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Cloning and Characterisation of Two H^+ Translocating Organic Pyrophosphatase Genes in *Salix* and Their Expression Differences in Two Willow Varieties with Different Salt Tolerances

Min Li[1,#], Chunmei Yu[2,#], Yaoyi Wang[2], Wentao Li[2], Ying Wang[1], Yun Yang[2], Huihui Liu[2], Yujuan Li[1], Feng Tan[1] and Jian Zhang[1,3,*]

[1]*Institute of Agricultural Science in the Regions along Yangtze River of Jiangsu, Rugao, Jiangsu Province 226541, China;* [2]*College of Life Science, Nantong University, Nantong, Jiangsu Province 226019, China;* [3]*Center for Computational Biology, Beijing Forestry University, Beijing 100083, China*

Abstract: Willows are one of the most important tree species for landscaping, biofuel and raw timber. Screening salt-tolerant willow varieties is an effective approach to balance wood supply and demand. However, more salt-tolerant willow varieties are required and little is known regarding the mechanism of salt tolerance at the gene expression level. In this paper, two willow varieties were studies in terms of their differences in salt-tolerances and mechanism of salt tolerance at the level of *VP1* gene expression. The results showed that *Salix* L0911 (L0911) had higher biomass than *Salix matsudana* (SM), and salt injuries were less severe in L0911 than in SM. The activities of peroxidase and superoxide dismutase, as well as the contents of soluble protein and proline, were higher in L0911 than in SM, whereas the contents of Na^+ and K^+, as well as the Na^+/K^+ ratio, were lower in L0911 than in SM. Two *VP1* genes (*VP1.1* and *VP1.2*) cloned in L0911 and SM had similar sequences and structures. *VP1.1* and *VP1.2* belonged to different subgroups. Total expression levels of the *VP1.1* gene in both roots and leaves of L0911 were higher than that in SM under normal conditions. Under salt stress, expression of *VP1* in SM roots initially increased and then decreased, whereas the expression of *VP1* in leaves of L0911 and SM, as well as in roots of L0911, decreased with increasing salt concentrations. This study increased our understanding of the salt-tolerance mechanism of willow and may facilitate the selection of salt-tolerant willow resources.

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Keywords: Willow, *VP1* gene, Salt tolerance, Gene expression, Phylogenetic analysis.

INTRODUCTION

China is facing a material shortage in the wood processing industry. According to the forestry statistical yearbook of China in 2012 published by the State Forest Administration, log imports reached 37.893 million m^3 and 349.036 million dollars in 2012, which increased by 48.9% and 42.6% from 2003 (25.456 million m^3 and 244.715 million dollars). Consequently, material shortages have restricted development of the wood processing industry in China.

Willows originate from Southern China and have a widespread global distribution, and are one of the most important energy, landscaping and environmentally friendly timber tree species [1-3]. However, the planting of willows in China has been restricted by shortages of land resources. Saline soils in China, accounting for ~10% of the total farmland area, cover an area of 20 million hectares, among which coastal saline soils account for 5 million hectares. Consequently, screening for salt-tolerant willows that can grow in saline soils has both economic and ecological significance in China, and is also an effective method to balance wood supply and demand.

Previous reports on willow salt tolerance have focused on screening natural salt-tolerant willow germplasms [4], analysing physiological characteristics such as relative conductivity, stomatal aperture, accumulation of Na^+ and Cl^- in stems and leaves [2, 3, 5-7] and the relationship between the accumulation of proline in leaves and salt tolerance of willows under salt stress [8]. The Na^+ content in plant cells are determined by multiple factors [9-11]. In general, glycophytes can compartmentalise excess Na^+ into vacuoles to decrease salt-induced cellular injury, but cells can be injured once Na^+ contents are beyond the tolerance levels; halophytes tolerate salt stress by pumping Na^+ out of the cells. Vacuolar H^+-translocating organic pyrophosphatase (H^+-PPase, VP1, EC 3.6.1.1) and vacuolar H^+-ATPase (H^+-ATPase, EC 3.6.1.3) bound in vacuolar membranes are two major proteins that pump protons from the cytoplasm to vacuoles and form H^+ gradients across vacuolar membranes. H^+ gradients promote compartmentalisation of Na^+ and K^+ into vacuoles through transporters and ion channels [11-14]. Many studies related to herb plant *VP1* genes and their functions have been performed [15-19]. However, more salt-tolerant willow varieties are required; *VP1* genes in willows have not been cloned, and their roles in salt tolerance among different willow varieties remain unclear.

*Address correspondence to this author at the Institute of Agricultural Science in the Regions along Yangtze River of Jiangsu, Rugao, Jiangsu Province 226541, China; Tel: 86- 513-87571253; Cell: 86-13358068755; E-mail: yjnkyy@163.com

[#] *Min Li and Chunmei Yu contributed equally to this work.*

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In this report, the salt tolerances of two willow varieties were determined and *VP1* genes from two willow varieties (*Salix* L0911 (L0911) and *Salix matsudana* (SM)) were cloned and sequenced. The expression of these genes was analysed in L0911 and SM under normal and salt stress conditions, respectively. Our goals were to provide a salt-tolerant willow variety, study the role of *VP1* genes in two willow varieties with different salt tolerances and provide a theoretical basis for selecting and evaluating willow germplasm resources.

MATERIALS AND METHODS

Plant Materials and Salt Stress Treatments

L0911 was a highly salt-tolerant variety among wild resources on saline land. SM, a native willow variety, showed slight salt tolerance in Jiangsu Province, China. L0911 and SM were used as raw materials in this report. Next, 2-3-year-old shoots from SM and L0911 with 0.5-2 cm diameters were cut into 14-16 cm in length and cultured in ½ Hoagland liquid medium. The materials were grown hydroponically in a plant growth chamber under the following conditions: 24ºC day/18ºC night, 60-80% relative humidity, light intensity 600-1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and photoperiod of 14 h day/10 h night. Biochemical reagents were ordered from Sigma and AMERASO (USA).

NaCl was added to the medium and the treatments were as follows: treatment 1 (T1), new roots with 4-5 cm in length and new shoots with 5-6 leaves were transferred to 0, 100, 200 and 300 mM NaCl solutions for 48 h; treatment 2 (T2), new roots and shoots as in T1 were transferred to 200 mM NaCl solutions for 0, 12, 24, 48, 72 and 96 h.

