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Molecular characterization of *PeSOS1*: the putative Na^+/H^+ antiporter of *Populus euphratica*

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Abstract *Populus euphratica* is a salt-tolerant tree species growing in semi-arid saline areas. A Na^+/H^+ antiporter gene was successfully isolated from this species through RACE cloning, and named *PeSOS1*. The isolated cDNA was 3665 bp long and contained a 3438 bp open reading frame that was predicted to encode a 127-kDa protein with 12 hypothetical transmembrane domains in the *N*-terminal part and a long hydrophilic cytoplasmic tail in the *C*-terminal part. The amino acid sequence of this *PeSOS1* gene showed 64% identity with the previously isolated *SOS1* gene from the glycophyte *Arabidopsis thaliana*. The level of protein expressed by *PeSOS1* in the leaves of *P. euphratica* was significantly up-regulated in the presence of high (200 mM) concentrations of NaCl, while the mRNA level in the leaves remained relatively constant. Immunoanalysis suggested that the protein encoded by *PeSOS1* is localized in the plasma membrane. Expression of *PeSOS1* partially suppressed the salt sensitive phenotypes of the EP432 bacterial strain, which lacks the activity of the two Na^+/H^+ antiporters *EcNhaA* and *EcNhaB*. These results suggest that *PeSOS1* may play an essential role in the salt tolerance of *P. euphratica* and may be useful for improving salt tolerance in other tree species.

Keywords *E. coli* mutant complementation · Na^+/H^+ antiporter · *Populus euphratica* · Salt tolerance

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Introduction

Excess salt (NaCl) disturbs intracellular ion homeostasis in plants, leading to membrane dysfunction, attenuation of metabolic activity, and secondary effects that cause growth inhibition and, ultimately, cell death (Hasegawa et al. 2000). In vitro studies have shown that 100 mM Na^+ may inhibit protein synthesis by competing with K^+ at carrier protein binding sites (Blumwald et al. 2000). Under salt stress, plants maintain high concentrations of K^+ and low concentrations of Na^+ in the cytosol by regulating the expression and activity of K^+ and Na^+ transporters, and of H^+ pumps that generate the proton motive force required for transport of various species across their membranes for transport (Zhu et al. 1993; Nass et al. 1997; Zhu 2002). Although Na^+-ATPase activity has been detected in some algae, there is no evidence that such an activity exists in cells of higher plants (Blumwald et al. 2000; Benito et al. 2002). At the molecular level, one of the most remarkable features of plant cells, compared to animal and fungal cells, is the absence of a Na^+ ATPase (Garciadeblas et al. 2003; Benito et al. 2002). Maintenance of low cytosolic Na^+ contents appears to be impossible without an efficacious Na^+ efflux system (Haro et al. 2005), thus the absence of such an enzyme is widely believed to prevent plant cells from maintaining low Na^+ and high K^+ cytosolic concen-

trations under salt stress (Banuelos et al. 2002; Haro et al. 2005). However, although Na^+ ATPases perform these important functions in animal and fungal cells (Benito et al. 2002; Benito Rodriguez-Navarro 2003), it is possible that *AtSOS1* or another Na^+/H^+ antiporter that has not yet been identified is responsible for Na^+ effluxes in higher plants (Shi et al. 2000; Qiu et al. 2003). Both physiological and biochemical data support the hypothesis that Na^+/H^+ antiporters in the plasma membrane mediate Na^+ effluxes (Xiong et al. 2002; Qiu et al. 2002, 2003), and it is assumed that the proton motive force created by P-type H^+-ATPases drives Na^+ effluxes from plant cells through Na^+/H^+ antiporters in their plasma membranes (Schachtman and Liu 1999).

Molecular analyses of physiological and metabolic adaptations at the cellular level have led to the identification of a large number of genes that are induced by salt stress (Ingram and Bartels 1996; Bray 1997; Shinozaki et al. 1998; Zhang and Blumwald 2001; Hamada et al. 2001). The gene *AtSOS1*, which encodes a plasma membrane Na^+/H^+ antiporter, homologous to bacterial and yeast Na^+/H^+ antiporters, has been identified in the glycophyte *Arabidopsis thaliana* (Shi et al. 2000, 2002; Qiu et al. 2002), but homologues have rarely been identified and functionally characterized in other plants. *Arabidopsis sos1* mutants have been isolated by genetic screening of plants that are hypersensitive to NaCl, and mutations in the *AtSOS1* gene rendered the mutant plants very sensitive to Na^+ (Wu et al. 1996). AtSOS1 encodes a plasma membrane-localized Na^+/H^+ antiporter that is preferentially expressed in epidermal cells at the root tip, and hence assumed to be involved in the control of net Na^+ fluxes across the plasma membrane (Shi et al. 2000, 2002) Overexpression of *AtSOS1* greatly improved the salt tolerance of *A. thaliana* by limiting Na^+ accumulation in its cells (Shi et al. 2003). Similar results were obtained when the plasma membrane Na^+/H^+ antiporters, SOD2 from *Schizosaccharomyces pombe* and nhaA from *Escherichia coli*, were overexpressed in *Arabidopsis* (Gao et al. 2003) and rice (Wu et al. 2005), respectively. Apse et al. (1999) further demonstrated that the overexpression of *AtNHX1* in transgenic *A. thaliana* both increased its salt tolerance and stimulated Na^+/H^+ antiporter activity, suggesting that this overexpression may have activated additional salt tolerance mechanisms.

