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# Cloning of a vacuolar $H^+$-pryophosphatase gene from emphemeral plant *Olimarabidopsis pumila* whose overexpression improve salt tolerance in tobacco

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***Olimarabidopsis pumila*** **is a close relative of the model plant *Arabidopsis thaliana* but, unlike *A. thaliana*, it is a salt-tolerant ephemeral plant that is widely distributed in semi-arid and semi-salinized regions of the Xinjiang region of China, thus providing an ideal candidate plant system for salt tolerance gene mining. The vacuolar $H^+$-translocating inorganic pyrophosphatase (V-$H^+$-PPase) is an electrogenic proton pump that play pivotal role in translocating protons into vacuoles in plant cells. A V-$H^+$-PPase gene, *OpVP*, was isolated from *O. pumila* in this study. The *OpVP* cDNA has an open reading frame of 2 313 bp, encoding a polypeptide of 770 amino acid residues with an estimated molecular mass of 80.7 kDa. The OpVP shows high amino acid similarity with other Brassicaceae V-$H^+$-PPase genes. Expression profiles under salt and drought treatment, abcisic acid (ABA), indole-3-aceticacid (IAA) and gibberellins (GA) induction were investigated, and the results reveal that the expression of *OpVP* was induced in leaves under treatment with salt, drought, ABA, IAA and GA3. Overexpression of the *OpVP* gene confers enhanced salt tolerance to the transformed tobacco. Transgenic tobacco grows well in the presence of 200 mM NaCl, while wild-type plants exhibit chlorosis and growth inhibition, even death. Compared with wild-type, transgenic plants accumulated more $Na^+$ in leaves. Moreover, the leaves of transgenic plants retain higher chlorophyll content during salt stress. This study shows that *OpVP* is a potential gene for salt tolerance, and can be used in future for developing salt tolerant crops.**

**Key words:** $H^+$-pyrophoshatase, *Olimarabidopsis pumila*, salt, abiotic stress.

## INTRODUCTION

Abiotic stress, such as drought, salinity and extreme temperature, is one of the primary causes of crop loss worldwide, reducing average production of major crop plants. Salinity is a major environmental factor limiting crop growth and productivity (Ashraf, 1994). It was estimated that 20% of all cultivated land, and nearly half of irrigated land, is affected by salinity (Rhoades and Loveday, 1990). In China, more than 90 million hectares of the land are exposed to salinity or secondary salinity. Xinjiang occupies one-sixth of the area of China, but more than one-third of the land in Xinjiang is salinized. Therefore, developing salt-tolerant varieties of crops is an important breeding goal in Xinjiang. Owing to extreme ecological environment, however, salt tolerant organisms are abundant in Xinjiang. An important research project is to mine salt-resistance genes from such plants which can

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**Abbreviation: PCR,** polymerase chain reaction; **qRT-PCR,** quantitative Real-time RT-PCR; **RACE,** rapid amplification of cDNA ends; **dNTPs,** deoxyribonucleotide tri phosphate; **ABA,** abcisic acid; **IAA,** indo-3-aceticacid; **GA,** gibberellins; V-$H^+$-PPase,vacuolar $H^+$-pyrophosphatase; **PPi,** inorganic pyrophosphate; CS, conserved segment; **ORF,** open reading frame.

be used in future crop improvement.

*Olimarabidopsis pumila* (Stephan) Al-Shehbaz, O'Kane & R. A. Price [synonym: *Arabidopsis pumila* (Stephan) N. Busch] habitats semi-arid and semi-salinized land in Xinjiang. *O. pumila* is a close relative of *Arabidopsis thaliana*, but it is more tolerant to salt stress than *A. thaliana* (Hoffmann et al., 2010; Roy et al., 2010). Although the size of 1 C DNA (173 Mbp) of *O. pumila* is comparable to that of *A. thaliana* (167 Mbp), its genome size is about twice as large as that of *A. thaliana* (Hoffmann et al., 2010). *O. pumila* has 2n = 32 chromosomes, but it has the lowest mean DNA content per chromosome of the four Brassicaceae species *A. thaliana*, *Arabis auriculata*, and *Arabis montbretiana*. The estimated time of divergence of *A. thaliana* and *O. pumila* was 10 - 14 million years (Mya) (Clauss and Koch, 2006; Roy et al., 2010).

To cope with salt stress, plants have developed multifarious adaptation mechanisms to tolerate high concentrations of salt in the environment (Hamada et al., 2001). In high salt stress environment, plant can survive in salt stress environment by exclusion of excess $Na^+$ from the cytoplasm and sequestration of $Na^+$ from cytosol to vacuole towards the maintenance of ion homeostasis inside the cell. Many studies concentrated on the $Na^+/H^+$ antiporter proteins in the plasma membrane and tono-plast which play essential roles in $Na^+$ exclusion and compartmentalization, for example SOS1 (Qiu et al., 2003), NHX1 (Pardo et al., 2006), HKT1 (Haro et al., 2005). Previous studies showed that the $Na^+/H^+$ anti-porter (Apse et al., 1999; Gaxiola et al., 2001), $H^+$-adenosine triphosphatase ($H^+$-ATPase) and $H^+$-inorganic pyro-phosphatase ($H^+$-PPase) (Maeshima, 2001) coordinately regulate sodium ion concentration on vacuole.

