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RESEARCH PAPER

Cloning of an H^+-PPase gene from *Thellungiella halophila* and its heterologous expression to improve tobacco salt tolerance

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Abstract

An H^+-pyrophosphatase (PPase) gene named *TsVP* involved in basic biochemical and physiological mechanisms was cloned from *Thellungiella halophila*. The deduced translation product has similar characteristics to H^+-PPases from other species, such as *Arabidopsis* and rice, in terms of bioinformation. The heterologous expression of *TsVP* in the yeast mutant *ena1* suppressed Na^+ hypersensitivity and demonstrated the function of TsVP as an H^+-PPase. Transgenic tobacco overexpressing TsVP had 60% greater dry weight than wild-type tobacco at 300 mM NaCl and higher viability of mesophyll protoplasts under salt shock stress conditions. *TsVP* and *AVP1*, another H^+-PPase from *Arabidopsis*, were heterologously expressed separately in both the yeast mutant *ena1* and tobacco. The salt tolerance of *TsVP* or *AVP1* yeast transformants and transgenic tobacco were improved to almost the same level. The *TsVP* transgenic tobacco lines TL3 and TL5 with the highest H^+-PPase hydrolytic activity were studied further. These transgenic tobacco plants accumulated 25% more solutes than wild-type plants without NaCl stress and 20–32% more Na^+ under salt stress conditions. Although transgenic tobacco lines TL3 and TL5 accumulated more Na^+ in leaf tissues, the malondialdehyde content and cell membrane damage were less than those of the wild type under salt stress conditions. Presumably, compartmentalization of Na^+ in vacuoles reduces its toxic effects on plant cells. This result supports the hypothesis that overexpression of H^+-PPase causes the accumulation of Na^+ in vacuoles instead of in the cytoplasm and avoids the toxicity of excessive Na^+ in plant cells.

Key words: *Arabidopsis thaliana*, H^+-PPase, salt tolerance, *Thellungiella halophila*.

Introduction

Salinity is an important factor limiting plant growth. About 20% of the world's irrigated lands are now affected by salinity (Rhoades and Loveday, 1990). The detrimental effects of salt on plants are a consequence of both water deficit that results from the relatively high solute concentrations in the soil and excessive Na^+ concentrations in the cytoplasm. Excessive Na^+ in the cytoplasm not only alters ion ratios and affects critical biochemical processes (Maathuis and Amtmann, 1999), but also increases plasma membrane injury and causes malondialdehyde (MDA) accumulation (Dionisio-Sese and Tobita, 1998). To deal with the injury, plants have developed multifarious strategies. The accumulation of compatible solutes and reduction of sodium ions in the cytoplasm are two common mechanisms in plants. Compatible solutes mainly include betaine; polyols and sugars, such as mannitol and sorbitol; and amino acids, such as proline (Chen and Murata, 2002). Mechanisms have been developed in plants to avoid the injury due to excessive sodium ion concentrations in the cytoplasm. There are two important ways among these mechanisms for reducing excessive Na^+ in the cytoplasm: one is to exclude Na^+ from cells by the Na^+/H^+ antiporter located in the plasma membrane; and the other is to pump Na^+ into vacuoles by the Na^+/H^+ antiporter located in the tonoplast (Zhu, 2003).

The compartmentalization of Na^+ into vacuoles provides an efficient mechanism for averting the toxic effects of Na^+ in the cytosol. The transport of Na^+ into vacuoles is

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Abbreviations: CaMV, cauliflower mosaic virus; DIG, digoxigenin; DTT, dithiothreitol; FDA, fluorescein diacetate; MDA, malondialdehyde; ORF, open reading frame; PMSF, phenylmethylsulphonyl fluoride; PPase, pyrophosphatase.

mediated by vacuolar Na^+/H^+ antiporters that are driven by the electrochemical gradient of protons. The proton-motive force generated by the vacuolar ATPase (V-ATPase) and vacuolar pyrophosphatase (V-PPase) can drive secondary transporters, such as the Na^+/H^+ antiporter and Ca^{2+}/H^+ antiporter, as well as organic acids, sugars, and other compound transporters to maintain cell turgor (Lincoln, 1992). The vacuolar H^+-PPase is a single subunit protein located in the vacuolar membrane (Maeshima, 2000). It pumps H^+ from the cytoplasm into vacuoles with PPi-dependent H^+ transport activity. Theoretically, overexpression of H^+-PPase should enhance the ability to form the pH gradient between the cytoplasm and vacuoles, resulting in a stronger proton-motive force for the Na^+/H^+ antiporter, Ca^{2+}/H^+ antiporter, and other secondary transporters. The accumulation of cations, such as Na^+, in vacuoles could increase the osmotic pressure of plants, while reducing the toxic effects of these cations (Gaxiola *et al*., 2001).

Apse *et al.* (1999) have reported that overexpressing *AtNHX1*, a vacuolar Na^+/H^+ antiporter from *Arabidopsis*, resulted in higher salt tolerance in transgenic *Arabidopsis*, and the salinity tolerance was correlated with the higher levels of *AtNHX1* transcripts and vacuolar Na^+/H^+ antiporter activity. In addition, evidence from Gaxiola *et al*. (2001) supports the role of the vacuolar electrochemical proton gradient in salt tolerance by overexpressing a vacuolar H^+-PPase in *Arabidopsis thaliana*. Transgenic plants overexpressing *AVP1*, coding for a single subunit protein for vacuolar H^+-PPase, displayed enhanced salt tolerance that was correlated with the increased ion content of the plants. These results suggest that the enhanced vacuolar H^+ pumping in the transgenic plants provided additional energy for vacuolar sodium accumulation via the vacuolar Na^+/H^+ antiporter.

Thellungiella halophila (salt cress; synonymous with *Thellungiella salsuginea*) (Al-Shebaz *et al.*, 1999), a classical halophyte living in the seashore saline soils in eastern China, is able to survive for several months and produce viable seeds, even in the presence of 500 mM NaCl (Inan *et al.*, 2004). *Thellungiella halophila* produces neither salt glands nor other complex morphological alterations either before or after salt adaptation. It appears that the salt tolerance comes from its basic biochemical and physiological mechanisms. In the present report, a vacuolar H^+-PPase gene involved in basic biochemical and physiological mechanisms was cloned from *T. halophila*. Then the H^+-PPase genes from *A. thaliana* and *T. halophila* were heterologously expressed in the yeast mutant *ena1* and in tobacco to study the function of the H^+-PPase and compare the different effects of H^+-PPase from *A. thaliana*, a typical glycophyte, and *T. halophila*, a typical halophyte on salt tolerance. The results indicated that the overexpression of *TsVP* or *AVP1* enhanced the salt tolerance of both the yeast mutant *ena1* and tobacco. The *TsVP* transgenic tobacco lines TL3 and TL5 that had higher H^+-PPase hydrolytic activity were further studied by analysing dry weight, solute potential, ion content, MDA content, and electrolyte leakage of leaf cells.

Materials and methods

Cloning the H^+-PPase gene from a cDNA library and sequence analyses

A pair of primers P1 (5′-GGCGTTCATTGTTGCATTCAGGT-3′) and P2 (5′-CCCAGGCTCCTCCAGTGTTAGAT-3′) were designed from the sequence of the *AVP1* gene from *A. thaliana*. A DNA fragment was amplified from *T. halophila* cDNA by reverse transcription–PCR (RT–PCR) with P1 and P2. This DNA fragment was cloned into pGEM®-T easy vector (Promega, USA) and sequenced by Bioasia, Inc. (Shanghai, China). This DNA fragment, labelled using the DIG-High Prime kit (Roche Inc.), was used to screen a cDNA library from 200 mM NaCl-stressed 8-week-old *T. halophila*. Several clones positive for the H^+-PPase gene named *TsVP* were isolated and sequenced. Alignment was performed using the deduced amino acid sequence of *TsVP* and H^+-PPases from other species.