Determination of Phenotypic Traits

Cuttings of SM and L0911 mentioned above were treated with 0, 17, 34, 51, 68, 85 and 102 mM NaCl solutions, respectively. 10 cuttings were randomly selected for both L0911 and SM. Phenotypic traits were measured 3 times for individual cutting. Days of sprouting were recorded after NaCl treatments, and the heights of shoots and lengths of lateral roots were recorded 30 days after treatment. In addition, cuttings grown in normal ½ Hoagland liquid medium for 30 days were transferred to salt solutions of 0, 17, 34, 51, 68, 85 and 102 mM NaCl solutions, respectively, and the scales of salt injury were collected 10 days after salt treatments. The grades of salt injury were determined 10 days after salt treatments, as described by Chen *et al.* [20].

Determination of Physiological Traits

10 cuttings were randomly selected for both L0911 and SM. Leaves and roots from each cutting were collected after T1 and T2 treatments for the determination of superoxide dismutase (SOD) and peroxidase (POD) activities, soluble proteins, proline, MDA and Na^+ and K^+ contents. The determination was conducted 3 times for each cutting. The activities of SOD and POD were measured according to previously reported methods [21, 22]. The contents of soluble proteins and proline were determined based on previous methods [23, 24]. The levels of lipid peroxidation were measured in terms of malondialdehyde (MDA) contents following the method of Zheng *et al.* [25]. The concentrations of Na^+ and K^+ were determined using the methods of Zhu *et al.* [26] using a Shimadzu AA-680 atomicabsorption/flame spectrophotometer (6400A).

Cloning of *Salix VP1* Genes

Total RNA was extracted from 50-100 mg of leaves and roots from 3 different cuttings for L0911 and SM after T1 and T2 treatments using the TaKaRa MiniBEST Universal RNA Extraction Kit (China) according to the manufacturer's instructions and converted into cDNAs using Moloney Murine leukemia virus (M-MLV) reverse transcriptase (Promega, USA). Primers used in this study are listed in Table **S1**.

Amino Acid Sequence Analysis and 3D Structural Prediction of *Salix VP1* Genes

The 3D structures of *Salix* VP1.1 and VP1.2 were constructed using the Swissmodel workspace (http://swissmodel.expasy.org/) with PDB: 4A01 as template [28-30].

Phylogenetic Analysis of *VP1* Genes

Clustering analysis was carried out using the ClustalX programme. Tree construction was performed using the Mega 5.02 programme and phylogenetic relationships among genes were analysed using neighbour-joining [27].

Expression Analysis of *Salix VP1* Genes

Leaves and roots from 10 different cuttings of L0911 and SM after T1 and T2 treatments were selected for RNA extraction using the same method as in cloning of *VP1* genes mentioned above. Determinations of quantitative polymerase chain reaction (qPCR)were conducted 3 times for each cutting. Reverse transcription qPCR was performed on an ABI 7500 Real-Time PCR system using 7500 Software v2.0.4 (Applied Biosystems, USA). All PCR reactions were mixed as follows: 2 μL of diluted cDNA, 10 μL of 2× SYBR® Premix Ex TaqTM II (TaKaRa, China), 0.4 μL of 50X ROX Reference Dye II and 400 $nmol \cdot L^{-1}$ of primers (Table **S1**) with a final volume of 20 μL. All PCR reactions were performed under the following programme: 95ºC for 30 s, followed by 40 cycles at 95ºC for 5 s and 60ºC for 34 s in 96-well optical reaction plates (Applied Biosystems, USA). The *Ubiquitin Q* gene was used as an internal control.

RESULTS

Phenotypic and Physiological Traits of *Salix Matsudana* and *Salix* L0911 Under Normal and Salt-stress Conditions

Phenotypic analysis demonstrated that roots and leaves of L0911 grew faster than those of SM under normal hydroponic culture conditions (Fig. **S1**). The results of hydroponic culture under salt stress demonstrated that the growth of new shoots (or leaves) and lateral roots were inhibited when salt concentrations were at or above 68 mM for L0911 and 34 mM for SM. Salt injuries were much more severe in SM than in L0911 under the same conditions (Table **S2**), demonstrating that L0911 had higher salt tolerance than SM. Physiological trait analysis demonstrated that the activities of POD (EC 1.11.1.7) and SOD (EC 1.15.1.1), as well as the contents

of soluble proteins and proline, were higher in L0911 than in SM (Fig. **S2** and Fig. **S3**), whereas the contents of MDA and Na^+ were lower in L0911 than in SM (Fig. **S4** and Fig. **S5**) under the same salt-stress treatment, especially under high levels of salt stress. In addition, no significant changes in the Na^+/K^+ ratios were observed in L0911, which were significantly lower than those in SM treated with different salt concentrations (Fig. **S6**). In general, these phenotypic and physiological characterisations showed that L0911 had higher salt tolerance than SM, especially when treated with high concentrations of salt solution, demonstrating that L0911 is a salt-tolerant willow variety and SM is a slightly salt-tolerant willow variety.

Cloning of *Salix VP1* Genes

With different salt tolerances, L0911 and SM were used as materials to clone *VP1* genes in *Salix*. *Arabidopsis AVP1* (*Atlg15690*) gene sequences were used as probes to search the database of *Populus trichocarpa* (www.phytozome.com), and oligonucleotide primers were designed according to the conserved sequences of four *P. trichocarpa VP1* genes (Table **S1**) to amplify *VP1* genes from *Salix*. The final nucleotide sequences of *VP1* genes were constructed based on the sequence information of at least three independent clones. Finally, two *VP1* genes (*VP1.1* and *VP1.2*) were cloned in this study. Fig. **1** shows the results of *VP1.1* cloning in *Salix*. The size of *VP1.1* was between 2000 and 3000 base pairs, and sequence analysis demonstrated that the similarity of nucleotide sequences between *VP1.1* and *VP1.2* was 72.83% (data not shown). The nucleotide sequences of the *VP1* genes cloned in this report were determined and will be submitted to GenBank.

