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ORIGINAL PAPER

Isolation of a gene encoding a novel atypical LEA protein from the halophyte *Prosopis strombulifera* with a sodium salt-specific expression

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Abstract Salinity is one of the major abiotic stresses that adversely affect the crop productivity and growth of the whole plant. The halophytic *Prosopis strombulifera* (Fabaceae) shrub is a useful plant model to study the molecular and physiological mechanisms involved in salinity tolerance. In this work, the isolation and characterization of the mRNA for a new atypical LEA protein (PsLEA3) is reported. PsLEA3 has alanine as the most abundant amino acid followed by serine. This characteristic is different from typical LEA proteins which have high glycine content. PsLEA3 shows a negative average of hydropathy and remains localized in the chloroplast. The phylogenetic relationship between related LEA proteins and PsLEA3 showed that this protein belongs to the same group as a LEA protein of Arabidopsis. Northern analysis revealed a differential expression of this atypical LEA mRNA in *P. strombulifera* plants under NaCl and Na_2SO_4 treatments. Over-expression in roots of Na_2SO_4-treated plants from Ψ_o −1.9 MPa was associated with abscisic acid (ABA) and its conjugate (ABA-glucose ester) accumulation as well as with the root length of these plants, showing an inhibition and toxicity induced by sulphate anion present in the medium.

Keywords Halophyte · Late-embryogenesis abundant (LEA) proteins · NaCl · Na_2SO_4

Abbreviations

ABA	Abscisic acid
ABA-GE	ABA-glucose ester
GRAVY	Grand average of hydropathy
LEA	Late-embryogenesis abundant
NES	Nuclear export signals
SLP-L	Subcellular localization of protein using local aligment

Introduction

Salinity is a major problem in agricultural production due to the toxic effects of salts on plant growth. Excessive salt accumulation in the soil can produce sodic (or alkaline) and saline soils. Sodic soils have a poor soil structure and are found in both arid and semi-arid regions. They retain high Na^+ levels at the exchangeable site of clay particles in the soil. Thus, sodic soils have a high exchangeable sodium percentage ESP > 15 and a high pH, greater than 8.5. Saline soils are found in several regions such as estuaries, coastal fringes and some arid regions. These regions are dominated by Na^+ ions causing a high electrical conductivity EC > 4 dS/m, corresponding to approximately

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40 mM NaCl (Munns and Tester 2008; Hauser and Horie 2010).

There are different strategies to solve the problem of salinity in the soils via remedial land management or by using salt-tolerant crops. Indeed, the ability of these crops to remove excessive salt from the soil and to regulate their water potential may result in a significant decrease of this economical burden. The existing salt-tolerant species have a genetic potential that can be used for plant adaptive responses. One of these species is *Prosopis strombulifera* (Lam.) Benth (Burkart 1976), a spiny shrub which grows in areas spreading from the Arizona desert (USA) to Patagonia (Argentina). This species is particularly abundant in salinized soils in central regions of Argentina. The major cations present in saline environments are typically Na^+, Ca^{2+}, and Mg^{2+}; the main anions are Cl^-, SO_4^{2-} and HCO^{3-}. NaCl and Na_2SO_4 are the most commonly found salts in soils of many countries (Manivannan et al. 2008). In saline soils of southern Cordoba and southwestern San Luis provinces in Argentina, these two salts are found at similar concentrations, although Sosa et al. (2005) found that Na_2SO_4 was up to three times more abundant in some soil samples. It is important to evaluate the effects of both salts, NaCl and Na_2SO_4 on the plant growth for a better understanding of the physiological responses that plants use to cope with these environments. Our previous results reported a halophytic response of *P. strombulifera* to NaCl surviving up to 1 M NaCl in hydroponic experiments. On the contrary, plants grown in the presence of Na_2SO_4 showed that root length and shoot height were more affected than those in NaCl-treated plants confirming previous observations on the inhibition generated when sulphate anion is present in the medium immediate and sustained reduction of growth parameters, accompanied by senescence symptoms such as chlorosis, necrosis and leaf abscission (Reinoso et al. 2005; Devinar et al. 2013). Seedlings grown in an increasing gradient of NaCl (250 up to 700 mmol l^{-1}) did not develop salt glands in the leaves. Thus, plants growing in NaCl solutions would be able to filter more efficiently soil solution to prevent passage of excess ions to the xylem (Reinoso et al. 2004). Accordingly, when *P. strombulifera* grows under high NaCl concentration, it shows adaptive responses that include a delicate and carefully-controlled balance. This balance involves the compartmentation of Cl^- and Na^+ in leaf vacuoles, exclusion of Na^+ in roots, and an increase of proline, pinitol, and mannitol synthesis in the cytoplasm. Instead, plants growing in Na_2SO_4 solutions show reduced capacities for ion compartmentalization and osmotic adjustment causing a water imbalance, and symptoms of toxicity due to altered carbon metabolism (Llanes et al. 2012). The *P. strombulifera* differential physiological responses to the major salts present in the soil described above provide an excellent model to study the underlying molecular mechanisms of salt tolerance in this species.

The integrated plasticity in plant development involves different genes playing an essential role in plant responses to abiotic stress. One group of these genes is the coding for late embryogenesis abundant (LEA) proteins which has an important role in plant adaptation to water and salt stress tolerance; they accumulate mainly in mature seeds or can be induced in several vegetative tissues under salinity, dehydration, cold and freezing stresses (Hanin et al. 2011). Different studies have defined at least six different groups of LEA genes according to the amino acid sequence and expression pattern (Dure 1993; Battaglia et al. 2008). Most LEA proteins belong to a widely group of proteins called "hydrophilins", characterized by a high hydrophilicity index, greater than 1, high glicine content and low Cys and Trp content (Wise and Tunnacliffe 2004). According to this classification, "typical" LEA proteins are those which show hydrophilic characteristics, whereas those that contain a significantly higher proportion of hydrophobic residues are called "atypical" LEA proteins. This group adopts a globular conformation and might be located in chloroplast/mitochondria, like four atypical LEA proteins reported from Arabidopsis (Hundertmark and Hincha 2008).

