De novo transcriptome analysis unveils regulatory pathways associated with stress tolerance in a promising C3 model of halophyte, Suaeda salsa

Shima Jamalirad
University of Zanjan
Mohammad Reza Azimi
University of Zanjan
Nayer Azam Khoshkholgh Sima
Agricultural Biotechnology Research Institute of Iran
Mehrshad Zeinalabedini
Agricultural Biotechnology Research Institute of Iran
Laleh Karimi Farsad
Agricultural Biotechnology Research Institute of Iran
Ghasem Hosseini Salekdeh
Macquarie University
Mohammad Reza Ghaffari (mrghaffari52@gmail.com)
Agricultural Biotechnology Research Institute of Iran

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Abstract

Suaeda salsa is a promising halophyte model to study the molecular mechanisms underlying salt tolerance in plants. To attain a thorough knowledge of transcriptomic profiles under salt stress during seedling establishment, we accomplished whole-transcriptome sequencing on the seedlings of *Suaeda salsa* at 30 days after exposure to 0 mM, 200 mM, 400 mM, and 800 mM NaCl. We observed that transcripts implicated in solute transport and nutrient uptake, protein synthesis, modification, hemostasis, transcriptional regulation, and phytohormone action prominently changed at different concentrations of salinity. Likewise, significant changes in the expression level of members of gene families such as MYB, bHLH, MADS/AGL, bZIP, NAC, C2C2, B3, ERF, WRKY, HB, NF-Y, C2H2 suggest them as key players in the salt tolerance of *Suaeda salsa* during seedling establishment. We additionally found the superfamilies of tyrosine-like protein kinase (TLK) linked to phosphorylation and Ca^{2+}calmodulin-dependent protein kinas, enabling the signal sequence for protein activity and gene transcription under salinity stress. The novel identified autophagy ATG members, and autophagic cargo receptor protein (NBR1) was observed under salt stress suggesting that autophagy regulates rapid protein turnover as a prerequisite for salt stress tolerance in *S. salsa*.

Introduction

Soil salinization affects 20% of the agricultural lands that produce 40% of the worldwide food, and this is a severe global problem in crop production and seed quality (.Xu et al. 2017). Salinity stress limits plant growth and productivity and has multiple damaging effects on plant cells, including ion injury and osmotic imbalance (.Munns and Tester 2008, Pan et al. 2020). Plants require a complex regulatory network to mediate transport of ions and partition of assimilates in organs within their cells to survive in high salinity environments (.Munns and Tester 2008, Zhao et al. 2017). Hence, for saline agriculture, developing and utilizing saline-alkali land by breeding salt-tolerant germplasm is a solution to improve saline soil (.Flowers and Colmer 2008, Xu, Zhao, Duan, Sui, Yuan and Song 2017). Halophytes display salt requirements for optimal growth and development and demonstrate no growth inhibition in response to salinity (.Furtado et al. 2019). Halophytes have evolved different salinity tolerance mechanisms compared to glycophytic plants, thus can grow normally in higher saline soils and exhibit multifarious adaptations to hypersaline environments (.Furtado, Nagy, Asp, Tyburski, Skorupa, Gołębiewski, Hulisz and Hrynkiewicz 2019, Han et al. 2020). In spite of glycophytic plants, halophytes prefer moderate salt conditions. Thus, the potential and need for halophytes in saline agriculture are continuously increasing (.Li et al. 2019, Zhao, Yang, Guo, Mao, Li, Sun, Wang and Yang 2017). *S. salsa* is an obligate halophyte belonged to Amaranthaceae family and is mainly distributed in Europe and Asia. This plant species has beneficial nutrient ingredients such as vitamins, proteins, and antioxidants and can be utilized as forage for domestic animals. Likewise, *S. salsa* could remediate the negative impacts of soils contaminated with salinity and toxic heavy metals (.Chen et al. 2010, Li, Zhang, He, Wang, Zhu and Ji 2019, Xu, Zhao, Duan, Sui, Yuan and Song 2017). The maximum ability of halophytes to adjust the specific ion toxicity and osmotic capacity makes them thrive under higher salinities. Physiological studies revealed that ion

accumulation and osmotic adjustments are fundamental constituents of the salt tolerance mechanism in halophytes (.Ajmal Khan et al. 2000, Diray-Arce et al. 2015). Several characteristics including, plant species, genotype, and developmental stage primarily, determine the salt tolerance of the plants (.Vicente et al. 2004). Typically, plants are more sensitive to salt stress at seed germination and seedling establishment phases, and the tolerant one overcomes these stages and can shape the natural habitat (.Ali Hassan et al. 2017, Donohue et al. 2010). The multiple regulatory mechanisms govern the salt tolerance in halophytes (.Jin et al. 2016). However, the lack of reference genome availability assembles difficulties for halophyte plant species to comprehend the molecular mechanism of salt stress tolerance (.Furtado, Nagy, Asp, Tyburski, Skorupa, Gołębiewski, Hulisz and Hrynkiewicz 2019). Regardless, transcriptome-wide gene expression analysis has been widely used to understand the molecular mechanisms of responding plants to abiotic stresses at the genetic level (.Jin, Dong, Yang and Zhu 2016, Younesi-Melerdi et al. 2020). The study of transcriptome profiles provide insight for functional studies of numerous genes involved in abiotic stress response (.Wang et al. 2020). Recently, most studies have been extensively focused on glycophytes regarding salinity (.Zhao, Yang, Guo, Mao, Li, Sun, Wang and Yang 2017). In the case of halophytes, most have focused on early vegetative growth, and different halophytes populations displayed substantial tolerance to salt stress (.Jin, Dong, Yang and Zhu 2016). Seed germination is the most critical developmental phase of plant establishment under saline soils. Hence, understanding the reasons underlying the differential responses among different salinity levels during the germination phase can be considered as a determinant approach to augment our knowledge around salt response from physical (at the embedding process) to biological (at the embryonic growth) point of views. Here, we investigated and compared gene expression on seedlings of germinated seeds at 0 mM and under moderate (200 mM) and severe (400 mM and 800 mM NaCl. Our focus was on phytohormone triggered salt stress response, signaling pathways and cell wall mechanism crucial for stress tolerance, transcription factors important for cell signal transduction, and ubiquitin-proteasome system enzymes vital for protein turnover and systems carrier implicated in transports and hemostasis.

Materials And Methods

Sample collection and salt stress treatment

The previously collected records by the Agricultural Biotechnology Research Institute of Iran (ABRII) were used to identify distribution localities for sampling (.Zeinalabedini et al. 2021). *Suaeda salsa* 's seeds were collected from the coastal area in BANDAR-E-Gaz, Golestan province, northeast of Iran at 36°47'16.1"N and 53°56'30.5"E. This study was a research project permitted by the Agricultural Biotechnology Research Institute of Iran, ABRII12-05-05-024-93001-971215. Thus, no further permissions were required to collect seed from the field. To evaluate the impacts of salinity stress, non-germinated seeds were planted at 0 mM, 200 mM, 400 mM, and 800 mM NaCl and grown under 28°C with a 16:8hour light-dark cycle for 30 days in a constant salinity in a greenhouse. Then, the seedlings were sampled with three biological replicates and immediately frozen in liquid nitrogen and kept at -80°C until RNA extraction.

