMT2a* in *cat2* could improve CAT activity under chilling stress. Moreover, the enzymatic activity of CAT in *mt2a* was higher than that in WT plants after cold treatment, suggesting that AtMT2a and CAT might complement each other in antioxidative process potentially in *Arabidopsis*. Taken together, our results provided a novel insight into the relationship between MTs and antioxidative enzymes in the ROS-scavenging system in plants.

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Introduction

During growth and development, plant has to cope with a range of different external stresses, such as low temperature, drought, and high salinity. To survive environmental changes, plants have developed elaborate mechanisms to perceive external signals and to manifest adaptive responses with the proper physiological and morphological changes (Mahajan and Tuteja 2005; Huang et al. 2009). The production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), and its more toxic byproducts, hydroxyl radicals (OH^-) and singlet oxygen ($^1O_2$), occurs at all times during plant growth and development, and increases when plants are exposed to various biotic and abiotic stresses (Xiong et al. 2002). Previous studies indicated that ROS are highly reactive and toxic and can lead to the oxidative destruction of cells (Asada 1988). In recent years, it has been identified that ROS play a novel role in the regulation of many biological processes, such as growth, cell cycle, programmed cell death, hormone signal, biotic and abiotic stress response, and development (Mittler et al. 2004; Kotchoni and Gachomo 2006). These studies provided evidences that ROS has a dual role in plant biology as both toxic byproducts of aerobic metabolism and key regulators of growth, development, and defense pathways.

It has been accepted that antioxidant defense systems, including non-enzymatic antioxidants such as ascorbate, reduced glutathione, and tocopherol, and enzymatic antioxidants such as superoxide dismutase (SOD) and catalase (CAT), play a crucial role in plants against various stresses

(Mittler et al. 2004). The regulation of the concentrations of antioxidants and of the activities of antioxidant enzymes is an important mechanism for combating oxidative stress (Kovtun et al. 2000). It has been reported that superoxide radicals (O_2^-) are scavenged through the catalytic activity of SOD, while hydrogen peroxide (H_2O_2) is scavenged through the catalytic action of ascorbate peroxidase (APX and CAT; Mittler et al. 1999). Most of these enzymes play their function by binding metal ions as metal co-factors in plant, such as protoporphyrin-bound iron which is essential for native catalase to degrade hydrogen peroxide into water and oxygen (Vetrano et al. 2005).

Metallothioneins (MTs) constitute a superfamily of evolutionally conserved, low molecular mass, cysteine-rich proteins that can bind metals via the thiol groups of their cysteine (Cys) residues (Cobbett and Goldsbrough 2002; Zhigang et al. 2006; Zhou et al. 2006; Thirumoorthy et al. 2007). In animals, MTs are not only involved in maintaining homeostasis of essential metals and metal detoxification (Thirumoorthy et al. 2007) but also implicated in a range of other physiological processes, including scavenging ROS, regulating cell growth, and proliferation (Davis and Cousins 2000). Recently, plant MTs have been proposed to be primarily involved in the response to metal toxicity and oxidative stress (Mir et al. 2004; Vetrano et al. 2005; Guo et al. 2008). It has been proposed that plant MTs might function as efficient scavengers of ROS production when plants were exposed to abiotic stress (Chatthai et al. 1997; Nishiuchi et al. 2007; Yuan et al. 2008). Additionally, plant MTs are also 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). Although increasing numbers of reports have indicated that plant MTs may play important roles as they do in animals, knowledge on the molecular functional mechanisms of plant MTs is limited, and the relationships between MTs and antioxidant enzymes in plant response to oxidative stress have remained largely unexplored.

In this paper, we showed that the expression of *AtMT2a* was induced effectively under H_2O_2 and low temperature stresses, and AtMT2a functions as an ROS scavenger in the cytosol under abiotic stress conditions. AtMT2a and CAT might be complementary to each other in antioxidative process. Our results provided interesting information to investigate the ROS-scavenging mechanism of MTs in plants.

Materials and Methods

Plant Materials and Stress Treatments

Plants (*Arabidopsis thaliana* ecotype Columbia) were grown on germination medium agar plates for 2 weeks, as described previously (Yamaguchi-Shinozaki and Shinozaki 1994). For different stress treatments, uniformly developed 14-day-old seedlings were transferred into liquid medium containing 150 mM NaCl for 6 h, 20 g/100 mL PEG for 2 h, and 10 mM H_2O_2 for 6 h. For the low temperature treatment, the seedlings were transferred to an incubator at 4°C for 24 h.