The overexpression of AtLEA3-3 in Arabidopsis enhances the tolerance to salt and osmotic stress (Zhao et al. 2011). Moreover, Arabidopsis plants deficient in LEA4 group genes were more susceptible than wild-type plants under water stress. Many mechanisms have been proposed for LEA proteins action, such as hydration buffer, molecular shield, enzyme protection and membrane interaction (Olvera-Carrillo et al. 2011). However, their precise function is still obscure. Two signaling pathways, ABA-dependent and ABA-independent can regulate the expression of LEA proteins. The ABA-dependent signaling pathways include bZIP transcription activators named ABFs or AREBs (ABRE binding factors), which bind to ABRE elements, homologs of *A. thaliana* CBF4/DREB1D transcription activator. On the other hand, the ABA-independent signaling pathways involve homologs of *A. thaliana* such as DREB2A and DREB2B transcription activators, which bind to CRT/DRE/LTRE elements (Yamaguchi-Shinozaki and Shinozaki 2006). It has been reported that exogenous applications of ABA induce the expression of many LEA genes (Galau et al. 1989). However, the importance of endogenous ABA as a signal for the stress- responsive induction of LEA gene expression varies extensively (Chandler and Robertson 1994). It has been known that the changes in endogenous ABA levels are regulated by ABA catabolism governed by two pathways: an oxidative pathway and sugar conjugation (Nambara and Marion-Poll 2005). ABA-glucose ester (ABA-GE) is the predominant ABA sugar conjugate, which may

function as a storage form of releasable free ABA. The mechanisms underlying transport of ABA and its conjugates remain not completely clear as well as the functions of conjugated forms of ABA (Schachtman and Goodger 2008).

In this study we report the isolation and characterization of a gene coding a LEA protein of the halophyte *P. strombulifera* and its differential expression under increasing concentrations of NaCl and Na_2SO_4, and their iso-osmotic mixture, in order to contribute to the knowledge of stress molecular markers and the putative function of PsLEA3.

Materials and methods

Plant materials

Seeds from *P. strombulifera* plants were collected in southwest area of San Luis province, Argentina. The area is located in the El Monte Phytogeographic Region, 33 43′S, 66 37′W, in a saline depression between annual 300–400 mm isohyets, altitude 400–500 m, and average annual temperature 15–20 °C. Pods were collected at random from 100 plants within the same population. Seeds were selected visually for uniformity in size and healthy appearance. After collection, seeds were scarified with sulphuric acid for 10 min and washed overnight under running water; then they were rinsed in distilled water. Finally, seeds were placed in Petri dishes with two layers of water saturated filter paper for 24 h at 37 °C before sowing. Germinated seeds with roots 20-mm long were grown under hydroponic conditions, in two black trays per treatment (200 seedlings per tray), with 25 % full-strength Hoagland's solution (osmotic potential −0.11 MPa) and were grown in a chamber with 16-h light (28 °C)/8-h dark (20 °C) cycle and, 70 % relative humidity. Hoagland solutions were changed at 7-day intervals and continuously aerated; pH of the medium was 6. Each experiment was performed three times.

Salt treatments

Salt treatments were applied after 21 days of plant growth using a simple randomized design. Pulses of NaCl alone (50 mmol l^{-1}) and Na_2SO_4 alone (38 mmol l^{-1}) were applied every 48 h until reaching final osmotic potentials (Ψ_o) = −1.0, −1.9, or −2.6 MPa, respectively (measured by a vapor pressure osmometer Model 5500, Wescor Inc., Logan, UT, USA). These Ψ_o values corresponded to age 29, 40 and 48 days, respectively (Devinar et al. 2013). Iso-osmotic bisaline solutions were obtained by mixing equal volumes of the respective monosaline solutions at each osmotic potential. For each sampling, 25 treated plants were collected at random at 24 h after the medium reached a final osmotic potential as indicated above; 25 control plants (no salt added; Ψ_o of medium = −0.11 MPa) were collected for each treatment. Plants were frozen with liquid nitrogen, and stored at −80 °C for a posteriori analysis. Each experiment was performed four times.

Determination of growth parameters

Root length and shoot height were measured weekly in 20 plants from each treatment from the time that salt pulses were started (21, 29, 40 and 48 days of culture).

Determination of Na^+ and K^+ ions

Samples containing 150 mg DW were ground in a mortar with liquid nitrogen, digested with concentrated HNO_3 at 200 °C, and dissolved in deionised water to a final volume appropriate for the standard curve. Contents of Na^+ and K^+ were determined by a Zeltec ZF250 IND flame photometer (Skoog et al. 2000).

Extraction and determination of ABA and ABA-GE

ABA and ABA-GE were extracted and purified as described by Zhou et al. (2003), with modifications. 150 mg of lyophilized plant material (leaves or roots) were ground with liquid nitrogen and ABA and ABA-GE were extracted with 3 ml extraction buffer pH 2.8. 50 ng of [2H6]-ABA and [2H5]-ABAGE (NRC-Plant Biotechnology Institute, Saskatoon, Canada), were added as internal standards. Extracts were transferred to 50 ml tubes, centrifuged at 8000 rpm for 15 min, and supernatants were collected and mixed with ethyl acetate. Then, the organic phase was extracted and evaporated at 37 °C. Dried extracts were dissolved in 1500 µl methanol and evaporated and immediately resuspended in 50 µl methanol (100 %). 10 µl of each sample were injected onto a liquid chromatograph (LC) with Electrospray Ionization (ESI) (Waters Corp., New York, NY, USA). Analyses were performed using an Alliance 2695 (LC Separation Module, Waters, USA) quaternary pump equipped with auto-sampler. A Restek C18 (Restek, USA) column (2.19 × 100 mm, 5 µm) was used at 28 °C. The binary solvent system used for elution gradient consisted of 0.2 % acetic acid in H_2O (solvent B) and MeOH (solvent A), at a constant flow-rate of 200 µl min^{-1}. A linear gradient profile with the following proportions (v/v) of solvent A was applied [t (min), % A]: (0, 40), (25, 80), with 7 min for re-equilibration. MS/MS experiments were performed on a Micromass Quatro UltimaTM PT double quadrupole mass spectrometer (Micromass, Manchester City, UK). Data were acquired and

analyzed using MassLynxTM 4.1 and QuanLynxTM 4.1 (Micromass, Manchester, UK) software. For quantification, values were obtained from a calibration curve previously constructed using each compounds and their pure standard/deuterated internal standards.

