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Cloning and characterization of gene encoding a Mn-containing superoxide dismutase in *Eutrema halophilum*

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Abstract

A gene encoding Mn-containing superoxide dismutase (Mn-SOD), designated as *ThMSD*, was cloned from salt cress (*Eutrema halophilum*) by reverse transcriptase - polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE). The full length of *ThMSD* (acc. No. EF413171) is 1 047 bp with an open reading frame (ORF) of 693 bp. The deduced 231-amino acid polypeptide had a predicted molecular mass of 25.5 kDa, an estimated pI of 9.08, and a putative Mn-binding site. Recombinant ThMSD protein was expressed in *Escherichia coli* and characterized. The SOD activity of ThMSD was inactivated by sodium azide but not by potassium cyanide or hydrogen peroxide confirming that ThMSD is a Mn-SOD. Real-time PCR revealed that *ThMSD* was expressed in roots, rosette leaves, stems, stem leaves, flowers, and siliques. *ThMSD* mRNA reached the highest content in roots and its content increased when plants were treated with NaCl (in a concentration dependent manner), ABA, and subjected to drought. *ThMSD* was transformed into *Arabidopsis* and the stress tolerance properties of transgenic lines were assayed. The seeds of transgenic lines exhibited significantly higher germination rate under 100 and 150 mM NaCl than the wild type. The root growth of transgenic lines was affected less obviously than the wild type under 100 mM NaCl. The above results indicate that ThMSD played an important role in *E. halophilum* tolerance to environmental stresses, especially NaCl stress.

Additional key words: gene expression, NaCl, RACE, RT-PCR, salt cress, *ThMSD*, transgenic plants.

Introduction

Salt cress, a close relative of *Arabidopsis*, is able to withstand high salinity, low temperature, and drought (Zhu 2001, Inan *et al*. 2004, Taji *et al*. 2004,). Research on the mechanism of salt stress tolerance causes extensive concern in recent years, including research on specific developmental, physiological, biochemical, and genetic characteristics (Inan *et al*. 2004). Plants accumulate excess reactive oxygen species (ROS) when exposed to stress. ROS cause oxidative stress resulting in cell damage (Halliwell and Gutteridge 1999). Superoxide dismutases (SODs, EC1.15.1.1.) play a vital role in preventing ROS-induced damage to plants (Alscher *et al*. 2002, Juarez *et al*. 2008, Poage *et al*. 2011). SODs are ubiquitously found in plants and differ by the metal cofactor, Cu/Zn, Fe, or Mn (Kliebenstein *et al*. 1998). The plants contain cytosolic Cu/Zn-SOD, mitochondrial Mn-SOD, and chloroplastic Cu/Zn-SOD and/or Fe-SOD (Del Rio *et al*. 2003). *Arabidopsis thaliana* genome encodes eight SOD genes, and *AtMSD1* (At3g10920) encodes a 25 kD protein found in the mitochondrial matrix (Herald *et al*. 2003). Plant mitochondria possess a highly conserved Mn-SOD (Gutteridge and Halliwell 2000), and no other mitochondrial enzyme can compensate for the Mn-SOD activity, but the specific effect of mitochondrial superoxide production on cellular function has not yet been examined.

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Abbreviations: IPTG - isoptopyl-β-D-galactopyranoside; MS - Murashige and Skoog; NBT - nitroblue tetrazolium; NJ - neighbor-joining; ORF - open reading frame; RACE - rapid amplification of cDNA ends; ROS - reactive oxygen species; RT-PCR - reverse transcription polymerase chain reaction; SOD - superoxide dismutase; TEMED - N,N,N′,N′-tetramethylethylenediamine; UTR - untranslated region; WT - wild type plant.
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We wonder to know if Mn-SOD in NaCl tolerant salt cress has some special characteristics in comparison to that from glycophytic plants like *Arabidopsis*. We cloned *ThMSD* from this plant species and demonstrated its function by expression in *E. coli*. The expression pattern of *ThMSD* was investigated in different tissues including roots, stems, leaves, flowers, siliques, and rossete leaves under different stresses. To further clarify the function of *ThMSD*, we overexpressed it in *Arabidopsis* and assayed the stress tolerence properties of the transgenic lines.

