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Gene expression and physiological analysis of *Atriplex halimus* (L.) under salt stress

Monther T. Sadder[1,2*], Firoz Anwar[1], Abdullah A. Al-Doss[1]

[1]Department of Plant Production, Collage of Food and Agricultural Sciences, King Saud University, PO Box 2460, Riyadh 11451, Saudi Arabia
[2]Department of Horticulture and Crop Science, Faculty of Agriculture, University of Jordan, Amman 11942, Jordan

***Correspondent author: msadder@ksu.edu.sa**

Abstract

Atriplex halimus (L.) is a xero-halophyte shrub adapted to extreme drought and salinity stresses. Ability of local *A. halimus* to tolerate salinity stress was investigated. Two-month-old *A. halimus* plants were exposed to salt stress (0, 150, 300 and 600 mM NaCl) in properly aerated liquid medium. Differential gene expression of stressed plants was investigated using real-time PCR analysis. Furthermore, stress related physiological changes including photosynthetic efficiency, chlorophyll, carotenoid, free proline and MDA content were measured. Stressed plants showed increased expression of salt response genes compared to the control (intermediate-associated protein, acetohydroxy acid reductoisomerase, early-responsive to dehydration stress-related protein, ER-lumenal protein and membrane protein). Their expression was much higher at 150 mM than 300 mM stress level, indicating their specificity for low level salt stress. Photosynthetic activity was slightly decreased with both extended stress exposure and increased salt concentration, while total chlorophyll and proline increased under saline stress. The MDA level was significantly lower after 70 h compared to 30 h stress. Adaptation to salt stress is a complex trait governs by molecular changes expressed as physiological processes during plant development.

Keywords: *Atriplex halimus*, gene expression, salinity, PSII, chlorophyll, proline, MDA.
Abbreviations: **A**rabinogalactan protein (AGP), chlorophylls (Chl), complex I intermediate-associated protein 30-like (CIA30), early-responsive to dehydration (ERD), expressed sequence tags (ESTs), malondialdehyde (MDA), photosystem II (PS-II), quantitative PCR (qPCR), reactive oxygen species (ROS).

Introduction

Limited water resources and salty water are major challenges for world food security, especially in many developing countries. The xero-halophyte saltbush (*Atriplex*) is well adapted to saline clay soils receiving less than 150 mm precipitation and is considered a highly palatable forage shrub (Le Houerou, 1980). Xero-halophyte species prevailing dry weather in Arab countries have adaptation mechanisms enabling them to evolve under these harsh environments (Hassine et al., 2008). Populations of *A. halimus* are found naturally in saline basins in several Arab countries, e.g. Saudi Arabia, Jordan, Egypt, Libya and Tunisia. The identification of physiological properties used by xero-halophyte species to cope with salt and/or drought is of great interest for plant scientists to identify traits associated with stress tolerance.

Plant strategies for stress tolerance comprise a number of physiological and biochemical processes including maintained water use efficiency, net carbon gain, osmotic adjustment (Bohnert et al., 2006; Ezawa and Tada, 2009; Laüchli and Grattan, 2007), lowering of water potential (Martínez et al., 2005), stomata closure, protection of endogenous enzymes and photosystem II (PSII), leaf shedding, leaf rolling, accumulation of compatible solutes and organic acids (Munns, 2002).

The accumulation of Na^+ and Cl^- reduces plant growth (Hassine et al., 2008; Yousif et al., 2010). However, lowering leaf water potential causes stomata closure (Nardini et al., 2001) and helps in maintaining turgor (Laüchli and Grattan, 2007). Turgor can be maintained by osmotic adjustment by both a net accumulation of inorganic solutes (Bohnert et al., 2006) and osmolytes (Munns and Tester, 2008). These osmolytes facilitate water uptake and stabilize enzymes in hyper-saline conditions (Hassine et al., 2008; Bohnert et al., 2006). At low concentrations organic solutes are reported to enhance the activity of antioxidant enzymes by direct scavenging of reactive oxygen species (ROS) (Banu et al., 2009; Miller et al., 2010; Parida and Jha, 2010).

