Differentially Expressed Long-Term Salinity Responsive Sequences in Halophyte *Suaeda maritima* (L.) Dumort

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ABSTRACT

Salinity is a major abiotic stress that affects plant growth and productivity. To cope with salt stress, plants express large number of salt responsive genes and proteins that are involved in a wide range of cellular functions. In the present study, halophytic plant *Suaeda maritima* (L.) Dumort. were hydroponically exposed to NaCl for understanding the molecular mechanisms behind salinity tolerance using PCR-based Suppressive Subtractive Hybridization (SSH). Two cDNA subtraction libraries were constructed between *Suaeda maritima* X *Sesuvium portulacastrum* and *Suaeda maritima* X *Salicornia brachiata* to identify differentially expressed genes from leaves exposed to 200mM NaCl treatment for 14 days. A total of 224 clones from both libraries were assembled into 109 unique-ESTs grouped into different functional categories. Based on GO functional annotation, the expressed sequences like Oxygen-evolving enhancer protein1, AARF domain-containing kinase protein, V-type proton ATPase subunit2, RMD5 homologA, and ABC transporter G35 that are involved in photosynthesis, cellular transport, cell rescue and defense, polyubiquitination and secondary metabolism played a significant role implying a complex response to salt in *S. maritima*. This is the first report that SSH could facilitate screening across species and family specific identification of salt responsive genes provides insight into biological mechanisms underlying salinity response.

Keywords: Abiotic stress, expressed sequence tags, salinity, salt tolerance.

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I. INTRODUCTION

Salinity is one of the most important abiotic stresses that adversely affect plant germination, growth and ultimately yield. Today, worldwide 20% of total cultivated lands and 33% of irrigated agricultural lands are afflicted by high salinity (Golldack *et al.*, 2014). The effects of salt on crops, there is a need to increase food production at a rate of 1.8% per year to feed the increasing world population, which is projected to be 9.5 billion by the year 2050. In this context, developing crop plants that can sustain productivity under salinity stress conditions becomes important (Hossain, 2019; Shahid *et al.*, 2020).

Halophytes, also called as salt loving plant of about 500 species, grow normally in heavily saline soils constituting approximately 0.14% of known plant species. It exhibits complex salt tolerance mechanism because of their unique specialized structures includes salt glands, bladder hairs, succulent tissues, and thick layers of suberin (Flowers & Colmer, 2015). The salt-tolerant mechanisms of halophytes can be divided into two main categories: (i) preventing or reducing the amount of salt being uptake by plant tissue and (ii) reducing the concentration of salt present in the cytoplasm. Thus, halophytes are ideal plants for studying the

mechanism of salt tolerance as they survive, grow normally for longer time spans and complete their life cycle in saline environments (Yuan *et al.*, 2019; Zhang *et al.*, 2020). *Suaeda maritima* (L.) Dumort is an herbaceous, facultative, annual halophyte with succulent leaves that grows up to 30 cm in height that is native to saline soils of arid and semiarid regions. This species is a self-pollinated bisexual dicot of the family Chenopodiaceae (now included in the family Amaranthaceae) with $2n = 36$ chromosomes that produces reddish brown seeds (Sahu & Shaw, 2009; Alhdad & Flowers, 2020; Certain *et al.*, 2021). It exhibits high resistance to alkali stresses and grows well with salt content even without salt glands or bladders on its leaves to modulate their tissue ion concentration. It not only performs exclusion of salt but also quite efficiently compartmentalizes the excess salt in to the cell vacuoles similar to *Suaeda salsa* (Li *et al.*, 2020). Therefore, in the present study, *S. maritima* has been selected as a model for the study of physiological and molecular characterization of salt tolerance in plants.

