Functional characterization of a type 2 metallothionein gene, SsMT2, from alkaline-tolerant Suaeda salsa

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A type 2 metallothionein gene, SsMT2, was cloned from Suaeda salsa, a salt- and alkali-tolerant plant, which is a dominant species on the saline/alkali soil of northeast China. The SsMT2 gene was expressed in all organs except the flower and its expression was induced by various stresses such as CdCl2, NaCl, NaHCO3, and H2O2 treatments. SsMT2-transgenic yeast (Saccharomyces cerevisiae) and plants (Arabidopsis thaliana) showed significantly increased resistance to metal, salt, and oxidant stresses. These transgenics accumulated more Cd, but less Na than their wild type counterparts. SsMT2 transgenic Arabidopsis maintained lower level of H2O2 than wild type plants did in response to the stress treatments. These results demonstrated that the SsMT2 gene plays an important role in reactive oxygen species scavenging and confers enhanced metal and oxidant tolerance to plants.

Saline-alkaline soils are widely distributed on earth, and the total global area of salt-affected soils, including saline-alkaline soils, is 8.31 × 108 ha1. The saline-alkaline soils in Northeast China contain a high concentration of NaHCO3.2 Very few plants survive in this area, and those that do have high tolerance to saline/alkali stress. The genus Suaeda consists of 110 species of which most are highly salt tolerant14. In saline/alkali communities of northeast China, S. salsa is typically the predominant vegetation. S. salsa accumulates salts within cells, therefore, significantly decreases the salt concentrations in the soil13. At a density of 15 plants/m2, S. salsa plants can remove 303–386 g/m2 of Na+ from saline soil during its growing season, which suggests that S. salsa could be used to improve the saline soil quality8. Several S. salsa communities have been developed as tourism resources in saline-alkali soil7. S. salsa also can regulate transportation or transformation of nutrients and heavy metals8. Because S. salsa can survive in soil with high NaHCO3 content, it may have a special mechanism to accommodate the formidable salt/alkali in the environment.

An extensive number of studies has been completed in plants addressing tolerance to salinity and/or alkalinity, leading to identification of a class of plant Metallothioneins (MTs) proteins, that are associated with plant resistance extreme environmental stress8. MTs are a family of low molecular weight (7–10 kDa), Cys-rich proteins that bind to metals in a range of organisms, such as Oryza sativa16, Arabidopsis11, Elsholtzia haichowensis is12, and Gossypium hirsutum13. MTs are divided into three classes based on the arrangement of Cys residues14. Plant MTs belong to class II and can be further subdivided into the following four types: MT1, MT2, MT3, and MT4, based on the Cys distribution pattern15.

MT function in plants can be triggered when plants suffer metal and/or salt stress. Several MT genes have been cloned. For example, EhMT1 was cloned from E. haichowensis under high Cu2+ concentration16, Hordeum vulgare MT from Fe-deficient roots17, Triticum aestivum MT from roots treated with Al3+18, tomato MT and cabbage MT from roots treated with Cd2+19,20, Silene nicaeensis SnMT2 from root of plants collected from area with higher metal pollution index (MPI)21, Oryza sativa rgMT and Chloris virgata Swartz ChlMT1 from seedlings treated with NaHCO322–24, and celery pAgMT2 and pAgMT3 were induced by salt stress25.

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The ectopic expression of OsMT1e-P enhanced tolerance of salt stresses in transgenic tobacco, and the resultant plants survived and set viable seeds under saline conditions. A SbMT gene was used to transform tobacco, and transgenic lines had better phenotypic performance under salt (NaCl) stress conditions compared to wildtype plants. Overexpression of OsIFL in transgenic tobacco plants conferred salinity stress tolerance. Screening of a rice cDNA library revealed OsIFL strongly interacted with metallothionein protein.

Cadmium (or Cd$^{2+}$), among the most toxic non-essential elements with high mobility in plants, directly or indirectly inhibits primary physiological processes. The photosynthetic apparatus appears to be particularly sensitive to Cd$^{2+}$ toxicity, even at very low concentrations. MTs were first isolated as Cd-binding protein from horse kidney in 1957. This family of proteins detoxifies metal ions through direct binding Cd$^{2+}$.

The production of reactive oxygen species (ROS) occurs at all times during plant growth and development, and increases when plants are exposed to biotic and abiotic stresses. The cysteines in MTs directly involved in the removal of ROS and thus, protect against cellular injury, and indirectly reduce the production of cellular ROS. MTs may act as an antioxidant by mitigating ROS-induced cellular injury independent of a function in metal sequestration.

Each kind of MT may have a unique function and plays an important role against abiotic stress. Because S. salsa grows in saline or alkaline soil habitat and persists, the biological function of MTs in anti-alkali plants has not been elucidated. Therefore, we cloned an open reading frame of a type 2 MT, designated as SsMT2, from S. salsa and investigated its function under the stress induced by Cd$^{2+}$, Na$^+$ and H$_2$O$_2$ in transgenic yeast (Saccharomyces cerevisiae) and Arabidopsis thaliana. The results enhance our insights into the SsMT2 gene function when halophyte plants are grown under environmental stresses.

