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Molecules and Cells

Molecular Cloning and Expression of a Cu/Zn-Containing Superoxide Dismutase from *Thellungiella halophila*

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Superoxide dismutases (SODs) constitute the first line of cellular defense against oxidative stress in plants. SODs generally occur in three different forms with Cu/Zn, Fe, or Mn as prosthetic metals. We cloned the full-length cDNA of the *Thellungiella halophila* Cu/Zn-SOD gene *ThCSD* using degenerate RT-PCR and rapid amplification of cDNA ends (RACE). Sequence analysis indicated that the *ThCSD* gene (GenBank accession number EF405867) had an open reading frame of 456 bp. The deduced 152-amino acid polypeptide had a predicted molecular weight of 15.1 kDa, an estimated pI of 5.4, and a putative Cu/Zn-binding site. Recombinant ThCSD protein was expressed in *Escherichia coli* and assayed for SOD enzymatic activity in a native polyacrylamide gel. The SOD activity of ThCSD was inactivated by potassium cyanide and hydrogen peroxide but not by sodium azide, confirming that ThCSD is a Cu/Zn-SOD. Northern blotting demonstrated that *ThCSD* is expressed in roots, stems, and leaves. *ThCSD* mRNA levels increased by about 30-fold when plants were treated with sodium chloride (NaCl), abscisic acid (ABA), and indole-acetic acid (IAA) and by about 50-fold when treated with UVB light. These results indicate that ThCSD is involved in physiological pathways activated by a variety of environmental conditions.

INTRODUCTION

When exposed to stress, plants accumulate excess reactive oxygen species (ROS), including superoxide (O_2^-), hydroxyl radicals (OH·), and hydrogen peroxide (H_2O_2) (Scandalios, 1997). ROS cause oxidative stress upon reaction with lipids, proteins, and nucleic acids, resulting in mutagenesis and cell death (Cadenas, 1989; Halliwell and Gutteridge, 1999). To minimize the damaging effects of ROS, plants have evolved both non-enzymatic and enzymatic antioxidant defenses. For instance, superoxide dismutases (SODs, EC1.15.1.1.) are enzymes that convert superoxide radicals into molecular oxygen and H_2O_2 (Bowler et al., 1992). SODs are ubiquitous in plant cells and represent the first line of cellular defense against oxidative stress. These enzymes play a vital role in preventing ROS-induced cell damage and death in aerobic organisms (Alscher et al., 2002; Desikan et al., 1996; Juarez et al., 2008).

Eukaryotic SODs are classified according to their metal prosthetic group: Cu/Zn, Fe, or Mn (Fridovich, 1969). Subcellular protein fractionation has shown that plants contain cytosolic Cu/Zn-SOD, mitochondrial Mn-SOD, and chloroplastic Cu/Zn-SOC and/or Fe-SOD (Bowler et al., 1992). Cu/Zn-SOD is a homodimeric enzyme (McCord and Fridovich, 1969) that requires zinc for its structural integrity and enzymatic activity and copper for its disproportion of superoxide (Beem et al., 1974).

Cu/Zn-SOD genes have been cloned in many plant species, including pea (Scioli, 1988), maize (Cannon and Scandalios, 1989; Kernodle and Scandalios, 1996), tomato (Perl-Treves R et al., 1990), sweet potato (Lin et al., 1993), tobacco (Herouart, 1993), rice (Sakamoto et al., 1995), *Arabidopsis* (Kliebenstein et al., 1998), and wheat (Wu et al., 1999). Cu/Zn-SOD is involved in stress responses in many plants. For instance, overexpression of this enzyme in tobacco plants confers increased resistance to oxidative stress (Gupta et al., 1993) and partial resistance to ozone-induced foliar necrosis (Pitcher and Zilinskas, 1996). In potato plants, overexpression of Cu/Zn-SOD increases tolerance against methyl viologen-induced stress (Perl et al., 1993) as well as against oxidative and high temperature stress (Tang et al., 2006). In cucumber leaves, targeting of Cu/Zn-SOD during light-chilling stress, a combination of low light and low temperature conditions, leads to the inactivation of photosystem (PS) I by ROS (Choi et al., 2002).

