RESEARCH ARTICLE

Identification of salt-responsive genes from C4 halophyte *Suaeda nudiflora* through suppression subtractive hybridization and expression analysis under individual and combined treatment of salt and elevated carbon dioxide conditions

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Abstract Salinization of soil is a prime abiotic stress that limits agriculture productivity worldwide. To study the mechanisms that halophytes take up to survive under high salt condition is important in engineering salinity stress tolerance in sensitive species. *Suaeda nudiflora* is a halophyte plant that grows in the saline environment and extreme high tidal belt. The species have high capability to produce high protein biomass in salty soils due to C4 photosynthesis. The physiological and biochemical changes in *S. nudiflora* under salinity stress were studied by measuring chlorophyll content, electrolytic leakage, level of lipid peroxidation and total soluble sugars. Increased lipid peroxidation and electrolytic leakage was observed in salt stressed *S. nudiflora* compared to control plants. A suppression subtractive hybridization strategy was employed to identify differentially expressed genes under salt treatment in *S. nudiflora*. A total of 333 positive clones were identified and screened. Of these, 250 expressed sequence tags were identified. cDNA subtraction library resulted in 33 contigs and 138 singletons. The functional annotation and metabolic pathways identification were performed using the Blast2GO program. In addition, we analyzed the expression patterns of 18 genes associated with salt stress-responsive pathways by semi-quantitative PCR under salt and elevated carbon dioxide (CO₂)

conditions. Several of the analyzed genes showed an increase in expression levels under different time points of salt treatment and at different concentrations of salt. When the same genes were studied for its expression under elevated CO₂ concentrations, most of the known salt responsive genes showed higher expression under the combined treatment of elevated CO₂ concentrations (500 ppm) and NaCl treatment (200 mM) compare to ambient condition. This implies that salt responsive genes are enhanced at elevated CO₂ concentrations.

Keywords *Suaeda nudiflora* · C4 photosynthesis · Suppression subtractive hybridization · Semi quantitative PCR · Salt and elevated carbon dioxide conditions

Introduction

The earth's climate has never been static, but the planet is currently experiencing a faster change in climate than it had in the past due to increasing anthropogenic activities. Since the beginning of industrial revolution, carbon dioxide concentration in the atmosphere has increased from 280 ppm to approximately 380 ppm (Prior et al. 2011). Increasing greenhouse effect caused by greenhouse gases cause earth's surface temperature to rise and climate change predictions point towards higher solar radiation increasing temperatures, soil degradation and desertification, and decreasing and/or more irregular precipitation patterns in coming years (Change 2007; van Ittersum et al. 2003; Yeo 1998). Desertification often results due to the high salinity in soil which affects 7% of the global total land area and 20–50% of the global agricultural land (Geissler et al. 2009).

Salinization of soil is one of the major abiotic stress that affects the agricultural productivity (Zhu 2001). Plants

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show varying responses to salt stress depending on species, organs and developmental stages (Low et al. 1996; Ratajczak 2000). Halophytes are the salt-tolerant plants that are tailored to grow in saline environments where the growth of most glycophytes are inhibited substantially (Ramani et al. 2006). The halophytic species viz., *Suaeda* sp., *Mesembryanthemum crystallinum*, *Sesuvium portulacastrum*, and *Lobularia maritime*, etc., gather excess sodium ions in the stem and leaves and hence increase the succulence under higher salt concentrations (Qi et al. 2009). Halophytes are ideal candidate species for isolation of novel promoters and genes with potential functions in salt tolerance which could be engineered to glycophytic plants.

Suaeda sp. that belongs to chenopodiaceae family, are the most common plants in saline and alkaline soils (Sun et al. 2011). More than hundred species belonging to the genera *Suaeda* occur along side sea coasts and in other habitats with high salinity throughout the world (Powell 1998). *Suaeda nudiflora* is a halophyte plant that grows in saline environment and extreme high tidal belt. As the seeds contain approximately 30–35% oil, the species have high capability to be a future oil crop and is suitable to produce high protein biomass in salty soils due to C4 photosynthesis (Singh et al. 2004). *S. nudiflora* leaves are small, linear and succulent. Succulence is believed to be the morphological adaptation for salinity tolerance.

The higher concentration of Na^+ compare to K^+ and increase in seedlings at higher salinity makes *S. nudiflora*, a natrophilic species. The two distinct traits of *S. nudiflora* are high sodium ions entry and/or low sodium ion exit on plasma membrane of root system and high K^+/Na^+ discrimination to select potassium from soils with increased sodium concentration. *Suaeda nudiflora* plant utilizes 2 systems for potassium ion acquisition, one is low-affinity and the other is high-affinity uptake mechanisms. Low- and high-affinity transport systems that are essential for potassium ion acquisition are involved in Sodium ion transport as well. Thus, sodium ions could enter the cell through low affinity channels called non selective cation channels or high affinity K^+ carriers (Patel and Pandey 2009).

