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Effects of salinity on expression of the salt overly sensitive genes in *Aeluropus lagopoides*

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Abstract

In this study, seeds of *Aeluropus lagopoides* were cultured in hydroponic medium supplemented by ½MS medium. After 21 days, seedlings were treated with NaCl (600 mM), ABA (abscisic acid) (50 µM), Ca_2SO_4 (5 mM), NaCl+Ca_2SO_4, NaCl+ABA and Ca_2SO_4+ABA. Expression analysis of the A. *lagopoides* salt overly sensitive genes (*SOS1*, *SOS2* and SOS3) was done by Semi-Quantitative RT-PCR. The *SOS* ESTs were isolated, cloned and sequenced. *SOS1* and *SOS2* expression were up- regulated in response to NaCl and NaCl+Ca_2SO_4 in roots and roots and shoots, respectively. *SOS3* expression was increased in almost all treatments in shoots. Results showed that ABA regulated the *SOS* pathway by enhancing *SOS2* and *SOS3* expressions in roots and shoots, respectively.

Keywords: ABA, *Aeluropus lagopoides*, Gene expression, Salt stress, Semi-Quantitative RT-PCR, *SOS* pathway.
Abbreviations: ABA_abscisic acid; CIPK-CBL_interacting protein kinases; EST_expressed sequenced tag; PKS_protein kinases; SNF1_ sucrose non-fermenting 1; SOS_salt overly sensitive.

Introduction

Aeluropus lagopoides is a stoloniferous, prennial halophytic grass with C_4 photosynthesis of Gramineae family which grows in high salt conditions. *Aeluropus lagopoides* (Poaceae) is distributed in the regions with intermediate salinity and semi-desert climate on Iranian plateau (Bor 1970; Breckle 1983; Watson and Dall witz 1992). *A. lagopoides* be able to survive up to 1500 mM NaCl (Bodla et al., 1995) with a relatively low accumulation of sodium and chloride ions in dry overgrown tissues (Barhoumi and Djebali 2007). In some halophytes, salt excretion is happened as an avoidance strategy that permits to control and regulation of salt content in plant organs (Atkinson et al., 1967). Under salt stress, *Aeluropus littoralis* excreted sodium and chloride from leaves special salt glands under salt stress (Barhoumi et al., 2006).Salt stress affects the plant growth and development in many different ways. Excess salt causes ion toxicity in the cell. High concentrations of salt in the root medium also create imbalance in osmotic pressure that reduces water absorption and nutrient rates and also transportation of them to shoots. Secondary stresses such as nutritional deficiency or toxicity and oxidative stress often occur as consequences of ion toxicity and hyperosmotic stresses (Zhu 2000). Plants remove excess Na^+ from the cytoplasm by transporting it into the vacuole or out of the cell using Na^+/H^+ antiporters which are located in the tonoplast and plasma membranes, respectively (Hasegawa et al., 2000; Blumwald et al., 2000). In plants, this exchange activity is driven by the H^+-electrochemical gradient generated by the H^+ pumps such as the plasma membrane and tonoplastic H^+- ATPase and H^+-pyrophosphatases, respectively. However, plant plasma membrane Na^+/H^+ exchangers had not been identified at the molecular level until several salt-stress mutants were found in *Arabidopsis thaliana*. In those research some experiments had been designed to identify the salt stress-related cellular machinery components in *A. thaliana*, three SOS genes (*SOS1*, *SOS2*, and *SOS3*) was characterized in a common pathway (Oh et al., 2007). In *Arabidopsis*, the plasma membrane Na^+/H^+ antiporter, *SOS1*, mediates Na^+ efflux and its activity is regulated by the *SOS3*/ *SOS2* kinase complex during salt stress (Chinnusamy et al., 2006). *SOS2* is a Ser-Ther protein kinase belonging to the SNF1-related Kinase (SnRK) 3 family. *SOS3* is a myristoylated Ca^{2+} sensor belonging to the recovering-like family of SOS-like Ca^{2+} sensor/binding proteins. Upon Ca^{2+} binding, *SOS3* undergoes dimerization and enhances the protein kinase activity of *SOS2*. Besides activating *SOS2*, *SOS3* was also shown to recruit *SOS2* to the plasma membrane to achieve efficient interaction with *SOS1*. Mutant plants deficient in either *SOS2* or *SOS3* share the salt-sensitive phenotype of *SOS1* plants (Martinez-Atienza et al., 2007). The *SOS3*/ *SOS2* pathway has been predicted to control the expression and activity of ion transporters such as *NHX1* (Chinnusamy et al., 2006). The expression of *SOS1* is ubiquitous but it is stronger in epidermal cells surrounding the root tip and in parenchyma cells bordering the xylem. This evidence shows that *SOS1* acts as a Na^+/H^+ antiporter in the plasma membrane and transports sodium from root to shoot so it plays a crucial role in sodium efflux from root cells (Chinnusamy et al., 2006; Shi et al., 2000). On the other hand, *SOS2* plays a critical role in regulating the duration and amplitude of cytosolic Ca^{2+} oscillations by regulating the vacuolar H^+/Ca^{2+} antiporter CAX_1 (Cheng et al., 2003). The wheat high affinity K^+ transporter, *HKT1*, functions as a Na^+/K^+ cotransporter which confers low- affinity Na^+ uptake at toxic Na^+ concentrations (Rubio et al. 1995). Thus, *HKT1* could represent one of the Na^+ uptake pathways in plant roots. The

Table 1. Specific primers designed for tubuline and *alaSOS1-4* genes.

