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MsPIP2;2, a novel aquaporin gene from *Medicago sativa*, confers salt tolerance in transgenic *Arabidopsis*

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ABSTRACT

Aquaporins (AQPs) are channel proteins that facilitate water transport across cell membranes and play important roles in many biological processes. However, most AQP functions are still poorly understood in the plant kingdom. Here, *MsPIP2;2* was isolated and identified from alfalfa (*Medicago sativa*). *MsPIP2;2* was localized to the plasma membrane, and its expression was induced by salt and abscisic acid (ABA) treatment. Overexpression of *MsPIP2;2* in *Arabidopsis* increased the seed germination rate, seedling root length, survival rate, proline content and antioxidant defence activity and decreased cell membrane damage and reactive oxygen species (ROS) accumulation compared to those in WT under salt stress. The salt tolerance of *MsPIP2;2* was affected by Ca^{2+} and pH in transgenic *Arabidopsis* plants. *MsPIP2;2*-overexpressing plants maintained a better K^+/Na^+ ratio and higher Ca^{2+} content under salt stress. The higher K^+/Na^+ maintenance in transgenic plants was mainly achieved by increasing Na^+ efflux and K^+ retention in roots via regulating the expression of the related ion channel genes. Stress-responsive genes, including *P5CS1*, *RD29A*, *DREB2* and *KIN2*, were upregulated in transgenic plants under salt stress. These results suggest that *MsPIP2;2* confers salt tolerance by regulating antioxidant defence system-mediated ROS scavenging, K/Na ion homeostasis and stress-responsive gene expression in plants.

1. Introduction

Soil salinity is one of the major environmental threats to the sustainable development of global agriculture. In the presence of excess salt, ionic toxicity and osmotic stress are the greatest challenges faced by plants, leading to nutrition disorders and oxidative stress (Serrano and Rodriguez, 2002; Türkan and Demiral, 2009). Therefore, it is critical to improve the salt tolerance of plants to accelerate agricultural production.

Plant growth and development are strongly dependent on root water uptake from the soil and the regulation of water movement in the plant body. Water diffusion across cell membranes is precisely regulated by water channels consisting of aquaporins (AQPs), which are small (23–34 kDa) integral membrane proteins that belong to the major intrinsic protein (MIP) family (Aharon et al., 2003; Chaumont and Tyerman, 2014). In addition to transporting water, AQPs also facilitate other small molecules' movement, such as glycerol, CO_2, NH_3, antimony, arsenite, ammonium, formamide, hydrogen peroxide, lactic acid, silicon, urea and boron, across membranes (Hachez et al., 2013; Sade et al., 2010; Srivastava et al., 2016; Uehlein et al., 2003). AQPs are involved in diverse physiological processes, including seed germination, cell elongation, stomatal movement, phloem loading and unloading, reproductive growth and stress responses (Eisenbarth and Weig, 2005). AQPs are classified into five subfamilies: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), NOD 26-like intrinsic proteins (NIPs), small basic intrinsic proteins (SIPs) and unclassified X intrinsic proteins (XIPs) (Kaldenhoff and Fischer, 2006). The subfamily of PIPs is further subdivided into PIP1 and PIP2 according to their homology (Chaumont et al., 2000). To date, many AQP genes have been found in different plant species, including 35 in *Arabidopsis* (Johanson et al., 2001), 33 in *Oryza sativa* (Sakurai et al., 2005) and 36 in *Zea mays* (Chaumont et al., 2001).

Abbreviations: AQP, aquaporin; PIP, plasma membrane intrinsic protein; GFP, green fluorescent protein; IL, ion leakage; MDA, malondialdehyde; ROS, reactive oxygen species; SOD, superoxide dismutase; POD, peroxidase; CAT, catalase; Fv/Fm, maximum photochemical efficiency

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Many AQPs can respond to abiotic stress and participate in various physiological processes. The expression of AQP genes varies depending on the organ, hormones and abiotic stresses (Heinen and Chaumont, 2009; Maurel et al., 2002). For example, the transcript of *MfPIP2-7* is induced by cold stress and ABA treatments in *Medicago falcata*. *MfPIP2-7* transgenic tobacco (*Nicotiana tabacum* L.) confers tolerance to freezing, chilling and NO_3^- deficiency (Zhuo et al., 2016). The transcripts of most *AtPIPs* are downregulated under cold stress (Jang et al., 2004). The expression of most *AtPIPs* is decreased in *Arabidopsis* in the presence of drought stress, while *AtPIP1;4* and *AtPIP2;5* are upregulated (Alexandersson et al., 2005). Overexpression of *AtPIP1;2* negatively impacts plant drought resistance in transgenic tobacco (Aharon et al., 2003). Most *AtPIP* genes are slightly regulated by high salinity in *Arabidopsis*, but *AtPIP1;5* and *AtPIP2;6* are downregulated in both roots and shoots (Jang et al., 2004). *TaAQP8*, a wheat PIP1 gene, was reported to be upregulated under salt stress. Heterologous expression of *TaAQP8* in tobacco promotes root growth under salinity, with an increased K^+/Na^+ ratio and Ca^{2+} content and reduced oxidative damage (Hu et al., 2012). Overexpression of rice *OsPIP1-1* and *OsPIP2-2* in transgenic *Arabidopsis* improves tolerance to salt and drought stress and alleviates inhibition of the main root growth (Guo et al., 2006). These studies indicate that overexpression of distinct PIPs can improve plant resistance to abiotic stresses, including salt stress.

Alfalfa (*Medicago sativa* L.), an adapted perennial forage crop cultivated worldwide, can grow in nitrogen-poor soils through symbiotic nitrogen fixation by rhizobia in root nodules (Wang et al., 2016). Alfalfa plays important roles in preventing soil erosion and livestock nutrition because of its large root system and high protein content (Samac et al., 2015). The growth and development of alfalfa are constrained by multiple environmental stress factors, such as drought, salt and extreme temperature (Behr et al., 2015; Mouradi et al., 2016). Therefore, genetic improvement of stress tolerance in alfalfa and better understanding of how these plants resist abiotic stresses are needed. Although the functions of many AQP genes have been demonstrated in other species, little is known about the AQPs in alfalfa. In this study, a full-length cDNA encoding *MsPIP2;2* was cloned from alfalfa and analysed. To understand the expression pattern of *MsPIP2;2* in response to abiotic stresses, the transcript levels in response to drought, salt and ABA were analysed. Moreover, the biological functions of *MsPIP2;2* under salt stress were also investigated by overexpressing the gene in *Arabidopsis*. Our results suggested that overexpression of *MsPIP2;2* enhanced salt tolerance mainly by modulating ion homeostasis, enhancing the antioxidant defence system and increasing stress-responsive gene expression in plants.

2. Materials and methods

2.1. Plant materials and stress treatments

Alfalfa (*Medicago sativa* L. cv. Baoding) seeds were surfaced sterilized with 75% (v/v) ethanol for 30 s and 5% sodium hypochlorite solution for 10 min and then washed with sterile water 4–5 times. Sterilized seeds were germinated on wet filter paper in Petri dishes. Five-day-old seedlings were transplanted into a hydroponic system with Hoagland's nutrient solution following the procedure described by Tocquin et al. (2003). Plants were placed in a growth chamber with 60% humidity and a 16-h photoperiod at 24 °C. Four weeks after incubation, the plants were subjected to different stress treatments. In the drought treatment, alfalfa plants were left to air dry in the container without nutrient solution for 0, 2, 4, and 6 h of dehydration stress (Yang et al., 2013). In the treatment with NaCl and ABA, the plants were transferred into a nutrient solution supplemented with 150 mM NaCl and 10 μM ABA, whereas the control contained normal nutrient solution. Leaves and roots were harvested from the treated and control plants. The tissues were instantly frozen in liquid nitrogen and stored at −80 °C for further analysis. The experiment was repeated three times.

2.2. Isolation and cloning of the MsPIP2;2 gene

Plant total RNA was extracted from alfalfa leaves with the MiniBEST plant RNA extraction kit (Takara Biotech Co., Ltd., Dalian, China). First-strand complementary DNA (cDNA) was synthesized using the PrimeScript™ II 1st Strand cDNA Synthesis Kit (Takara). Based on the sequence alignment with *Medicago truncatula*, the EST sequence belongs to the major intrinsic protein (MIP) family. Two primers (PIP2-F/PIP2-R, Table S1) were designed for amplification of the alfalfa partial cDNA sequence. The amplified products were inserted into the pMD19-T vector (Takara, Japan) by TA cloning and were sequenced by Sangon Biotech Co. (Shanghai, China). To obtain the full-length *MsPIP2;2* cDNA, a SMARTer RACE cDNA Amplification Kit (Clontech, Palo Alto, CA) was used according to the manufacturer's instructions. Specifically, 5′ GSP (Table S1) was used as the 5′ RACE PCR primer, and 3′ GSP (Table S1) was used as the 3′ RACE PCR primer. Using ordinary PCR, the amplified products were obtained and cloned into the pMD19-T vector (Takara). After sequencing, the full-length cDNA sequence was assessed using DNAMAN software and was amplified with primers LPIP2-F and LPIP2-R (Table S1).

2.3. Sequence analysis

The full-length nucleotide sequence of the *MsPIP2;2* gene was analysed using the NCBI website (http://www.ncbi.nlm.nih.gov/ and https://web.expasy.org/protparam/), and its deduced amino acid sequence and open reading frame (ORF) were obtained. A multiple-amino-acid alignment of the MsPIP2;2 protein was performed using DNAMAN software, and the phylogenetic tree was constructed using neighbour-joining method with 1000 bootstraps using MEGA6 software. For the prediction of the transmembrane helices of the MsPIP2;2 protein, the protein sequence was analysed using TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

2.4. Quantitative real-time PCR (qPCR) analysis

Quantitative real-time PCR (qPCR) was performed to detect the transcript levels of *MsPIP2;2* and genes involved in specific stress signalling pathways in various plant materials. Total RNA was extracted from each tissue harvested. Subsequently, the first-strand cDNA was reverse transcribed using the PrimeScript RT Reagent Kit (Takara). The alfalfa *β-actin* gene was used as an internal reference control in alfalfa (Long et al., 2014). The *Arabidopsis* actin gene (*AtActin*) was used as a reference gene in *Arabidopsis* plant materials. Three independent biological replicates and three replicate reactions for each sample were performed in the experiment. The relative expression levels of genes were calculated using the $2^{-\triangle\triangle CT}$ comparative method as described by Livak and Schmittgen (2001). The gene-specific primers were designed using the online NCBI Primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primers used in this study are listed in Table S1.