An increase in CO_2 availability evokes a broad range of physiological and morphological responses in plants and directly impact photosynthetic processes. The response to increased CO_2 concentration varies among different plant species based on the photosynthetic pathways, inherent growth rates and other characteristics. At elevated atmospheric CO_2 concentrations, reduced transpiration and improved water potential might lead to increased tolerance to water stress in plants (Pérez-López et al. 2009). In addition, reduced stomatal conductance might lead to more efficient use of water (Roden and Ball 1996; Sebastiani et al. 2002). Thus elevated CO_2 concentrations have been

shown to have implications in water stress tolerance. Since water and salinity stress induce many common mechanisms of tolerance in plants, plant responses under the combined treatment of elevated CO_2 and salinity assumes importance.

The gene expression profile provides a worthy starting point for understanding the molecular mechanism of salinity tolerance in plants (Fu et al. 2005). SSH is a potent tool for identifying stress responsive genes. This method reduces false positive rate, gives higher degree of target sequence enrichment and higher consistency (Diatchenko et al. 1996).

Large-scale expressed sequence tags (EST) sequencing and investigation have been carried out in a number of halophytic species, such as *S. salsa* (Zhang et al. 2001), *Thellungiella halophila* (Wang et al. 2004), *M. crystallinum* (Kore-eda et al. 2004) and *Avicennia marina* (Mehta et al. 2005). Zeng et al. (2006) studied the salt responsive genes by using SSH library in *Sesuvium portulacastrum* subjected to sea water. However, to our knowledge, no reports are available on large scale identification and analysis of the salt responsive genes from *S. nudiflora*.

In the present study, we employed SSH to identify genes that are expressed differentially under salt stress conditions in *S. nudiflora*. We further analyzed the expression patterns of 18 salt responsive genes under; (a) varying salt concentrations, (b) varying time points of salt treatment and (c) ambient and elevated CO_2 with/without salt treatment.

Materials and methods

Plant material and salt treatment

The seeds *S. nudiflora* were collected from Kakinada, Andhra Pradesh, India. The seedlings were germinated in the pots filled with vermiculite, placed in plastic trays and transferred to hydroponics containing modified Hoagland's medium (Wang et al. 2007). The plants were maintained at 25 °C at the photoperiod of 16 h light and 8 h dark. The plants were acclimatized for 7 days and then treated with 200 mM NaCl for 14 days. The plants grown with NaCl-free medium served as control.

Determination of malondialdehyde, soluble sugars, chlorophyll content and electrolyte leakage

Malondialdehyde (MDA), soluble sugars, chlorophyll content and extent of electrolyte leakage in the control as well as NaCl treated plants were measured.

MDA content was measured according to Hodges et al. (1999). The Shoot tissues were homogenised in 5% of TCA

solution. A 3 ml of 0.5% Thiobarbituric acid (TBA) and 5% Trichloroacetic acid (TCA) were added to 2 ml of the extract. The mixtures were heated to 90 °C for 30 min and cooled in ice to room temperature. The samples were centrifuged at 5000 rpm for 15 min. The absorbance of the supernatants was measured at 450 nm, 532 nm and 600 nm.

Total soluble sugars were determined following the method of Yadav et al. (2012). 500 mg of tissue was crushed in 5 ml of ethanol and centrifuged at 3500×g for 10 min. A 3 ml of freshly prepared anthrone reagent was added (150 mg of anthrone in 100 ml of 72% H₂SO₄) to 0.1 ml of supernatant. The mixture was placed in a boiling water bath for 10 min. The reaction was stopped by transferring tube to an ice bath. Then absorbance was measured at 625 nm.

The chlorophyll content was measured spectrophotometrically using Arnon's method (Arnon 1949). The shoots were homogenized thoroughly in 80% acetone and incubated for 1–2 h with shaking at 120 rpm in the dark. The homogenate was centrifuged at 3000 rpm for 5 min in the dark and the OD of the supernatant was measured at 645 and 665 nm.

The electrolyte leakage was calculated as a measure of electrical conductivity as per (Lutts et al. 1996). Leaves of similar size were washed thoroughly with deionised water. The discs were kept in closed vials containing 10 ml of deionised water and incubated at 25 °C on a rotary shaker for 24 h. The electrical conductivity (EC) was determined using conductivity meter (Hanna DiST®5 Waterproof EC/TDS/°C/°F Tester). Samples were autoclaved (120 °C for 20 min), cooled up to 25 °C and the electrical conductivity was measured.

Statistical analysis

Each experiment was carried out in triplicates. All the data were expressed as mean, Standard deviation (SD) and subjected to analysis of variance (ANOVA) to determine the significance of difference between the means of control and treated plants for each set of experiments. A Tukey HSD multiple comparison of mean test was used, significant differences were considered at $P < 0.05$.