Identification of Insertion Mutation

The T-DNA insertion line, *mt2a* (SALK_059712), was ordered from the *Arabidopsis* Biological Resource Center (ABRC; http://www.arabidopsis.org/abrc/). The homozygous alleles were determined by PCR amplification with the promoter-specific primers (2ap5: ctgcagcggttcttgctcg and 2ap3: ggatccctcgagaaaattcaaa) and T-DNA specific primers homologous to the left border (LBb1: gcgtggaccgcttgctg caact; Zsigmond et al. 2008). Another homozygous alleles of *cat2* (SALK_057998) which was also ordered from the ABRC were confirmed using gene-specific primers (cat25: gtccagctagttcttacaactc and cat23: tgcttggtctcacgttcagac) as described above in this section.

Freezing Tolerance

Arabidopsis seeds were surface-sterilized and water-imbibed in the dark for 1 day at 20°C and then were treated with −6°C for 12 h. Seeds were transferred to 0.5 Murashige and Skoog medium (pH 5.7) and germinated with a photoperiod of 16 h of light and 8 h of dark for 2 days. Germination kinetics was determined by measuring the proportion of seeds at different time points in a sample where the radicle had begun to emerge from repeated experiments with duplicate plates of approximately 25 seeds each.

Conductivity Test

Arabidopsis seeds were surface-sterilized and water-imbibed in the dark for 1 day at 20°C and then were treated with −6°C for 12 h. The conductivity of the seed leachate was determined using the YSI 3200 (Yellow Springs, OH, USA) conductivity meter after swirling the contents for 10 to 15 s. Conductivity was calculated as conductivity for each liter mass of seeds=μS $cm^{-1}g^{-1}$.

Northern Bolt Analysis

Total RNA of the whole seedlings was isolated using the RNeasy Plant Mini Kit (Qiagen Fremont, CA, USA). The 3′UTR of *AtMT2a* was used as probe which was amplified by the two primers P5: gcacctgcaagtgaagaagcct and P3: ccactggaagtacaaactcatcac. The specific *AtMT2a* fragment

was labeled with [α-^{32}P]dCTP using the hybridization procedure described by Yamaguchi-Shinozaki and Shinozaki (1994). The blots were exposed to the phosphorimager.

H_2O_2 Staining with DAB

H_2O_2 accumulation in plant was visualized by DAB staining. Fourteen-day-old *Arabidopsis* seedlings were vacuum-incubated for 2 min with DAB solution (1 mg mL^{-1}, pH5.8) and incubated in the dark at 22°C 20 h. The stain was poured off and chlorophyll removed by incubating overnight in 96% (*v*/*v*) ethanol. DAB is polymerized locally in the presence of H_2O_2 giving a visible brown stain.

Subcellular Localization of the MT2a Protein

The *AtMT2a* cDNA with the termination codon removed was fused in frame to the GFP reporter gene in the pBINmGFP5-ER vector and verified by DNA sequencing. The fusion construct for AtMT2a-GFP and the GFP control plasmid were introduced into *Arabidopsis* as described previously (Sakuma et al. 2006). The GFP fluorescence in the young roots of 14-day-old transgenic plants was analyzed with the fluorescence microscope (Olympus BX50). All photographs were taken at the same magnification.

Measure of H_2O_2 Production

Fourteen-day-old *Arabidopsis* seedlings (about 0.1 g FW) were homogenized in 1 mL cold acetone in a mortar with silica sand (Xue et al. 2009). The extract and washings were centrifuged at 1,250 g for 10 min, and the chlorophyll contents were absorbed by activated carbon. Then, 200 μl supernatant were added to 1 mL of reaction buffer [0.25 mM $FeSO_4$, 0.25 mM $(NH_4)_2SO_4$, 25 mM H_2SO_4, 1.25 mM xylenol orange, and 1 mM sorbitol] at room temperature for 1 h. H_2O_2 level were quantified at 560 nm absorbance, and H_2O_2 level were calculated by reference to standards.