*AtNHX1* gene was first identified from Arabidopsis genome, encoding $Na^+/H^+$ antiporter on tonoplast (Gaxiola et al., 1999). *NHX1* have been cloned from several plant species and its overexpression showed enhanced tolerance to salinity stress in transgenic plants. $H^+$-ATPase (V-ATPase) and $H^+$-inor-ganic pyrophosphatase (V-PPase) are two major electrogenic proton pump existed in plant vacuolar membrane. Both pumps create an $H^+$ electrochemical gradient that energize the activity of secondary active transporters, including tonoplast $Na^+/H^+$ antiporters. Vacuolar $H^+$-pyrophosphatase (V-$H^+$-PPase) is an enzyme that acidifies vacuole in plant cells, which maintaining vacuolar pH and catalyzing the hydrolysis of inorganic pyrophosphate (PPi) to energize proton transport from the cytoplasm into vacuoles (Maeshima, 2000). Comparison of V-$H^+$-PPase from different organisms has identified three conserved segment (CS). The first conserved segment (CS1) exposed to the cytosol includes the catalytic domain DVGADLVGKVE. The conserved segment (CS2) is also located in a hydrophilic loop. The third conserved segment (CS3) is in the carboxyl-terminal part containing a dozen residues. Research indicated that CS3 may be exposed to the cytosol and play a critical role in the catalytic function together with CS1 and CS2 (Rea et al., 1992; Mimura et al., 2004).

Transgenic plants overexpressing *Arabidopsis* V-$H^+$-PPase gene, *AVP1*, are much more tolerant to high concentrations of NaCl and to water deprivation than the non-transgenic plants (Gaxiola et al., 2001). Overexpression of *AVP1* in a commercial cultivar of tomato enhanced root system development which helps confer water deficit stress resistance in transgenic plants (Park et al., 2005). Multi-year field-trial data indicate that *AVP1*-expressing cotton leads to at least 20% more fiber yield than wild-type control plants in dry-land conditions, because of a large shoot mass in dryland conditions (Pasapula et al., 2011).

In addition to maintaining vacuo-lar pH, *AVP1* overexpression appears to facilitate auxin transport and lead to larger root system (Li et al., 2005). Recent research provided a surprising role for V-$H^+$-PPase gene which showed that the major function of *AVP1* in early seedling development is removal of inhibitory PPi rather than proton pump and vacuole acidi-fication (Bertoni et al., 2011; Ferjani et al., 2011). Other groups have also demonstrated that over-expression of similar genes encoding V-$H^+$-PPase can increase both salt- and drought tolerance in heterologous systems, including rice (Zhao et al., 2006), tobacco (Gao et al., 2006), and maize (Li et al., 2008).

In this study, we report the isolation, sequence analysis and functional characterization of *OpVP*, the V-$H^+$-PPase gene from *O. pumila*, and demonstrate that overexpression of *OpVP* can obviously increase salt tolerance of transgenic tobacco.

## MATERIALS AND METHODS

### Plant material and treatments

Seeds of *O. pumila* were collected from natural semi-arid and semi-saliferous soil located in the north of Xinjiang of China. The seeds were surface-sterilized for 20 min with 10% sodium hypochlorite, rinsed several times with sterile water and plated on square petri dishes with 0.5 × Murashige and Skoog (MS) medium (pH = 5.7) supplemented with 1% (w/v) sucrose and 0.1% (w/v) agar at 4°C for 3 days in the dark to synchronize germination. The plates were incubated in a growth chamber under a phototperiod of a 14-h/10-h light/dark cycle at 22°C. After 7 days, the seedlings were transplanted into soil and kept in a growth room with a 14-h photoperiod. When plants were 4 weeks old, the soil were watered with 0.5 × MS nutrient solution containing either 100 μM abcisic acid (ABA), or 30% PEG8000 (w/v, drought stress treatment), respectively. The leaves of these plants were collected at 0, 6, 12, 24 and 48 h after initiation of stress treatment, respectively. For the expression studies under different concentrations of NaCl treatment, 4-week-old seedlings were harvested from *O. pumila* plants after three days treatment with 0, 50, 100, 150, 200, 250, 300 and 500 mM NaCl, respectively. In the second set of experiment, 500 mM NaCl was given for different time period (0, 6, 12, 24, 48 and 72 h). All the samples were frozen immediately in liquid nitrogen and stored at -80°C until use.

### Isolation of *OpVP* from *O. pumila* by RT-PCR and 5′ and 3′ RACE

To isolate the V-H$^+$-PPase gene form *O. pumila*, degenerate primers were designed based on the conserved sequenced of all known dicot V-H$^+$-PPase genes. The forward primer was 5′-SWCCWGARTGTTRTSYSTAA-3′ and reverse primer is 5′-AWGTRRCGTASTGGCWRR-3′, where R represents A or G, Y represents C or T, S represents G or C, and W represents A or T. Total RNA was isolated from the leaves of *O. pumila* plants treated with 200 mM NaCl for 8 h using the plant RNA Mini-Prep Kit (Qiagen, Hilden, Germany). Total RNA (1 μg) was used for first-strand synthesis using the M-MLV reverse transcriptase according to manufacture protocol (Promega, USA). The reverse transcription products were amplified by polymerase chain reaction (PCR) under the following thermal cycle conditions: 94°C for 2 min, followed by 35 cycles of 94°C for 40 s, 55°C for 40 s, and 72°C for 90 s.