DNA isolation

For the isolation of total DNA, 150 mg of young seedling roots was homogenized under liquid nitrogen and 15 ml of extraction buffer (100 mM Tris–HCl, pH 8.0, 50 mM EDTA, pH 8.0, 500 mM NaCl, 10 mM B-mercaptoethanol and 1 ml of 20 % SDS) and incubated at 65 °C for 15 min. Samples were incubated at 0 °C for 30 min with 5 ml of 5 M potassium acetate and centrifugated for 15 min at 17,000 rpm. 10 ml of isopropanol were added to the supernatant, mixed and incubated at −20 °C for 30 min. The pellet was washed with 70 % ethanol, dried at room temperature and resuspended in 450 µl TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, pH 8.0) plus 50 µl of 3 M NaCl followed by incubation at 37 °C for 30 min with 2 µl of RNAse (10 mg ml^{-1}). The solution was transferred to an eppendorf tube and the extract was washed once with 1 volume phenol and twice with 1 volume chloroform. Then, DNA was precipitated with 0.8 volume of isopropanol, centrifuged for 1 min; the pellet was washed with 70 % ethanol, dried and resuspended in 200 µl of TE buffer.

Isolation and amplification of the LEA sequence

The CODEHOP program was used to design the primers; forward 5′-GAAGGAAGAAGAGTTGGGAAAGA-3′ and reverse 5′-CGGCGACGTCGATCTCCTTGGTA-3′ primers were designed for amplification of the LEA gene. PCR amplification was performed using 1 µM of each primer, genomic DNA and Taq-polymerase (Promega, Madison, WI, USA). The PCR conditions were: 4 min at 94 °C; 40 cycles of 30 s at 94 °C; 1 min at 45 °C; 1 min at 72 °C; 7 min at 72 °C. The PCR amplified products were analyzed on 1 % agarose gel, then DNA bands were removed and purified with the Qiagen Gel Extraction kit (Qiagen GmbH, Hilden, Germany). Purified DNA amplified sequences were cloned into the vector pCR 2.1-TOPO (Invitrogen, San Diego, CA, USA) and sequenced on ABI3130 Sequencer (Applied Biosystems, Life Technologies Corp., Carlsbad, CA, USA).

Isolation of total RNA and Northern blot analysis

Total RNA from *P. strombulifera* leaves and roots was isolated using RNAqueous Kit (Ambion/Applied Biosystems). 10 µg of total RNA was separated by electrophoresis on an agarose and formaldehyde gel, denaturated and blotted onto a nylon membrane. Membranes were then hybridized with α-32P labeled probe (corresponding to a cDNA fragment of *P. strombulifera*) on Amersham Rediprime II DNA Labeling System (GE Healthcare). Filters were washed twice for 30 min at 65 °C with 5X SSC-0.5 % SDS, and twice with 1X SSC-0.5 % SDS. Autoradiograms were exposed on autoradiography films. Ethidium bromide dyeing was used to equalize loading of RNA in the samples. Northern blot images were analyzed using ImageJ software http://rsb.info.nih.gov/ij/.

Protein bioinformatics analyses

Amino acid sequences were translated to fasta format and then analyzed by checking different databases. Pfam http://pfam.sanger.ac.uk/ and InterPro Scan http://www.ebi.ac.uk/Tools/InterproScan/ were used to analyzed the protein sequence. The subcellular localization of the protein was predicted using WolF PSORT http://wolfpsort.org/ and SLP-L http://sunflower.kuicr.kyoto-u.ac.jp/smatusuda/splocal.html. The nuclear export signals were analyzed with NetNES database (http://www.cbs.dtu.dk/services/NetNES/) and the PSORTII (http://psort.ims.utokyo.ac.jp/) was used to infer the nuclear localization signals. In addition, protein secondary structure was predicted by a system of neural networks rating (PROFsec) at an expected average accuracy >72 % for the three states helix, strand and loop. Disulfind program was used for predicting the disulfide bonding state of cysteines and their disulfide connectivity starting from sequence alone. The isoelectric point and molecular mass predictions were obtained with compute pI/Mw tool by ExPASy Server. Protein hydropathy analyses were performed by using the Kyte and Doolittle algorithm hydropathy plots (Kyte and Doolittle 1982).

Sequence alignment and phylogenetic tree

The identities and similarities of the isolated sequence were analyzed with the BLAST program and the GenBank database on the NCBI web server.

The multiple sequence alignment of the full-length LEA proteins was performed by ClustalX using a MEGA5 program. Tree reconstructions were predicted by the neighbor joining method with a Kimura2-parameter correction model and a bootstrap test of 1000 replicates.

Statistical analysis

Data were analyzed using INFOSTAT (v 2011). A two factorial experiment: osmotic potential (Ψ_o, −1.0, −1.9, or −2.6 MPa) and treatment (Control, NaCl, Na_2SO_4 and NaCl + Na_2SO_4) was set up in a completely randomized design. Two way ANOVA was performed and significant

differences among treatments were calculated by the use of pair-wise comparisons using Duncan significant difference test ($p < 0.05$).

Results

Results of root length showed an increase from the beginning of the experience in all treatments and no significant differences were detected between them. However, Na_2SO_4 treatment caused a shoot growth inhibition from −1.9 MPa, while NaCl treated plants showed the maximum growth with no significant differences compared to non treated plants. No significant differences were observed in bisaline treated plants compared to monosaline treatments (Fig. 1).