Construction of yeast expression vectors and yeast transformation

The complete open reading frames (ORFs) of *TsVP* and *AVP1* (GenBank accession no. NM_101437) were amplified with primers *TsVP*-P3 (5′-GGAGGAGAGATGGTGGCGTC-3′) and *TsVP*-P4 (5′-CAAAACGAAAATAAGAATGG-3′), and primers *AVP*-P1 (5′-GAGAAGATGGTGGCGCCTGC-3′) and AVP-P2 (5′-TCACTGG-GGAGTTGCTGGAG-3′), respectively, using Pyrobest® polymerase (Takara Inc., Japan), These full-length cDNA fragments were cloned into pGEM®-T easy vector and sequenced by Bioasia, Inc (Shanghai, China). The DNA fragments of *AVP1* and *TsVP* were digested with *Eco*RI and cloned into the *Eco*RI site in the multiple cloning site (MCS) of the pYES2 vector (Invitrogen Inc., USA) between the GAL1 promoter and the CYC1 terminator sequences. These plasmids were named pYES2-TsVP and pYES2-AVP. Ura^- yeast strains were transformed by the LiAc/polyethylene glycol method.

Functional assays using the yeast mutant

The *ena1* yeast mutant was transformed with three different plasmids, pYES2-*TsVP*, pYES2-*AVP*, and pYES2 as a control. Three *ena1* yeast mutant strains and the wild type (W303) were grown at 28 °C for 16 h to reach OD_{600} 1.0–2.0 in YPD medium. About 10^5 yeast cells in 2 μl and two 5-fold serial dilutions of the yeast cells were spotted onto YPGAL plates with different NaCl concentrations (0.25, 0.5, and 0.75 M). The strains were cultured at 28 °C for 60 h. The growth status of these yeast strains was used to identify the *TsVP* function and compare the salt tolerance.

Construction of plant expression vectors and tobacco transformation

TsVP and *AVP*1 full-length cDNAs were cloned into the plant expression vector pROKII under the control of the cauliflower mosaic virus (CaMV) 35S promoter with standard protocols described in Sambrook *et al.* (2000). Leaf discs from *Nicotiana tabacum* Wisconsin38 were transformed with *Agrobacterium tumefaciens* strain LBA4404 (Voelker *et al.*, 1987) carrying the above recombinant plasmids. Regenerated shoots with kanamycin resistance were selected in MS medium (Murashige and Skoog, 1962) supplemented with 170 μM kanamycin, 4.44 μM 6-benzyladenine (6-BA), and 0.571 μM indoleacetic acid (IAA), and rooted in

MS medium without growth regulators. These kanamycin-resistant plantlets were confirmed by PCR with primers *TsVP*-P5 (5′-CAGAACTCGCCGTAAAGACT-3′) and *TsVP*-P6 (5′-GCAGAAACCGAAGATAACG-3′) for *TsVP* transgenic tobacco, and primers *AVP*-P3 (5′-GCCGTAAAGACTGGCGAACA-3′) and *AVP*-P4 (5′-AGCACCAAGCACGAAAGCAA-3′) for *AVP1* transgenic tobacco. The PCR-positive plantlets were transplanted into soil and grown in a greenhouse at 27/23 °C (day/night) with a 16 h light (600 μmol m^{-2} s^{-1})/8 h dark cycle and ~70% relative humidity. Eight weeks later, these plants were collected for Southern and northern blotting analysis. The transgenic tobacco plants with different levels of *TsVP* gene expression were used for determination of PPi hydrolysis activity and salt tolerance assays.

Germination and growth of transgenic tobacco

Sterilized seeds from the homozygous T_2 transgenic and wild-type plants of tobacco were germinated on MS medium. The seedlings were grown under conditions of 25/20 °C (day/night) temperature, 16 h photoperiod with 200 μmol m^{-2} s^{-1} light intensity. Twenty days later, some seedlings were transferred to MS medium supplemented with different concentrations of NaCl (0, 100, 200, or 300 mM, respectively). After 25 d salt treatment, the seedlings were used to measure the parameters such as dry weight, ion content, MDA content, electrolyte leakage of leaf cells, and the protoplast viability. Other seedlings were transplanted into soil and grown in a greenhouse under the same conditions as above. Eight weeks later, these plants were collected for measurement of PPi hydrolysis activity and proton transport assays.

Southern and northern blotting

DNA of tobacco plants was isolated from 1 g of leaves by the cetyltrimethyl ammonium bromide (CTAB) method. Southern blotting was performed with the digoxigenin (DIG)-labelled full-length *TsVP* cDNA probe as described in the DIG System Manual (Roche, Inc.). RNA was extracted with Trizol reagent from the leaves of transgenic and non-transgenic plants, and treated with RNase-free DNase. Northern blotting was performed with denatured total RNA (20 μg per lane) in the standard protocol with the [α-^{32}P] dCTP-labelled full-length cDNA of *TsVP* as a probe in Church buffer at 65 °C.

Real-time RT–PCR

The 6-week-old plants of *A. thaliana* and 8-week-old plants of *T. halophila* were treated with half-strength MS salt solution containing 200 mM NaCl. The roots and aerial parts of these plants were collected at different times after salt treatment. Total RNAs were extracted by Trizol reagent from these samples and treated with RNase-free DNase. cDNA synthesis was performed with the RT reagent kit (Takara, Dalian, China) according to the manufacturer's protocol. Real-time quantitative RT–PCRs were done on chromsome 4 (MJ Research, USA) with the SYBR® RT–PCR Kit (Takara, Dalian, China), in a 10 μl reaction volume, which contained 5 μl of SYBR® Green I PCR mix, 0.2 μM of each forward and reverse primer, 1 μl of diluted cDNA template, and appropriate amounts of sterile ddH_2O. Amplification conditions were: 2 min at 95 °C; 40 cycles of 15 s at 95 °C, 30 s at 58 °C, and 30 s at 72 °C. Fold changes of RNA transcripts were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with β-tubulin as an internal control. The entire experiments were repeated at least three times.

Membrane vesicle isolation

Tobacco tonoplast vesicles were prepared by sucrose density gradient ultracentrifugation as described previously by Lüttge *et al.* (2000) with minor modifications. About 30 g of leaf slices were homogenized in 90 ml of ice-cold buffer containing 100 mM Tricine/Tris, pH 8.0, 300 mM mannitol, 3 mM $MgSO_4$, 3 mM EGTA, and 0.5% (w/v) polyvinylpolypyrrolidone. Prior to use, 5 mM dithiothreitol (DTT) and 1 mM phenylmethylsulphonyl fluoride (PMSF) were added to the buffer. After filtration and pre-centrifugation of the homogenate at 4200 *g* for 10 min, the pellets were resuspended in a small volume of ice-cold buffer containing 100 mM Tricine/Tris, pH 8.0, 300 mM mannitol, 3 mM $MgSO_4$, 3 mM EGTA, and 5 mM DTT. The suspension containing the vesicles was layered over a 10/25% (w/w) discontinuous sucrose gradient solution with 5 mM HEPES/Tris, pH 7.5, and 2 mM DTT. After centrifugation at 100 000 *g* for 2 h, the vesicles at the interface between the 10% and 25% sucrose solutions were collected, and diluted with 3 vols of ice-cold buffer containing 10 mM HEPES/NaOH, pH 7.0, 3 mM $MgSO_4$, and 1 mM DTT. Membranes were collected by centrifugation at 100 000 *g* for 60 min and resuspended in storage buffer with 10 mM HEPES/NaOH, pH 7.0, 40% (v/v) glycerol, 1 mM DTT, and 1 mM PMSF, and then were stored at –70 °C, ready for use.