Quantification of K^+ and Na^+ content

The ion content of freeze-dried samples (0.10 gr) was placed in an electric furnace for ash production heating at 500°C for 4 h. The ash was mixed with 10 mL HCOOH (10%) and incubated at 100°C for 2 h. Then the samples were filtered and diluted 10 times. Then, K^+, $Na^{+,}$ and Ca^+ concentrations were measured using a PFP7 flame photometer with three independent biologically replicates (Jenway Inc., Burlington, VT, USA).

RNA extraction

Total RNA was extracted using the CTAB-LiCl method as previously described (.Zhang et al. 2013). The extracted RNA was measured using ND2000 spectrophotometer and the Agilent 2100 Bioanalyzer was used to estimate RNA sample QC. Library construction and HiSeq 2000 sequencing were carried out according to standard protocols. The libraries were sequenced on the Illumina HiSeq 2000 platform for generating 150-bp paired-end reads. The raw data were then filtered with an NGS toolkit with 90% above QC and stated by FastaQC.

De novo assembly of transcripts

Trimmomatic software version 0.36 was used to remove illumine adaptor sequences and low-quality bases (Q-value < 20) (.Bolger et al. 2014) with the following parameters: SLIDING WINDOW: 4:15 LEADING:5 TRAILING:5 MINLEN:50. Then, the clean forward and reverse reads were pooled separately and assembled into transcripts with Trinity version 2.2.0 software (.Haas et al. 2013) based on the default parameters. Transcripts shorter than 200 bp were discarded. The benchmarking universal single-copy orthologs (BUSCO, V.2.0) was used to evaluate the completeness of the assemblies (.Simão et al. 2015). The functional annotation of unigenes was then searched using BLASTX against Nr and mercator4 (V.2.0) for functional annotation with a cut-off E-value of 10^{-5} and MapMan as described previously (.Usadel et al. 2009).

Differential expression analyses

The clean reads were aligned to the assembled transcripts with Bowtie2 (.Langmead and Salzberg 2012) and the expression levels of transcripts were calculated and normalized via Expectation Maximization in RSEM (.Li and Dewey 2011). The differentially expressed genes between salinity treatments (200 mM, 400 mM and 800 mM and control (0 mM) and between treatments were calculated using EdgeR package. The p-value and FDR were obtained and the log2 fold change (log2FC) was calculated with the edgeR package (.Robinson et al. 2010). The $|log2FC| \geq 1$, and p-value < 0.01 were considered to identify differentially expressed genes for further analysis.

Functional pathway enrichment analyses using gene ontology and MapMan functional annotation

The Cytoscape plugins ClueGO V2.5.7 and CluePedia V1.5.7 were used for Gene Ontology (GO) enrichment analysis. A p-value of 0.05 with Benjamini–Hochberg false discovery rate correction with the two-sided hypergeometric test was applied along with the kappa coefficient threshold of 0.4. In addition, the gene expression profiles in each bin were determined using MapMan software (version 3.6.0). Mapman provides significant gene list of integrated data to diverse metabolic overviews as previously described (.Ghaffari et al. 2016) .

Statistical analysis

Unpaired Student's t-test was conducted to compare the mean (n = 3). The P-value < 0.05 showed data statistically significant.

Quantitative real-time PCR (qRT-PCR)

Purified RNA of seedlings in three independent biological replicates was used to synthesized cDNA using oligo dT primer and iScrip cDNA Synthesis kit (Takara), based on the manufacturer protocol. Quantitative real-time PCR (qPCR) was performed using designed primers (S2 Table). Real time PCR system (Roche Life Science, Germany) was performed using SYBR Green Real Time Master Mix (Takara). The relative expression levels were obtained by normalization to the transcript level of a housekeeping gene, actin as previously (.Koobaz et al. 2020). The relative gene expression was calculated using the comparative cycle threshold methods as previously described (.Neuhoff et al. 1988).

Results

Physiological changes of seedlings under salinity treatment

S. salsa seeds were subjected to control (0 mM), low (200 mM), moderate (400 mM) and high salinity (800 mM) for 30 days. Compared to control (0 mM NaCl), seedlings of *S. salsa* remarkably had no injury symptoms after imposing 200 mM and 400 mM NaCl stress after 30 days. However, a significant inhibitory growth effect of salt on seedlings growth was observed from the 200 mM NaCl after 30 days. In the 800 mM NaCl treatment, the growth of seedlings was significantly reduced (Fig. 1).

Na^+ and K^+/Na^+ ratio in seedlings under salt stress

The Na^+ contents in seedlings started to accumulate and ranged 5.4 mmol/gr at 200 mM and rose to the maximum level of 13.2 mmol/gr at 800 mM ($P < 0.05$). Moreover, the Na^+/K^+ ratio was 1.6-fold in control plants and significantly increased up to 4.5-fold at 800 mM NaCl ($P < 0.05$), indicating that Na^+ accumulated in seedlings might decline plant growth under 800 mM NaCl (Fig. 2).

De novo transcriptome assembly

RNA-seq (Illumina HiSeq X platform with paired-end sequencing, Beijing Genomics Institute, BGI) was performed on eight samples (Table 1). After, the quality control steps, 1.345 million high-quality clean

reads were de novo assembled into 237,192 transcripts and 136515 unigenes with an N50 length 1717 bp and 38.46% GC content. The average mapping rate per library was up to 75.77%. The sequencing data has been submitted to SRA of NCBI with accession no. PRJNA802064.