**Results**

**Cloning of an open reading frame of SsMT2 in S. salsa.** The open reading frame (ORF) of SsMT2 was obtained from the cDNA in the S. salsa. The full-length fragment contains of 234 bp and encodes a 77-amino acid polypeptide (GenBank accession number MF447531). The amino acid sequence of this transcript had the highest similarity (91%) with that of the SbMT protein (GenBank accession number: JF780913) from Salicornia brachiata, followed by AcMT from Amaranthus cruentus (AF268027) (79%), SnMT from Silene niceensis (ADP92404) (75%), and SmMT from Salvia miltiorrhiza (ABR92329) (60%) (Fig. S1).

**SsMT2 gene expression in S. salsa.** Northern blot detected strong signals in roots, leaves, stems and seed, but no signal in flowers, indicating that the SsMT2 gene expressed in all organs except flowers (Fig. 1A). The expression of the SsMT2 gene was significantly induced under CdCl$_2$ and H$_2$O$_2$ stresses in S. salsa. NaCl stress caused a moderate increase of the transcript and NaHCO$_3$ stress caused slight increase of the transcript (Fig. 1B). The results indicated that different stresses affect the SsMT2 expression differentially in S. salsa.

**SsMT2-transgenic yeast responses to Cd$^{2+}$, Na$^+$ and H$_2$O$_2$ stresses.** Northern blot showed that one distinct band was detected in the transgenic yeast and no signal in the control and indicated that the SsMT2 gene was expressed in the transgenic yeast (Fig. S2A). The quantification of SsMT2 protein in yeast was analyzed using Western blot (Fig. S2B). Stronger signals were detected in SsMT2 transformed yeast, compared to weak signal in WT yeast (non-SsMT2 transformed). This result indicated that some other MT proteins present in the yeast, and SsMT2 transformed yeast has more MT protein than WT yeast.

The cell growth of transgenic and non-transgenic yeasts was compared at five serial dilutions for 48 h showed in Northern blot. No treatment (CK = 0) is a control. Cropped images were displayed and original blots are shown in the Supplementary 3.

**Figure 1.** Organ distribution of SsMT2 expressionin S. salsa and detection of SsMT2transcripts in stress-treated S. salsa. (A) Northern blot analysis showed the differential expression of SsMT2 in different organs of S. salsa. (B) Gene expression in S. salsa after different stresses treatments for 48 h showed in Northern blot. No treatment (CK = 0) is a control. Cropped images were displayed and original blots are shown in the Supplementary 3.
concentration was increased to 160 µM CdCl₂, 1 M NaCl, 26 mM NaHCO₃, or 3.2 mM H₂O₂, the transgenic yeasts grew, but non-transgenic yeasts did not grow (Fig. 2).

**SsMT2**-transgenic Arabidopsis responses to Cd²⁺, Na⁺ and H₂O₂ stresses. The copy numbers of the **SsMT2** gene in the transgenic lines were indicated by one or more distinct bands in the transgenic Arabidopsis. There were four plants (#1, #3, #5 and #6 in Fig. S2C) that had one copy, one plant (#2) that had three copies (Fig. S2C), and one plant (#4) that had nine copies (Fig. S2C). No positive signal was detected in WT Arabidopsis plants (Fig. S2C). The expression of **SsMT2** gene in transgenic Arabidopsis was detected by Northern blot. Of these transgenic Arabidopsis plants, three (#1, #5 and #6 in Fig. S2D) were positive, and indicated the **SsMT2** gene was highly expressed in these transgenic plants.

The effects of CdCl₂, NaCl, NaHCO₃ and H₂O₂ on seed germination were examined in the above three selected transgenic Arabidopsis and wild type plants (Fig. 3A). Seeds of wild type and transgenic plants were germinated on medium, each containing 100 µM CdCl₂, 100 mM NaCl, 2 mM NaHCO₃ or 1 mM H₂O₂, with 3 days later for wild type than transgenic lines. In the presence of 150 mM NaCl or 4 mM NaHCO₃, only 40% or 20% wild type lines seed germination respectively, while 100% transgenic lines seed germination. In the presence of 180 µM CdCl₂ or 5 mM H₂O₂, no wild type seeds were germinated. Although transgenic plant seeds were also heavily affected, 48% or 72% seeds were germinated respectively. The transgenic lines extended germination until the cotyledon turned white under 5 mM H₂O₂. On the control (no stress) media, seed germination showed no significant difference between wild type and three selected transgenic lines (Fig. 3A).