The PCR products were cloned into the pGEM T-easy vector (Promega, USA) and sequenced. Specific primers were then designed for rapid amplification of cDNA ends (RACE) according to the sequence information of the partial cDNA fragment to obtain the full-length sequence of the gene. For 5′ RACE, gene specific primers were GSP1 (5′-CAGAGGTAACAGCACCAAGAACG-3′), and GSP2 (5’-GTGCTGAATCCCTCAACAGAGCC-3′). Primers for 3′-RACE were: GSP1, 5′-TTCGCAGGCAGTTCAACACCATC-3′; and GSP2, 5′-GATTCCTCCTGGTTGCCTTGTCA-3′. The RACE reactions were performed using SMART$^{TM}$ RACE cDNA Amplificatin Kit (Clontech, USA) according to the manufacturer’s protocol. The RACE products were cloned and sequenced. A full-length cDNA was obtained by splicing 5′-RACE fragment, partial cDNA fragment and 3′-RACE fragment into together. The full-length cDNA was used to search putative open reading frame (ORF) with ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The longest open reading frame was amplified by RT-PCR using the primers OpVPF (5′-ATGGTGGCGACAGCTTTACTACCGG-3′) and OpVPR (5′-TTAGAGGTACTTGAAAAGGATACC-3′) under the following thermal cycle conditions: 94°C for 2 min, followed by 35 cycles of 94°C for 45 s, 58°C for 45 s, and 72°C for 2 min. The resulting PCR products were cloned into the pGEM T-easy vector and positive clone was sequenced. Multiple alignments were generated using Clustal W. Phylogenetic analysis were conducted using MEGA version 4 (Tamura et al., 2007), based on the Neighbor-Joining method. Robustness of the conducted phylogentic tree was tested using 1000 bootstrap repetitions.

### Expression of *OpVP*

Total RNA (1 μg) was reverse-transcribed in a sterile RNase-free microcentrifuge tube in a total volume of 10 μL with 0.5 μg oligo (dT) 18 primer, 1.0 μL dNTPs (10 mM), 2 μL 5 × M-MLV buffer, 0.25 μL RNase inhibitor (40 U/μL), and 0.5 μL reverse-transcriptase M-MLV (200 U/μL). Semi-quantitative RT-PCR was used to analyze the relative expression levels of *OpVP*. The gene-specific primers were OpVP-RTF (5′-GCCTGGGACAACGCCAAGAAGTA-3′, forward) and OpVP-RTR (5′-CACCGTGAGTGGCAAAGAAGGGA-3′, reverse). Actin-F (5′-GGTAACATTGTGCTCAGTGGTGG-3′) and Actin-R (5′-AACGACCTTAATCTTCATGCTGC-3′) were used to amplify the *Actin2* gene as an internal control. Amplification was performed for 28 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s. PCR products were subsequently separated on a 1.2 % (w/v) agarose gel, then stained with ethidium bromide and photographed under UV light.

For quantitative real-time polymerase chain reaction (qRT-PCR), gene-specific primers and *Actin2* gene primers were the same as above. qRT-PCR was performed as follows: 10 ng cDNA, 5 pM of each primer and SYBR® *Premix Ex Taq* ™ (Perfect Real Time) mix (TaKaRa, China) were mixed and amplified in light cycler® 480 real-time PCR system (Roche, Swtzerland) in a 20 μL final reaction volume. The threshold cycles (Ct value) of the target gene and control in different samples were obtained after qRT-PCR. Relative difference (N) was the number of treated target gene transcript copies relative to the untreated gene transcript copies, and was calculated as follows: $N = 2^{-\Delta\Delta Ct} = 2^{-(\Delta Ct\ treated\ -\ \Delta Ct\ control)}$, where $\Delta\Delta Ct$ = $\Delta Ct$ of the treated sample minus $\Delta Ct$ of the untreated control sample, and $\Delta Ct$ is the difference in threshold cycles for the *OpVP* target and the *Actin2* internal reference. For statistical analyses of gene expression among different treatments, one-way ANOVA was performed to assess significant difference between control and each treatment.