Salt cress (*Thellungiella halophila*), a close relative of *Arabidopsis*, is able to withstand dramatic salinity shock, low temperature, and drought (Inan, 2004; Taji, 2004; Zhao et al., 1999; Zhu, 2001). In order to determine whether Cu/Zn-SOD plays a role in the defense against oxidative stress in *T. halophila*, we cloned and characterized the *T. halophila* Cu/Zn-SOD gene,

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ThCSD. We demonstrate by recombinant protein expression in *E. coli* and Northern blotting analysis that ThCSD is a bona fide Cu/Zn-SOD that is expressed in *T. halophila* roots, stems, and leaves and is upregulated in response to sodium chloride (NaCl), abscisic acid (ABA), indole-acetic acid (IAA), and UVB light.

MATERIALS AND METHODS

Plant materials and NaCl treatments

Four-week-old *T. halophila* plants (collected from Dongying, Shandong Province, China) were grown at 22°C under 12-h photoperiod illumination. NaCl, ABA, and IAA treatments were performed as described by Yamaguchi-Shinozaki (1994) for 48 h. UVB treatment was performed as described by Lo et al. (2004) for 30 min and 60 min. After treatment, fully expended plant leaves were collected, immediately frozen in liquid nitrogen, and stored at -80°C until use.

Preparation of RNA and reverse transcription

Total RNA was extracted from seedlings of *T. halophila* treated with NaCl and Trizol reagent (Invitrogen, USA) according the manufacturer's instructions. Using total RNA as the template, reverse transcription (RT) was performed with oligo-dT(16), 5′CDS [5′-(T)25VN-3′, N=A, C, G, or T; V=A, G, or C], or 3′CDS [5′-AAGCAGTGGTATCAACGCAGAGTAC(T)30VN-3′, (N=A, C, G, or T; V=A, G, or C)] primers from a RACE kit (Cat. 634914, Clontech) using M-MLV reverse transcriptase (Pro-mega, Madison, WI). The resulting cDNA was used as a template for subsequent PCR, 5′-RACE, and 3′-RACE.

Amplification of *ThCSD* gene fragment

The cDNA reverse transcribed from oligo-dT(16) was used as the template for amplification of a *T. halophila* Cu/Zn-SOD gene (*ThCSD*) with two degenerate primers, ThCSDP1 [5′-CATGTCCATGCTCT(T/C)GGTGA-3′] and ThCSDP2 [5′-TC(A/G)TGGCC(T/A)CCCTTTCC-3′], which were designed to recognize the conserved regions of plant Cu/Zn-SOD genes. PCR cycling conditions were as follows: 94°C for 3 min for initial denaturation; 30 cycles of 94°C for 45 s, 56°C for 1 min, and 72°C for 1 min; and 72°C for 10 min for a final extension. The amplified PCR products were cloned into the pGEM-T Easy Vector and transformed into bacteria (Promega). Recombinant bacteria were identified by blue/white screening and confirmed by PCR. Plasmids containing the insert were purified and used as a template for DNA sequencing by Sangon (China). Nucleotide sequences were analyzed with the Blast search program.

Rapid amplification of cDNA ends PCR (RACE)

Primers were designed to amplify the 5′ end (ThCSDGSP1, 5′-GCAGTTCCATCATCTCCAACAACGATG-3′) and the 3′ end (ThCSDGSP2, 5′-CCGGTCCACATTTTAACCCTGATGG-3′) of *ThCSD*. For 5′ RACE, the Universal Primer Mix (UPM, long primer: 5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3′, short primer: 5′-CTAATACGACTCACTATAGGGC-3′) and ThCSDGSP1 were used to reverse transcribe cDNA using RNA prepared from *T. halophila* seedlings as a template. The PCR reaction was performed with 28 cycles of 94°C for 1 min, 68°C for 1 min, and 72°C for 1 min. For 3′ RACE, the Universal Primer Mix (UPM) and ThCSDGSP2 were used to reverse transcribe cDNA using *T. halophila* seedling RNA as a template. The PCR reaction conditions were the same as for 5′ RACE, except the annealing temperature was 66°C. The 5′ and 3′ RACE PCR fragments with the correct lengths were cloned into the pGEM-T Easy Vector and subsequently sequenced.