Table 1
Summary of transcriptome reads

Sample name	Sample description	Total reads	%GC
C_0_1	Control replication (0mM) 1	51,833,302	43.2%
C_0_1	Control replication (0mM) 2	39,066,724	43.0%
S_200_1	Salt stress (200mM) replication 1	45,490,996	43.1%
S_200_2	Salt stress (200mM) replication 2	44,813,556	43.3%
S_400_1	Salt stress (400mM) replication 1	43,017,018	43.3%
S_400_2	Salt stress (400mM) replication 2	61,176,666	43.5%
S_800_1	Salt stress (400mM) replication 1	69,660,290	43.6%
S_800_2	Salt stress (400mM) replication 2	41,274,952	43.3%

Functional annotation of transcripts

For functional annotation, a total number of 112455 transcripts (~ 48%) were annotated against the NR database using NCBI BLASTX. *Chenopodium quinoa*, *Beta vulgaris* and *Spinacia Oleracea* were found to be the top hit species (Fig. 3). We then compared the transcripts against the gene ontology (GO) database. A total of 237,193 were assigned at least one GO term. The GO terms were categorized into 23 functional groups, which were divided into three categories including cellular component, biological process and molecular function (Fig. 3). We first checked the prominent GO terms corresponding to total transcripts in seedlings profiling under salinity stress. The majority of GO terms assigned included ATP binding, metal ion binding and DNA binding in molecular function category; obsolete-oxidation reduction process, transmembrane transport and regulation of transcription in biological process category; integral component of membrane, nucleus and membrane in cellular component category (Fig. 3). MapMan functional annotation suggested that transcripts fall under 28 bins. The top 10 bins included photosynthesis (1634 transcripts), phytohormone (1239 transcripts), chromatin organization (1229 transcripts), Cell cycle organization (1673 transcripts), RNA synthesis and processing (7926 transcripts), protein synthesis, modification and hemostasis (1075 transcripts), cell cycle (1116 transcripts), vesicle trafficking (1378 transcripts), solute transport (3920 transcripts) and lipid metabolism (1511 transcripts) (Table 2).

Table 2
The top 10 bins in MapMan functional annotation

Biological process	Number of transcripts
RNA synthesis and processing	7926 transcripts
Solute transport	3920 transcripts
Chromatin organization	1229 transcripts
Cell cycle organization	1673 transcripts
Phytohormone	1239 transcripts
Protein synthesis, modification and hemostasis	1075 transcripts
Cell cycle	1116 transcripts
Photosynthesis	1634 transcripts
Lipid metabolism	1511 transcripts
Vesicle trafficking	1378 transcripts

Identification and functional annotation of differentially expressed genes (DEGs)

DEGs under salt treatment were computed in two pairwise contrasts between salinity treatments and salinity treatments with the control (S1 Table). The number of DEGs varied between control and treatments and within salinity treatments. The most DEGs belong to the comparison of control and 400 mM NaCl treatment (1893 DEGs), and the lowest belongs to the comparison of 400 mM NaCl treatment and 800 mM NaCl treatment (508 DEGs) (Fig. 4a). Further, the comparison between the DEGs showed that the 80.3% proportion of genes was unique to the control vs salt treatment, while 81.6% combined salt treatments uniquely expressed genes. Between 16.3% (control and salt treatments) and 21.6% (combined salt treatments) of DEGs that were differentially expressed had common responsive indicating regulatory changes unaffected by different treatments. In comparison with control in low salinity (200 mM NaCl), 623 genes (FC $\geq$ 2) were identified and assigned to 256 up-regulated and 262 down-regulated genes (Fig. 4a). Most of the up-regulated assigned genes were classified as being involved in photosynthesis and subcategory of Calvin cycle (11 transcripts). In contrast the majority of the down-regulated genes were mostly involved in TF families (10 transcripts) and protein modification (6 transcripts). In the 400 mM of salt stress, the differentially expressed genes were much larger including 1955 genes which were assigned into categories of protein modification (58 transcripts), solute transport carrier (56 transcripts) and transcription factor (52 transcripts). The number of up-regulated genes was 1113 genes which mostly belonged to protein modification (45 transcripts), solute transport carrier (41 transcripts) and transcription factor (36 transcripts) categories. Meanwhile, the bulk of 708 genes that were down-regulated involved in the cell wall organization (24 transcripts) category. However, in high salinity of 800 mM NaCl, 1754 differentially expressed genes were assigned as being mostly involved in categories of

transcription factor (76 transcripts), and protein biosynthesis (68 transcripts). The majority of 802 up-regulated transcripts were more included as being involved in TFs (57 transcripts) and solute transport carriers (42 transcripts). While many of the 889 down- regulated genes were more occurred in protein biosynthesis (66 transcripts) photosynthesis and chromatin organization (30 transcripts) which support the idea of limited growth and photosynthesis performance in high salinities. The lowest down-regulated candidate genes linked to solute transport carrier a plasma membrane intrinsic protein (PIP) which might be a sign of drought stress in the higher salinity treatment.

Identification of salt stress response genes S.salsa **at seedlings stage**

Protein synthesis, modification and hemostasis altered under salinity

The most significant impact was observed on the genes involved in protein synthesis, modification, and hemostasis under salinity treatment in *Suada salsa* (Fig. 5a). Of the significantly changed genes were 146 up-regulated DEGs aligned to protein synthesis, modification, and hemostasis under all NaCl treatments. In protein modification, the most significant up-regulated genes were 124 members belonging to superfamilies of tyrosine-like protein kinase (TLK) linked to upstream and downstream regulators by phosphorylation Ca^{2+}/calmodulin-dependent protein kinas functioning as metabolic sensors for kinase function and substrate definition. Besides, proteins involved in hemostasis are linked to the ubiquitin-proteasome system like members of ubiquitin-fold protein (UBQ), ubiquitin-conjugating E2 protein (UBC), RING-v-class E3 ligase, and BTB/POZ substrate adaptor components of LR and proteolysis-cysteine-type peptidase activities. The members of autophagy, like component ATG18, ATG8-maturation peptidase (ATG4), and autophagic cargo receptor protein (NBR1), were up-regulated, particularly by increasing salinity treatments whereas, several genes involved in the protein synthesis and ribosome biogenesis, like large and small ribosomal subunit-component were down-regulated in response to high salinities.

Complex phytohormone triggered by salt stress

Under salt stress, complex gene regulation networks are influenced via plant hormones, impacting downstream genes' expression responsible for osmoregulation and cell protection. Under all salinity treatment, 41 genes involved in phytohormone action were up-regulated by increasing salinity. Many genes were key signaling peptide regulators that belonged to CRP (cysteine-rich-peptide) category like RALF-peptide receptor and NCRP (non-cysteine-rich-peptide) like IMA/FEP precursor polypeptide. Besides, genes encode essential enzymes of auxin biosynthesis, flavin-containing monooxygenases (YUCCAs), auxin perception and signal transduction, auxin transporter, auxin transporter (PIN), and auxin efflux transporter (PILS) were induced mostly by increasing of salinity treatments. A signaling molecule like ethylene signal transducer (EBF) and gene encode essential enzymes of ethylene biosynthesis such as aminocyclopropane carboxylate (ACC) oxidase up-regulated under salinity.

Solute transport carrier and nutrient uptake activated under salinity

Solute transport carrier-related genes were a large number of 96 up-regulated DEGs in all salt stress treatments (Fig. 5b). APC MFs, ABC, and ZIP superfamilies were up-regulated under salt stress treatments. APC members facilitate amino acids, auxin, borate, nitrate, fluoride, and ammonium transport, while MFs play a significant role in anion transporter (NRT1/PTR). Zip family encourages cation transport under salinity treatment. Moreover, our results indicated nitrogen, phosphorus, iron, and sulfur assimilation were induced by up-regulation of nitrate reductase (NR), ATP sulfurylase and APS reductase, ubiquitin-conjugating E2 protein (PHO2), and ferric cation-chelate reductase, which dynamically changed in salt stress treatment, indicating the critical and complex roles of nutrients under salt stress.