### Construction of plant expression vectors and tobacco transformation

The complete ORF was amplified with the primers OpVP-P3 (5’-CGGGATCCATGGTGGCGACAGCTTTACTACCGG-3’, underline indicate the site of restriction enzyme *Kpn* I ) and OpVP-P4 (5’-GCTCTAGATTAGAGGTACTTGAAAAGGATACC-3’, underline indicate the site of restriction enzyme *Xba* I ) under the following cycle parameters: 94°C for 2 min, followed by 35 cycles of 94°C for 45 s, 56°C for 45 s, and 72°C for 2 min. The resulting PCR products were cloned into the pGEM T-easy vector and positive clone was sequenced. The confirmed plasmid of pGEM T-OpVP were cut by *Kpn* I and *Xba* I restriction enzymes, then was inserted into the binary vector pCAMBIA2300-35S-OCS in which transgene expression was under the control cauliflower mosaic virus 35S promoter containing the neomycin phosphotransferase (*npt* II ) gene conferring resistance to kanamycin as a selectable marker. The resulting plasmid was mobilized into *Agrobacterium tumefaciens* GV3101 and transformed in tobacco (*Nicotiana tabacum* L.) with standard protocol (Horsch et al., 1986). We generated several independent homozygous transgenic tobaccos of T3 generation to be used in the following assays.

### Chlorophyll content

Chlorophyll content of T3 transgenic plants grown under salt stress was estimated according to the method described by Arnon (1949). 0.1 g plant tissue was homogenized in 80% acetone and incubated in dark for 6 h. The homogenate was centrifuged at 10 000 rpm for 10 min. Supernatant obtained was read at 649 and 665 nm in Spectra Max plus-384 (Molecular device, USA) and total chlorophyll amount was calculated.

### Measurement of $Na^+$ content

The WT and T3 transgenic tobacco plants were grown and salt stressed as described above. Leaves tissue were harvested after 7 days and 16 days of salt treatment, respectively. The leaves were dried in an oven at 60°C and their dry weights were measured till the constant weight was achieved. The dried leaves were extracted with 1 M $H_2SO_4$ as described by Storey (1995). The supernatants were determined using an atomic absorption spectrophotometer (Shanghai Precision & Scientific Instrument Co., China) to quantify $Na^+$ content.

## RESULTS

### Isolation and characterization of V-H$^+$-PPase (*OpVP*) gene from *O. pumila*

The full-length cDNA of *OpVP* was obtained by RT-PCR

and RACE. The *OpVP* cDNA consists of 2 698 bp, which includes 130 bp 5′ leader sequence, and 208 bp 3′-noncoding region. The open reading frame (ORF) of *OpVP* is 2 313 bp encoding a protein of 770 amino acids (GenBank accession number: KF557584) with a therore-tical molecular mass of 80.7 kDa. Phylogenetic analysis (Figure 1) showed that OpVP formed a clade with the most closely related plant $H^+$-PPase homologs. The highest identity was found to be 98.3% similarity with *Arabidopsis lyrata*. OpVP also showed high similarity to V-$H^+$-PPase from *A. thaliana* AVP1 (98.2%), AVP3 (98.2%), *Brassica rapa* BrVP1 (95.2%), and *Thellungiella salsuginea* TsVP (95.2%).

## Expression analysis of *OpVP* in *O. pumila*

For studying the expression analysis of *OpVP*, qRT-PCR was carried out using cDNA from different NaCl concentrations (0, 50, 100, 200, 250, 300 and 500 mM) treated plants (Figure 2A). In the absence of NaCl condition, expression of *OpVP* was low. When treated with different concentrations of NaCl for three days, *OpVP* transcripts increased significantly as the NaCl concentration raised ($P < 0.05$), indicating that the expression of *OpVP* was induced by salt stress (Figure 2A). The expression of *OpVP* in leaves of *O. pumila* plants corresponding to the different time period was also measured at 500 mM NaCl treated (Figure 2B). The results showed that the transcript did not increased at 6 h, but gradually increased from 12-72 h, and reached at a maximum at 48 h ($P < 0.05$).

The expression profile of *OpVP* in seedlings of *O. pumila* was investigated in the presence of 20% PEG8000, 1 μM abcisic acid (ABA), 1 μM indo-3-aceticacid (IAA) and 2 μM gibberellins (GA3) by semiquantitative RT-PCR. The result demonstrated that drought stress, ABA application, IAA and GA3 induced transcription accumulation of *OpVP* (Figure 3). In the drought stress treatment, *OpVP* transcripts were detected at 0 h and induced at 1 h, and reached a peak at 2 h of treatment, after that gradually decreased (Figure 3A). Interestingly, the transcript level of *OpVP* also increased in response to exogenous ABA, which functions as a signaling molecule and plays an important role in stress tolerance.

Figure 3B showed that the transcript level of *OpVP* under 1 μM ABA induction was up-regulated, and reached a maximum at 2 h of 1 μM ABA treatment, and decreased only slightly after 48 h of treatment. In addition, the expression profiles of *OpVP* response to different concentration of IAA and GA3 were also invest-tigated. To our surprise, both IAA and GA3 can intensively induce the expression of *OpVP*. *OpVP* transcripts arrived at high value at 2 h of 1 μM IAA or 2 μM GA3 of treatment, and maintained high expression level until 12 h (Figure 3C and D).