Construction of *ThCSD* expression vector

The entire *ThCSD* ORF was PCR amplified using the following primers: forward, 5′-AGAATTC**ATG**GCCAAGGGAGTT-3′ (*Eco*RI restriction site underlined and translation start codon in boldface) and reverse, 5′-AGTCGACTTAGCCCTGAAGACCA-3′ (*Sal*I site underlined). The resulting 546-bp PCR product (*ThCSD*) was digested with *Eco*RI and *Sal*I, gel purified, and ligated into the *Eco*RI and *Sal*I sites in the pET30a vector. The pET30-*ThCSD* expression vector, which produces a recombinant fusion protein with an N-terminal 49-amino acid S/His-tag, was transformed into the *E. coli* strain BL21 (DE3).

Expression and purification of ThCSD

Six independent transformant BL21 cell lines carrying pET30-*ThCSD* were screened for protein expression using small-scale cultures. Transformants were inoculated into 5 ml LB medium (0.5% yeast extract, 1% peptone, 1% NaCl) and incubated at 37°C overnight in a shaking incubator (250 rpm). When the cultures reached an optical density of 0.5 at 600 nm, 1 μmol/L isoptopyl-β-D-galactopyranoside (IPTG) was added to induce expression, and the cells were grown for an additional 3 h. Cells were harvested and suspended in 1× SDS sample buffer (15 mM Tris-HCl, pH 6.8; 6% glycerol; 0.5% SDS; 3.6 mM 2-mercaptoethanol; 0.025% bromophenol blue). After heating and centrifugation, the supernatant was run on a 12.5% SDS polyacrylamide gel to detect the target protein.

A single BL21 transformant yielding a high expression level was selected for large-scale (200 ml) production of recombinant ThCSD. The cells were collected by centrifugation, resuspended in 4 ml binding buffer (5 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.8), and sonicated three times for 30 s. The resulting suspension was centrifuged at 14,000 × *g* for 30 min. The supernatant was then applied to 2 ml His-Bind (Shanghai Kaiji, China), which was then washed with 10 ml binding buffer and 10 ml wash buffer (60 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.8). The protein was eluted with 6 ml of 100 mM imidazole buffer (100 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.8) followed by 6 ml of 1 M imidazole buffer (1 M imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.8). The eluate from this step was examined for the presence of ThCSD using 12.5% SDS polyacrylamide gel electrophoresis (SDS-PAGE).

Western blotting

Proteins were separated by SDS-PAGE according to Laemmli (1970), using a 5% stacking gel and a 15% separating gel. After SDS-PAGE, the proteins were transferred to a nitrocellulose membrane (Sartorius, Germany), and 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, USA), following the manufacturer's instructions.

Enzyme assay and staining for SOD activity

BL21 cells transformed with *pET30-ThCSD* or empty vector were grown in a 50 ml culture. Cells were resuspended in 5 ml phosphate-buffered saline (PBS, pH 7.8) and sonicated as described above. The SOD activity of supernatant proteins was determined by the nitroblue tetrazolium (NBT) reduction assay based on the method of Stewart and Bewley (1980). The assay mixtures (3 ml) contained 50 mmol/L PBS (pH 7.8), 13 μmol/L Met, 75 μmol/L NBT, 10 μmol/L EDTA-Na_2, 2 μmol/L riboflavin,

and 25 μl supernatant sample. The A_{560} was monitored with a spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme required to cause 50% inhibition in the rate of NBT reduction 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). Briefly, proteins were separated in a 15% (w/v) native-PAGE gel and then soaked in a solution of 0.2% nitroblue tetrazolium, 0.028 M N,N,N′,N′-tetramethylethylenediamine (TEMED), and 2.8×10^{-5} M riboflavin in 50 mM PBS (pH 7.8) for 30 min at room temperature. The gels were then illuminated until chromatic zones indicating SOD activity were visible in a uniformly blue background.