Transcription factors pattern changed under salinity

We extracted the expression of the transcription factors families to understand how transcription factors amplify gene regulation signals and trigger protective mechanisms through the induction or repression of functional genes under salt stress. The changes in the gene expressions of 143 DEGs of 16 TFs families of MYB, bHLH, MADS/AGL, bZIP, NAC, C2C2, B3, ERF, WRKY, HB, NF-Y, C2H2 are shown in Table 3. In general, upon all salt treatment, most TFs and prominently WRKYs family followed by NACs and NYs families showed up-regulations (Fig. 5c).

Table 3
The transcription factors' families and their regulations under different salinity level in *S. salsa*

TF Family	0 vs 200		0 vs 400		0 vs 800		Total	
	Up	Down	Up	Down	Up	Down	Up	Down
MYB	1	1	4	1	6	5	11	4
bHLH	1	1	1	4	0	3	2	5
MADs	1	0	7	0	5	0	8	0
bZIP	0	0	1	0	2	0	3	0
NAC	0	1	3	0	7	0	11	1
C2C2	0	0	1	0	2	1	3	1
B3	1	0	4	0	4	0	5	0
ERF	0	0	5	2	5	1	8	3
WRKY	0	1	4	1	6	0	9	2
HB	0	5	1	8	7	3	9	10
NF-Y	0	1	1	0	7	0	7	1
C2H2	0	0	1	0	3	0	4	2
Up = Up-regulated; Down = Down-regulated								

Cell wall metabolism modified under salinity

We identified 13 up-regulated DEGs in all NaCl treatments cell related wall and cell membrane composition (Fig. 5d). These up-regulated genes related to lignin-monolignol biosynthesis, homogalacturonan, hydroxyproline-rich glycoprotein activities and cutin polyester biosynthesis pathway of which 3 membranes of lignin-monolignol biosynthesis like caffeoyl-CoA 3-O-methyltransferase (CCoA-OMT) were more prominent.

Validation by quantitative real-time PCR analysis

The technical and biological variations were inspected by performing qRT-PCR of three independent biological replicates using ten salt-responsive genes. These transcripts were belonged to different functional groups and significantly expressed at the higher salinity stress levels. The qRT-PCR results highly correlated with RNA sequencing, implying that identified DEGs can be considered high accuracy in this work (Fig. 6).

Discussion

Salt stress significantly influences growth and crop production worldwide (.Ghaffari, Ghabooli, Khatabi, Hajirezaei, Schweizer and Salekdeh 2016). The investigation of halophytes expands our knowledge of the crucial adaptations needed for survival in harsh environmental conditions and their experiences in regulating cellular ion homeostasis and osmotic adjustment, and alterations of the cell wall and membrane composition (.Khoshkholgh Sima et al. 2019). However, the metabolic adjustments and transcriptional regulation through extensive transcriptomic reprogramming remain unclear, especially in high salinities. Accordingly, we employed a transcriptome-wide and physiological data analyses on seedlings halophyte *S. salsa* under different stress treatments. Our differentially expressed transcripts data revealed diverse expression patterns associated with many genes, suggesting their critical positions in salt stress responses at the seedling stage of the C3 model of euhalophyte, *S. salsa*.

Solute transport carrier and nutrient uptake

The APC family is essential for long-distance transport and absorption of amino acids from the soil and element uptake (.Tian et al. 2020). In this study, encoding genes participating in the uptake and transport of essential nutrient, like sulfate (sulfate transporter (SULTR)), molybdate (molybdate transporter (MOT)), and to a greater degree borate (Borate transporter), amino acids (amino acid transporter permeases (AAP) and proline (proline transporter (ProT)) were induced under salt stress in *S. salsa*. Boron (B) stabilizes the plant cell wall and leaf expansion (.Saouros et al. 2021) and reduces cell membrane damage during abiotic stresses (.Saouros, Mohan, Cecchetti, Lehmann, Barrit, Scull, Simpson, Alguel, Cameron, Jones and Byrne 2021). It was previously shown amino acid transporter permeases contribute to proline uptake during salt stress in *Arabidopsis thaliana* (.Wang et al. 2017). Likewise, Proline-specific transporters 2 (AtProt2) induced by salt stress in Arabidopsis. The accumulation of proline and betaine provides

osmotic effects for plants to cope with salt stress (.Rentsch et al. 1996). Notably, major facilitator superfamily (MFS) members, particularly nitrate or di/tri-peptide transporters (NRT1/PTR), PO43− (phosphate transporter PHT1, PHT2, and PHT5), Fabaceae-N70, and metal chelator transporter (TCR) were significantly induced in higher salinity levels explaining a mechanism by which *S. salsa* may sequestrate Cl^- and Na^+ into the vacuole for salt stress tolerance. Other necessary transporters which highly induced in *S. salsa* ATP were binding cassette proteins (ABC) of ABCC and ABCG which act as regulators of ion channels and gatekeepers of cells preserving nutrients within the cell and expelling toxins (.Saha et al. 2015). Together, this data indicates macro-and microelements are controlled to balance various nutrients and ions to combat salinity stress at different levels in *S. salsa*.

Phytohormones

Phytohormones regulate various metabolic and transcriptional processes using signaling network, and alter plant growth adaptation in response to stress. Auxin accumulates and redistributes and changes the plant growth and architecture adaptations in response to salt stress (.Ribba et al. 2020). According to our data, the transcripts encoding enzyme of auxin biosynthesis, such as flavin monooxygenase (YUCCA), auxin transporter (PIN) and the auxin-responsive genes, auxin/indole acetic acid (AUX/IAA) was induced under salt stress. It was previously indicated that auxin transporter mutants of aux1, pin1 and pin2 in *Arabidopsis thaliana* were more sensitive to salt and high-temperature stresses and accumulated higher ROS than the wild type (WT) (.Krishnamurthy and Rathinasabapathi 2013). Furthermore, the reduced expression of PIN-FORMEDs repressed the PIN2 protein and indole-3 acetic acid inducer 17 (IAA17) suggesting that root system architecture significantly affected by auxin signaling under salinity stress. Elevating ethylene biosynthesis increase plant tolerance to salinity stress. The ethylene-responsive element binding factors (EBFs) are essential transcriptional regulators and implicated in various stresses (.Yin et al. 2019). Aminocyclopropane carboxylate oxidase (ACO) that converts ACC into ethylene was induced in response to salinity. The exogenous application of ethylene enhanced salt tolerance in *Arabidopsis thalian*. Sugimoto et al. (2018) showed the T3 generation of transgenic rice with barley ACO genes, improved tolerance to salt stress. indicating that ethylene contributes to plants' salt stress tolerance (.Sugimoto et al. 2018). The differential expression of different hormone-related genes may indicate that plant hormones triggered expression of genes and shape plant growth and morphology enabling adaption to salt stress in *S. salsa*.

