INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
16–0610/2018/20–1–62–70
DOI: 10.17957/IJAB/15.0362
http://www.fspublishers.org

Full Length Article

MsPP2C, a Protein Phosphatase 2C Gene of Alfalfa, Confers Enhanced Salt Tolerance in *Arabidopsis*

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Abstract

Protein phosphatase 2C (PP2C), one of the protein serine/threonine phosphatases, plays a negative role in plant development, organ formation, hormone regulation and stress resistance. However, fewer studies have shown that the PP2C gene is positively regulated by abiotic stresses in plants. In this study, one PP2C gene, *MsPP2C*, was isolated from *Medicago sativa* L. cv. 'Gongnong No. 2' (alfalfa Zhongmu). *MsPP2C* contains an 1176-bp open reading frame that encodes a 391-amino acid protein. Fluorescence quantitative PCR showed that salt stress, abscisic acid (ABA), and drought stress treatments induced the expression of *MsPP2C*. To further investigate the salt-stress resistance in the *MsPP2C*-overexpressing plants, *35S:MsPP2C* transgenic Arabidopsis plants were obtained by transforming a constructed vector through *Agrobacterium*-mediated transformation. Compared with the wild type (WT) line, in the *MsPP2C*-overexpressing plants, Fv/Fm increased by 50–52%, the chlorophyll content elevated by 31–63%, the MDA content decreased by 17–21%, and the proline content increased by 17–23% under salt stress. Additionally, the sodium ion (Na^+) content in both the leaves and roots increased. However, the aboveground tissues in the transgenic plants had a lower Na^+ content, while the roots had a higher Na^+ content. In conclusion, our results suggest that the overexpression of the *MsPP2C* gene isolated from alfalfa Zhongmu conferred salt-tolerance in Arabidopsis. The salt tolerance may due to osmolytes accumulation in the expanded cells instead of protection of the cell membrane from the damage by the accumulation of superoxide anion radicals. This study provides a basis for improving biotechnological approaches for alfalfa to adapt salt stress driven by probably future climate change.

Keywords: *Medicago sativa; MsPP2C;* Protein phosphatase; Salt stress; Characterization function study

Introduction

Plants resist to harsh and adverse environments. Among the abiotic factors threating to plants, salinity is of special concern because approximately 6% of the world's total land and 20% of the irrigated land are greatly affected by salinity (Rahman *et al.*, 2013). Salinity has attracted more attention than other environmental constraints (Ahmad *et al.,* 2010). In some regions, salinity has arisen as an ecology threat at the national level. For example, the effect of soil salinization has been evaluated on the agricultural development and the stability of ecological systems and biodiversity in China (Lorenzo *et al.*, 2009; Long *et al.*, 2012).

After a long-term evolution, a series of processes within a plant are activated as a response to external salt stress (Abba *et al.*, 2006; Antoni *et al.*, 2013; Cui *et al.*, 2013). Therefore, identification of key mechanisms would be helpful to develop new approaches to overcome salt stress. Transcriptional regulation of gene expression is a critical step during plant responses to salt stress, which is related to plant adaption on survival and sustainability. Protein reversible phosphorylation has been observed during plant responses to abiotic stress, indicating protein phosphatase may play an important role (Abba *et al.*, 2006; Antoni *et al.*, 2013). To date, 80 and 78 PP2Cs have been discovered in Arabidopsis and *Oryza sativa*, respectively (Hu *et al.*, 2016). Studies have shown the PP2C gene can be induced by diverse stimuli in response to abscisic acid (ABA) regulation.

Under abiotic stresses, the ABA content dramatically increases in plants, which regulates stomatal closure and activates the antioxidative system to protect vegetative tissues from injury (Kathuria *et al.*, 2009; Farooq *et al.*, 2015). The core ABA signaling pathway consists of three important elements: ABA receptors (PRY/PYL/RCAR proteins), PP2Cs, and SNF1-related protein kinases (SnRK2s) (Bielewicz *et al.*, 2013). It is widely accepted that the group A PP2C, composed of nine members (ABI1, ABI2, HAB1, HAB2, AHG1, AGH3/AtPP2CA, HAI1, HAI2 and HAI3), plays a key role in the ABA signaling pathway (Barton *et al*., 2015). Studies have demonstrated that the PP2Cs in Group A, except the three HAI PP2Cs, are

negative regulators of the ABA signaling (Bielewicz *et al.*, 2013). However, the positive regulatory role of PP2C under salt and drought stress is still unclear. Defining their molecular and physiological functions will therefore enrich our understanding of the stress-related signaling networks.

Alfalfa (*Medicago sativa* L.) is an important leguminous forage crop with about 32 million hectares under cultivation globally (Anower *et al.*, 2013). It not only supplies forage for animals, but also improves soil fertility and protects the environment with significant economic, ecological and social effects (Liu *et al.*, 2014). However, the production and yield of alfalfa under salt stresses are not stable because of the high variety of the sensitivity of alfalfa cultivars to salt stress (Anower *et al.*, 2013). In the present study, an alfalfa cultivar, *Medicago sativa* L. cv. 'Gongnong No. 2', was selected to study the PP2C gene, *MsPP2C,* in response to exogenous ABA and other abiotic stresses. The transgenic plants were also investigated for the physiological changes and the expressions of stress-related genes under stresses. The results demonstrate that *MsPP2C* played an important role under salt stress, and the overexpression of *MsPP2C* in *Arabidopsis thaliana* can be used as an effective strategy to study the function of *MsPP2C*.