As shown in Fig. 2, the Na^+ content in roots and leaves of salt-treated plants was consistently higher than in non-treated plants throughout the whole experiment, with no significant differences between them. The K^+ content was not altered by any of the salt treatments (Supplemental Fig. 1).

ABA levels in roots was higher in salt-treated plants than in non-treated plants at Ψ_o −1.0 MPa, showing a sharp increase of ABA when Na_2SO_4 was present in the medium. Similarly, in leaves, Na_2SO_4 treatment caused the maximal ABA levels from −1.9 MPa (Fig. 3). At high salinity (−2.6 MPa), the ABA-GE levels increased in roots of salt treated plants compared to non-treated plants, but the highest ABA-GE levels were observed in Na_2SO_4-treated plants. Similarly, leaves of Na_2SO_4-treated plants showed an increase of ABA-GE levels that was 12-fold higher than the other treatments at −2.6 MPa (Fig. 3).

Results of the isolation and characterization of a *P. strombulifera* gene showed that it encoded a novel LEA protein. Kyte and Doolittle hydropathy plot showed that the predicted PsLEA3 protein is strongly hydrophobic throughout the length of the protein except for the C-terminal region, with a high hydropathy (GRAVY) average value of −0.471 (Fig. 4a). Based on the sequences analysis of this protein, a conserved domain was detected by both databases. A domain of late embryogenesis abundant (LEA3) family from this plant is encoded at amino acids 2–67 (Pfam 03242). This indicates that the new protein isolated belongs to LEA3 group and it was named PsLEA3 (Fig. 4b).

The molecular mass of the predicted protein was 8.2062 kDa, and the theorical isolelectric point (pI) was calculated to be 7.99. PsLEA3 protein showed preponderance of Ala, Val and Ser that constitute 14.3, 10.4 and 11.78 %, respectively. Remarkably, this protein does not contain cysteine. The predicted secondary structure composition of PsLEA3 showed a 23.38 % of α helices and 2.6 % β sheets with a high percentage of loops (74.03 %).

The subcellular localization predicted by WoLFPSORT program was analyzed. This program generated highly reliable scores for both chloroplast (RI: 9) and nuclear (RI: 5) localizations. Therefore, these data suggest the possibility for a nucleo-cytoplasmatic transport. Similarly, the Subcellular Localization of Protein using Local aligment (SLP-L) program indicated that this protein has chloroplast localization. NetNES predicted nuclear export signals (NES) in PsLEA protein with a higher value than 0.5 (threshold value) of the terminal portion of the sequence. Based on sequence analyses of PSORT II, PsLEA does not have nuclear localization (Supplemental Table 1).

A multiple amino acid sequence alignment of PsLEA3 from *P. strombulifera* was constructed (Supplemental Fig. 2). Phylogenetic relationships between PsLEA3 and related LEA proteins from different species were also analyzed. As depicted in Fig. 4c, an unrooted dendrogram showing clustering of PsLEA3 with other proteins was constructed. Based on the bootstrap consensus tree inferred from 1000 replicates, PsLEA3 is orthologue of the Arabidopsis At4g15910 with a high bootstrap support of 96 % as a quantifier for its robustness. PsLEA3 of *P. strombulifera* was found to be similar to the Arabidopsis LEA protein of group 3, with a moderate bootstrap of 76 %. Notably, results of this tree clearly separated PsLEA3 from *P. juliflora* LEA (PjLEA3) with a mediocre bootstrap of 51 %. A radiation neighbor joining phylogenetic tree exhibited three different clades. Notably, the PsLEA3 and At4g15910 were grouped in the clade I (Supplemental Fig. 3).

Northern analysis of this PsLEA3 mRNA from *P. strombulifera* (Fig. 5) showed a high mRNA expression only in leaves of NaCl treated plants at Ψ_o −1.0, −1.9 and −2.6 MPa. The expression in leaves of Na_2SO_4 treated plants was low at −1.0 MPa and not detected at −1.9 and −2.6 MPa. In roots, Na_2SO_4 strongly induced the PsLEA3 mRNA expression at Ψ_o −1.9 and −2.6 MPa. When both salts were mixed up, the lowest expression was found in leaves at Ψ_o −1.0 MPa, while at Ψ_o −1.9 and −2.6 MPa the PsLEA3 mRNA expression was not detected. In roots of these plants, no differences were found in PsLEA3 mRNA expression compared to NaCl treatments along the experiment.

Discussion

This is the first study in which a cDNA encoding LEA protein from *P. strombulifera* was isolated and characterized. Furthermore, the different patterns of LEA mRNA

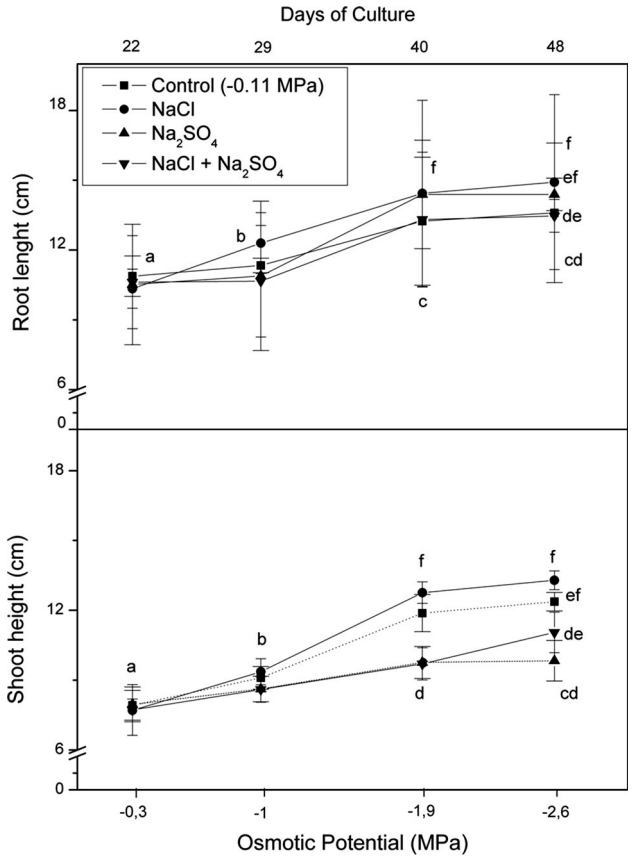

Fig. 1 Effects of NaCl, Na_2SO_4 and their iso-osmotic mixture on root length and shoot height of *P. strombulifera*. Data were from three replicated experiments (total n = 20 plants per treatments), and represent mean ± SE. *Different letters* above data indicate significant differences among treatments ($p < 0.05$)