Determination of the metallic cofactor of SOD

The identification of the SOD type was based on differential inhibition of SOD activity in native gels (as described above) following preincubation of purified protein with potassium cyanide (KCN, 3 and 6 mM), sodium azide (NaN_3, 5 and 10 mM), or hydrogen peroxide (H_2O_2, 5 and 10 mM) at 37°C for 30 min (Rubio et al., 2001).

Northern blot hybridization

Total RNA samples (10 μg/lane) were denatured and separated in a 15% (v/v) formaldehyde-1.2% (w/v) agarose gel and transferred onto a Zeta-Probe membrane (Bio-Rad, Hercules, CA) in 10× SSC. After rinsing in 2× SSC for 5 min, the membrane was UV cross-linked and air-dried. A 500-bp DNA fragment specific for ThCSD was PCR amplified using the ThCSDPP1 (5′-AACTGGAAACGCAGGCGGC-3′) and ThCSDPP2 (5′-CTTAACTTAAATGCATGGTGCCAC-3′) primers and *T. halophila* genomic DNA as a template. The PCR fragment was purified and used as a Northern blot probe. The probe was labeled with [α-^{32}P]-dCTP (Yahui, China) using a random-primed DNA labeling kit (Promega). Hybridization and washing were performed as described by Church and Gilbert (1984). The hybridization signal was imaged by a phosphorimager (GE Healthcare, USA).

RESULTS AND DISCUSSION

Cloning of full-length *ThCSD* cDNA

Degenerate primers were used to amplify a 250-bp Cu/Zn-SOD cDNA fragment from *T. halophila* seedlings. To obtain full-length cDNA, 5′- and 3′-RACE were used to amplify 320-bp and 500-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 accession number EF405867.

Full-length *ThCSD* cDNA is 733 bp long and contains a 44-bp 5′-untranslated region (UTR), a 456-bp open reading frame (ORF), and a 233-bp 3′-UTR with an AATAAA putative polyadenylation signal and a poly(A) tail (Fig. 1). The ORF encodes a 152-amino acid polypeptide with a predicted molecular mass of 15.1 kDa and a pI of 5.4. Conserved amino acid residues thought to be responsible for copper/zinc binding (His45, His47, His62, His70, His79, His119, and Asp82) (Bannister, 1987; Tainer, 1983) and an arginine residue (Arg142) thought to guide the superoxide anion to the active site (Fridovich, 1986; Bannister, 1987) are present (Figs. 1 and 2). Two cysteine residues (Cys56 and Cys145) that are believed to form a disulfide bond and residues involved in Cu/Zn-SOD dimer formation (Gly36, Leu37, Gly40, His42, Arg78, Gly84, Ile112) are also conserved in this species (Deng et al., 1993).