Type	Name	Sequence	Length	Melting temperature(˚C)
(Forward)	*Tub*	5´- GCTTTCAACAACTTCTTCAG- 3´	20	56
(Reverse)	*Tub*	5´- GGGGCGTAGGAGGAAAGC- 3´	18	60
(Forward)	SOS_1	5´- GGGGGTTCCTTCTTCTGCTCTATG- 3´	24	74
(Reverse)	SOS_1	5´- CTGACTTGTCCACTTTACTATTCC- 3´	24	68
(Forward)	SOS_2	5´-TCGCCAAGGTCAGGTTCGG- 3´	19	62
(Reverse)	SOS_2	5´- CGCGCTCAGCCCAAAATCA- 3´	19	60
(Forward)	SOS_3	5´- TGGAGGCCCTCTACGAGTTGTT- 3´	22	68
(Reverse)	SOS_3	5´- AAAGCTGGGGAATGACATGGTTAT- 3´	24	68

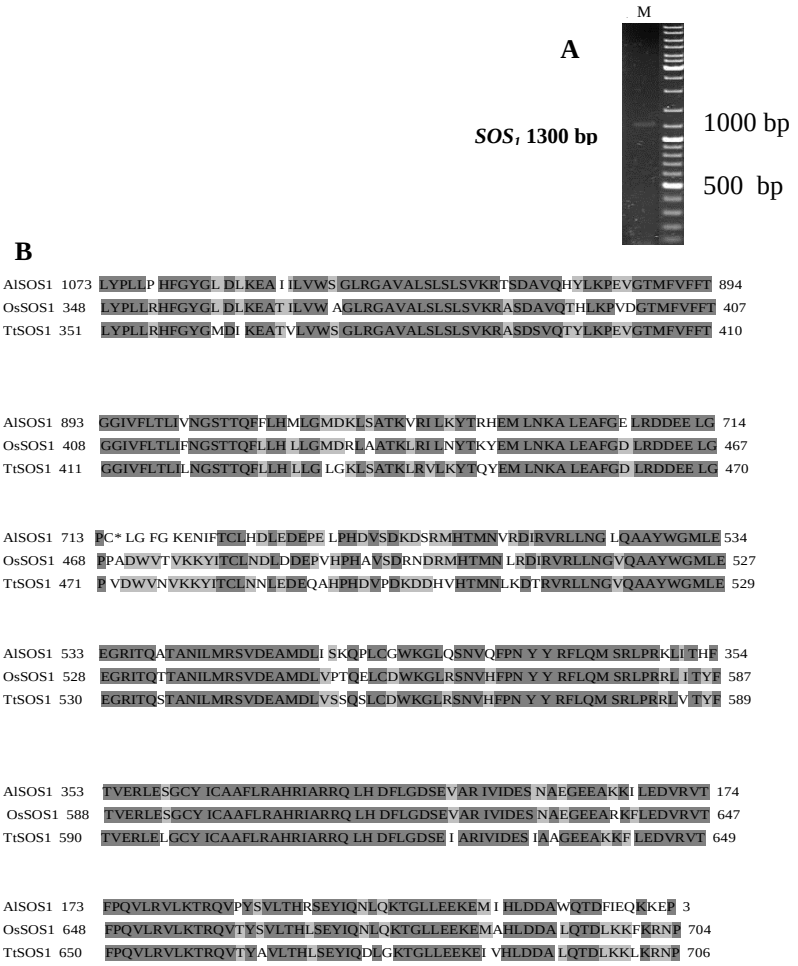

Fig 1. *alaSOS1* isolation from *A. lagopoides* by RT-PCR: a) line 1 relates to the *alaSOS1* amplification at 55 ºC, and lane 2 represents 100bp DNA marker b) The alignment of amino acid sequences from putative SOS1 proteins of different plants. Amino acids identical in at least two proteins are highlighted in dark gray and conservative substitutions are highlighted in gray. The alaSOS1 was aligned to the OsSOS1- Na^+-H^+ antiporter (*Oryza sativa*) (GenBank accession No. AAW33875.1) and TrSOS1- Plasma membrane Na^+-H^+ antiporter (*Triticum turgidum*) (GenBank accession No. ACB47885.1).