Differential expression of some gene in saltbush under salt stress was investigated by research groups, including DREB in *A. hortensis* (Shen et al., 2003), DREB in *A. halimus* (Khedr et al., 2011), GH3.3, CAT1/2, TIP1, SIHP1 and EXP1 in *A. centralasiatica* (Xu et al., 2011). This study is part of project that aims to carry out and analyze salt stress induced genes in *A. halimus*. Plant performance was assessed based on physiological parameters and stress related gene expression.

Results

Expression of salt responsive genes

PCR screening showed seven genes out of ten were positive using *A. halimus* cDNA generated from RNA isolated from stress treatments. The stressed plants showed increased expression level of all seven salt stress responsive genes compared to control, when analyzed by real-time PCR (Fig. 1). The expression increase ranged from 2 (HO089224) to 10

folds (HO089205) at 150 mM NaCl. However, the expression was dramatically lower at 300 mM reaching around 2 folds. On the other hand, the gene expression levels at 600 mM NaCl were lower at 150 mM but higher at 300 mM.

Photosynthesis efficiency

The capacity of the *A. halimus* to withstand different degrees of salt/osmotic stress was tested by evaluating its effect on photosynthesis, especially primary photochemical events and chlorophyll (Chl) content. The primary photosynthetic events associated with light reaction were determined through fluorescence analysis.

The activity of PSII was slightly affected during salt stress compared to the control (Fig. 2). Photosynthetic efficiency measured in terms of F_v/F_m (indicating PSII efficacy) varied between 0.6 and 0.8 in both control and salt stressed plants, respectively. Among each salt stress level (150, 300, or 600 mM), there was a slight drop of PSII activity after 50 h compared to 30 h point, followed by recovery at 70 h point.
The change in the total chlorophyll content in leaves from plants exposed to different degrees of osmotic stress was determined. Total chlorophyll content decreased for all treatments with time (from 30 h to 70 h points) (Fig. 3). The dramatic decrease was associated with chlorophyll a (around 60%) not chlorophyll b. Carotenoid content decreased at 70 h point compared to 30 h point for 300 and 600 mM NaCl. Similarly, it was decreased in the control (Fig. 4). At 150 mM NaCl, carotenoid content did not vary between 30 and 70 h points, while increasing salt concentration resulted in increase in carotenoids as measured at 30 h point.

Free proline and MDA

During present investigations the change in the levels of free proline in *A. halimus* plants under salt stress was compared with control plants. The proline content in *A. halimus* leaves increased gradually by increasing salt concentration (Fig. 5). This was true at both 30 and 70 points. The proline content was at 70 h point higher in all salt concentrations than 70 h point, although it was significantly higher only in 600 mM NaCl stress treatment.

The overall level of toxic oxygen species was determined by measuring the level of malondialdehyde (MDA), a cytotoxic product of lipid peroxidation that is generally taken as an index for the level of ROS. Therefore, during present investigation the change in MDA levels in *A. halimus* plants under salt stress was compared to the control. For control plants, the MDA level increased slightly at 70 h point compared to 30 h point (Fig. 6). The 150 mM NaCl stressed plants had MDA levels similar to the control at 30 h point. However, MDA levels increased slightly in both 300 and 600 mM salt treatments at 30 h point. On the other hand, MDA levels dropped dramatically for all salt concentrations (150, 300 and 600 mM) at 70 h point.

Discussion

Halophyte plants have adapted physiological responses to tolerate the constraint of salinity. Soil salinity and drought compromise water uptake, leading to osmotic adjustment in xero-halophyte plant species. Na+ and Cl- ions are often used as indicators of plant efficacy to withstand salinity stress (Martínez et al., 2005; Djerroudi et al., 2011; Boughalleb and Denden 2011). During the present investigation, two-month-old *A. halimus* plants were grown in mineral growth medium supplemented with 0, 150, 300 and 600 mM NaCl for different time interval (30, 50 and 70 h). A group of randomly selected salt responsive expressed sequence tags (ESTs) from the related species, *A. centalasiatica* (NCBI, 2011), were subjected to annotation using Blast2Go server (Blast2Go, 2011). The first five ESTs listed in Table (1) did not give any BLAST hit, presumably novel to *Atriplex*, while the second five ESTs in the same table gave similarity hits. The EST HO056119 was annotated as classical arabinogalactan protein (AGP) 26-like (Synonym: salt overly sensitive 5 or SOS5). AGP from *Salicornia europaea* was shown to increase salt tolerant in tobacco (Yamada et al., 2010). Furthermore, AGP was recorded to be massively up-regulated in salt stressed cells (Lamport et al., 2006). The rice genome has 69 AGPs, which display differential expression patterns in response to salt stress (Ma and Zhao, 2010). However, the quantitative PCR (qPCR) primes designed from *A. centalasiatica* did not work using *A. halimus* cDNA in this study. Comparison of AGP genes of both *A. centalasiatica* and *A. halimus* (unpublished data), revealed high sequence divergence. In fact, we could detect two different ESTs coding AGP from *A. halimus* cDNA, which have sequence similarities of 80.6% and 51% to Arabidopsis homologs AGP 41 and AGP 24, respectively (unpublished data).