## Overexpression of *OpVP* confers enhanced salt tolerance in transgenic tobacco

To investigate the function of *OpVP*, after transformation of tobacco plants with overexpression vector *p35S:OpVP* and selection with the kanamycin, several individual T3 transformants were produced. The expression of *OpVP* gene in all transgenic lines has been monitored by semi-quantitative RT-PCR. Figure 4A showed that *OpVP* were all expressed in 5 independent transgenic lines, however, L4 displayed much higher level of expression of *OpVP* than the four other lines. L2 and L4 were used for subsequent analysis. To assess the effect of salt tolerance, the seedling of both WT and transgenic plants were planted on soil pots. 4-week plants were subjected to incubation of 200 mM NaCl. Seven days later, all the plants showed no obvious effect, but some base rosette of WT turned white and died whereas the leaves in transgenic plants could continue to expand leaves and greening (figures were not supplied). After treated consecutively by 200 mM NaCl for 45 days, the WT plants grown weak and flowering, and the leaves became chlorotic, whereas the transgenic plants still remained in vegetative period and the leaves were green (Figure 4B).

To further evaluate the increased salt tolerance of transgenic tobacco overexpressing *OpVP*, chlorophyll contents of wild-type and both transgenic lines was determined. Chlorophyll contents were measured both in WT and transgenic plants of L2 and L4 stressed by 200 mM NaCl for 0, 7, 16 and 45 day, respectively. The wild type and the transgenic line L4 showed the same chlorophyll content, however, the transgenic line L2 showed higher chlorophyll content before salt stress ($P < 0.05$) (Figure 5). The contents of chlorophyll in all the experiment lines increased on 7 days. There were more chlorophyll contents in wild-type than both transgenic lines ($P < 0.05$). When stressed from 7 to 45 days, the chlorophyll content in WT and L2 plants reduced significantly with the stress time increase. However, the chlorophyll content in L4 plants reduced gradually, but it increased significantly when stressed to 45 days ($P < 0.05$). For example, after seven days of salt stress, there was 77% increase of the chlorophyll in wild-type, whereas only 40 and 61% in transgenic L2 and L4, respectively. However, after 45 days of salt stress, there was 50% increase of chlorophyll in L4, whereas there was 66% reduction in wild-type, and 50% reduction in L2. Comparatively, the chlorophyll content in L4 plants were highest at 45 days of salt stress, which is also correlated with the expression levels of *OpVP*.

## $Na^+$ accumulation in transgenic plants

The contents of $Na^+$ were determined in the leaves of plants from transgenic lines and wild-type grown under 200 mM NaCl treatments (0 and 16 days). In the absence