Suppression subtractive hybridization (SSH) is a simple and versatile technique for selectively amplifying cDNA fragments of differentially expressed genes or an effective tool for functional genomics study. It enables researchers to compare two populations of mRNAs and obtain clones of

genes that were expressed in one population but not in the other. This method can develop libraries that include both scarce and abundant genes, potentially yielding a more diverse gene pool than other techniques to identify specific genes (i.e. stress responsive genes). By using two specific hybridizations and two PCR specific amplifications, SSH has the advantage of decreasing false positive rate, improve the screening efficiency, higher degree of target sequence enrichment and higher consistency (Diatchenko *et al.*, 1996). It has been successfully applied in the identification of salt responsive genes in various plants such as *Thellungiella halophila* (Wang *et al.*, 2004), *Aeluropus littoralis* (Zouari *et al.*, 2007), *Bruguiera cylindrica* (Wong *et al.*, 2007), *Chenopodium album* (Gu *et al.*, 2011), *Mesembryanthemum crystallinum* (Roeurn *et al.*, 2016) and *Suaeda nudiflora* (Jothiramshekar *et al.*, 2020).

In the present study, two SSH cDNA libraries were targeted salt responsive transcripts of *Suaeda maritima* (L.) Dumort., as experimental tool of *Suaeda maritima* X *Sesuvium portulacastrum* and *Suaeda maritima* X *Salicornia brachiata* (hereafter refereed as SM X SP and SM X SB library) that will result in family specific and genus specific ESTs without being stressed to the plants [as all of them are halophytes]. The optimal salt concentration for its growth is 200mM NaCl, but it grows well at a range of 100mM to 400mM NaCl were observed in our earlier experiments (Shrikanth *et al.*, 2017). This study provides new insights into complex biological phenomena and thereby allows a better understanding of salt response pathways in non-model plant species.

II. MATERIALS AND METHODS

A. Plant Material and Salt Treatment

Seedlings of *Suaeda maritima* (L.) Dumort., and *Salicornia brachiata* Roxb., were collected from the Pichavaram mangrove wetlands, Chidambaram district in Tamilnadu. *Sesuvium portulacastrum* (L.) L., stem cuttings were obtained from Pichavaram mangrove area and maintained under field conditions inside the MSSRF campus, Chennai. Seeds were sown in sterile vermiculite and allowed to germinate under greenhouse conditions ($32 \pm 3^\circ\text{C}$ daytime and $29 \pm 2^\circ\text{C}$ night-time temperatures; 80-90% RH). Pots were sprinkled with tap water every morning. Seedlings were later carefully removed from the vermiculite pots using water without disturbing the root system and hydroponically acclimatized in a growth chamber maintained at $24 \pm 3^\circ\text{C}$, 70-75% relative humidity with 14 h light ($200 \mu\text{mol m}^{-2} \text{s}^{-1}$)/10 h dark cycle. The hydroponic medium constituted Modified Hoagland's Solution during acclimatization for 7 days and in the same medium with 200mM NaCl salt treatment for 14 days.

B. Total RNA Extraction and mRNA Purification

TRI reagent-based extraction procedure (Sigma aldrich) were used for *S. maritima* and *S. brachiata*. A modified CTAB extraction procedure that included extraction with guanidium hydrochloride were employed for total extraction from *S. portulacastrum*. mRNA purifications were performed using Machery and Nagel mini-prep kit following the manufacturer's instructions with minor changes. Quantity of mRNA was verified using 1.2% agarose gel electrophoresis

by comparing with known concentration of mammalian mRNA supplied with Clontech's cDNA library kit.

C. cDNA Synthesis and Suppression Subtractive Hybridization (SSH)

Based on the quantity of RNA obtained, a cDNA synthesis step using SMARTer cDNA synthesis kit (Clontech) to generate cDNAs from limited amounts of total RNA or mRNA. SSH kit (Clontech) was used to prepare the cDNA libraries using cDNAs containing *rsa* I restriction site at their ends. Two cDNA subtraction libraries between *Suaeda maritima* (SM) X *Sesuvium portulacastrum* (SP) and *Suaeda maritima* (SM) X *Salicornia brachiata* (SB) were constructed from 200mM NaCl treated plants for 14 days. All the procedures were according to the manufacturer's instructions with slight modifications. Subtracted cDNAs were cloned in T-vector (MBI Fermentas) and transformed into *E. coli* Omnimax RT1 (Invitrogen) chemically competent cells. Recombination efficiency of the clones were analyzed using colony PCR with the help of M13 forward and reverse universal primers.