Sequence and Structural Analysis of *Salix VP1* Genes

The amino acid sequences in *Arabidopsis* (Atlg15690), *Vigna radiata* (VrVP1), *P. trichocarpa* (potri) and two *Salix* varieties were compared. Multiple alignment (Fig. **1**) showed that although some minor differences existed, the majority of amino acid sequences related to *VP1* genes among and within the species studied were relatively conserved. The similarity of amino acid sequences between VP1.1 and VP1.2 was 81.21% (data not shown). Membrane-related regions were highly conserved in the studied genes, demonstrating that these regions are required for VP1. Mutations in these regions may cause a loss of function of VP1. Amino acid sequences at important activity sites associated with VP1 function, such as PPi binding sites and acidic sites, were the same in all species studied, indicating that these sites were extremely conserved and vital for the normal function of VP1.

The 3D structure of VP1.1 and VP1.2 proteins in *Salix* are given in Fig. **S7**. Our results showed that VP1.1 and VP1.2 proteins in *Salix* had similar 3D structures, with obvious differences only at the protein termini (Fig. **S7 A-C**). This suggested that the amino acid sequences of VP1.1 and VP1.2 were highly conserved and differences were found only in regions unrelated to normal protein function. Characterisation of the active sites and regions that play a role in in the formation of salt bridges and hydrogen bonds supported the idea that VP1.1 and VP1.2 proteins had similar structures (Fig. **S7 D-G**).

Phylogenetic Analysis of *VP1* Genes

The cDNA sequences of VP1 genes from start to stop codons in *Arabidopsis thaliana, Glycine max, Vitis vinifera, Medicago truncatula, P. trichocarpa, Solanum lycopersicum, V. radiata* (PDB: 4A01) and *Salix* obtained in this study were clustered. The *S. lycopersicum* (Solyc04G071889) and *Arabidopsis* Atlg78920 (AVP2) VP1 sequences were used as outgroups to construct the tree. Phylogenetic analysis results are shown in Fig. **2**. Based on these results, VP1 genes in these species could be divided into two groups (Type I and Type II). Type I could be further divided into two subgroups (Type Ia and Type Ib). VP1.1 and VP1.2 cloned in this study belonged to the same group (Type I) but different subgroups (Type Ia and Type Ib, respectively).

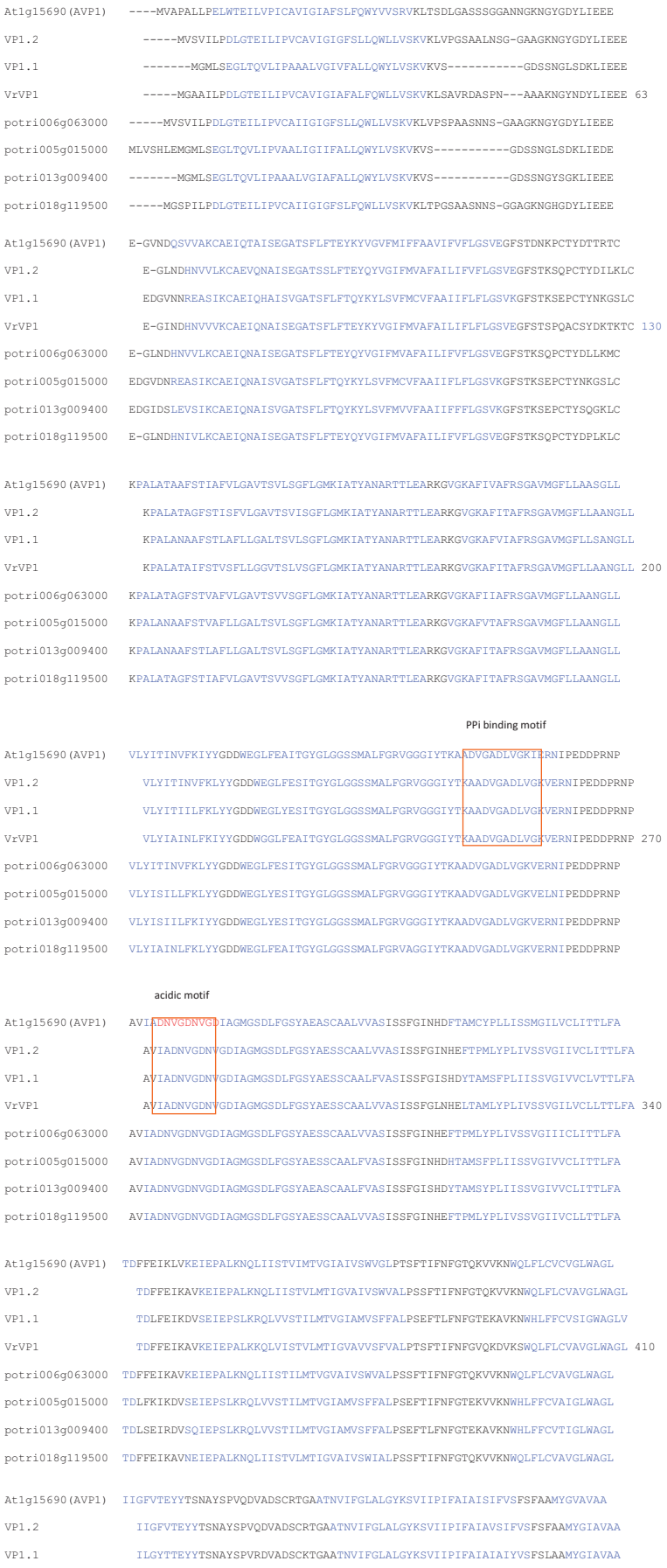
Expression of *VP1* Genes in *Salix* Under Normal and Salt-stress Conditions

Under normal conditions, the expression of *VP1.1* in L0911 was ~1.7-fold higher than that of SM, while *VP1.2* expression in roots of L0911 decreased by ~20% compared to SM. The expression in leaves was not significantly different between the two varieties (Fig. **3**). Under salt-stress treatments, the expression of two *VP1* genes in SM roots initially increased and then decreased, and peaked in the presence of 200 mM NaCl. The expression of two *VP1s* in roots of L0911 showed a decreasing trend (Fig. **3A**). The expression of *VP1.1* and *VP1.2* in leaves of two varieties was negatively correlated with NaCl concentrations (Fig. **3B**). When treated with 200 mM NaCl solution, the expression of *VP1.1* and *VP1.2* in roots of SM changed slightly before 72 h, and they were two-fold higher than controls after 96 h. The expression of *VP1.1* and *VP1.2* in roots of *Salix* L0911 decreased first and then increased, and the lowest expression was observed after 72 h for *VP1.1* and 48 h for *VP1.2* (Fig. **3C**). Under the same conditions, the expression of *VP1.1* and *VP1.2* in leaves of SM decreased first and then changed slightly. The expression patterns of *VP1.1* and *VP1.2* in leaves of L0911 were the same as in roots, but the lowest expression was observed after 24 h (Fig. **3D**).

