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Full Length Article

Induction of Salt Tolerance in Transgenic Alfalfa (*Medicago sativa*) through Overexpression of the *MsERECTA* Gene

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Abstract

Alfalfa is an important crop for large-scale improvement of saline-alkali land and development of modern animal husbandry in China. However, the mild salt tolerance of alfalfa limits further promotion of planting. Therefore, it is of great significance to cultivate alfalfa varieties with high salt tolerance. With the aim to improve the tolerance of alfalfa to high levels of salt stress, a receptor-like kinase gene, *MsERECTA*, was analyzed for homology, isolated from alfalfa (*Medicago sativa* L.) cv. Zhongmu No. 1, and transferred back to alfalfa Zhongmu No. 1. Expression of the *MsERECTA* gene can be induced by salt and ABA, and the most responsive expression was found in immature leaves of wild-type plants. Subsequently, the transgenic lines were compared with the wild type (WT) plants without any introduced genes for salt tolerance after being treated with 250 mM NaCl for 26 d. Transgenic alfalfa plants over-expressing *MsERECTA*, *OXERECTA-2*, *-6*, and *-10*, showed slower water loss rates and lower malondialdehyde and Na^+ contents in the leaves and roots compared to the WT. However, these lines showed higher levels of chlorophyll and free proline. These results suggest that over-expression of the *MsERECTA* gene could efficiently improve salt tolerance of alfalfa Zhongmu No. 1 at a high salt level (250 mM NaCl). The present study provides a solid foundation for transgenic breeding of super salt-tolerant alfalfa.

Keywords: *Medicago sativa*; Salinity stress; *ERECTA*; Over-expression; Water use efficiency; Receptor-like kinase; Salinity

Introduction

Alfalfa (*Medicago sativa* L.) is an important leguminous forage crop with about 32 million hectares under cultivation globally (Anower *et al.* 2013). Compared with other crop plants, alfalfa is relatively salt tolerant (Wang and Han 2009; Anower *et al*. 2013; Tang *et al*. 2013; Wang *et al.* 2014; Al-Farsi *et al.* 2020a) despite that some alfalfa cultivars and half-sib families are sensitive to soil salinity (Anower *et al.* 2013). Salt tolerance of alfalfa plants can be improved by two strategies. For salt-sensitive alfalfa cultivars, salt tolerance can be enhanced by over-expressing exotic genes from other salt-tolerant plants (Bai *et al.* 2013; Wang *et al.* 2014; Tang *et al*. 2013, 2014). It has been found that the expression of genes of *GsZFP1* (Tang *et al.* 2013), *AtNDPK2* (Wang *et al.* 2014), *GsCBRLK* (Bai *et al.* 2013), and *WRKY20* (Tang *et al.* 2014) are able to improve salt tolerance of transgenic alfalfa (Al-Farsi *et al.* 2020a). In contrast, over-expression of key functional genes from salt-tolerant alfalfa cultivars has also been proven to confer salt tolerance to other plants. For example, one study reported that over-expression of *MsERF8* (a gene from apetla 2/ethylene response factors super-family) and *GDP-mannose 3,5-epimerase* (the catalyst for the conversion of GDP-D-mannose to GDP-L-galactose) enhances salinity tolerance in tobacco (*Nicotiana benthamiana* L.) (Chen *et al.* 2012) and *Arabidopsis thaliana* (Ma *et al.* 2015), respectively. However, both approaches are only effective in moderating salt stress when the Na^+ concentration is lower than 200 m*M*.