The effects of CdCl₂, NaCl, NaHCO₃ and H₂O₂ on seedling growth were examined at the early stage of growth of transgenic plants #1 and #5 (#5 and #6 had very similar phenotype, so only #5 plant was selected for analysis) (Fig. 3B). No significant phenotypic difference was observed between the transgenic lines and WT plants on the control medium. However, the growth of transgenic and WT plants was inhibited when the medium contained 100 µM CdCl₂, 100 mM NaCl, 2 mM NaHCO₃, or 1 mM H₂O₂. However, the transgenic plants grew better than their WT counterparts. The growth of young leaves of the **SsMT2**-transgenic lines were less affected under the 180 µM CdCl₂, 150 mM NaCl, or 4 mM NaHCO₃ stress compared to the wild type plants. There were no significant differences in the dry weights of the **SsMT2** transgenic lines and WT plants without stresses and 5 mM H₂O₂. However, green leaves in transgenic plant and white leaves in WT plants were observed when grown on the medium with 5 mM H₂O₂. Dry weight (Table 1) of the **SsMT2** transgenic lines was higher than WT plants under other stress conditions. Additionally, there was no significant difference with root length among plants between transgenic and wild type plants under stress (data not shown). These results showed that the **SsMT2** gene expression in Arabidopsis transgenic plants increased metal, salt or oxidant tolerance during early stage of seedling growth.

The effects of CdCl₂, NaCl, NaHCO₃, and H₂O₂ on #1 and #5 transgenic plants were examined during adult stage of plant growth (Fig. 3C). No phenotypic differences were observed between the transgenic and WT plants under normal conditions. After exposing both sets of plants to 100 mM CdCl₂, 400 mM NaCl, 500 mM NaCl, 400 mM NaHCO₃ or 500 mM NaHCO₃, 1.5 M H₂O₂, or 2 M H₂O₂ stress, **SsMT2**-transgenic plants had a significantly higher survival rate than WT plants (Table 2).

**Metal ion uptake in** **SsMT2**-transgenic yeast. **SsMT2**-transgenic yeast accumulated higher amounts of Cd²⁺ (Table 3) and lower amounts Na⁺ (Table 4) than non-transgenic yeast (control) when exposed to 140 µM CdCl₂, 160 µM CdCl₂, 600 mM NaCl, 1 M NaCl, 22 mM NaHCO₃, or 26 mM NaHCO₃ stresses. No significant differences in the amount of Cd²⁺ and Na⁺ accumulation were observed between transgenic and non-transgenic yeast on the YPG (1% yeast extract + 2% peptone + 2% galactose) medium without any stresses.
Figure 3. Seed germination and plants growth of transgenic plants under different stresses. (A) Seed germination on medium supplemented with 0 (CK), 100 µM CdCl₂, 180 µM CdCl₂, 100 mM NaCl, 150 mM NaCl, 2 mM NaHCO₃, 4 mM NaHCO₃, 1 mM H₂O₂ or 5 mM H₂O₂ in the Arabidopsis wild type (WT) and transgenic plants (#1, #5, #6). (B) Relative stress tolerance of WT and SsMT2-overexpressed third generation transgenic Arabidopsis plants (#1 and #5) at the seedling stage. 14-day-old seedlings were grown on medium supplemented each of 0 (CK), 100 µM CdCl₂, 180 µM CdCl₂, 100 mM NaCl, 150 mM NaCl, 2 mM NaHCO₃, 4 mM NaHCO₃, 1 mM H₂O₂ or 5 mM H₂O₂. C. Relative stress tolerance of wild type and SsMT2-overexpressed third generation transgenic Arabidopsis plants (#1 and #5) at the adult stage. 28-day-old plants were grown on soil supplemented each of 0 (CK), 50 mM CdCl₂, 100 mM CdCl₂, 400 mM NaCl, 500 mM NaCl, 400 mM NaHCO₃, 500 mM NaHCO₃, 1.5 M H₂O₂ or 2 M H₂O₂.
Table 1. Dry weigh (mg/10 plants) of Arabidopsis under different stress treatments. Results are presented as means ± SE (n = 3). Low case letters a and b indicate significant differences among mean values within each plant at p ≤ 0.05. CK, control; WT, wild type; #1 and #5 are SsMT2-transgenic plants.

| Plant | CdCl2 | NaCl | NaHCO3 | H2O2 |
|-------|-------|------|--------|------|
|       | 0.01 M | 0.05 M | 0.01 M | 0.05 M |
| WT    | 14.1 ± 0.8 | 7.2 ± 0.5 | 8.7 ± 0.4 | 1.9 ± 0.1 |
| #1    | 14.8 ± 1.0 | 10.6 ± 0.9 | 10.3 ± 1.0 | 5.2 ± 0.4 |
| #5    | 14.4 ± 0.7 | 11.1 ± 1.0 | 11.4 ± 0.9 | 5.4 ± 0.4 |

Table 2. Survived rate under different stress treatments. Results are presented as means ± SE (n = 3). Low case letters a and b indicate significant differences among mean values within each plant at p ≤ 0.05. CK, control; WT, wild type; #1 and #5 are SsMT2-transgenic plants.

| Yeast | CdCl2 | NaCl | NaHCO3 |
|-------|-------|------|--------|
| pYES2 | 31.7 ± 2.3 | 79.76 ± 4.33 | 99.73 ± 8.91 |
| pYES2-SsMT2 | 29.12 ± 2.17 | 119.42 ± 20.2 | 149.71 ± 13.12 |