The expression of the EST HO089198, complex I intermediate-associated protein 30-like (CIA30) was found to be very high, specifically at salt concentration of 150 mM (Fig. 1). CIA30 is a mitochondrial protein present in human, mouse and fission yeast (Janssen et al., 2002). Furthermore, it was recorded to be associated with heat stress in the seaweed *Seagrass zostera* (Reusch et al., 2008).

Two additional salt responsive ESTs had similarities to the vegetable crop *Spinacia oleracea*. Both *S. oleracea* and *A. triplex* are related species belong to the Amaranthaceae family. The EST HO089198 encodes plastid protein acetohydroxy acid reductoisomerase, while the EST HO089205 encodes ER-lumenal protein. Their expression at 150 mM salt stress increased 7 and 10 folds, respectively. On the other hand, both had low expression levels at 300 mM (Fig. 1). Acetohydroxyacid synthase reductoisomerase is a key enzyme in the biosyntheses of valine and isoleucine in plants (Dumas et al., 1989).

The expression of EST HO056123 in *A. halimus* was increased in response to salt stress at 150 mM (6 folds) and at 600 mM (3 folds). It encodes early-responsive to dehydration (ERD) stress-related protein, which is one of late-embryogenesis-abundant proteins induced by osmotic stress in different plant species (Zhu, 2002; Meili et al., 2012). Arabidopsis plants transformed with maize ERD gene showed elevated salt tolerance (Liu et al., 2009).

Chlorophyll is a key component for photosynthesis, which in turn, is a key metabolic event essential for growth and development of plants. However, photosynthesis is extremely sensitive to abiotic stresses such as salt, drought, frost and temperature (Dubey, 1997; Ashraf and McNeilly, 2004). The loss of chlorophyll content accompanied by inactivation of photochemical reactions, especially those mediated by PSII, was recorded in plants exposed to salt, drought and low temperature stresses (Sharma and Hall, 1992; Fedina et al., 1993). Chlorophyll content is widely used as an indirect indicator for PSII activities under osmotic stress condition. In addition, decline in the PSII activity could be caused by destruction of the chloroplasts through dilation of thylakoids and envelope breakdown, destabilization of pigment protein complexes, low CO_2 fixation, increase in ribulose 1,5-

Table 1. Top hits for *A. centalasiatica* investigated ESTs using BLASTx with search limit to Viridiplantae (NCBI, 2011).

#	Query	Annotation	Subject	Organism	Positives
1	HO089224	Predicted protein	XP_001763374	*Physcomitrella patens subsp. patens*	32/74 (43%)
2	HO089140	Predicted protein	BAK00705	*Hordeum vulgare subsp. vulgare*	55/108 (51%)
3	HO089233	Hypothetical protein	EEC67940	*Oryza sativa Indica Group*	18/29 (62%)
4	HO089144	No significant similarity found			
5	HO056119	Classical arabinogalactan protein 26-like	XP_002269710	*Vitis vinifera*	28/45 (62%)
6	HO089198	Probable complex I intermediate-associated protein 30-like	XP_003547847	*Glycine max*	208/226 (92%)
7	HO089196	Acetohydroxy acid reductoisomerase	CAA40356	*Spinacia oleracea*	197/204 (97%)
8	HO056123	Early-responsive to dehydration stress-related protein	NP_192343	*Arabidopsis thaliana*	120/146 (82%)
9	HO089205	ER-lumenal protein	AAA21806	*Spinacia oleracea*	183/184 (99%)
10	HO089211	Uncharacterized membrane protein YOL092W-like	XP_002274448	*Vitis vinifera*	92/109 (84%)