Receptor-like protein kinases (RLKs) are signaling proteins sharing a common structure composed of a signal peptide, a ligand-binding extracellular domain, a transmembrane region, and a cytoplasmic kinase domain. RLKs belong to a large gene family with at least 610 members in Arabidopsis, which represent nearly 2.5% of *Arabidopsis* protein coding genes and more than 1100 members in rice (*Oryza sativa* L.) (Shiu and Bleecker 2001). A number of RLK genes have been identified to be involved in growth, developmental functions, and resistance to abiotic stress (Song *et al.* 1995; Becraft *et al.* 2001; Li and Chory 2016). Many genes from the RLK family, such as *LysM*

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(Gao *et al.* 2013), have been found to be up-regulated under salinity stress. Recently, as a membrane-located RLK, the ERECTA protein has attracted more attention than before (Zheng *et al.* 2012) for its function in regulation and modification of plant development and resistance to exogenous stresses (Shpak *et al.* 2005; Xing *et al.* 2011). Several studies about water use efficiency (WUE) have detected the function of the *ERECTA* family receptors on regulating the stomatal development pathway (Shpak *et al.* 2005; Zheng *et al.* 2012; Pillitteri and Torii 2015). To date, the homology of Arabidopsis *ERECTA* has been reported in wheat (*Triticum aestivum* L.) (Zheng *et al.* 2015), *Arabidopsis thalian* (Masle *et al.* 2016), rice (*Oryza sativa*) (Ouyang *et al.* 2010), and poplar NE19 [*Populus nigra* × (*Populus deltoides* × *Populus nigra*)] (Xing *et al.* 2011). However, to our knowledge, little is known about the responses of plants expressing the ERECTA gene to salt stress, except for one study on rice (Ouyang *et al.* 2010; Liu *et al.* 2015). As a model plant, alfalfa is an ideal candidate to study super tolerance of transgenic plants over-expressing self-harbored key genes to severe salinity because some alfalfa cultivars are resistant to not only moderate levels salt stress but also high levels of salt stress.

Medicago sativa L. cv. Zhongmu No. 1 ('alfalfa Zhongmu No. 1' for abbreviation) is a well-known salt-tolerant cultivar resistant to moderate levels of salt stress, which is abundantly grown in middle and western areas of China (Wang *et al.* 2013). It is of great significance to identify functional genes responsible for salt tolerance in alfalfa Zhongmu No. 1. When being subjected to salt stress, the alfalfa Zhongmu No. 1 contains more macro- and micro-nutrients and antioxidative enzymes compared with other low salt-tolerant alfalfa cultivars (*e.g.*, *Medicago sativa* cv. Defor) (Wang and Han 2009; Wang *et al.* 2011). Microarray analysis of two alfalfa cultivars with contrasting tolerance to salt stress showed a remarkable difference between them in growth and physiology under normal growing conditions, which is highly related to the expression of RLK genes. However, when subjected to a higher level of spissated alkali salts, the tolerance difference between alfalfa Zhongmu No. 1 and other cultivars disappears (Wang *et al.* 2014; Ma *et al.* 2014). In the present study, one of the salt-tolerance-related genes, the *MsERECTA* gene, was identified from alfalfa Zhongmu No. 1 and subject to further analysis on the basis of its annotation as a RLK encoding gene and its sequence homology with the *Arabidopsis ERECTA* gene. Therefore, we overexpressed the *MsERECTA* gene in alfalfa Zhongmu No. 1 to investigate the ability of salt tolerance under high salinity levels. Additionally, we measured the effect of overexpression of the *MsERECTA* gene on the salt tolerance of alfalfa, and then determined the potential function of this gene in the enhancement of salt tolerance in alfalfa plants. Our results would provide a solid basis for breeding salt-resistant alfalfa cultivars which are able to survive and grow well under severe salt conditions.

Materials and Methods

Plant materials and growth conditions

Seeds of alfalfa Zhongmu No. 1 were sterilized with 6% sodium hypochlorite solution for 5 min before sowing. Following germination in a sand medium at 25/20^{o}C for 8 h/16 h in a dark room, four seedlings from one of the holes of a foam quadrat were bulked and transplanted into a plastic pot (13 cm in height, 7 cm in top diameter and 5 cm in bottom diameter). Seventy-grit silica sand was used as the growing medium, which is recommended for testing salinity tolerance in alfalfa (Anower *et al.* 2013). Seedlings were fed with nutritional solutions with the composition adapted from Wei *et al.* (2013).