Measurement of ATPase and H⁺-PPase activity

ATPase and PPase activity were measured as the release of Pi from ATP and PPi, respectively, during an incubation period of 30 min at 37 °C according to the method of Wang *et al.* (2001). Inorganic phosphate was determined using the method of Lin and Morale (1977). V-ATPase hydrolytic activity is presented as the difference of the measured values in the absence or in the presence of 50 mM NO_3^-. V-PPase hydrolytic activity was calculated as the difference in activity measured in the presence or absence of 50 mM KCl (K^+-stimulated PPase activity).

Proton transport assays

An inside-acid pH gradient (ΔpH) in vacuolar membrane vesicles produced by the vacuolar H^+-PPase was measured at 25 °C by quenching of quinacrine fluorescence using a spectrofluorometer at 495 nm after excitation at 420 nm (Churchill and Sze, 1983). The complete 300 μl reaction mixture contained 10 mM HEPES/Tris (pH 7.5), 0.33 mM EGTA, 2.0 μM quinacrine, 250 mM mannitol, 50 mM KCl, 1 mM PPi, and 50 μg of membrane protein. Proton pumping was initiated by the addition of 3 mM $MgSO_4$. The rate of fluorescence quenching was used as a relative estimate of the rate of H^+ pumping.

Tobacco leaf disc salt tolerance assay

The fourth fully expanded leaves acrofugally of wild-type and transgenic plants were briefly washed twice in distilled water. Leaf discs of 1 cm in diameter were cut and floated on 400 mM NaCl and 600 mM NaCl solution for 60 h (Veena *et al.*, 1999) under continuous white light at 25 °C. The chlorophyll was extracted with 80% acetone and the content of chlorophyll was measured by a spectrometer. The experiment was performed with three *TsVP* transgenic lines TL2, TL3, and TL5, two *AVP1* transgenic lines, AL3 and AL7, and one line of wild-type tobacco, and repeated three times on every plant.

Tobacco protoplast isolation and protoplast viability under salt tolerance

The healthy mature leaves of sterilized tobacco seedlings without salt stress were used to isolate mesophyll protoplasts; these leaves were sliced into ~2 mm wide strips and added to 5 ml volumes of CPW9M (Patat-Ochatt *et al.*, 1988) solution containing 9% mannitol, 1.5% Onozuka R-10 cellulase (Yakult Honsha Co., Japan), and 0.5% macerozyme. The pH of the CPW9M solution was adjusted to 5.8. The incubation was carried out in darkness for 1.5–2.0 h at 27 °C. After digestion, the enzyme mixtures were filtered through nylon mesh, and the protoplasts were purified as described by Pan *et al.*

(2003). Finally, the mesophyll protoplasts were collected and re-suspended in CPW9M solution with 9.0% mannitol. These purified mesophyll protoplasts were treated with CPW9M solution containing different concentrations of NaCl (100, 200, 300, and 400 mM) for 5 min. The viability of mesophyll protoplasts was assessed with the fluorescein diacetate (FDA) staining method (Widholm, 1972). The viability of mesophyll protoplasts was calculated by the following formula: viability of mesophyll protoplasts=the number of protoplasts with green fluorescence after FDA staining/ the number of total mesophyll protoplasts × 100%.

Quantification of solutes in leaf tissue

Extended leaves from non-NaCl-stressed tobacco seedlings were frozen in liquid nitrogen and thawed to extrude sap by a glass syringe. The osmotic potential of the leaf sap was determined with a cryoscopic osmometer. The readings (mmol kg^{-1}) were used to calculate the solute potential (ψ_S) in MPa (mega Pascales) using the formula ψ_S=moles of solute×RK, where R = 0.008314 and K = 298.

Na^+ absorption by the leaf discs of tobacco

Na^+ absorption was performed as described by Smith and Epstein (1964*a*, *b*) with minor modifications. Healthy and fully expanded leaves from wild-type and transgenic plants were washed briefly in distilled water twice. The leaf discs of 1 cm in diameter were cut and floated on distilled water for 2 h, then they were floated on 300 mM NaCl for various times in continuous white light at 25 °C; five discs as a group were picked out every 2 h. These groups of discs were washed twice with distilled water and transferred into the solution containing 100 mM KCl and 200 mM mannitol for 20 min to remove Na^+ in the outer space of mesophyll cells, then these discs were again rinsed twice with distilled water. These leaf discs were used for Na^+ content assays. The Na^+ content of leaf discs was determined by atomic absorption, and the speed of uptake of the Na^+ was calculated by the following formula: Na^+ uptake speed (μmol h^{-1} per disc)=(T_{N+2} Na^+ content–T_N Na^+ content)/2, where T_N Na^+ content presents the Na^+ content per disc at different time points.

Measurement of ion concentrations

The amounts of Na^+, K^+, Ca^+, and Cl^- in tobacco leaves and roots were measured. All leaves without midribs of the tobacco seedlings were used to measure the Na^+ concentration. The roots were rinsed in deionized water for 10 s and then washed with cold $LiNO_3$ solution isotonic with MS medium containing different concentrations of NaCl according to the method described by Flowers *et al.* (1986). Tobacco leaves and roots were dried for 48 h at 70 °C, and the dry weight was measured. The dried leaves and roots were extracted with 1 N HNO_3 as described by Storey (1995). The supernatants were analysed by atomic absorption for Na^+, K^+, and Ca^{2+}. The amount of Cl^- was determined by silver titration according to the method described previously by Chen *et al.* (2001).

Assay of malondialdehyde concentration

The MDA content was determined using the protocol described by Peever and Higgins (1989). Tobacco leaves (100–300 mg) were used to measure MDA content. The absorbance at 450, 532, and 600 nm was determined with a spectrometer. The concentration of MDA was calculated by the following formula:

$$C\ (\mu mol\ l^{-1}) = 6.45(OD_{532} - OD_{600}) - 0.56OD_{450}$$

Determination of leaf cell membrane damage

Leaf cell membrane damage was measured as the leakage of electrolytes from leaf cells using a conductivity meter, according to the method described by Gibon *et al.* (1997). The cell membrane damage represented as electrolytes leakage (%) was calculated using the following equation described by Premachandra *et al*. (1991): electrolytes leakage (%)=[1–(1−S1/S2)/(1−C1/C2)]×100, where the conductivity measurements correspond to NaCl-treated leaves (S1), boiled NaCl-treated leaves (S2), non-NaCl-treated leaves (C1), and boiled non-NaCl-treated leaves (C2).

Results

Cloning of the H^+-PPase gene from the T. halophila *cDNA library*

A pair of primers, P1 and P2, was designed to amplify the *T. halophila* double-stranded cDNA. A DNA fragment was obtained and sequenced. The result indicated that the fragment was a part of the H^+-PPase gene in *T. halophila*. Then the fragment was used to screen the *T. halophila* cDNA library. A positive clone containing a full-length cDNA for the putative H^+-PPase gene named *TsVP* was isolated. The full-length cDNA of *TsVP* was registered in GenBank (accession no. AY436553).

TsVP *gene sequence analyses*

Sequence analyses by the NCBI ORF finder revealed an ORF of 2316 bp with a 5′-untranslated region of 100 bp and a 3′-untranslated region of 344 bp. The deduced amino acid sequence is 771 amino acids and contains 14 transmembrane domains predicted by the online analyses tool, TopPred software from http://us.expasy.org. The topological model was very similar to that of a previous report on H^+-PPase by Maeshima (2000). Multiple alignments of the deduced amino acid sequence with vacuolar H^+-PPases from other species were performed with the Clustal W program. The results indicated that the TsVP protein shared 96% identity with the AVP1 protein of *A. thaliana* at the amino acid level, and the five conserved domains reported by Drozdowicz and Rea (2001) were also present in the deduced amino acid sequence of *TsVP* (Fig. 1).