Populus euphratica is a poplar species noted for its high salinity tolerance (Wang et al. 2003). It is mainly distributed in deserts with extremely hot and dry summers, possibly due to its phreatophytic habit. However, other species of this genus are known to be drought sensitive (Wang et al. 1996; Vinocur and Altman 2005). The physiological salt-resistant traits of *P. euphratica* have recently been investigated. For example, in vitro experiments have revealed that this species can tolerate up to 450 mM NaCl (Gu et al. 2004a). In addition, comparative studies suggest that this species is more salt-resistant than any other poplar cultivars in terms of growth, photosynthesis and plant mortality (Ma et al. 1997; Chen et al. 2003; Hukin et al. 2005). To date, molecular studies of salt resistance in plants have centered on herbaceous species (Bohnert and Cushman 2000; Inan et al. 2004), however, because of their long lifespan trees may have evolved adaptive strategies differing from those of annual plants, to survive long periods of stress (Ottow et al. 2005). Thus, there is interest in studying salt resistance in trees, and *P. euphratica* appears to be one of the most promising model systems for such studies (Chen et al. 2003; Gu et al. 2004b; Zhang et al. 2004).

In order to investigate the molecular mechanisms of stress tolerance in *P. euphratica*, we have isolated a Na^+/H^+ antiporter gene through rapid amplification of cDNA ends (RACE) cloning. This gene is predicted to encode a transmembrane protein, similar to plasma membrane Na^+/H^+ antiporters from *A. thaliana.* The functional expression of the recovered gene, which encodes a homolog of the *AtSOS1* antiporter, was further analyzed by mutation complementation in bacteria.

Materials and methods

Plant material and growth conditions

Populus euphratica plants (two years old) were collected from Neimeng Ejinaqi (42°01′N, 101°14′E) in China and grown in a greenhouse with a light:dark regime of 16:8 h (approximately 250 μmol m^{-2} s^{-2} Photosynthetically Active Radiation, PAR, in the light cycles) at 22°C. To study the expression pattern of *PeSOS1* under salt stress, these plants (grown in soil) were transferred to hydroponic culture (1/4 MS) without NaCl. After 12 days of cultivation, the concentrations of NaCl were increased stepwise by 50 mM every four days to a maximum of 200 mM NaCl.

Cloning of *PeSOS1*

Degenerate primers were designed for cloning the Na^+/H^+ antiporter from *P. euphratica* mRNA based on an alignment of amino acid sequences of yeast, microbes, moss and plant Na^+/H^+ antiporters (Fig. 1). The two corresponding degenerate primers used were: P1: 5′-GG(ATCG)GA(AG)(AT)(GC)(ATCG)(CT)T(ATCG)ATGAA(CT)GA(CT)GG -3′ and P2: 5′-CAT(CT)TCCCA(AG)AA(AG)TG(AG)TG -3′.

RNA was extracted from the leaves of *P. euphratica* using the CTAB reagent method (Chang et al. 1993). One

SoD2	172	AESGCNDG----------W----	292
NhaP	166	GESL F N DG----------DKFWE L	322
PpSOS1	182	GESLMNDG-----GESAQSM----HHFWEM	304
OsSOS1	170	GESLMNDG----------QSL----HHFWEM	292
CnSOS1	194	GESLMNDG-------E-------SL----HHFWEM	316
AtSOS1	195	GESLMNDG----------HHFWEM	317

Fig. 1 Consensus amino acid sequences of Na^+/H^+ antiporters used for primer design. Amino acid sequences of Na^+/H^+ antiporters of SOD2 (*Schizosaccharomyces pombe*, CAA77796), NhaP (*Pseudomonas aeruginosa*, BAA31695), PpSOS1 (*Physcomitrella patens*, CAD91921), OsSOS1 (*Oryza sativa*, AY785147), CnSOS1 (*Cymodocea nodosa*, CAD20320) and AtSOS1 (*Arabidopsis thaliana*, AAF76139) are aligned. Two regions separated by suitable lengths are shown with the corresponding numbers for each of the initial methionine residues. Shaded amino acid sequences represent those used for the mixed synthetic primers

μg of total RNA was used in RT-PCR reactions and 2-step cDNA synthesis was carried out at 42°C for 30 min using Oligo d $(T)_{15}$ primers. The two degenerate primers were then added for PCR amplification using the following temperature cycling conditions: five cycles of 95°C for 30 s, 37°C for 30 s, 72°C for 30 s, 40 cycles of 95°C for 30 s, 42°C for 30 s, 72°C for 30 s, and an additional polymerization step of 72°C for 10 min. The amplified fragments were purified from agarose gels and ligated into the pMD18-T Vector (Takara, Japan) and transformed into JM109 chemically competent *Escherichia coli.* On the basis of the sequences of this RT-PCR fragment, two additional primers were designed to amplify the full-length cDNA by rapid amplification of cDNA ends (RACE) reactions.

For the 5′-RACE reactions, the first strand of cDNA was synthesized using a gene-specific primer and AMV reverse transcriptase (Takara, Japan). After the first strand of cDNA synthesis, the original mRNA template was removed using RNase H, and a homopolymeric tail was then added to the 5′-end of the cDNA. PCR amplification was performed using a nested, gene-specific primer and a deoxyinosine-containing dG-anchor primer P3 (5′-GGCCACGCGTC GACTAGTAC$(G)_{16}$ -3′).

For 3́-RACE reactions, the first strand of cDNA synthesized was initiated at the poly (A) tail of the mRNA using a dT adapter primer P4 (5′-CTGATCTAGAGGTACCGGATCC$(T)_{16}$-3′). Following the first strand of cDNA synthesis, PCR amplification was performed using a gene specific primer that annealed to a site located within the cDNA molecule. A universal amplification primer (P5: 5′-CTGATCTAGAGGTACCGGATCC-3′) was used to target the complementary 3′-end of the mRNA. The 5′- and 3′-RACE products were cloned into a pMD18-T cloning vector. The cloned products of the original PCR and the 5′- and 3′-RACE reactions were sequenced, and contiguous sequences were assembled to obtain the full-length of the *PeSOS1* cDNA. After determining the open reading frame, a fragment containing this reading frame was re-obtained by PCR from the *P. euphratica* leaves' cDNA using primers P6 (5′-GTCGACATGGGGAGCGCGATAGAAAAAGGAGTAGG-3′) and P7 (5′-GAGCTCCTAA GAAGCATGATG GAACGACAGCGTAC -3′).

Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the leaves of poplar plants subjected to salt stress. Reverse transcription was carried out in 25 μl reaction mixtures containing 2 μg of total RNA as a template, 1 μl of 10 μM Oligo $(dT)_{15}$ primer, 5 μl of 5 × reverse transcription buffer, 2.5 μl of 10 mM dNTPs, 30 U of RNase inhibitor and 30 U of AMV reverse transcriptase (Promega, USA). In the control reactions, reverse transcriptase was omitted. Semi-quantitative RT-PCR was performed with the primer pair P8 (5′-TTGT GGTCTATCAGCTATTCTATCGG -3′) and P9 (5′-CCA TCAC CCTTGAAAGCAGTCCT -3′), flanking 306 bp sequence of *PeSOS1* from TM6-TM8 (Figs. 2, 3). RT-PCR amplification of the house-keeping *Actin* gene, was performed using the specific primers P10 (5′-CCTCCAA TCCAGACACTGTA-3′) and P11 (5′-AACTGGGATGA TATGG AGAA -3′) under the same conditions as those for the *PeSOS1* reaction.

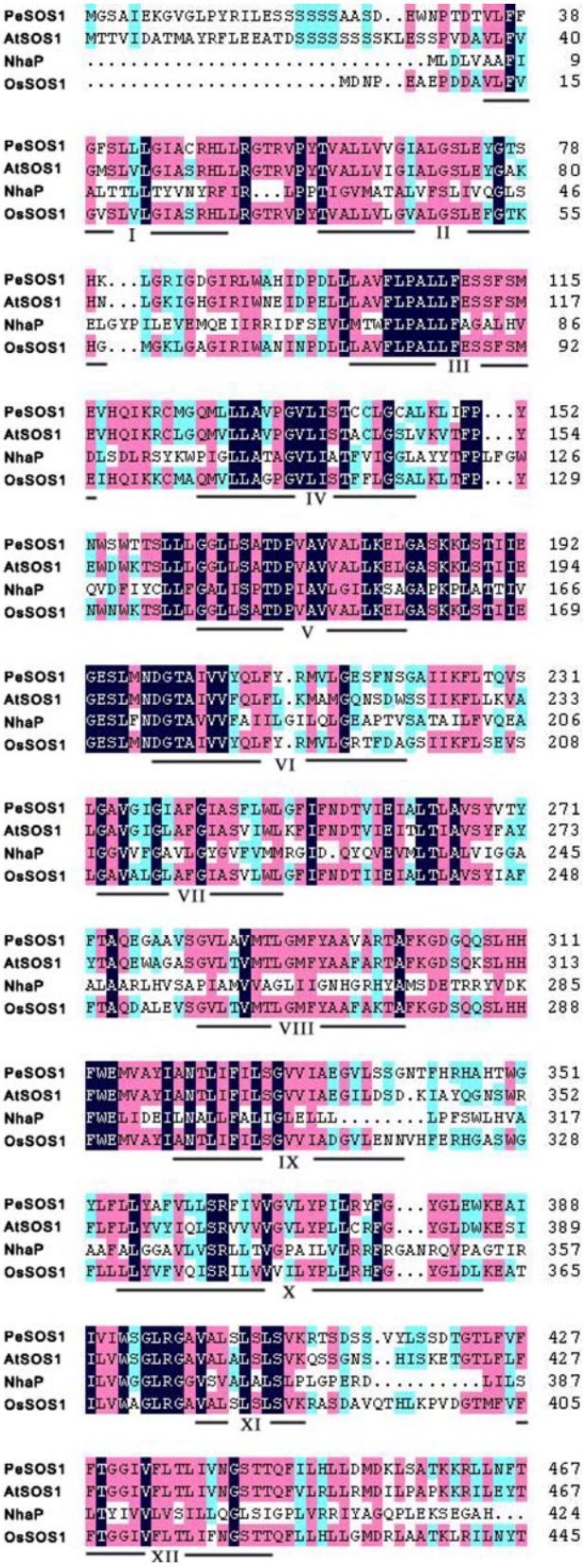
Preparation of *PeSOS1* antibodies and Western blotting analysis

Anti-*PeSOS1* rabbit polyclonal antibodies were raised against the COOH-terminal peptide containing 170 amino acids of *PeSOS1* (Fig. 2A). The EcoRI and HindIII fragments of the PCR product amplified from the cDNA template using the PCR primers P12 (5′-CTGAGAATTC GCGGATGGTGCTTTGAGGAGAGGA-3′) and P13 (5′-CTGGAAGCTT CTAAGAAGCATGAT GGAAC GACAGC -3′) were ligated into the same sites of the pET28a vector which carry an *N*-terminal His.Tag plus an optional *C*-terminal His.Tag sequence. These fusion proteins were produced in *E. coli* (strain BL21pLysS) that had been transformed with pET28a, into which the region encoding the final 170 amino acids of *PeSOS1* had been subcloned. Induction and purification of the recombinant His.Tag-fusion was performed according to the manufacturer's instructions (Pharmacia). Polyclonal antibodies were raised in rabbits with purified antigen.