Salt treatments

Five seedlings were randomly sampled from one pot and then separated into immature leaves (I-leaves), mature leaves (M-leaves), stem, and root. Eighty seedlings were sampled as 20 bulks (four seedlings per bulk) for the NaCl treatment, and the remaining 200 seedlings were subjected to the ABA treatment. For the NaCl treatment, the sampled seedlings were transplanted into plastic vessels (column, 9 cm × 2.2 cm, inner diameter × height). The four bulked seedlings in one group were transplanted into the same vessel. The transplanted seedlings were fixed by sponge to make sure the roots were soaked thoroughly. Each vessel was filled with 100 mL of a nutrition solution composed of 2.5 mmol/L $Ca(NO_3)_2$, 2.5 mmol/L KNO_3, 1 mmol/L $MgSO_4$, 0.5 mmol/L $(NH)_4H_2PO_4$, 2×10^{-4} mmol/L $CuSO_4$, 1×10^{-3} mmol/L $ZnSO_4$, 0.1 mmol/L EDTA Fe Na, 2×10^{-2} mmol/L H_3BO_3, 5×10^{-6} mmol/L $(NH)_4Mo_{27}O_4$, and 1×10^{-3} mmol/L $MnSO_4$. Additionally, NaCl was added to solutions at a rate of 250 m*M* in each vessel to impose salt stress. Seedlings were sampled for gene expression analyses before the hydroponic study and 2, 4, and 6 h after the commencement. One of the four transplanted seedlings was randomly selected from one vessel, and five seedlings were sampled as five replicates (n=5).

The ABA treatment was conducted *in situ* on the 200 seedlings. ABA was applied by spraying seedling leaves with 200 μM ABA solution. The ABA-treated seedlings were sampled at 0, 1, 3, and 5 h after the test commencement. Sampling of the ABA-treated seedlings followed the same method used in the salt treatment. All the harvested leaves, stems, and roots were washed by distilled water, wiped, dropped immediately into liquid nitrogen and stored at -80°C for RNA extraction.

Isolation of the *MsERECTA* gene and quantitative real-time PCR analysis

A full-length cDNA, namely, *MsERECTA*, was isolated. The cDNA sequence was amplified by PCR using the primer sets 5'-ATGTCGGGTCTGGATCAACCTGCC GTCA-3' and

5'-TCACTCACTGTTCTGGGAGA TAACTTC-3'.

The relative quantification value for the *MsERECTA* gene was calculated by the 2-DDCT method, and the quality of the cDNA was assessed by PCR using the GPDH gene as an internal control. The gene-specific primers used in QRT-PCR were: *q MsERECTA*-F (5'-CAATTGGAAT TTCTGGTTTT GAGGAAT-3'), *q MsERECTA*-R (5'-CACAATCCAG TTAATTGGCA CATG-3'); GPDH-F (5'-GTGGTGCCAAGAAGGTTGTTAT-3') and GPDH-R (5'-CTGGGAATGATGTTGAAGGAAG-3'). Five biological and three technical replicates were performed for QRT-PCR analyses.

Vector construction

The *MsERECTA* gene was connected to a pMD18-T vector. *Escherichia coli* were transformed to extract plasmids for PCR and enzyme digestion. Positive clones inserted by the forward direction were screened, and the pMD18-T-*MsERECTA* and pCAMBIA1304 were digested by *Spe* I and *Bgl* II with T4DNA as the ligase for 6 h at 16°C. After the transformation of *E. coli*, the single colony was picked and tested through PCR and for positive cloning sequencing. Plasmids were extracted from the bacterial colony containing positive clones and identified by dual-enzyme digestion.

Transformation

The hypocotyls were sliced off and incubated in the pre-medium containing 2.0 mg/L 2,4-D, 0.25 mg/L kinetin, 250 mg/L cefotaxime, and 50 mg/L kanamycin for 2 d, and then soaked in the solution of the transformed bacteria (*Agrobacterium tumefaciens*) (OD600 = 0.5) for 10 min. After being dried by a tissue paper, the hypocotyls were incubated in the culture medium (50 mg/L Kan, 300 mg/L Cef, 2.0 mg/L 2,4-D, and 0.25 mg/L KT) until the formation of callus cells. After the regeneration of new buds in a length of about 1 cm, buds were sliced off and incubated in 1/2 medium to induce new root egress. The trans-gene was selected by digesting the dual-loci of *Spe* I and *Bgl* II to the two tips of the amplification primer prior to the ligation of *MsERECTA* to the expression vector pCAMBIA1304. When the shoot length grew up to 10 cm, the rooted explants were acclimated in pots in the greenhouse for one week and transferred to the growing medium. Throughout the experiments, the cultures were maintained in a growth chamber at 25 ± 2°C under a 16 h photoperiod. The T_2 generation was employed as the transgenic material.