Analyses of expression patterns under salt stress

Northern blotting analyses indicated that *TsVP* mRNA was present in both the leaves and roots of 8-week-old *T. halophila* seedlings. As shown in Fig. 2A and B, the level of mRNA accumulation in the leaves was relatively higher than in the roots. Total RNA was isolated at various times (0, 2, 4, 8, 16, and 24 h) under salt stress. The accumulation of *TsVP* mRNA increased over time during the period 0–16 h, and reached a maximum at 16 h. *TsVP* mRNA decreased from 16 h to 24 h.

The expression patterns of *AVP1* and *TsVP* under salt stress were compared by real-time RT–PCR with β-tubulin as an internal control. The fold change in gene expression is shown in Fig. 2C for *AVP1* and Fig. 2D for *TsVP*. The increase in *AVP1* expression was <2.5-fold, while

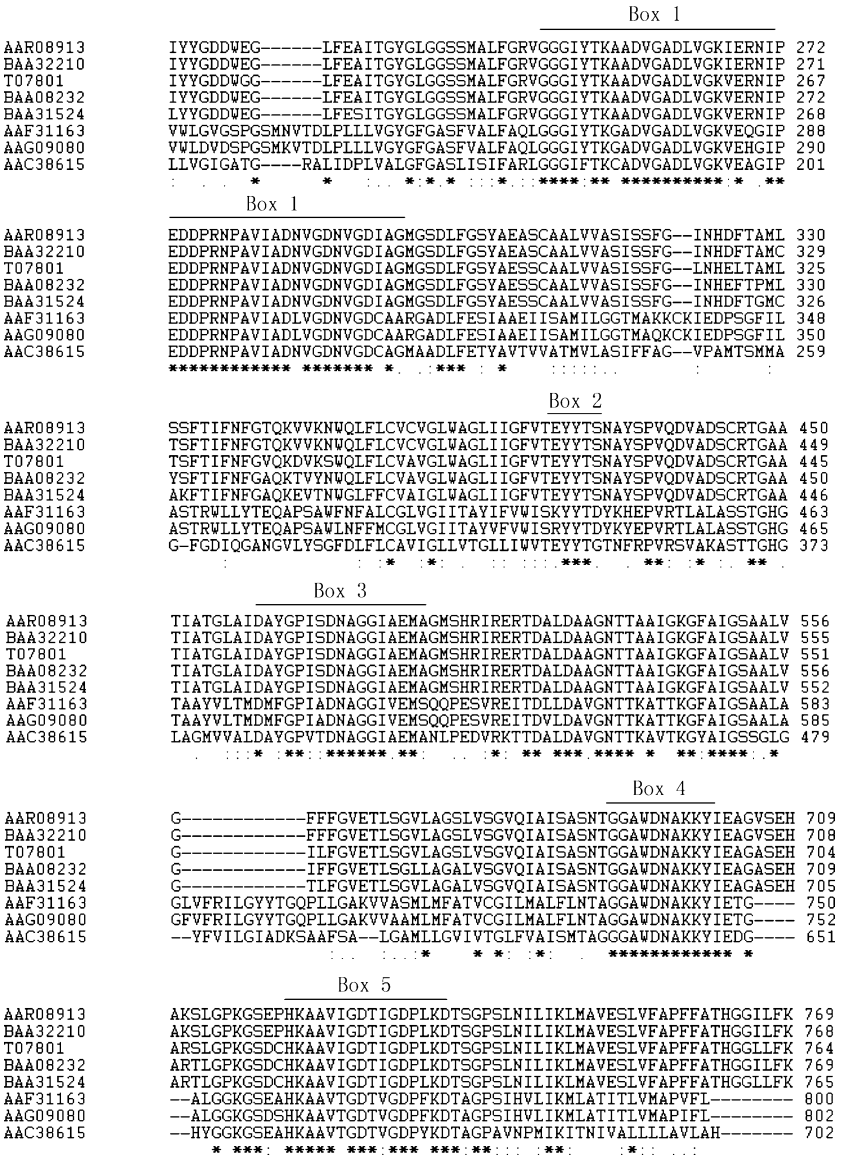

Fig. 1. The alignment of the deduced amino acid sequence of *TsVP* and H^+-PPases from other species by the Clustal W program. The amino acid sequences are as follows: AAR08913 (TsVP) from *T. halophila*; BAA32210, AAF31163, and AAG09080 from *Arabidopsis*; T07801 from *Vigna radiata*; BAA08232 and BAA31524 from *Oryza sativa*; and AAC38615 from *Rhodospirillum rubrum*. An asterisk indicates that the residues in that column are identical in all sequences; a colon indicates that conserved substitutions have been observed; and a full stop indicates that semi-conserved substitutions are observed. There are five conserved amino acid sequence boxes marked by lines above the amino acid sequence shown in the sequence alignment which have been reported to be used in identification of V-PPase homologues by Drozdowicz and Rea (2001).

TsVP expression increased 3-fold and 5-fold during the first 16 h in the aerial parts and roots, respectively.

Functional assays of TsVP *and* AVP1 *using the yeast mutant* ena1

In *Saccharomyces cerevisiae*, the primary pathway for Na^+ extrusion is mediated by ENA1 (Haro *et al.*, 1991; Rios *et al.*, 1997), a plasma membrane Na^+-ATPase. The yeast mutant strain *ena1* lacks the plasma membrane sodium efflux pump; therefore, it must rely on an internal detoxification system to overcome sodium toxicity. Growth of the *ena1* strain is sensitive to a low concentration of sodium (200 mM), whereas the growth of the wild-type strain is not inhibited by such low NaCl concentrations. Gaxiola *et al.* (1999) have reported that overexpression of the E229D (*AVP1-D*) gain-of-function mutant of the *AVP1* gene (Zhen *et al.*, 1997) with enhanced H^+ pumping capability in the yeast Na^+ hypersensitive strain *ena1* can restore the salt tolerance to salt-sensitive *ena1* mutants. The work of Gaxiola *et al.* (1999) supports a model whereby expression of a plant vacuolar H^+-PPase could contribute to better growth of *ena1* in the presence of a high external Na^+ concentration by pumping more Na^+ from the cytosol into vacuoles. This work demonstrates that the yeast *ena1* mutant strain can be