Table 3. Cd2+ accumulation (µg/g dry weight) in yeast and SsMT-transgenic yeast under CdCl2 stresses treat. Results are presented as means ± SE (n = 3). Low case letters a and b indicate significant differences among mean values within each plant at p ≤ 0.05. pYES2, yeast cell without SsMT2; pYES2-SsMT2, yeast cell containing SsMT2.

| Yeast | Ca2+ | Na2+ | NaHCO3 |
|-------|------|------|--------|
| pYES2 | 0.80 ± 0.72 | 24.43 ± 21.21 | 352.18 ± 21.15 |
| pYES2-SsMT2 | 73.51 ± 7.36 | 199.48 ± 17.44 | 228.12 ± 20.17 |

Table 4. Na+ accumulation (µg/g dry weight) in yeast and SsMT-transgenic yeast under NaCl or NaHCO3 treatment. Results are presented as means ± SE (n = 3). Low case letters a and b indicate significant differences among mean values within each plant at p ≤ 0.05. pYES2, yeast cell without SsMT2; pYES2-SsMT2, yeast cell containing SsMT2.

| Plant | Shoot | Root |
|-------|-------|------|
|       | CdCl2 | CdCl2 |
|       | 0.01 M | 0.05 M | 0.01 M | 0.05 M |
| WT    | 0.25 ± 0.01 | 1.72 ± 0.13 | 0.12 ± 0.01 | 2.83 ± 0.25 |
| #1    | 0.20 ± 0.01 | 2.61 ± 0.21 | 0.17 ± 0.14 | 4.90 ± 0.39 |

Table 5. Cd2+ accumulation (µg/g dry weight) in shoots and roots of wild-type and transgenic Arabidopsis lines in the presence of CdCl2. Results are presented as means ± SE (n = 3). Low case letters a and b indicate significant differences among mean values within each plant at p ≤ 0.05. CK, control; WT, wild type; #1 is SsMT-transgenic plants.

Metal ion uptake in SsMT2-transgenic Arabidopsis plants. Cd2+ and Na+ concentrations in transgenic and WT plants were measured to determine whether or not overexpression of the SsMT2 gene affected the Cd2+ and Na+ accumulation in transgenic Arabidopsis plants. On Murashige and Skoog basal (MS) medium, the concentrations of Cd2+ (Table 5) and Na+ (Table 6) in the shoots and roots did not differ significantly between transgenic and WT seedlings. The concentration of Cd2+ in SsMT2-transgenic and WT plants increased dramatically, with a relatively higher level in roots and shoots of SsMT2-transgenic plants when seedlings were grown on medium containing either 100 µM CdCl2 or 180 µM CdCl2. When exposed either to 100 or 150 mM NaCl, 2 or 4 mM NaHCO3, the Na+ concentrations in the transgenic and WT plants dramatically increased, but with a relatively lower level in both roots and shoots of SsMT2-transgenic lines.
Effects of treatments on the production of H$_2$O$_2$ in plant leaves. Hydrogen peroxide in leaves was detected in situ using 3, 3′-Diaminobenzidine (DAB) histochemical staining method (Fig. 4A). The DAB staining results directly ‘visualized’ the H$_2$O$_2$ content in the plants based on the density of staining. The color of the rosette leaf showed no difference between WT and SsMT2-transgenic plants without heavy metal or salt stresses (Fig. 4A). The accumulation of H$_2$O$_2$ in plants under stress conditions was detected in both transgenic and non-transgenic plants. The color of the WT leaf was darker than that of the leaves of the SsMT2-transgenic line under different stress, which indicated that the H$_2$O$_2$ content in the transgenic line was lower than that of the WT plant after 48 h treatment (Fig. 4B). SsMT2 increased the H$_2$O$_2$ scavenging function of the transgenic plants, indicating that the transgenic plants had better tolerance to oxidative stresses.

### Table 6. Na$^+$ accumulation (mg/g dry weight) in shoots and roots of wild-type and transgenic Arabidopsis lines in the presence of NaCl or NaHCO$_3$. Results are presented as means ± SE (n = 3). Low case letters a and b indicate significant differences among mean values within each plant at p ≤ 0.05. CK, control; WT, wild type; #1 is SsMT2-transgenic plants.

| Organ | Plant | CK | NaCl 100 mM | NaCl 150 mM | NaHCO$_3$ 2 mM | NaHCO$_3$ 4 mM |
|-------|-------|----|-------------|-------------|--------------|---------------|
| shoot | WT    | 0.92 ± 0.01 | 43.10 ± 4.11$^a$ | 55.00 ± 3.11$^b$ | 7.32 ± 0.52$^a$ | 8.81 ± 0.41$^b$ |
|       | #1    | 0.88 ± 0.02 | 32.41 ± 2.51$^a$ | 48.11 ± 2.12$^b$ | 5.20 ± 0.42$^a$ | 7.52 ± 0.31$^b$ |
| root  | WT    | 0.62 ± 0.02 | 14.11 ± 1.10$^b$ | 16.72 ± 1.20$^b$ | 4.21 ± 0.21$^a$ | 6.00 ± 0.52$^b$ |
|       | #1    | 0.58 ± 0.01 | 5.91 ± 0.31$^a$  | 8.31 ± 0.91$^b$  | 2.92 ± 0.10$^a$ | 4.01 ± 0.26$^a$ |