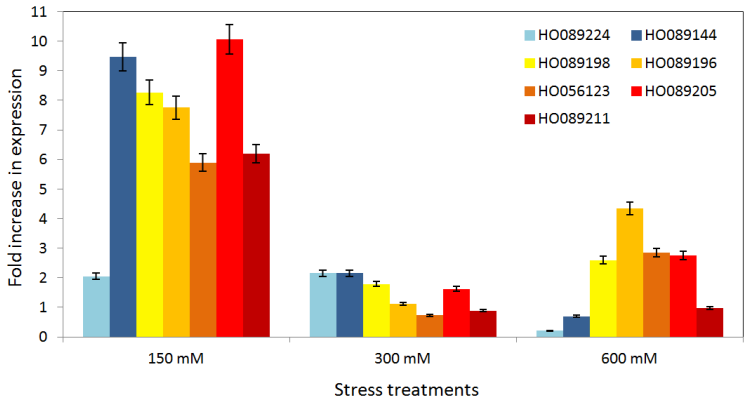

Fig 1. Real-time PCR of selected ESTs using cDNA from salt stressed *A. halimus* showing fold increase in expression compare to the control. Bars represent standard error.

biphosphate oxygenase activity or dissociation of extrinsic proteins 18 and 23 kDa from water oxidation complex (Sharma and Hall, 1992; Papageorgiou and Murata, 1995; Sakamoto and Murata, 1998; Prasad and Pardha Saradhi, 2004). We determined the photosynthetic efficiency, in terms of chlorophyll content and PSII activity in leaves of *A. halimus* plants exposed to osmotic stress (imposed by using different concentrations of salt for different time exposures). At any given time and under any degree of osmotic stress/salt stress imposed (up to 600 mM for 70 h), PSII mediated electron transport activity (measured in terms of F_v/F_m) and Chl content of leaves of salt stress plants was maintained compared to corresponding control (Fig. 1 and 2).

This mild salt response in the presented data of stressed *A. halimus* plants reflects enormous adaptation capabilities under stress conditions as compared to sensitive plant species. Hussaine et al. (2008) reported that chlorophyll content was almost maintained under 160 mM salt stress for 10 days but interestingly PSII activities decreased 8-12%. This was in agreement with our results. On the other hand, carotenoids are important in harvesting light energy during photosynthesis. Therefore, the decrease in carotenoids, mainly after extended exposure to salt stress, would hinder photosynthesis and, in turn plant growth and development.

Several investigations carried out by different research groups showed that free proline accumulation is an adaptive strategy to counteract negative impact of abiotic stresses such as salinity and drought (Ashraf and Foolad, 2007; Hussaine et al., 2008). Compatible solutes such as proline have been shown to accumulate in plants under osmotic (salinity as well as drought) stress as an adaptive means to counteract stress-induced deleterious effects (Ashraf and Foolad, 2007; Zhu et al., 2011). It helps in maintaining desired osmotic potential (i.e. involved in osmotic adjustment), protect various cellular components (membranes, proteins/enzymes and nucleic acids) and maintain cellular redox (Verdoy et al., 2006). Hussaine et al. (2008), applied moderate (40 and 160 mM) salt stress for 10 days and found no significant increase in proline level from day one and day ten. This could be due to low level of salt stress treatment. However, during the present investigations, an increase in the levels of free proline was recorded in leaves of *A. halimus* exposed to salt/osmotic stress. The increase in proline was

Table 2. Designed qPCR primers of salt responsive genes ESTs from *A. centalasiatica* and reference gene rbcL from *A. halimus* (AY270059).