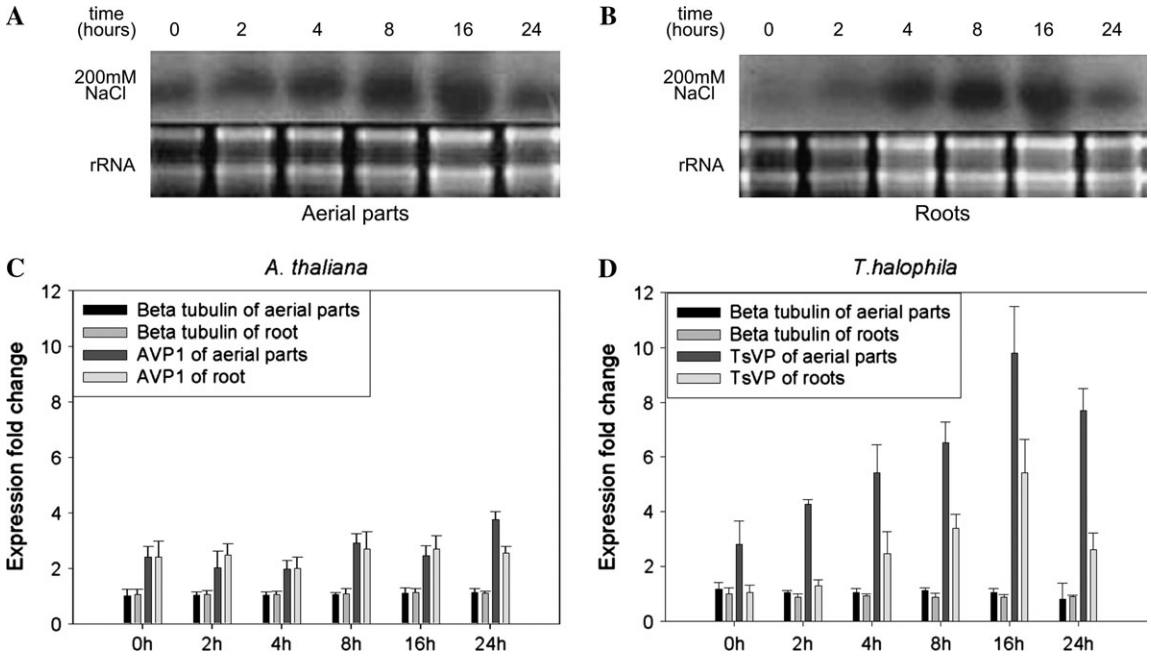

Fig. 2. Expression patterns of *AVP1* and *TsVP* under salt stress. Plants were irrigated with 200 mM NaCl solution, and total RNA was isolated from the leaves and roots of *T. halophila* at six different time points (0, 2, 4, 8, 16, and 24 h). A 20 μg aliquot of total RNA was subjected to northern blotting analysis with ^{32}P-labelled full-length TsVP cDNA as probe, shown in (A) and (B). Expression patterns of *AVP1* in *Arabidopsis* and *TsVP* in *T. halophila* under salt stress were analysed by real time RT–PCR with β-tubulin as internal control. The fold change in expression of *AVP1* and *TsVP* is shown in (C) and (D), respectively.

used to identify the function of the plant vacuolar H^+-PPase (Brini *et al.*, 2005). In the present work, overexpression of *TsVP* can restore the salt tolerance of the salt-sensitive *ena1* mutant in a similar way to *AVP1*. As shown in Fig. 3, all yeast strains grew well on YPGAL medium without NaCl. When these yeast strains grew on YPGAL medium that had different concentrations of NaCl (0.25, 0.50, and 0.75 M), the *ena1* mutants carrying the pYES2-*TsVP* or pYES2-*AVP1* plasmids showed a similar growth status, which was much better than the *ena1* mutants carrying empty pYES2 plasmids but still weaker than the wild-type yeast. These findings were consistent with previous work on *AVP1-D* heterologous expression in the yeast *ena1* mutant, suggesting that TsVP is an H^+-PPase similar to AVP1.

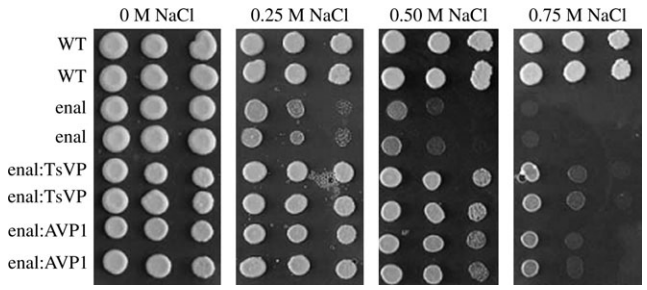

Fig. 3. Functional characterization of TsVP from *T. halophila* using the yeast mutant *ena1*. Growth of wild-type yeast (WT), the *ena1* mutant carrying the pYES2 vector (*ena1*) and *ena1* carrying pYES2-TsVP (*ena1*: TsVP) or pYES2-AVP1 (*ena1*: AVP1) on YPGAL (1% yeast extract, 2% peptone, 2% galactose) medium without NaCl or with different concentrations of NaCl (0.25, 0.50, and 0.75 M). The heterologous expression of *TsVP* or *AVP1* in *ena1* can partly restore the salt tolerance of the yeast mutant *ena1*, and the similar growth status of *ena1* carrying pYES2-TsVP or pYES2-AVP1 on YPGAL medium supplemented with different concentrations of NaCl is shown.

Generation of TsVP *and* AVP1 *transgenic tobacco plants*

TsVP and *AVP1* were each inserted into the plant expression vector pROKII under the control of the CaMV 35S promoter and with the plant selective marker gene nptII. The resulting plasmids were named pROKII-*TsVP* and pROKII-*AVP1*. *Nicotiana tabacum* Wisconsin38 was transformed with *A. tumefaciens* (LBA4404) carrying pROKII-*TsVP* or pROKII-*AVP1*, and 15 independent *TsVP* transformants were identified by PCR. Eleven *TsVP* transgenic tobacco lines were subjected to Southern and northern blotting to test DNA integration and RNA expression further. The hybridization results from six transgenic tobacco plantlets are shown in Fig. 4A and B. Three *TsVP* transgenic tobacco lines TL2, TL3, and TL5 are shown in lanes 2, 3, and 5 in Fig. 4A and B. Two *AVP1* transgenic tobacco lines AL3 and AL7 with the highest levels of expression were used in further assays.

ATPase and PPase activity

The *TsVP* gene was transferred into tobacco and expressed at different levels. To confirm the function of TsVP, the

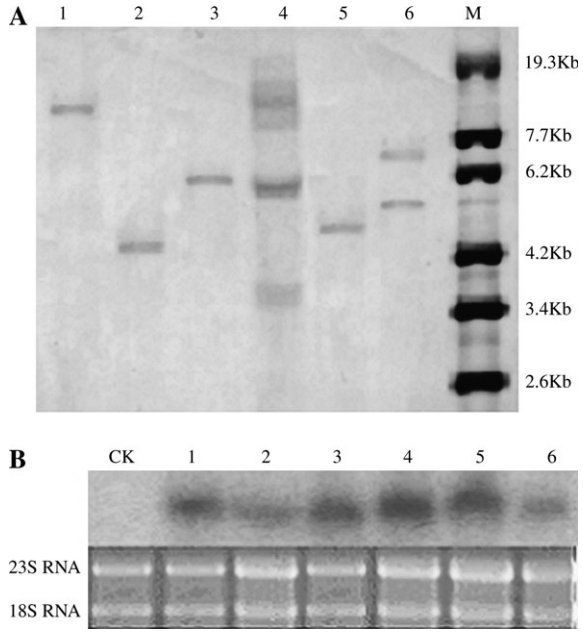

Fig. 4. Southern and northern blot analysis of transgenic tobacco plants. (A) M presents the λDNA/*Hin*dIII molecular weight marker; lanes 1–6 show genomic Southern blotting analysis of different transgenic tobacco plants. Genomic DNA from the leaves of transgenic tobacco was digested with *Hin*dIII, which has one cut site in the T-DNA region and no cut site in the *TsVP* cDNA region, and hybridized with labelled full-length *TsVP* cDNA as probe. (B) CK, an untransformed tobacco plant; lanes 1–6, different transgenic tobacco plants corresponding to transgenic tobacco lines 1–6. Northern blotting analysis was performed using total RNA from the extended leaves of the plants. Hybridization was performed with the ^{32}P-labelled full-length *TsVP* cDNA as probe.

hydrolytic activity of H^+-PPase was measured using tobacco tonoplast vesicles. The purity of the tonoplast vesicles was between 52% and 63% as determined by the hydrolytic activity of V-ATPase in the presence or absence of the specific V-ATPase inhibitor 50 mM NO_3^-. The hydrolytic activity of ATPase and PPase, which was determined by measuring the release of inorganic phosphate, is shown in Fig. 5A and B. The V-ATPase activity of wild-type tobacoo and the three TsVP transgenic tobacco lines was comparable, whereas the H^+-PPase hydrolytic activity in transgenic tobacco lines was higher than that of wild-type tobacco. The level of H^+-PPase hydrolytic activity in the transgenic tobacco lines was correlated with the level of *TsVP* gene expression. The H^+-PPase hydrolytic activity was significantly higher in transgenic tobacco lines TL3 and TL5, which had higher levels of *TsVP* gene expression than TL2, and was significantly different from that in wild-type tobacco (P <0.05) by t-test, while the hydrolytic activity of H^+-PPase in TL2 was only modestly higher than that in the wild-type tobacco. PPi-dependent proton transport of homozygous T_2 transgenic tobacco lines TL3 and TL5 was measured using a quinacrine fluorescence assay in which the decrease (quenching) of fluorescence intensity accompanied the formation of inside-acid ΔpH. The rate and magnitude of ΔpH formation were always higher in reactions with vesicles isolated from *TsVP* transgenic tobacco (Fig. 5C).