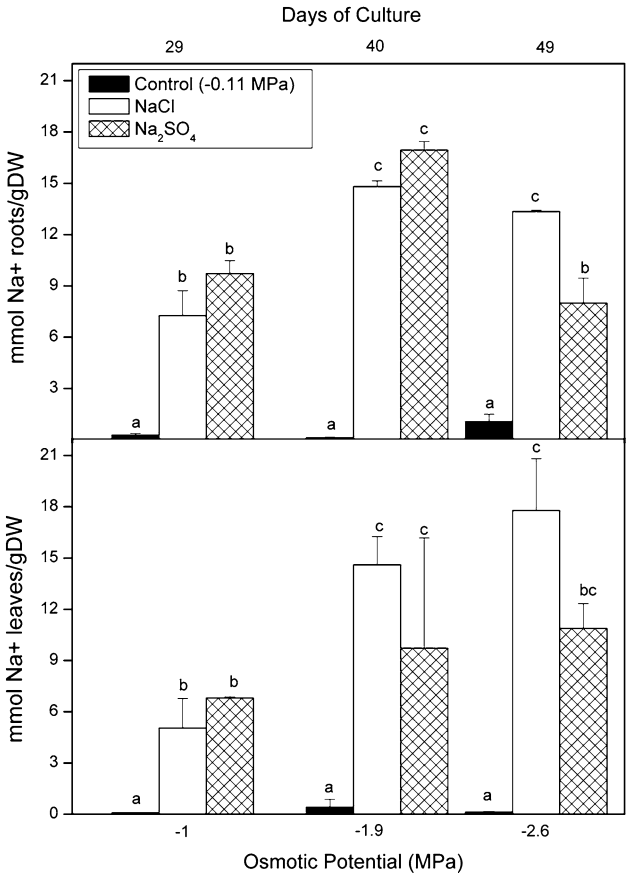

Fig. 2 Effects of NaCl, Na_2SO_4 and their iso-osmotic mixture on Na^+ content in roots and leaves of *P. strombulifera* plants. Values are means from three replications bars represents the mean ± SE. *Different letters* above *bars* indicate significant differences among treatments ($p < 0.05$)

expression and ABA interaction were evaluated in plants growing under NaCl, Na_2SO_4 and their isoosmotic mixture.

Previous studies have demonstrated that *P. strombulifera* is less tolerant to Na_2SO_4 than to NaCl and their isoosmotic mixture. Na_2SO_4-treated plants showed a reduction of growth parameters, accompanied by senescence symptoms such as chlorosis, necrosis, and leaf abscission (Reinoso et al. 2005). In this study, root length and shoot height of these plants were also more affected than NaCl-treated plants, confirming the growth inhibition and toxicity induced by Na_2SO_4-treatment. Moreover, NaCl-treated plants accumulated similar levels of Na^+ and K^+ both, in leaves and roots than Na_2SO_4-treated plants. The high Na^+ accumulation observed in NaCl-treated plants demonstrate that they are able to use this ion for osmotic adjustment and efficient compartmentalization in vacuoles.

The LEA protein of this halophyte was classified as LEA3 group. This group is characterized by a repeating motif of 11 amino acids (Finn et al. 2007). LEA3 proteins show a wide variation in the number of tandem repeats, ranging from 5 to more than 30. It has been proposed that the 11-mer motifs are linked by ionic bridges and trigger some important functional activities (Quevillon et al. 2005).

The pI of this protein correlates with the characteristic of LEA3 group members which are mainly basic proteins with a pI ranging from 8 to 10 (Dure 1993).

This protein has not preponderance of glycine, unlike typical LEAs, and does not contain cysteine. The absence of cysteine in PsLEA3 could be associated to the molecular basis of the primary structure affecting the protein folding type and the spatial conformation.

The analysis of PsLEA3 shows a nonregular secondary structure for group 3 because the secondary structure prediction programs indicate a high degree of amphipathic α helices for this group. Contrarily, several proteins from group 1 and group 2 LEA have been inferred to have a high proportion of loop regions (Tunnacliffe and Wise 2007). However, the information about the function of protein structure remains unclear. Similar to other atypical LEA proteins (Hundertmark and Hincha 2008), results of the PsLEA3 localization showed that this protein could be