2.5. Subcellular localization of the MsPIP2;2 protein

The coding region of the *MsPIP2;2* gene without the stop codon was amplified using specific primers (MsPIP2-F/MsPIP2-R) containing the EcoRI restriction site and was cloned into the binary vector pCAMBIA-1300, generating the pCAMBIA1300-MsPIP2;2-GFP (green fluorescent protein) fusion plasmids. The MsPIP2;2 fusion protein and a plasma membrane-localized marker, pm-rk, were co-transformed into tobacco (*Nicotiana benthamiana*) epidermal cells by *Agrobacterium tumefaciens* infiltration. After incubation at 25 °C for two days, fluorescence signals were observed with a confocal laser scanning microscope (Leica TCS-SP8, Germany). The primers used for plasmid construction are shown in Table S1. For subcellular localization, the seeds of transgenic *Arabidopsis* plants containing pCAMBIA1300-MsPIP2;2-GFP were

germinated on half-strength MS (1/2 MS) medium for 5 d. Fluorescence signals were examined by confocal microscopy.

2.6. Plant transformation and generation of transgenic plants

The recombinant vector pCAMBIA1300-MsPIP2;2-GFP was introduced into *Agrobacterium tumefaciens* strain GV3101 and then transformed into wild-type *Arabidopsis* (Col-0, WT) by the floral dip method (Clough and Bent, 1998). More than 80 independent lines were selected for DL-phosphinothricin (PPT) resistance. Homozygous transformants (T3) were further confirmed by semi-quantitative RT-PCR using specific primers (RTPIP2-F/RTPIP2-R, Table S1) for *MsPIP2;2*. The *Arabidopsis* actin gene was used as an internal reference gene (AtActin-F/AtActin-R, Table S1). The T3 generation homozygous lines (Line 22 (L22) and Line 25 (L25)) and Col-0 (WT) were used for further phenotypic analysis and salt stress assays.

2.7. Stress tolerance assays

Seeds of *Arabidopsis* WT and transgenic lines were sown on 1/2 MS medium plates, supplemented with 0, 100, 125 or 150 mM NaCl and incubated at 4 °C for 2 d before being placed at 22 °C. The seed germination rates were scored daily for 7 d after transfer to 22 °C. For root elongation assays of WT and transgenic lines, seedlings were vertically grown on 1/2 MS medium plates for 4 d and were then transferred to 1/2 MS plates containing 0, 100, 125 or 150 mM NaCl. The length of the primary root was measured 7 d after transfer. For the salt stress tolerance assay in soils, plants from the transgenic lines and WT were cultured on 1/2 MS medium plates for 10 d and were then transplanted into soil. For the survival assays under salt stress, two-week-old plants were subjected to 300 mM NaCl solution for 10 d and were then watered for 5 d to recover after the salt treatment. Growth performance was monitored, and the survival rates were detected after recovery. Chlorophyll fluorescence was measured, and maximum photochemical efficiency (Fv/Fm) ratios were calculated. In addition, seedlings with a consistent growth state were watered with 150 mM NaCl solution, while the control was treated with normal nutrient solution. After 10 d of salt treatment, plants were harvested and used for physiological analyses. For testing the Ca^{2+} and pH effects on the salt tolerance of transgenic plants, 4-day-old seedlings were transferred to 1/2 MS plates containing 125 mM NaCl with various concentrations of Ca^{2+} (0, 0.75, 1.5, 3, 6 or 9 mM $CaCl_2$) or different pH values (4.0, 4.5, 5.0, 5.7, 6.5. 7.5 or 8.5). The phenotype was photographed and the root length was measured after 7 d of growth.

2.8. Measurement of ion leakage (IL), malondialdehyde (MDA), hydrogen peroxide (H_2O_2), superoxide anion radical ($O2^{\cdot -}$) and proline contents and the activities of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT)

IL was measured, and cell death was detected using trypan blue staining based on the method described by Dahro et al. (2016). The MDA content was determined by a modified thiobarbituric acid (TBA) method as described by Puckette et al. (2007). The contents of H_2O_2 and $O_2^{\cdot -}$ were measured following the method described by Jiang and Zhang (2001), with some modifications. The proline content was spectrophotometrically determined according to Zhao et al. (2009). The antioxidant enzyme activities were spectrophotometrically measured. Total SOD activity was assayed by the nitroblue tetrazolium (NBT) method (Giannopolitis and Ries, 1977) with minor modifications. The POD activity was determined using the guaiacol method described by Polle et al. (1994). CAT activity was measured following the method described in a previous study (Jiang and Zhang, 2001).

2.9. Measurement of Na^+, K^+ and Ca^{2+} contents

To measure the contents of Na^+, K^+ and Ca^{2+}, plants were washed with ultrapure water, treated at 105 °C for 10 min and baked at 80 °C for 48 h. Then, 20–50 mg (dry weight basis) was weighed and dissolved with 5 ml of acetic acid and heated at 100 °C for 1 h. The digested samples were chilled to room temperature and centrifuged at 15,000 rpm for 15 min. Then, the supernatant was transferred into new tubes and diluted to a total volume of 50 ml with ultrapure water. The Na^+, K^+ and Ca^{2+} contents of the samples were determined by atomic-absorption spectrophotometry.

2.10. Measurement of Na^+ and K^+ fluxes in the roots of Arabidopsis with non-invasive micro-test technology (NMT)

Seeds of both *Arabidopsis* WT and transgenic lines were sown on 1/2 MS medium plates, incubated at 4 °C for 2 d, and then incubated at 22 °C for 7 d. The seedlings were transferred to 1/2 MS medium with 0 or 150 mM NaCl for 24 h to detect the net fluxes of Na^+ and K^+ in the root elongation zone (550–600 μm, apart from the root tip). The steady state ion fluxes were continuously detected for 8 min with the NMT technique of USA Younger (Lanzhou University, China) as described by Sun et al. (2009a). All measurements were repeated with at least six samples independently.

2.11. Statistical analysis

The salt treatment was repeated four times with three replicates, giving rise to consistent results. Statistical analyses were performed with SPSS Statistical 20.0 software, and significant differences were calculated based on Student's *t*-test; $P < 0.05$ (*) and $P < 0.01$ (**) were considered significant.

3. Results

3.1. Isolation and characterization of MsPIP2;2

The full-length cDNA of *MsPIP2;2* (MK109796), which was cloned by 5′ RACE and 3′ RACE, contained a coding sequence of 864 bp nucleotides, encoding a protein of 287 amino acids with a calculated molecular weight of 30.58 kDa and a theoretical isoelectric point (pI) of 7.74. The protein contained a conserved domain of the MIP superfamily (31–266) (Fig. S1). MsPIP2;2 shared high sequence similarity with other plant PIPs, including 2 Asn-Pro-Ala signature motifs (NPA motif) (107–109, 228–230) and 6 transmembrane helices (TMHs) (40–62, 77–99, 120–142, 164–186, 198–220, 248–270) (Fig. S2). An unrooted phylogenetic tree analysis showed that *MsPIP2;2* was clustered into a branch with other legume plants, such as *Cicer arietinum* and *Pisum sativum,* and shared the highest homology with *Medicago truncatula* (Fig. S3).

3.2. Expression of MsPIP2;2 in response to abiotic stresses

The native expression pattern of *MsPIP2;2* in different alfalfa tissues was examined by qRT-PCR. The results showed that the expression abundance of *MsPIP2;2* was lower in roots than in leaves (Fig. 1A). To confirm the response of *MsPIP2;2* to abiotic stress, the transcript levels were determined after different treatments. *MsPIP2;2* was not significantly altered in root tissue under drought treatment, but the transcript level was significantly downregulated in leaves at 2 and 4 h (Fig. 1B). The expression of *MsPIP2;2* was upregulated by NaCl in both roots and leaves at 2 and 4 h, followed by a decline after 8 h. In contrast to the control, the accumulation of *MsPIP2;2* increased 1.76- and 3.01-fold in roots and 3.38- and 2.39-fold in leaves at 2 and 4 h, respectively (Fig. 1C). ABA treatment also resulted in a change in *MsPIP2;2* gene expression, followed by a similar pattern compared to the NaCl

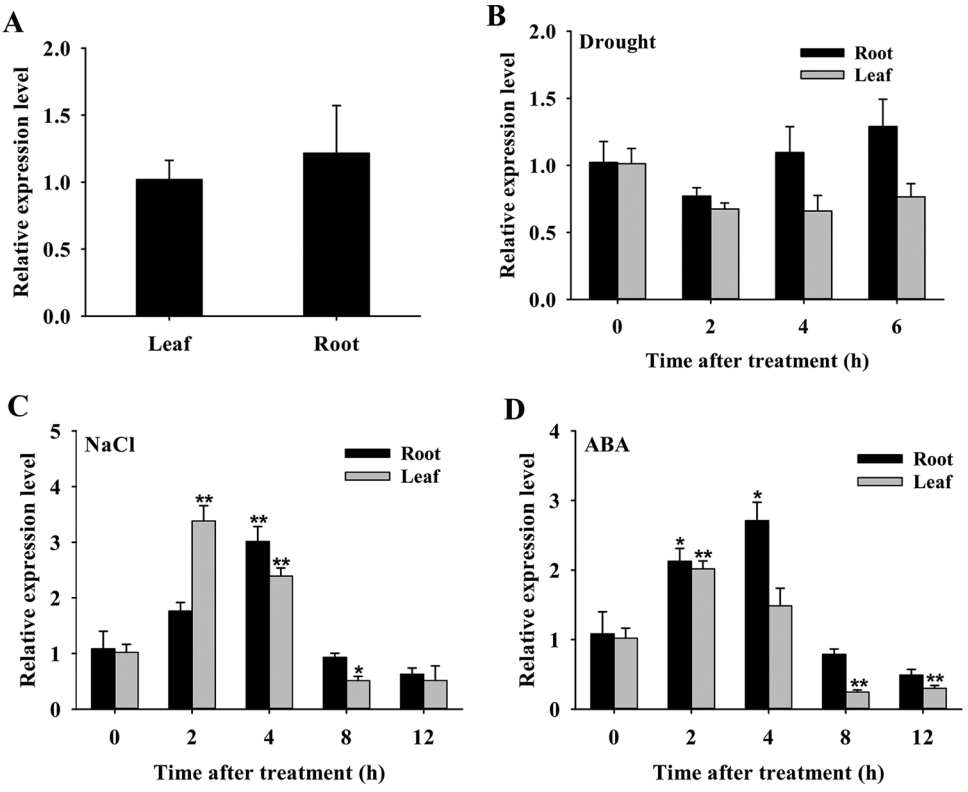


Fig. 1. Tissue-specific expression of *MsPIP2;2* (A) and expression analysis of *MsPIP2;2* in roots and leaves of alfalfa subjected to different stresses (B–D). Relative expression levels in roots and leaves under drought (B), NaCl (C) and ABA treatment (D). The expression levels were normalized to the *Medicago Actin* gene. Bars represent the mean ± SE (n = 3). Asterisks and double asterisks above the bars indicate significant differences: *, $P < 0.05$; **, $P < 0.01$.

treatment in all tissues (Fig. 1D). These results suggested that *MsPIP2;2* expression is partly positively regulated by salt stress.