Cell wall metabolism

The lignin synthesis and its composition are regulated efficiently by Caffeoyl-coenzyme A O-methyltransferase (CCoAOMT) which transfers of the methyl group from S-adenosylmethionine (SAM) to the hydroxyl group during lignin and flavonoids biosynthesis (.Hafeez et al. 2021). The transcriptomic data showed CCoAOMT significantly induced by increasing salt levels Halophytic *Eutrema salsugineum* and Gossypium species (.Li et al. 2021). This result may indicate that lignin accumulation in an important mechanism for tolerance to salt stress in *S. salsa*. The two isoforms of GAUT1 and GAUT7 which are a type transffered galacturonic acid from uridine 5'-diphospho-galacturonic acid into the pectic polysaccharide homogalacturonan of α-1,4-galacturonosyltransferase constituting the main components

of cell wall pectin biosynthetic homogalacturonan galacturonosyltransferase complex induced under salinity in S. salsa. Homogalacturonan (HG) synthesis is mediated biosynthetically by GAUT1 in plants and increased up to five-fold under salinity suggesting an enhancement in lignin production that may solidify the cell wall as a sign for tolerance to salt stress in *S. salsa*.

Degradation, modification and redox homeostasis proteins

The turnover, sub-localization and post-translational modification of proteins appear to be essential parts of salt resistance in halophytes (.Zhang, Zhang, Lin, Zheng and Yang 2013). The ubiquitin-proteasome system positively regulator stress tolerance in plants (.Dametto et al. 2015). According to our data from the seedlings, the highly significant induction of transcripts encoding ubiquitin-fold protein conjugation connected to the ubiquitin-proteasome system was observed, indicating a positive regulator of salt stress tolerance. Phosphorylation is post-translational modifications (PTMs) processes, that enables fast improvement, regulation, or determination of protein function in response various environmental changes (.Ruggiero et al. 2019). Protein kinases (PKs) of receptor-like kinases (RLKs), mitogen-activated protein kinases (MAPKs) and receptor protein kinases (RPKs) activate and sense external signals playing crucial roles in response to salt stress. The Ca^{2+} signaling pathway-related proteins like calcineurin B-like proteins (CBLs), calcium-binding proteins (CMLs), calcium-dependent protein kinases (CDPKs), and CBL-interacting protein kinases, (CIPKs) activate intracellular signaling cascades complex and generation of Ca^{2+} as the second messengers. It has been shown that Arabidopsis CPKs regulate ABA signaling and the activate of ion channels in seedling growth as a mechanism, by which Arabidopsis responds to salt stress. In the present work, most of the signal transduction-related transcripts encoding PKs and RPKs were induced under salinity. It was previously shown that PKs and PRKs activated intermediate proteins that regulate H_2O_2 homeostasis and thereby enhancing salt tolerance in rice (.Zhou et al. 2018). These regulations control reactive oxygen species (ROS) generation, sense deviation from and readjust of the cellular redox state (.Huang et al. 2009, Jiang et al. 2016). The GPD-L-galactose phosphorylase (GGP) is a candidate gene for engineering ascorbate accumulation in plants. Given, overexpression of GGP genes significantly increased ascorbate accumulation in potato (*Solanum tuberosum*) and rice and alleviated salt- and drought-induced oxidative stress. Herein, salt stress induced biosynthesis of ascorbate catalyzing by two enzymes GDP-L-galactose phosphorylase 1 (GGP1) and GDP-L-galactose phosphorylase2 (GGP2) suggesting the important roles of these genes in sensing of external factor and readjustment of the cellular redox for the effective removal of ROSs in *S. salsa*. Several autophagy components such as ATG18, ATG8-maturation peptidase (ATG4), and autophagic cargo receptor protein (NBR1) were up-regulated under salt stress. Autophagy maintains cellular homeostasis in response to stress and contributes to nutrient remobilization in plants (.Luo et al. 2017). Several autophagy-specific ubiquitin-like proteins members such as ATG18 were shown to be improved salt stress in *Arabidopsis thaliana* and emmer wheat (.Sun et al. 2018). Further study indicated that RNAi-ATG18A Arabidopsis are more sensitive to salt and osmotic treatments indicating that ATGs may alleviate stress damages in *S. salsa* under salt stress condition.

Transcription factors

TFs as sequence-specific DNA binding proteins regulate the expression pattern of genes in response to specific signals of various stressors. In this work, various transcription factors (TFs) related to families of WRKY, AP2-like ethylene-responsive transcription factor (AP2/ERF), MYB, MADS-box transcription factor (MADS-box), NAC, and nuclear transcription factor Y (NF-Y) TF families were induced under salinity stress. Several ethylene-responsive transcription factor (ERFs) with conserved AP²/ERF DNA-binding domain were prominently induced under salt stress that suggest ERF TF family positively regulates *S. salsa* responses to salt stress (Table 3). The role of MADS-box genes in regulation of gene regulatory metabolic pathways was previously described in response to different stresses in plants (.Castelán-Muñoz et al. 2019, Saha, Sengupta, Gupta and Gupta 2015). It has been previously shown that the expression of some plant MADS-box genes confers enhancements of various stress tolerances in transgenic plants by improving water content capacity and decreasing the water loss rate (.Castelán-Muñoz, Herrera, Cajero-Sánchez, Arrizubieta, Trejo, García-Ponce, Sánchez, Álvarez-Buylla and Garay-Arroyo 2019). In this study, many MADS-box genes were highly induced upon salinity stress that suggests their crucial roles in salt tolerance. WRKYs can be induced various abiotic stresses and phytohormones of ethylene and abscisic acid (ABA) (.Gao et al. 2020). Overexpression of WRKY genes has been shown that successfully enhanced drought and salt stresses tolerance in Chrysanthemum. Niu et al (2012) showed that transgenic Arabidopsis carrying the *TaWRKY2* genes conferred to higher salt and drought tolerance (.Niu et al. 2012). We found here that several members of WRKY's family were induced indicating that WRKY family may play important role in responding to different salinity levels in *S. salsa* (Table 3). Many reports have shown that nuclear transcription factor (NF-Y) regulate growth, development and respond to a variety of abiotic stresses in *Paspalum vaginatum* (.Wu et al. 2018), maize (.Kimotho et al. 2019), and wheat (.Chen et al. 2015, Ma et al. 2015). Further roles of NF-Y were shown through modulating gene regulation in both ABA-dependent and ABA-independent pathways in bermudagrass in response to salt and drought stress (.Chen, Zhao, Zhuo, Lu and Guo 2015). Several NF-Y transcripts were induced in our analysis under salt stress suggesting that the induction of a series of NF-YC1 TF probably enhanced ABA sensitivity and subsequently the salt tolerance response in *S. salsa* under salinity stress. NAC proteins families are involved in regulating responses to high salinity stress (.Dudhate et al. 2021, Yuan et al. 2020). NACs have been shown to be involved in salt stress response in wheat and *Arabidopsis thaliana* (.Dudhate, Shinde, Yu, Tsugama, Gupta, Liu and Takano 2021). In current study, NAC transcription factors were highly induced under salinity which is consistent with previous observation in the halophyte *S.liaotungensis* and indicating an engagement in salt stress response under salinity stress *S. salsa*.