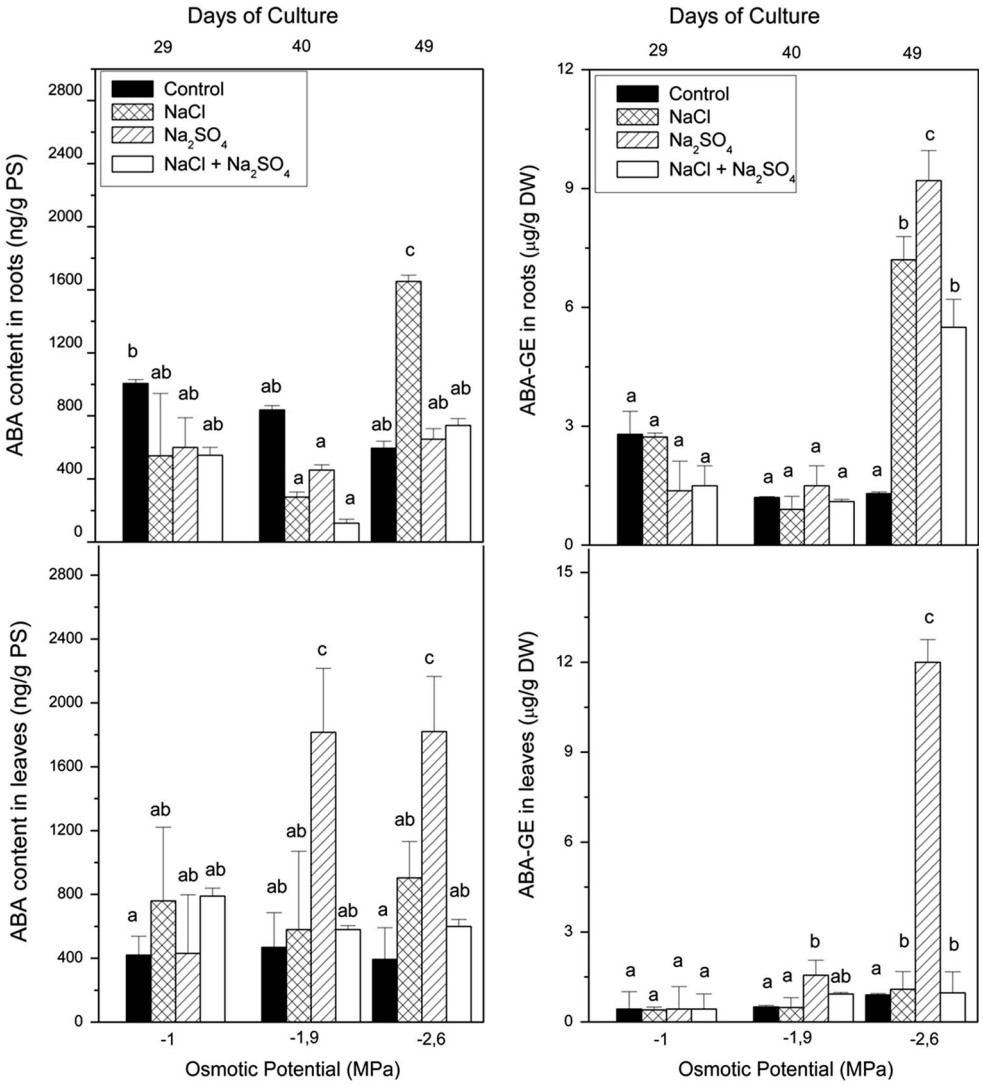

Fig. 3 Effects of NaCl, Na_2SO_4 and their iso-osmotic mixture on ABA and ABA-GE levels in roots and leaves of *P. strombulifera* plants. Values are means from three replications bars represents the mean ± SE. *Different letters* above *bars* indicate significant differences among treatments ($p < 0.05$)

exported out of the nucleus and remain in the chloroplast, possibly in the thylakoid membrane. Both computer predictions and experimental observations suggest that LEA proteins are expressed in different subcellular compartments (Tunnacliffe and Wise 2007). Localization of PsLEA3 could be related to subcellular functions of LEA proteins protecting the cellular components from desiccation induced damage. For example, the maintenance of the chloroplasts integrity in the dry state, the membrane stabilization and the photosynthetic system. However, further studies should be carried out to elucidate the localization sites as well as the physiological functions of these proteins.

Hydropathy analysis showed that the predicted PsLEA3 protein has a high hydropathy average value (−0.471). This correlates with other LEA members such as CaLEA6 and IbLEA14, which have the C-terminal domain more hydrophilic than the N-terminal. In fact, PsLEA3 contained a lower proportion of polar (hydrophilic) and small residues but a higher proportion of non-polar residues in comparison to those LEA members. These features more likely contribute to the hydrophobic character, which is opposite to the extensive hydrophilic characteristics of other LEA proteins. Moreover, the average hydropathy score of 30 members of LEA3 group from plants is −0.97 ± 0.3 (Goyal et al. 2005). However, the hydropathy score for PsLEA3 protein was −0.46, which is moderately hydrophilic. Hydrophilicity is a common characteristic of the most abundant LEA proteins, while atypical LEA proteins exhibit a preponderance of hydrophobicity showing a globular conformation. Therefore, these proteins have been proposed to be involved in cellular functions somehow different from those of hydrophilic LEA proteins (Tunnacliffe et al. 2010). Hydrophobic character shown by GRAVY (−0.46) of PsLEA3 and the presence of alanine as the major amino acid are opposite to the characteristics of