Materials and Methods

Plant Materials and Treatments

Cultivars of alfalfa Gongnong (*M. sativa* L. cv. 'Gongnong No. 2') were provided by Chinese Academy of Agricultural Sciences. Seeds were transported to the Laboratory of Environment and Resources College in Dalian Nationalities University (39º02' N, 121º46' E). The seeds were surface-sterilized with 0.1% (w/v) $HgCl_2$ for 5 min, washed with 75% ethanol for 10 min, and rinsed with sterilized water for three times. The cleaned seeds were soaked in darkness for 24 h, and then placed in a growth chamber with a 16 h/8 h light/dark cycle at 23ºC. During the illumination, the ambient photosynthetic photon flux density was adjusted to 200 μmol m^{-2} s^{-1} by plant growth lamps (Oudi® Illumination Ltd., Huzhou City, Zhejiang Province, China). The germinated seedlings were grown in Hoagland nutrient solutions, which were replaced every 2 d. The nutritional composition of the solutions was adapted from a previous study by Wei and Pan (2013), which mainly includes NH_4NO_3 (4 mmol), K_2HPO_4 (0.5 mmol), KCl (0.5 mmol), $CaCl_2$ (1 mmol), and $MgSO_4.7H_2O$ (0.6 mmol). The stress treatments were applied 20 d after being planted.

For the salt stress treatment, seedlings were watered with the Hoagland nutrient solution supplemented with 250 mM NaCl for 0, 2, 4 and 6 h, respectively. The exogenous ABA treatment was performed by spraying seedling leaves with 200 μM ABA solution for 0, 2, 4 and 6 h, respectively. The drought treatment was conducted by watering seedlings with 20% PEG6000 (polyethylene glycol; PEG is a hydrophilic macromolecule with strong water absorption ability, which can cause plant dehydration) to imitate hydropenia for 0，2, 4 and 6 h, respectively. After the treatments, the third entirely expanded leaves from the bottom in healthy condition were sampled and immediately frozen in liquid nitrogen until further analysis. Four independent biological replicates were performed for each treatment (*n*=4).

Gene Cloning and Sequence Analysis

Total RNA in the foliar samples was extracted using the CTAB method (Chang *et al.*, 1993). A total of 400 ng total RNA was evaluated on an Agilent 2100 Bio-analyzer. A reverse transcription reaction was carried out with 20 μl RNA to synthesize cDNA using a 1st strand cDNA synthesis Kit (D6110A) following the manufacturer's protocol. The full-length cDNA sequence of the *MsPP2C* gene was identified using the Arabidopsis *SPDS1* as the template via the Blastn program (http://www.ncbi.nlm.nih.gov/BLASTn/). The specific primers for thefluorescence quantitative PCR were *ER5*: 5′-ATGGCTGAAA AGAATAGCCGTGTAGTTG-3′ and *ER3*: 5′-CTTCATCAGT ACTTGATTCACAGCTATC-3′. The internal reference primers were *actin 5*: 5′-TGGGCTGCCACAGAACATTTGA-3′ and *actin 3*: 5′-GCTGTGGTTGCTTTTTTGGTGTCTC-3′. Four independent biological replicates were performed for each treatment (*n*=4).

Cloning of *MsPP2C*

Using the total RNA of alfalfa as the template, reverse transcription was carried out by M-MLV reverse transcriptase to synthesize the first-strand cDNA for further PCR amplification. The primers used for the PCR amplification were *MsPP2C-F*: 5′-ATGGCTGAAAAGAATAGCCGTGTAGTTG-3′ and *MsPP2CR*: 5′-TTATTGTTGACGTTGATCTCTCCTCA-3'. The PCR system was set up following the manufacturer's protocol (TaKaRa). After an initial denaturation at 94°C for 5 min, the reaction was subjected to 34 cycles of amplification consisting of 50 s at 94°C, 50 s at 57°C, and 60 s at 72°C with a final extension at 72°C for 10 min. The PCR products were detected by 1% agarose gel electrophoresis and sequenced.

Constructing a *MsPP2C*–Pcambia1304 Vector and Producing Transgenic Plants

The primers containing restriction enzyme sites were designed as follows: *MsPP2C–Spe*I-*FP*: 5′-ACTAGTATGGCTGAAAAGAATAGCCGTGTAGTTG-3' and *MsPP2C–Bgl*II-*RP*: 5′-AGATCTTTATTGTTGACGTTGATCTCTCCTCA-3′. *MsPPP2C* was amplified using *MsPP2C–PMD18T* as a

template. After an initial denaturation at 94°C for 5 min, the reaction was subjected to 34 cycles of amplification consisting of 50 s at 94°C, 50 s at 57°C and 60 s at 72°C with a final extension at 72°C for 10 min. The PCR products were collected and inserted into a *PMD18-T* vector; and the constructed vectors were then sequenced. The plasmids with correct sequencing were digested by the restriction enzymes, *Spe*I and *Bgl*II, separately. The recombination expression vector (*Pcambia1304–35S*:*MsPP2C)* was constructed by ligating the *MsPP2C* gene fragments with a *pCambia1304* vector by T4 ligase, and transformed into Arabidopsis through the *Agrobacterium* transformation system. The transgenic Arabidopsis lines were selected by growing on Murashige and Skoog (MS) plates containing kanamycin. The transgenic lines at T_3 generation were obtained by a kanamycin selection method.