The leaves were ground with liquid N_2 and then resuspended in extraction buffer (50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 320 mM sucrose, 1 mM DTT, 1 μM pepstatin and 1 mM PMSF). After centrifuging at 10 000 *g* for 30 min at 4°C, the supernatant was collected for analysis. Membrane-enriched vesicles were prepared as described by

Fig. 2 *PeSOS1* is predicted to encode a transmembrane protein. (**A**) The deduced amino acid sequence of *PeSOS1*. The 12 putative transmembrane domains (TM) are underlined. The region of the COOH-terminal peptide containing the 170 amino acids sequence used to raise polyclonal antibodies is shaded. B: Hydrophilic plot of *PeSOS1*. Hydrophobicity values were calculated using the program TMPRED available at http://www.ch.embnet.org/software/ TMPRED form.html

Yang et al. (2003, 2004). All steps were carried out at 4°C. The leaves were ground with liquid N_2 and immediately homogenized in isolation medium (1:5 w/v) containing 250 mM sucrose, 25 mM Hepes-Tris, pH 7.6, 1 mM EDTA, 1.5% PVP, 1 mM PMSF. The homogenate was filtered through four layers of cheesecloth and centrifuged at 15,000*g* for 30 min. The supernatant was then centrifuged for 30 min at 80,000*g*, crude membrane microsomes were collected and re-suspended in a buffer containing 250 mM sucrose, 1 mM DTT, 1 mM EDTA, 2 mM Hepes-



◀**Fig. 3** *PeSOS1* is similar to Na^+/H^+ antiporters. Alignment of *PeSOS1* (DQ517530) with Na^+/H^+ antiporters AtSOS1 from *Arabidopsis thaliana* (AF256224); NhaP from *Pseudomonas aeruginosa* (BAA31695) and OsSOS1 from *Oryza sativa* (AY785147). The sequences were aligned using the program DNAman. Identical amino acids are highlighted in black, while conservative substitutions are highlighted in red

Tris, pH 7.2, then layered onto a discontinuous sucrose gradient consisting of 32/46 % (w/w) sucrose and centrifuged at 100,000*g* for 80 min. The toloplast fraction was collected from the top of 32% layer of the gradient; The PM-enriched membrane fraction was collected from the 46% sucrose interface, diluted and centrifuged, the pellet was collected. The PM fraction was isolated by adding the microsomal suspension to a two-phase partition system consisted of 6.3% PEG3350, 6.3% DextranT-500, 8.7% sucrose, 0.014% KCl, 0.049% phosphate buffer (pH 7.8). The resulting upper phase was diluted and centrifuged at 100,000*g* for 30 min and the pellet was collected. The purity of the plasma membrane was estimated by assay of the H^+-ATPase activity, The H^+-ATPase activity was determined by measuring the release of Pi (Qiu and Su 1998; Yang et al. 2003). Na_3VO_4, KNO_3 and NaN_3 caused about 72%, 1.5% and 2.9% inhibition in the H^+-ATPase activity of the upper phase membrane fractions from two-phase partition method, respectively.

The protein concentration was determined according to the method of Bradford (1976), using BSA as the calibration standard. The proteins were separated by SDS-PAGE, then transferred to nitrocellulose membranes. The membranes were blocked with TTBS (25 mM Tris–HCl, pH 7.5, 137 mM NaCl and 0.2 % Tween 20) containing 5% non-fat dry milk for at least 1 h (Zhao et al. 2007) and incubated with polyclonal *PeSOS1* antibody for 2 h in TTBS containing 1% non-fat dried milk. After multiple washes with TTBS, bound antibodies were detected with a horseradish peroxidase:anti-rabbit solution (1:10 000 ratio) and a chemiluminescence kit (Pharmacia).

Bacterial strains and salt tolerance determination

Experiments were carried out using the *E. coli* double mutant strain EP432 bearing deletions in the *nhaA* and *nhaB* Na^+/H^+ antiporter genes (Padan and Schuldiner 1994; Ivey et al. 1993). The open reading frame of *PeSOS1* was amplified by PCR with primers P14 (5′-GGCCATTACGGCCATGGGGAGCGCGATAGAAAAAG-3′- SfiI site underlined) and P15 (5′-CTGAGAATTCCTAAGAAGCA TGATGATGGAACG ACAG-3′ - EcoRI site underlined). The resulting fragment

encoding the mature protein of *PeSOS1* was cloned into the SfiI and EcoRI site of the shuttle expression vector pDL2xN-STE (Biotech, Germany) and transformed into the *E. coli* strain $DH_5\alpha$.

E. coli EP432 cells were grown on plates containing modified Luria-Bertani medium, LBK (1% Bacto Tryptone, 0.5% Yeast Extract and 50 mM KCl) supplemented with 50 mM MES-Tris, 30 μg/ml kanamycin, 34 μg/ml chloramphenicol and 1.5% agar. Single colonies formed were used as inocula for a liquid culture of LBK with 30 μg/ml kanamycin and grown overnight at 37°C under aerobic conditions. Competent *E. coli* EP432 cells were prepared (modified after Dower et al. 1988) and transformed by $CaCl_2$ with the plasmid pDL2xN-STE -*PeSOS1* and the empty vector pDL2xN-STE. Clones were selected on LBK plates with 100 μg/ mL ampicillin and sequenced.

For bacterial salt stress experiments, *E. coli* EP432 expressing *PeSOS1* and control *E. coli* EP432 (the negative control carried the empty vector pDL2xN-STE) strains were used. Preparatory cultures were grown under selective conditions in LBK medium and 100 μg/ml ampicillin at 37°C under aerobic conditions until a pre-defined cell density had been reached. Subsequently, equal cell densities were used to inoculate the LBK with 100 μg/ml of ampicillin medium in sterile tubes. Isopropyl *β*-D-thiogalactopyranoside (IPTG) (1 mM) was added to induce for 3 h and 200 mM NaCl then added for the final culturing (Ottow et al. 2005). These bacterial cells were incubated at 37°C with shaking (200 rpm) and used for tests at the different periods. Bacterial cultures without added NaCl were used as controls. Bacterial growth was measured photometrically as the optical density (OD) recorded at 600 nm (Biophotometer, Japan). Data were analyzed by a nonlinear logistic curve fitting using Origin 7.0 (Microcal Software Inc., USA).