wheat low affinity cation transporter, *LCT1*, may also mediate Na^+ influx in to plant cells (Schachtman et al., 1997). Salt stress induces ABA accumulation in addition to cytosolic Ca^{2+} may also regulate the SOS pathway through the ABI2 protein phosphatase 2C. ABI2 may negatively regulate salt tolerance by inactivating *SOS2* or *SOS2*- regulation channels such as *HKT1*, *SOS1* and *NHX1*. *SOS2* regulates tonoplast Na^+/H^+ exchange activity that it is independent of *SOS3* (Chinnusamy et al., 2006). In this study, *SOSs* ESTs were isolated, characterized and analyzed in shoots and roots under different treatments to investigate *SOS* genes regulation by salinity and ABA in *A. lagopoides.*

Results

A. lagopoides showed physiological and molecular responses to salinity. Previously we showed that *A. lagopoides* could tolerate more than 600 mM NaCl *in vitro* by transferring excess NaCl to the upper parts of the plant. Additionally, the relationship between ion homeostasis and the related transporters and potential correlated mechanisms were

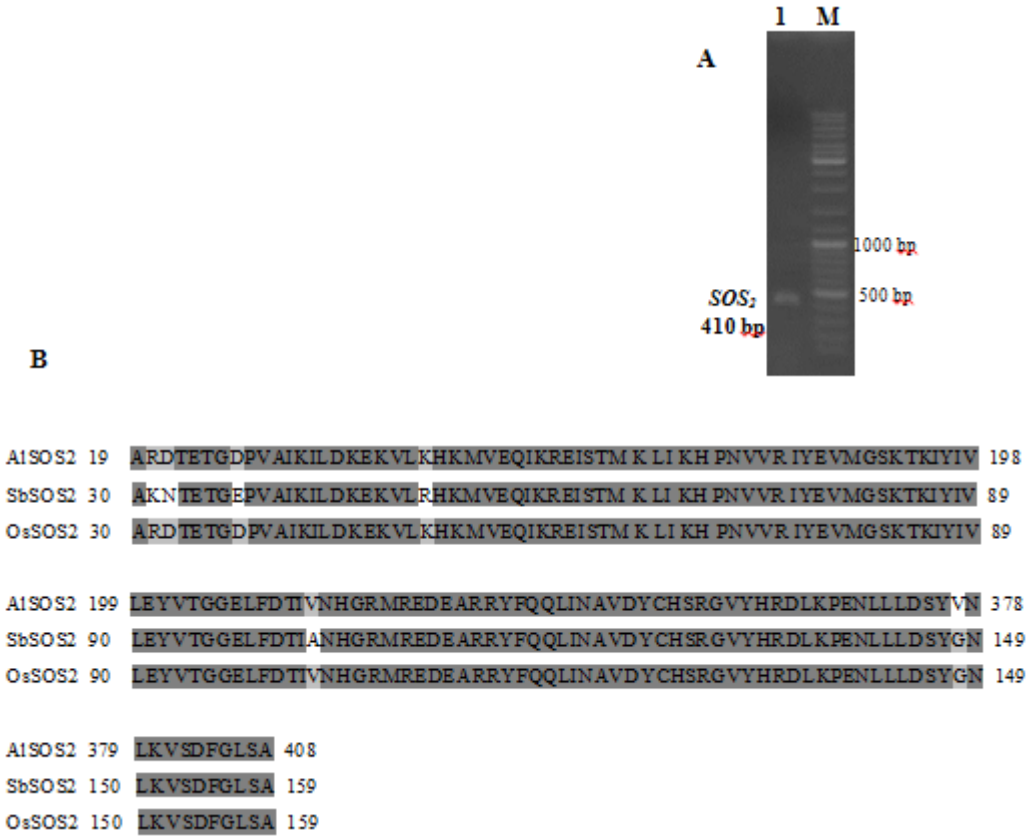

Fig 2. *alaSOS2* isolation from *A. lagopoides* by RT-PCR: a) line 1 relates to the *alaSOS2* amplification at 59 ºC, and lane 2 represents 100bp DNA marker *b*) The alignment of amino acid sequences from putative SOS2 proteins of different plants. Amino acids identical in at least two proteins are highlighted in dark gray and conservative substitutions are highlighted in gray. The alaSOS2 was aligned to the OsSOS2- CIPK- like protein (*Oryza sativa* Japonica Group) (GenBank accession No. ABG21866.1) and SbSOS2- Serine/ Threonine kinase (*Sorghum bicolar*) (GenBank accession No. CAA73068.1).

studied (unpublished data) and of them SOS pathway genes were selected as candidate genes to evaluate *A. lagopoides* salt tolerance in various conditions. It was reported that Ca^{2+} and ABA, increase and or inhibit some transporters function, together with NaCl this was used to be tested on SOS pathway genes.

SOS ESTs Cloning

AlaSOS1, SOS2 and SOS3 ESTs were cloned in pTZ57R/T vector, their sizes were 1330, 410 and 510 bp, respectively, (Figs. 1, 2, 3 and 4). After sequencing and alignment, results showed a strong homology, 85%, 88% and 85% , between *SOS1*, *SOS2* and *SOS3* encoded proteins and rice, sorghum and maize *SOS* proteins, respectively (Figs. 1, 2, 3 and 4). Consequently, we submitted them in GenBank as *alaSOS1* to *alaSOS3* with GT734407 to GT734409 accession numbers, respectively.