CAT Extraction and Assays

Soluble proteins of 14-day-old *Arabidopsis* seedlings (about 0.1 g FW) were extracted by homogenizing the powder in 1 mL of potassium phosphate buffer (0.1 M, pH 7.8) containing 1 mM EDTA, 0.3% (*v*/*v*) Triton X-100, and 1 g/100 mL polyvinylpolypyrrolidone. The homogenate was centrifuged at 15,000 g for 20 min at 4°C, and the supernatant was used for the following enzyme assays. Protein content was determined according to the method of Bradford with bovine serum albumin (0.563 mg mL^{-1}) as standard (Bradford 1976). Catalase activity was determined by spectrophotometrically following H_2O_2 decomposed min^{-1}mg^{-1} protein and corresponds to the mean±SD of the values obtained with three measurements per extract (Jiang and Zhang 2002).

Statistical Analysis

Data were analyzed for significant differences using the statistical software SASS for windows. Differences at the 5% level were considered significant. These data presented are the means from three replications.

Results

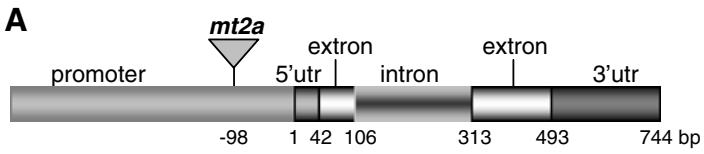
AtMT2a Involved in the Mediation of H_2O_2 Level during Abiotic Stresses

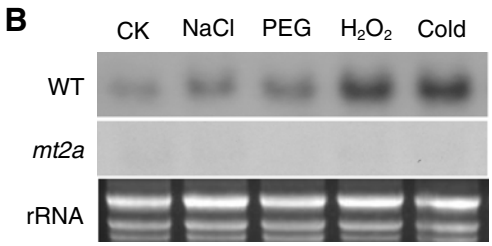
To analyze the function of *AtMT2a* (At3g09390) in *Arabidopsis*, *a* T-DNA insertion line which was designed as *mt2a* (SALK_059712) was obtained in the *A. thaliana* Col-0 background. By screening the Genbank and *Arabidopsis* Biological Resource Center (http://www.arabidopsis.org/abrc/), we found that the T-DNA insertion is in the promoter region of *AtMT2a* (Fig. 1a). The homozygote lines of *mt2a* were identified using three primers by PCR analysis (data not shown).

To get clue of the role of *AtMT2a* in abiotic stress, we detected its transcription level in WT plants treated with various abiotic stresses including cold, drought, salinity, and H_2O_2. The northern blot analysis indicated that the expression of *AtMT2a* could be induced by the four kinds of stresses, especially by low temperature and oxidative stresses (Fig. 1b). Moreover, the cold and other stresses resulted in more accumulation of intercellular H_2O_2 in WT *Arabidopsis* seedlings (Fig. 1c, d). In *mt2a* mutant, no *AtMT2a* transcripts were detected under normal and all stress conditions (Fig. 1b), whereas H_2O_2 accumulation was significantly increased by measuring H_2O_2 contents and histochemical 3,3-diaminobenzidine (DAB) staining in cold stress (Fig. 1c–e). These results revealed that a linear relation between the expression of *AtMT2a* and the H_2O_2 level is possible in *Arabidopsis* plants.

mt2a Displays Sensitivity to Cold Stress during Seed Germination

To elucidate the in vivo functions of the *AtMT2a* gene during cold stress in plants, we performed the seed germination of *mt2a* mutant and WT exposed to cold stresses. As shown in Fig. 2a to c, the seeds of *mt2a* germinated later than WT by analyzing germination ratio and root length, though the germination rates of the seeds

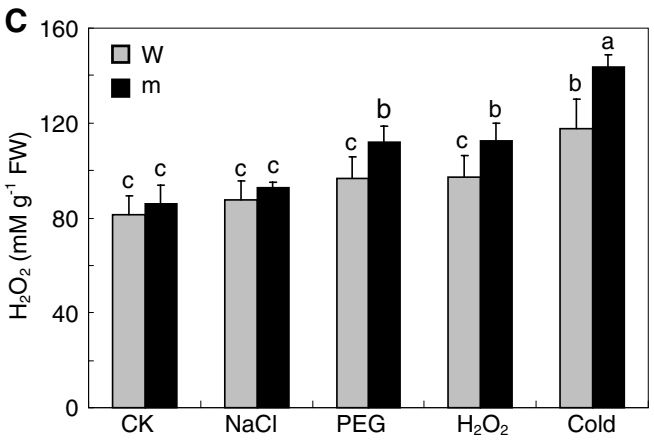




D

H_2O_2 contents (mM g^{-1} FW)