**Figure 4.** 3,3′-Diaminobenzidine (DAB) staining (A) and H$_2$O$_2$ content (B) in leaves in wild type and transgenic Arabidopsis under different stresses. Seedling leaves of WT and transgenic (#1) Arabidopsis plants were grown on medium supplemented with no treatment (CK), 100 µM CdCl$_2$, 180 µM CdCl$_2$, 100 mM NaCl, 150 mM NaCl, 2 mM NaHCO$_3$, 4 mM NaHCO$_3$, 1 mM H$_2$O$_2$ or 5 mM H$_2$O$_2$ for 48 h. H$_2$O$_2$ accumulation in leaves was detected by DAB staining and H$_2$O$_2$ content in leaves in wild type and transgenic Arabidopsis under different stresses was measured with Plant H$_2$O$_2$ Kit. Data are means of three replicates ± SE.
Discussion

The expression of the SsMT2 gene was increased significantly after S. salsa plants were grown under various stresses and indicated that the SsMT2 gene may be involved in adaptation to these stresses. Similar expression patterns of MTs were induced when CdCl₂ was applied 16,19,23, and when salt stresses presented in plants 41,42. The SsMT2 transgenic yeast showed higher tolerance to CdCl₂, NaCl, and NaHCO₃ stress than the non-transgenic yeast in present study. In plants, different MTs often showed different expression patterns in different plant organs. For example, type 2 MTs were preferentially expressed in the leaves 11,38, type 1 MTs were found mainly in roots 39,40. SsMT2 was expressed in most organs of Arabidopsis, including leaves and seeds, and its expression level increased when the S. salsa plants were exposed to the stresses conditions. Increased expression implies the SsMT2 gene transcript may affect plant seed germination and development, which were inhibited under the stressful environments 41,42. MTs had significant impacts on plant growth when the plant suffered various abiotic stresses 40,42. In this study, transgenic Arabidopsis plants had significantly higher seed germination rates and more vigorous seedling growth than non-transgenic plants under high concentrations of metals, salts or hydrogen peroxide. These results indicated that the SsMT2 gene was involved in the transgenic Arabidopsis accommodation of metal, salt and/or oxidant stresses.

SsMT2 transgenic yeast and Arabidopsis plants increased tolerance to CdCl₂ stress. However, Cd²⁺ accumulation in cells was elevated and indicated that the SsMT2 expression and Cd²⁺ accumulation have positive linear correlation. The SsMT2 gene has the same function with the CcmT2b gene, which greatly increased Cd²⁺-tolerance and Cd²⁺ accumulation in E. coli and tobacco 45. Arabidopsis MT1 knock-down lines were hypersensitive to Cd²⁺ and accumulated lower amounts of Cd²⁺ when compared with WT plants 46. Compared with the wild type, transgenic plants of Ziziphus jujuba overexpressing the ZjMT gene and accumulate more Cd²⁺ in the roots 47. However, there are some exceptions; for example, BcMT26 48 and TcMT47 49, transgenic lines did not increase tolerance to Cd²⁺ nor did they increase Cd²⁺ accumulation. In this study, the Cd²⁺ accumulation was higher in the transgenic yeast and Arabidopsis, and more tolerance to Cd²⁺ than WT plants. The SsMT2 protein chelates the Cd²⁺ in the cytoplasm, and thus blocks Cd²⁺ from freely interacting with cytoplasmic components or entering into organelles. Via this mode of action, decreased Cd²⁺ does limited damage transgenic yeast cells and plants, whereas Cd²⁺ damages WT yeast and plants. The full function of MTs to influence Cd²⁺ tolerance and Cd²⁺ accumulation in cells requires further investigation to elucidate its function.

Sodium ion accumulation in SsMT2-overexpressed yeast and plants was significantly lower than that in WT plants under high NaCl or NaHCO₃ environments. There are three mechanisms to prevent excess Na⁺ accumulation in the plant. First, Na⁺ in plant cells may be reduced once Na⁺ influx transporter genes are activated. Second, Na⁺ can be transported and stored in vacuoles. Third, Na⁺ in the cytoplasm can be exported to external medium or the apoplastic via plasma membrane Na⁺/H⁺ antiporters 46. The plant MTs do not contain signal peptides and do not have Na⁺ transportation function. The reason for resulting in lower Na⁺ concentration in SsMT2-transgenic lines and enhancing the tolerance of transgenic organism to salt stress may be that the SsMT2 gene interacted with transporter genes. Overexpression of SsMT2 in transgenic lines induced the transport Na⁺ out of plant. Lower Na⁺ concentration in the SsMT2-transgenic lines probably decreased damage to the plant and increased the tolerance of transgenic yeasts and plants to Na stress.