Imposition of salt stress on the WT plants and the transgenic lines

Three transgenic lines were cultured with the WT plants of alfalfa Zhongmu No. 1 as the control. Briefly, four plantlets were planted in one pot (13 cm in height, 7 cm in top-diameter, and 5 cm in bottom-diameter) filled with seventy-grit silica sand. When the shoot height of 90% population was approximately 20 cm, the time point was marked as d0, and the plants were transplanted into large pots (25 cm in top diameter, 20 cm in bottom diameter, and 20 cm in height). For each pot, only one plant was transplanted. Five uniformly-sized plants were transplanted for one transgenic line or WT as replicates (n=5). A mixture of vermiculite and perlite (v : v, 3:1) was used as the medium (80 cm^3) for each one potted plant. All plants were raised under a 16-h photoperiod with an irradiance of 450 μmol/ (m^2 s), a temperature of 24°C and a relative humidity of 65%. Plants were irrigated every 2 d with 3 L of nutritional solution (Wei *et al.* 2013) throughout the experiment. To test the tolerance of the transgenic lines to salt stress, all plants received an additional 250 m*M* NaCl. From d0, the experiment was carried out for 26 d until the plants showed apparent symptoms of salt toxicity (marked as "d26").

Water loss measurements

Water loss was measured using the procedure described by Wang *et al.* (2014) with the following formula: WLM (%) = $[(W_F - W_D) / (W_T - W_D)]$, where W_F is the weight of freshly excised rosette leaves (weighed immediately), W_T is the turgid weight of leaves after incubation in water for 6 h at 20°C on a bench at room temperature and at 60% relative humidity in dim light, and W_D is the dry weight of the same leaves after drying at 80°C for 48 h. WLM% was measured using 1 g of fresh rosette leaves.

MDA, chlorophyll and proline content of leaf tissues

The malondialdehyde (MDA), chlorophyll and proline contents were measured using a modified TBA method (Kim and Nam 2013).

Results

Structural analysis of *MsERECTA*

A *Medicago sativa* gene showing homology to *ERECTA* in *Arabidopsis* was designated as *MsERECTA*. The Genebank number of *MsERECTA* in alfalfa is KM277792.1, and *ERECTA* in *Arabidopsis* is U47029.1. The *MsERECTA* cDNA is 2,937 bp in length and encodes 825 amino acid residues with a predicted molecular mass of 104.5 kDa and a pI of 5.56 (Fig. 1A). In the C-terminal cytoplasmic region (amino acids 605–783), a serine/threonine protein kinase domain was predicted (Fig. 1B).

Phylogenetic analyses

Phylogenetic analyses demonstrate that the *MsERECTA* gene had evolved during or before early angiosperm evolution. No function has been assigned to this gene (Fig. 1C).