	WT	*mt2a*
CK	81.21 +/- 8.12	85.71 +/- 8.05
NaCl	87.41 +/- 8.37	93.23 +/- 2.31
PEG	96.53 +/- 8.94	112.78 +/- 7.15
H_2O_2	97.52 +/- 9.18	112.64 +/- 7.54
Cold	117.81 +/- 12.11	143.71 +/- 5.59



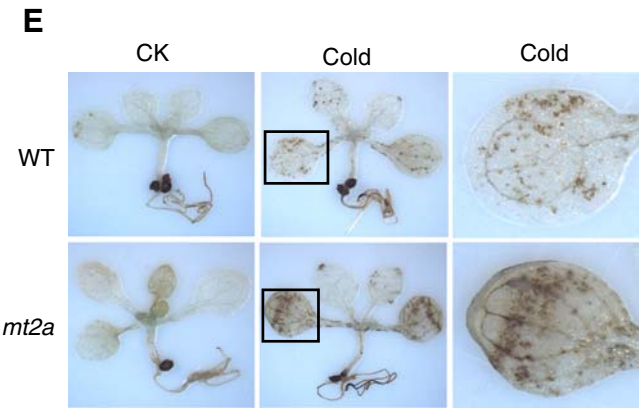


Fig. 1 The expression of *AtMT2a* and mediation of ROS balance of AtMT2a under abiotic stress conditions. **a** Position of T-DNA insertion in the *At3g09390* gene. The promoter sequence is disrupted by a T-DNA located upstream of the ATG codon at −98 bp in the *mt2a* allele (SALK_059712). **b** Northern blot analysis of *AtMT2a* in wild-type (WT) and *mt2a* seedlings. RNAs were prepared from 14-day-old WT and *mt2a* seedlings treated with 150 mM NaCl for 6 h (NaCl), 20% PEG for 2 h (PEG), 10 mM H_2O_2 for 6 h (H_2O_2), 4°C for 24 h (Cold), respectively. About 20 μg of total RNA was analyzed by RNA gel blotting. The 3′UTR was used as probe for *MT2a* gene, and the ethidium bromide-stained rRNA is shown as a loading control. **c** H_2O_2 contents of 14-day-old WT (W) and *mt2a* (m) seedlings grown under normal condition (CK) and treated with above abiotic stresses, respectively. Values are the mean obtained from three experiments, and *different letters* indicate significant differences at $P<0.05$. **d** Mean H_2O_2 contents (±SE) of 14-day-old WT and *mt2a* seedlings grown under normal condition (CK) and treated with above abiotic stresses, respectively. **e** Detection H_2O_2 contents in 14-day-old WT and *mt2a* seedlings by DAB histochemical assay under normal condition (CK) and treated with 4°C for 24 h (cold)

of *mt2a* and WT seedlings were similar following 40 h of germination in 0.5 MS medium after freezing treatment, and membrane permeability of *mt2a* seeds was obviously higher than that in WT seeds after cold stress treatment by conductivity test (Fig. 2d). These above results strongly supported the idea that AtMT2a functions in the resistance against cold stress in *Arabidopsis*.

To identify the cellular compartment in which AtMT2a functions, transgenic *Arabidopsis* plants were produced carrying transcriptional fusions of *MT2a* and GFP open reading frame under the control of 35S promoter. The 35S: GFP was also overexpressed in transgenic *Arabidopsis* plants as a control (Fig. 3b). As shown in Fig. 3d and f, AtMT2a-GFP was localized to the cytosol of young roots of transformed *Arabidopsis* under both normal and cold stress conditions, suggesting that AtMT2a mainly functions in mediating the ROS balance in the cytosol.