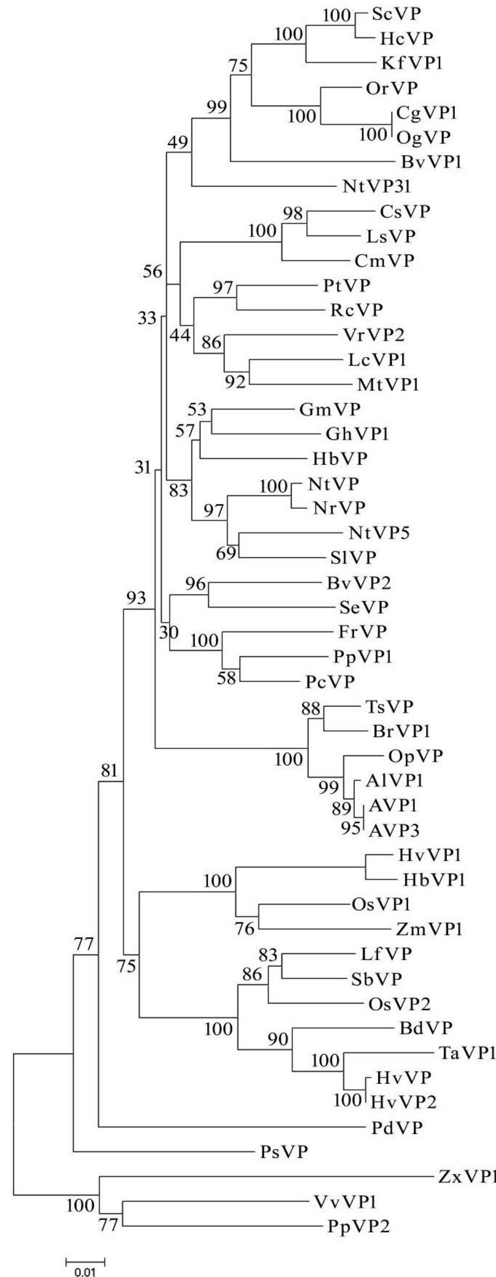


**Figure 1.** Phylogentic analysis of OpVP with other known plant H$^+$-pyrophosphatase homologs. Phylogentic analysis was based on typical vacuolar proton-pumping PPase from plants. The gene abbreviation and GenBank accession number are as the follows: *Suaeda corniculata* (ScCP; ADQ00196), *Halostachys caspica* (HcVP; ABO45933), *Kalidium foliatum* (KfVP1, ABK91685), *Oxybasis rubra* (OrVP; AAM97920), *Chenopodium glaucum* (CgVP1; ABD98735), *Oxybasis glauca* (OgVP; ABD98735), *Beta vulgaris* (BvVP1; AAA61610), *Beta vulgaris* (BvVP2; AAA61609), *Nicotiana tabacum* (NtVP; CAA58701), *Nicotiana tabacum* (NtVP5; CAA54869), *Nicotiana tabacum* (NtVP31; CAA58700), *Cucumis sativus* (CsVP; XP_004150506), *Lagenaria siceraria* (LsVP; AET95912), *Cucurbita moschata* (CmVP; BAA33149), *Populus trichocarpa* (PtVP; XP_002325187), *Ricinus communis* (RcVP; XP_002530755), *Vigna radiata* (VrVP2; BAA23649), *Lotus corniculatus* (LcVP1; ABS01290), *Medicago truncatula* (MtVP1; ACI22377), *Glycine max* (GmVP; XP_003531725), *Gossypiym hirsutum* (GhVP1; ADN96173), *Hevea brasiliensis* (HbVP; AAS66771), *Nicotiana rustica* (NrVP; ABF85694), *Solanum lycopersicum* (SlVP; XP_004241690), *Salicornia europaea* (SeVP; AEI17666), *Fragaria vesca* (FrVP; XP_004303283), *Prunus persica* (PpVP1; AAL11506), *Prunus persica* (PpVP2; AF367447), *Pyrus communis* (PcVP; BAC41250), *Thellungiella salsuginea* (TsVP; AAR08913), *Brassica rapa* (BrVP1; AET95910), *Olimarabidopsis pumila* (OpVP; KF557584), *Arabidopsis lyrata* (AlVP1; XP_002890120), *Arabidopsis thaliana* (AVP1; NP_173021), *Arabidopsis thaliana* (AVP3; AAA32754), *Hordeum vulgare* (HvVP; ACA63883), *Hordeum vulgare* (HvVP1; BAB18681), *Hordeum vulgare* (HvVP2; BAA02717), *Hordeum brevisubulatum* (HbVP1; AAP06752.1), *Oryza sativa* (OsVP1; BAA08232), *Oryza sativa* (OsVP2; BAA08233), *Zea mays* (ZmVP1; NP_001105380), *Leptochloa fusca* (LfVP; ACT98610), *Sorghum bicolor* (SbVP; ADJ67258), *Brachypodium distachyon* (BdVP; 003564217), *Triticum aestivum* (TaVP1; AAP55210), *Potamogeton distinctus* (PdVP; BAF63470), *Picea sitchensis* (PsVP, ABR18024), *Zygophyllum xanthoxylum* (ZxVP1; ABU92563), *Vitis vinifera* (VvP1; AAF69010).

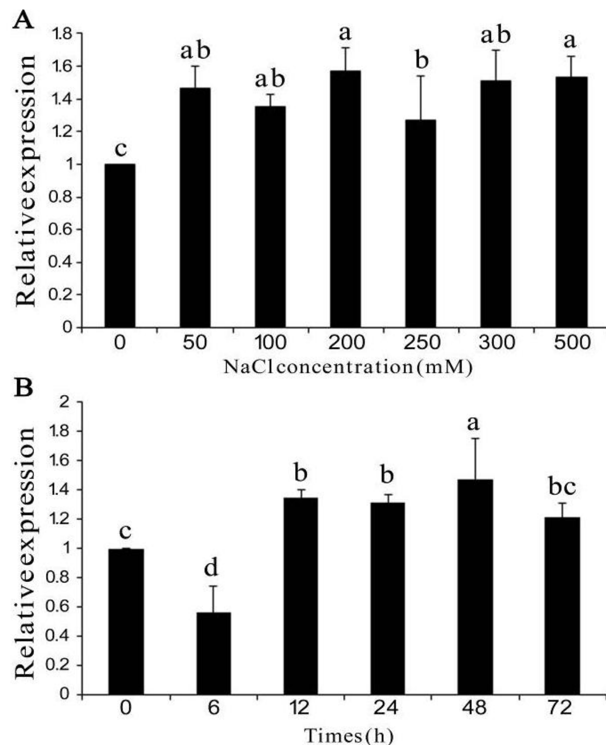


**Figure 2.** qRT-PCR analysis of the *OpVP* gene under NaCl stress condition. **A,** Different concentration of NaCl was given for 72 h. **B,** 500 mM NaCl for different time periods (h). Values are means ± SE. Similar letters indicate no significant difference at $P < 0.05$ using Duncan test.

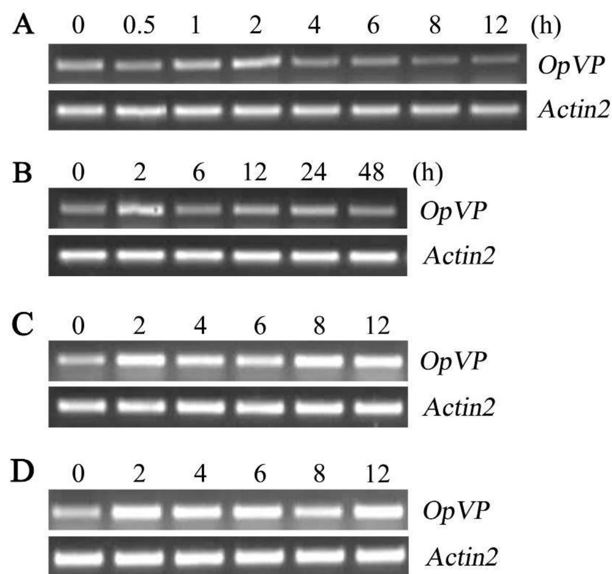


**Figure 3.** Expressing profiles of *OpVP* in *O. pumila* seedlings in reponse to different stress and hormone induction. **A,** Expressing pattern of *OpVP* in the whole plant under dehydration 20% PEG8000. **B,** Expressing pattern of *OpVP* under 1 μM ABA. **C,** Expressing pattern of *OpVP* under 1 μM IAA; D, Expressing pattern of *OpVP* under 2 μM GA3.