SSH library construction and EST sequencing

Total RNA was extracted from *S. nudiflora* shoot using TRI reagent (Sigma). The total RNA quality was checked on 1.2% agarose gel electrophoresis. Two micrograms of mRNA were used as starting material for cDNA synthesis. cDNAs were prepared using the SMART PCR cDNA synthesis kit and subtracted with the PCR-Select DNA Subtraction Procedure (Clontech Laboratories, Inc.

A Takara Bio Company). The subtraction library was done using cDNA synthesized from 14 days NaCl-treated shoot samples categorized as 'tester' and control shoot samples as 'driver'. cDNA library was first cloned into InsTAclone T-vector (MBI Fermentas) and later used to transform chemically competent *E. coli* (OmniMAX 2 T1 Phage-Resistant, Invitrogen).

Plasmid DNA extraction and sequencing of differentially expressed ESTs

The resultant library was plated on ampicillin selection medium and grown overnight. Individual colonies were grown on Luria–Bertani (LB) liquid medium and plasmid DNA was isolated following the method of Lyozin et al. (2014). The isolated plasmid DNAs were then proceeded for EST sequencing (BigDyeTM Terminator v3.1 Cycle Sequencing Kit in 3130xl Genetic Analyzer, Applied Biosystems). About three hundred clones were sequenced using M13 forward primer.

Sequencing and analysis

To eliminate the DNA of vector origin and the adaptors used for the differentially expressed fragments' amplification, the obtained sequences were analyzed using VecScreen (<http://www.ncbi.nlm.nih.gov/VecScreen/>) and ESTs were submitted to NCBI's EST database. Sequences corresponding to the same genes were assembled using the CAP3 Sequence Assembly Software (Huang and Madan 1999). The contigs and singletons generated were analyzed by the BLAST program using the algorithms BlastN (Zhang et al. 2000) and BlastX (Altschul et al. 1997). EST sequence data (250) was submitted to NCBI's dbEST database. The sequences have been published with the accession number starting from JZ896709 to JZ896958. To assign biological functions to the transcripts encoded by the differentially expressed genes, a functional annotation of contigs and singletons was performed using the BLAST2GO (B2GO) software version 3.0 (Tufan et al. 2015). A Blast2GO InterPro Scan (Zdobnov and Apweiler 2001) was performed to search for additional GO terms corresponding to functional domains. The ESTs with GO annotations received enzyme codes (EC), and the Blast2GO KEGG module retrieved the maps of metabolic pathways in which the tracked EC numbers participated.

Free air CO₂ enrichment (FACE) treatment

The CO₂ enrichment experiments were conducted using the mini-Free Air CO₂ Enrichment (FACE) facilities at M. S. Swaminathan Research Foundation (MSSRF). *S. nudiflora* seedlings were germinated in vermiculite filled pots

kept in plastic trays, filled with modified Hoagland's medium which was changed once a week, under green house conditions with average temperature of 30/22 °C (day/night). After a month, the germinated plants were divided into two sets. One set was transferred to the ambient CO₂ ring of 380 ppm (Ambient Control, AC) and the other was transferred to elevated CO₂ ring of 500 ppm (Elevated CO₂ treatment, EC) in the FACE facility. After 1 month of acclimatization, a set of plants from each ring were subjected to 200 mM NaCl treatment (Ambient CO₂ + salt – ACS, Elevated CO₂ + salt – ECS). The treatments were given for duration of 90 days and the shoot samples were collected for further gene expression analysis.

Semi-quantitative reverse transcription (RT) PCR

Total RNA from shoot and root tissues was isolated as described before and expression of 18 salt responsive genes from the SSH library was analyzed further under; (a) different salt concentrations (0, 100, 200, 300, 400 and 500 mM), (b) different time points of salt treatment (0, 48 h, 7 days and 14 days of 200 mM NaCl treatment) and (c) under Ambient and Elevated CO₂ with and without salt stress (AC, EC, ACS, ECS). Primers for semi-quantitative RT–PCR were designed using NCBI's Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Two microgram of RNA samples were treated with RNase-free DNase I (Promega) to remove DNA contamination. The cDNAs from each sample were synthesized using a M-MLV Reverse Transcriptase enzyme (Invitrogen). Actin was used as an internal standard. The cDNA concentration was standardized by equalizing the expression of the actin gene in both control and treated samples. The PCR products were visualised on 2% Tris–acetate-EDTA (TAE) agarose gels.

Results and discussion

The physiological and biochemical responses of *S. nudiflora* under salt stress were studied by measuring chlorophyll content, electrolytic leakage, lipid peroxidation (MDA content) and total soluble sugars (Fig. 1). The level of lipid peroxidation and electrolytic leakage are well-known stress indicators to assess the severity of the salt-induced oxidative stress (Katsuhara et al. 2005). In this study, the MDA content was found to be slightly increased in salt-treated plants compared to control plants but the difference was not significant. While there was significant increase (10%) in electrolyte leakage was observed in salt treated plants.