Expression profile of *MsERECTA*

The expression of the *MsERECTA* gene was up-regulated by

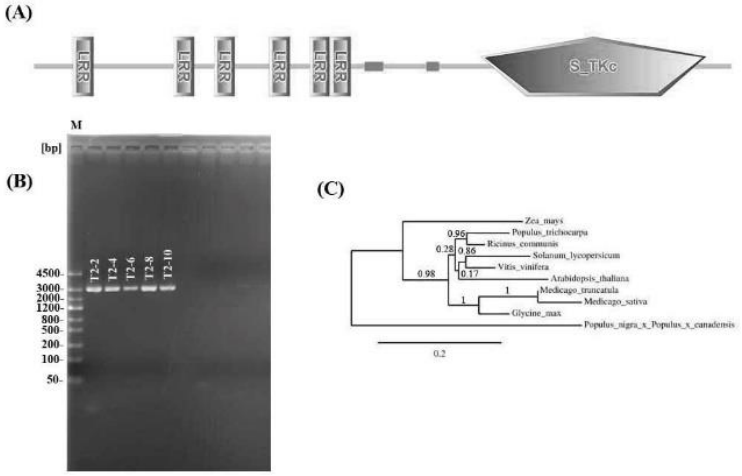

Fig. 1: Structure of the predicted *MsERECTA* protein and conserved features of the amino acid sequence. (**A**) The schematic of the *MsERECTA* protein. *LRR*, the rich leucine repeat; red color, transmembrane region; S_TKc, kinase domain. The SMART website (http://smart.embl-heidelberg.de/) was used to simulate the protein structure. (**B**) PCR amplification with designed primers of the DNA extracted from each of the five lines (T2-2, T2-4, T2-6, T2-8, and T2-10) of the transgenic alfalfa plants over-expressing the *MsERECTA* gene with a DNA marker as the reference. The primers of 5' RACE was employed with the ones at 35s in pCAMBIA1304 carriers. The primers of 3' RACE was employed with those of 3' RACE of *MsERECTA*. M, DNA marker. (**C**) Phylogram of the deduced full-length protein sequences of *MsERECTA* homologs constructed with the MEGA4 software. The bootstrapping values (out of 10,000 samples) were presented for each node

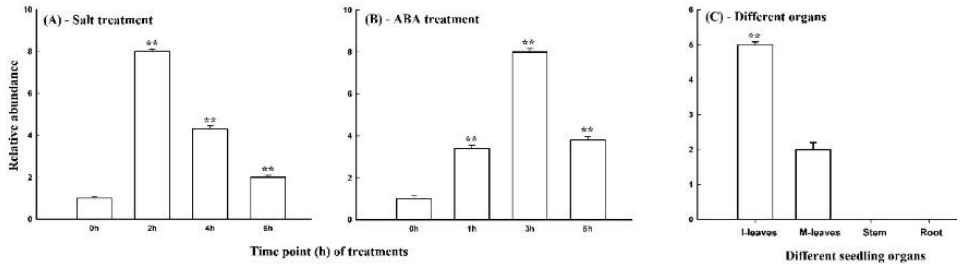

Fig. 2: Expression patterns of the *MsERECTA* gene in alfalfa leaves detected by real-time PCR analysis in response to salt treatment (**A**) and exogenous ABA (**B**). The relative expression was quantified using the g*lyceraldehyde-3-phosphate dehydrogenase* (GPDH) gene as an internal reference, and the unstressed expression level was assigned to be the value of 1. Columns present the mean values from replicated experiments and error bars presents the standard error. (**C**) Tissue-specific expression of the *MsERECTA* gene under regular conditions without salt stress. M-leaf, mature leaf; I- leaf: immature leaf

exogenous salt treatment for the first 2 h and subsequently, it was down-regulated with time (Fig. 2A). In the ABA treatment, transcripts of the *MsERECTA* gene accumulated rapidly for the first 3 h and then declined with time (Fig. 2B). The tissue-specific difference of the expression pattern indicated that the *MsERECTA* gene was mainly expressed in immature leaves. No expression was detected in stem and root (Fig. 2C).

Gene expression level in the transgenic lines

Three independent T_3 35S: *MsERECTA* transgenic lines were observed, including *Ox MsERECTA-2, Ox MsERECTA-6* and *Ox MsERECTA-10*. The expected amplification profiles were acquired from the WT and 3 transgenic lines, suggesting that the *MsERECTA* gene is integrated in the alfalfa Zhongmu No. 1. The expression levels of the *MsERECTA* gene among the WT and the 3 transgenic lines were different, though the difference was not significant (Fig. 3A). Due to the most apparent white color for protein accumulation by the gene expression of the transgenic lines, the transgenic line *Ox MsERECTA-6* showed more apparent integration relative to the other lines and WT.