DISCUSSION

L0911 and SM Showed Different Salt Tolerances

Phenotypic analysis demonstrated that L0911 could tolerate a salt concentration of 68 mM, whereas SM could only tolerate 34 mM. The activities of POD and SOD [33-37], as well as the contents of soluble protein and proline [35, 36, 38], MDA [39-41] and Na^+ and K^+ [10, 11, 47], are commonly used to determine the salt tolerance of various species. Physiological trait analysis showed that the activities of POD and SOD, as well as the contents of soluble protein and proline, were higher in L0911 than in SM, whereas the contents of Na^+ and K^+, as well as the Na^+/K^+ ratio, were lower in L0911 than in SM. Many previous studies exploring the salt tolerances of different species [10, 11, 33-41] showed that salt-tolerant species can increase the contents of POD, SOD, soluble proteins and proline to tolerate salt and maintain low Na^+ levels. Our results corroborated these characteristics, demonstrating that L0911 and SM have different salt-tolerant abilities, and that L0911 is a variety with high salt tolerance.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VP1.1 -------MGMLSEGLTQVLIPAAALVGIVFALLQWYLVSKVKVS----------GDSSNGLSDKLIEEE
VrVP1 -----MGAAILPDLGTEILIPVCAVIGIAFALFQWLLVSKVKLSAVRDASPN---AAAKNGYNDYLIEEE 63
potri006g063000 -----MVSVILPDLGTEILIPVCAIIGIGFSLLQWLLVSKVKLVPSPAASNNS-GAAGKNGYGDYLIEEE
potri005g015000 MLVSHLEMGMLSEGLTQVLIPVAALIGIIFALLQWYLVSKVKVS----------GDSSNGLSDKLIEDE
potri013g009400 -------MGMLSEGLTQVLIPAAALVGIAFALLQWYLVSKVKVS----------GDSSNGYSGKLIEEE
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VP1.2 E-GLNDHNVVLKCAEVQNAISEGATSSLFTEYQYVGIFMVAFAILIFVFLGSVEGFSTKSQPCTYDILKLC
VP1.1 EDGVNNREASIKCAEIQHAISVGATSFLFTQYKYLSVFMCVFAAIIFLFLGSVKGFSTKSEPCTYNKGSLC
VrVP1 E-GINDHNVVVKCAEIQNAISEGATSFLFTEYKYVGIFMVAFAILIFLFLGSVEGFSTSPQACSYDKTKTC 130
potri006g063000 E-GLNDHNVVLKCAEIQNAISEGATSFLFTEYQYVGIFMVAFAILIFVFLGSVEGFSTKSQPCTYDLLKMC
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VP1.2 KPALATAGFSTISFVLGAVTSVISGFLGMKIATYANARTTLEARKGVGKAFITAFRSGAVMGFLLAANGLL
VP1.1 KPALANAAFSTLAFLLGALTSVLSGFLGMKIATYANARTTLEARKGVGKAFVIAFRSGAVMGFLLSANGLL
VrVP1 KPALATAIFSTVSFLLGGVTSLVSGFLGMKIATYANARTTLEARKGVGKAFITAFRSGAVMGFLLAANGLL 200
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potri018g119500 KPALATAGFSTIAFVLGAVTSVVSGFLGMKIATYANARTTLEARKGVGKAFITAFRSGAVMGFLLAANGLL
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VP1.2 VLYITINVFKLYYGDDWEGLFESITGYGLGGSSMALFGRVGGGIYTKAADVGADLVGKVERNIPEDDPRNP
VP1.1 VLYITIILFKLYYGDDWEGLYESITGYGLGGSSMALFGRVGGGIYTKAADVGADLVGKVERNIPEDDPRNP
VrVP1 VLYIAINLFKIYYGDDWGGLFEAITGYGLGGSSMALFGRVGGGIYTKAADVGADLVGKVERNIPEDDPRNP 270
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potri013g009400 VLYISIILFKIYYGDDWEGLYESITGYGLGGSSMALFGRVGGGIYTKAADVGADLVGKVERNIPEDDPRNP
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acidic motif
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VP1.2 AVIADNVGDNVGDIAGMGSDLFGSYAESSCAALVVASISSFGINHEFTPMLYPLIVSSVGIIVCLITTLFA
VP1.1 AVIADNVGDNVGDIAGMGSDLFGSYAESSCAALFVASISSFGISHDYTAMSFPLIISSVGIVVCLVTTLFA
VrVP1 AVIADNVGDNVGDIAGMGSDLFGSYAESSCAALVVASISSFGLNHELTAMLYPLIVSSVGILVCLLTTLFA 340
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At1g15690(AVP1) TDFFEIKLVKEIEPALKNQLIISTVIMTVGIAIVSWVGLPTSFTIFNFGTQKVVKNWQLFLCVCVGLWAGL
VP1.2 TDFFEIKAVKEIEPALKNQLIISTVLMTIGVAIVSWVALPSSFTIFNFGTQKVVKNWQLFLCVAVGLWAGL
VP1.1 TDLFEIKDVSEIEPSLKRQLVVSTILMTVGIAMVSFFALPSEFTLFNFGTEKAVKNWHLFFCVSIGWAGLV
VrVP1 TDFFEIKAVKEIEPALKKQLVISTVLMTIGVAVVSFVALPTSFTIFNFGVQKDVKSWQLFLCVAVGLWAGL 410
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potri013g009400 TDLSEIRDVSQIEPSLKRQLVVSTILMTVGIAMVSFFALPSEFTLFNFGTEKAVKNWHLFFCVTIGLWAGL
potri018g119500 TDFFEIKAVNEIEPALKNQLIISTVLMTIGVAIVSWIALPSSFTIFNFGTQKVVKNWQLFLCVAVGLWAGL
At1g15690(AVP1) IIGFVTEYYTSNAYSPVQDVADSCRTGAATNVIFGLALGYKSVIIPIFAIAISIFVSFSFAAMYGVAVAA
VP1.2 IIGFVTEYYTSNAYSPVQDVADSCRTGAATNVIFGLALGYKSVIIPIFAIAVSIFVSFSFAAMYGIAVAA
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(Fig. 1) contd….