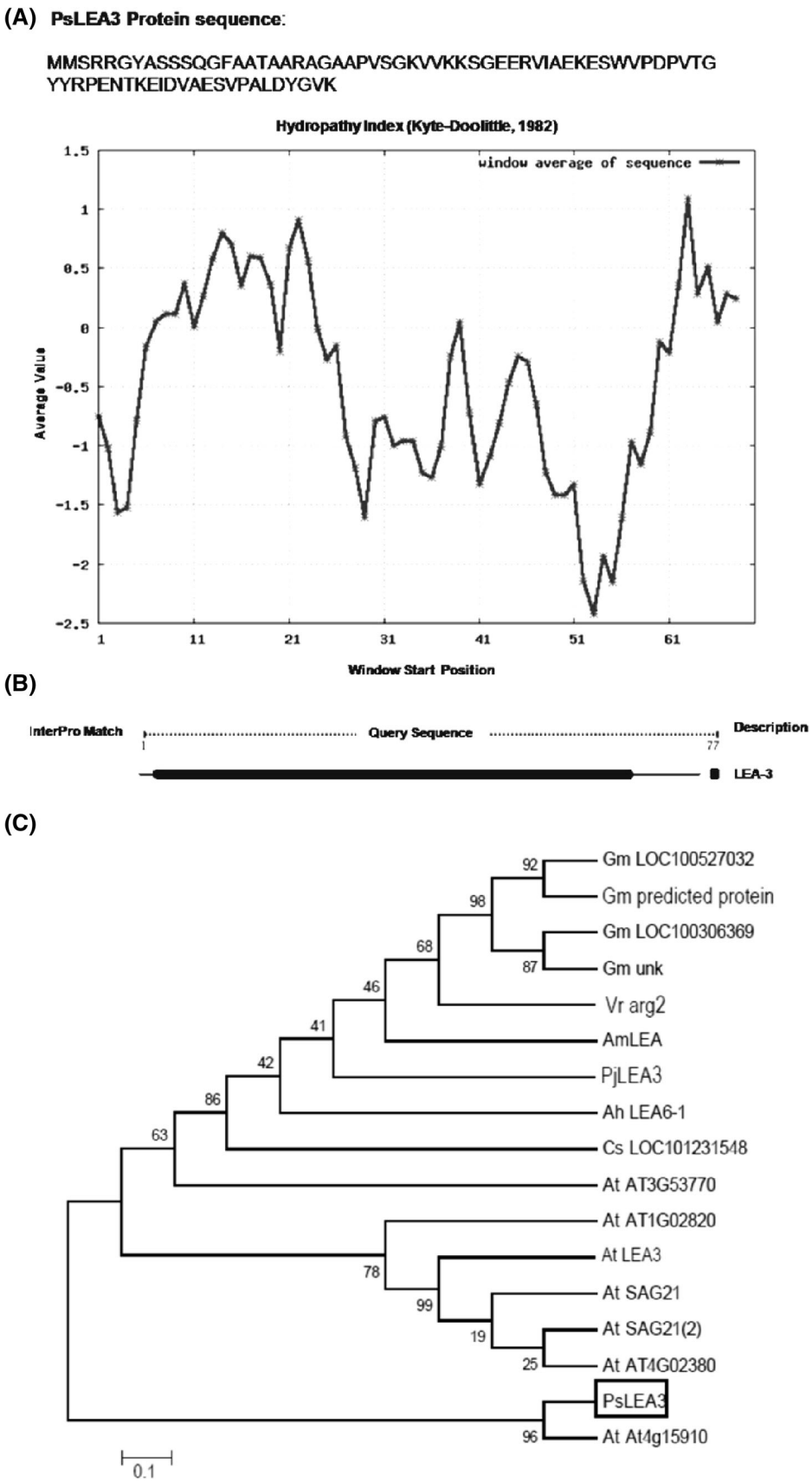

Fig. 4 **a** PsLEA protein sequence and hydropathic index plot of the deduced PsLEA3 amino acid sequence analyzed using the Kyte-Doolittle algorithm. Amino acid position is plotted on the x axis beginning with the C terminus. Regions above a hydropathy score of zero are hydrophobic. **b** Secondary structure prediction with conserved domain analyzed by using InterProScan. **c** Unrooted phylogenetic tree between *P. strombulifera* (PsLEA3) and related LEA proteins. GenBank accession numbers for *Arabidopsis thaliana* LEA proteins are At4g15910, At1G02380, At3G53770 and At4G02380. Other proteins are shown in this figure by their accession number, Gm (*Glycine max*), Am (*Ammopiptanthus mongolicus*), Pj (*Prosopis juliflora*), Cs (*Cucumis sativus*), Vr (*Vigna radiate*). Bootstrap support on the *left* of each node was inferred from 1000 replicates