Phenotypes associated with *MsERECTA* overexpression in alfalfa

The WT plants and three transgenic lines did not show any difference in the initial growth at d0 (Fig. 3B). At 26 d, more than half of the leaves were still green in the transgenic

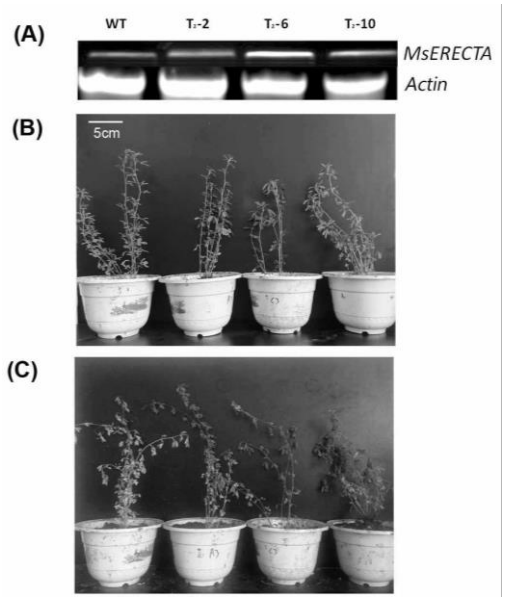

Fig. 3: (A) Transcript level analyses of *MsERECTA* in transgenic and WT alfalfa plants by semi-quantitative RT-PCR and real-time RT-PCR. WT represents nontransgenic plants, and T_2-2, T_2-6, and T_2-10 represent transgenic lines of *Ox MsERECTA-2*, *Ox MsERECTA-6*, and *Ox MsERECTA-8* overexpressing *MsERECTA*, respectively. Overexpression of the *MsERECTA* gene confers salt tolerance in transgenic alfalfa plants. (**B**) Initial status of the transplanted WT alfalfa plants and three transgenic lines without any salt treatment, which was marked as d0. (**C**) Shoot performance of senescence and chlorosis to different extents for WT alfalfa plants and transgenic lines subjected to the 250 m*M* NaCl treatment for 26 d

plants, but nearly all leaves of the WT plants turned yellow and showed severe chlorosis (Fig. 3C). These results indicated that the overexpression of the *MsERECTA* gene was apparently related to the enhanced tolerance of alfalfa to salt stress.

Physiological and biochemical performances

Although the WLMs declined at d26 relative to those at d0, the WLMs were higher in the transgenic plants than that of the WT plants (Fig. 4A). Consistent with these results, the chlorophyll content was also declined at d26 relative to that at d0. The chlorophyll content in the transgenic plants was higher than that of the WT plant at d26 (Fig. 4B). Nevertheless, compared to the initial status at d0, the proline content was increased by salt stress at d26; at this stage, the proline content was higher in the transgenic plants than that in the WT plants (Fig. 4C). In general, the MDA content increased in response to the salt treatment for 26 d for the WT and transgenic plants. However, the MDA content was increased to a greater extent in the controlled plants than that in the transgenic plants (Fig. 4D).

The sodium contents in the leaves and roots were almost the same in both the WT and transgenic plants under