These results are in accordance with those of Lokhande et al. (2011) were increased level of lipid peroxidation and

electrolyte leakage in *S. portulacastrum* clones under high salt stress reported. Similar results have also been observed in other halophytes such as the *Beta maritima* (Bor et al. 2003).

Plants reduce osmotic potential by increasing mineral ions content and other solutes synthesis to enhance uptake of water under salinity. Total soluble sugars are an important osmotic adjustment solutes (Jia et al. 2011) which accumulate during salt stress. The total soluble sugar content in *S. nudiflora* was reduced in salt-treated plants when compared to control plants (Fig. 1d).

Salinity causes reduction in total chlorophyll content which in turn leads to reduced photosynthesis activity in plants. The total chlorophyll content in *S. nudiflora* under salinity slightly reduced compared to control plants with no significant difference. The reduced inhibitory effect of salt stress on the chlorophyll content of *S. nudiflora* could be attributed to increased activity of antioxidative enzymes and reduced chlorophyllase activity. Similar results have been observed in many salt tolerant species. For instance, the chlorophyll content in remained unchanged when exposed to salt stress.

EST sequencing and analysis

Of 333 positive clones, 250 ESTs met the quality requirements subsequent to sequence cleaning (Supplementary file 1). The CAP3 Assembly ends up in 33 contigs and 138 singlets. The Blast search showed 79.6% of the reads with similarities to characterized proteins, while 7.6% matched to hypothetical proteins, and 11.2% had no similarity to known sequences. In the functional annotation process, 62% of the reads set were assigned GO terms in Blast2GO, whereas no results were obtained for 38% of the sequences. ESTs were categorized based on biological processes (Fig. 2a) in which they were involved into either molecular functions (Fig. 2c) or as cellular components (Fig. 2b). Based on the molecular function, more transcripts were consigned to binding (92) and catalytic activity (70) tagged on by transporter activity (7). Based on the biological process, the keyclusters were metabolic and cellular process (119 and 118 GO terms respectively), followed by single organism process (75 GO terms) and response to stimulus (55 GO terms).

Transcripts with enzymatic functions involved in various metabolic pathways were identified based on the EC codes assigned and the KEGG maps salvaged by B2GO. The exposure of *S. nudiflora* to salinity induced enzymes involved in the metabolism of histidine, starch, sucrose, nitrogen, pyruvate, cysteine and methionine, methane, glycine, serine, and threonine. Moreover, the enzymes involved in catalyzing steps in glycolysis, gluconeogenesis, citrate cycle, oxidative phosphorylation, carbon fixation

Fig. 1 Effect of 200 mM NaCl on *S. nudiflora* at 14 days interval. Graphs showing measurement of MDA content, chlorophyll content, electrolyte leakage, and amount of total soluble sugars

Fig. 2 Functional classification of salt induced clones in *S. nudiflora* shoots identified from subtractive cDNA library. Classification of ESTs based on **a** biological process, **b** cellular components, and **c** molecular function, using Blast2Go software

pathways and those involved in microbial metabolism in diverse environments, were also detected.

Gene expression analysis

Eighteen differentially regulated ESTs (Table 1) were randomly selected from SSH library and their expression

patterns in response to salt stress were examined using the semi-quantitative RT PCR. To gain further insights into the role of these genes during salinity, the expression patterns were also investigated under varying salt concentrations, varying time points and under ambient and elevated CO₂ with and without salinity. The expression levels of all the selected genes in both shoots and roots varied in

Table 1 List of genes used in semi quantitative RT-PCR and the PCR conditions

Gene	Genbank accession no.	Primer sequences (F: forward and R: reverse)	Annealing temperature in °C	Expected product (number of PCR size (bp))
Ascorbate peroxidase	JZ896712	F 5'-TCAGCGAAACCAAGTTCGGA-3' R 5'-CTTTCTGGAGGCCACACCTT-3'	60	272
Photosystem i reaction center subunit chloroplastic	JZ896722	F 5'-AATGGGCTGGATGTCACTGG-3' R 5'-GCCAACTAAAGAGTGGGGCT-3'	52	221
Water channel protein MipH	JZ896733	F 5'-AAGGCCCTTGGATCTTTCAGG-3' R 5'-AAACCTGGGACGCCTTTCCT-3'	60	149
Chitinase-like protein 1	JZ896780	F 5'-TTCCCACAAACAAGGTCACCA-3' R 5'-ACCGATGAAGAAGGGACAACC-3'	60	141
Betaine aldehyde dehydrogenase	JZ896782	F 5'-AGCTCATCGTGCTCGTTACT-3' R 5'-TTATTGTCCAGCGCTTCTGC-3'	60	180
Heat shock cognate 70 kDa protein 2-like	JZ896783	F 5'-AAGTGCATGGAGCCAGTTGA-3' R 5'-CGAGCAAGTTGTTGTCACGG-3'	60	438
Serine hydroxymethyltransferase 4	JZ896790	F 5'-CATTGCGGGAGCACCAATTC-3' R 5'-CAGGCCATGTCTCCTGGTTT-3'	60	273
Mannose glucose-specific lectin-like	JZ896793	F 5'-CATACTAATTTGCGCCCCCG-3' R 5'-CCCTGCAAGCCTTTTCAACA-3'	60	180
magnesium-chelatase subunit chloroplastic	JZ896795	F 5'-ATCGGAGTGAGACCGATTGC-3' R 5'-CTCATCAAGCTCCGCTACCA-3'	60	186
NAD-dependent malic enzyme 59 kDa mitochondrial	JZ896833	F 5'-GTCCTTAACATGGCAACGCA-3' R 5'-TGCAGCAGCTGGGTCTAAAT-3'	60	135
Sedoheptulose-chloroplastic	JZ896849	F 5'-ACGGCCATATCATTGAGCCA-3' R 5'-ACTGTCGCCAGGTTACATT-3'	52	243
Phosphoinositide phosphatase sac6	JZ896854	F 5'-AGTCCTGACAACGCCACTTT-3' R 5'-TTCATTTTGAGCGCCTGTCG-3'	56	122