D. Differential Screening of the Subtracted Clones

Fifty nanogram of plasmid DNA from each subtracted clone and the appropriate probes for blots were generated through PCR amplification of the cDNA inserts (for which sequence information is already available) using M13 forward and reverse universal primers. The probes were gel eluted using Eppendorfs Perfectprep® Gel Cleanup kit, following the manufacturer's protocol. The denatured probe DNA was added to the tube of Amersham Rediprime random primer labelling kit and the mixed contents was purified using Pharmacia ProbeQuant G-50 column. Hybridization using the 32P labelled probe DNA was done in DIG hybridization buffer and incubated overnight. The membrane was washed using SSC and the washed membranes were saran wrapped and exposed to an X-ray film in a cassette for 3-4 days at -80°C . The X-ray film was processed in the developer solution then rinsed in water and fixed.

E. Sequencing and Bioinformatics Analysis

Randomly selected plasmid clones from subtracted library were sequenced using M13 forward and reverse sequencing primers. Sequencing reactions were performed using Applied Biosystems automated sequencing kit according to the manufacturer's instructions. Sequences longer than 100bp were treated for vector trimming and deletion of adaptors, poly A/T ends and low-quality regions using VecScreen software. The cleaned sequences were collected and assembled by CAP3, using default parameters to generate contigs and singletons (Huang & Madan, 1999). After analysis, unique-ESTs sequences were submitted to dBEST database of NCBI. The obtained sequence information was queried against the non-redundant (nr) peptide sequences in NCBI database (<http://www.ncbi.nlm.nih.gov/BLAST>) with blastn and tblastx (Altschul *et al.*, 1990). Functional annotation and analysis was performed by using Blast2GO tool (www.blast2go.com), web-based freeware following the standard procedure of BLASTX for unigenes dataset (parameters: nr database, high scoring segment pair (HSP) cutoff length 33, report 20 blast hits, maximum E-value $1.0\text{E}-3$), followed by mapping and annotation (parameters: E-value

hit filter 1.0E-6, annotation cutoff 55, GO weight 5, HSP-hit coverage cutoff 20). GO terms were summarized according to their molecular functions, biological processes, and cellular components. Enzyme mapping of annotated sequences was performed by using direct GO to Enzyme mapping and used to query the Kyoto Encyclopaedia of Genes and Genomes (KEGG) to define the KEGG orthologs (KOs) and metabolic pathways.

III. RESULTS AND DISCUSSION

A. Effect of Salt Treatment on *S. maritima*

The plants grew well in vermiculite containing medium. However, when they were transferred to a hydroponic medium, some of the plants wilted. It is possible that during transfer from solid vermiculite medium roots were damaged and those plants with damaged roots were not able to survive. Plants that survived and were transferred to different NaCl concentrations resulted in various morphological changes in the leaves overtime. The plants displayed a linear decrease in leaf number and size under salt concentrations of 300 to 500mM. At 500mM NaCl, the plants showed signs of severe stress at later stages of treatment. At 100 and 200mM NaCl, the plants did not show signs of salt injury, were able to produce new leaves, and continued to grow comparatively similar or better than the control plants (Fig.1). Thus, our results show that all the species can withstand lower salt concentrations, higher concentrations above 200mM affect its growth over long periods.

Fig. 1. Effect of long-term salt treatment on halophytes. A, B – *S. portulacastrum* cuttings in acclimatization medium (MHS) and MHS+200mM NaCl containing medium respectively; C, D – *S. maritima* seedlings in acclimatization medium (MHS) and MHS+200mM NaCl containing medium respectively; E, F – *S. brachiata* seedlings in acclimatization medium (MHS) and MHS+200mM NaCl containing medium respectively.