Element analyses of bacteria

Bacteria were first centrifuged (14000 rpm, 10 min, 4°C), the supernatant was discarded and the pellet was rinsed with deionized water five times, then dried at 70°C for 48 h and weighed. The resulting samples were digested with HNO_3, and the Na^+ and K^+ concentrations were determined using an atomic absorption spectrometer (HITACHI 180-80, Polarized Zeeman Atomic Absorption Spectrophotometer, Japan).

Results

PeSOS1 encodes a putative Na^+/H^+ antiporter

The DNA fragment covering the whole region of the open reading frame was cloned and sequenced (GenBank accession number: DQ517530). The recovered cDNA was 3665 bp long, and contained a 3438 bp open reading frame. This gene was named *PeSOS1* and is predicted to encode a polypeptide of 1,145 amino acid residues (Fig. 2A) with a theoretical molecular mass of 127 kDa. Hydrophilicity plot analysis showed that the *N*-terminal portion of *PeSOS1* is highly hydrophobic and has 12 predicted transmembrane domains (Figs. 2B, 3). Database searches revealed substantial similarities between the transmembrane region of *PeSOS1* and the Na^+/H^+ antiporters from plant and microbial origins (Fig. 3). The amino acid sequence of this *PeSOS1* gene showed 64% identity with the previously isolated *SOS1* gene from the glycophyte *Arabidopsis thaliana*. We found the highest sequence similarity with the 'glycophytic' type Na^+/H^+ antiporters (which have been functionally characterized by Shi et al. (2000)) in a 347 amino acid residue sequence (99–445), located between TM3 and TM12 of the *PeSOS1* gene (Figs. 2, 3). The *PeSOS1* gene has 77% identity and 89% similarity with the *AtSOS1* gene from *A. thaliana* in the transmembrane region. In addition, this *PeSOS1* sequence exhibited 31% identity and 49% similarity with the NhaP sequence from the bacterium *Pseudomonas aeruginosa* across a region of 347 amino acids. NhaP has also been functionally characterized (Utsugi et al. 1998). The *C*-terminal portion of *PeSOS1* is hydrophilic and predicted to reside in the cytoplasm (Fig. 2B).

A phylogenetic tree including Na^+/H^+ antiporter sequences from higher plants, animals, fungi and *E. coli* was created. This phylogenetic analysis indicated two distinct clusters, supported by high bootstrap values: a "vacuolar" cluster and a "plasma membrane" cluster. The relationships between these clusters were consistent with their phylogenetic distributions. Figure 4 shows that *PeSOS1* clusters with the plasma membrane Na^+/H^+ antiporters including AtSOS1, SOD2, NHA1, NhaA, and NhaP. Phylogenetic analysis suggests that *PeSOS1* may be localized at the plant-cell plasma membrane.

Expression of *PeSOS1* under salt stress in *P. euphratica*

To examine the expression of the PeSOS1 gene under salt stress, RT-PCR and Western blotting analyses were performed. The relative amounts of the mRNA in salt-resistant *P. euphratica* leaves remained relatively constant with the increasing time under 200 mM NaCl in the growth media (Fig. 5A). Western blotting analysis showed that *PeSOS1* was significantly up-regulated under salt stress and expressed up to 5 and 10 times more highly in the leaves of *P. euphratica* after treatment with 200 mM NaCl for 24 h and 48 h, respectively, than in control plants (Fig. 5B).

PeSOS1 was immunodetected to assess its cellular localization in the leaves of *P. euphratica* after treatment

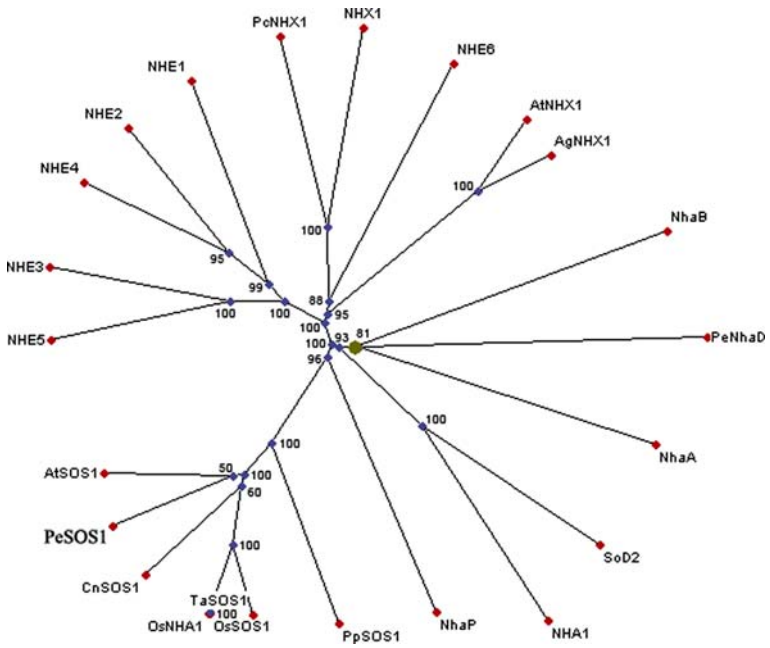


Fig. 4 Position of the *PeSOS1* in the phylogenetic tree of Na^+/H^+ antiporters. Multiple sequence alignment was performed with CLUSTALW 1.83 and a neighbor-joining tree was constructed using the program PhyloDraw. The accession numbers (in brackets) and sources of the Na^+/H^+ antiporters are as follows: AgNHX1 (AB038492), *Atriplex gmelini*; AtNHX1 (AF510074), AtSOS1 (AF256224), *Arabidopsis thaliana*; NHX1 (NP010744), *Saccharomyces cerevisiae;* PcNHX1 (AAG38538), *Pneumocystis carin*; NHE1 (P19634), NHE2(AAD41635), NHE3 (U28043), NHE4 (P26434), NHE5 (AAC98696), NHE6 (Q92581), *Homo sapiens*; CnSOS1 (CAD20320), *Cymodocea nodosa*; OsNHA1 (AY328087), OsSOS1 (AY785147), *Oryza sativa*; TaSOS1 (AY326952), *Triticum aestivurn*; PpSOS1 (AJ564258), *Physcomitrella patens*; PeNhaD1 (AJ561195), *Populus euphratica*; NhaP (BAA31695), *Pseudomonas aeruginosa*; NHA1 (NP-013239) , *Saccharomyces cerevisiae*; SOD2 (CAA77796, *Schizosaccharomyces pombe;* NhaA (P13738), NhaB (P27377), *Escherichia coli*. Bootstraps were indicated in each node