3.3. Subcellular localization of the MsPIP2;2 protein

To test the subcellular localization of *MsPIP2;2*, the MsPIP2;2-GFP fusion protein and pm-rk (a plasma membrane marker) were transiently co-expressed in tobacco leaves via *Agrobacterium*-mediated transformation. As shown in Fig. 2A, the MsPIP2;2-GFP signal was clearly observed in the plasma membrane and essentially overlapped with the pm-rk in epidermal cells (Fig. 2A). Furthermore, GFP signals were observed at the plasma membrane of tobacco protoplasts (Fig. 2B). The MsPIP2;2-GFP signals were also detected at the plasma membrane of transgenic *Arabidopsis* seedling roots (Fig. 2C). Our results indicated that the MsPIP2;2 protein was localized mainly in the plasma membranes of plant cells.

3.4. Overexpression of MsPIP2;2 improved the salt tolerance of transgenic Arabidopsis

To investigate the role of *MsPIP2;2* in the plant response to salt stress, the *MsPIP2;2* gene was overexpressed in *Arabidopsis* under the control of the Cauliflower mosaic virus 35S (CaMV 35S) promoter by the floral dip method. Transgenic plants (T3 generation) were generated and confirmed by fluorescence signals in seedling roots and semi-quantitative RT-PCR analysis. As shown in Fig. 2C, a green fluorescent signal was observed in the *MsPIP2;2* transgenic *Arabidopsis* seedling roots. qPCR indicated that *MsPIP2;2* RNA was detected in two transgenic lines but not in the wild type (WT) (Fig. S4). Line 22 (L22) and Line 25 (L25) were selected for further analysis.

To explore the function of *MsPIP2;2* during seed germination, seeds from WT and transgenic lines were sown on 1/2 MS medium containing 0, 100, 125 or 150 mM NaCl for 7 d to determine the germination rates. The germination rates were inhibited in both WT and transgenic lines by NaCl treatment. While transgenic lines and WT plants showed comparable germination rates under normal growth conditions, transgenic lines showed better germination rates than WT under NaCl treatment (Fig. 3A). The root growth of transgenic lines was less inhibited than that of WT plants under 100, 125 or 150 mM NaCl treatment (Fig. 3B, C). In addition, the cotyledon greening rates were significantly higher in the transgenic lines than in the WT under 150 mM NaCl (Fig. 3D); without salt stress, the difference was indistinguishable (Fig. 3C, D). In addition, we detected the response of transgenic plants to drought. No phenotypic differences were observed in response to 200 mM or 300 mM mannitol medium (Fig. S5).

To further understand the function of *MsPIP2;2* at the vegetative stage, two-week-old plants of each line grown in soil were exposed to 300 mM NaCl stress for 10 d. The transgenic plants exhibited less necrosis than the WT plants after salt treatment and after recovery from salt stress for 5 d (Fig. 4A). The survival rates of the transgenic lines were noticeably higher than those of WT *Arabidopsis* (Fig. 4B). Although the Chl fluorescence decreased in both WT and transgenic plants under salt stress, the transgenic plants showed more intensive Chl fluorescence than WT plants (Fig. 4C). The Fv/Fm ratios of the transgenic lines were significantly higher than those of the WT after salt stress (Fig. 4D). These results indicated that overexpression of *MsPIP2;2* enhanced the salt tolerance of transgenic *Arabidopsis*.

3.5. Overexpression of MsPIP2;2 alleviated cell membrane damage and decreased ROS accumulation in transgenic Arabidopsis plants under salt stress

The different phenotypes between transgenic plants and the WT under salt stress prompted us to analyse the physiological changes in these lines. To detect the cell membrane injury and oxidative damage caused by salt stress in WT plants and transgenic lines, we measured IL, MDA, H_2O_2, and $O_2^{\cdot -}$ under normal growth conditions and 150 mM NaCl treatment. As shown in Fig. 5A and B, IL and MDA in the WT were significantly higher than those in the transgenic lines under NaCl treatment, implying that *MsPIP2;2* overexpressors showed better cell membrane integrity than WT under salt stress. In addition, transgenic lines exhibited lower accumulation of H_2O_2 and $O_2^{\cdot -}$ than WT plants

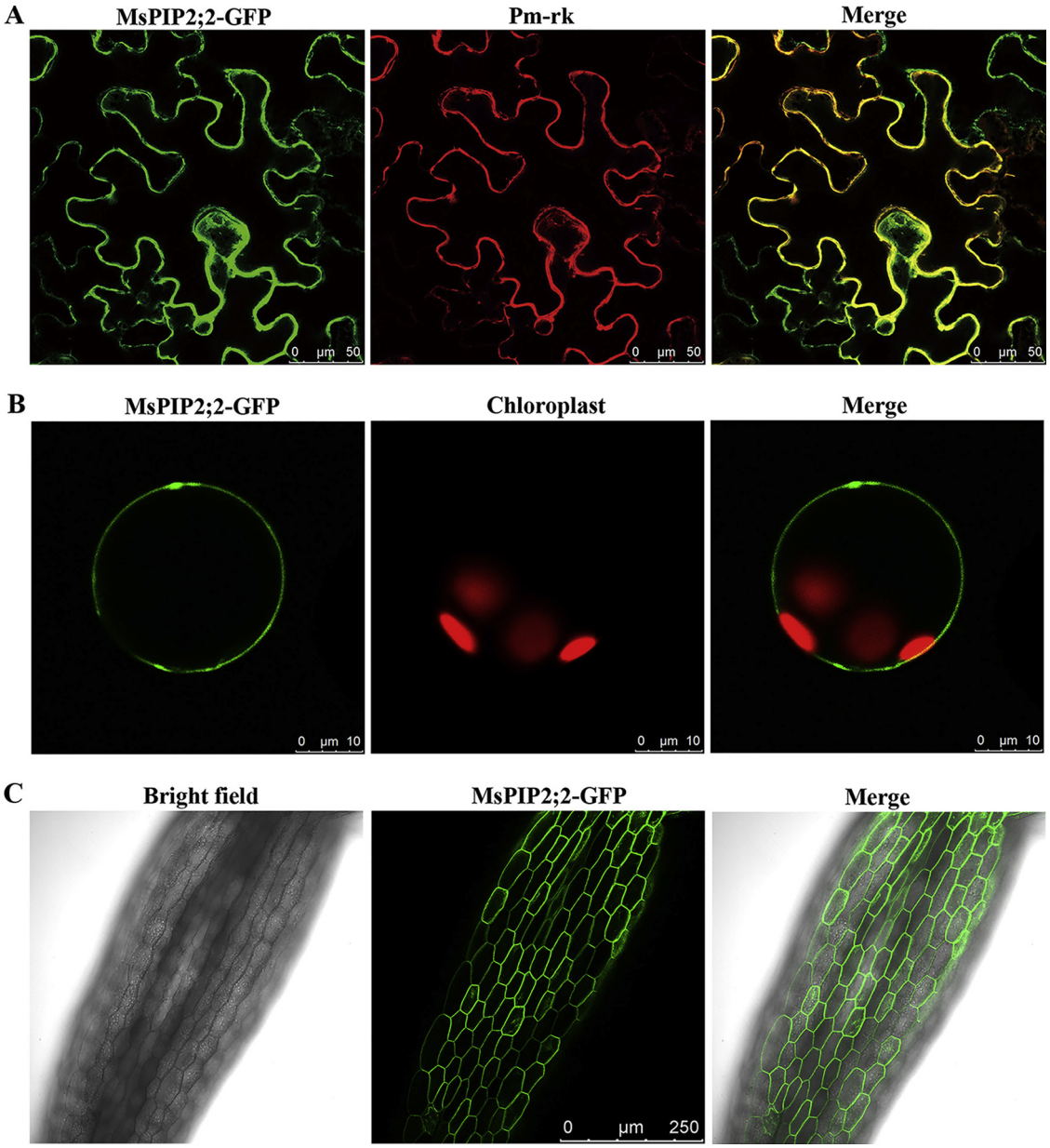


Fig. 2. Subcellular localization of the alfalfa MsPIP2;2 protein. (A) MsPIP2;2-GFP signal overlaid with a pm-rk in the epidermal cells. Images from left to right represent MsPIP2;2-GFP (green), pm-rk (red) and an overlay (of green and red) from the same sample. Scale bar, 50 μm. (B) A mesophyll protoplast isolated from infiltrated tobacco leaves. Images from left to right represent MsPIP2;2-GFP (green), chloroplast (red) and an overlay (of green and red) from the same sample. Scale bar, 10 μm. (C) Fluorescence images of transgenic *Arabidopsis* seedling roots. Images from left to right represent bright field, MsPIP2;2-GFP (green) and an overlay (of bright field and green) from the same sample. Scale bar, 250 μm (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

(Fig. 5C, D), suggesting that salt stress induced less oxidative damage in transgenic lines. Trypan blue staining was performed in plant leaves to examine the cell death of each line under salt stress. Both WT and transgenic plant leaves showed similar light staining under normal growth conditions. However, salt stress caused intense blue staining in both WT and transgenic plants. The blue staining in the transgenic lines was noticeably lighter than that in the WT leaves, implying less cell death in transgenic plants than WT plants under salt stress (Fig. 5E). These results suggested that overexpression of *MsPIP2;2* can significantly alleviate membrane and oxidative damage caused by salt stress.