Table 1 continued

Gene	Genbank accession no.	Primer sequences (F: forward and R: reverse)	Annealing temperature in °C	Expected product (number of PCR size (bp))
Target of myb protein 1	JZ896867	F 5'- TGGAGTGCTGTCCTAGCAATG-3' R 5'- GAGGCTGATGGAAAGACCCAA-3'	60	177
Jasmonate-induced protein homolog	JZ896896	F 5'- TCTCACCATCGACGCAGTAAC-3' R 5'- CTTGGGACAAGCCTGAAAACC-3'	60	150
Protein of unknown function	JZ896912	F 5'- ATGTGAAAGCGCGTTGAAA-3' R 5'- AACGGTCCTGATCATCTCGTC-3'	60	308
Oxygen-evolving enhancer protein chloroplastic	JZ896918	F 5'- AGGTAGCAAGAGCAAAGCCA-3' R 5'- ACCCCCAAAGTAGCATCAAGG-3'	60	178
26 s proteasome non-atpase regulatory subunit 4 homolog	JZ896922	F 5'- ATTGGGCCACCTGAATACCAG-3' R 5'- GATGGCTGGAAAAGGTGTTTCG-3'	60	129
Cytochrome c oxidase assembly protein mitochondrial-like	JZ896941	F 5'- GGCGACCACAGCATCATAAC-3' R 5'- AACGGTTCCGAACACGGTAA-3'	60	216
Actin	BE231408	F 5'- AAACCACAAGCCCCTAAACC-3' R 5'-TTGCATCACTCAGCACCTTC-3'	60	250

response to different salt concentration and at different time points. Under 200 mM salt stress, compared to control, seven genes (ascorbate peroxidase, heat shock cognate 70 kDa protein 2-like, mannose glucose-specific lectin-like, phosphoinositide phosphatase sac6, oxygen-evolving enhancer protein chloroplastic, 26 s proteasome non-atpase regulatory subunit 4 homolog and cytochrome c oxidase assembly protein) showed highest level of expression in the shoots at 14 days of salt stress. While nine genes (photosystem I reaction center subunit, water channel protein, chitinase-like protein 1, betaine aldehyde dehydrogenase

(BADH), serine hydroxymethyltransferase 4, magnesium-chelatase chloroplastic subunit, target of myb protein 1, NAD-dependent malic enzyme 59 kDa mitochondrial and a protein of unknown function) showed highest level of expression at 7 days of salt stress. The other two genes viz., sedoheptulose-chloroplastic and jasmonate-induced protein homolog, showed higher expression at 48 h of salt stress. In addition to this, we analyzed the expression patterns of these genes under varying salt concentrations, varying time points of salt treatment and under ambient and

elevated CO₂ with and without salinity to further characterize them (Fig. 3).

Under 200 mM salt treatment, all the selected genes showed changes in expression levels at different time points in shoot as well as root tissue. Seven genes, (ascorbate peroxidase, heat shock cognate 70 kDa protein 2-like, mannose glucose-specific lectin-like, phosphoinositide phosphatase sac6, oxygen-evolving enhancer protein chloroplast, 26 s proteasome non-atpase regulatory subunit 4 homolog and cytochrome c oxidase assembly protein) showed highest level of expression at 14 days of salt treatment in shoot. Nine genes (photosystem I reaction center subunit, water channel protein, chitinase-like protein 1, betaine aldehyde dehydrogenase (BADH), serine hydroxymethyltransferase 4, magnesium-chelatase chloroplast subunit, target of myb protein 1, NAD-dependent malic enzyme 59 kDa mitochondrial and a protein of unknown function) showed highest level of expression at 7 days of salt stress in the shoot compared to control. The other two genes viz., sedoheptulose-chloroplast and jasmonate-induced protein homolog, showed higher expression compared to control at 48 h time point in the shoot.