Materials and methods

Plants and treatments: Salt cress (*Eutrema halophilum* (Pall.) Al-Shehbaz & Warwick, also named *Thellungiella halophila*, Shandong ecotype) seeds were stratified at 4 °C for 3 weeks. After germination, plants were grown in pots with *Vermiculite* moistened with Hoagland nutrient solution in a growth room at temperature of 22 °C, relative humidity of 80 %, a 12-h photoperiod and irradiance of 400 μmol m^{-2} s^{-1}. Four-week-old plants were used for stress treatments. For a NaCl treatment, the plants were irrigated with a nutrient solution containing 300 mM NaCl. For drought treatments, the pots were either deprived of water until wilting of lower leaves was observed or 20 % (m/v) polyethylene glycol (PEG 6000) was added. ABA treatment consisted of spraying the plants with 100 μM ABA solution. For NaCl treatments with different concentrations, 3-week-old plants grown *in vitro* on Murashige and Skoog (MS) medium were transferred to MS media containing 0, 50, 100, 200, 300, 400, 500, and 600 mM NaCl for 24 h. Samples from rosette leaves of *E. halophilum* grown in the pots and the whole plants grown *in vitro* were collected, immediately frozen in liquid nitrogen, and stored at -80 °C until use.

Isolation of *ThMSD* cDNA and sequence analysis: Total RNA was extracted by *Trizol* reagent (*Invitrogen*, Carlsbad, CA, USA) according the manufacturer's instructions. 5'-RACE-ready-cDNA and 3'-RACE-ready-cDNA were synthesized using *SMART RACE* cDNA amplification kit (*Clontech*, Palo Alto, USA). Two degenerate primers, ThMSDP1 [5′-CAACAATGGCGATTCGTT-3′] and ThMSDP2 [5′-TCA(CG)TTG(TC)(AT)GTTCCTTCT CAT-3′], which were designed to recognize the conserved regions of plant Mn-SOD genes, were used to amplify a *E. halophilum* Mn-SOD gene fragment. Two specific primers were designed to amplify the 5′ end (ThMSDGSP1, 5′-TGGCAGTTGTGTCAACCACAAG CTT-3′) and the 3′ end (ThMSDGSP2, 5′-GGATCA GGCCGTGAACAAGGGA-3′) of *ThMSD*. 5′ RACE and 3′ RACE were done.

The full-length cDNA sequence was subjected to a similarity search in the *NCBI* database using the *BLASTX* algorithm with default parameters. A neighbor-joining (NJ) tree was constructed using the *MEGA4.0* software package and the *CLUSTAL* algorithm in conjunction with the amino acid sequences of known plant Mn-SOD (Tamura *et al*. 2007).

Expression, purification, and Western-blot analysis of the recombinant ThMSD: The entire *ThMSD* open reading frame (ORF) was amplified by PCR using the following primers: forward, 5′-CCATGGAA**ATG** GCGATTCGTTCT-3′ (*Nco*I restriction site underlined and the translation start codon in bold) and reverse, 5′-GTCGACCTTGCATTCCTTCTCAT-3′ (*Sal*I site underlined). The resulting about 700 bp PCR product (*ThMSD*) was digested with *Nco*I and *Sal*I, gel purified, and ligated into the *Nco*I and *Sal*I sites in the pET30a vector. The pET30-*ThMSD* expression vector, which produces a recombinant fusion protein with an N-terminal 49-amino acid S/His-tag and an C-terminal 19-amino acid His-tag, was transformed into the *Escherichia coli* strain BL21 (DE3).

Six independent transformed cell lines BL21(DE3) carrying pET30-*ThMSD* were screened for protein expression using small-scale cultures. A single transformant yielding a high expression was selected for large-scale (200 cm^3) production of recombinant ThMSD. The recombinant protein was purified by *His-Bind* (*Kaiji*, Shanghai, China) according to the instructions. The eluate was examined for the presence of ThMSD using 12.5 % (m/v) sodiumdodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Western blotting was performed as described by Towbin (1979). The membrane was incubated with an anti-His monoclonal antibody (1:4 000 dilution), washed three times, and then incubated with alkaline phosphatase-conjugated goat anti-mouse antibody (1:10 000 dilution). Protein bands were visualized using a *BCIP/NBT* Western blotting substrate kit (*Sigma*, St Louis, MO, USA).

Assays of SOD activity: The *E. coli* or leaf tissues extracts were used. SOD activity in supernatant or in purified proteins was determined by the nitroblue tetrazolium (NBT) reduction assay based on the method of Stewart and Bewley (1980). One unit of enzyme activity was defined as the amount of enzyme required to cause 50 % inhibition in the rate of NBT photoreduction under the conditions of the assay. To identify SOD activity on a native protein gel, the gel was stained *via* the riboflavin-nitroblue tetrazolium method (Stewart and Bewley 1980). The identification of the SOD type was based on differential inhibition of SOD activity in native gels following preincubation of purified protein with KCN (5 and 10 mM), NaN_3 (5 and 10 mM), or H_2O_2 (5 and 10 mM) at 37 °C for 30 min (Rubio *et al*. 2001).

Real-time RT-PCR: RNA was isolated from *E. halophilum* samples using the *Trizol* reagent (*Invitrogen*) according to the standard protocol. Purified RNA was treated with *DNase I* (*Promega*, Carlsbad, CA, USA).