Ala-SOS genes were induced by Salinity

NaCl treatment resulted in up-regulation of *alaSOS1* expression in roots but not in a considerable issue in shoots (Fig.5). At the same time *alaSOS2* expression was elevated significantly in shoots and roots by NaCl (Fig.5). Expression pattern of *alaSOS3* was different, it was increased in shoots and roots but its level of changes was more considerable in shoots rather than roots (Fig.5).

Ca^{2+} changed ala-SOS gene expression

While *alaSOS1* expression was not changed considerably by applying Ca^{2+} in shoots and roots, *alaSOS2* and *alaSOS3* expressions were increased noticeably in both tissues (Fig.5). Interestingly, the level of *alaSOS3* changes was more significant in shoots than roots (Fig.5).

ABA influence on alaSOS genes expression pattern

Contrary to expectations, *alaSOS1* expression in shoots was not affected by ABA but it was inhibited in roots by supplying the phytohormone (Fig.5). Simultaneously, ABA increased *alaSOS2* expression considerably in the both tissues (Fig.5). On the other hand the expression pattern of *alaSOS3* was increased by ABA in shoots and roots, but its amplifying in shoots was greater than roots (Fig.5).

Effect of salinity and Ca^{2+} on alaSOS genes expression

$NaCl+Ca_2SO_4$ treatment resulted in up-regulation of *alaSOS1* expression in roots (Fig.5). Conversely, *alaSOS1* expression level was changed by $NaCl+Ca_2SO_4$ in shoots but not in a considerable issue (Fig.5). At the same time *alaSOS2* expression was increased under $NaCl+Ca_2SO_4$ treatment in the roots and its expression was elevated significantly in the shoots in response to $NaCl+Ca_2SO_4$ (Fig.5). Expression pattern of *alaSOS3* was dissimilar, it was increased by

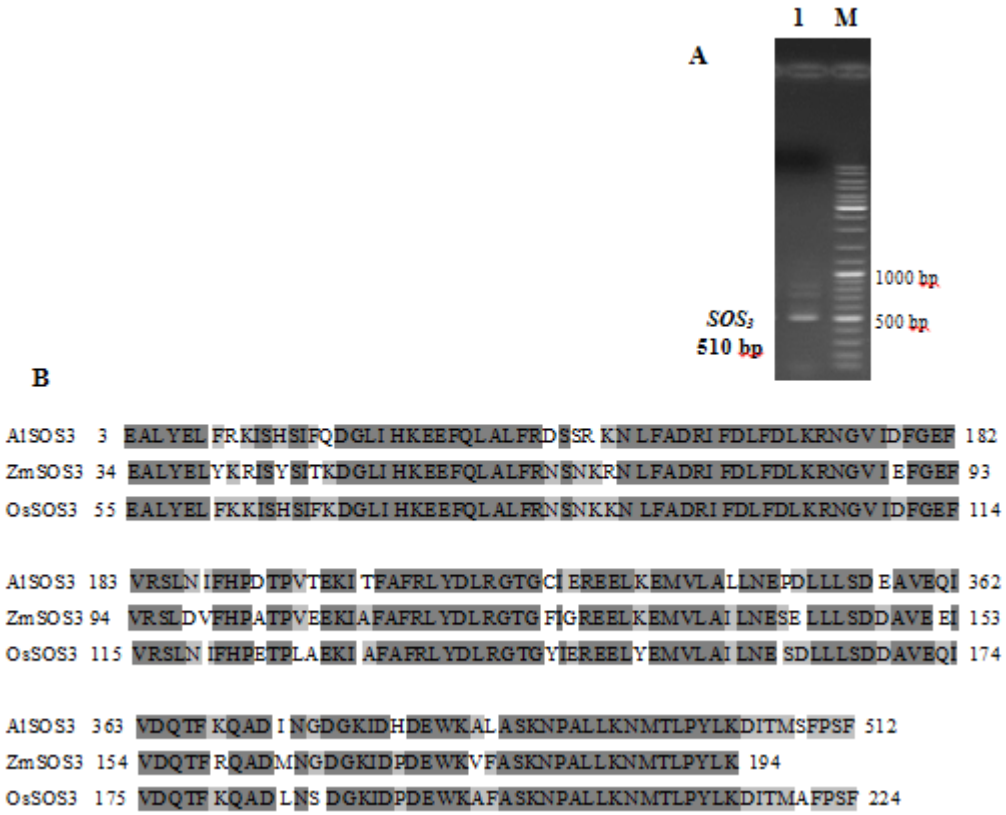

Fig 3. *alaSOS3* isolation from *A. lagopoides* by RT-PCR: a) line 1 relates to the *alaSOS3* gene amplification at 51 ºC, and lane 2 represents 100bp DNA marker b) The alignment of amino acid sequences from putative SOS3 proteins of different plants. Amino acids identical in at least two proteins are highlighted in dark gray and conservative substitutions are highlighted in gray. The alaSOS3 was aligned to the OsSOS3- Putative Calcineurin B (*Oryza sativa* Japonica Group) (GenBank accession No. D21753.1) and ZmSOS3- Calcineurin B- like protein (*Zea mays*)- (GenBank accession No. ACJ65321.1).