The exposure of plants to heavy metals and salts can induce ROS to be produced and thus change the balance between ROS production and scavenging 49,50. SsMT2-transgenic lines improved H₂O₂ tolerance in both transgenic yeast and Arabidopsis plants. Compared with WT plants, SsMT2-transgenic Arabidopsis plants produced less H₂O₂. This observation was consistent with the results of MTs in other plant species, such as Arabidopsis T-DNA insertion mutant mt2a 41, E. haichowensis EhMT1 gene 42, Casuarina glauca CgMT1 gene 43, and Gossypium hirsutum GhMt3a 44, the transgenic seedlings of these species had less H₂O₂ than that in control plants under various stresses. The SsMT2 gene is involved in the mediation of H₂O₂ scavenging during the abiotic stress and resulted in much lower level of H₂O₂ accumulated in the transgenic plants. Therefore, the SsMT2 gene plays an important role in reactive oxygen species scavenging under the stresses imposed in this study. The present study also provided evidence that SsMT2 may decrease the impact by induced H₂O₂ and protected plants from damage.

In conclusion, SsMT2 was expressed from seed germination and increased tolerance to stress in transgenic plants. H₂O₂ content in transgenic lines was lower than the control. These results suggest that the role of SsMT2 to influence plant or yeast tolerance to heavy metal and salt stresses may directly bind ion and trigger other genes’ function, or indirectly improve ROS-scavenging ability.

Materials and Methods

Cloning of full-length open reading frame (ORF) region of SsMT2. We have identified some candidate salt-responsive genes in S. salsa using the full-length cDNA over-expressing gene (FOX)-hunting system 54. The SsMT2 gene was one of those genes identified. Seeds of S. salsa plants were collected from an alkaline soil area in Northeast China and germinated on MS medium at 28 °C under 2000 Lux irradiation with a 16 h light/8 h dark photoperiod in an illuminated incubator. Total RNA was isolated from 4-week-old seedlings using RNeasy Plant Micro Kit (Qiagen, Hilden, Germany). cDNA was synthesized from 1 μg of the total RNA with Prime-Script Reverse Transcriptase (Takara, Tokyo, Japan) using an oligo (dT) primer. MT cDNA sequence from FOX-hunting system was obtained and open reading frame (ORF) was found by blasting in the NCBI database. A transcript fragment was amplified by PCR from the cDNA with the forward primer (5'-ATGTTCTGCTGTTGGTGAATGTTG-3') and reverse primer (5'-TCATTGGAAGTWGACTGAGGTGAGT-3'), which were designed from the MT ORF sequence. The PCR product was purified from agarose gel using the DNA Gel Extraction Kit (Generay, Shanghai, China) and cloned into plasmid pMD18-T (Takara, Tokyo, Japan) and sequenced. A new gene was designated as SsMT2 and its ORF nucleotide sequence and protein sequence was deposited to GenBank database (MF447531).
Construction of expression and transformation vectors. The coding region of the *SsMT2* gene was amplified from pMD18T-*SsMT2* plasmid DNA with BamHI sense primer 5′-GGATCCTAGTTCGTTGGATCCGTTAA-3′ (restriction site underlined for all restriction enzymes below) and XhoI antisense primer 5′-GAGCTCTGCACAGTTGACGAGGTT-3′. The PCR amplified fragments were digested with two restriction enzymes BamHI and XhoI and then ligated into the BamHI/XhoI sites of the vector pYES2 (Takara, Tokyo, Japan) to get pYES2-*SsMT2* construct. The plasmid DNA of pYES2-*SsMT2* was transformed into competent yeast strain INVSc1 (*S. cerevisiae*) (Takara, Tokyo, Japan) using the electric impulse method following the manufacturer's instructions (InVitrogen) and the transformants were selected based on their growth on uracil deficient synthetic complete (SC-Ura) solid medium (6.7 g/L Yeast Nitrogen Base, 0.77 g/L-Ura Do supplement, PH = 5.8).

Construction of plant expression and transformation vectors. The coding region of *SsMT2* gene was amplified from pMD18T-*SsMT2* plasmid DNA with the previously described BamHI sense primer and SacI antisense primer 5′-GGAGGTCCTCTTTGAGGTTGACGAGGTT-3′. The PCR fragments were digested with BamHI and SacI and then ligated into the BamHI/SacI site of pBI121 binary vector (Takara, Tokyo, Japan), the plasmid DNAs of pBI121-*SsMT2* was transformed into the *Agrobacterium tumefaciens* strain EHA105 (Takara, Tokyo, Japan) and then *Arabidopsis* (ecotype: Columbia) was transformed using the floral dip method.

Northern blot analysis for the *SsMT2* gene expression in *S. salsa*. To examine the expression pattern of the *SsMT2* gene in different organs of *S. salsa* plant, total RNA was isolated from roots, leaves, shoots, flowers and seeds respectively using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Amounts of 5μg total RNA were fractionated on 1% agarose-formaldehyde gel and transferred onto Hybond N+ membranes (Amersham Pharmacia). Hybridizations were carried out at 50 °C using a DIG-labeled probe in hybridization buffer (7% SDS, 50 M NaHCO3, 0.9 M NaCl, 0.09 M Sodium citrate), which is the PCR production of the *SsMT2* ORF full length sequence amplified with the forward primer (1μl,10μM 5′-ATGCTCTTGCTGGTGTAAGTGTTG-3′) and reverse primer (1μl,10μM 5′-TCATTTGCAGGTGCATGGGT-3′), using 10× PCR digoxigenin (DIG) Labeling Mix (Roche Diagnostics, Switzerland), 0.5μL Ex-tag, 5μl Ex-tag buffer, 35.5μL ddH2O. Hybridization signals were detected with CDP-Star (Tropix) using Biotech Image Master VDS-CL Multi-function Bio-imaging Station.