Salt Tolerance in the *MsPP2C*-Overexpressing Lines

Transformation of the *MsPP2C* gene was performed using an Agrobacterium-mediated leaf disc transformation method. Regenerated shoots were rooted on a MS medium containing 200 μg mL^{-1} kanamycin and 250 μg/mL carbenicillin and grown in the laboratory condition as described above. The T1 and T2 regenerations of the transgenic Arabidopsis lines were germinated on a 1/2 MS medium containing 200 μg/mL kanamycin in a growth chamber under 18 h illumination per day at 24ºC. The T-3 generation was used for transgenic determination.

After grown on MS plates for 7 d, both the transgenic and WT lines were transplanted into pots for the NaCl treatment, which was conducted at two different seedling growth stages. During the first stage, the seedlings were treated with NaCl one week after the transplant when juvenile leaves appeared. During the second stage, the seedlings produced their vegetative shoots two weeks after the transplant. The toxic responses of the seedlings to the NaCl addition were closely monitored during these two growing stages to ensure the differences between the WT and transgenic lines were definite and significant. These two stages of the seedlings were treated as two separated experiments, during which both the transgenic and wild type lines were watered using 250 mM NaCl solution every 2 d for two weeks. The seedlings showed distinct responses and were recorded for visual performance by photographing images. Four independent biological replicates were performed for each treatment (n=4).

Physiological Analysis of the Transgenic Arabidopsis Lines

After the 250 mM NaCl treatment, the chlorophyll content, maximal photochemical efficiency (Fv/Fm), proline content, malondialdehyde (MDA) content, and sodium ion (Na^+) content were measured for the T3 transgenic and wild type lines.

The chlorophyll content was determined using the leaf samples with similar weight (~0.01 g) of the transgenic and WT lines. The chlorophyll content was calculated using the formulae Ca (mg L^{-1}) = $12.21A_{663} - 2.81A_{645}$, C$b$ (mg L^{-1}) = $20.13A_{645} - 5.03A_{663}$, and C$t$ (mg g^{-1}) = (Ca + Cb) × V / m, where Ca is the content of chlorophyll a, Cb is the content of chlorophyll b, Ct is the total chlorophyll content from plant tissues, V is the volume of the liquid extract and m is the weight of the sample. Two technical and five biological replicates were performed.

The chlorophyll fluorescence measurements were recorded with a Plant Efficiency Analyzer (PEA) (Hansatech Instruments Ltd., Norfl, England, UK). For each measurement of Fv/Fm, a randomly selected shoot was dark-adapted for 15 min using the manufacturer's plastic/foam clips. The shoot was acclimated to room temperature for at least 1 h prior to the fluorescence measurement. A fluorometer actinic light level of 1200 μmol m^{-2} s^{-1} was determined to be sufficient for our preliminary tests per manufacturer's instructions. An algorithm was used to determine the line of best fit through the initial 8–24 data points at the onset of illumination. This line of best fit was then extrapolated from time zero to determine F_0 (initial or minimal fluorescence) and F_m (maximum fluorescence) was obtained at the same light intensity when the primary electron acceptor from PSII (Q_A) became fully reduced. Variable fluorescence (Fv) was automatically calculated by subtracting F_0 from Fm and Fv/Fm was calculated sequentially.

The proline content was determined using fresh leaves (500 mg), which were extracted with 5 mL 3% sulfosalicylic acid by shaking at 100ºC for 10 min. The extracts were filtered through glass wool and analyzed for the proline content using the acid ninhydrin method. Briefly, 2 mL aqueous extract was mixed with 2 mL glacial acetic acid and 2 mL acid ninhydrin reagent (1.25 g ninhydrin, 30 ml glacial acetic acid and 20 mL 6 M orthophosphoric acid) and incubated at 100ºC for 30 min. After cooling, the reaction mixture was partitioned against toluene (4 mL), and the absorbance of the organic phase was measured at 520 nm. The proline concentration was determined by comparing with a standard curve constructed using proline solutions with known concentrations (Sigma, St Louis, MO, USA). Two technical and five biological replicates were performed.

The MDA content was measured using a modified thiobarbituric acid (TBA) method (Lowe and Eddy, 1997). In this experiment, approximately 0.1 g leaf tissues were ground in 10 mL 10% trichloroacetic acid (TCA) using a mortar and pestle.

The Na^+ content in plants was determined by a flame photometer method (Lowe and Eddy, 1997). The dried samples were ground into powder. In a 15 mL scale tube, 50 mg powder and 10 mL ddH_2O were placed. The mixture was topped up to 50 mL with ddH_2O, and incubated in a boiling water bath for 2 h. After filtration, the sodium

ion content in the filtrate was determined. Four independent biological replicates were performed in these treatments (*n*=4).