B. SSH Library of *S. maritima* under 200mM Salt

RNA extraction from NaCl treated plants resulted in good concentrations of RNA. SMARTer cDNA synthesis kit were used to synthesis cDNA and were purified using Chroma-spin column which were used for *rsa* I digestion. The SSH library were constructed using cDNA from leaves exposed to 200mM NaCl *S. maritima* as tester and that 200mM NaCl of *S. portulacastrum* and *S. brachiata* as driver. Subtraction procedures were verified for efficiency according to the manufacturer's recommendations at each step (Fig. 2). Efficiency of adaptor ligation is crucial for good cDNA subtraction were verified using actin specific primers.

Subtracted cDNA fragments were cloned, and randomly selected for sequencing 224 clones (162 in SMXSP + 62 in SMXSB) from both the libraries and checked by colony PCR amplification using M13 universal primer to detect the presence of cDNA inserts and to estimate the size. This resulted in the identification of 109 ESTs were putatively identified and found to contain inserts ranging from 50 to 900 bp were submitted to NCBI. Differential screening was performed for the two libraries using subtracted cDNA probes across species and families clearly resulted in common and specific clones in individual libraries (Fig.3).

Fig. 2. Evaluation of subtraction efficiency by PCR. A, B, C – Leaf total RNA extracted from *S. portulacastrum* (SP), *S. maritima* (SM) and *S. brachiata* (SB) respectively; D, E, F – cDNA synthesized from SP, using SM (mRNA template) and SB respectively using SMARTer cDNA synthesis kit; G, H, I – Purified cDNAs from SP, SM and SB respectively; J, K, L – *Rsa* I digested cDNAs from SP, SM and SB respectively; M, N, O – Unabstracted and subtracted cDNA from SP, SM and SB respectively after secondary nested PCR.

Fig. 3. Dot-blot differential expression screening from subtracted libraries of *S. maritima*. Forward - Specific to *SM* leaf induced during NaCl treatment; Reverse - Specific to *SP* and *SB* leaf induced during NaCl treatment.

TABLE I: DIFFERENTIALLY EXPRESSED SALT RESPONSIVE SM X SP LIBRARY ESTS THAT WERE ISOLATED BY SUPPRESSION SUBTRACTION HYBRIDIZATION (SSH)

GenBank Accession	Putative Annotated Protein	ESTs Clones	Length (bp)	Homology Species	Identity (%)	E-Value
JZ905155	RMD5 homolog A	2	256	<i>Chenopodium quinoa</i>	98	3.75E-10
JZ905160	Stress up-regulated Nod 19 protein	1	912	<i>Beta vulgaris</i> subsp. <i>vulgaris</i>	90	3.18E-32
JZ905161	ABC transporter G family member 35	2	87	<i>Beta vulgaris</i> subsp. <i>vulgaris</i>	93	2.16E-9
JZ905163	Oxygen-evolving enhancer protein 1	2	281	<i>Salicornia europaea</i>	94	1.67E-55
JZ905174	CAAX amino terminal protease	1	518	<i>Beta vulgaris</i> subsp. <i>vulgaris</i>	94	7.87E-108
JZ905176	Thylakoid assembly 8	1	335	<i>Spinacia oleracea</i>	73	3.84E-22
JZ905184	aarF domain-containing protein kinase At1g79600	5	583	<i>Beta vulgaris</i> subsp. <i>vulgaris</i>	80	1.34E-55
JZ905203	Probable glutathione S-transferase	1	140	<i>Beta vulgaris</i> subsp. <i>vulgaris</i>	89	4.52E-12
JZ905217	Trifunctional UDP RHM1	1	286	<i>Chenopodium quinoa</i>	93	3.50E-53

TABLE II: DIFFERENTIALLY EXPRESSED SALT RESPONSIVE SM X SB LIBRARY ESTS THAT WERE ISOLATED BY SUPPRESSION SUBTRACTION HYBRIDIZATION (SSH)