3.6. Overexpression of MsPIP2;2 increased osmotic solute contents and antioxidant enzyme activities in transgenic Arabidopsis plants under salt stress

The proline content increased in both WT and transgenic plants under salt stress (Fig. 6A). However, the transgenic lines exhibited significantly higher proline than the WT plants. Antioxidant enzyme activities were increased under salt stress in all lines, but the transgenic lines displayed higher activities of POD, SOD and CAT than the WT plants (Fig. 6B, C and D). These results suggested that overexpression of *MsPIP2;2* enhanced the salt tolerance of transgenic plants by improving plant osmotic solute contents and antioxidant enzyme activities.

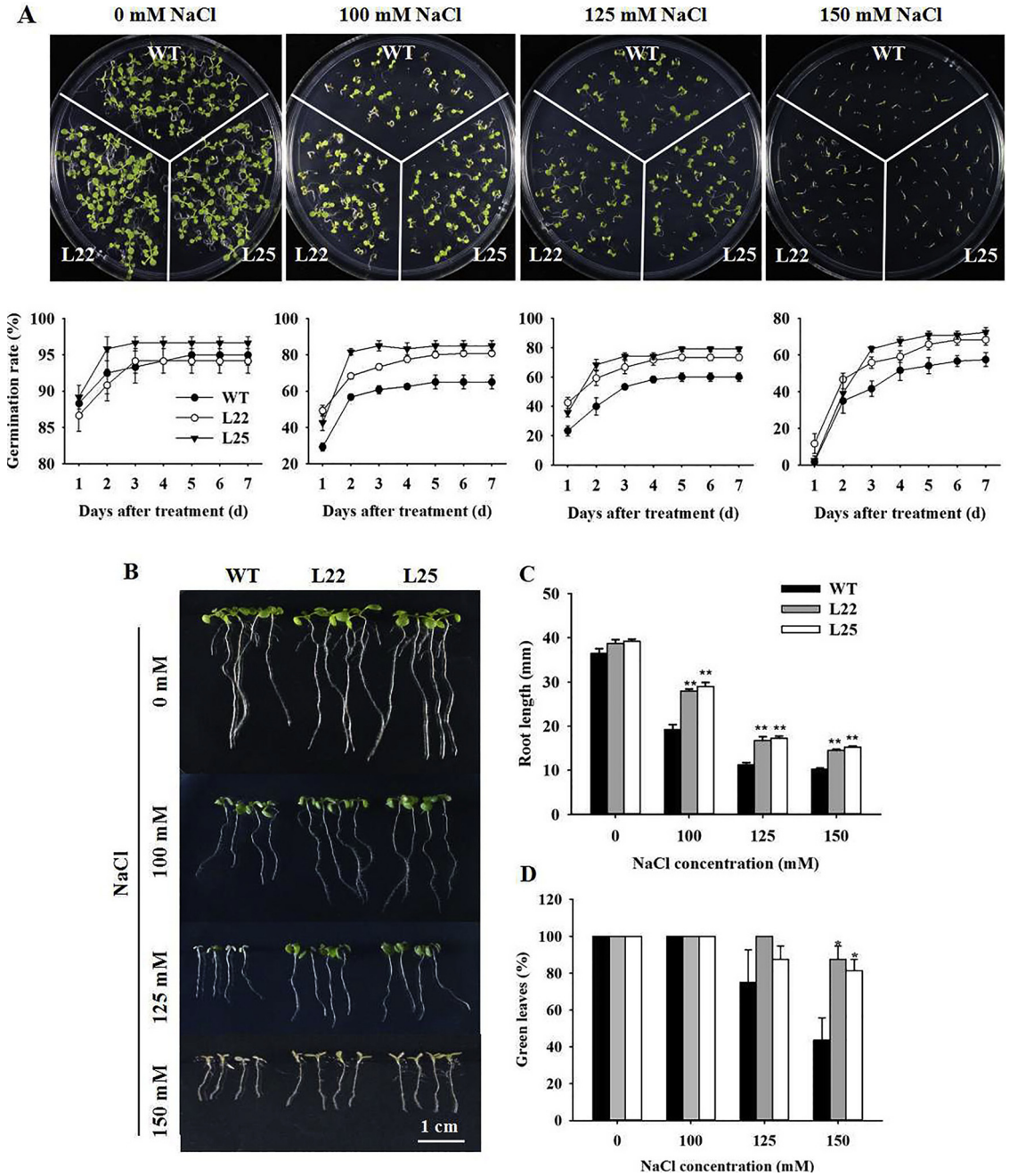


Fig. 3. Salt stress response of *MsPIP2;2*-overexpressing *Arabidopsis* during the seedling stage. (A) Seed germination phenotype (upper panels) and germination rates (lower panels) of WT and transgenic lines on MS medium supplied with 0, 100, 125 or 150 mM NaCl for 7 d. (B) Root growth phenotypes of WT and transgenic plants on MS medium supplied with 0, 100, 125 or 150 mM NaCl for 7 d. (C) The root length was calculated. (D) The green leaf rate was calculated. Bars represent the mean ± SE (n = 3). Asterisks and double asterisks above the bars indicate significant differences between the transgenic lines and WT under the same growth conditions: *, $P < 0.05$; **, $P < 0.01$.

3.7. The salt tolerance of MsPIP2;2 was affected by Ca^{2+} and pH in transgenic Arabidopsis plants

Given that AtPIP2;1 possesses the ability to transport cations and is a candidate for a previously reported non-selective cation channel (NSCC) responsible for Ca^{2+}- and pH-sensitive Na^+ entry into roots (Byrt et al., 2017), we detected whether the activity of *MsPIP2;2* was regulated by Ca^{2+} and pH. As shown in Fig. 7, the phenotypes and root length of transgenic plants were superior to those of WT under salt stress or with high Ca^{2+} (6 mM or 9 mM) and low pH (4.5 or 5). However, there was no significant difference between transgenic plants and WT under salt stress with low Ca^{2+} ($\leq$1.5 mM) or high pH ($\geq$6.5) conditions (Fig. 7). These results indicated that the salt tolerance of *MsPIP2;2* was affected by low Ca^{2+} and high pH.

3.8. Overexpression of MsPIP2;2 increased the K^+/Na^+ ratio and Ca^{2+} content in transgenic Arabidopsis plants under salt stress

To determine whether the accumulation of Na^+, K^+ or Ca^{2+} is involved in the salt tolerance of *MsPIP2;2* transgenic plants, the contents of Na^+, K^+ and Ca^{2+} in shoots and roots were analysed under normal growth conditions and 150 mM NaCl treatment. No significant differences were observed in the Na^+, K^+ or Ca^{2+} content in the transgenic lines relative to the WT under normal conditions (Fig. S6).

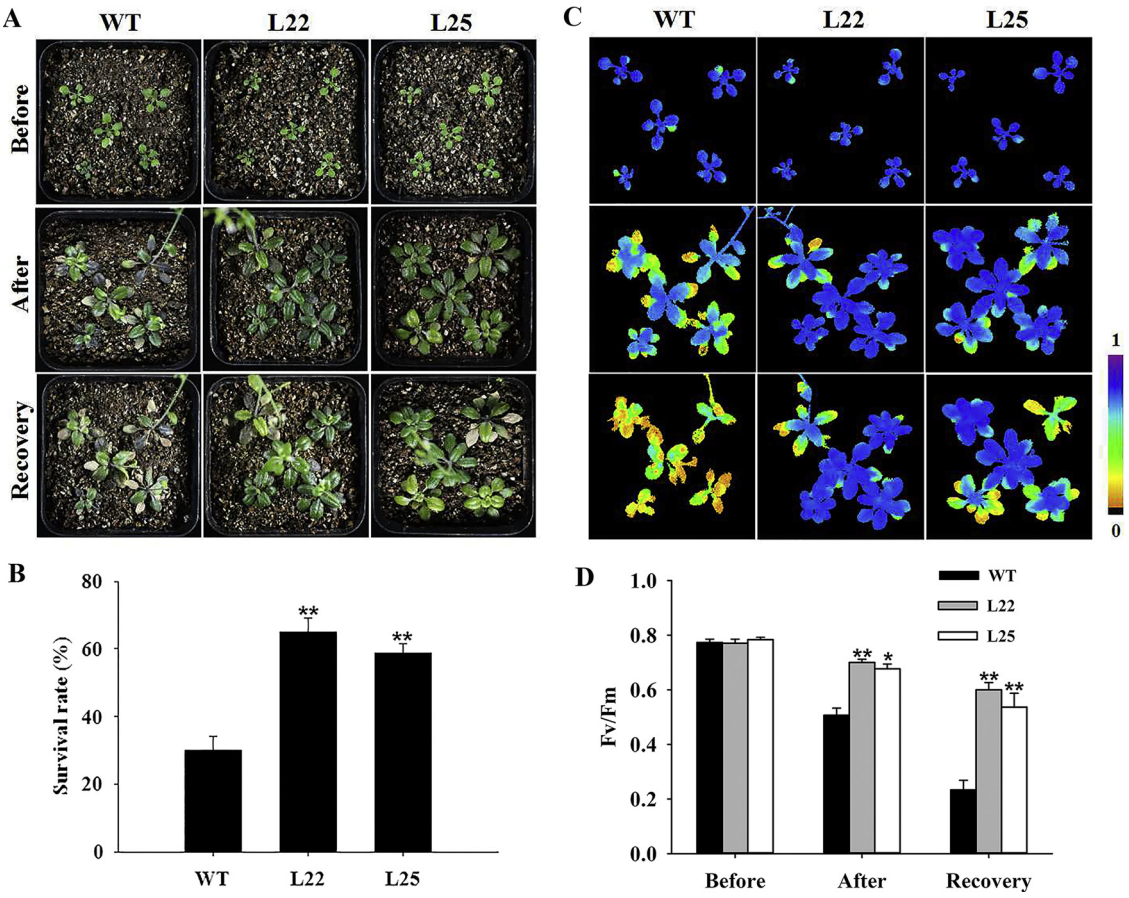


Fig. 4. Salt tolerance analysis of *MsPIP2;2*-overexpressing *Arabidopsis* at the vegetative stage. (A) Phenotypes of WT and *MsPIP2;2* transgenic plants grown in soil treated with 300 mM NaCl for 10 d followed by recovery for 5 d. (B) The survival rate was calculated. (C) Images of Chl fluorescence and (D) Fv/Fm ratios of WT and *MsPIP2;2* transgenic *Arabidopsis*. Four independent experiments were conducted and produced similar results. Bars represent the mean ± SE (n = 3). Asterisks and double asterisks above the bars indicate significant differences between the transgenic lines and WT under the same growth conditions: *, $P < 0.05$; **, $P < 0.01$.