First-strand cDNA was prepared from 1 μg of total RNA with the *M-MLV* reverse transcriptase (*Promega*) and oligo-dT(18) according to the manufacturer's instructions. The cDNA was diluted 50 to 100 times and 0.001 cm^3 aliquot of the total reaction volume (0.02 cm^3) was used as a template in real-time RT-PCR amplification. The real-time PCR was conducted with *SYBR Premix Ex Taq*^{TM} (*Takara*, Dalian, China) and carried out using a *Biorad* system. The reaction procedures were as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s. *Tubulin* (forward primer: 5′-GCCAATCCGGTGCTGGTAACA-3′ and reverse primer: 5′-CATACCAGATCCAGTTCCTCCTCCC-3′) was used as an internal control. Relative fold expression changes were calculated using the relative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Generation of 35S::*ThMSD* transgenic plants and the effect of NaCl on seed germination and root growth: The *ThMSD* ORF was PCR amplified using the following primers: forward, 5′-CCATGGAAA**ATG**GCGATTCGT TCT-3′ (*Nco*I restriction site underlined and the translation start codon *in bold*) and reverse, 5′-GTCGACCTT GCATTCCTTCTCAT-3′ (*Bst*E II site underlined). The PCR product (*ThMSD*) was digested with *Nco*I and *Bst*E II, gel purified, and ligated into the *Nco*I and *Bst*E II sites in the p3301 plant expression vector. In p3301-*ThMSD*, *ThMSD* was cloned in a sense orientation behind the 35S promoter. p3301-*ThMSD* was transferred to *Agrobacterium tumefaciens* GV3101 with a helper plasmid pMP90. Construct 35S::*ThMSD* was introduced into *Arabidopsis thaliana* L. ecotype Columbia (Col 0) with a floral dip method mediated with GV3101.

Homozygous transgenic lines were used for further investigation. To obtain evidence that overexpression of *ThMSD* conferred resistance to salt stress, we tested their ability to germinate and growth under different NaCl concentrations. In the test of germination, approximately 100 seeds from the wild type (WT) and several homozygous transgenic lines were surface sterilized and placed on MS medium containing 0, 100, and 150 mM NaCl. The seeds were stratified at 4 °C for 3 d and then grown under temperature of 22 °C, air humidity of 80 %, a 16-h photoperiod, and irradiance of 400 μmol m^{-2} s^{-1}. Germination was scored after 7 d. All experiments were conducted in triplicate. For root growth measurement, 5-d-old seedlings from vertical MS plates were transferred onto vertical MS plates supplemented with 100 mM NaCl. Each plate contained 10 WT and 10 transgenic seedlings. At least 5 plates were included in each experiment. Increases in root lengths were measured 7 d after transfer in each of the three independent experiments. The significance of differences between WT and transgenic plants was tested by one-way *ANOVA* and by Student's *t*-test.

Results and discussion

To clone the full-length *ThMSD* cDNA, degenerate primers were used to amplify a 700 bp Mn-SOD cDNA fragment from *E. halophilum* seedlings. 5′- and 3′-RACE were used to amplify 700 and 600 bp cDNA fragments, respectively, and the three cDNA fragments were assembled. RT-PCR using a primer pair designed to the ends of the assembled sequence confirmed the presence of the full-length cDNA. The cDNA and deduced amino acid sequences were submitted to *NCBI* GenBank under acc. No. EF413171. Full-length *ThMSD* cDNA is 1 047 bp long and contains a 693 bp ORF, a 111 bp 5′-untranslated region (UTR), a 243 bp 3′-UTR with an AATAAA putative polyadenylation signal, and a poly(A) tail (Fig. 1). The ORF encodes a 231-amino acid polypeptide with a predicted molecular mass of 25.5 kDa and a pI of 9.08. Conserved amino acid residues thought to be responsible for Mn binding (His55, His100, Asp192, and His196) (Soon and Chung 2003). ThMSD had putative mitochondrial targeting sequences with 27 - 30 amino acids in the N-terminal end (Fig. 2).

The deduced amino acid sequence of *E. halophilum* Mn-SOD shows significant sequence identity with Mn-SOD sequences from other plants (Fig. 2). Specifically, this protein exhibits 90 - 93 % identity with those from *Raphanus sativus*, *Brassica napus*, *Brassica juncea*, and *A. thaliana*, and 72 - 78 % identity with those from *Gossypium hirsutum*, *Oryza sativa*, *Zea mays*, and *Triticum aestivum*. Phylogenetic analysis of Mn-SODs indicates that they can be divided into two major groups (Fig. 3). ThMSD is grouped with those from *R. sativus*, *A. thaliana*, *B. napus*, *B. juncea*, and *G. hirsutum*, whereas the other group comprises those from *O. sativa*, *Z. mays*, and *T. aestivum* confirming the general agreement between taxonomic groupings and Mn-SOD evolution (Kliebenstein *et al*. 1998, Soon and Chung 2003). This may be due to the significant evolutionary separation of Mn-SOD between dicotyledons and monocotyledons, similarly as it was found in Cu/Zn-SOD (Kim and Triplett 2008).