$NaCl+Ca_2SO_4$ in the shoots and roots but its level of changes was more considerable in the shoots rather than roots (Fig.5).

Effect of ABA & NaCl on alaSOS genes expression

While we expected the enhancing effect of NaCl on *alaSOS* genes was compensated by ABA, the results were relatively different. *AlaSOS1* expression in shoots and roots changed but insignificantly compared with NaCl or ABA treatments, independently (Fig.5). Also, we found that *alaSOS2* expression in the shoots was not changed meaningfully compared with NaCl or ABA treatments but it was increased significantly in the roots compared to ABA treatment (Fig.5). The results of *alaSOS3* expression were similar to the *alaSOS2* except for the roots which we observed a distinguished reduction relative to ABA treatment (Fig.5).

Alternation of alaSOS genes by supplying the exogenous ABA and Ca^{2+}

Consistent with the predicted protein structure of alaSOS3 we expected the meaningful changes in its expression pattern upon supplying exogenous Ca^{2+} and ABA together. But its consequence on *alaSOS3* was not considerable in the shoots and roots (Fig.5). Although *alaSOS1* and *alaSOS2* expression was changed significantly in roots, their differences in shoots were not meaningful (Fig.5).

Discussion

Studies have identified salt tolerance determinants in organisms ranging from cyanobacteria to fungi and from algae to higher plants. However, complete understandings of these factors in plants that are naturally salt tolerant (halophytes) have not been understood. Research with halophytic species has provided a glimpse of these adaptive components, but has been limited by the lack of molecular genetics in any of the species studied. *A. lagopoides* was used to address the questions about the existence and probable role of the SOS pathway in a halophyte contains salt glands. More than five genes are included in the SOS pathway and *SOS1*, *SOS2* and *SOS3* were introduced at first by Zhu and colleagues (Zhu et al., 1998). ABA and Ca^{2+} play important roles in plant growth and development, as well as in the responses of the plants to the environmental stresses. There is accumulating evidence that changes in protein phosphorylation may be an important part of ABA signaling. Because ABA is known to activate Ca^{2+} signaling, it seems likely that PKS kinases and SCaBPs also play a role in the response of the plant to ABA. Also, there is further evidence that SCaBP5 and closely related Ca^{2+} sensors and interacting protein kinases may be global negative regulators of ABA signaling in plants (Guo et al., 2002). SOS_1 functions as a Na^+/H^+ antiporter on the plasma membrane and plays a crucial role in sodium efflux from root cells and the long distance Na^+ transport from root to shoot (Shi and Zhu 2002). Despite Shabala et al. (2005) explanation about *SOS1*

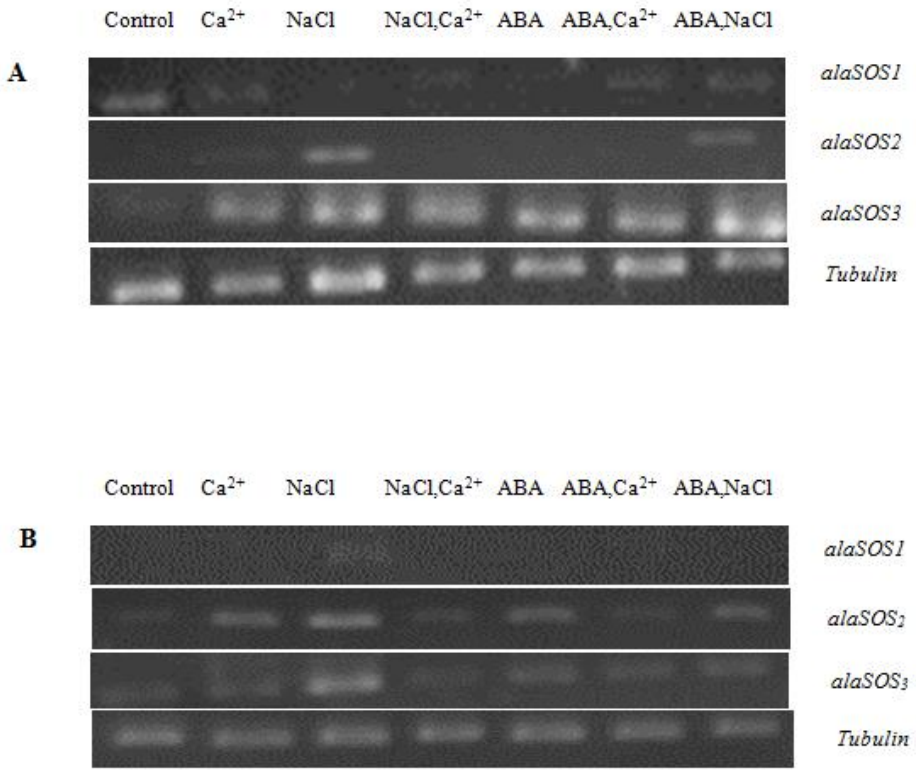

Fig 4. *alaSOS1-3* expression patterns in the shoot and root of *A. lagopoides* **A**) Expression analysis by semi-quantitative RT-PCR under NaCl (600 mM), ABA (50 µM), Ca_2SO_4 (5mM), NaCl+Ca_2SO_4, NaCl+ABA and Ca_2SO_4+ABA treatments in the shoot **B**) expression analysis by semi-quantitative RT-PCR in the root under the same condition as shoot.