However, the transgenic lines showed significantly lower Na^+ but higher K^+ content in shoots and increased Na^+ and K^+ in roots compared with the WT plants under salt stress, resulting in a significantly higher K^+/Na^+ ratios in the transgenic lines compared to the WT plants (Fig. 8). In addition, Ca^{2+} content was increased in the transgenic plants under salt stress. These results suggested that the improved salt tolerance of *MsPIP2;2* transgenic plants is associated with the homeostasis of Na^+, K^+ and Ca^{2+}.

3.9. Overexpression of MsPIP2;2 altered the net K^+ and Na^+ fluxes in the roots of transgenic Arabidopsis under salt stress

The results of the NMT indicated that Na^+ and K^+ were in flux in the elongation zone of the root tips (Fig. 9). As shown in Fig. 9A and D, both the transgenic and WT plants showed little fluctuation in the Na^+ and K^+ fluxes under normal conditions. However, after being subjected to 150 mM NaCl, Na^+ efflux was significantly increased in both transgenic and WT plants, and the efflux of Na^+ decreased gradually with time. However, the Na^+ flux was higher in transgenic plants compared to WT (Fig. 9B). Meanwhile, the efflux of K^+ was reduced obviously in the transgenic lines relative to WT after salt treatment (Fig. 9E). The average net fluxes of Na^+ and K^+ did not differ significantly between transgenic and WT seedlings under normal growth conditions. However, when the seedlings were treated with salt stress, the average net Na^+ effluxes of the two transgenic lines were 2.02- and 2.04-fold higher than that of WT, while the average net K^+ effluxes of the two transgenic lines were 1.66- and 1.77-fold lower than that of WT, respectively (Fig. 9C, F). These results indicated that the *MsPIP2;2* gene could promote salt tolerance by increasing Na^+ exclusion and reducing K^+ efflux.

3.10. Overexpression of MsPIP2;2 altered the expression levels of ion homeostasis genes

The observed changes in Na^+ and K^+ in transgenic plants prompted us to analyse the transcript levels of several genes involved in the regulation of ion homeostasis. The expression levels of *AtSOS1*, *AtHKT1*, *AtGORK*, *AtAKT1*, *AtAKT2*, *AtKAT1*, *AtKAT2*, *AtHAK5*, *AtKUP4* and *AtKUP7* were detected in roots of 15-day-old seedlings treated with 150 mM NaCl for 4 h. The results indicated that all tested genes showed similar expression levels between transgenic and WT plants under normal conditions (Fig. 10). However, when the seedlings were treated with 150 mM NaCl for 4 h, the expression levels of *AtSOS1*, *AtHKT1*, *AtKAT1*, *AtKAT2* and *AtHAK5* were elevated to different degrees in the transgenic plants compared with WT plants, while *AtGORK* expression was decreased significantly in the transgenic plants (Fig. 10). In addition, the expression levels of *AtAKT1*, *AtAKT2*, *AtKUP4* and *AtKUP7* remained unchanged between the transgenic lines and the WT (Fig. S7).

3.11. Overexpression of MsPIP2;2 upregulated stress-responsive genes

To further understand the role of *MsPIP2;2* overexpression in stress response pathways, the expression of four stress-responsive genes, i.e., *P5CS1*, *RD29A*, *DREB2* and *KIN2*, from *Arabidopsis*, was analysed using qPCR. As shown in Fig. 11, the expression levels of all four tested genes

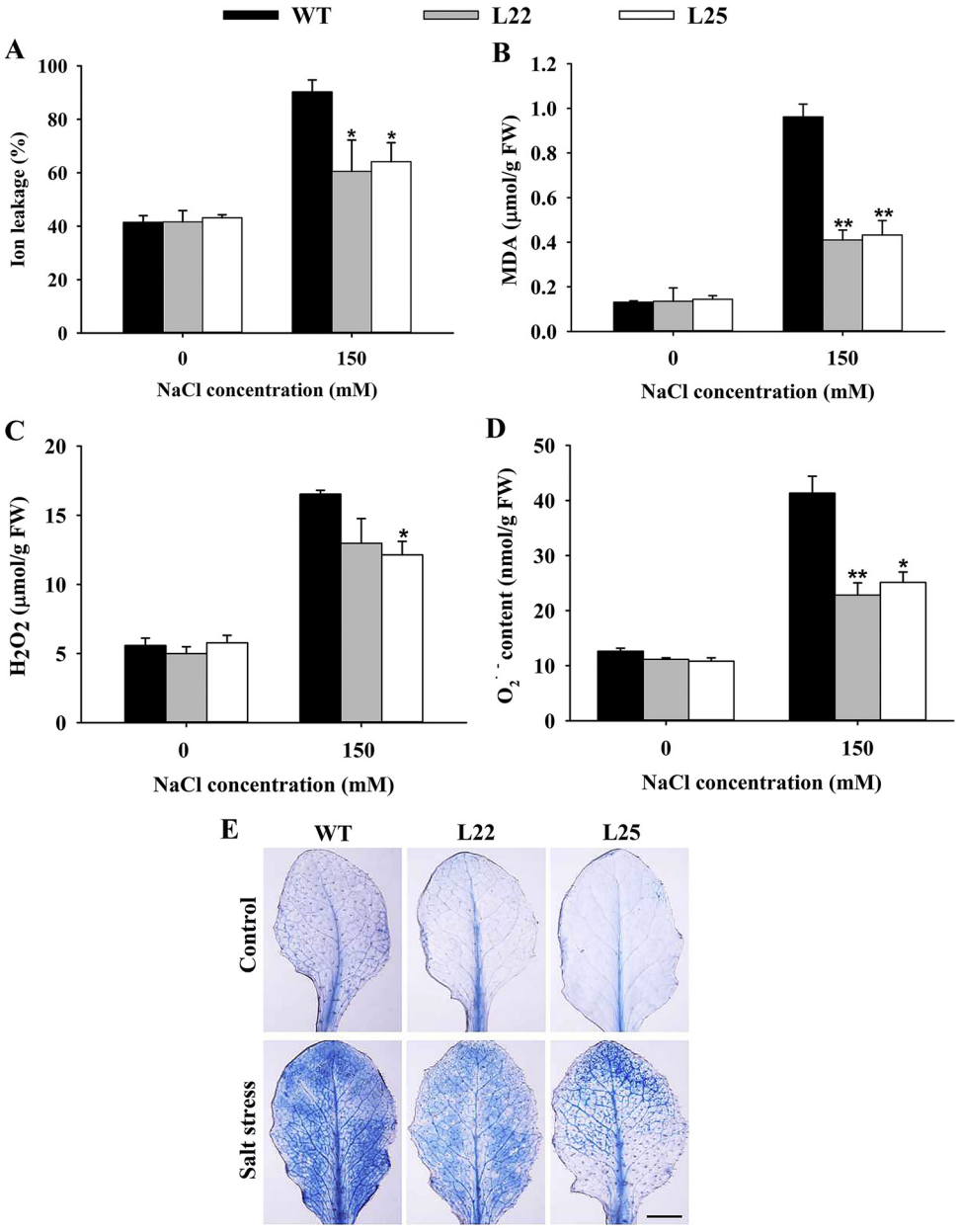


Fig. 5. Effects of salt stress on (A) ion leakage (IL), (B) the MDA content, (C) H_2O_2 accumulation, (D) $O_2^{\cdot -}$ accumulation and (E) cell death shown by trypan blue staining of leaves of the WT and *MsPIP2;2* transgenic *Arabidopsis*. Scale bar, 2.5 mm. Bars represent the mean ± SE (n = 3). Asterisks and double asterisks above the bars indicate significant differences between the transgenic lines and WT under the same growth conditions: *, $P < 0.05$; **, $P < 0.01$.

were markedly higher in the *MsPIP2;2* transgenic lines than in the WT plants under salt stress. In addition, the *AtP5CS1* gene had higher expression in transgenic plants than in the WT in the absence of salt stress. These results indicated that overexpression of *MsPIP2;2* upregulated the expression of stress-responsive genes under salt stress.

4. Discussion

AQPs play an important role in maintaining cellular water homeostasis, which is crucial for the growth of plants under osmotic stresses, such as drought stress and salt stress. Many plant AQP genes that participate in drought and salt stresses have been characterized (Xu et al., 2014; Sade et al., 2010; Guo et al., 2006) but not in alfalfa. Although the initial salt stress response and metabolite production have been extensively studied, the mechanism by which AQPs enhance plant salt tolerance has not been well understood. Herein, the enhanced activities of the antioxidant defence system and maintenance of Na^+ and K^+ homeostasis due to increased Na^+ efflux and reduced K^+ loss observed in *MsPIP2;2*-overexpressing plants accounted for its better salt tolerance. The upregulation of stress-responsive genes in *MsPIP2;2*-overexpressing plants under salt stress indicated that *MsPIP2;2* participates in ABA-dependent signal transduction and improves salt stress tolerance.