To further characterize *ThMSD*, we constructed a pET30a-*ThMSD* His-tagged expression plasmid and tested it for expression in BL21 cells. Total protein lysates of the transformants were analyzed by SDS-PAGE (Fig. 4*A*). Cells transformed with pET30a-*ThMSD* expressed a His-tagged protein of approximately 32 kDa, corresponding well with the predicted 25.5 kDa molecular mass of *E. halophilum* Mn-SOD combined with the 6.5 kDa size of the S/His-tag.

Protein extracts of the transformants were run on a native gel and stained using the riboflavin-nitroblue tetrazolium method. Control cells showed three SOD activity bands (Mn-SOD, Fe-SOD, and the putative hybrid Mn/Fe-SOD) (Moran *et al*. 2003), whereas the extracts from pET30a- *ThMSD* transformed cells showed one additional band (Fig. 4*B*). SOD activity was also quantitated using a NBT reduction assay. Though protein

extracts from cells transformed with an empty vector exhibited high SOD activity (234.8 U mg^{-1}) due to the presence of Mn-SOD, Fe-SOD, and putative hybrid Mn/Fe-SOD (Fig. 4*B* and Moran *et al*. 2003), the extracts from *ThMSD*-expressing cells exhibited much higher SOD activity (642.1 U mg^{-1}). These data confirm that the cloned cDNA encoded a SOD protein.

Recombinant ThMSD protein from a large-scale culture of the *ThMSD*-expressing transformants was recovered in soluble fractions using a Ni^{2+} chelating sepharose fast flow column and then analyzed by SDS-PAGE. The presence of the ThMSD protein was confirmed by the 32-kDa band observed by Western blotting with an anti-His monoclonal antibody. The purified recombinant ThMSD was mixed and dialysed in 50 mM phosphate-buffered saline (PBS; pH 7.5) and activity assayed by NBT reduction was about 2 000 U mg^{-1}, more than three times as high as in the crude extract from BL21 cells with pET30a-*ThMSD*. To determine which metals bind to ThMSD, purified recombinant ThMSD was then run on a native gel to examine SOD activity. The SOD activity was sensitive to NaN_3 but resistant to H_2O_2 and KCN (Fig. 4*C*) indicating specific Mn-SOD activity (Moran *et al*. 2003).

Real-time PCR result shows that *ThMSD* was expressed in roots, stems, leaves, flowers, and siliques of the 7-week-old plants of *E. halophilum*, and it reached the lowest expression in the stems. Among all tissues assayed, the amount of *ThMSD* mRNA was the highest in roots, about 12 times as that in the stems (Fig. 5*A*). In small radish, all the *SOD* genes (*Mn*-, *Fe*- and *Cu/Zn-SOD*) are expressed and *Mn-SOD* even highly in the roots (Soon and Chung 2003). *Mn-SOD* is also highly expressed in the roots of the rubber tree (Miao and Gaynor 1993).

Compared to *ThMSD*, *ThCSD* (*Cu/Zn-SOD*) transcripts from *E. halophilum* are also detected in roots, stems and leaves but the lowest amount in the roots (Xu *et al*. 2009). *ThMSD* was expressed more in the roots than in other tissues and this suggest that *ThMSD* might be one of key SODs in *E. halophilum* that protect roots against salinity since *E. halophilum* is mainly distributed in sea coast areas with high NaCl content. Mn-SOD activity was