The *SsMT2* gene expression level in *S. salsa* seedling under different stresses was detected by Northern blot. The seeds of *S. Salsa* were sown onto the MS medium, then the 4-week-old *S. Salsa* seedlings were treated with various stresses (100 μM CdCl2, 180 μM CdCl2, 100 mM NaCl, 150 mM NaCl, 2 mM NaHCO3 or 4 mM NaHCO3, 1 mM H2O2 and 5 mM H2O2) for 48 h. Total RNA was isolated from leaves. Northern blot was conducted as above procedure.

Stress tolerance of the transgenic yeast. The expression of *SsMT2* gene in transgenic yeast was analyzed using Northern blot. Total RNA from yeast was extracted using the RNeasy Yeast Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instruction. Northern blot was conducted as above procedure.

Western blot was used to investigate the *SsMT2* protein amount in yeast. Protein extraction from yeast followed Zhang's protocol. Yeast cells (1.5 mL in YPG) were harvested prior to stationary phase (OD600 = 1.0) by centrifugation. Cells were first pre-treated with 2 M LiAc and then treated with 0.4 M NaOH for 5 min on ice. Finally, cells were centrifuged and yeast whole proteins were extracted with SDS-PAGE sample buffer. Western blot was conducted according to Ohkuni's protocol. Equal volume of samples was lysed in SDS sample buffer. These samples were separated by 12% SDS-PAGE and subsequently transferred the proteins from gel to a polyvinylidene difluoride (PVDF) membrane using a transfer apparatus at 30 V for 90 min. After blocked in PBST (Phosphate Buffered Saline with Tween20) containing 5% skimmed milk for 1 h at room temperature, membrane was incubated with MT antibody (1: 3,000) overnight at 4 °C and wash membrane 3 times for 10 min each time with 1x PBST; then incubated with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulins (1: 5,000; Sigma) at 37 °C for 1 h. Wash membrane 3 times for 10 min each time with 1x PBST. The signals were detected with CDP-Star detection reagent using Biotech Image Master VDS-CL Multifunction Bio-imaging Station.

Cells of transgenic yeast harboring pYES2-*SsMT2* and pYES2 (control) were respectively incubated in YPG medium at 30 °C overnight. The concentration of overnight culture was adjusted to OD600 = 0.5. Culture solutions with serial dilutions (10, 10−1, 10−2, 10−3, and 10−4) were spotted onto YPG agar plates which were supplemented with different concentrations of metals (140 μM CdCl2 or 160 μM CdCl2), salts (600 mM NaCl, 1 M NaCl, 22 mM NaHCO3, or 26 mM NaHCO3), and oxidant (2.8 mM H2O2 or 3.2 mM H2O2), respectively. Photos were taken between the 3rd and 7th day after the stress treatments.

Stress tolerance of the transgenic *Arabidopsis*. The southern hybridization of genomic DNA of transgenic *Arabidopsis* was conducted to investigate the copy number of the *SsMT2* gene in the transgenic lines. Genomic DNA from 2-week-old *Arabidopsis* (wild type, transgenic lines#1, #2, #3, #4, #5 and #6) leaves was isolated using the CTAB method and then digested with HindIII at 37 °C for 60 min. The digested fragments were separated on 1% (w/v) agarose gel and then transferred to the hybrid. Expression of *SsMT2* gene in transgenic *Arabidopsis* was analyzed using Northern blot. The *SsMT2* gene expression level in transgenic *Arabidopsis* lines (#1, #5 and #6) was detected by Northern blot.

The seeds of wild type and the third generation (homozygous) transgenic *Arabidopsis* plants (#1, #5, #6) were surfaced-sterilized with 70% ethanol for 1 min, followed by 1% NaClO solution for 3 min. and then rinsed three times in sterile water. The seeds were sown onto agar plates that contained MS basal medium, 1% (w/v) sucrose, and 0.8% (w/v) agar, supplemented with either filter-sterilized 100 μM CdCl2, 180 μM CdCl2, 100 mM NaCl, 150 mM NaCl, 2 mM NaHCO3, 4 mM NaHCO3, 1 mM H2O2 or 5 mM H2O2. Seeds germinated on MS
medium were used as control, three times repeat. Photos were taken on the 14th day after the stress treatments. Germination rate was calculated when the transgenic and wild type Arabidopsis no longer sprouted.