Leaf discs from transgenic tobacco with overexpression of TsVP *or* AVP1 *are salt tolerant*

Leaf discs from transgenic tobacco TL2, TL3, TL5, AL3, and AL7, and wild-type tobacco were floated on 400 and 600 mM NaCl solution, respectively, for 60 h. The amount of chlorophyll was compared between the transgenic and wild-type tobacco plants. The results indicated that chlorophyll was lost from the *TsVP* and *AVP1* transgenic tobacco leaves at a slower rate than from leaves of wild-type tobacco under two different NaCl concentrations (Fig. 6A), and no distinct difference between *TsVP* and *AVP1* transgenic tobacco plants was observed. The result also reflected that the transgenic plants with higher expression of *TsVP* and higher hydrolytic activity of vacuolar H^+-PPase possessed a better salt tolerance, and the transgenic tobacco TL2 that had lower expression of *TsVP* was less salt tolerant than TL3 and TL5. The transgenic tobacco lines TL3 and TL5 were used for further assays.

Viability of tobacco mesophyll protoplasts under salt tolerance

Tobacco mesophyll protoplasts were treated with CPW9M containing different concentrations of NaCl (100, 200, 300, and 400 mM). Under NaCl shock stress, the protoplasts showed changes in appearance and some of them lost the ability to fluoresce after staining with FDA. The viability of mesophyll protoplasts decreased with higher NaCl concentrations (Fig. 6B). Both the *TsVP* and the *AVP1* transgenic tobacco had higher viability (P <0.01) of mesophyll protoplasts at different NaCl concentrations, which indicated a higher salt tolerance of the cells. The assay for viability of mesophyll protoplasts also reflected that *TsVP* and *AVP1* transgenic tobacco plants have the same level of salt tolerance.

More biomass of transgenic plants under salt treatment

To study further the difference between transgenic and wild-type tobacco under salt treatment, biomass (dry weight) was measured under three different NaCl concentrations. Biomass decreased gradually with increased salt concentrations. The differences in biomass between wild type and TL3, TL5, AL3, and AL7 at the same NaCl concentration became more distinct at higher salt concentrations (Fig. 6C). For example, at 300 mM NaCl, the biomass of transgenic tobacco discs was 60% greater than that of wild type, with P <0.01 (n=6) by t-test.

Faster Na^+ uptake in transgenic tobacco than in the wild type

Leaf discs from transgenic lines TL3 and TL5 showed faster uptake of Na^+, and the speed of Na^+ uptake by the leaf discs

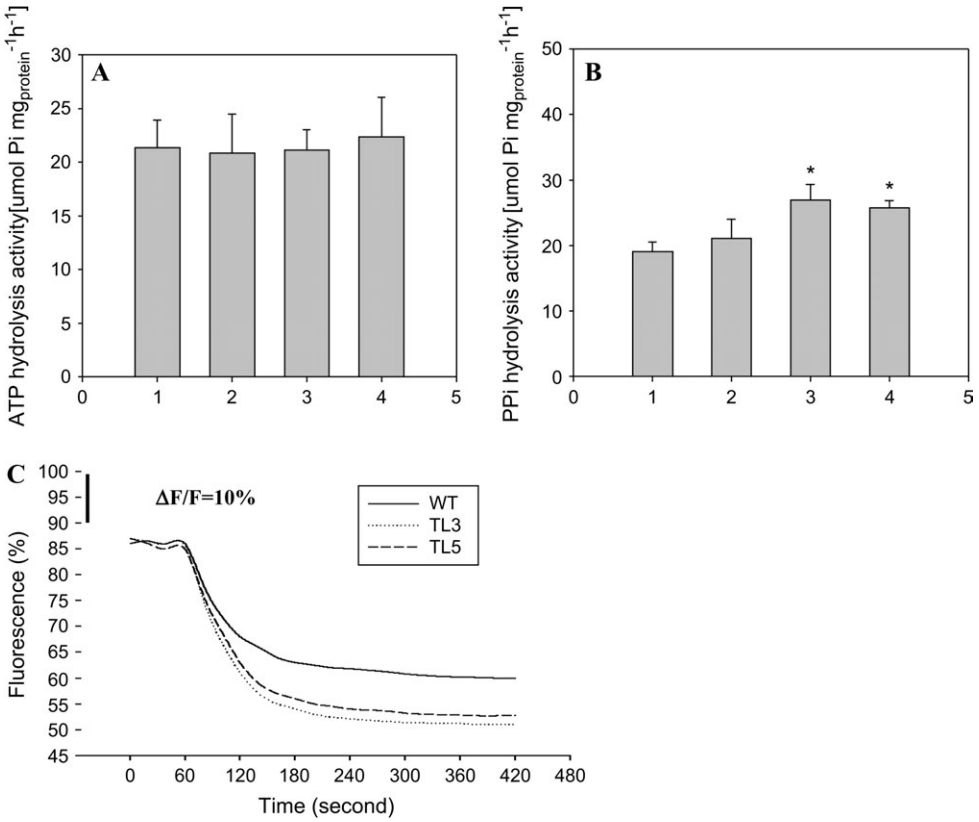

Fig. 5. Higher vacuolar H^+-PPase hydrolytic and H^+ pumping activity depended on PPi in transgenic tobacco plants not under salt stress. (A) Vacuolar V-ATPase activity is expressed as the difference between the values from measurement in the absence and presence of 50 mM NO_3^-. (B) H^+-PPase hydrolytic activity represents K^+-stimulated hydrolysis of PPi substrate. Numbers (1, 2, 3, and 4) on the abscissa represent different tobacco lines: 1 represents wild-type tobacco (WT), and 2, 3, and 4 represent TL2, TL3, and TL5 transgenic tobacco lines, respectively. Date are the means ± SE of three samples from a plant. An asterisk indicates a significant difference at the 0.05 level by *t*-test. (C) PPi-dependent quenching of quinacrine fluorescence by tonoplast vesicles. $MgSO_4$ (3 mM) was added to initiate the reaction. F=total fluorescence; ΔF=change in fluorescence. The rates of H^+ pumping are shown as fluorescence quenching per second. The data are the results of three independent samples from every tobacco line.

was significantly different compared with wild-type tobacco from 2 to 8 h, with $P < 0.05$, as shown in Fig. 7.

Transgenic plants retain more solutes under non-salt stress conditions

The amount of solutes in the leaves was measured under non-NaCl stress conditions. The leaves of transgenic tobacco lines TL3 and TL5 retained 25% more solutes than those of wild-type tobacco. The osmotic pressure or solute potential (ψ_S, refers to the concentration of osmotically active particles dissolved in water) of the wild-type plants was less negative than that of the transgenic lines TL3 and TL5 under non-NaCl stress conditions. The ψ values of the wild-type plants, and transgenic lines TL3 and TL5 were –0.807 MPa (SE=0.026, n=6), –0.986 MPa (SE=0.068, n=6), and –1.012 MPa (SE=0.067, n=6), respectively. The data showed that a significant difference existed in solutes between the wild-type and transgenic plants, with $0.01 < P < 0.05$ by *t*-test under non-NaCl stress conditions.