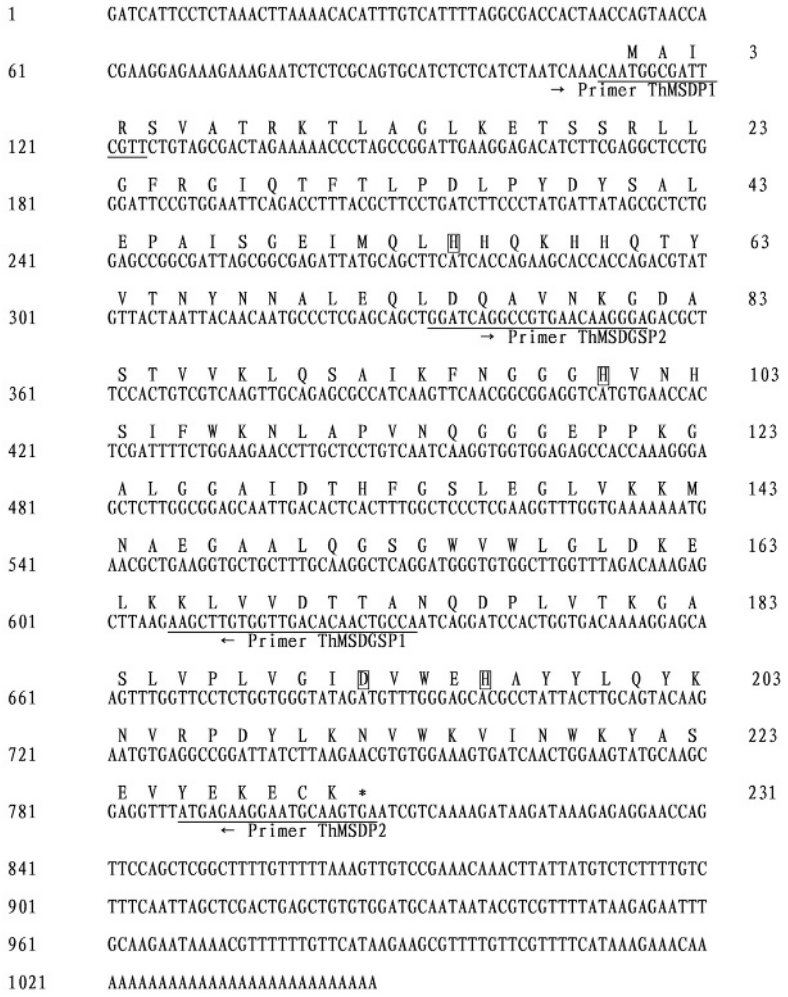

Fig. 1. Nucleotide and deduced amino acid sequences of *E. halophilum* Mn-SOD cDNA. Conserved amino acid residues for Mn binding and active site formation are *boxed*. The positions of the primers used in this study are *underlined*. ThMSDP1 and ThMSDP2 were used to amplify the 700 bp *ThMSD* fragment, and ThMSDGSP1 and ThMSDGSP2 were used for 5′- and 3′-RACE.