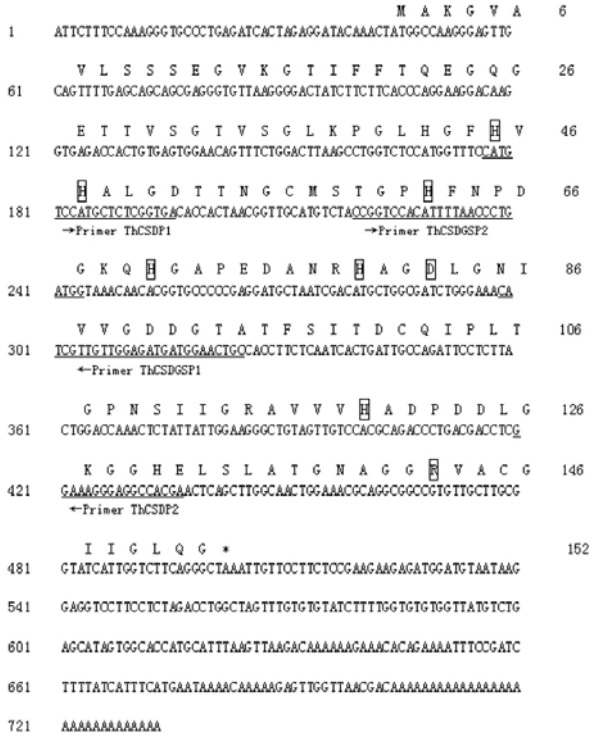

Fig. 1. Nucleotide and deduced amino acid sequences of *T. halophila* Cu/Zn-SOD cDNA. Conserved amino acid residues for Cu/Zn binding and active site formation are boxed. The positions of the primers used in this study are underlined. ThCSDP1 and ThCSDP2 were used to amplify the 250-bp *ThCSD* fragment, and ThCSDGSP1 and ThCSDGSP2 were used for 5′- and 3′-RACE.

The deduced amino acid sequence of *T. halophila* Cu/Zn-SOD shows significant sequence identity with Cu/Zn-SOD sequences from other plants (Fig. 2). Specifically, this protein exhibits 94.7% identity with *A. thaliana* Cu/Zn-SOD (CSD1), 92.1% identity with *Raphanus sativus* Cu/Zn-SOD, and ~85 to 91% identity with Cu/Zn-SOD from *Zea mays*, *Brassica napus*, *Brassica juncea,* and *Mesembryanthemum crystallinum*.

Expression of *ThCSD* cDNA in *E. coli*

To further characterize *ThCSD*, we constructed a pET30a-*ThCSD* His-tagged expression plasmid and tested for expression in BL21 *E. coli* cells. Six independent transformants were cultured, and total protein lysates were analyzed by SDS-PAGE (Fig. 3 showed transformants a, b, and c). Cells transformed with pET30a-*ThCSD* express a His-tagged protein of approximately 21 kDa, corresponding well with the predicted 15.2 kDa molecular weight of *T. halophila* Cu/Zn-SOD combined with the 5.5 kDa size of the S/His-tag. To test for SOD enzymatic activity, protein extracts were run on a native gel and stained using the riboflavin-nitroblue tetrazolium method (Fig. 4). Control cells showed three SOD activity bands (Mn-SOD, Fe-SOD, and Cu/Zn-SOD) (Moran et al., 2003), whereas extracts from pET30a-*ThCSD* transformed cells showed one additional band. SOD activity was also quantitated using a NBT reduction assay. Though protein extracts from cells transformed with an empty vector exhibited high SOD activity (166.7 U/mg) due to the presence of Mn-SOD, Fe-SOD, and Cu/Zn-SOD (Fig. 4; Moran et al., 2003), extracts from pET30a-*ThCSD*-ex-pressing

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T. halophila    MAKGVAVLSSSEGVKGTIFFTQEGQGETTVSGTVSGLKPGLHGFHVHALGDTTNGCMSTG  60
A. thaliana     MAKGVAVLNSSEGVTGTIFFTQEGDGVTTVSGTVSGLKPGLHGFHVHALGDTTNGCMSTG  60
R. sativus      MGKGVAVLNSSEGVKGTIFFTQEGNGSTTVTGTVSGLKPGLHGFHVHALGDTTNGCMSTG  60
Z. mays         MAKGVAVLNSSEGVKGTIFFTQEGDGATTVTGTVSGLKPGPHGFHVHALGDTTNGCMSTG  60
B. napus        MAKGVAVLNSSEGVKGTIFFTQEGDGATTVTGTVSGLKPGPHGFHVHALGDTTNGCMSTG  60
B. juncea       MGKGVRVLNSSEGVKGTIFFTQEGNGTTTVTGTVSGLKPGLHGFHVHALGDTTNGCMSTG  60
M. crystallinum MVKAVVVLSSSEGVSGTVQFTQEGSGPTTVTGNVSGLRPGLHGFHVHALGDTTNGCMSTG  60
                * * * ** ***** **.  ***** * ***.*.****.** ******************