Ion concentrations in plant tissues

The amounts of Na^+, K^+, Ca^{2+}, and Cl^- were measured in the leaves and roots of plants under different NaCl concentrations (0, 100, 200, and 300 mM). In the leaves, the Na^+ and Cl^- concentration increased**,** and the K^+ or Ca^{2+} concentration decreased with increasing NaCl concentrations from 0 to 300 mM. The Na^+ content of leaves was from 20% to 30% higher in transgenic lines TL3 and TL5 than that in wild-type plants under different NaCl treatments, and the difference between the transgenic and wild-type plants was significant with the 300 mM NaCl treatment ($P < 0.05$) (Table 1). Furthermore, the K^+ or Ca^{2+} content was also slightly higher than that in the wild type with different salt treatments, but the difference was not significant. In the roots, the Na^+ or Cl^- content increased with the increase of salinity. However, the K^+ or Ca^{2+} content increased with the increase of salinity, which is different from the situation in leaves.

To study further the effects of excessive Na^+ on plants, leaf cell electrolyte leakage (%), that represents the degree

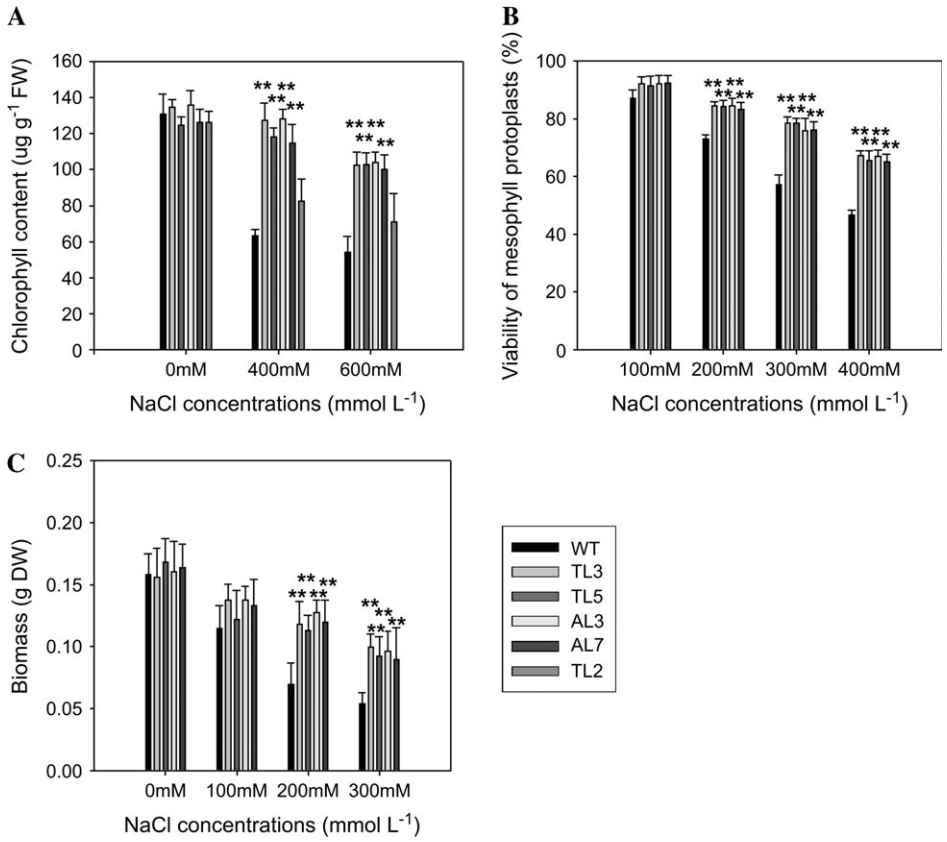

Fig. 6. Transgenic tobacco plants showed enhanced salt tolerance. Chlorophyll content of NaCl-treated tobacco leaf discs. The leaf discs of transgenic tobacco TL2, TL3, TL5, of TsVP, AL3, AL5, of AVP1, and wild-type tobacco (WT) were floated on solutions with different concentrations of NaCl (0, 400, and 600 mM) for 60 h under continuous light at 25 °C. Data are the means±SE (n=3). (B) The viability of tobacco mesophyll protoplasts under salt shock stress. The viability of mesophyll protoplasts was assessed by the fluorescein diacetate (FDA) staining method. The percentages of viable protoplasts in CPW9M solution supplemented with different concentrations of NaCl were counted. (C) Biomass (dry weight, DW) of tobacco seedlings after 25 d salt treatment with different concentrations of NaCl. Data are the means ±SE (n=6). The biomass of transgenic tobacco TL3 and TL5 for *TsVP*, and AL3 and AL7 for *AVP1* is significantly different from wild type (WT), with P <0.01 by t-test, under 200 and 300 mM NaCl stress conditions. Asterisks indicate a significant difference from the WT under the same NaCl concentration at *P <0.05 or **P <0.01 by the t-test, respectively.

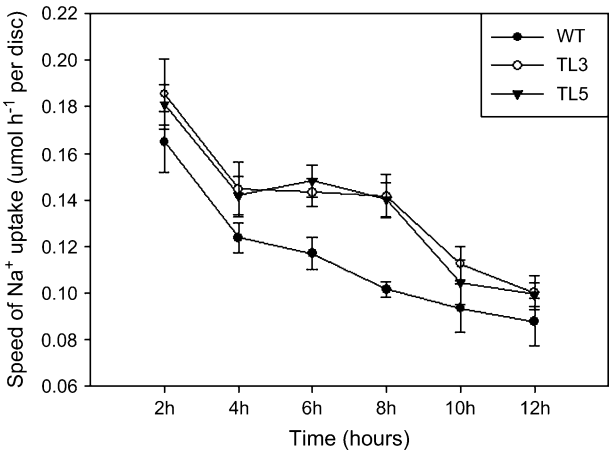

Fig. 7. Instant uptake of Na^+ by tobacco leaf discs. In transgenic lines TL3 and TL5, the speed of Na^+ uptake was quicker than that of wild-type tobacco (WT). The speed of Na^+ uptake from 4 h to 8 h was significantly different from wild-type tobacco at P <0.05 by t-test. The x-axis represents the incubation times of leaf discs in 300 mM NaCl solution.

of cell membrane damage under stress, and MDA content were determined. MDA content and leaf cell membrane damage increased with the increase in salt concentration. In transgenic tobacco lines TL3 and TL5, the amount of MDA was 43% and 76%, respectively, less than that of wild-type plants (Fig. 8A). Leaf cell electrolytes leakage was 29% and 34% respectively, less than that of wild-type plants at 300 mM NaCl (Fig. 8B).

Discussion

Thellungiella halophila is closely related to *Arabidopsis* and its genome size is approximately twice that of *Arabidopsis*. In recent years, *T. halophila* has become an important model for abiotic stress research (Amtmann *et al.*, 2005). The present work focuses on an H^+-PPase, which is located in tonoplasts and plays an important role in the plant's basic biochemical and physiological

systems. The gene *TsVP* from *T. halophila* is the first gene encoding an H^+-PPase isolated from a halophyte. The deduced amino acid sequence contains 771 amino acids and encodes 14 transmembrane domains. The transmembrane model is identical to H^+-PPases of other species (Maeshima, 2000), and five conserved domains used to identify H^+-PPase homologues by Drozdowicz and Rea (2001) were also found in the deduced amino acid sequence. TsVP protein shares 96% identity with the AVP1 protein at the amino acid level; the different amino acids were mainly clustered near the N-terminus from residue 4 to 150. The function of *TsVP* was characterized by heterologous expression in the yeast salt-sensitive mutant *ena1*, which was also used to demonstrate the function of *Arabidopsis AVP1* by Gaxiola *et al*. (1999).