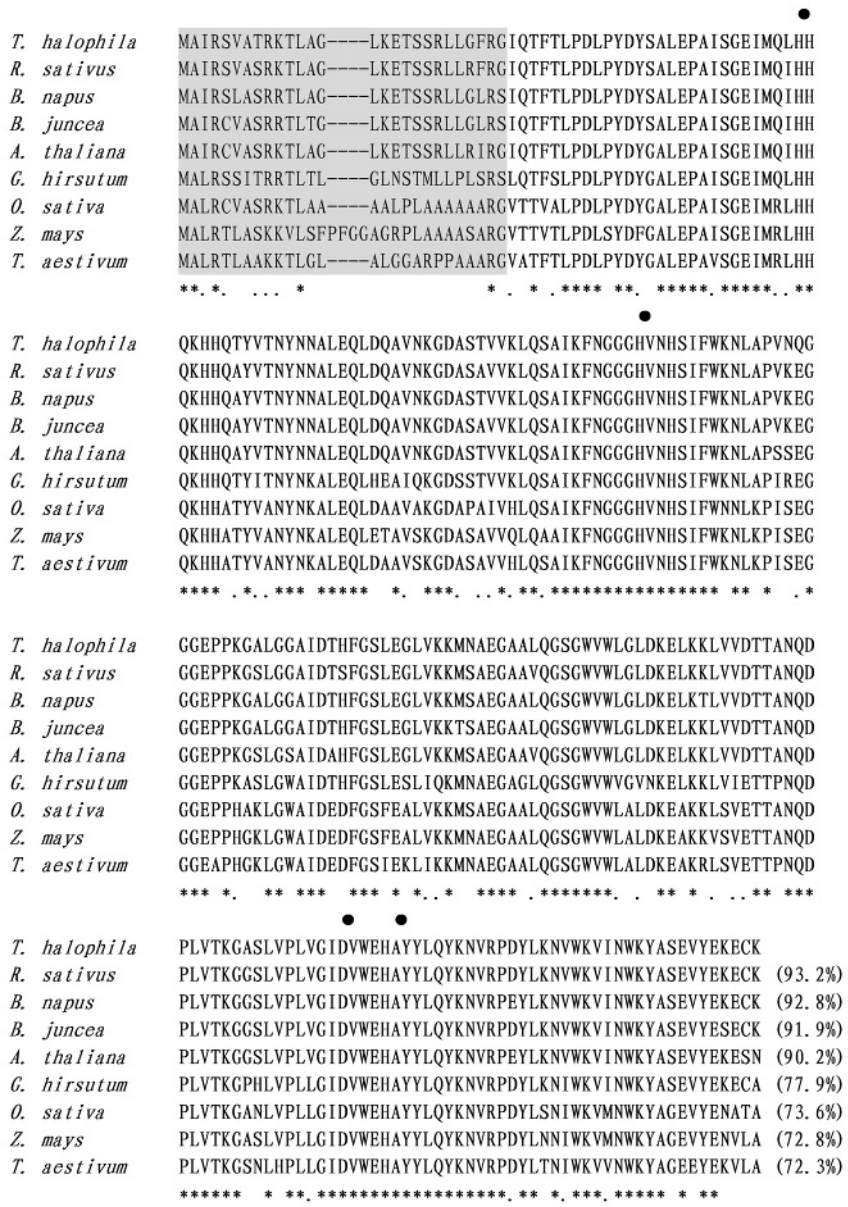

Fig. 2. Alignment of predicted amino acid sequences of ThMSD and Mn-SODs from other plants. GenBank accession numbers are as follows: *R. sativus* (AF263920), *B. napus* (EU487185), *B. juncea* (HQ404362), *A. thaliana* (AY085319), *G. hirsutum* (DQ088820), *O. sativa* (L34039), *Z. mays* (NM_001112272), and *T. aestivum* (U72212). The homology percentage of the deduced amino acid sequence of ThMSD with those of other plants are presented at the end of the sequence comparisons. Shaded boxes are putative mitochondrial targeting sequences. (●) indicates an Mn-binding site. Conserved amino acids and similar residues are marked with *asterisks* and *dots*, respectively.

found to be parallel to the activity of the mitochondrial respiratory electron transport chain (Møller 2001, Camacho *et al*. 2004).

ThMSD expression increased after treatments with NaCl and ABA, or after the drought treatment (including the PEG 6000 and water deficit occurring naturally). *ThMSD* mRNA expression increased by more than 12-fold after NaCl and PEG 6000 treatments, compared to 6-fold and 3-fold ones when water deficit was induced naturally or after the ABA treatment, respectively (Fig. 5*B*). In our test, *ThMSD* could be induced by NaCl, ABA, and drought. Similarly, *Mn-SOD* is induced by NaCl and ABA in small radish (Soon and Chung 2003) and by ABA in maize (Zhu and Scandalios 1994). Although the *ThMSD* mRNA content increased less compared to *ThCSD* (Cu/Zn-SOD) (Xu *et al*. 2009), the similar result has been reported in *Arabidopsis* (Kliebenstein *et al*. 1998). Different expression profiling between Mn-SOD and Cu/Zn-SOD may relate with their different functions.

E. halophilum plants can withstand high NaCl stress but we do not know how the concentration of NaCl affects mRNA expression. We performed the real-time PCR

analysis of 3-week-old plants in MS media with different concentrations of NaCl. Under all NaCl concentrations (50, 100, 200, 300, 400, 500, and 600 mM), the amount of the *ThMSD* transcripts was higher than in the control plants but the highest was at 200 mM NaCl, more than 10 times of that under 600 mM (Fig. 5*C*), the highest NaCl concentration that *E. halophilum* can survive (our unpublished data). We assume that too high concentration of NaCl can hurt plant badly (Gao *et al*. 2003) and thus the *ThMSD* transcripts decrease to a low level.

ThMSD-overexpressing lines M5-2, M6-2, M9-1, and WT of *Arabidopsis* were germinated on MS medium containing 0, 100, and 150 mM NaCl. On the MS without NaCl, all the germination rates were around 93 - 96 %. On

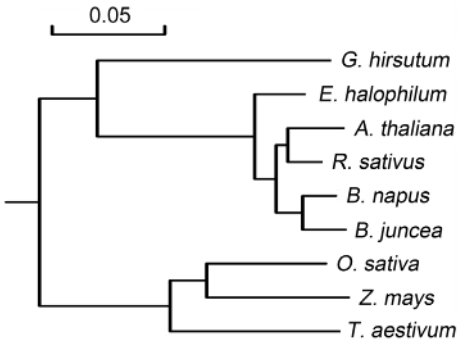

Fig. 3. Phylogenetic tree of plant mitochondrial Mn-SODs. The tree was constructed by neighbor-joining method using *MEGA4.0*. The scale bar of 0.05 is equal to 5 % sequence divergence. The reliability of the tree is measured by bootstrap analysis with 1000 replicates. Aligned sequences and GenBank accession numbers are as in Fig. 2.