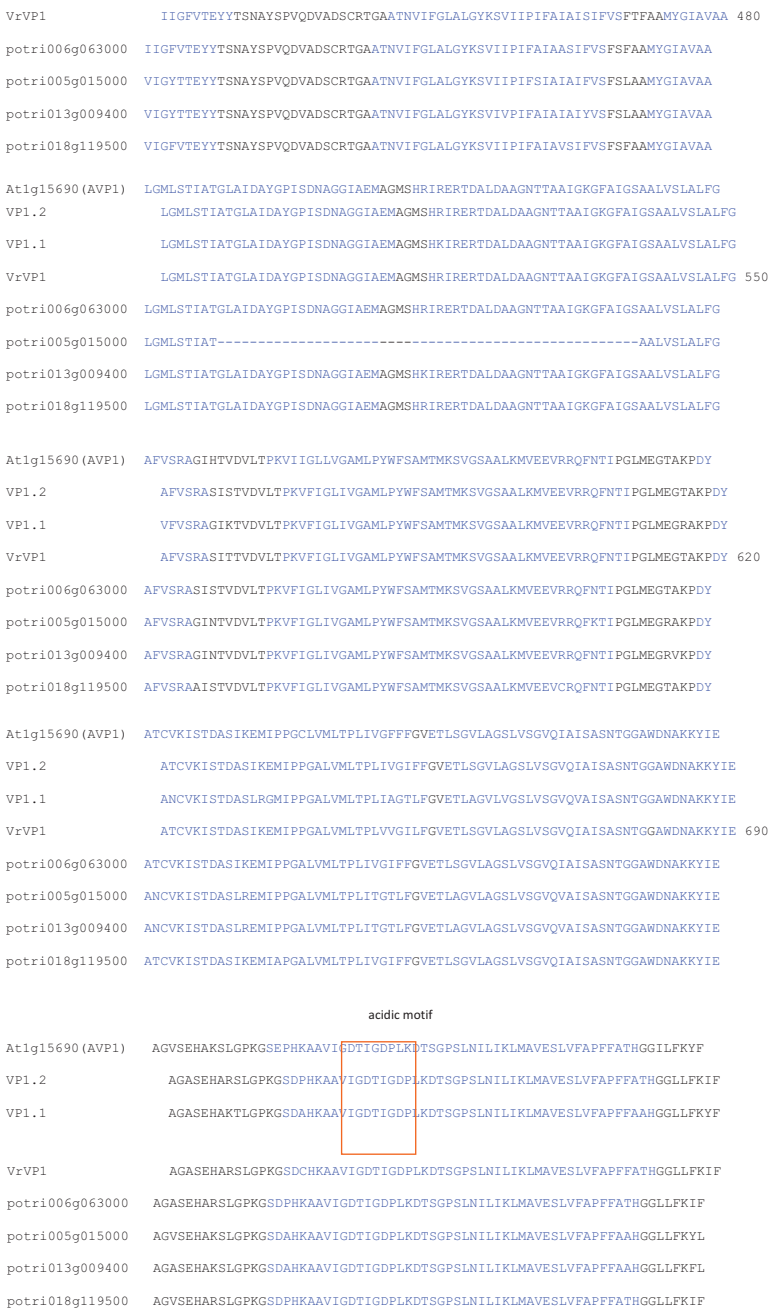

Fig. (1). Multiple alignments of VP1 amino acid residues in *Arabidopsis* (At1G15690), *Vigna radiate* (VrVP1), *Populus triocarpa* (potri) and two *Salix* VP1 genes (VP1.1 and VP1.2) obtained in this study. Sequences shown in black bold fonts are membrane region related.

Cuttings in saline soils (Dongling Reclamation Area, Rudong, Jiangsu Province, China, with salt solution concentrations of ~60-68 mM) showed that L0911 could grow normally, which further supported its high salt tolerance.

Constitutive High Expression of the *VP1.1* Gene Causes the Larger Root Systems of *Salix* L0911

Compared to SM, L0911 developed more lateral roots, grew faster and tolerated higher levels of salt stress (Fig. **S1** and Table **S2**). These phenotypes in L0911 were very similar to plants such as *Arabidopsis*, barley and rice overexpressing *VP1* genes [42-44]. *AVP1* was shown to positively regulate organ size in *Arabidopsis* [31, 42, 45, 46]. Our results demonstrated that constitutive expression of the *VP1.1* gene was higher in L0911 than in SM. Consequently, the high expression of *VP1.1* may be associated with the higher biomass of *Salix*, which was in accordance with studies in other species [31, 42-46]. In addition, some reports have suggested that the larger biomass and root systems in *VP1* transgenic lines are the result of increased auxin transport and PPi hydrolysis [42, 45, 46]. At this time, the contents of auxin and PPi in the two *Salix* varieties remain unknown. However, exploring the relationships among *VP1* gene expression, auxin transport and PPi hydrolysis should provide interesting results in future studies.

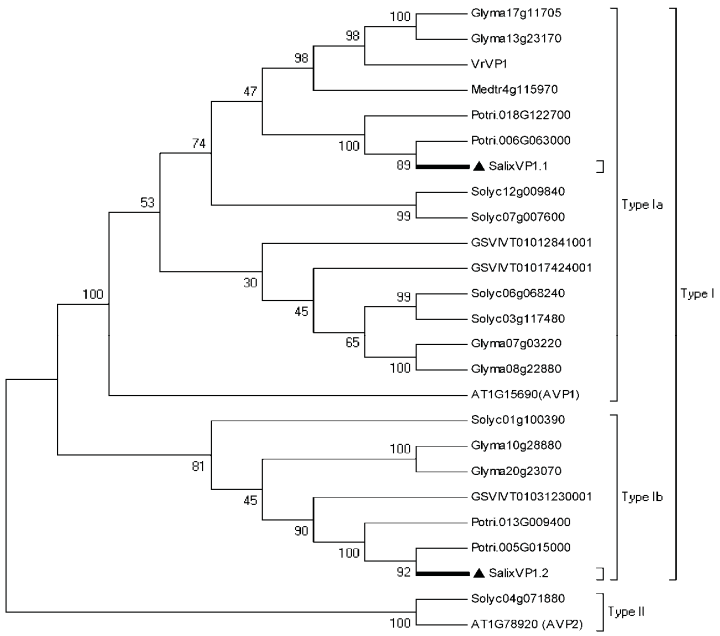

Fig. (2). Phylogenetic tree of *VP1* sequences. Amino acid sequences were searched from website www.phytozome.com using *Arabidopsis* AVP1 as probe. Bold line and "▲" show the two *Salix VP1* genes cloned in this study. AT, Glyma, GSVIVT, Medtr, Potri., Solyc, and Vr stand for *Arabidopsis thaliana*, *Glycine max*, *Vitis vinifera*, *Medicago truncatula*, *Populus triocarpa*, *Solanum lycopersicum* and *Vigna radiate* (PDB: 4A01), respectively.