To find out whether the SsMT2 gene impacts the early seedling development under the different stresses, the seeds of wild type and transgenic Arabidopsis (#1, #5) were germinated on MS medium. The 14-day-old seedlings were transplanted onto MS medium (as a control) and MS medium supplemented with different concentrations of metals (100 μM CdCl₂, 180 μM CdCl₂), salts (100 mM NaCl, 150 mM NaCl, 2 mM NaHCO₃, 4 mM NaHCO₃), or oxidant (1 mM H₂O₂, 5 mM H₂O₂), respectively. The plates were positioned vertically on shelves in order to compare root growth visually. Root length and dry weight were measured after stresses applied 7th and 14th day, three times repeat. Photos were taken between the 7th and 14th day after the stress treatments.

In addition, we examined the stress tolerance at the plant adult stage. Briefly, wild type and transgenic seeds (#1, #5) were grown on MS medium. One-week-old plants were transferred to pots filled with 3:1 mixture of nutrition soil: peat in a chamber (22 °C, 100 M photons·m⁻²·s⁻¹, 60% relative humidity, 16/8 h day-night cycles). The soil-grown plants were watered with 50 mM CdCl₂, 100 mM CdCl₂, 400 mM NaCl, 500 mM NaCl, 400 mM NaHCO₃, 500 mM NaHCO₃, 1.5 M H₂O₂, or 2 M H₂O₂ solution respectively every 4 days for a total of 12 days. The plants survived rate was calculated on the 12th day after treatment and we took photos at the same time.

**Ion uptake in transgenic yeast.** To examine whether the SsMT2 gene involves in the accumulation of metals in yeast cells, the Cd²⁺ or Na⁺ content was measured with the method previously described. In brief, yeast cells cultured in the YPG liquid medium containing 140 μM CdCl₂, 160 μM CdCl₂, 600 mM NaCl, 1 M NaCl, 22 mM NaHCO₃, or 26 mM NaHCO₃ and maintained at 30 °C with shaking at 160 rpm for 12 h. After treatment, 200 mg (dry weight) of cells were collected and analyzed using atomic absorption spectrophotometer (AA800, Perkin Elmer America). Blank sample was used 10 times to calculate the standard deviation, then the measured standard deviation value was put into the regression equation to figure out that Atomic Absorption Spectrometry (AAS) detection limitation for Cd²⁺ was 0.0003 μg/g and Na⁺ was 0.0005 μg/g. The samples were divided into two groups, two samples per group. In each group, one sample was added the standards, another one as a control. Every time the two samples were measured in parallel. The recovery rate was calculated according to the additive amount and the detectable quantity of the ions. The recovery rate for Cd²⁺ in the standard reference material (GB 04-1721-2004 Beijing, China) was 95% and Na⁺ in the standard reference material (GB 04-1738-2004 Beijing, China) was 98%, indicating that this method is accurate.

**Ion uptake in Arabidopsis plants.** Fourteen-day-old WT and transgenic Arabidopsis plants (#1 transgenic plant) were treated without (control) or with each of following solution: 100 μM CdCl₂, 180 μM CdCl₂, 100 mM NaCl, 150 mM NaCl, 2 mM NaHCO₃, or 4 mM NaHCO₃ respectively for 48 h. Roots and shoots were harvested and washed in deionized water. Desorption of shoot and root was performed with 1 mM MES-Tris (pH 6.0) containing 0.5 mM CaCl₂. The samples were dried at 80 °C for 2 days for dry weight measurement. The dried plant materials were digested in a 5 mL mixture of HNO₃ and HClO₄ (87:13, v/v) overnight at room temperature, diluted with 5 mL of 2.5% HNO₃, and then measured for ion contents by an atomic absorption spectrophotometer.

**Reaction to H₂O₂ stress in transgenic Arabidopsis plants.** Fourteen-day-old WT and transgenic Arabidopsis plants (#1) were treated without (control) or with each of 100 μM CdCl₂, 180 μM CdCl₂, 100 mM NaCl, 150 mM NaCl, 2 mM NaHCO₃, or 4 mM NaHCO₃, respectively for 48 h. H₂O₂ accumulation in plant leaves was visualized by histochemical staining with 3, 3′-Diaminobenzidine (DAB). DAB accumulation in plant leaves was visualized by histochemical staining with 3, 3′-Diaminobenzidine (DAB). DAB solution, vacuum-infiltrated for 10 min, and then incubated at room temperature for 12 h in the absence of light until the appearance of blown spots. The stain solution was poured off and the chlorophyll was removed by incubating the samples in absolute ethanol overnight. Staining of the rosette leaf was photographed with a microscope (Olympus). The H₂O₂ content was also measured using Plant H₂O₂ ELISA Kit (America Rapid Bio).

**Statistical analysis.** All treatments were arranged in a randomized complete block design with three replicates and subjected to analysis of variance. The differences among the mean values of different treatments were compared using Duncan’s Multiple Range tests at significant difference level of P ≤ 0.05 using SPSS (Statistical Product and Service Solutions) for Windows version 11.5.

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Author Contributions
J.S.M. and L.S.K. Conceived and designed the experiments; J.S.M., X.C., L.G.L., S.D. and L.Y. Performed the experiments; W.X.W. Analyzed the data; L.S.K. Contributed reagents/materials/analysis tools; J.S.M., X.C. and W.X.W. wrote the manuscript.

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