Results

Cloning and Homology Comparison of the *MsPP2C* Gene

The complete encoding sequence of *MsPP2C* was identified by homology searching in GenBank with the Arabidopsis *SPDS1* (GenBank accession No. KT592510.1). The *MsPP2C* cDNA is 1,169 bp in length, and encodes a 391 amino acid protein with a predicted molecular mass of 44.5 kDa and a pI of 5.46. Compared with the phosphatases in other plants (Fig. 1A), MsPP2C showed high similarity at the C terminal, which is considered as the conserved domain of the phosphatases. In addition, the C terminal of MsPP2C had a high similarity with that of many genes in *Ricinus communis*, *M. truncatula*, *A. thaliana*, *Solanum lycopersicum*, *Zea mays*, *Glycine max* and *Vitus vinifera* (Fig. 1B). These results suggest that *MsPP2C* may play a similar role with the Arabidopsis *SPDS1*.

Expression Patterns of *MsPP2C* in Response to ABA and Abiotic Stresses

To further understand the expression level of MsPP2C under different stresses, fluorescence quantitative PCR was carried out using *actin* as the internal control. The results show that the *MsPP2C* gene could be induced by abiotic stresses and exogenous application of ABA. Under the salt treatment, the *MsPP2C* expression reached the maximum at 2 h, which was approximately seven-fold higher than that of the control (Fig. 2A). When treated by ABA, the *MsPP2C* expression reached the maximum at 4 h, which was approximately seven-fold higher than that of the control (Fig. 2B). The expression of *MsPP2C* induced by the drought treatment was similar to that by ABA, which peaked at 4 h with an expression six times higher than that of the control (Fig. 2C).

RT-PCR Analysis of the Transgenic Plants

To study the characteristics of the exogenous gene in the transgenic Arabidopsis, the T_1 transgenic plants were selected by kanamycin resistance and analyzed by RT-PCR. Compared with the internal control *actin*, the expression level of *MsPP2C* was higher in the transgenic plants than that in the wild type plants (Fig. 3), which means that the exogenous gene *MsPP2C* was completely integrated into the chromosomes of Arabidopsis, and could pass to the next generation. The *MsPP2C* band had the highest brightness in the seventh transgenic samples and was absent from the wild type. Among the transgenic lines, the *MsPP2C* band in the fourth

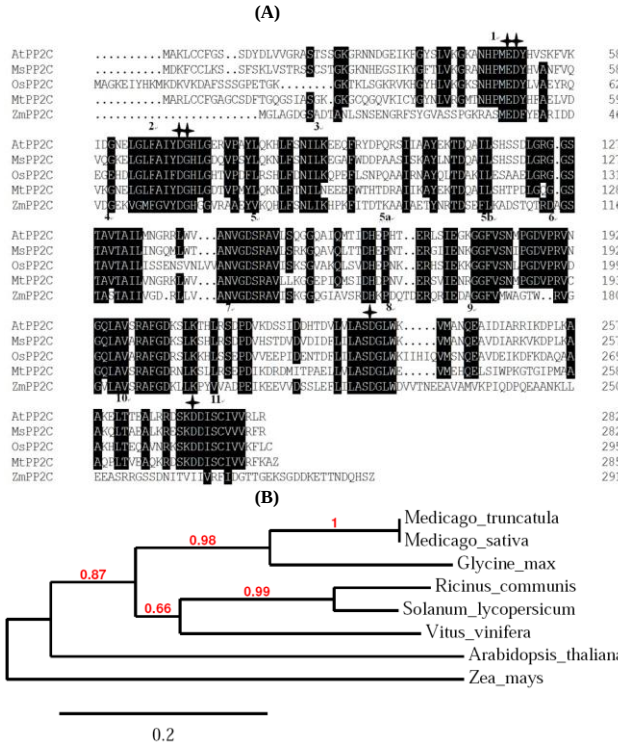

Fig. 1: Multiple sequence alignment and phylogenetic analysis of *MsPP2C* and its homologs from other plant species. (A) Sequence alignment of *MsPP2C* and its homologs. Identical amino acids are shaded in black, while dashes indicate gaps introduced to optimize the alignment. Residues putatively involved in the coordination of the phosphate and metal ions are marked with stars. The conserved motifsin the *PP2C* family are indicated in Arabic numbers above each region. The alignment was done using DNAman version 5.2. (B) A phylogenetic tree of *MsPP2C* with other homologs constructed using the neighbor-joining method by MEGA 4.0. All protein sequences were retrieved from GenBank and the *PP2C* sequence accession numbers were as follows: NP_973883 (*AtPP2C*, Arabidopsis), XP_003588942.1(*MtPP2C*, M*edicago truncatula*), NP_874564 (*OsPP2C)*, BAC16709 (*Oryza sativ*), XP_973865 (*ZmPP2C)*, and AY621066 (*Zea mays*)

transgenic samples had the weakest brightness, while that of the ninth transgenic samples was moderate. To understand the *MsPP2C* overexpression, seeds from the T_1-4, T_1-7 and T_1-9 transgenic lines with different expressing intensities were selected. After the T_3 homozygous lines were obtained from these three lines, the physiological indexes were determined.