GenBank Accession	Putative Annotated Protein	ESTs Clones	Length (bp)	Homology Species	Identity (%)	E-Value
JZ905223	CBS domain-containing protein CBSX3	1	439	<i>Cucurbita moschata</i>	96	8.22E-80
JZ905228	V-type proton ATPase subunit d2	4	372	<i>Camellia sinensis</i>	99	2.70E-40
JZ905236	Magnesium-chelatase subunit ChlH,	1	257	<i>Papaver somniferum</i>	96	8.68E-50
JZ905248	Probable choline kinase 1	1	356	<i>Beta vulgaris</i> subsp. <i>vulgaris</i>	88	2.83E-31

D. Possible Roles of Identified ESTs in *S. maritima* Salinity Response with Respect to Their Functional Classification

The identified unique-ESTs were classified into nine functional groups based on Gene Ontology annotation of cellular component, molecular function and biological processes includes energy metabolism (2), photosynthesis (1), protein metabolism (2), stress and defense response (3), membrane transporters (1), structural genes (2), gene expression and RNA metabolism (1), secondary metabolism (2) and unknown Fig. 4. Biological processes, location, role and their specific function of the identified salt responsive ESTs as shown in Fig. 5 and Table III.

C. Putative Identification of Salt Responsive ESTs in *Suaeda maritima*

Out of 224 sequences 109 ESTs showed similarity with known or unknown category of genes in NCBI database. The genes identified in the study includes RMD5 homolog A, Stress up regulated Nod 19 protein, ABC transporter G family member 35, Oxygen-evolving enhancer protein 1, CAAX amino terminal protease, aarF domain-containing protein kinase, Thylakoid Assembly 8, Probable glutathione S-transferase, Trifunctional UDP-glucose 4,6 dehydratase/UDP-4-keto-6-deoxy-D-glucose3,5epimerase /UDP-4-keto-L-rhamnose reductase RHM1 belongs to SMXSP library (Table I) whereas CBS domain-containing protein CBSX3, V-type proton ATPase subunit d2, Magnesium-chelatase subunit ChlH, Probable choline kinase 1 belongs to SMXSB library (Table II).

E. Photosynthesis and Energy metabolism

Oxygen evolving enhancer protein 1 (OEE1) and Magnesium chelatase ChlH were under the category of photosynthesis and energy metabolism. Oxygen evolving enhancer protein 1 (OEE1) is a major nuclear-encoded chloroplast protein subunit of 33 Kilo Daltons (kDa). It plays a crucial component of photosystem II (PSII) which provides the binding site for other subunits peripherally located on the luminal side of the thylakoid membrane. Previous studies shown in various halophytes like *Bruguiera gymnorhiza*, *Suaeda aegyptiaca* and *S. maritima*, enhanced levels of OEE1 under salinity leads to the restoration of oxygen evolution, stabilize the ligation of Mn cluster and promoting rapid redox cycling in plants (Koichi *et al.*, 2000; Askari *et al.*, 2006; Alhdad *et al.*, 2013). A similar result was obtained in our

study (clone no.MP 34) implies that OEE1 is the essential protein for oxygen evolution and PSII stability under NaCl treatment (Fig. 5). Another photosynthetic protein was upregulated in the present study is Magnesium chelatase ChlH (clone no. MB 23), an ATP-dependent subunit of three component enzyme that catalyzes the insertion of magnesium ion into protoporphyrin IX to yield Mg-protoporphyrin IX

during synthesis of chlorophyll (Table III). In *Arabidopsis thaliana* and salt-treated halophyte *Tangut nitraria*, it involves in the binding of ChlH subunit and porphyrins on chloroplast membranes, thereby photosensitizing the chlorophyll intermediates that leads to contribute photo oxidative stress tolerance in plants (Begcy *et al.*, 2011; Cheng *et al.*, 2015).

Fig. 4. Top hits Blast species with Go classification of unisequences in the SSH cDNA library. Functional assignment of the unisequences were performed with significant hits in the database. The bar graph shows the distribution of unisequences in three principal GO categories: biological process, cellular component and molecular function A– SMXSP library; B –SMXSB library.