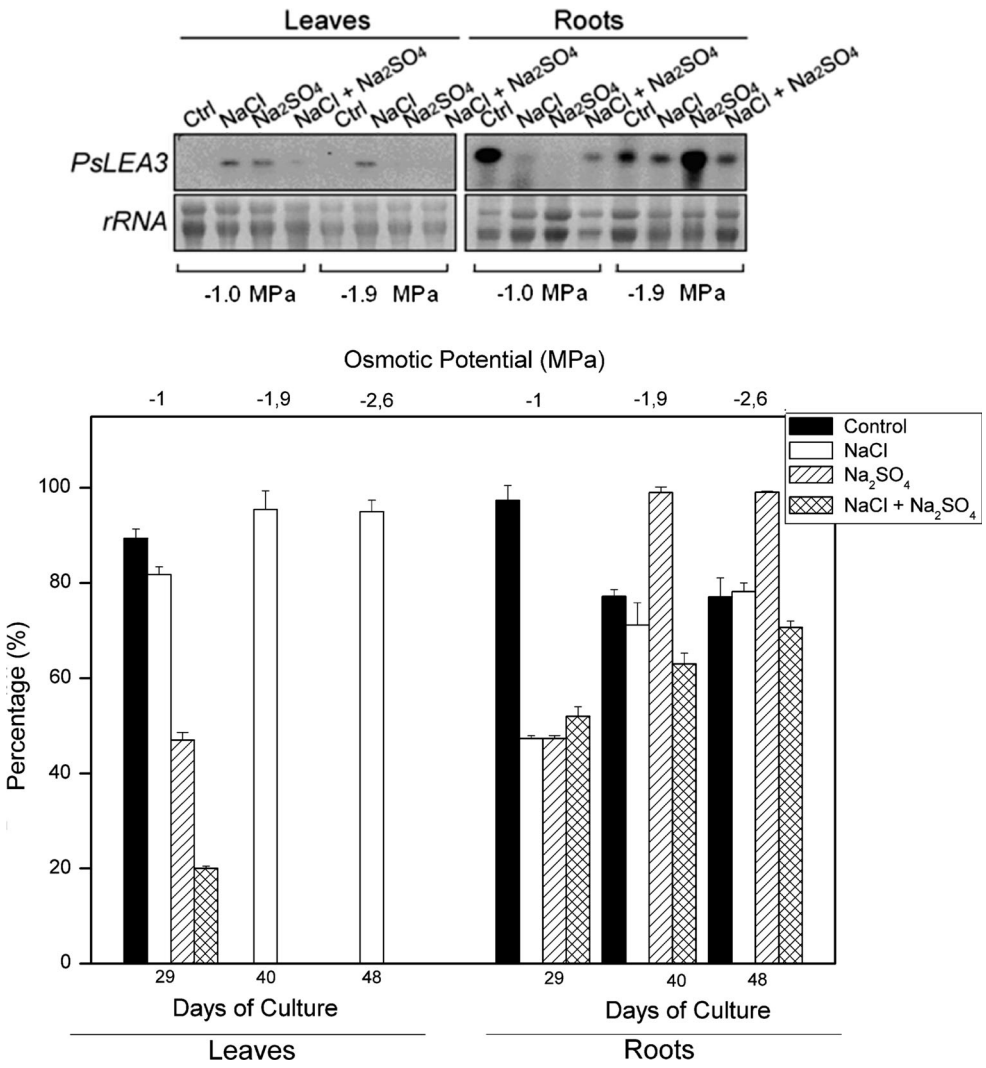

Fig. 5 Expression analysis of *P. strombulifera* LEA (PsLEA3) gene. RNA gel blot analysis of LEA transcripts in leaves and roots. The Y-axis in the Northern blot graph refers to background-corrected intensities (as calculated by ImageJ). For Northern blot data, the signal corresponding to the mRNA with the highest signal intensity was set to 100 %, and signals for the additional mRNAs were calculated as a percentage thereof

typical LEA proteins rendering this protein an atypical LEA member.

LEA proteins constitute a phylogenetically heterogeneous group unlikely to share common ancestry. This extensive divergence between LEA proteins, even within the same plant species, does not allow obtaining confident phylogenetic inference for this group of proteins. However, the results of this study clearly separated PsLEA3 from other LEA, and placed it in the same group as LEA of Arabidopsis At4g15910. This protein was shown to increase under progressive drought stress in root and leaves of Arabidopsis, and the regulation of this gene seems to be mediated by different signals, one of which is ABA (Pouchkina-Stantcheva et al. 2007).

Similarly to other types of LEA proteins, the accumulation of LEA3 proteins could be regulated by ABA during some specific developmental stages, and/or under adverse environmental conditions (Tunnacliffe et al. 2010; Hand et al. 2011). It has been demonstrated that some LEA3 proteins are accumulated in scutellar tissues and shoots of young wheat plants under a severe drought stress with more than 90 % water loss. Then, when plants were rehydrated, shoots and scutellar tissues resumed growth. The roots of rehydrated plants showed mRNA accumulation but not increased LEA protein content and they did not resume growth. In addition, these roots were replaced by new developing roots from scutellar tissue. Hence, LEA3 accumulation in severely dehydrated seedlings was associated with tissue dehydration tolerance (Leprince and Buitink 2010).

A high LEA protein accumulation is correlated with stress tolerant of some crops. A comparison of protein profiles after ABA treatment showed a significant increase in LEA3 protein content in roots from salt-tolerant rice varieties compared to salt-sensitive ones (Moons et al. 1995). In addition, Kim et al. (2005) showed that the increase of a pepper hydrophobic LEA, CaLEA6, in transgenic tobacco induced dehydration and salt tolerance.

Results of this study indicate that NaCl treatment showed a high mRNA expression while Na_2SO_4 treatment did not induce PsLEA3 mRNA expression in leaves. Increased expression of this mRNA at lower osmotic potentials suggests a role in protecting biomolecules from dehydration, ion sequestration, etc. This differential PsLEA3 mRNA expression correlates with Na^+ and K^+ contents, which showed that NaCl-treated plants accumulated similar Na^+ levels than Na_2SO_4-treated plants in their leaves, but they succeed in ion compartmentalization and osmorregulation with direct consequences on their growth. However, these results are still inconclusive regarding to the inhibitory effect of sulphate and some alternative interpretations should be considered. When the sulphate anion is present in the culture medium, the capacities for ion compartmentalization and osmotic adjustment are reduced. Therefore, this response results in water imbalance and toxicity symptoms caused by an alteration in the carbon metabolism, for example, production of sorbitol instead of mannitol, reduced sucrose production and protein levels (Llanes et al. 2012).

Endogenous ABA and ABA-GE levels in the halophyte *P. strombulifera* are consistent with previous studies (Devinar et al. 2013; Llanes et al. 2013). ABA and ABA-GE levels work together to create and intensify the salt-specific stress signal. This response is correlated with high ABA-GE glucosidase activity, demonstrating the ABA-GE transport from roots to leaves (Llanes et al. 2013). Accordingly, results of this study show that the exposure to different concentrations of Na_2SO_4 and NaCl altered the patterns of LEA mRNA expression both in leaves and roots of *P. strombulifera*.

Expression of specific LEA genes during water or cold stress require high ABA levels as demonstrated in studies using ABA-deficient mutants of tomato, maize and Arabidopsis. It has been also demonstrated that other LEA genes respond to ABA treatments, showing the existence of ABA-independent induction pathways (Nordin et al. 1991). Consistently, our results showed that in leaves of NaCl-treated plants, ABA levels were low and similar to those of non-treated plants. However, the PsLEA3 mRNA expression was the highest in this treatment, suggesting an ABA-independent pathway. Moreover, Na_2SO_4-treated plants showed the highest free ABA and ABA-GE levels from −1.9 MPa, but did not showed a PsLEA3 mRNA expression.

On the other hand, in roots of Na_2SO_4-treated plants, the high ABA levels observed at the beginning of the experiment (−1.0 MPa) may induce the subsequent PsLEA3 mRNA expression, as was detected from −1.9 MPa. These results suggest that PsLEA3 gene and its differential expression pattern may be ABA-regulated, reflecting the different stress levels sensed by the root system when plants grow in the presence of sulphate sodium salts.

It has been proposed that LEA proteins are part of the complex and large pool of molecules involved in biochemical mechanisms for cell protection together with compatible solutes synthesis, ion compartmentation and others. Thus, the results of this study reinforce our previous demonstrations that a single species such as *P. strombulifera* can be highly tolerant to one of the salts present in the soil (NaCl) but intolerant or scarcely tolerant to another (Na_2SO_4). Therefore, the understanding of the interplay between LEA proteins and stress tolerance in model systems that mimics what really happens in the field, is crucial for identifying key genes involved in stress defense of economically important species.

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