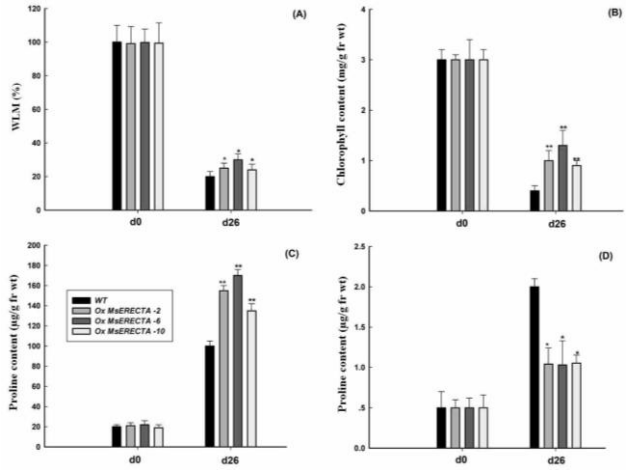

Fig. 4: Water loss measurement (WLM) (**A**) and contents of chlorophyll (**B**), free proline (**C**), and MDA (**D**) in leaves of wild type (WT) alfalfa plants and three transgenic lines, *MsERECTA-2*, *Ox MsERECTA-6*, and *Ox MsERECTA-8* at different levels of salt stress. Each value is the mean of five independent measurements, and error bars indicate standard deviation (SD). Significant differences are calculated by the Student's t-test and denoted by one or two stars corresponding to $P < 0.05$ and $P < 0.01$, respectively

normal conditions at 0 d, while at 26 d after the NaCl treatment, the Na^+ content was significantly higher in the WT plants than that of the transgenic plants (Fig. 5).

Discussion

Soil salinization has become a severe problem that restricts agricultural development in China and affects the stability of the ecosystem and the biodiversity of organisms (Li *et al.* 2014). As a glycophyte, alfalfa exhibits reduced biomass under severe salt stress with varying responses among different cultivars (Wang and Han 2009; Al-Farsi *et al.* 2020b). Additionally, the dose of 250 m*M* NaCl used in this study is higher than the dose (210 m*M* NaCl) by Wang and Han (2009), which was already evaluated to be a high level of salt. Therefore, 250 m*M* NaCl could fully mimic the salt stress in severe saline-alkali lands, and results obtained under this condition could be useful for improving salt tolerance of alfalfa Zhongmu No.1.

Protein phosphorylation is one of the central signaling events in response to environmental stresses in plants. In this study, we identified and isolated the *MsERECTA* gene from leaves of alfalfa Zhongmu No. 1, and found that its gene structure was similar to its counterparts in Arabidopsis, rice, and maize. These results implied that the *MsERECTA* gene was evolved prior to the separation between monocots and dicots. Additionally, we identified that the *MsERECTA* gene was a typical RLK gene. It can be surmised that up-regulation of this gene expression in response to exogenous salt and ABA applications may be harbored and ready to be loaded in many other salt-tolerant alfalfa cultivars and plant

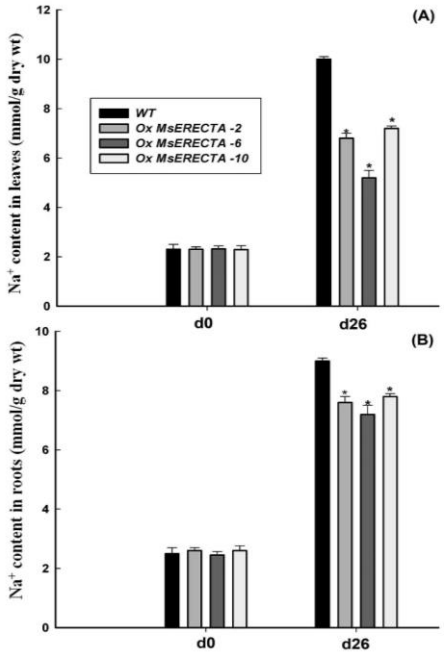

Fig. 5: Content of Na^+ ions in leaves (**A**) and roots (**B**) of wild type (WT) alfalfa plants and three transgenic lines, *MsERECTA-2*, *Ox MsERECTA-6*, and *Ox MsERECTA-8* at different levels of salt stress. Each value is the mean of five independent measurements, and error bars indicate standard deviation (SD). Significant differences are calculated by the Student's t-test and denoted by one or two stars corresponding to $P < 0.05$ and $P < 0.01$, respectively

species, wherein the *MsERECTA* gene may have been inherited as monogenic recessive alleles like other reported genes responsible for salt tolerance (Rai *et al.* 2003).