Fig. 5. Functional categorization of unisequences in the SSH cDNA libraries. All of the unisequences were clustered into different functional categories after BLASTing against the NCBI database. A, B, C – Biological process, molecular function and cellular component of SMXSP cDNA library; D, E, F – Biological process, molecular function and cellular component of SMXSB cDNA library; G, H – Combination of all (three) categories of SMXSP and SMXSB cDNA library.

TABLE III: LOCATION AND FUNCTIONAL ROLE OF DIFFERENTIALLY EXPRESSED SALT RESPONSIVE ESTS IN THE LEAVES OF *S. MARITIMA* COMPARED WITH THOSE FOUND IN OTHER PLANTS

Processes	Putative annotated protein	Location	Role	Function
Photosynthesis and energy metabolism	Oxygen-evolving enhancer protein 1	Chloroplast	Stabilization and assembly	Protect PSII from oxidative photo damage and responsible for photosynthesis and generation of precursor metabolites
Chlorophyll metabolism	Magnesium-chelatase ChlH	Chloroplast	Chlorophyll metabolism	Act as an enzyme involved in chlorophyll biosynthesis pathway
Carbohydrate metabolism	Trifunctional RHM1	Integral component of membrane	Protein binding	Acts as a co-enzyme involves in Carbohydrate biosynthesis
Lipid metabolism	Probable choline kinase 1	Membrane	Proton catalytic activity	Involved in phospholipid biosynthesis
Stress response	Stress up-regulated Nod 19 protein	Integral part of membrane	Transport and Ion binding	Involves ATP binding oligomerisation transmembrane activity and helps for root nodule development
Defense mechanism	ABC transporter G family member 35	Integral component of membrane	ATP Binding	Involved in ATPase activity and act as a self-defense i.e. resistance to heavy metals
Oxidative stress / defense mechanism	Probable glutathione S-transferase	Chloroplast	Binding	Plays a vital role in protecting from oxidative and nitrative stress
Redox metabolism	CBS domain-containing protein CBSX3	Integral component of membrane	Regulate redox homeostatic metabolism activity	Act as antioxidants by activating enzymes that scavenge reactive oxygen species, such as H ₂ O ₂ and involves in positive regulation of calcium-mediated signalling
Cellular component biogenesis	aarF domain-containing protein kinase Atlg79600	Plastoglobule	Tocopherol cyclase activity, plastoglobule integrity and regulatory network	Highly conserved protein and also responsible for kinase activity and embryo development in seed dormancy condition
Gene expression and RNA metabolism	Thylakoid assembly 8	Chloroplast	Protein Phosphorylation	Plays a vital role in posttranscriptional processes within organelles
Protein synthesis / turnover	CAAX amino terminal protease	Integral component of membrane	Peptidase activity	Metal-dependent proteases involved in anchoring of proteins in eukaryotes
Protein degradation	RMD5 homolog A	Nucleus	Proteasome and ligase activity	Ubiquitin dependent protein is necessary for polyubiquitination
Cell structure and transport	V-type proton ATPase subunit d2	Membrane	Hydrogen ion transporter	Plays a significant role in signal transduction and is expressed during matured /germinated pollen stage

F. Secondary Metabolite Biosynthesis

Trifunctional UDP RHM1 and Choline kinase 1 (CK1) were under the category of secondary metabolism. Trifunctional UDP RHM1 is one of the major secondary metabolic enzymes used for flavonol rhamnosylation and mainly involved in UDP-beta-L-rhamnose biosynthesis, a precursor of the primary cell wall components. UDP-rhamnose is synthesized from UDP-glucose through the consecutive three-step reaction catalyzed by a single structure enzyme UDP-rhamnose synthase (RHM), which has UDP-D-glucose 4,6- dehydratase, UDP-4-keto-6-deoxy- D-glucose 3,5-epimerase, and UDP-4- keto-rhamnose 4-keto-reductase activities. In *A. thaliana*, *Chenopodium album*, *Ornithogalum caudatum* and *Populus euramericana*, this enzyme protein defined as a trifunctional plays a significant role in supplying UDP-rhamnose for modification of flavonols (Gu *et al.*, 2011; Kim *et al.*, 2013; Han *et al.*, 2015; Yuan *et al.*, 2018) were identified in the present study (clone no.MP 157) might be involved for carbohydrate biosynthesis in *S. maritima* plants (Fig.5). Another key enzyme is Choline kinase I (CK1), is a primary Cho kinase involved in the phosphatidylcholine subpathway that synthesizes phosphocholine from choline. It encodes kinases with a strict