Gaxiola *et al.* (2001) have reported that the overexpression of the vacuolar H^+-PPase gene *AVP1* in transgenic *Arabidopsis* plants resulted in drought and salt tolerance, and ascribed these properties to the increased accumulation of solutes. In the study of Gaxiola *et al*., the transgenic plants accumulated more solutes without salt stress and more Na^+ and K^+ in their leaf tissues under salt stress conditions. In the present experiments, H^+-PPase genes from *A. thaliana* or *T. halophila* were heterologously expressed in the yeast mutant *ena1* and tobacco. Salt tolerance in transgenic tobacco was correlated with *TsVP* gene expression and H^+-PPase activity. The results also indicated that the salt tolerance produced by heterologous expression of *TsVP* or *AVP1* was at a similar level. With no NaCl stress, the transgenic tobacco leaves accumulated more solutes than the wild-type leaves, which was consistent with Gaxiola's report. The higher viability of tobacco mesophyll protoplasts under NaCl stress in the transgenic tobacco plants may be due to more solutes in leaf tissues. Under salt stress conditions, more Na^+ accumulated in the leaves of the transgenic plants than in the leaves of the wild type. The difference was significant when treatment was with 300 mM NaCl. K^+ and Ca^{2+} were only modestly increased in transgenic tobacco leaf tissues. With increasing salinity, the K^+ and Ca^{2+} content decreased in the leaves and increased in the roots, especially for K^+. This may be due to increased uptake of K^+ and Ca^{2+} by the roots under salt stress, and the excess Na^+ may have disrupted the transport of K^+ and Ca^{2+} from the roots to the leaves. In the case of Cl^-, there was no significant difference between the wild-type and transgenic tobacco.

To study further the effects of excessive Na^+ on tobacco, MDA content and cell membrane damage were measured in leaf tissues. MDA content and cell membrane damage were less in transgenic tobacco than in wild-type tobacco under salt stress conditions. These results demonstrated that transgenic tobacco was healthier or subjected to less damage under salt stress. The correlation in our results between higher Na^+ and less damage in transgenic tobacco leaf tissues supports the hypothesis that the overexpression

Table 1. *Na^+ concentration (mmol g^{-1} DW) in transgenic and wild-type tobacco leaves at different concentrations of NaCl*
Data are means ±SE, (n=3). An asterisk represents a significant difference from the wild type under the same NaCl concentration at the 0.05 level by t-test.

NaCl concentration (mM)	Na^+ concentration in leaves (mmol g^{-1} DW)		
	Wild-type plants	TsVP transgenic TL3	TsVP transgenic TL5
0	0.0329±0.00806	0.0339±0.00458	0.0366±0.00263
100	1.28±0.0745	1.52±0.136	1.45±0.0965
200	1.67±0.131	2.21±0.191	2.13±0.0963*
300	1.72±0.148	2.74±0.100*	2.24±0.0742*

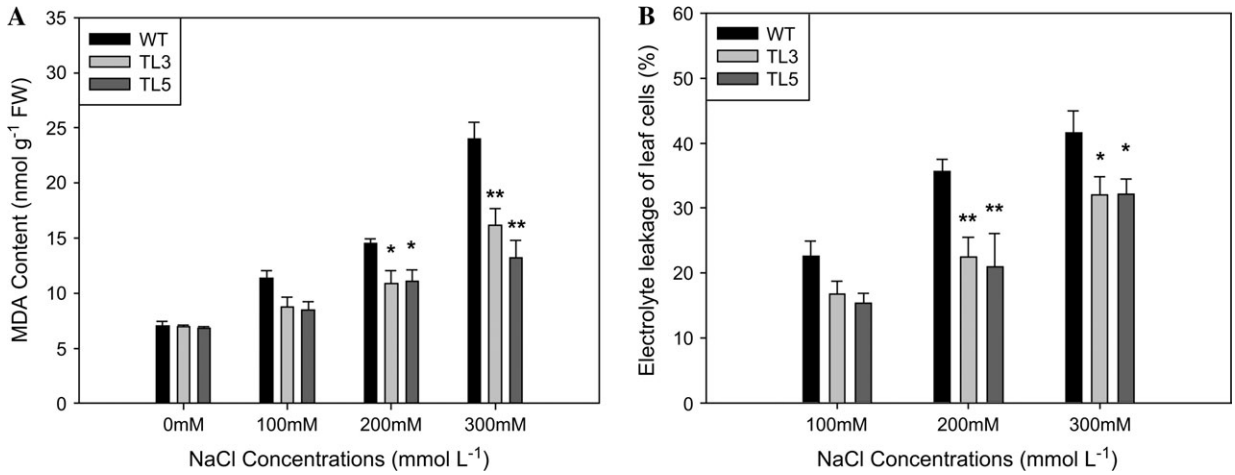

Fig. 8. Membrane damage of transgenic tobacco under salt stress. The tobacco plantlets were cultured on MS medium supplemented with different concentrations of NaCl for 25 d; MDA content and the electrolyte leakage of leaf cells were measured. (A) The MDA content of the leaves; (B) the electrolyte leakage of leaf cells. Data were means ±SE (n=6). The asterisks indicate a significant difference from the WT under the same NaCl concentration at *0.01< P <0.05 and **P <0.01 in t-test, respectively.

of H^+-PPase can enhance the accumulation of Na^+ in vacuoles. Accumulation of Na^+ in vacuoles instead of in the cytoplasm can avoid the toxicity of excessive Na^+ to plant cells. In view of the higher viability of tobacco mesophyll protoplasts in the transgenic tobacco plants under NaCl shock stress, it is concluded that higher salt tolerance in the transgenic tobacco was the result of better adaptability to salt stress for shorter times and more Na^+ accumulation in vacuoles instead of in the cytoplasm. The successful heterologous expression of *TsVP* in tobacco and enhanced tobacco salt tolerance demonstrate that this gene from a halophyte can be beneficial for plant genetic engineering to enhance plant salt tolerance.

A comparative study of the genetic effects of salt tolerance between *T. halophila* and *A. thaliana* could reveal differences in the mechanisms of plant salt tolerance between the glycophyte and halophyte. In *T. halophila*, the expression pattern of *TsVP* was analysed under salt stress at different time points. *TsVP* mRNA levels in both roots and leaves increased during the first 16 h and decreased after 16 h. The expression of *AVP1* in *Arabidopsis* showed no distinct change under salt stress. This suggests that the regulation of expression of the H^+-PPase gene is different between *T. halophila* and *A. thaliana* under salt stress. This difference might result from two possibilities: one is that some transcription factors are different in *T. halophila* and *A. thaliana*, the other is that the promoter sequence or enhancer sequence of the H^+-PPase gene is different in *T. halophila* and *A. thaliana*.

In the present report, the salt tolerance from heterologous expression of *TsVP* or *AVP1* was at similar levels; this might suggest that TsVP and AVP1 have almost the same biochemical function. However, the expression patterns were different between *T. halophila* and *A. thaliana* under salt stress. Although it is not confirmed that different gene expression patterns contribute to the difference in salt sensitivity between *T. halophila* and *A. thaliana*, it is concluded that the differences in salt sensitivity between *A. thaliana* and *T. halophila* cannot be attributed to the function of H^+-PPase. It could be the result of some subtle regulatory networks correlated with basic biochemical and physiological mechanisms in plants under salt stress. Of course, it cannot be absolutely excluded that there are some specialized genes for salt tolerance in *T. halophila*.

It was reported that the hydrolytic activity of H^+-PPase firstly increased and then decreased in the leaves of halophytic *Suaeda salsa* L. under 400 mM NaCl stress (Wang *et al.*, 2001). Recently, mRNA from a tonoplast H^+-PPase gene was found to increase in the roots for a short time and there was no detectable increase in the level of transcript in the leaves under salt stress in barley (Fukuda *et al.*, 2004). Combining *TsVP* expression patterns in *T. halophila* in the present report, it is concluded that the changes of H^+-PPase activity were different in different tissues of different species under salt stress conditions.

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