The *MsERECTA* gene was strongly expressed in immature leaves, but feebly expressed in mature leaves. The expression of *MsERECTA* was not expressed in stems and roots under normal conditions (Fig. 2C), indicating that *MsERECTA* tended to be expressed in newly-grown organs. This expression feature concurs with some other RLK genes, such as *PdERECTA* in the poplar genotype NE19 and *OsSIK1* in rice (Ouyang *et al.* 2010). Therein, the expression of both genes of *PdERECTA* and *OsSIK1* also resulted in enhanced salt-tolerance in *Arabidopsis thaliana* and *Oryza sativa*, respectively. The high expression of the *MsERECTA* gene induced by ABA and salt treatments also concur with the expression of *PdERECTA* and *OsSIK1*. In addition to the salt treatment, other factors also induced the expression of RLK members, such as H_2O, H_2O_2, PEG, heat, drought, and cold (Ouyang *et al.* 2010; Zheng *et al.* 2012).

Transgenic plants exhibited enhanced salt tolerance compared with the WT plants (Fig. 3C). Both the growth and productivity of the plants were depressed under salt stress due to the decline of the cell division rate and the loss of turgor during cell expansion (Navarro *et al.* 2017). Therefore, the chlorosis of leaves of the WT plants was a typical water loss symptom caused by the high-level salt stress, whilst overexpression of the *MsERECTA* gene in the transgenic alfalfa resulted in an enhanced capacity of reserving foliage water as revealed by the WLMs of the transgenic plants (Fig. 4A). Wang *et al.* (2014) reported that transgenic alfalfa plants expressing the *AtNDPK2* gene have a higher WLM than that of the WT plants despite their plants were subjected to drought. Salt-sensitive and -tolerant alfalfa cultivars had contrasting chlorophyll contents when facing salt stress (Anower *et al.* 2013). The higher chlorophyll content in the transgenic plants than that in the WT plants (Fig. 4B) clearly suggested that overexpression of the *MsERECTA* gene in alfalfa preserved more photosynthetic facilities in chlorophyll cells, which probably resulted in subsequent promotions of energy supply and protein synthesis.

High levels of salt cause an imbalance of the cellular ions, leading to ion toxicity and osmotic stress (Tang *et al.* 2014). Rapid adjustment of the proline concentration is believed to be a functional mechanism for salt tolerance in alfalfa (Petrusa and Winicov 1997). Remarkably increased proline content in the transgenic alfalfa Zhongmu No. 1 under salt stress has been widely considered as a crucial factor to evaluate the enhancement of gene over-expression on salt tolerance (Tang *et al.* 2013, 2014; Zhang *et al.* 2014). Similarly, the MDA content has been performed as an available tool to evaluate the validity of gene expression in transgenic alfalfa for salt tolerance (Bai *et al.* 2013, 2014; Zhang *et al.* 2014). Physiological and biochemical results revealed that expression of the *MsERECTA* gene can maintain membrane permeability with improved photosynthetic activities, more osmoprotectants, and less membrane damage through alleviating lipid peroxidation. As a result, less Na^+ was accumulated in the transgenic plants compared with the wild type ones. At present, the physiological mechanism of salt resistance of alfalfa is mainly focused on two aspects of osmotic regulation and ion regionalization, including water balance, absorption, transport, distribution and regulation of salt, photosynthetic respiration, proline accumulation, membrane permeability, hormone action, enzyme activity and so on (Li *et al.* 2009; Ma *et al.* 2015; Soulages *et al.* 2016). Salt related genes of Alfalfa mainly include ion balance related genes, such as Na^+/H^+ reverse transporter gene NHX1 (Kerepesi *et al.* 2011), osmotic regulation related genes such as the key gene P5CS in proline synthesis pathway, P5CS (Lee *et al.* 2013).

Conclusion

Over-expression of the *MsERECTA* gene in alfalfa Zhongmu No. 1 at a high salinity helped in the enhancement of salt tolerance in alfalfa plants. The present study provides a solid foundation for transgenic breeding of super salt-tolerant alfalfa. These findings provide a solid basis for breeding salt-resistant alfalfa cultivars which are able to survive and grow well under severe salt conditions.

Acknowledgments

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Author Contributions

Ruiheng Lyu, Peng Guo conducted the experiments and prepared the manuscript, Jiali Chen and Yajing Bao analyzed the experimental data, Baoling Yang and Peng Guo designed the experiment.

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