In the current study, a PIP2 subfamily gene, named *MsPIP2;2*, was cloned and functionally characterized from alfalfa. MsPIP2;2 protein contained 2 NPA motifs and 6 putative transmembrane helices (TMHs), which are shared by PIP members and are involved in proton exclusion and channel transport selectivity (Fig. S2) (Li et al., 2009; Tajkhorshid et al., 2002). MsPIP2;2 protein was localized mainly in the plasma membrane (Fig. 2), indicating that MsPIP2;2 may be involved in regulation of transmembrane transport, thereby modulating the cellular ion homeostasis to some extent. *MsPIP2;2* was rapidly induced within 4 h in both roots and leaves of alfalfa under salt stress (Fig. 1C, D), and it may contribute to reducing the damage caused by salt stress in alfalfa.

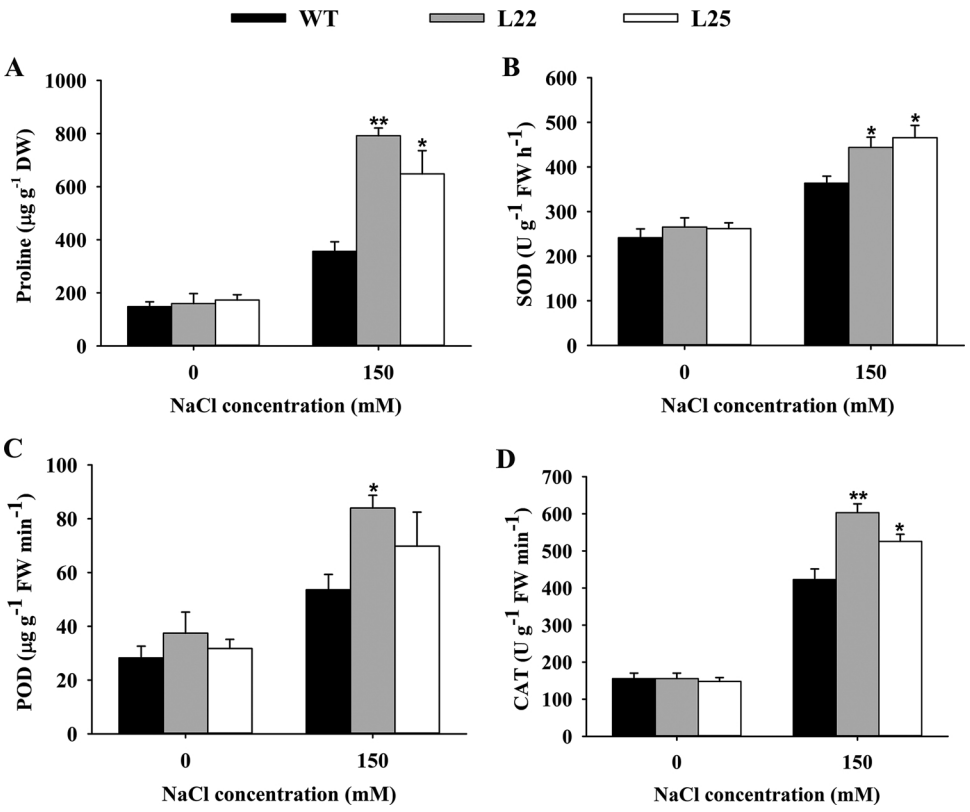


Fig. 6. Effects of salt stress on (A) the proline content, (B) SOD activity, (C) POD activity and (D) CAT activity of the WT and *MsPIP2;2* transgenic *Arabidopsis*. Bars represent the mean ± SE (n = 3). Asterisks and double asterisks above the bars indicate significant differences between the transgenic lines and WT under the same growth conditions: *, $P < 0.05$; **, $P < 0.01$.

In addition, the expression of the *MsPIP2;2* gene was also affected by ABA treatment, suggesting that it may be associated with the ABA signalling pathway.

Salt stress can cause physiological and biochemical changes in plants, which to some extent, reflect the capacity of plants to cope with adversities. Salt stress has been previously demonstrated to induce rapid accumulation of reactive oxygen species (ROS) and lipid peroxidation, which lead to cell membrane injury and oxidation damage of proteins, DNA and lipids (Miller et al., 2010; Xu and Shi, 2007). Common ROS include $^1O^2$, H_2O_2, $O_2^{\cdot -}$ and $^{\cdot}OH$ (Apel and Hirt, 2004). Herein, we measured the accumulation of H_2O_2 and $O_2^{\cdot -}$ to assess the extent of oxidative injury in plant cells. IL and MDA were also examined as important indicators of membrane injury under salt stress conditions (Ashrafi et al., 2015; Xu et al., 2014). Our data showed that *MsPIP2;2*-transgenic *Arabidopsis* exhibited lower H_2O_2, $O_2^{\cdot -}$, IL and MDA contents compared with those of WT plants under salt stress (Fig. 5), implying that overexpression of *MsPIP2;2* can alleviate injuries in plants by reducing the accumulation of ROS. The cell death test further confirmed this conclusion. ROS can alter the structure of AQPs, resulting in channel closure through a direct oxidative mechanism (Kourie, 1998), but this may be stopped by AQP overexpression. Here, *MsPIP2;2* facilitated H_2O_2 diffusion and reduced ROS accumulation. Some AQPs function to transport H_2O_2 in plants (Rhee et al., 2017; Tian et al., 2016).

Plants can trigger antioxidant defence systems to scavenge excess ROS and protect cells against oxidative stress under stressful conditions. This process depends on some functionally correlative antioxidant enzymes, such as superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT), to detoxify stress-induced ROS (Miller et al., 2010). Herein, we observed that the transgenic plants displayed higher SOD, POD and CAT activities than the WT plants under salt stress conditions (Fig. 6B–D), implying that *MsPIP2;2*-overexpressing *Arabidopsis* may have a more powerful capacity for ROS scavenging. This may be a reason the transgenic lines had lower ROS accumulation under salt stress conditions. Plant osmotic solute content acts as an important stress indicator (Mansour and Ali, 2017; El-Esawia et al., 2019). The proline content was drastically elevated in transgenic plants compared with WT plants in the presence of salt stress (Fig. 6A). Obviously, the accumulation of proline is inseparable from the salt-tolerance capacity of plants. *P5CS1* is a key enzyme involved in proline synthesis, and it was upregulated in transgenic plants under salt stress (Fig. 10A). A recent study by Dai et al. (2018) also revealed the protective role of proline through *FcWRKY40*-mediated salt tolerance. Taken together, our findings provide convincing evidence that overexpression of *MsZPIP2;2* can enhance salt tolerance by improving physiological and biochemical processes.

High salt stress can induce ionic toxicity and hyperosmotic stress in plants. Ionic toxicity is caused mainly by high concentrations of Na^+ in the cytosol, which lead to ionic imbalance and dysfunction in plants (Yang and Guo, 2018). Therefore, maintenance of cellular ion homeostasis, in particular a favourable K^+ to Na^+ ratio, is critical for plants to alleviate salt stress (Dai et al., 2018). A previous study reported that AtPIP2;1 possesses the ability to transport cations and is a candidate for the non-selective cation channel (NSCC) responsible for Na^+ entry into roots. The Na^+/K^+ ratio of AtPIP2;1-injected oocytes is significantly lower than that of controls at high external NaCl (0.5M), and the ionic conductance of AtPIP2;1 can be inhibited by external Ca^{2+} and low pH (Byrt et al., 2017). In the current study, *MsPIP2;2* transgenic plants was more salt-tolerant than WT plants under high Ca^{2+} (6 mM or 9 mM) or low pH (4.5 or 5), but the salt tolerance of *Arabidopsis* under salt stress with low Ca^{2+} ($\leq$1.5 mM) or high pH ($\geq$6.5) conditions was not altered (Fig. 7). These results imply that the activity of *MsPIP2;2* can be affected by Ca^{2+} and pH and that *MsPIP2;2* may have ion permeability or the function of regulating the activity of some proteins responsible for Na^+ efflux, thus conferring a degree of salt tolerance. Therefore, the ionic conductance of *MsPIP2;2* needs further examination by voltage clamp experiments to explain the underlying mechanisms.

AtPIP2;1 conductances are relatively small compared to those of other transporters, such as HKTs (Munns et al., 2012; Byrt et al., 2014). Under salt stress, plants maintain a favourable cytosolic K^+/Na^+ ratio