#	Accession	Primer Code	Primer Sequence	Tm	bp
1	HO089224	F-001Ace	CCTACCACTGTATCTGCTCTC	45.6	527
2		R-001Ace	CAGACAAAGACATGGTTTCC	46.7	
3	HO089140	F-002Ace	CTATTGAGCTGCTGCTCAAAC	49.7	314
4		R-002Ace	CTTCACGCTTTCACCACC	48.4	
5	HO089233	F-003Ace	GCATTTATTAGCCCTGAACC	48.5	439
6		R-003Ace	CCACACAAAGCCACCAAC	48.9	
7	HO089144	F-004Ace	CCACCATGCGAGCATAGTCC	55.3	301
8		R-004Ace	GGCCCCTTTCAAAATCCCTC	56.2	
9	HO056119	F-005Ace	GACCAAATGATTCTAGGGGG	50.1	342
10		R-005Ace	GAGAAAGAGGCGATCAATTG	49.0	
11	HO089198	F-201Ace	ACTTACTCGGTCCTAATTGC	45.5	641
12		R-201Ace	GCTACCAAGAAAGCTCTCAC	45.9	
13	HO089196	F-202Ace	CCGAAGTGTTGTTTTGGCTGG	56.9	395
14		R-202Ace	CAGCCACCAAGGCTTGTTGG	57.7	
15	HO056123	F-203Ace	GGAGTATGAAAGTGCTGGG	46.8	502
16		R-203Ace	GCACTAAGCTCACTCTACCC	45.7	
17	HO089205	F-204Ace	TCGTGGGTGGCTTTTACC	50.6	347
18		R-204Ace	TGGGCATCGTTAAAGTAAGC	49.9	
19	HO089211	F-205Ace	TATAGAACTGGGTAGGTAGCG	46.1	376
20		R-205Ace	TCACAAATCATATTCACCCC	47.0	
21	AY270059	rbcL_F A.h.	TCGGGAGGTATTCACGTTTGGC	59.7	186
22		rbcL_R A.h.	CTCACGAGCAAGATCACGTCCC	58.3	

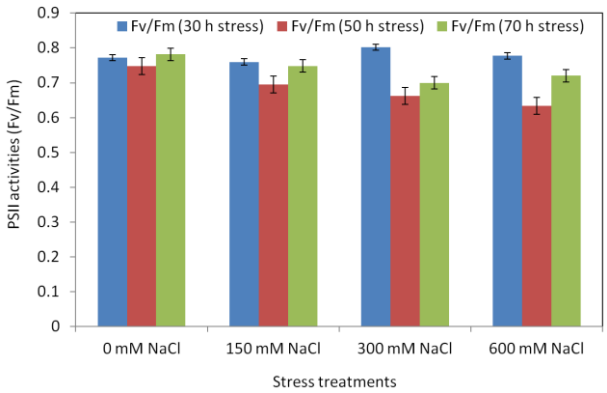

Fig 2. PS II activities under different time exposure and salt stress. Bars represent standard error.

proportional to the degree of imposed stress, both time wise and concentration wise (Fig 5). Leaves of *A. halimus* plants exposed to 600 mM NaCl for 30 h and 70 h showed ~2.55 and 3.21 folds higher proline content compared to control plants, respectively.

There are four major constraints of salinity on plant growth namely osmotic effects, restriction of CO_2 gas exchange, ion toxicity, and nutrient imbalance (Greenway and Munns 1980; Koyro 2003). MDA accumulation is considered to be a marker of oxidative damage (Weisany et al., 2012). However, lipid peroxidation can also be induced via an enzymatic pathway by the activity of lipoxgenases, which have been observed to be induced by salt stress (Mittova et al. 2002; Molina et al. 2002). Sai et al. (2011) observed continues increase in MDA levels in *A. hortensis* up to 260 mM salt stress. Rubio et al. (2009) observed greater oxidative damage in *Lotus japonicus* exposed to a high saline concentration, despite the maintenance of antioxidant levels. They suggested two possible explanations; MDA was accumulated due to the fact that cellular membranes are particularly sensitive to ROS attack and the oxidative damage is the result of an excess of ROS production rather than insufficient antioxidant protection. Moreover, it has been well documented that biotic and abiotic stresses are responsible for the increase in cell wall lignification (Chazen and Neumann 1994; Polle et al., 1994; Katerji et al., 1997) which could be associated with decreased plant growth, nutrient content, and digestibility (Guenni et al., 2002). The MDA content was increased more than 2 fold in *A. halimus* under 160 mM salt stress (Hussaine et al., 2008). Our date showed slight increase in MDA concentration in 300 and 600 mM stress levels at 30 h point (Fig 6), which may be an extensive lipid peroxidation of cell membrane components. This increase could be due to ROS produced by oxidative stress (Sairam et al., 2002). The present results indicated a half fold decrease in MDA, when expose to 70 h stress compared to 30 h exposure in all three salt concentrations. This is additional evidence supporting the tolerant capabilities of *A. halimus* plants for scavenging of ROS.