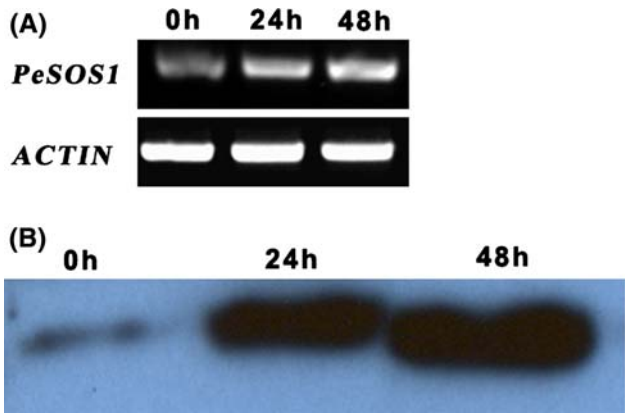


Fig. 5 Induced expression of *PeSOS1* mRNA and *PeSOS1* product by NaCl expression of the *PeSOS1* gene and *PeSOS1* products at 24 and 48 h after treating leaves with 200 mM NaCl. (**A**) RT-PCR expression analysis of *PeSOS1* transcripts. A 306 bp *PeSOS1* fragment was amplified by 28 PCR cycles. As control, a 762 bp *Actin* fragment was simultaneously amplified. B: Protein was extracted from leaves from the same plants as described in Materials and methods. Twenty μg of protein per well were separated on a 10% polyacrylamide electrophoresis gel and used for Western blot analysis (*PeSOS1*)

with 200 mM NaCl for 48 h. Our results suggest that *PeSOS1* may mediate Na^+ effluxes at the plant-cell plasma membrane, which is consistent with the non-existence of the *PeSOS1* band in the vacuolar fraction observed in the Western blot analysis (Fig. 6).

Increased salt resistance in a salt-sensitive *E. coli* mutant after complementation with *PeSOS1*

To assess the role of *PeSOS1* in haloresistance, the cDNA was cloned into a shuttle expression vector (pDL2xN-STE) and expressed as a recombinant protein in the *E. coli* strain EP432, which is deficient in the two main Na^+/H^+ anti-

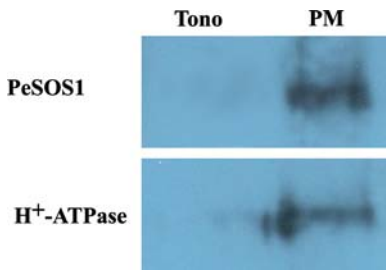


Fig. 6 Immunoblot of *PeSOS1* proteins in membrane fractions of *Populus euphratica*. Localization of *PeSOS1* in leaf membranes after treatment with 200 mM NaCl for 48 h. Ten µg of protein per well was separated on a 10% polyacrylamide electrophoresis gel and used for Western blot analysis (*PeSOS1*).Tono, tonoplast fraction of the gradient; PM, plasma membrane fraction

porters, *EcNhaA* and *EcNhaB*. Under controlled conditions, the presence of the eukaryotic *PeSOS1* gene did not have any obvious effect on the general growth behavior of the *E. coli* cells (Fig. 7). The expression of the *PeSOS1* products in the transformants was confirmed by Western blotting analysis (data not shown). The growth of the reference strain carrying the empty vector in the presence of 200 mM NaCl at pH 7.0 was severely inhibited (Fig. 7). In contrast, the recombinant strain with the *PeSOS1* gene continued to grow in the presence of 200 mM NaCl, reaching optical densities ca. 70% as high as those of cells grown in the absence of salt stress after 16 h (Fig. 7).

Effects of NaCl on elemental ratios in salt-sensitive E. *coli* mutants expressing *PeSOS1*

When EP432 cells were grown in the presence of 200 mM NaCl, the bacteria carrying the *PeSOS1* gene accumulated significantly less sodium after 7 h and 15 h than the bacteria without the putative Na^+/H^+ antiporter (Fig. 8). At the end of the exponential growth phase (after 15 h; Fig. 8), the cells lacking the *PeSOS1* gene accumulated more than twice as much sodium as cells protected by the putative Na^+/H^+ antiporter *PeSOS1*. In the presence of 200 mM NaCl, the concentration of K^+ in the EP432 cells was lower than in the cells protected by the *PeSOS1* gene. Although increases in the level of accumulated potassium were observed during the course of the experiment in both bacteria carrying the *PeSOS1* gene and bacteria lacking the putative Na^+/H^+ antiporter, the former accumulated significantly more potassium after 7 h and 15 h, than the latter (Fig. 8),