specificity for Cho substrate, suggesting a crucial role of the CDP-Cho pathway in regulating phosphatidylcholine (PC) biosynthesis under abiotic conditions in plants such as salinity, cold and drought. In *Arabidopsis* and soybean, CK1 were increased under salt treatment which in turn to enhance the synthesis of PC (Begora *et al.*, 2010; Guschina *et al.*, 2014) were upregulated in our study (clone no. MB 51) might be involved in the phospholipid biosynthesis and metabolism in *S. maritima* (Fig.5).

G. Stress and Defense Mechanism

Stress upregulated Nod19, Glutathione S-transferases and Cystathionine b-synthase were under the category of stress and defense-related proteins. Nod19 is a serine endopeptidase transcriptional regulator protein encoded by the nod genes are highly responsible for the synthesis and export of specific rhizobial lipochitooligosaccharides were collectively known as Nod factors (NF). It triggers a series of host responses such as root hair deformation, early nodulin gene expression, ion flux changes and transmembrane activity under salinity were reported in *Medicago truncatula*, pea and other legumes (Doss, 2005; Kelly *et al.*, 2018). This transcript was upregulated in the present study (clone no. MP 28) might be involved stress regulation and transport mechanism in *S.*

maritima plants (Fig. 5). Glutathione S-transferases (GSTs) is a major class of Phase II detoxification enzymes acts as potential component of catalyzing the conjugation of glutathione (GSH) to electrophilic compounds through thioether linkages. It served as a cellular regulatory role through maintaining intracellular H₂O₂ levels, particularly with respect to antioxidant protection of cell membranes. The upregulation of GST upon salt treatment were reported in the differential expression analysis of various halophytes like *Aeluropus litoralis*, *Salicornia brachiata*, *Suaeda salsa*, *Suaeda maritima*, and *Thellungiella hallophila* plays a significant role in protecting cells against stress (Wang *et al.*, 2004; Zouari *et al.*, 2007; Sahu & Shaw, 2009; Tiwari *et al.*, 2016; Li *et al.*, 2020) were identified in our study (clone no. MP 125) might be involved in altering structure and function of proteins from oxidative damage implies its potential role in salt tolerance in *S. maritima* (Fig. 5). In addition, Cystathionine b-synthase (CBS) were also upregulated in the present study (clone no. MB 4) has a characteristic arrangement of three beta sheets and two alpha helices that binds adenosine-containing ligands such as AMP, ATP, or NADPH (Bertoni, 2011). It serves antioxidants by activating thioredoxin enzymes that scavenge reactive oxygen species and involves in positive regulation of calcium-mediated signaling in *S. maritima* plants (Table III). A similar trend was previously reported in *Arabidopsis thaliana* annotated as CBSX1 and CBSX2 and in transgenic tobacco over expressing rice by Yoo *et al.*, (2011) and Singh *et al.*, (2012).

H. Cell Organization and Assembly

Among the identified clones, AARF domain-containing kinase protein were found the maximum number of ESTs clones (clone nos. MP 57, 62, 70, 77, 81) in the present study is found to be characteristic feature of ABC1 Kinase family (Table 1). Previous studies reported that six isoforms of ABC1-like kinases identified in *Arabidopsis* under light and salt conditions (Yang *et al.*, 2012) might be involved in plastoglobule morphology, thylakoid integrity or chloroplast regulatory network in *S. maritima* (Fig.5).