Characteristics of the Transgenic Plants

One week after transplant, all seedlings were germinated

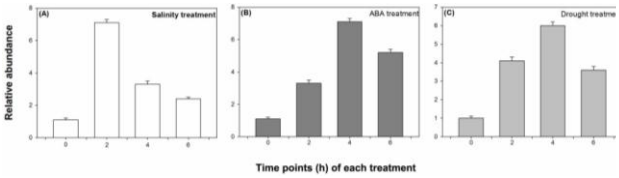

Fig. 2: Expression patterns of the *MsPP2C* gene in alfalfa leaves detected by real-time PCR analysis in response to drought treatment of exogenous manipulations of salinity (A), abscisic acid (ABA) (B), and drought (C) treatments. The relative expression was quantified using actin as an internal reference, and the expression level in the unstressed plants was assigned a value of 1. The columns present the mean values from the replicated experiments (n=4) and the error bars presents the standard error

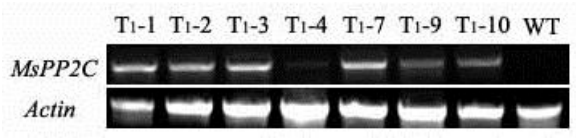

Fig. 3: Fluorescence quantitative RT-PCR analysis of the *MsPP2C* gene in the first generation of the transgenic lines of T_1-1, T_1-2, T_1-3, T_1-4, T_1-7, T_1-9, T_1-10, and the wild type (WT) line in *Arabidopsis thaliana* subjected to the salt stress treatment. Actin is used as an endogenous control in all cDNA samples

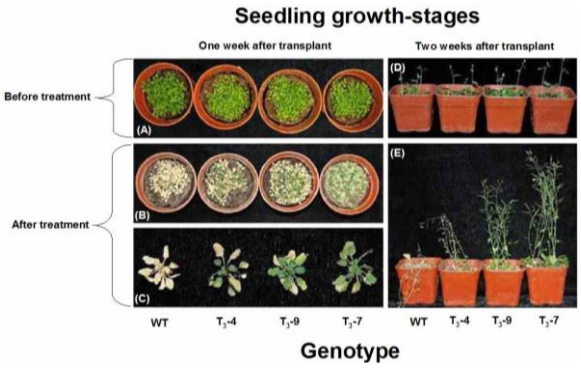

Fig. 4: The growth performance of the seedlings of the T3-generation of the wild type (WT) and the transgenic lines T_3-4, T_3-7, and T_3-9 at different growth stages in response to salt stress. Seedlings in (A), (B) and (C) were treated with NaCl one week after transplant when only juvenile leaves came out; and seedlings in (D) and (E) were treated with NaCl two weeks after transplant when vegetative shoots had grown up. The T_3 transgenic Arabidopsis seedlings in (A) and (D) did not receive any treatment; and those in (B), (C), and (E) had been treated with 250 mM NaCl for two weeks

(Fig. 4A). After treated with 250 mM NaCl for two weeks, all germinated seedlings from the WT line died, but those from the transgenic line survived (Fig. 4B). The foliars of the dead WT seedlings turned gray, while only half of the leaves of the T_3-4 transgenic seedlings turned gray. In contrast, nearly all leaves of the T_3-7 transgenic seedlings were still green (Fig. 4C). These results suggest that the overexpression of the *MsPP2C* gene could promote the salt resistance in the transgenic Arabidopsis. The results also indicate the NaCl treatment at 250 mM could lead to different responses in the WT and transgenic lines. Because the seedlings in some treatments had died, and no shoot has grown during the current growing stage, these seedlings were not used for the physiological measurement.

On the other hand, many Arabidopsis seedlings had grown vegetative shoots (Fig. 4D). These seedlings also received 250 mM NaCl treatment for two weeks, after which nearly all WT seedlings died. However, the seedlings from the T_3-4, T_3-9 and T_3-7 transgenic lines showed gradually improved growth (Fig. 4E). These morphological results indicate that the transgenic Arabidopsis seedlings from different lines had different physiological responses to the NaCl treatment. Hence, the seedlings at this growing stage were used for the physiological measurement.

Determination of Physiological Indexes in the Transgenic Arabidopsis

The seedlings without the NaCl treatment had similar Fv/Fm among the WT and transgenic lines. In response to the NaCl treatment at 250 mM, the overall Fv/Fm declined by 6–26% relative to those without the NaCl treatment (Fig. 5A). The Fv/Fm value in the WT line was about ~21% lower than that in the T_3-7 and T_3-9 lines. This result indicates there was less injury to the photosynthetic apparatus in the transgenic lines under salt stress. Compared to the initial seedlings, the chlorophyll content in the NaCl-treated seedlings dropped by 31–63% (Fig. 5B). In response to the NaCl treatment, the chlorophyll content in the seedlings from the T_3-7 transgenic line was one-fold higher than that in the WT line. This result suggests that the transgenic Arabidopsis seedlings could sustain fairly sufficient photosynthesis in response to the NaCl treatment.