constitutively expression in the root under salinity, we observed an altered *alaSOS1* expression pattern and concluded that *alaSOS2-alaSOS3* complex played an exclusive role in controlling the H^+/Na^+ exchange activity. Our results showed that *alaSOS1* expression was hardly ever in roots under control condition and was induced by salinity, so we concluded that its expression was critical for osmotic regulation and nutrients uptake. SOS1, the final element of the SOS signal- transduction chain, has been found predominantly in the root tip (Shi and Zhu 2002; Shabala et al., 2005). Consequently, it was assumed that this is the region, where this gene is functionally expressed but Shabala et al., (2005) showed that the function of the entire root has been altered in *sos* (and particularly *sos1*) mutants. This strongly suggests that location of the gene expression does not always portray the location of gene function. The SOS1 (Na^+/H^+) antiporter is a specific Na^+ exchanger and has been reported to be unable to transport K^+ (Shabala et al. 2005). *AlaSOS1* expression was increased by NaCl in both roots and shoots and it got to the highest level in shoots of *A. lagopoides* (Figs. 6 and 7). This is in agreement with the Oh et al., (2009) report about *SOS1* increasing expression level in response to salt treatments in Arabidopsis and Thellungiella. Additionally, transcript abundance of *alaSOS1* in all treatments except salt treated root was not detectable while it showed relatively constitutive and high abundance in all treatments in the shoots and it was in contrast to Thellungiella. Our previous study showed that one of the probable salt tolerance mechanisms in *A. lagopoides* is sodium transferring from root to shoot or out of plant by ion transporters such as *alaSOS1* which perform functions in excess Na^+ and helps excrete of the salt by salt glands (Jannesar et al., 2009). By enhancing external Na^+ content, increased mRNA levels encoding plasma membrane H^+-translocating ATPase in roots of tobacco and rice was distinguished (Niu et al., 1996; Zhang et al., 1999) and it might be related to an increased requirement for H^+-extrusion to sustain activity of the Na^+/H^+ antiporter (Tester and Davenport 2003). Furthermore, mRNA levels of endodermal H^+- ATPase was increased by adding NaCl to the growth medium of a halophyte, *Atriplex nummularia* (Tester and Davenport 2003). While we found recently that the expression pattern of a plasma membrane H^+-ATPase isoform encoding gene from *A. littoralis* (*AliHA1*) was up-regulated by NaCl in shoots without any significant changes in the roots (not published data). Some researchers have shown that one of the potential strategies for salt tolerance in halophytes is loading of excess Na^+ to the xylem and transporting it to the shoots (Tester and Davenport 2003). Salt excretion was recognized by salt glands located on the *A. littoralis* leaf surfaces which were grown under high salinity (200- 800 mM NaCl) (Barhoumi et al., 2006). Previously, we demonstrated high Na^+ contents in *A. lagopoides* shoot and root under NaCl (600 mM) treatments (Sobhanian et al., 2010, Jannesar et al., 2009). In fact, halophytes control Na^+ uptake effectively than salt- sensitive plants. It is probably only the fact that they live longer than glycophytes at higher salinities that leads to the high concentrations of shoot Na^+ often observed (Tester and Davenport 2003). Moreover, we defined *alaSOS1* ABA-independent regulation in both tissues (Figs. 4 and 5). While Chinnusamy et al., (2006) reported ABA negatively regulating effect on salt tolerance either by inactivating *SOS2* or by regulating Na^+/H^+ antiporters such as *SOS1* and *NHX1*. *alaSOS1* expression level in shoots under control condition was higher than roots and it was not changed significantly in response to different treatments, so the real function of the alaSOS1 protein remains to determine. Cheng et al., (2003) reported that *SOS2* interacted