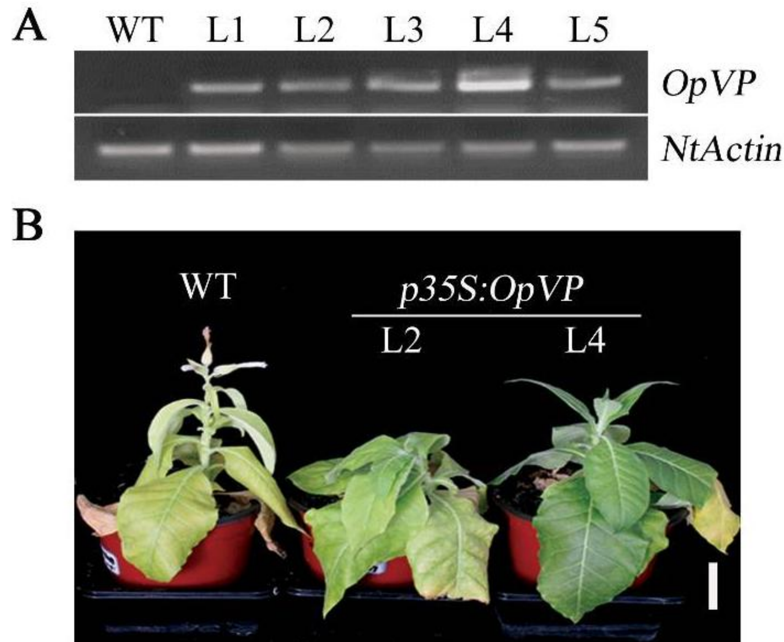


**Figure 4.** Analysis of *OpVP* transgenics (T3). **A,** Analysis of *OpVP* expression in tobacco transgenic lines by semiquantitative RT-PCR. **B,** Growth comparison of 45-day-old seedlings stressed by 200 mM NaCl. The photograph shows plants at the 45th day after treatment with 200 mM NaCl. WT: non-transgenic controls; L2, L4: transgenic plants overexpressing *OpVP*.

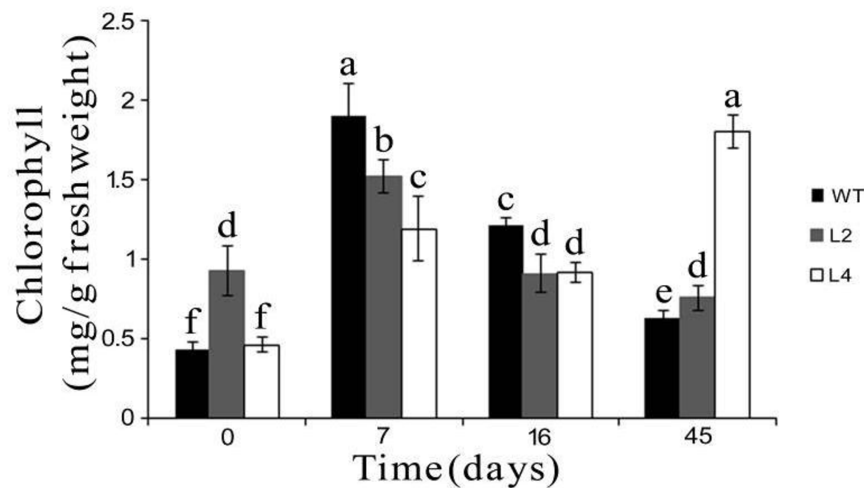


**Figure 5.** Chlorophyll content in the leaf of transgenic and wild type (WT) under 200 mM NaCl stress.The chlorophyll contents were measured 0, 7, 16 and 45 days salt treatments. Values represents means ± S.E. Columns with different letters indicate significance at $P < 0.05$ (Duncan test). WT, Non-transgenic controls; L2, L4: transgenic plants overexpressing *OpVP*.

of NaCl, there were no significant differences in $Na^+$ concentration between WT and transgenic plants. When stressed at 200 mM NaCl, $Na^+$ content significantly increased in the leaves of both transgenic and WT plants (Figure 6). When stressed to 16 days, however, there was significantly more $Na^+$ in both L2 and L4 than wild-