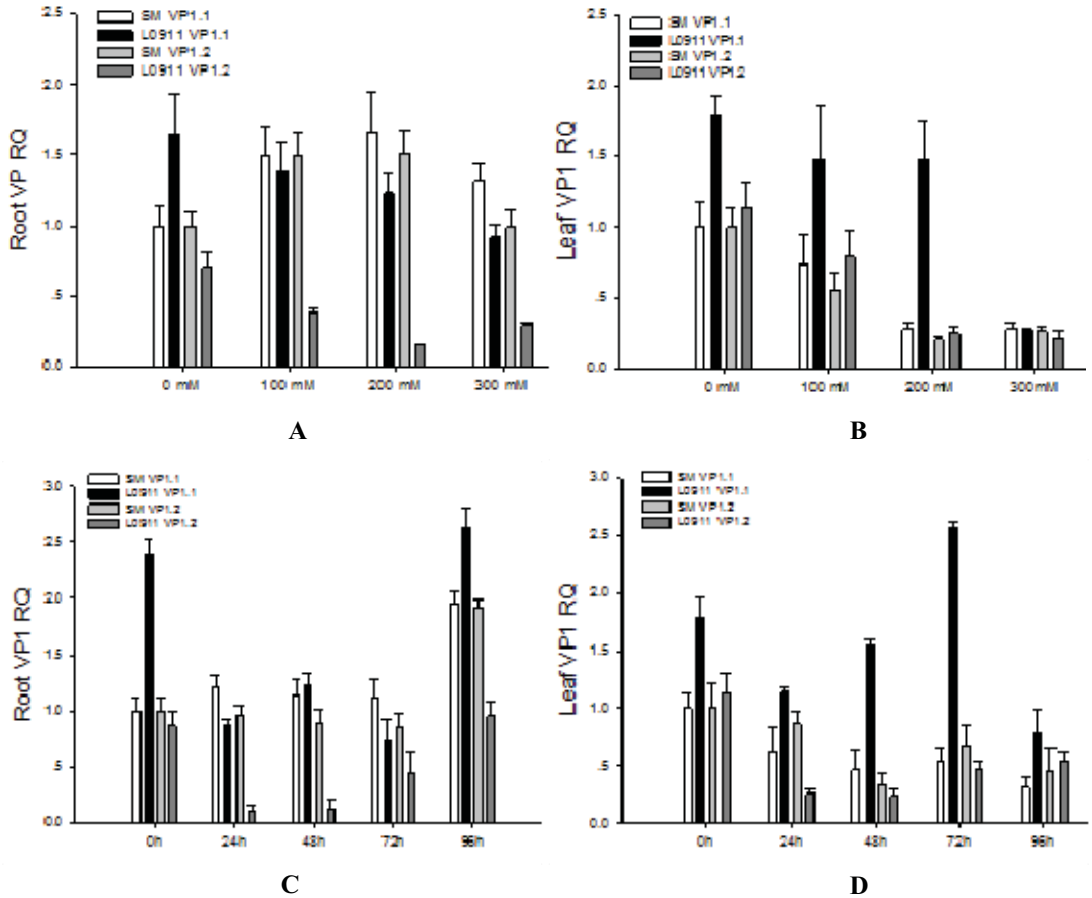

Fig. (3). Expression of two *Salix VP1* genes under salt stress. **A**. Root expressions of *VP1* genes in 100, 200, 300 mM NaCl solution. **B**. Leaf expressions of *VP1* genes in 100, 200, 300 mM NaCl solution for 48 hours. **C**. Root expressions of *VP1* genes treated with 200 mM NaCl solution for 12, 24, 48, 72 and 96 hours. **D**. expressions of *VP1* genes treated with 200 mM NaCl solution for 12, 24, 48, 72 and 96 hours. The Ubiquitin gene was used as an internal control, and all samples were compared with the untreated controls.

VP1 Genes Contribute Unequally to Salt Tolerance of L0911 and SM

Our results demonstrated that Na^+ contents in L0911 were significantly lower than that in SM under salt stress, and that the ratios of Na^+/K^+ were well maintained under normal and salt-stress conditions (Fig. **S5**). Expression of the *VP1.1* gene was significantly higher in L0911 than in SM under normal conditions, but little difference existed between L0911 and SM in the expression of *VP1.2* (Fig. **3**). The expression of *VP1* first increased and then decreased in roots of SM when treated with different concentrations of salt solution. In contrast, the expression of *VP1.1* and *VP1.2* decreased in leaves of L0911 and SM, as well as in roots of L0911 with increasing concentrations of salt solution.

The Na^+ content in roots is affected by many factors [10, 11, 47]. SM tolerated salt stress by upregulating *VP1* gene expression. In contrast, L0911 did not maintain low Na^+ contents in the cytoplasm by increasing *VP1* gene expression and compartmentalising Na^+ into vacuoles. Many studies have shown that overexpression of *VP1* enhances the salt tolerance of plants [16, 19, 32, 42, 44]. Our results were contradictory to those conclusions, but were consistent with a report in wheat [15]. The reason for the low Na^+ content in roots of L0911 may be that the roots of L0911 could prevent Na^+ from entering cells and that Na^+ in the roots of L0911 could be pumped out of cells. In this study, the levels of lipid peroxidation in L0911 were lower than that in SM, which maintains cell membrane integrity. Relatively high soluble protein contents could alleviate osmotic stress caused by Na^+ around the roots of L0911 and maintain normal cell shapes. Furthermore, high activities of POD and SOD alleviated the injuries of salt stress to L0911 cells. These factors may contribute to preventing the entry of Na^+ into cells and pumping Na^+ out of cells. Our future studies will focus on V-ATPases, the NHX antiporter, SOS pathways and HKT to explore the mechanisms of salt tolerance in L0911 at the molecular level.