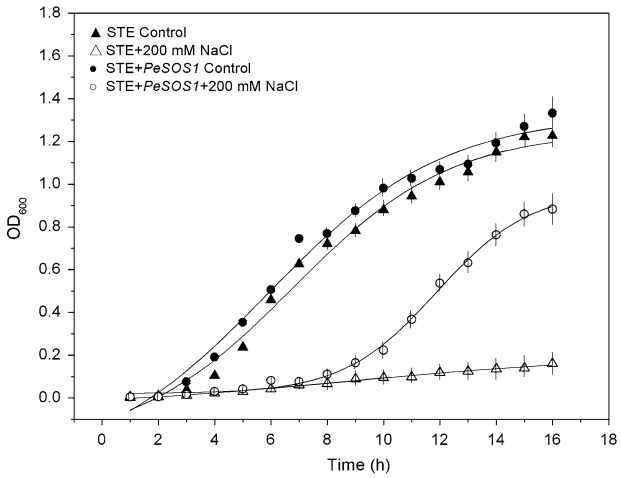


Fig. 7 Growth of the *E. coli nhaAnhaB* mutant strain EP432 transformed with the putative plant antiporter gene PeSOS1 (circles) or the empty expression vector STE (triangles). Bacteria were grown aerobically in LBK medium supplemented with or without 200 mM NaCl at pH 7.0 for 16 h. Error bars (n = 3) ± SD are shown

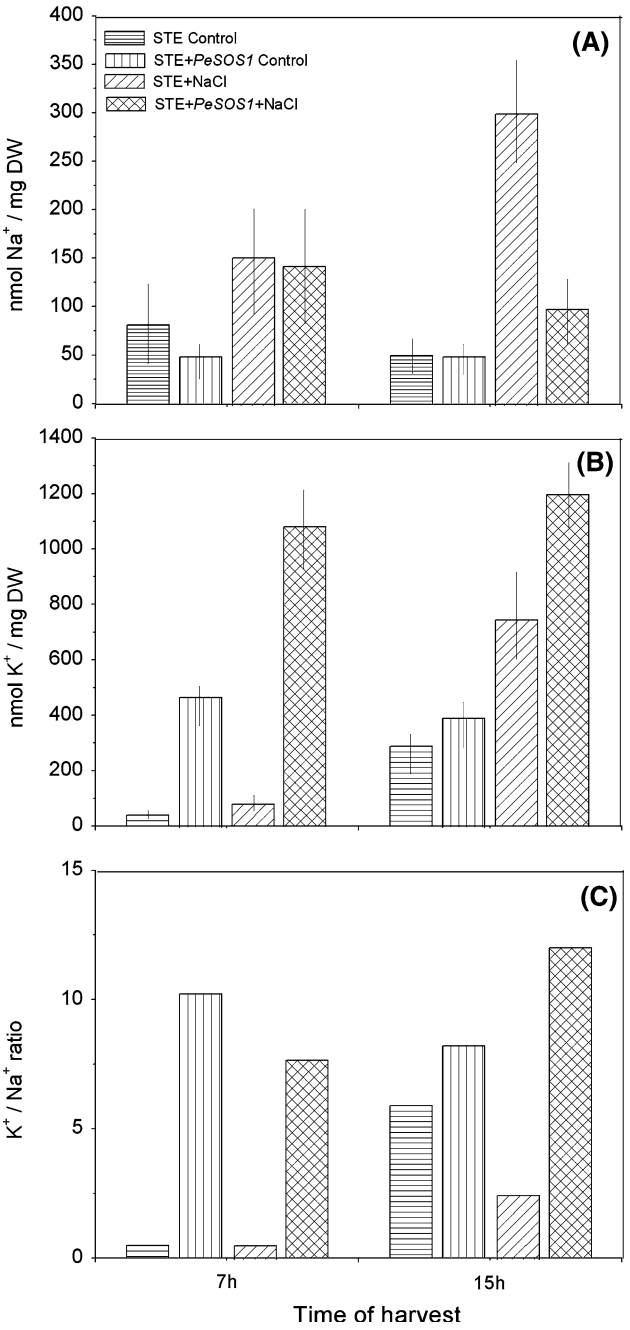


Fig. 8 Changes in Na^+ and K^+ concentrations in the *E. coli nhaAnhaB* mutant strain EP432 carrying the putative plant antiporter gene *PeSOS1* or the empty expression vector STE. (**A**) Na^+ concentrations; (**B**) K^+ concentrations; (**C**) K^+/Na^+ ratios. Cells were grown with and without 200 mM NaCl as described in *Material and methods* and were harvested after 7 h (left) and 15 h (right). Error bars (n = 3) ± SD are shown

and thus had higher K^+/Na^+ ratios. These results suggest that *PeSOS1* mediates Na^+ effluxes in the EP432 cells, and the Na^+ tolerance conferred by the *PeSOS1* gene is correlated with lower Na^+ contents and higher K^+ concentrations.

Discussion

When plants are exposed to salt stress, cellular ion homeostasis may be disturbed. To avoid the toxic effects of salt, mechanisms limiting Na^+ uptake, and promoting Na^+ compartmentalization into vacuoles and/or extrusion to the external medium are used to maintain low sodium (Na^+) concentrations in the cytosol of cells (Zhu 2002; Fukuda et al. 2004; Horie and Schroeder 2004; Nakayama et al. 2005). It has been reported that Na^+/H^+ antiporters play important roles in these processes (Niu et al. 1996; Ohta et al. 2002; Qiu et al. 2004). Thus, we cloned the *PeSOS1* gene from *P. euphratica* to elucidate its function in salt tolerance.