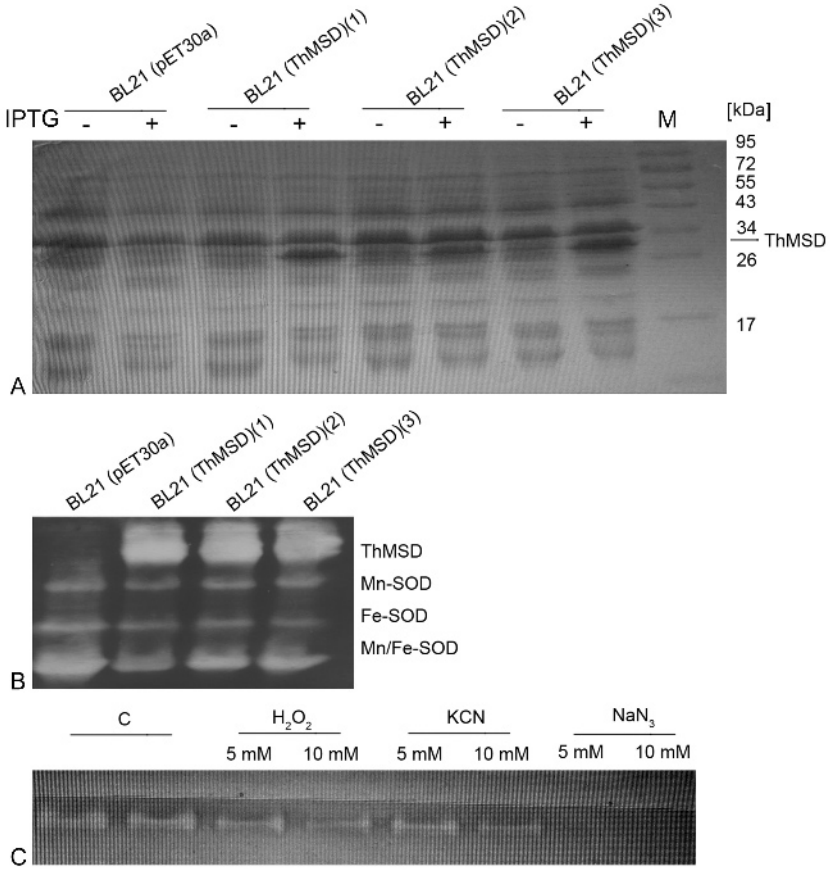

Fig. 4. Expression of *ThMSD* in *E. coli* and its characterization. *A* - SDS-PAGE analysis of ThMSD expressed in *E. coli*. Protein extracted from cells of the transformants 1, 2, and 3 included the recombinant ThMSD protein (32 kDa) induced by IPTG. M - molecular mass markers. *B* - SOD activity of ThMSD was visualized on a native polyacrylamide gel. Protein extracted from cells of the transformants 1, 2, and 3 had four SOD activity bands, whereas control cells had three SOD bands. *C* - Sensitivity of SOD activity of purified recombinant ThMSD to H_2O_2, KCN, and NaN_3. C - purified ThMSD only.

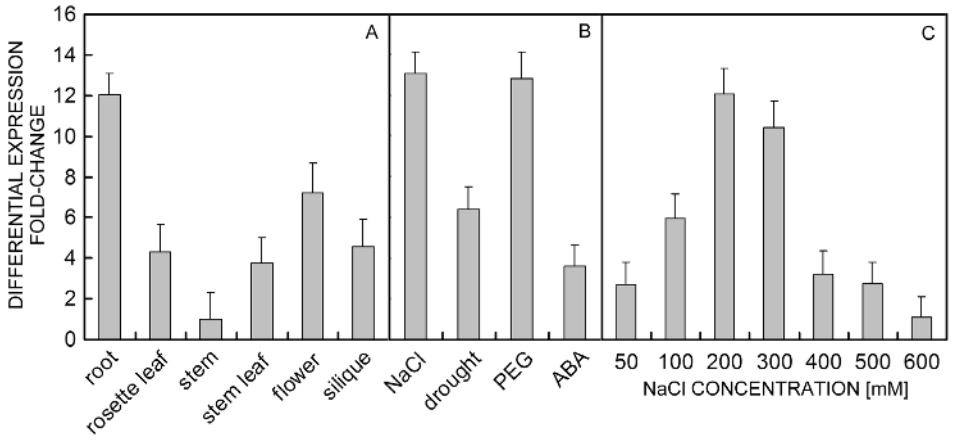

Fig. 5. *ThMSD* transcripts profiling by real-time PCR analysis. Results are presented as differential relative transcript abundance. The data represent means ± SD of three replicates. *A* - Total RNA was prepared from different tissues of 7-week-old plants. Y-axis shows the transcript fold-change to that in stems. *B* - Total RNA was prepared from stress treatments of 4-week-old plants subjected to different stress conditions including 300 mM NaCl for 24 h, 100 μM ABA for 24 h, 20 % PEG 6000 for 1 week, and natural drought. Y-axis shows the transcript fold-change to that in the control. *C* - Total RNA was prepared from NaCl treatments of 3-week-old plants in MS media with 0, 50, 100, 200, 300, 400, 500, and 600 mM NaCl for 24 h. Y-axis shows the transcript fold-change to that in the control.