CONCLUSION

L0911 is a fast-growing, salt-tolerant willow variety, while SM has slight salt tolerance. The mechanisms of salt tolerance between L0911 and SM varied at the level of *VP1* gene expression. SM tolerated salt stress by increasing the expression of *VP1*, whereas *VP1* gene expression in L0911 were relatively low under salt stress.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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SUPPLEMENTARY MATERIALS

Supplementary material is available on the publisher's web site along with the published article.

REFERENCES

[1] Hanley, S.; Barker, A.; Van, Ooijen, W.; Aldam, C.; Harris, L,.; Ahman, I.; Larsson, S.; Karp, A. A genetic linkage map of willow (Salix viminalis) based on AFLP and microsatellite markers. *Theor. Appl. Genet*., **2002**, *105*(6-7), 1087-1096.

[2] Mirck J, Volk TA: Response of three shrub willow varieties (Salix spp.) to storm water treatments with different concentrations of salts. *Biores. Technol.,* **2010**, *101*(10), 3484-3492.

[3] Dong, Y.; Ma, Y.; Wang, H.; Zhang, J.; Zhang, G.; S.Y.M. Assessment of tolerance of willows to saline soils through electrical impedance measurements. *For Sci. Pract.,* **2013**, *15*(1), 32-40.

[4] Hangs, R.D.; Schoenau, J.J.; Van, Rees, K.C.J.; Steppuhn, H. Examining the salt tolerance of willow (Salix spp.) bioenergy species for use on salt-affected agricultural lands. *Can. J. Plant Sci*., **2011**, *91*(3), 509-517.

[5] Dimitriou, I.; Aronsson, P.; Weih, M.; Stress tolerance of five willow clones after irrigation with different amounts of landfill leachate. *Bioresource Technol.* **2006**, *97*(1), 150-157.

[6] Aronsson, P.; Dahlin, T.; Dimitriou, I. Treatment of landfill leachate by irrigation of willow coppice--plant response and treatment efficiency. *Environ. Pollut.,* **2010**, *158*(3), 795-804.

[7] Mirck, J.; Volk, T.A. Mass balances and allocation of salt ions from Solvay storm water for shrub willow (Salix spp.). *Biomass Bioenerg.,* **2012**, *39*, 427-438.

[8] Stolarska, A.; Klimek, D. Free Proline Synthesis in Leaves of Three Clones of Basket willow (salix viminalis) as a Response to Substrate Salinity. *Environ. Protect. Eng.,* **2008**, *34*(4), 97-101.

[9] Foyer CH, Shigeoka S: Understanding oxidative stress and antioxidant functions to enhance photosynthesis. *Plant Physiol.,* **2011**, *155*(1), 93-100.

[10] Munns, R. Comparative physiology of salt and water stress. *Plant, cell Environ.,* **2002**, *25*(2), 239-250.

[11] Munns, R.; Tester, M. Mechanisms of salinity tolerance. *Ann. Rev. Plant Biol.,* **2008**, *59*, 651-681.

[12] Isayenkov, S.; Isner, J.C.; Maathuis, F.J. Vacuolar ion channels: Roles in plant nutrition and signalling. *FEBS Lett.,* **2010**, *584*(10), 1982-1988.

[13] Janicka-Russak, M.; Kabala, K.; Wdowikowska. A.; Klobus, G. Modification of plasma membrane proton pumps in cucumber roots as an adaptation mechanism to salt stress. *J. Plant Physiol.,* **2013**, *170*(10), 915-922.

[14] Hasegawa, P.M. Sodium (Na+) homeostasis and salt tolerance of plants. *Environ. Exp. Botany,* **2013**, *92*, 19-31.

[15] Brini, F.; Gaxiola, R.A.; Berkowitz, G.A.; Masmoudi, K. Cloning and characterization of a wheat vacuolar cation/proton antiporter and pyrophosphatase proton pump. *Plant Physiol. Biochem.,* **2005**, *43*(4), 347-354.

[16] Guo S, Yin H, Zhang X, Zhao F, Li P, Chen S, Zhao Y, Zhang H: Molecular cloning and characterization of a vacuolar H+ -pyrophosphatase gene, SsVP, from the halophyte Suaeda salsa and its overexpression increases salt and drought tolerance of Arabidopsis. *Plant Mol. Biol.,* **2006**, *60*(1), 41-50.

[17] Liu, Q.; Zhang, Q.; Burton, R.A.; Shirley, N.J.; Atwell, B.J. Expression of vacuolar H+-pyrophosphatase (OVP3) is under control of an anoxia-inducible promoter in rice. *Plant Mol. Biol.,* **2010**, *72*(1-2), 47-60.

[18] Wang, Y.; Xu, H.; Zhang, G.; Zhu, H.; Zhang, L.; Zhang, Z.; Zhang, C.; Ma, Z. Expression and responses to dehydration and salinity stresses of V-PPase gene members in wheat. *J. Genet. Genom.,* **2009**, *36*(12), 711-720.

[19] Dong, Q.L.; Liu, D.D. An XH, Hu DG, Yao YX, Hao YJ: MdVHP1 encodes an apple vacuolar H(+)-PPase and enhances stress tolerance in transgenic apple callus and tomato. *J.Plant Physiol.,* **2011**, *168*(17), 2124-2133.

[20] Chen, C.Y.; Tao, C.X.; Peng, H.; Yi, D. Genetic Analysis of Salt Stress Responses in Asparagus Bean (Vigna unguiculata (L.) ssp. sesquipedalis Verdc.). *J. Heredity,* **2007**, *98*(7), 655–665.

[21] Beauchamp, C.; Fridovich, I. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.,* **1971**, *44*(1), 276-287.

[22] Chance, B.; Maehly, A.C. Assay of catalases and peroxidases. *Methods Enzymol.,* **1955**, *2*, 764–817.

[23] Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.,* **1976**, *72*, 248-254.

[24] Bates, L.S.; Waldren, R.P.; Teare, I.D. Rapid determination of free proline for water-stress studies. *Plant Soil.,* **1973**, *39*, 205-207.

[25] Zheng, Y.H.; Li, X.; Li, Y.G.; Miao, B.H.; Xu, H.; Simmons, M.; Yang, X.H. Contrasting responses of salinity-stressed salt-tolerant and intolerant winter wheat (Triticum aestivum L.) cultivars to ozone pollution. *Plant Physiol. Biochem.,* **2012**, *52*, 169-178.