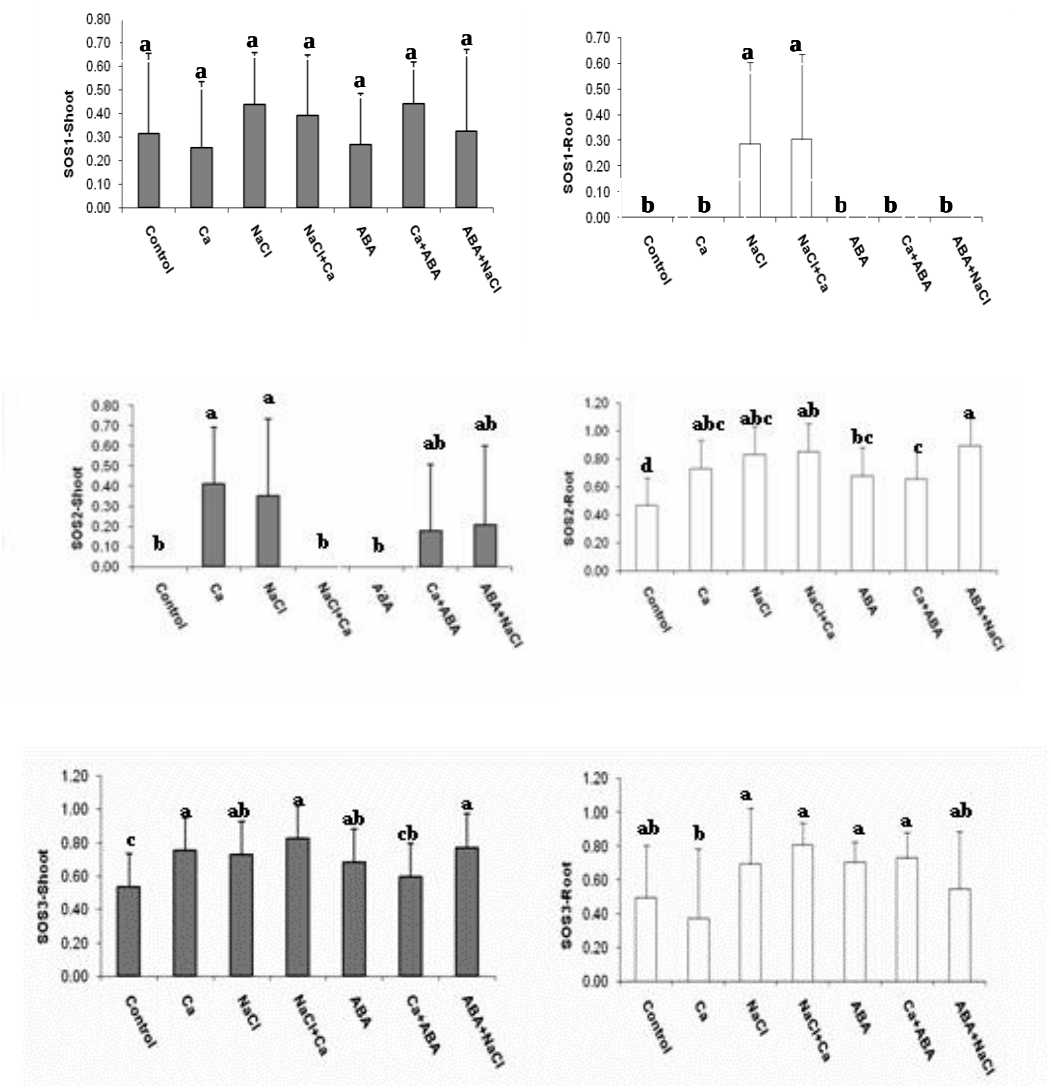

Fig 5. *alaSOS1-3 expression* pattern analysis in the shoot and root of *A. lagopoides.* Relative expression levels of the bands were measured by Total Lab software, intensity of the each bands are means of at least 3 replicates ± standard error. Same letters above each column represent no significant difference based on Dancans' test at $P< 0.05$.

with vacuolar Na^+/H^+ antiporter and significantly influenced the Na^+/H^+ exchange activity. It was observed in the present study that *alaSOS2* expression was up- regulated in the roots under all treatments, while its increasing level was only observed in response to NaCl and Ca^{2+} treatments in the shoots (Fig. 5). Presumably, Ca^{2+} is able to compensate the NaCl effects on the *alaSOS2* expression level in *A. lagopoides* shoots treating by NaCl and Ca_2SO_4 , concurrently. We suggest that Na^+ homeostasis in *A. lagopoides* is regulated by *alaSOS2* encoded protein and Ca^{2+} involves in its signal transduction. Bertorello and Zhu (2009) reported that roots were exposed to sodium elicited an increase in the cytosolic free Ca^{2+}. Exogenous ABA was able to stimulate cytosolic Ca^{2+} content and as a result the $alaSOS_2$ expression level was increased in the roots treated by ABA too. Furthermore, it seems that *SOS3*- SOS_2 complex functions in sequestration of excess Na^+ in the intracellular compartments by influencing NHX activity and it controls the expression and activity of SOS_1 antiporter and Na^+ efflux levels (Liu et al., 2000). *AlaSOS3* expression level was not significantly changed in *A. lagopoides* roots exposed to different conditions except for Ca^{2+} treatment (Figs. 4 and 5). Liu et al (2000) reported that *SOS3* takes part in salt tolerance in shoots and roots and plays a critical regulatory role in plants, so essentially its high expression level was not required. As shown in Fig.5, *alaSOS3* expression level was changed slightly in salt or control conditions in the roots with the exception of Ca^{2+} treatment. While ABA increases the cytosolic calcium content, *SOS3* up- regulation under ABA (50 µM), Ca_2SO_4 (5mM) and NaCl (600 mM) treatments in shoots was expected. Wang et al., (2007) showed that *SOS3* expression level was low and varied in different maize organs and it was regulated by NaCl and ABA. SOS3 is a small myristoylated protein that appears to have no enzymatic activity by itself; Ca^{2+} binding and myristoylation are required for SOS3 function in salt tolerance (Gong et al., 2004). SOS3 activates SOS2 kinase in a Ca^{2+} dependent manner and sos3/sos2 double-mutant analysis also indicated that SOS3 and SOS2 function in the same pattern. The fist target of the SOS3-SOS2 regulatory pathway to be identified is the plasma membrane Na^+/H^+ exchanger (antiporter) encoded by the *SOS1* gene. Although there is currently no experimental evidence for SOS3 binding to Na^+, the possibility cannot be excluded that SOS3 might serve as a Na^+ sensor based on the ability of Na^+ to bind within the EF-hand motifs of other proteins. SOS2 regulation of Na^+/H^+