Materials and methods

Salt stress treatments

Seeds of *A. halimus* were collected from Al-Jouf province, northern of Saudi Arabia from one population. Seeds were germinated in trays (Size 30 × 12 × 4 $inch^3$) containing soil

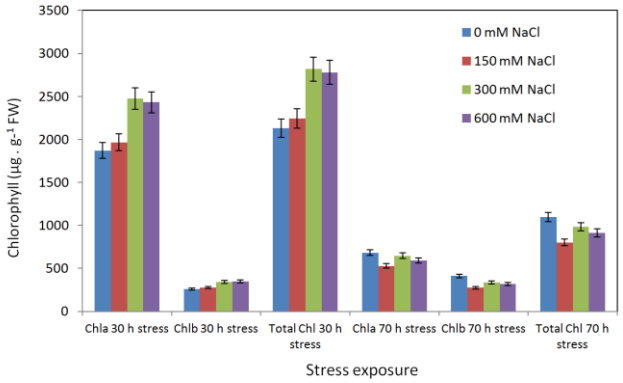

Fig 3. Chlorophyll content under different time exposure and salt stress. Bars represent standard error.

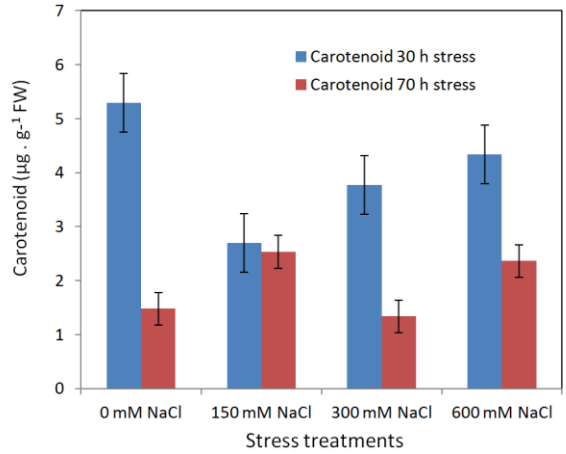

Fig 4. Carotenoid content under different time exposure and salt stress. Bars represent standard error.

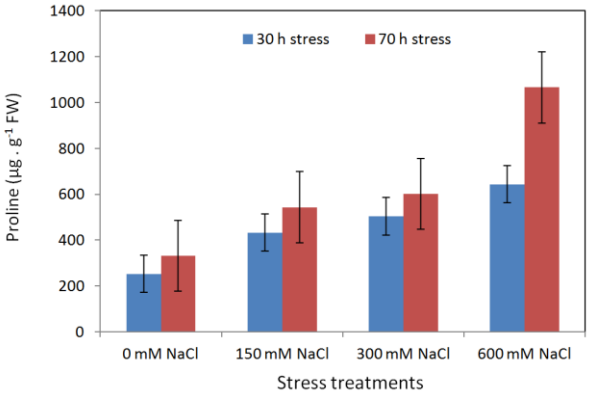

Fig 5. Free proline content under different time exposure and salt stress. Bars represent standard error.

mix (Peat moss: Sand: Soil of 1:1:1). Two-month-old plants were taken from trays and roots were washed thoroughly but gently with distilled water. Plants were subsequently transferred into plastic culture vessels (8 inch height), containing 400 ml liquid medium (5 mM KNO3, 1 mM $NH_4H_2PO_4$, 0.5 mM $MgSO_4$, and 5.5 mM Ca $(NO_3)_2$, 25 µM KCl, 10 µM H_3BO_3, 1 µM $MnSO_4$, 1 µM$ZnSO_4$, 0.25 µM $CuSO_4$, 10 µM Na_2MoO_4, 50 mg/l FeEDTA). The culture vessels were wrapped with aluminum foil to enforce darkness on roots. Plants were affixed from crown area with kitchen sponges to vessel opening, while roots were submersed in the liquid media. The culture vessels were properly aerated using