On analyzing the expression pattern of these genes in root at different time points after salt treatment, the genes such as photosystem I reaction center subunit chloroplast, sedoheptulose-chloroplast, jasmonate-induced protein homolog, target of myb protein 1 and phosphoinositide

phosphatase sac6 showed highest level of expression in 14 days of treatment compared to control and all the other genes showed highest expression levels at 7 days of stress. When the same genes were investigated for their expression patterns at different concentrations of NaCl in *S. nudiflora* shoot tissue, the expression of photosystem I reaction center subunit chloroplast, water channel protein, mannose glucose-specific lectin-like and 26 s proteasome non-atpase regulatory subunit 4 homologs increased with increasing salt concentrations. Several genes (ascorbate peroxidase, chitinase-like protein 1, serine hydroxymethyltransferase 4, jasmonate-induced protein homolog, magnesium-chelatase subunit chloroplast, oxygen-evolving enhancer protein chloroplast and protein of unknown function) showed highest levels of expression in plants treated with 500 mM NaCl. While genes such as BADH, heat shock cognate 70 kDa protein 2-like, sedoheptulose-chloroplast, cytochrome c oxidase assembly protein mitochondrial-like, NAD-dependent malic enzyme 59 kDa mitochondrial and phosphoinositide phosphatase sac6 were upregulated at 200 mM NaCl.

Several of the genes analyzed in our study have been previously shown to be upregulated under salt stress in various plant species. Salinity leads to increased production of Reactive Oxygen Species (ROS) in plants, which can be deleterious for lipids, protein and nucleic acids and negatively affect plant survival. Ascorbate peroxidase (APX) is

Fig. 3 Semiquantitative PCR of selected genes. i) shoot treated with 200 mM NaCl at different time points, ii) root treated with 200 mM NaCl at different time points, iii) treated with different concentration

of NaCl at 14 day time point and iv) shoot treated with/without 200 mM NaCl and 500 ppm carbon dioxide at 90 day time point

an antioxidant enzyme involved in ROS removal. APX has been reported to increase under salt treatment of various concentrations in halophyte plant *Aeluropus littoralis* (Modarresi et al. 2014). Upregulation of an APX gene under salinity and oxidative stress has been observed in mangrove tree *Avicennia marina* (Kavitha et al. 2008). In our study, the expression of APX gene was increased in the shoots under salt stress, while its expression was unaltered in response to elevated CO₂ with or without salt stress. Glycine betaine synthetic pathway is activated in many plant species in response to various abiotic stresses and lead to the synthesis of glycine betaine, an osmolyte. Betaine aldehyde dehydrogenase (BADH), is a primary enzyme in this pathway and has been reported to be upregulated in response to water deficit, osmotic stress, and salinity in many species (Wang et al. 2015). Similar to our results, BADH was found to be upregulated under salinity stress in the leaves of halophyte *Halogeton glomeratus* (Wang et al. 2015). In our studies, a gene encoding a heat shock protein was found to be upregulated under salinity in shoot and root tissues. Similar results were obtained for monocot halophyte *Spartina alterniflora* where leaf and root transcriptome profiling revealed several heat shock proteins and heat shock factors as upregulated under salt stress (Bedre et al. 2016).

We further analyzed the expression levels of these genes under elevated CO₂ concentrations in the presence and absence of NaCl. Our results showed that the combined treatment of both 200 mM NaCl and elevated CO₂ (500 ppm) resulted in increased expression levels for all the selected ESTs except target of myb protein 1, which showed higher expression levels in plants at ambient CO₂, treated with salt. Besides, enrichment of CO₂ may possibly augment the tolerance of plants to a number of environmental stresses viz light, temperature, salt stress etc., (Azam and Farooq 2003).

The results observed here is similar that in *Aster tripolium*, in which the expression levels of ascorbate peroxidase and heat shock proteins in leaf tissue were found to be upregulated under the combined treatment of salinity and elevated CO₂ compared to salinity stress treatment under ambient CO₂ conditions (Geissler et al. 2010). In *Solanum lycopersicum*, the expression of Respiratory Burst Oxidase was found to be increased under salt stress with elevated CO₂ condition (Yi et al. (2015). Increased enzyme activities and enhanced removal of reactive oxygen species has been reported in Mediterranean oak species under combined salt water stress and elevated CO₂ treatments (Marabottini et al. 2001). Enhanced amount of heat shock proteins has been observed in *Aster tripolium* leaves under combined salt and elevated CO₂ treatments (Geissler et al. 2010).

Conclusions

Using SSH approach, two hundred and fifty salt-responsive ESTs were identified and validation of 18 randomly selected ESTs using semi-quantitative PCR revealed differential expression patterns of these genes during salt stress with ambient and elevated CO₂. Importantly, expression of 17 genes except target of myb protein 1 genes were found to be increased under the combination of salt and elevated CO₂, suggesting that elevated CO₂ concentrations may improve salt tolerance in *S. nudiflora* and point towards a potential use for this plant as a source of biofuel under the changing climates. Further studies in this species may be helpful in understanding the salt tolerance mechanisms of plants and in developing salt tolerant crop plants through breeding or transgenic approaches.