                ●       ●       ●                                       ●
T. halophila    PHFNPDGKQHGAPEDANRHAGDLGNIVVGDDGTATFSITDCQIPLTGPNSIIGRAVVVHA 120
A. thaliana     PHFNPDGKTHGAPEDANRHAGDLGNITVGDDGTATFTITDCQIPLTGPNSIVGRAVVVHA 120
R. sativus      PHFNPDGKTHGAPEDANRHAGDLGNITVGDDGTASFTITDSQIPLDGPNSIVGRAVVVHA 120
Z. mays         PHFNPDGKTHGAPEDANRHAGDLGNIIVGDDGTATFTITDSQIPLTGPNSIVGRAVVVHA 120
B. napus        PHFNPDGKTHGAPEDANRHAGDLGNIIVGDDGTATFTITDSQIPLTGPNSIVGRAVVVHA 120
B. juncea       PHFNPEGKTHGAPEDANRHAGDLGNITVGDDGTATFTITDSQIPLDGPNSIVGRAVVVHA 120
M. crystallinum PHFNPAGKEHGAPEDETRHAGDLGNITVGDDGTATFTIIDSQIPLTGPNSIVGRAVVVHA 120
                ***** ** ****** .********* *******.*.* *.**** *****.********

                                        ◆
T. halophila    DPDDLGKGGHELSLATGNAGGRVACGIIGLQG 152
A. thaliana     DPDDLGKGGHELSLATGNAGGRVACGIIGLQG 152 (94.7%)
R. sativus      DPDDLGKGGHELSLATGNAGGRVACGIIGLQG 152 (92.1%)
Z. mays         ERDDLGKGGHELSLSTGNAGGRVACGIIGLQG 152 (91.4%)
B. napus        ERDDLGKGGHELSLSTGNAGGRVACGIIGLQG 152 (91.4%)
B. juncea       EPDDLGKGGHELSLTTGNAGGRVACGIIGLQG 152 (90.1%)
M. crystallinum DPDDLGRGGHELSKATGNAGGRVACGVIGLQG 152 (84.9%)
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Fig. 2. Alignment of predicted amino acid sequences of ThCSD and Cu/Zn-SODs from other plants. GenBank accession numbers are as follows: *T. halophila* (EF405867), *A. thaliana* (X60935), *B. napus* (AY970822), *Z. mays* (DQ245740), *R. sativus* (AF009735), and *M. crystallinum* (U80069). The percentage homologies of *T. halophila* Cu/Zn-SOD (ThCSD) with Cu/Zn-SODs from other plants are shown at the ends of the sequences. Conserved amino acids and similar residues are marked with asterisks and dots, respectively. (●) indicates amino acid residues known to be responsible for Cu/Zn binding. (◆) indicates the arginine residue (Arg142) that is thought to be necessary to guide the superoxide anion to the active site.

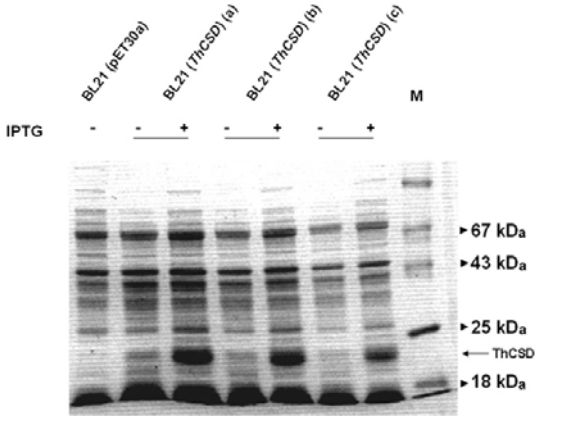

Fig. 3. SDS-PAGE analysis of ThCSD expressed in *E. coli*. Protein extracted from cells of the transformants a, b, and c included the recombinant ThCSD protein (the molecular mass of ThCSD fused to an N-terminal S/His-tag is approximately 21 kDa) induced by IPTG. M, molecular weight markers.