The Complementary Relationship between AtMT2a and CAT in ROS Scavenging

It is well-known that CATs, as a kind of enzymatic antioxidants, play an important role in reducing the accumulation of hydrogen peroxide (H_2O_2), thereby protecting cells against oxidative damage (Mittler et al. 2004; Kotchoni and Gachomo 2006). To investigate the relationship of AtMT2a and CATs, a mutant homozygote of a CAT2 gene (At4g35090), *cat2* (SALK_057998), was obtained. Northern blot analysis showed that the mRNA levels of *AtMT2a* were obviously increased in *cat2* seed-

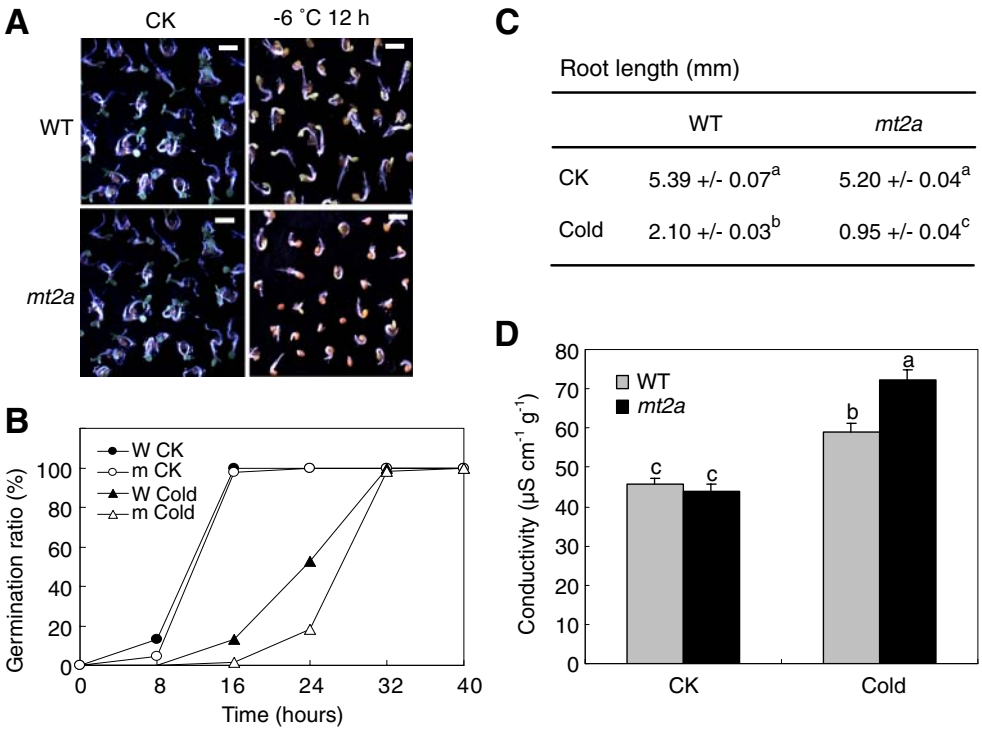


Fig. 2 Cold stress sensitivity of *mt2a* mutant. **a** WT and *mt2a* seedlings following 2 days of germination in 0.5 Murashige and Skoog (MS) medium after freezing treatment (−6°C, 12 h). *Bars*, 285 μm. **b** Germination kinetics of WT (W) and *mt2a* (m) seeds in the absence (CK) and presence of freezing treatment (Cold). **c** The root lengths of WT and *mt2a* seedlings in the absence (CK) and presence of freezing treatment (cold). The data shown are the average of three independent replicates. **d** Conductivity tests of WT and *mt2a* seeds in the absence (CK) and presence of freezing treatment (cold). Values are means (±SE). *Different letters* indicate significant differences at $P<0.05$

lings after cold treatment compared to WT seedlings (Fig. 4a). Interestingly, overexpression of *AtMT2a* in *cat2* mutant (designed as *cat2-MT2a*) resulted in an increase of the CAT activity under cold stress condition, and the activity of CAT in *cat2* was much lower than that in WT seedlings because of the absence of *CAT2* in *Arabidopsis*, indicating that higher level of AtMT2a could complement the lack of *CAT2* gene under cold stress condition (Fig. 4b). On the other hand, the CAT activity in *mt2a* was significantly higher than that in WT seedlings after cold treatment (Fig. 4b). These results suggested that AtMT2a and CAT might complement each other, which lead to keep the balance of ROS in *Arabidopsis* when plants were exposed to cold stress.