[26] Zhu, H.; Ding, G.H.; Fang, K.; Zhao, F.G.; Qin, P. New perspective on the mechanism of alleviating salt stress by spermidine in barley seedlings. *Plant Growth Regul.,* **2006**, *49*, 147-156.

[27] Tamura, K.; Peterson, D.; Peterson, N.; Stecher, G.; Nei, M.; Kumar, S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.,* **2011**, *28*(10), 2731-2739.

[28] Arnold, K.; Bordoli, L.; Kopp, J.; Schwede, T. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. *Bioinformatics,* **2006**, *22*(2), 195-201.

[29] Guex, N.; Peitsch, M.C. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis,* **1997**, *18*(15), 2714-2723.

[30] Schwede, T.; Kopp, J.; Guex, N.; Peitsch, M.C. SWISS-MODEL: An automated protein homology-modeling server. *Nucleic Acids Res.,* **2003**, *31*(13), 3381-3385.

[31] Gonzalez, N.; De Bodt, S.; Sulpice, R.; Jikumaru, Y.; Chae, E.; Dhondt, S.; Van Daele, T.; De Milde, L.; Weigel, D.; Kamiya, Y. Increased leaf size: different means to an end. *Plant Physiol.,* **2010**, *153*(3), 1261-1279.

[32] Gaxiola, R.A.; Sanchez, C.A.; Paez-Valencia, J.; Ayre, B.G.; Elser JJ: Genetic manipulation of a "vacuolar" H(+)-PPase: from salt tolerance to yield enhancement under phosphorus-deficient soils. *Plant Physiol.,* **2012**, *159*(1), 3-11.

[33] Yan, D.H.; Fenning, T.; Tang, S.; Xia, X.; Yin, W. Genome-wide transcriptional response of Populus euphratica to long-term drought stress. *Plant Sci*., **2012**, *195*, 24-35.

[34] Foyer, C.H.; Noctor, G. Redox signaling in plants. *Antioxid. Redox Signaling,* **2013**, *18*(16), 2087-2090.

[35] Jin, X.; Huang, Y.; Zeng, F.; Zhou, M.; Zhang, G. Genotypic difference in response of peroxidase and superoxide dismutase isozymes and activities to salt stress in barley. *Acta Physiologiae Plantarum.* **2009**, *31*(6), 1103-1109.

[36] Gao, C.; Wang, Y.; Liu, G.; Wang, C.; Jiang, J.; Yang, C. Cloning of Ten Peroxidase (POD) Genes from Tamarix Hispida and Characterization of their Responses to Abiotic Stress. *Plant Mol. Biol. Rep.,* **2009**, *28*(1), 77-89.

[37] Wang, Y.C.; Qu, G.Z.; Li, H.Y.; Wu, Y.J.; Wang, C.; Liu, G.F.; Yang, C.P. Enhanced salt tolerance of transgenic poplar plants expressing a manganese superoxide dismutase from Tamarix androssowii. *Mol. Biol. Rep.,* **2010**, *37*(2), 1119-1124.

[38] Foyer CH, Noctor G: Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. *Plant Cell,* **2005**, *17*(7), 1866-1875.

[39] Widodo, J.H.; Newbigin, E.; Tester, M.; Bacic, A.; Roessner, U. Metabolic responses to salt stress of barley (Hordeum vulgare L.) cultivars, Sahara and Clipper, which differ in salinity tolerance. *J. Exp.Bot.,* **2009**, *60*(14), 4089-4103.

[40] Winger, A.M.; Taylor, N.L.; Heazlewood, J.L.; Day, D.A. Millar AH: The Cytotoxic lipid peroxidation product 4-hydroxy-2-nonenal covalently modifies a selective range of proteins linked to respiratory function in plant mitochondria. *J. Biol. Chem.,* **2007**, *282*(52), 37436-37447.

[41] Yin, L.; Mano, J.; Wang, S.; Tsuji, W.; Tanaka, K. The involvement of lipid peroxide-derived aldehydes in aluminum toxicity of tobacco roots. *Plant Physiol.,* **2010**, *152*(3), 1406-1417.

[42] Zhang, J.; Li, J.; Wang, X.; Chen, J. OVP1, a vacuolar H+-translocating inorganic pyrophosphatase (V-PPase), overexpression improved rice cold tolerance. *Plant Physiol. Biochem.,* **2011**, *49*(1), 33-38.

[43] Schilling, R.K.; Marschner, P.; Shavrukov, Y.; Berger, B. Tester M, Roy SJ, Plett DC: Expression of the Arabidopsis vacuolar H(+) -pyrophosphatase gene (AVP1) improves the shoot biomass of transgenic barley and increases grain yield in a saline field. *Plant Biotechnol. J.,* **2014**, *12*(3), 378-386.

[44] Gaxiola, R.A.; Li, J.; Undurraga, S.; Dang, L.M.; Allen, G.J.; Alper, S.L.; Fink, G.R. Drought- and salt-tolerant plants result from overexpression of the AVP1 H+-pump. *Proc. Natl. Acad. Sci.*, **USA, 2001**, *98*(20), 11444-11449.

[45] Li, J.; Yang, H.; Peer, W.A.; Richter, G.; Blakeslee, J.; Bandyopadhyay, A.; Titapiwantakun, B.; Undurraga, S.; Khodakovskaya, M.; Richards, E.L. Arabidopsis H+-PPase AVP1 regulates auxin-mediated organ development. *Science,* **2005**, *310*(5745), 121-125.

[46] Ferjani, A.; Segami, S.; Horiguchi, G.; Muto, Y.; Maeshima, M.; Tsukaya, H. Keep an eye on PPi: the vacuolar-type H+-pyrophosphatase regulates postgerminative development in Arabidopsis. *Plant Cell,* **2011**, *23*(8), 2895-2908.

[47] Undurraga, S.F.; Santos, M.P.; Paez-Valencia, J.; Yang, H.; Hepler, P.K.; Facanha, A.R.; Hirschi, K.D.; Gaxiola, R.A. Arabidopsis sodium dependent and independent phenotypes triggered by H(+)-PPase up-regulation are SOS1 dependent. *Plant Sci*., **2012**, *183*, 96-105.