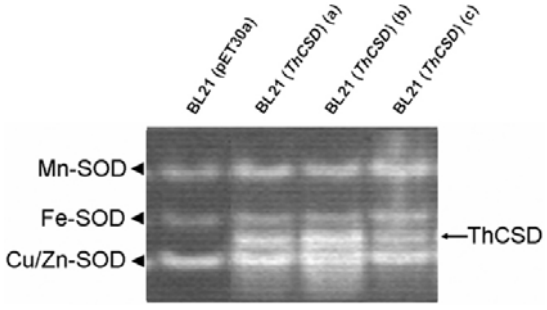

Fig. 4. SOD activity of ThCSD was visualized on a native polyacrylamide gel. Protein extracted from cells of the transformants a, b, and c had four SOD activity bands, compared to control cells showed three SOD activity bands (Mn-SOD, Fe-SOD, and Cu/Zn-SOD).

cells exhibited 3-fold greater SOD activity (487.9 U/mg). These data confirm that the cloned cDNA encodes a bona fide SOD protein.

Determination of the metallic cofactor of SOD

To determine which metals bind to ThCSD, protein extracts from a large-scale culture of pET30a-*ThCSD*-transformed BL21 cells were analyzed by SDS-PAGE followed by Coomassie blue staining. Recombinant ThCSD protein was recovered in soluble fractions using a Ni^{2+} Chelating Sepharose Fast Flow column and then analyzed by SDS-PAGE (Fig. 5A, lanes 1-6). The presence of the ThCSD protein was confirmed by the 21-kDa band observed by western blotting with an anti-His monoclonal antibody (Fig. 5B). Purified recombinant ThCSD (lanes 4 and 5) was then run on a native gel to examine SOD activity. The SOD activity was sensitive to KCN and H_2O_2 but resistant to NaN_3, indicating specific Cu/Zn-SOD activity (Fig. 6).

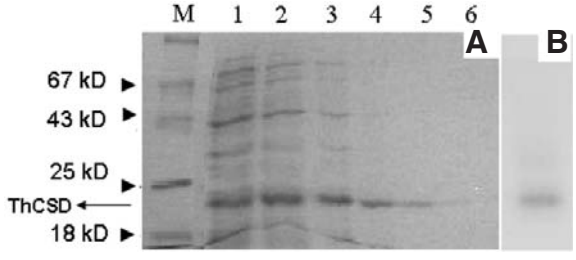

Fig. 5. (A) SDS-PAGE showing the purification of the recombinant ThCSD. Lane 1, total soluble protein from pET30a-*ThCSD* BL21 cells; lanes 2-6, protein eluate from an affinity column along with the time elapse. Lanes 4 and 5 contain recombinant ThCSD protein with more than 90% purity. M, marker proteins. (B) Western blot analysis with an anti-His antibody. The band corresponding to recombinant ThCSD protein is indicated.

Expression of *ThCSD* gene

To examine whether *ThCSD* expression is tissue-specific, we performed northern blot analysis of total RNA prepared from

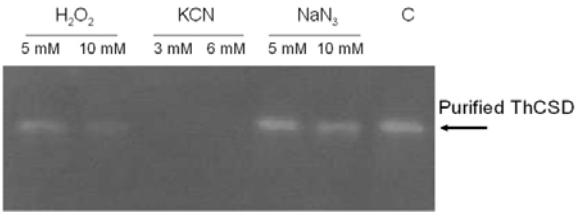

Fig. 6. Sensitivity of SOD activity of purified recombinant ThCSD. C, purified ThCSD only.