References

- Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25:3389–3402
- Arnon DI (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiol* 24:1
- Azam F, Farooq S (2003) Elevated CO₂ and stress tolerance in crop plants with particular. *Pak J Biol Sci* 6:1096–1107
- Bedre R, Mangu VR, Srivastava S, Sanchez LE, Baisakh N (2016) Transcriptome analysis of smooth cordgrass (*Spartina alterniflora* Loisel), a monocot halophyte, reveals candidate genes involved in its adaptation to salinity. *BMC Genom* 17:657
- Bor M, Özdemir F, Türkan I (2003) The effect of salt stress on lipid peroxidation and antioxidants in leaves of sugar beet *Beta vulgaris* L. and wild beet *Beta maritima* L. *Plant Sci* 164:77–84
- Change IC (2007) Impacts, adaptation and vulnerability. In: Working group II contribution to the intergovernmental panel on climate change fourth assessment report. Edited by Policymakers Sf Geneva, Switzerland: Intergovernmental Panel on Climate Change
- Diatchenko L et al (1996) Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc Natl Acad Sci* 93:6025–6030
- Fu X et al (2005) Construction of a SSH library of *Aegiceras corniculatum* under salt stress and expression analysis of four transcripts. *Plant Sci* 169:147–154
- Geissler N, Hussin S, Koyro H-W (2009) Elevated atmospheric CO₂ concentration ameliorates effects of NaCl salinity on photosynthesis and leaf structure of *Aster tripolium* L. *J Exp Bot* 60:137–151
- Geissler N, Hussin S, Koyro H-W (2010) Elevated atmospheric CO₂ concentration enhances salinity tolerance in *Aster tripolium* L. *Planta* 231:583–594
- Hodges DM, DeLong JM, Forney CF, Prange RK (1999) Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. *Planta* 207:604–611
- Huang X, Madan A (1999) CAP3: a DNA sequence assembly program. *Genome Res* 9:868–877