Conclusion

Based on our findings, we have proposed a model for salt stress tolerance in *S. salsa* in responding to salinity stress (Fig. 7). Our transcriptome-wide analysis of *S. salsa* seedlings focused on phytohormones that trigger the salt stress response, signaling pathways and cell wall mechanism responding to salt stress, transcription factors necessary for cell signal transduction, and ubiquitin-proteasome system enzymes vital for protein turnover and systems carrier implicated in transports and hemostasis. *S. salsa* triggers signal downstream through induction of transcripts encoding ACO, flavin monooxygenase

(YUCCA), and auxin transporter (PIN) involved phytohormones biosynthesis and signal molecule such as Ca^{2+} and also ROS (reactive oxygen species). The induction of transcripts encoding protein kinases (PKs) and Ca^{2+} signaling pathway-related proteins such as CBLs, CMLs, CDPKs, and CIPK suggest active signal transduction triggered by ROS production under salt stress conditions in *S. salsa*. The DEGs were observed by genes coding transcription factors such as ERFs, WRKYs, NACs, and bZIPs regulating ROS generation and scavenging, nutrient and solute transport, protein hemostasis, and turnover at the transcriptional level. Additionally, the expression level of genes related to lignin-monolignol biosynthesis, homogalacturonan, hydroxyproline-rich glycoprotein activities, and cutin polyester biosynthesis increased under salinity stress suggesting the solidification of the *S. salsa* cell wall as a critical factor for tolerance to salt stress. These abilities suggest that *S. salsa* is an excellent candidate for genes to be implemented in breeding programs to enhance crop stress tolerance.

Declarations

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Ethical Approval

not applicable

Competing interests

The authors declare no conflict of interest.

Author contribution

Conceptualization (MRG), performance of the research (SJ), data analysis (SJ, MRG, GHS, NAKS, MZ), writing—original draft preparation (SJ), writing—review and editing (MRG, MRA, NAKS, MZ, GHS), data visualization (SJ, MRG, LKF). All authors have read and agreed to the published version of the manuscript.

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Figures

Figure 1

S. salsa seedlings in response to salinity stress at 0 mM, 200 mM, 400 mM, 600 mM, and 800 mM NaCl levels 30 days after treatment

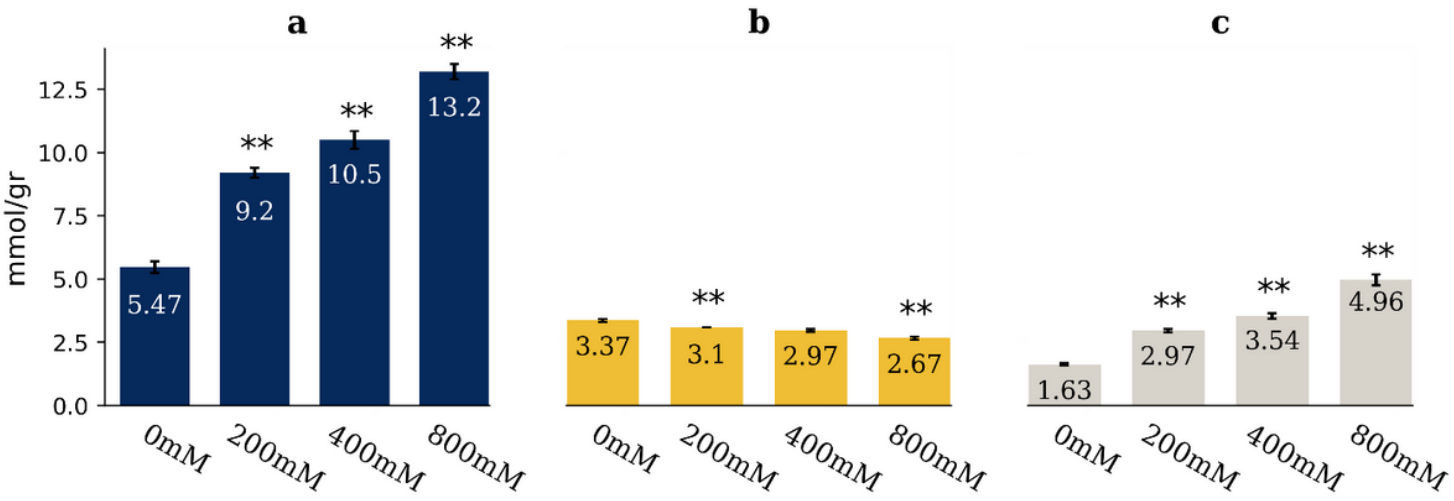


Figure 2

Impacts of salinity stress on the Na^+ content (a), K^+ content (b), and Na^+ to K^+ ratio (c) in *S. salsa*. Seedlings were exposed to 0 mM, 200 mM, 400 mM, and 800 mM NaCl for 30 days

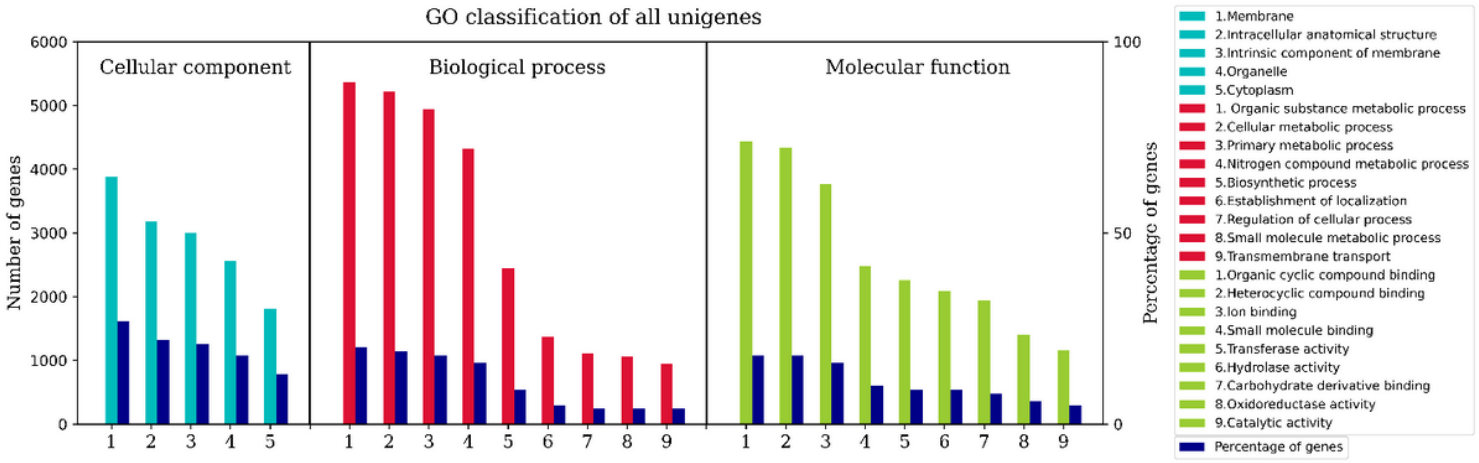


Figure 3

Gene ontology (GO) classifications in *S.salsa*. The percentage indicate the proportion of transcripts with the GO annotations