Both the proline and MDA contents elevated after the NaCl treatment by 2.7 and 1.9 fold on average, respectively (Fig. 5C, D). In response to the NaCl treatment, the proline content in the T_3-9 and T_3-7 transgenic lines was 0.9 and 1.4 fold higher than that in the WT line (Fig. 5C). This result suggests that the ability to accumulate osmoregulation substances was improved in the transgenic lines under salt stress. The MDA content is related to the ability to accumulate reduced oxygen and the antioxidant capacity in cells. The MDA content was much higher in the WT line than the transgenic lines. The MDA content in the WT line was 1.25-fold higher than that in the T_3-7 line (Fig. 5D). This result indicates that, under slat stress, the expression of *MsPP2C* relieved the accumulated reactive oxygen species in the transgenic Arabidopsis.

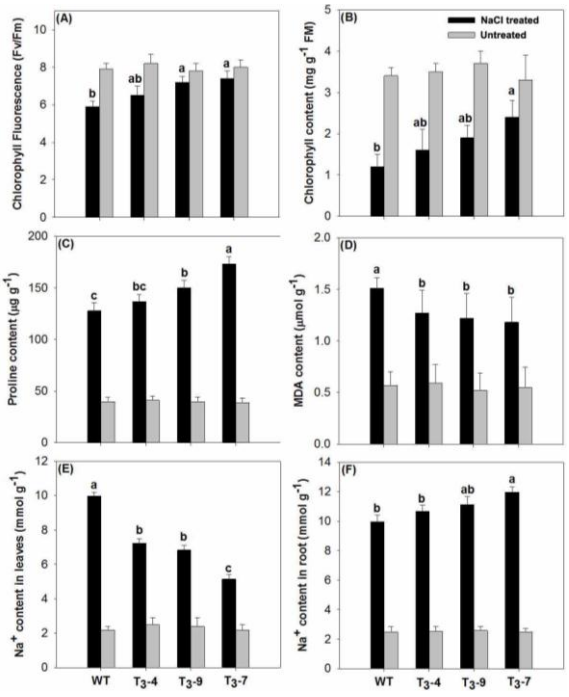

Fig. 5: Parameters of maximal photochemical efficiency (Fv/Fm) (A), chlorophyll content (B), proline content (C), MDA content (D) and sodium ion (Na^+) content (E), (F) in leaves and roots of the T_3 transgenic Arabidopsis seedlings in response to 250 mM NaCl solution for two weeks. Different letters indicate significant difference according to the Duncan test at the 0.05 level. The untreated control refers to the initial status of seedlings without any NaCl treatment

After the NaCl treatment, Na^+ was heavily accumulated in both the aboveground (leaves) and underground parts (roots) (Fig. 5E, F). However, the Na^+ content in the aboveground parts of the WT seedlings was 1.6 times higher than that in the T_3-7 line (Fig. 5E), while the Na^+ content was 1.2 fold higher in the roots of the seedlings from the T_3-7 transgenic lines than that in the WT line (Fig. 5F). These results suggest that the transgenic Arabidopsis seedlings had a higher ability to detain Na^+ in the roots, while Na^+ was mobilized upwards to the shoot part in the WT line.

Discussion

Alfalfa is the most widely distributed leguminous forage plant in the world, and represents the largest cultivated forage crops in China. It is considered the 'king of forage' due to its high productivity, wide ecological adaptability and ability to provide high quality protein to livestock. Alfalfa is also an important plant to improve the saline-alkaline soil as well as to develop agriculture on the saline soil (Peng *et al.*, 2008). Salt-resistant alfalfa cultivars have been developed mainly depending on the physiological determination by traditional methods (Endo *et al.*, 2014; Ashrafi *et al.*, 2015; Rahman *et al.*, 2015). Several alfalfa cultivars have been successfully developed, but are limited to regional availability or inexplicable responses. Biotechnical approaches have yet to be employed to improve the salt resistance of alfalfa. Previous studies indicate that the potential slat-tolerance genes in the transgenic alfalfa mainly include nucleoside diphosphate kinases (KDPKs) (Wang *et al.*, 2014), WRKY proteins (Tang *et al.*, 2014), calcium/calmodulin-dependent kinases (Cheng *et al.*, 2013), and so on. A great number of genes encoding other key enzymes may also play a role in the resistance to salt stress in alfalfa. The lack of salt-tolerant lines has greatly limited the development of alfalfa plantation (Xu *et al.*, 2008; Senadheera *et al.*, 2009; Pillitteri and Torii, 2012). PP2C is a key signaling molecule as it is common in plants and contains conserved structural characteristics as well as physicochemical properties. PP2C participates in different signaling pathways, including the ABA signaling pathway and other pathways for stress tolerance (Nakashima and Yamaguchi-Shinozaki, 2013; Lim *et al.*, 2015), development (Brock *et al.*, 2010; Singh *et al.*, 2015), and disease resistance (Hu *et al.*, 2006). In this study, a PP2C gene homolog, *MsPP2C*, from the leaves of alfalfa was transformed to Arabidopsis, and the transgenic performance of Arabidopsis was evaluated.