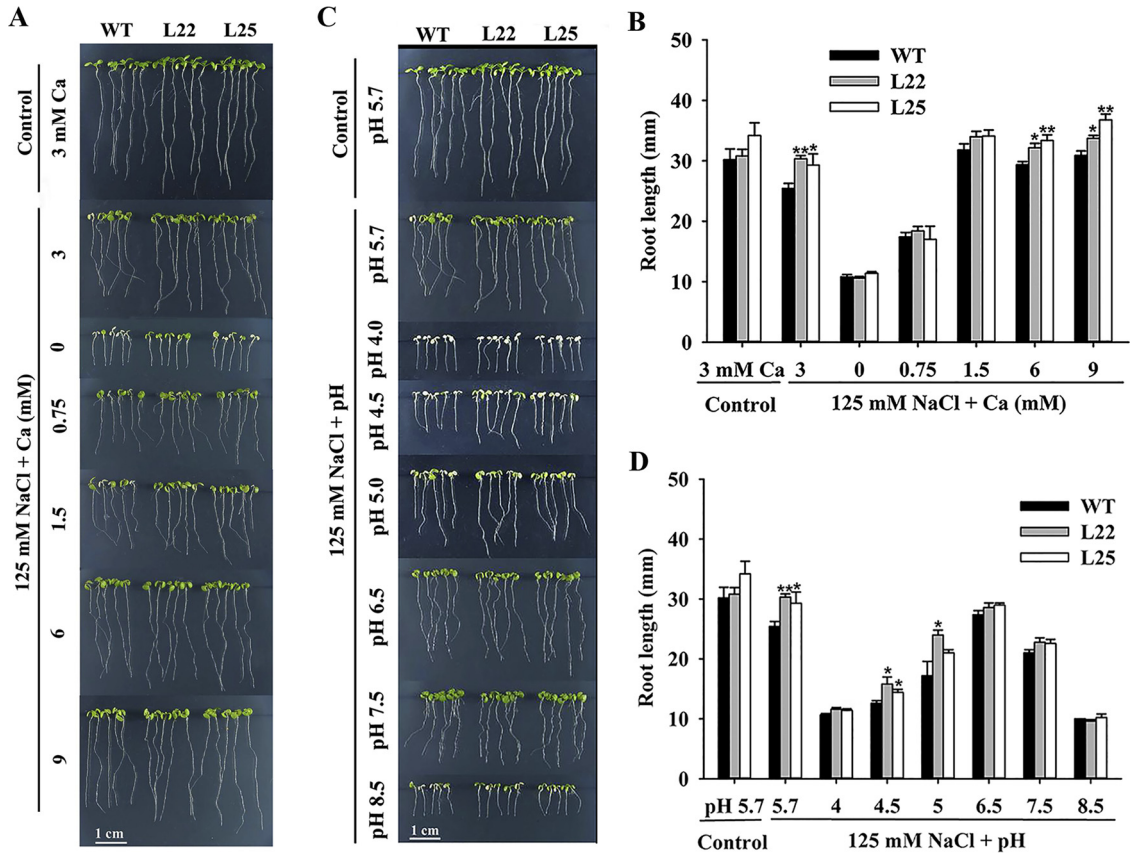


Fig. 7. Effects of external Ca^{2+} or pH on the salt tolerance of WT and *MsPIP2;2* transgenic *Arabidopsis*. (A) Root growth phenotypes of WT and transgenic plants on 1/2 MS medium supplied with 0 or 125 mM NaCl with different concentrations of Ca^{2+} (0, 0.75, 1.5, 3, 6 or 9 mM $CaCl_2$) for 7 d. (B) Root length was calculated for the plants in (A). (C) Root growth phenotypes of WT and transgenic plants on 1/2 MS medium supplied with 0 or 125 mM NaCl with different pH (4.0, 4.5, 5.0, 5.7, 6.5. 7.5 or 8.5) for 7 d. (D) Root length was calculated for the plants in (C). Bars represent the mean ± SE (n = 3). Asterisks and double asterisks above the bars indicate significant differences between the transgenic lines and WT under the same growth conditions: *, $P < 0.05$; **, $P < 0.01$.

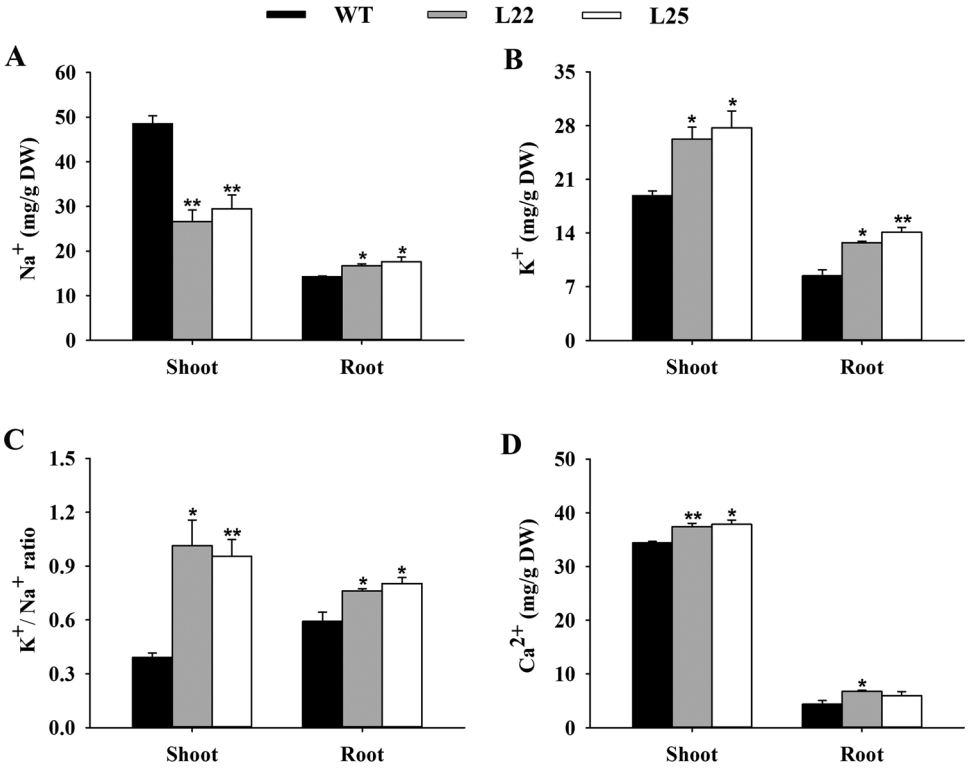


Fig. 8. Ion contents of *MsPIP2;2*-transgenic *Arabidopsis* and WT under salt stress. (A) Na^+ content. (B) K^+ content. (C) K^+/Na^+ ratio. (D) Ca^{2+} content. Bars represent the mean ± SE (n = 3). Asterisks and double asterisks above the bars indicate significant differences between the transgenic lines and the WT under the same growth conditions: *, $P < 0.05$; **, $P < 0.01$.

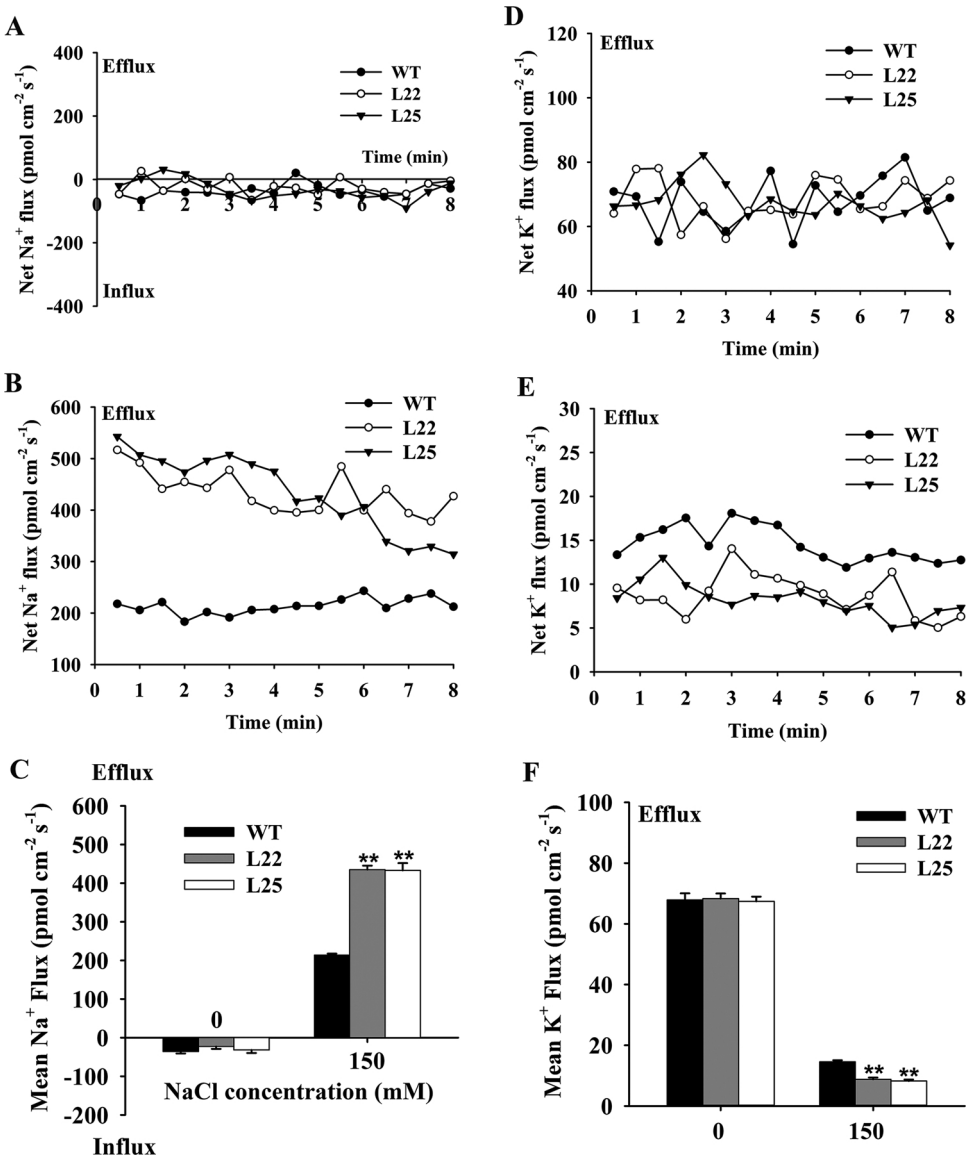


Fig. 9. The net flux of Na^+ and K^+ at the root tip elongation zones of 7-day-old *Arabidopsis* seedlings. (A) The net Na^+ flux under normal conditions (0 mM NaCl). (B) The net Na^+ flux after 150 mM NaCl stress. (C) The mean Na^+ flux. (D) The net K^+ flux under normal conditions (0 mM NaCl). (E) The net K^+ flux after 150 mM NaCl stress. (F) The mean K^+ flux. Bars represent the mean ± SE (n = 3). Asterisks and double asterisks above the bars indicate significant differences between the transgenic lines and the WT under the same growth conditions: *, $P < 0.05$; **, $P < 0.01$.

by restricting Na^+ uptake, promoting Na^+ exclusion or compartmentalization and restricting K^+ leak (Li et al., 2018; Shabala and Pottosin, 2014). In this study, *MsPIP2;2* transgenic plants had lower Na^+ and higher K^+ contents than WT in both shoots and roots, resulting in a strong ability to maintain the K^+/Na^+ balance under salt stress (Fig. 8). The regulation of Na^+ distribution between the root and shoot by AtHKT1 resulted in reduced Na^+ accumulation and enhanced plant salt tolerance (Zhu, 2016). *AtHKT1* was significantly upregulated in transgenic plants compared with WT, thus protecting plants from Na^+ toxicity (Fig. 10B). In addition, the effluxes of Na^+ and K^+ in the roots of the transgenic plants indicated that the transgenic plants maintained K^+/Na^+ balance through discharging more Na^+ and retaining more K^+ (Fig. 9). Li et al (2018) reported that overexpression of the *TsPIP1;1* gene increased the salt tolerance of rice via enhancing Na^+ efflux and decreasing K^+ efflux to modulate ion homeostasis. Plant Na^+ exclusion is regulated by the plasma membrane (PM) H^+-ATPase and Na^+/H^+ antiporter (SOS1) (Shi et al., 2003; Cuin et al., 2011), and K^+ leak is restricted by depolarization-activated outward-rectifying K^+ (GORK) channels and non-selective cation channels (NSCCs) (Anschutz et al., 2014; Sun et al., 2009b). Indeed, here evidence was presented that *SOS1* expression was upregulated and *GORK* expression was downregulated in the roots of *MsPIP2;2*-overexpressing plants (Fig. 10A, C). This result suggested that the increased K^+ in the transgenic plants under salt stress was probably due to reduced K^+ loss from the roots. However, we cannot completely exclude the possibility that *MsPIP2;2* facilitates the uptake of K^+ under salt stress. Previous studies also confirmed the uptake and translocation of K in rice under salt stress (Li et al., 2018). In our study, the remarkable up-regulation of *AtKAT1*, *AtKAT2* and *AtHAK5* in the transgenic lines might promote the uptake of K^+, thus altering the ratio of K/Na.