- Jia J, Cui X, Wu J, Wang J, Wang G (2011) Physiological and biochemical responses of halophyte *Kalidium foliatum* to salt stress. *Afr J Biotech* 10:11468–11476
- Katsuhara M, Otsuka T, Ezaki B (2005) Salt stress-induced lipid peroxidation is reduced by glutathione S-transferase, but this reduction of lipid peroxides is not enough for a recovery of root growth in *Arabidopsis*. *Plant Sci* 169(2):369–373
- Kavitha K, Venkataraman G, Parida A (2008) An oxidative and salinity stress induced peroxisomal ascorbate peroxidase from *Avicennia marina*: molecular and functional characterization. *Plant Physiol Biochem* 46:794–804
- Kore-eda S, Cushman MA, Akselrod I, Bufford D, Fredrickson M, Clark E, Cushman JC (2004) Transcript profiling of salinity stress responses by large-scale expressed sequence tag analysis in *Mesembryanthemum crystallinum*. *Gene* 341:83–92
- Lokhande VH, Nikam TD, Patade VY, Ahire ML, Suprasanna P (2011) Effects of optimal and supra-optimal salinity stress on antioxidative defence, osmolytes and in vitro growth responses in *Sesuvium portulacastrum* L. *Plant Cell Tissue Organ Cult* (PCTOC) 104:41–49
- Low R et al (1996) Early salt stress effects on the differential expression of vacuolar H⁺-ATPase genes in roots and leaves of *Mesembryanthemum crystallinum*. *Plant Physiol* 110:259–265
- Lutts S, Kinet J, Bouharmont J (1996) NaCl-induced senescence in leaves of rice (*Oryza sativa* L.) cultivars differing in salinity resistance. *Ann Bot* 78:389–398
- Lyozin GT et al (2014) Isolation of rare recombinants without using selectable markers for one-step seamless BAC mutagenesis. *Nat Methods* 11:966–970
- Marabottini R, Schraml C, Paolacci A, Raschi A, Rennenberg H, Badiani M (2001) Foliar antioxidant status of adult Mediterranean oak species (*Quercus ilex* L. and *Q. pubescens* Willd.) exposed to permanent CO₂-enrichment and to seasonal water stress. *Environ Pollut* 115:413–423
- Mehta PA, Sivaprakash K, Parani M, Venkataraman G, Parida AK (2005) Generation and analysis of expressed sequence tags from the salt-tolerant mangrove species *Avicennia marina* (Forsk.) Vierh. *Theor Appl Genet* 110:416–424
- Modarresi M, Moradian F, Nematzadeh G (2014) Antioxidant responses of halophyte plant *Aeluropus litoralis* under long-term salinity stress. *Biologia* 69:478–483
- Patel NT, Pandey AN (2009) Effect of soil salinity on growth, water status and nutrient accumulation in seedlings of *Suaeda nudiflora* (Chenopodiaceae). *Anales de Biologia* 31:61–70
- Pérez-López U, Robredo A, Lacuesta M, Mena-Petite A, Muñoz-Rueda A (2009) The impact of salt stress on the water status of barley plants is partially mitigated by elevated CO₂. *Environ Exp Bot* 66:463–470
- Powell AM (1998) Trees & shrubs of the trans-pecos and adjacent areas. University of Texas Press, Austin
- Prior SA, Runion GB, Marble SC, Rogers HH, Gilliam CH, Torbert HA (2011) A review of elevated atmospheric CO₂ effects on plant growth and water relations: implications for horticulture. *HortScience* 46:158–162
- Qi C-H, Chen M, Song J, Wang B-S (2009) Increase in aquaporin activity is involved in leaf succulence of the euhalophyte *Suaeda salsa*, under salinity. *Plant Sci* 176:200–205
- Ramani B, Reeck T, Debez A, Stelzer R, Huchermeyer B, Schmidt A, Papenbrock J (2006) *Aster tripolium* L. and *Sesuvium portulacastrum* L.: two halophytes, two strategies to survive in saline habitats. *Plant Physiol Biochem* 44:395–408
- Ratajczak R (2000) Structure, function and regulation of the plant vacuolar H⁺-translocating ATPase. *Biochimica et Biophysica Acta (BBA)-Biomembranes* 1465:17–36
- Roden JS, Ball MC (1996) The effect of elevated [CO₂] on growth and photosynthesis of two eucalyptus species exposed to high temperatures and water deficits. *Plant Physiol* 111:909–919
- Sebastiani L, Minnoccini A, Tognetti R (2002) Genotypic differences in the response to elevated CO₂ concentration of one-year-old olive cuttings (*Olea europaea* L. cv. Frantoio and Moraiolo). *Plant Biosyst Int J Deal Aspects Plant Biol* 136:199–207
- Singh A, Chikara J, Pandya J (2004) Plant regeneration from callus cultures in *Suaeda nudiflora* (Wild.) Moq. *Indian J Biotechnol* 3:454–456
- Sun Y, Wang Q, Lu X, Okane I, Kakishika M (2011) Endophytic fungi associated with two *Suaeda* species growing in alkaline soil in China. *Mycosphere* 2:239–248
- Tufan F, Uçarlı C, Gürel F (2015) Analysis of expressed sequence tags from cDNA library of *Fusarium culmorum* infected barley (*Hordeum vulgare* L.) roots. *Bioinformation* 11:34
- van Ittersum MK, Leffelaar PA, Van Keulen H, Kropff MJ, Bastiaans L, Goudriaan J (2003) On approaches and applications of the Wageningen crop models. *Eur J Agron* 18:201–234
- Wang Z-I et al (2004) Expressed sequence tags from *Thellungiella halophila*, a new model to study plant salt-tolerance. *Plant Sci* 166:609–616
- Wang S-M, Zhang J-L, Flowers TJ (2007) Low-affinity Na⁺ uptake in the halophyte *Suaeda maritima*. *Plant Physiol* 145:559–571
- Wang J et al (2015) Transcriptomic profiling of the salt-stress response in the halophyte *Halogeton glomeratus*. *BMC Genom* 16:169
- Yadav NS, Shukla PS, Jha A, Agarwal PK, Jha B (2012) The SbSOS1 gene from the extreme halophyte *Salicornia brachiata* enhances Na⁺ loading in xylem and confers salt tolerance in transgenic tobacco. *BMC Plant Biol* 12:1
- Yeo A (1998) Predicting the interaction between the effects of salinity and climate change on crop plants. *Sci Hortic* 78:159–174
- Yi C et al (2015) High atmospheric carbon dioxide-dependent alleviation of salt stress is linked to RESPIRATORY BURST OXIDASE 1 (RBOH1)-dependent H₂O₂ production in tomato (*Solanum lycopersicum*). *J Exp Bot* 66(22):7391–7404
- Zdobnov EM, Apweiler R (2001) InterProScan: an integration platform for the signature-recognition methods in InterPro. *Bioinformatics* 17:847–848
- Zeng HC, Deng LH, Zhang CF (2006) Cloning of salt tolerance-related cDNAs from the mangrove plant *Sesuvium portulacastrum* L. *J Integr Plant Biol* 48:952–957
- Zhang Z, Schwartz S, Wagner L, Miller W (2000) A greedy algorithm for aligning DNA sequences. *J Comput Biol* 7:203–214
- Zhang L et al (2001) Expressed sequence tags from a NaCl-treated *Suaeda salsa* cDNA library. *Gene* 267:193–200
- Zhu J-K (2001) Plant salt tolerance. *Trends Plant Sci* 6:66–71

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