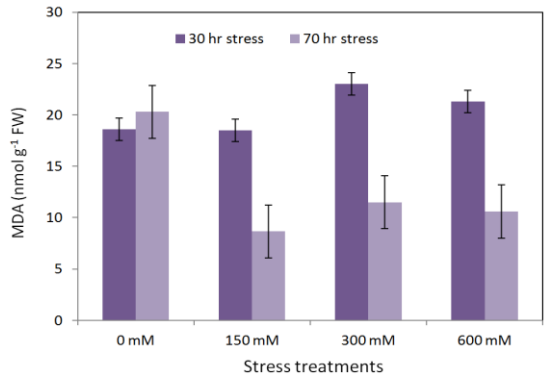

Fig 6. MDA content under different time exposure and salt stress. Bars represent standard error.

air pump connecting to flexible tubing and submersed air stones. After nine days of adaptation period, roots were washed carefully and thoroughly with distilled water. Subsequently, plants were transferred into new vessels containing fresh 400 ml medium supplemented with either 0, 150, 300 or 600 mM NaCl (four treatments). Each treatment consisted of six replicates, and each replicate was a vessel with two plants. Leaf samples were collected after 30, 50 and 70 h of stress exposure for further analysis. The data were presented as means with standard error bars.

Real-time of salt responsive genes

The gene expression was investigated using real-time PCR. Salt responsive genes as reference markers from the related *A. centalasiatica* were retrieved from the Genbank (NCBI, 2011) and qPCR primers were designed (Table 2), for normalization the rbcL gene from *A. halimus* was used (NCBI accession number AY270059). RNA was isolated using RNA isolation kit (Qiagen, USA). First strand cDNA was generated using reverse transcriptase (Promega, USA). Gene expression was indirectly assessed using the SYBR green dye in ABI real-time PCR machine (ABI, USA).

Photosynthesis measurement

The PSII activity in leaves was determined by measuring transient chlorophyll fluorescence using Handy PEA (Hansatech, UK) with an excitation light energy of 3000 µmol m^{-1} s^{-1}. An array of light emitting diodes with peak at 650 nm was used as light source. Plant leaves were dark adapted (in order to ensure that all the components of treatment PSII are in oxidized stress i.e. PS II reaction centers are fully open) for 30 min. Initial (F_o) and maximal (F_m) fluorescence yields were measured. The variable fluorescence yield (F_v) was defined as F_m-F_o. The variation in PSII activity was calculated as the ratio of variable to maximum fluorescence (F_v/F_m). Chlorophylls (Chl *a*, Chl *b*) and carotenoid content in leaves were determined according to the procedure of Arnon (1949) and calculated as µg/g fresh weight (FW).

Free proline and MDA

Free proline content was estimated using the protocol developed by Bates et al. (1973). Leaf samples of 100 mg were homogenized in 5 ml of 3% aqueous sulfosalycylic acid. The homogenate was centrifuged at 10,000 xg for 10 min at room temperature. Two ml of supernatant was

transferred into fresh test tube followed by addition of 2 ml of acid ninhydrin (1.25 g ninhydrin in 30 ml of glacial acetic acid and 20 ml of 6 M Orthophosphoric acid) and 2 ml of glacial acetic acid. The test tubes were incubated in water bath at 100°C for 1 h. The reaction was terminated by chilling on ice and 4 ml of toluene was added to each tube. The solutions were vigorously mixed on for 20 sec in order to facilitate quick diffusion/movement of chromophore from aqueous phase to non-aqueous phase. The toluene layer was separated from the aqueous layer and its absorbance was measured at 532 nm using toluene as blank. Concentration of proline in the sample was calculated from standard curve of L-proline and expressed in µg/g FW.

MDA was determined using Heath and Packer (1968) method. Fresh leaf tissue was homogenized in 5% TCA. One ml of tissue homogenate was mixed with 4 ml of 0.5% TBA (in 20% TCA). As blank, tissue homogenate was replaced by 1 ml of 5% TCA. The assay mixture was heated at 95°C water bath for 30 min, then cooled and centrifuged at 12000 xg for10 min. The absorbance of the supernatant was measured at 532 nm and was corrected for nonspecific absorbance at 600 nm. MDA concentration was calculated as nmol g^{-1} FW.

Conclusion

A. halimus plants not only could survive several degrees of imposed salt (osmotic) stresses but also illustrated sustainability of vitals plant physiological processes. The results convincingly demonstrated that the *A. halimus* plants possess in-built potential to regulate osmotic strength in their cells and hence have superior tolerant capacity under stress condition up to 600 mM NaCl. This result will help as an important basis for future investigations to functionally analyze the complex gene network behind this outstanding tolerance.

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