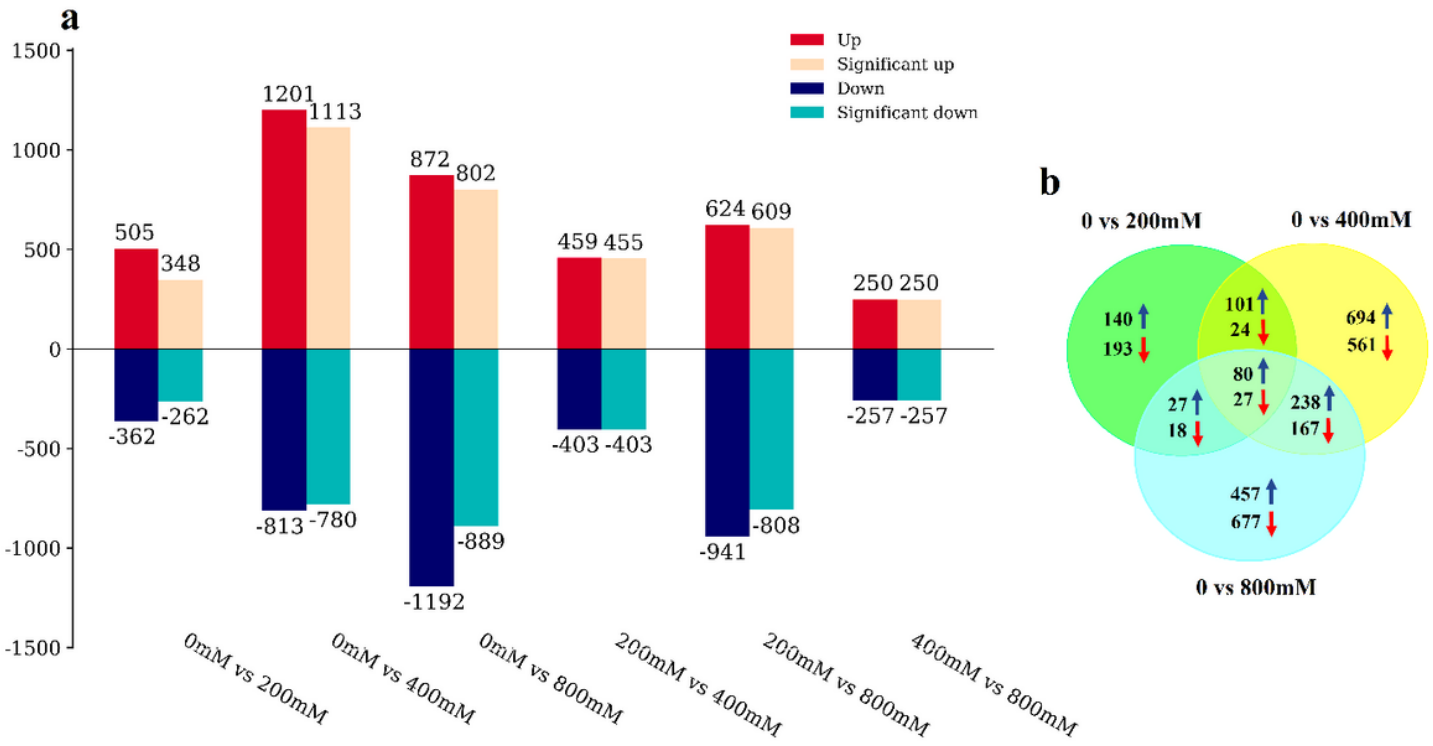


Figure 4

The number of differentially expressed genes. (a) The number of upregulated and downregulated differentially expressed genes (DEGs) are shown in blue and red colors respectively at 0 mM, 200 mM, 400 mM and 800 mM and their comparisons. (b) Total up and down-regulated expressed genes at 0 mM vs 200 mM, 0 mM vs 400 mM and 0 mM vs 800 mM