High salinity leads to increased production of ROS, which directly activate both GORK and NSCCs and further contribute to K^+ efflux (Shabala and Pottosin, 2014). As long as plants can control K^+ leak from the cytosol or are capable of compensating K^+ loss from the vacuolar K^+ pool, root cells are able to survive under salt stress. The findings of our study showed that the transgenic plants accumulated less ROS than WT, suggesting that overexpression of *MsPIP2;2* resulted in less K^+ leak due to ROS. This may be one explanation for

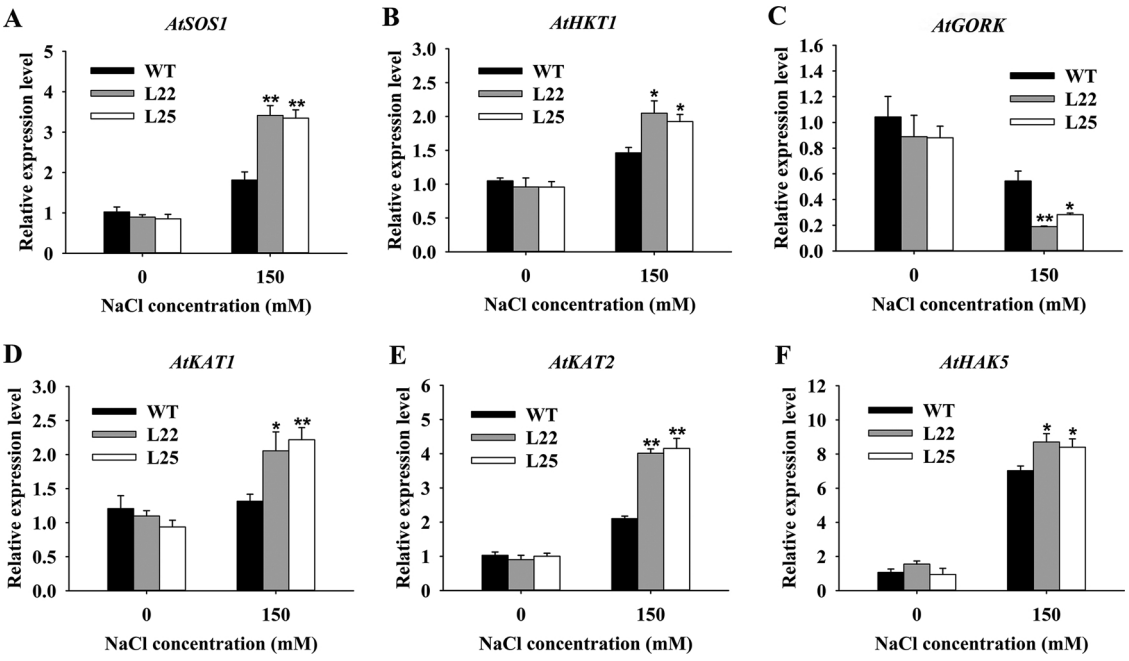


Fig. 10. Expression levels of ion homeostasis genes in roots of 7-day-old *Arabidopsis* seedlings after 150 mM NaCl treatment for 4 h. Expression patterns of *AtSOS1* (A), *AtHKT1* (B), *AtGORK* (C), *AtKAT1* (D), *AtKAT2* (E) and *AtHAK5* (F). Bars represent the mean ± SE (n = 3). Asterisks and double asterisks above the bars indicat'"e significant differences between the transgenic lines and the WT under the same growth conditions: *, $P < 0.05$; **, $P < 0.01$.

maintaining K^+ levels in transgenic plants, which is critical for plant growth under salt stress. In addition, AQPs play an important role in regulating rapid transmembrane water flow during plant growth (Gao et al., 2010). As a result, *MsPIP2;2*-overexpressing plants absorbed water more quickly and decreased the concentration of Na^+. Taken together, our results suggested that overexpression of *MsPIP2;2* may be conducive to maintaining and reconstructing cellular ion homeostasis, thus mitigating salt stress damage.

Abiotic stresses can, to some extent, induce the transcriptional modulation of stress-responsive genes (Xiong et al., 2002). Zhang et al. (2016) reported that overexpression of *MsZEP* increased the expression levels of *DREB* and *P5CS* in transgenic tobacco under drought and salt stress. In our study, the expression levels of *P5CS1*, *RD29A*, *DREB2* and *KIN2* were substantially elevated in transgenic lines compared with WT plants when subjected to salt stress (Fig. 11). *DREB2* belongs to the DREB1/CBF (C-repeat binding factor) transcription factor family, which

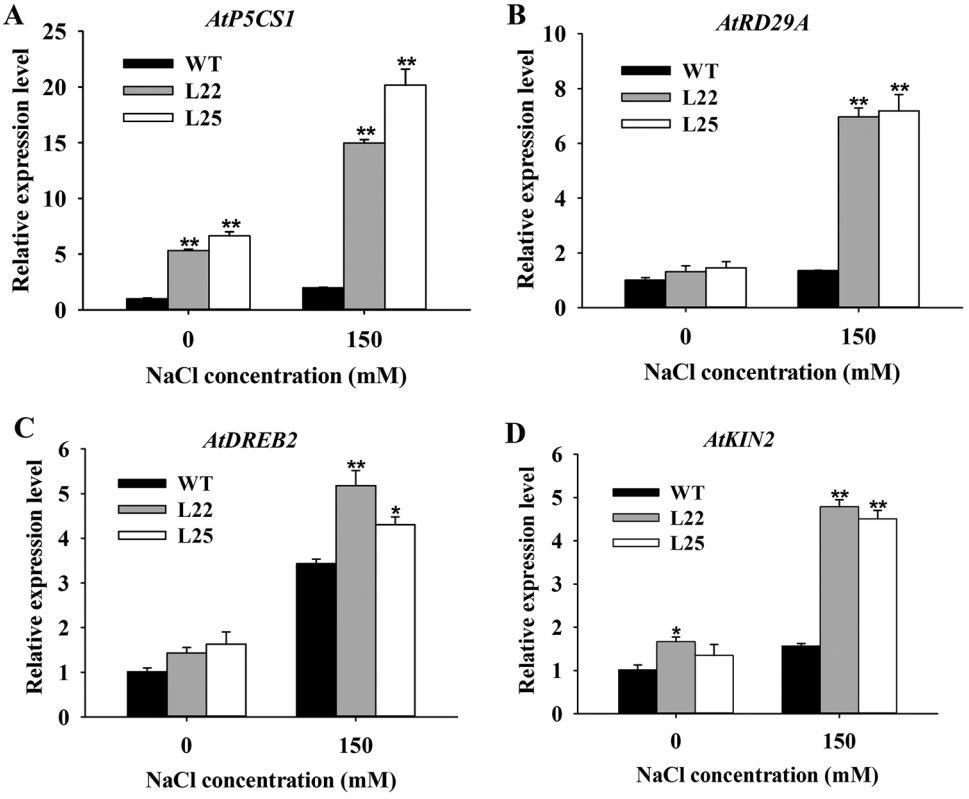


Fig. 11. Expression levels of stress-responsive genes in transgenic *Arabidopsis* and WT plants after 150 mM NaCl treatment for 10 days. Expression patterns of *AtP5CS1* (A), *AtRD29A* (B), *AtDREB2* (C) and *AtKIN2* (D). Bars represent the mean ± SE (n = 3). Asterisks and double asterisks above the bars indicate significant differences between the transgenic lines and the WT under the same growth conditions: *, $P < 0.05$; **, $P < 0.01$.

is highly induced by abiotic stressors (Zhuo et al., 2016). In addition, ABA-dependent signalling pathways play crucial roles in the response of plants to osmotic stress (Yoshida et al., 2014). The transcripts of ABA-responsive genes such as *RD29A* and *KINI* are induced in *MaPIP1;1*-transgenic *Arabidopsis* plants under salt stress (Xu et al., 2014). These data suggested that *MsPIP2;2* responses to salt stress may be regulated by the ABA signalling pathway, which corresponds to the upregulation of *MsPIP2;2* under ABA treatment. Notably, the AQP gene *MsPIP2;2* is supposed to be located at the end of the signalling pathway, but how it regulates the expression of upstream genes needs further study.

5. Conclusion

In conclusion, we identified the positive role of the *MsPIP2;2* gene in the improvement of salt stress tolerance. Overexpression of *MsPIP2;2* enhances salt tolerance in plants, leads to a reduction in ROS accumulation by enhancing the antioxidant defence system, and maintains ion homeostasis by increasing K^+ retention and Na^+ exclusion. Moreover, *MsPIP2;2* mediates signal transduction by increasing stress-responsive gene transcript levels. Our study provides new insight into how AQPs improve salt tolerance in plants and how *MsPIP2;2* can be targeted in future breeding programmes to impart salt tolerance to alfalfa.

6. Conflict of interest

The authors have no conflicts of interest to declare.

7. Author contributions

SL and JL designed the research; SL, YA, YC and JZ performed the experiment; SL, YL, JG analyzed data and wrote the manuscript; JL, PY and TH provided ideas, and revised the manuscript; all authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.envexpbot.2019.05.020.

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