Discussion

Plant growth and productivity are affected by various abiotic stresses, such as drought, high salinity, and low temperature (Mahajan and Tuteja 2005; Shan et al. 2007). Oxidative stress occurs as an essential response when plants are challenged with these abiotic stresses and results from the disturbance in balance between ROS production and scavenging (Mittler 2002). It has been accepted that ROS play a central role in many signaling pathways in plants involved in stress perception, photosynthesis regulation, pathogen response, and programmed cell death (Yuan et al. 2008). However, the excessive accumulation of ROS can lead to the oxidative destruction of cells (Mittler et al. 2004; Kotchoni and Gachomo 2006). Consequently, plants have developed a variety of ROS-scavenging mechanisms by which they respond to oxidative stress. These mechanisms include the production of both non-enzymatic antioxidants such as ascorbate and glutathione and enzymatic antioxidants such as SOD and CAT (Gajewska and Sklodowska 2007). In recent studies, the regulation of the concentrations of antioxidants and 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 antioxidative mechanisms may exist in plant cells and need to be clarified.

MTs are a class of low molecular mass (4 to 8 kD), cysteine-rich proteins that can bind metals via the thiol groups of their Cys residues. This property endows them with wide-ranging functional capabilities in biosystems (Thirumoorthy et al. 2007; Guo et al. 2008). Recently, a number of investigations have demonstrated MTs as being efficient scavengers of ROS production in animals (Dong et al. 2007; Peng et al. 2007). In mammalian cells, MTs may act in zinc trafficking and/or zinc donation to apoproteins, including antioxidant enzymes and zinc finger proteins (Guo et al. 2009). These numerous zinc coordination sites of proteins provide the opportunity for the cellular MT to influence oxidative damage caused by oxidative stress and other key processes (Davis and Cousins 2000; Maret 2004; Li et al. 2006). Based on our results, we proposed that plant MTs may also function as an ROS scavenger involved in the response to oxidative stress (Navabpour et al. 2003;

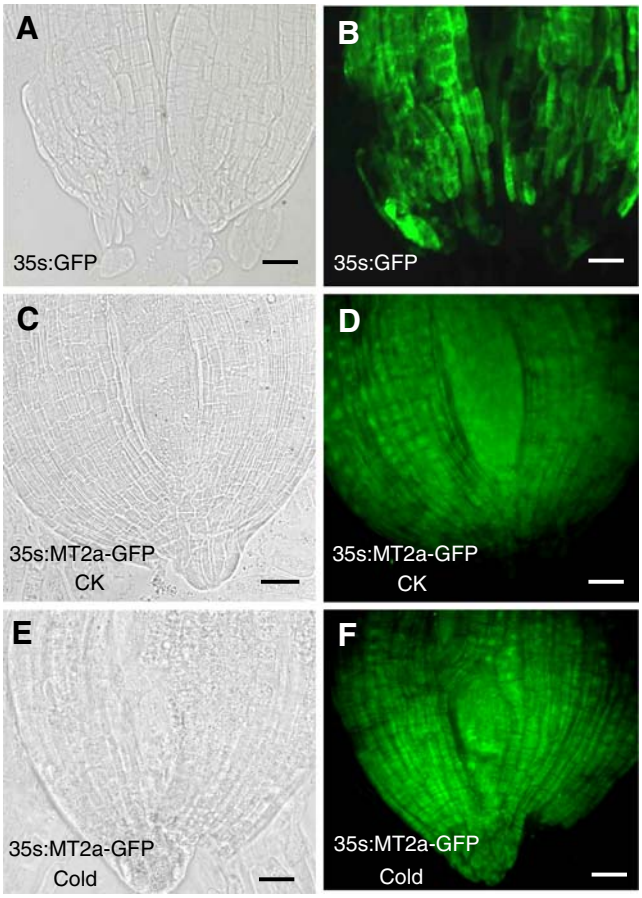


Fig. 3 Subcellular accumulation patterns of AtMT2a fused to GFP. Bright field images (**a**, **c**, and **e**) and fluorescence images (**b**, **d**, and **f**) of young roots from 14-day-old transgenic *Arabidopsis* plants carrying 35S: GFP (**a** and **b**) and 35S: AtMT2a-GFP (**c** to **f**) under normal condition (CK) and treated with 4°C for 24 h (cold). *Bars*, 50 μm