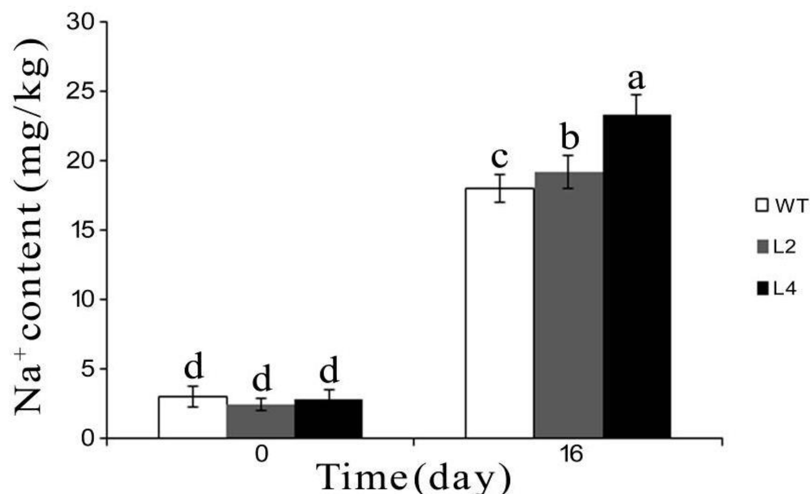


**Figure 6.** $Na^+$ contents in the leaf of transgenic and wild type (WT) under 200 mM NaCl stress. The $Na^+$ contents were measured 0 and 16 days of salt treatments. Values are means ± S.E. Columns with different letters indicate significance at $P < 0.05$ (Duncan test). WT, Non-transgenic controls; L2, L4: transgenic plants overexpressing *OpVP*.

type ($P < 0.05$). Comparatively, the contents of $Na^+$ in L4 plants were highest under salt stress, which is also correlated with the expression levels of *OpVP*. These results suggest that the enhanced salt tolerance in transgenic plants might be related to an increased $Na^+$ accumulation capacity.

## DISCUSSION

V-$H^+$-PPase plays an important role in the maintenance of the pH gradient across the vacuole membrane in plant stress response. In this study, a novel gene encoding a V-$H^+$-PPase, *OpVP*, was isolated from ephemeral plant *O. pumila*. An amino acid comparison between other Brassicaceae revealed that the polypeptides are well conserved. This indicates the V-$H^+$-PPase is one of the most highly conserved polypeptides among higher plants.

Expression of *OpVP* in *O. pumila* seedlings was significantly induced by salinity, drought and ABA application (Figures 2 and 3). This pattern of expression is similar to that of *TsVP* in *T. halophila* (Lv et al., 2008), *ScVP* in *S. corniculata* (Liu et al., 2011), *HVP1* and *HVP10* in barley (Fukuda et al., 2004). In addition, the expression level of *OpVP* was also significantly induced by IAA and GA3 (Figure 3), suggesting that gene may function differently under hormone treatment. Further studies are now in progress in our laboratory to investigate this relationship between hormone and *OpVP*.

To avoid the toxic effect of salt, plants have developed mechanisms to limit $Na^+$ uptake, to increase $Na^+$ exclusion, or to sequester $Na^+$ into vacuoles (Amtmann and Sanders, 1999). The vacuolar $H^+$-pumps play a key role in the maintenance of the $H^+$ electrochemical gradient across the vacuolar membrane. $H^+$-PPase can enhance the accumulation of $Na^+$ in vacuoles. Accumulation of $Na^+$ in vacuoles instead of in the cytoplasm can avoid the toxic effect of excessive $Na^+$ in plant cells (Gaxiola et al., 2001). V-$H^+$-PPase is a primary electrogenic proton pump that translocates protons across the tonoplast into vacuoles, thereby supporting strong ion differences and membrane polarization (Felle 2005), and alleviating the acidification of the cytosol (Stitt, 1998; Maeshima, 2000). Proton pumps coordinating with $Na^+/H^+$ antiporters on tonoplast enhance the ability of $Na^+$ uptake, as a result of accumulating more $Na^+$ on vacuole (Apse et al., 1999). Overexpression of *OpVP* gene in tobacco leads to increasing the activity of a $H^+$ pump on the vacuolar membrane of tobacco to move more $H^+$ into the vacuoles, therefore generating a higher proton electrochemical gradient ($\Delta\mu H^+$) that can be used to energize $Na^+/H^+$ antiporters. Enhanced expression of the vacuolar proton pumps should increase vacuolar solute accumulation by increasing the availability of protons. The sequestration of ions such as sodium in the vacuole could confer salt tolerance. In the present work, overexpressing *OpVP* transgenic tobacco showed a tendency to accumulate more $Na^+$ under salt stress conditions than non-transgenic wild type (Figure 6). The sequestration of $Na^+$ in the vacuole instead of in cytoplasm may prevent $Na^+$ toxicity, thus increases salt tolerance of transgenic tobacco plants (Figure 4). Moreover, *OpVP*-expressing tobacco plants maintained more chlorophyll content than wild-type control plants did under salt stress condition (Figure 5).

In conclusion, we successfully isolated and characte-

rized the *OpVP* cDNA from emphemeral plants *O. pumila* and developed transgenic *OpVP* expressing tobacco plants. The results indicated that overexpression of *OpVP* gene can increase salt tolerance by increasing the capacity to accumulate $Na^+$ in the vacuole. The overexpression of *OpVP* gene in economically important crop plants might be a strategy for engineering cultivars of agriculturally important plants to improve salinity tolerance in crops in salinization areas of the world.

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