The open reading frame of *MsPP2C* contained 1176 bp and encoded a 391-amino acid protein, which contained a typical domain of phosphatase (98–278 aa). Its catalytic domain contained a 13-amino acid conserved sequence. The expanded domains determine the functions of PP2C. When independent regulatory subunits are not present, the expanded domain at the N terminal functions in recognizing substances and modulating the enzymatic activities of the catalytic subunits. In addition, PP2C contained a secretory peptide following seven homocysteines at the N terminal. Studies have shown that cysteine plays an important role in signal transduction (Barton *et al.*, 2015). This also implies that *MsPP2C*, as a dehydrin gene, may act as a molecular co-chaperone such as heat shock proteins (HSPs), which regulates protein activities through interacting with target proteins (Shi *et al.,* 2014). *MsPP2C* of alfalfa Zhongmu shared a high sequence similarity at the C terminal with its homologs in other species, such as *Ricinus communis*, *Vitus vinifera*, *Glycine max*, *A. thaliana*, *Sorghum bicolor*, *Zea mays*, and *M. truncatula*. *MsPP2C* shared 100% homology with the PP2C in *M. truncatula*. In a genome-wide analysis of the heat shock transcription (HST) factors, Wang *et al.* (2012a, b) reported 16 HSFs in *M. truncatula*. In another study, So *et al.* (2013) reported that the deduced amino acid sequence of *GmDjp1*, a soybean gene encoding a type III J-protein induced by abiotic stress, showed a high homology with a J-protein in *M. truncatula*. However, little about *MsPP2C* in alfalfa Zhongmu has been discovered. This study should be beneficial to the community because this

alfalfa cultivar has been planted in many saline-alkali areas in North China (Peng *et al.*, 2008), and is expanding to other regions of eastern Asia.

To further understand the molecular mechanism of *MsPP2C*, the *MsPP2C* expression in alfalfa Zhongmu was evaluated by fluorescence quantitative PCR. The expression of *MsPP2C* was enhanced under stresses of salinity, ABA, and drought. Under the salt treatment, the *MsPP2C* expression was apparently increased. After 2 h of the treatment, the expression of *MsPP2C* reached the maximum, which was earlier than another PP2C, *OsPP108*. The expression of the latter reaches the maximum at 6 h upon the salt treatment (Singh *et al.*, 2015). Another PPC2, *AtMYB20,* has the highest expression at 4 h upon the application (Cui *et al.*, 2013). These findings suggest that *MsPP2C* is more sensitive to salt stress than other PP2Cs. However, *MsPP2C* quickly accumulated upon the ABA treatment and reached the maximum after 4 h of the treatment, which is in consistent with previous studies (Cui *et al.*, 2013; Singh *et al.*, 2015). These results indicate that *MsPP2C* may participate in the ABA signaling transduction at an asynchronous rate. The expression of *MsPP2C* also reached the maximum at 4 h after the drought treatment, indicating *MsPP2C* may participate in osmotic regulation. The RT-PCT results show *MsPP2C* was expressed in the T1 transgenic line, but not in the WT line. These results demonstrate that the *MsPP2C* gene was completely integrated into the Arabidopsis chromosomes, and thus able to pass to the offspring generations.

The phenotypes of the *MsPP2C* expressing Arabidopsis were evaluated upon salt stress (Fig. 4). Both one-week-old and two-week-old seedlings showed dramatic responses to the 250 mM NaCl treatment. Nearly all WT Arabidopsis seedlings showed repressed growth feature under salt stress. However, the seedlings from the transgenic lines, especially the T_3-7 line, grew at a normal rate. These results concur with the expression pattern of *MsPP2C*, both of which indicate the expression of *MsPP2C* was up-regulated by salt stress. It is well known that salt stress induces the endogenous ABA production (Liu *et al.*, 2005; Sripinyowanich *et al.*, 2013). Hence, it can be easily speculated that the expression of the *MsPP2C* gene was up-regulated through the ABA signaling pathway. The results can be explained by at least two reasons according to other similar studies (Hu *et al.*, 2006; Reyes *et al.*, 2006; Brock *et al.*, 2010; Liu *et al.*, 2012). Firstly, most plant PP2Cs have a conserved catalytic domain and a highly divergent N-terminal extension, which is usually less than 120 amino acids and typically acts as a negative regulator of the ABA signaling (Hu *et al.*, 2006). However, we found that *MsPP2C* encoded a 391 amino acid protein with a long N-terminal extension, which has been reported to repress the PP2C enzymatic activity of the C-terminal (Yu *et al.*, 2003). Similar genes, such as *FsPP2C2*, positively regulate ABA signaling by suppressing the expression of the GA-inducible genes (Reyes *et al.*, 2006). The other probable explanation may refer to the function of *MsPP2C*, which works as a kinase-interacting domain, such as HSPs. However, the mechanism remains to be understood at the biochemical level.

Stable photosynthesis is critical to a plant under salt stress. Sustainable photosynthetic production provides nutrition and building blocks for repairing the carbohydrate metabolism system under salt stress. Indeed, many studies have shown that salt stress apparently enhances the photoinhibition and photo damage of proteins of photosystem II (PSII) (for a summary, see Zhang *et al.* (2009). Therefore, to further determine the effects of the overexpression of the *MsPP2C* gene on salt tolerance in the transgenic Arabidopsis, Fv/Fm and chlorophyll content were measured under salt stress. Although both parameters declined after the salt treatment compared to those of the initial seedlings, they were higher in the T3-7 transgenic line than the WT line (Fig. 5A, B). These results illustrate that the photosynthetic capacity was higher in the transgenic lines than the WT line under salt stress due to the control of photoinhibition. Another PP2C (*OsPP108*) was reported to improve salt tolerance of rice through enhancing photosynthesis (Singh *et al.*, 2015), wherein the mechanism was demonstrated to be involved in the ABA regulation.