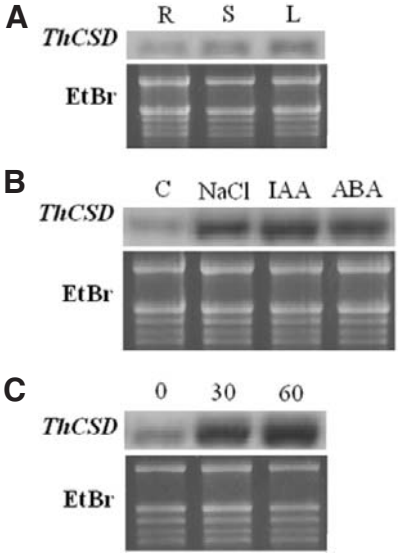

Fig. 7. Northern analysis of *ThCSD* expression. (A) Total RNA was prepared from roots (R), stems (S), and leaves (L). (B) The 4-week-old seedlings were treated with 200 mM NaCl for 48 h or sprayed with 50 μM of ABA or IAA for 48 h. (C) The 4-week-old seedlings were irradiated under UVB light for 30 min and 60 min. An ethidium bromide-stained gel was used to ensure equal loading of samples.

roots, stems, and leaves of 4-week-old plants grown under normal conditions. *ThCSD* is expressed in the roots, stems, and leaves of *T. halophila*, although the mRNA levels were slightly greater in leaves than in roots and stems (Fig. 7A). In *Arabidopsis*, three Cu/Zn-SOD genes, *AtCSD1*, *AtCSD2*, and *AtCSD3*, have been identified (Kliebenstein et al., 1998). Activity of AtCSD1 and AtCSD2 is detected in roots, leaves, stems, and siliques, with AtCSD1 localized to the cytosol and AtCSD2 to chloroplasts. AtCSD3 is thought to be peroxisomal, because its carboxyl terminus contains an Ala-Lys-Leu tripeptide, which is a typical peroxisomal targeting signal (Chu et al., 2005). Since *ThCSD* has the highest homology with *AtCSD1* (74%), the ThCSD protein likely occurs mainly in the cytosol.

To examine whether environmental stress affects *ThCSD* mRNA expression, we performed northern blot analysis of total RNA prepared from the leaves of *T. halophila* plants treated with NaCl, ABA, and IAA. The *ThCSD* mRNA levels increased in all cases by about 30-fold (Fig. 7B). In rice and small radishes, *Cu/Zn-SOD* expression increases progressively in response to phytohormones such as ABA and IAA (Kwon and An, 2003; Sakamoto et al., 1995), but tobacco *Cu/Zn-SOD* levels are not affected by either ABA and IAA (Kurepa et al., 1997). It is not known whether salt cress and other plants differ in their coordination of phytohormone-mediated changes in SOD gene expression with other metabolic processes.

When exposed to UVB radiation, *T. halophila* plants exhibited a dramatic increase in *ThCSD* mRNA levels after only 30 min. In addition, transcript levels increased by about 50-fold within 1 h (Fig. 7C). A similar induction of Cu/Zn-SOD mRNA during light exposure was reported in *N. plumbaginifolia* (Tsang Ed et al., 1991). UVB-induced formation of fatty acid hydroperoxides is likely to occur in all membranes (Lo et al., 2004). The effects of UVB on plants are mediated by a number of signal transduction pathways that produce ROS via NADPH oxidase and peroxidase (Jordan, 2002; Jordan et al., 1998). The loss of membrane function by lipid peroxidation is known to result from UVB damage (Bassman et al., 2001). The upregulation of *ThCSD* in response to UVB raises the possibility that *ThCSD* may be involved in protecting membranes from lipid peroxidation.

In conclusion, our results show that *ThCSD* transcripts are upregulated in plants following treatment with NaCl, ABA, IAA, and UV light. Future studies examining the effect of *ThCSD* overexpression or knock-down on stress responses will provide important insight into the anti-stress mechanisms in *T. halophila*.

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