The *PeSOS1* protein product is predicted to contain 12 transmembrane regions (TM) at the amino-terminus and a large cytoplasmic region at the carboxyl terminus (Fig. 2). Throughout this transmembrane region, *PeSOS1* shows substantial sequence similarities with Na^+/H^+ antiporters from *Arabidopsis*, microbes and rice. The transmembrane regions TM3-TM12 share identity with other Na^+/H^+ antiporters (Fig. 3). The TM6 and TM7 regions are highly conserved (Fig. 3). The amino acid sequence of the *PeSOS1* gene showed 64% identity with that of the previously isolated *SOS1* gene from the glycophyte *Arabidopsis thaliana*. The sequence similarities together with the results showing that the expression of *PeSOS1* suppresses some of the salt sensitive traits of the EP432 strain, strongly indicates that *PeSOS1* is a Na^+/H^+ antiporter (Figs. 7 and 8). Phylogenetic analysis showed that *PeSOS1* is more closely related to plasma membrane Na^+/H^+ antiporters from *Arabidopsis* than to the vacuolar antiporters from either plants or fungi (Fig. 4). In this phylogenetic tree, SOD2 and NHA1 have been shown to be active in the plasma membrane of *Saccharomyces pombe* and *S. cerevisiae* respectively, exporting Na^+ from the cytosol to the extracellular space (Dibrov et al. 1997; Prior et al. 1996). NhaA and NhaP are Na^+/H^+ antiporters that have important functions in Na^+ efflux in *Escherichia coli* and *Pseudomonas aeruginosa*, respectively (Padan et al. 1989; Utsugi et al. 1998). *PeSOS1* does not cluster with Na^+/H^+ antiporters such as AtNHX1, NHX1 or NHE6 (Fig. 4). AtNHX1 acts in the tonoplast, where it compartmentalizes Na^+ into the vacuole of *Arabidopsis* cells (Gaxiola et al. 1999, Apse et al. 1999). NHX1 plays an important role in transporting Na^+ to the yeast prevacuolar compartment (Nass et al. 1997; Nass and Rao 1998). Human NHE6 was found to be localized to secretory endosomes (Brett et al. 2002; Nakamura et al. 2005). Our localization studies using antibodies further revealed that *PeSOS1* is localized in the plasmalemma (Fig. 6). These results suggest that *PeSOS1* is probably a plasma membrane Na^+/H^+ antiporter in *P. euphratica.* Therefore, *PeSOS1* is expected to function in exporting Na^+ from the cytosol to the extracellular space and thus prevent rapid accumulations of Na^+ in the cytoplasm.

PeSOS1 gene expression is up-regulated by NaCl stress in leaves (Fig. 5). It has been reported that NaCl stress also up-regulates the expression of genes encoding plasma membrane H^+-ATPases in *P. euphratica* (Ma et al. 2002). Increased H^+-ATPase expression would provide the additional proton motive force that is required to enhance Na^+/H^+ antiporter activity (Niu et al. 1996), and this up-regulation is consistent with the role of AtSOS1 in Na^+ tolerance (Shi et al. 2002, 2003; Quintero et al. 2002; Qiu et al. 2004). Most higher plants are very sensitive to Na^+, but it is difficult to fully comprehend the processes involved in plants because the presence of Na^+ salts in soil solutions produce many complex physiological effects in them (Tester and Davenport 2003; Benito and Rodriguez-Navarro 2003). However, antiporter activity may be necessary for controlling ion balance and regulating pH and must, therefore, be regarded a house-keeping function. Under salt stress, plants maintain high concentrations of K^+ and low concentrations of Na^+ in the cytosol by regulating the expression and activity of K^+ and Na^+ transporters and of H^+ pumps that generate the proton motive force required for transport processes (Zhu 2003). *PeSOS1* expression was observed in the absence of leaf exposure to salt (Fig. 5) and therefore may play a role in maintaining ion balance even at normal conditions.

E. coli possesses at least three types of Na^+-transport antiporters: NhaA (Karpel et al. 1988), NhaB (Pinner et al. 1992) and ChaA (Ivey et al. 1993). Deletion of NhaA and NhaB renders *E. coli* sensitive to the Na^+ concentrations found in standard culture medium (Nozaki et al. 1998). Expression of VcNhaD, VpNhaD and PeNhaD1 in a *nhaAnhaB* deletion mutant restores the salt resistance of the mutant by the Na^+/H^+ antiporter activity of NhaD (Nozaki et al. 1998; Dzioba et al. 2002; Ottow et al. 2005). The expression of the poplar *PeSOS1* gene in the *E. coli nhaAnhaB* deletion mutant EP432 reestablished growth in the presence of 200 mM NaCl (Fig. 7). This complementation experiment serves as a functional test for the Na^+/H^+ exchange activity of *PeSOS1* genes.

When grown in the presence of 200 mM NaCl, *PeSOS1*-expressing cells maintained lower Na^+ and higher K^+ levels than the control cells without antiporter activity (*nhaA nhaB*) (Fig. 8). The Na^+ content increased in both bacteria carrying the *PeSOS1* gene and controls, but after 15 h of NaCl treatment the Na^+ content of the negative control bacteria was nearly three times higher than that of the bacteria containing the *PeSOS1* gene (Fig. 8A). In contrast, the K^+ content in the STE was approximately half of that reported for *PeSOS1* (Fig. 8B), resulting in an increase in the K^+/Na^+ ratios. Concomitant with the maintenance of

low Na^+ is the maintenance of high K^+. In fact, it is possible that a higher K^+/Na^+ ratio is more important for many species than simply maintaining a low concentration of Na^+ (Tester et al. 2003). The cause of the accumulation of K^+ in EP432 cells is unclear and this may be due to the coordination of physiological activities between the K^+/Na^+ exchangers on the membranes (Qiu et al. 2004). Enhanced influx of Na^+ and the inhibition of K^+ uptake by plants under salt stress may disturb K^+/Na^+ homeostasis, thus exerting toxic effects on plants (Zhu 2003). The ability to control net Na^+ influx into the cytoplasm and maintain a high level of K^+/Na^+ in the cytoplasm is of great importance in determining plant responses to salinity (Inan et al. 2004). In addition, our results indicate that *PeSOS1* functioned as a Na^+/H^+ antiporter that partly compensated for loss of the function of *E. coli nhaAnhaB*. Since bacterial growth was correlated with significantly decreased sodium concentrations in the strains expressing the *PeSOS1* gene, compared to the non-transformed deletion mutants (Fig. 8), our data also show that this protein plays a role in mediating sodium tolerance.

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