I. RNA Metabolism and Protein Ubiquitination

Thylakoid assembly 8 (THA8) and RMD5 Homolog A were upregulated in the present study under the category of RNA processing and protein polyubiquitination. Thylakoid assembly 8 is a major subfamily of plant pentatricopeptide repeat (PPR) proteins preceded by a short, conserved N-terminal region and have the potential to mediate specific intermolecular interactions in the processing of mitochondria and chloroplast organelles (Table III). Previous studies shown THA8 should be relatively conserved in maize, rice, and *Arabidopsis* were highly involved for the splicing of group II introns (Asakura & Barkan, 2006; Khrouchtchova *et al.*, 2012) which infers TH8 (clone no. MP 58) protein might play a role in RNA editing and processing in *S. maritima*. Another one is RMD5 Homolog A, an ubiquitin dependent protein plays an important role in E3 ligase activity (Fig.5). It was reported in *Arabidopsis* and *Chenopodium* as Gid2/Rmd5, a component of the GID complex plays a key role in regulation and degradation of proteins (Stone *et al.*, 2005; Santt *et al.*, 2008; Gu *et al.*, 2011). In the present study, we have identified 2 clones (clone nos. MP 13 & 23) shows

high homology (98%) with *Chenopodium* might play a vital role in polyubiquitination in *S. maritima* (Table I).

J. Signal Transduction and Vesicular Transport

Both CAAX proteins and Vacuolar ATPase (V-ATPase) were upregulated in the present study as CAAX proteins (clone no. MP 56) is defined as a group of posttranslational modification proteins encompasses a wide range of molecules such as Ras and a multitude of GTP-binding proteins (G proteins), proteins with DEAD/H (Asp-Glu-Ala-Asp/His) box, Ser/Thr protein kinase family, GPRK subfamily, heat shock proteins, etc.(Michaelson *et al.*, 2005) whereas Vacuolar ATPase (V-ATPase) (clone nos. MB 9, 10, 11 & 31), a highly conserved multisubunit enzyme (Table II) provides the crucial proton motive force necessary for the formation of synaptic vesicles and maintain the balance of vesicular system by interacting with SNAREs and GTPase (Forgac, 2007). Previous studies shown that CAAX in *Arabidopsis* and V-ATPase in *Suaeda salsa* plays an important role in cellular signaling processes and regulatory events like transport of molecules, membrane integrity and stability, cell to cell communication and recognition, vesicular trafficking, and indirect regulation of signaling pathways in response to salinity (Bracha *et al.*, 2002; Qiu *et al.*, 2007; Roberts *et al.*, 2008). Hence, both transcripts might play a significant role to protect cell membrane through transport and anchoring of proteins in *S. maritima* plants (Fig.5).

K. Membrane Transporter

ATP-binding cassette transporter G 35, which is a subfamily of large, ubiquitous, and diverse group of ABC transporter super family proteins. It acts as a key player in regulating the physiology and development of plants against biotic and abiotic stress conditions (Hwang *et al.*, 2016). It predominantly assists substrate derivatives of strigolactone plays a significant role for the specificity of secondary metabolite compounds and turns as a potential candidate gene for stress tolerance were reported in *Arabidopsis* (Liu *et al.*, 2018). This transcript identified in the present study (clone no. MP 29) might be involved in ATPase activity and self-defense mechanism in *S. maritima* (Fig.5).

IV. CONCLUSION

In the present study, we provided a comprehensive salt responsive dynamics in the leaves of halophyte *Suaeda maritima* (L.) Dumort., for long term salt treatment using SSH method. Our results indicated that potential salt responsive candidate genes like Oxygen-evolving enhancer protein 1, AARF domain-containing kinase protein, RMD5 homolog A, V-type proton ATPase subunit d2, ABC transporter G 35 that were mainly involved in photosynthesis, stress and defense response, protein ubiquitination, cellular transport, and secondary metabolites in *S. maritima*. This will provide a basis study for the mechanism of salt tolerance and may serve as a reference sequence for study of other succulent halophytes. Further analysis of salt responsive genes from this species would unravel novel mechanisms of salt stress tolerance in plants.

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CONFLICT OF INTEREST

Authors declare that they do not have any conflict of interest.

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