antiporters on the tonoplast and plasma membrane provides additional support that this kinase plays an important role in the maintenance of cellular Na^+ homeostasis and is a critical component of the salt tolerance machinery in Arabidopsis (Gong et al. 2004). Several reports were revealed that *SOS* pathway is not regulated by ABA in *Arabidopsis* (Boudsocq and Lauriere 2005) but this pathway is regulated by ABA in maize and somehow in *A. lagopoides*. Furthermore, *SOS3* expression was quickly and highly up- regulated by ABA in maize (Wang et al. 2007; Shi and Zhu 2002) and we illustrated that ABA plays an important role in SOS pathway regulation in *A. lagopoides*. Consequently, *alaSOS3* and *alaSOS2* expression levels by exogenous ABA were significantly increased in shoots and roots, respectively. In summary, as a halophyte, *A. lagopoides* tolerates high concentrations of salt and because of the salt-induction of *alaSOS* genes; it is believed that these genes have essential functions in salt tolerance. Continued research must be done on the roots and shoots for *alaSOS1*gene and *alaSOS2* gene since the results showed very different roles between both genes.

Materials and methods

Plant materials and treatments

A. lagopoides seeds were provided by Pakan Bazr Company (Isfahan, Iran) and were separated from inflorescense and stored at 4ºC. Seeds were sterilized by commercial sodium hypochlorid 20% and Triton X- 100 1% for 10 minutes and were cultured on the legged metal net in the hydroponic medium supplemented by ½ MS medium and transferred to cold room (4 ºC) for 2 days and then they were moved to growth chamber for 21 days with 16/8 h light/dark at 23±2 ºC. After 21 days seedlings were treated by NaCl (600 mM), ABA (50 µM), Ca_2SO_4 (5mM), NaCl (600mM) +Ca_2SO_4 (5mM), NaCl (600mM) +ABA (50 µM) and Ca_2SO_4 (5mM) +ABA (50 µM) for 10 days. Then samples shoots and roots were collected and stored at -70 ºC. Each treatment was included 3 biological repetitions and at least 3 technical replicates.

RNA extraction and gene cloning

Total RNA was extracted from shoots and roots by RNax plus kit (Cinnagene Company,Tehran, Iran). Then RNAs were treated by *DNase* 1 *RNase* free kit (Fermentas Company, Ukraine) for eliminating of genomic DNA. The first strand cDNA synthesis and RT-PCR were carried out with 5 µg of total RNA using RevertAid^{TM} First Strand cDNA Synthesis kit (Fermentas Company, Ukraine). Then *SOS* ESTs (*SOS1*, *SOS2* and *SOS3*) were isolated from *A. lagopoides* shoot and root by genes specific primers (Table 1) designed based on conserved regions of wheat *SOS* genes which found by alignment in DNASTAR MegAlign software (Madison. WI 53705, USA). These segments were ligated to pTZ57R⁄ T transmitting plasmids and transformed plasmids were extracted by miniprep method (Sambrook and Russell 2001) and digested by *Hind*III and *Eco*RI enzymes (Fermentas Company, Ukraine) and clones were sequenced by Genefanavarn company in Tehran, Iran using M13 forward and reveres primers.

Expression analysis

Expression pattern analysis of *SOS* genes was carried out by semi-quantitative RT-PCR in contrast to β- tubuline, a house keeping gene, in shoots and roots of *A. lagopoides* under different treatments and each RT-PCR performed in 6 different repetitions. Relative intensity of bands was measured by Total Lab software and the data was analyzed statistically by SPSS version 10. Variance analysis was carried out to determine the existence of significant differences between means and Duncan´s tests executed to compare means at $p<0.05$. Data entry, sequence management, and sequences alignment were performed by DNASTAR software (Madison. WI 53705, USA). Sequence similarity and several structural features were studied by use of online databases including BLASTN and X (Gish and State 1993), pfam (Bateman et al. 2000) and Blocks (Pierrokovski et al. 1996).

Acknowledgements

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