the medium with 100 mM NaCl, the germination rate of WT was 73.2 %, whereas the germination rates of the lines M5-2, M6-2, and M9-1 were 91.1, 86.3, and 80.0 %, respectively. When NaCl concentration increased to 150 mM, the germination rate of the WT decreased to 41.3 %, but the transgenic lines still had germination rates of 76.0, 67.2, and 68.1 %, respectively (Table 1). This result shows that the overexpresssion of *ThMSD* could alleviate NaCl stress and improve seed germination in *Arabidopsis*.

Table 1. Effect of different concentrations of NaCl [mM] in MS medium on germination of the WT and some transgenic plants determined after 7 d. Means ± SE of three replicates. *, **, and *** - significantly different from WT at $P < 0.05$, 0.01, and 0.001, respectively (Student's *t*-test).

NaCl	WT	M5-2	M6-2	M9-1
0	93.33±3.10	93.33±3.10	96.26±2.10	95.76±1.52
100	73.10±1.10	91.12±3.20***	86.33±3.80**	80.33±5.00*
150	41.32±3.54	76.63±2.62***	67.67±4.31***	68.37±5.23***

To obtain further evidence that overexpression of *ThMSD* conferred resistance to NaCl stress, we measured the root growth of transgenic lines M5-2, M6-2, M9-1, and WT plants under NaCl stress (Table 2). The trangenic lines showed nearly the same root growth as WT under control conditions. However, under the NaCl stress, the transgenic lines grew more rapidly. The relative growth (root length under the stress/root length under the control conditions) of the transgenic lines was higher than that of the WT. These results indicate that overexpression of *ThMSD* resulted in enhanced early seedling tolerance to NaCl stress in *Arabidopsis*.

From real-time PCR or SOD activity test, we found that M5-2, M6-2, and M9-1 possessed about 446, 256, and 362 % higher *ThMSD* transcription compared to *AtMSD1* transcription leading to a 142, 97, and 154 fold higher SOD activity, respectively. These data are in accordance with plant tolerance to the NaCl stress (Tables 1, 2). An increase in Mn-SOD activity would result in lower content of superoxide and other reactive oxygen species and would relieve oxidative stress in the mitochondria (Polle 2001). We conclude that *ThMSD* played an important role in tolerance to the NaCl stress. Gene transformation is a good method to demonstrate involvement of Mn-SOD in stress tolerance (Wang *et al*. 2005, 2010).

Table 2. *ThMSD* overexpression impacts on root length [mm] and relative root growth (RG = root length at 100 mM NaCl/root length at 0 mM NaCl) [%] under NaCl stress. The transgenic lines M5-2, M6-2, M9-1, and the WT were grown on MS plates for 5 d and transferred onto vertical MS plates supplemented with 0 and 100 mM NaCl. The root growth was measured 7 d after transfer. Means ± SE of five replicates. *, **, *** - significantly different from the WT at $P < 0.05$, 0.01, and 0.001, respectively.

NaCl	WT	M5-2	M6-2	M9-1
0	32.4±2.0	33.8±1.8	30.8±1.3	34.4±1.5
100	23.2±0.8	30.0±2.7***	27.8±1.3**	29.4±1.1***
RG	71.6	88.8	90.3	85.5

Furthermore, some reports have shown that mitochondrial ROS production has an important impact upon plant growth and development. Inhibition of root growth was reported in *Arabidopsis* mutants lacking the mitochondrial PrxIIF (Finkemeier *et al*. 2005). Transgenic *Arabidopsis* plants in which expression of *AtMSD1* is suppressed show retarded root growth even under control

growth conditions (Morgan *et al*. 2008). There are two conceivable ways in which Mn-SOD suppression may impinge plant growth: oxidative inhibition of mitochondrial function (Sweetlove *et al*. 2002) or perturbation of redox signaling (Foyer and Noctor 2003). In our study, the transgenic *Arabidopsis* plants had obviously no longer roots in the control conditions but present longer roots under the NaCl stress in comparison with the WT.

In summary, this is the first conclusive report of the presence of a high amount of Mn-SOD in *E. halophilum*. Our results show that *ThMSD* was very important to the high *E. halophilum* tolerance to the extreme NaCl stress. We wonder to know if *ThMSD* has some special characteristics in comparison to respective genes from glycophytic plants like *Arabidopsis*. The answer to this question needs further research. Future studies examining the effect of *ThMSD* knock-out mutant on stress responses or on growth and development will provide important insight into the function of *ThMSD*.

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