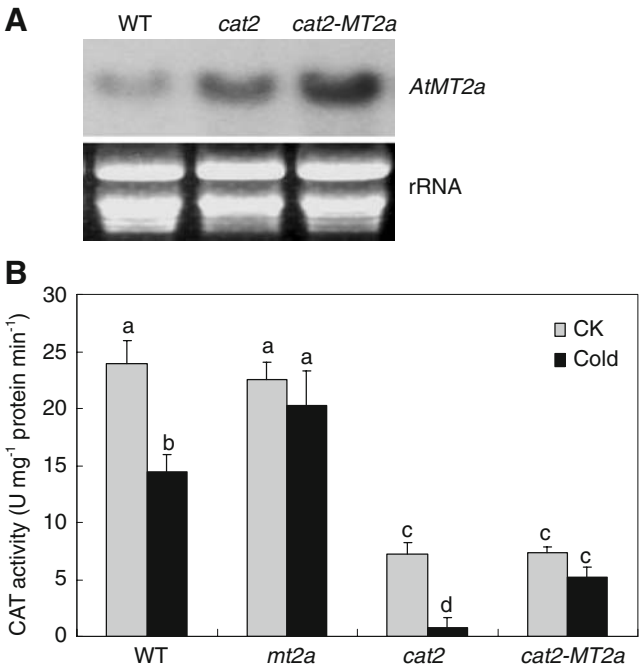


Fig. 4 The relationship between AtMT2a and CAT in *Arabidopsis*. **a** Northern blot analysis of *AtMT2a* expression in 14-day-old WT, *cat2* and *cat2-MT2a* (*AtMT2a* overexpressing line in *cat2*) seedlings treated with 4°C for 24 h. The 3′UTR was used as probe for *AtMT2a* gene. **b** The CAT activity of 14-day-old WT, *mt2a*, *cat2*, and *cat2-MT2a* seedlings under normal and cold stress (4°C for 24 h) conditions. The data shown are the average of three independent replicates. *Different letters* indicate significant differences at $P<0.05$

Nishiuchi et al. 2007; Yuan et al. 2008). More recently, a type 2 MT in rice, OsMT2b, was also showed to be involved in ROS scavenging and signaling (Yuan et al. 2008).

In our studies, the expression of *AtMT2a* could be induced by drought, high salinity, cold stress, and H_2O_2, especially under condition of oxidative stress and low temperature (Fig. 1b), and all above abiotic stresses resulted in increased intercellular H_2O_2 levels in *Arabidopsis* seedlings, especially cold stress (Fig. 1c, d). On the other hand, H_2O_2 content of *mt2a* was obviously higher than WT plants during cold stress, and only slightly higher H_2O_2 was accumulated in *mt2a* than WT plants under salinity and drought treatments (Fig. 1c–e). Moreover, the seeds of *mt2a* germinated slower than WT plants under cold condition (Fig. 2). Taken together, these results indicated that AtMT2a could scavenge redundant H_2O_2 effectively during abiotic stresses, especially under cold condition. By mediating the ROS balance, AtMT2a could contribute to low temperature tolerance of *Arabidopsis*.

CAT is the major H_2O_2-scavenging enzyme in all aerobic organisms. Protoporphyrin-bound iron exists in a high spin ferric state in native CAT, which is essential for its ability to degrade hydrogen peroxide into water and oxygen (Vetrano et al. 2005). Compared with WT seedlings, higher H_2O_2 level in *mt2a* leads to the increased CAT activity under cold stress condition (Fig. 4b), which is consistent with previous studies (Jiang and Zhang 2002). Our results also indicated that *AtMT2a* gene expression is obviously induced in *cat2* mutant in which the H_2O_2 level is higher than that in WT plants during cold stress (Fig. 4a). Moreover, a considerable recovery of enzymatic activity from 5.3% to 35.3% was detected when overexpressing *AtMT2a* in *cat2* mutant under cold stress condition (Fig. 4b). In general, organelles with a highly oxidizing metabolic activity or with an intense rate of electron flow, such as chloroplasts, mitochondria, and microbodies, are a major source of ROS production in plant cells, and then ROS could diffuse rapidly from plastids to cytoplasm (Henzler and Steudle 2000). Previous study demonstrated that CATs have been mainly found in peroxisomes (Mittler et al. 2004). However, we revealed that MT2a are located in cytoplasm under both normal and cold stress conditions (Fig. 3). In view of these observations, it is suggested that

MT2a and CAT complement each other in different compartment of cell in the ROS-scavenging process.

In summary, our results provided a novel insight into the relationship between MTs and antioxidative enzymes in plants in the ROS-scavenging system. MTs might play a role in the tolerance against cold stress by inhibiting ROS accumulation in plant. The complementary relationship between MTs and CAT in different compartment of cell under low temperature stress was observed for the first time. Our results described here establish a foundation for future studies of the ROS-scavenging mechanism in plants.

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