Plant adaptation to salt stress involves the accumulation of low molecular mass osmolytes, such as proline (Chen *et al.*, 2013). Proline acts as an osmo-protectant, and plays an important role in osmotic balancing, protection of sub-cellular structures and enzymes, and increasing cellular osmolarity (turgor pressure), which provides the turgor necessary for cell expansion under stress conditions (Kumar *et al.*, 2010). In the present study, the proline content increased to a higher level in the T3-7 line overexpressing the *MsPP2C* gene than that in the WT line (Fig. 5C), illustrating an improvement of salt resistance of Arabidopsis. Interestingly, the proline content in the T_3-9 transgenic line was significantly different from that in the T_3-7 line. However, the difference was not significant for the chlorophyll content and fluorescence. This maybe because the cells expanded with the utilization of the structural carbohydrate in the T_3-9 line, but the non-structural carbohydrate production from the photosynthetic apparatus did not change.

MDA is produced when polyunsaturated fatty acids in the cell membrane undergo peroxidation (Zhang *et al.*, 2009). The measurement of the MDA content is a practical approach to evaluate cell membrane damage. In the present study, the MDA content was higher in the WT line than that in all three transgenic lines under salt stress (Fig. 5D). This illustrates that the overexpression of the *MsPP2C* gene inhibited the accumulation of superoxide anion radicals in the transgenic lines. No difference was found among the transgenic lines, suggesting that the overexpression of the *MsPP2C* gene protected the transgenic seedlings from cell membrane injury. However, this protective effect was not

related to the expression level of *MsPP2C*. The MDA content is also lowered in the transgenic plants with enhanced salt tolerance by overexpression of *WRKY20* (Tang *et al.*, 2014), *AhCMO* (Zhang *et al.*, 2009), and *TFT7* (Xu *et al.,* 2008). Although the difference of the MDA content among the transgenic lines is insignificant, the proline content in the T_3-7 line is higher than that in other lines, indicating the main mechanism of salt tolerance through *MsPP2C* is by the accumulation of osmolytes instead of cell membrane production.

Under salt stress, the Na^+ content increased in both the aboveground (leaves) and underground parts (roots) in all lines. However, the Na^+ content was much lower in aboveground parts, but obviously higher in the underground parts in the transgenic lines, than that in the WT line (Fig. 5D, E). This result concurs with our hypothesis that the expression of the *MsPP2C* gene can induce Na^+ accumulation in the roots to prevent delivery to the aboveground parts, thus minimizing salt injury to plants. This also shows that the transgenic plants had a better coordination ability in absorbing saline ions than the wild type plants. They are essential parts of cellular signaling transduction processes including all physiological and disease resistance processes, such as glucose metabolism, photosynthesis, cellular development and gene expression. PP2C, as one protein phosphatase, regulates a variety of signaling pathways through competing with protein kinases. Protein phosphatases in the PP2C family have different physiological metabolic functions through interacting with different protein kinases (Benson, 1999). As our work has progressed, the discovery of the receptor protein kinase counterpart of PP2C has become the research focus of the protein phosphatase study, which will provide a better understanding of the function of PP2C in the signaling transduction. At present, we have cloned two protein kinase genes, *MsERECTA* and *MsSIKI*, from *M. sativa*. Experiments, such as yeast two hybrid assays, have been performed to elucidate the mechanism of the interaction between PP2C protein phosphatase and the corresponding protein kinases.

Finally, the overexpression of the gene *MsPP2C* augmented the salt-tolerance in Arabidopsis, suggesting *MsPP2C* acted as a positive regulator in the salt- and drought-induced adaptive responses which protect the plants from damage. Similarly, the transgenic plants were less sensitive to ABA during the germination and root growth. These results confirmed the roles of *MsPP2C* in the plant's responses to salt, drought, or ABA, and indicated that *MsPP2C* might mediate distinct mechanisms for these responses. In plants, stress responses are very complicated, and many signaling pathways are involved, including the calcium signaling, ABA signaling, and many others, such as phosphatidic acid (PA) Zhang *et al.*, 2009) and hormone interaction (Reyes *et al.*, 2006). The function of *MsPP2C* in salinity tolerance should be further studied. Such as, the salt resistance of MsPP2C gene transferred into Alfalfa.

Conclusion

MsPP2C was induced under different stress conditions and exogenous ABA application. Overexpression of *MsPP2C* in Arabidopsis improved salt tolerance and increased its chlorophyll and proline content. This study provides an important theoretical basis for improving breeding of alfalfa through genetic techniques.

Acknowledgments

This work was supported by the Natural Science Foundation of China (31771695,31170168), the Fundamental Research Funds for the Central Universities (DC201502070403), the National Spark Program Project (2013GA651006), The National Key Research and Development Program of China(2016YFC050707), and Liaoning Province Science and Technology Plan (2011209001). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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(Received 10 October 2016; Accepted 10 April 2017)