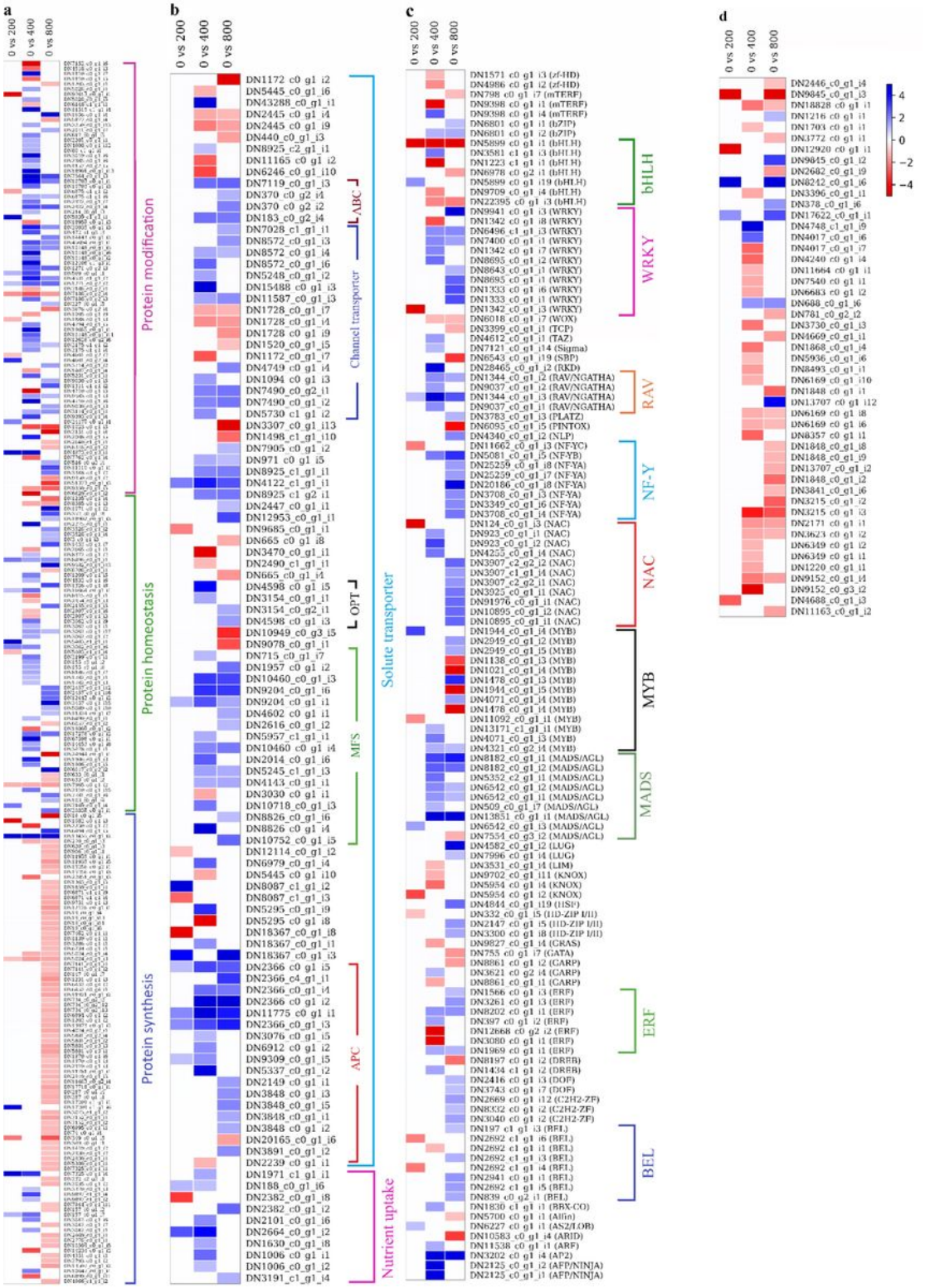
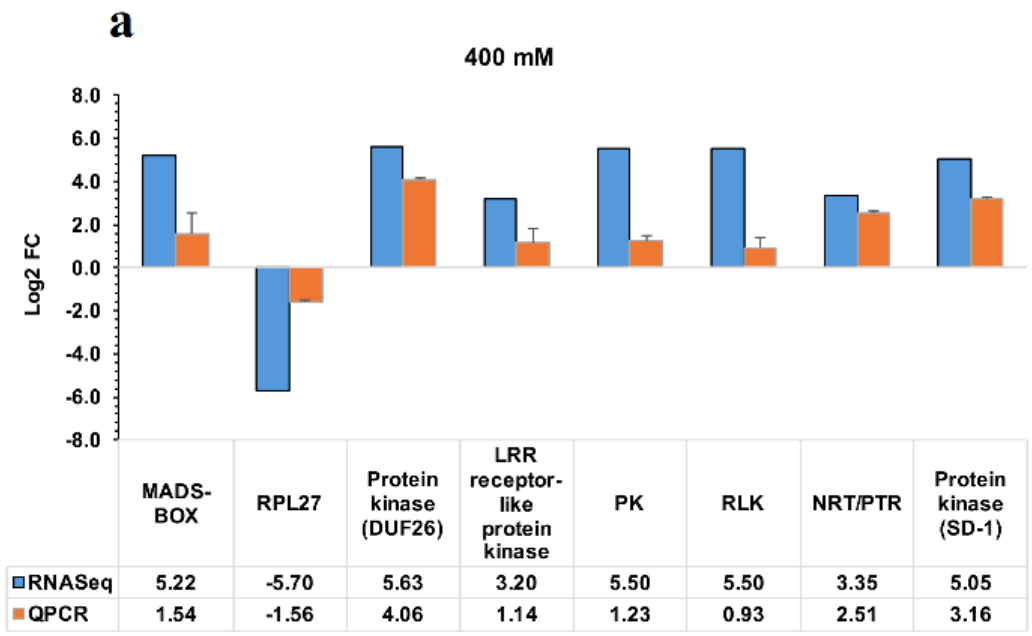
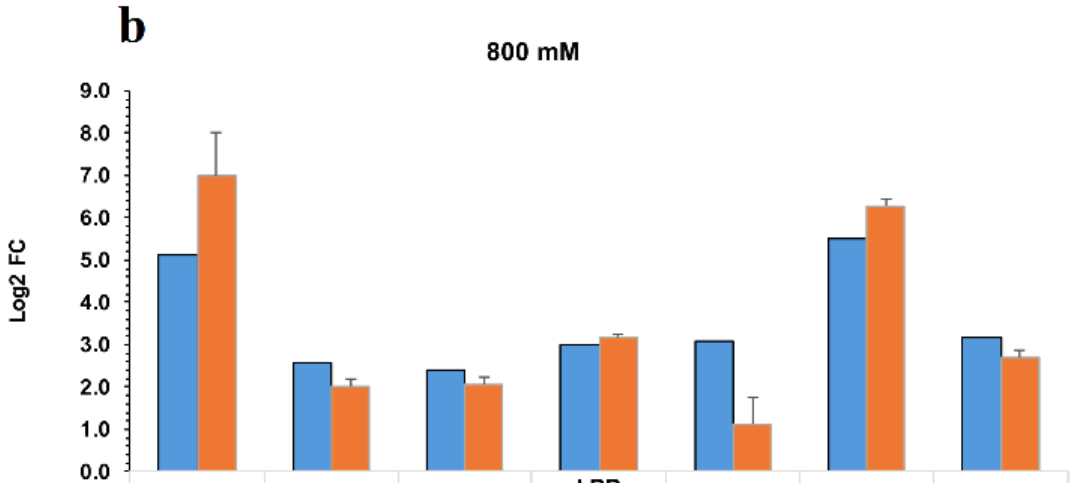


Figure 5

Differentially expressed genes in five functional pathways based on Mapman ontology under salt stress. a) Protein metabolism b) solute transport c) transcription factor d) Cell wall organization. Red and blue color show up-regulation and down-regulation respectively



	MADS-BOX	RPL27	Protein kinase (DUF26)	LRR receptor-like protein kinase	PK	RLK	NRT/PTR	Protein kinase (SD-1)
RNASeq	5.22	-5.70	5.63	3.20	5.50	5.50	3.35	5.05
QPCR	1.54	-1.56	4.06	1.14	1.23	0.93	2.51	3.16



	MADS-BOX	WRKY	Protein kinase (DUF26)	LRR receptor-like protein kinase	RLK	endopeptidase	NRT/PTR
RNASeq	5.1	2.6	2.4	3.0	3.1	5.5	3.2
QPCR	6.99	2.03	2.04	3.15	1.11	6.26	2.69

Figure 6

Validation of RNA-Seq results by qRT-PCR. Differentially expressed genes are included MADS-Box, RPL27, protein kinase (DUF-26), LRR receptor like protein kinase, PK, PLK, NRT/PTR, protein kinase (SD-1), WRKY and endopeptidase

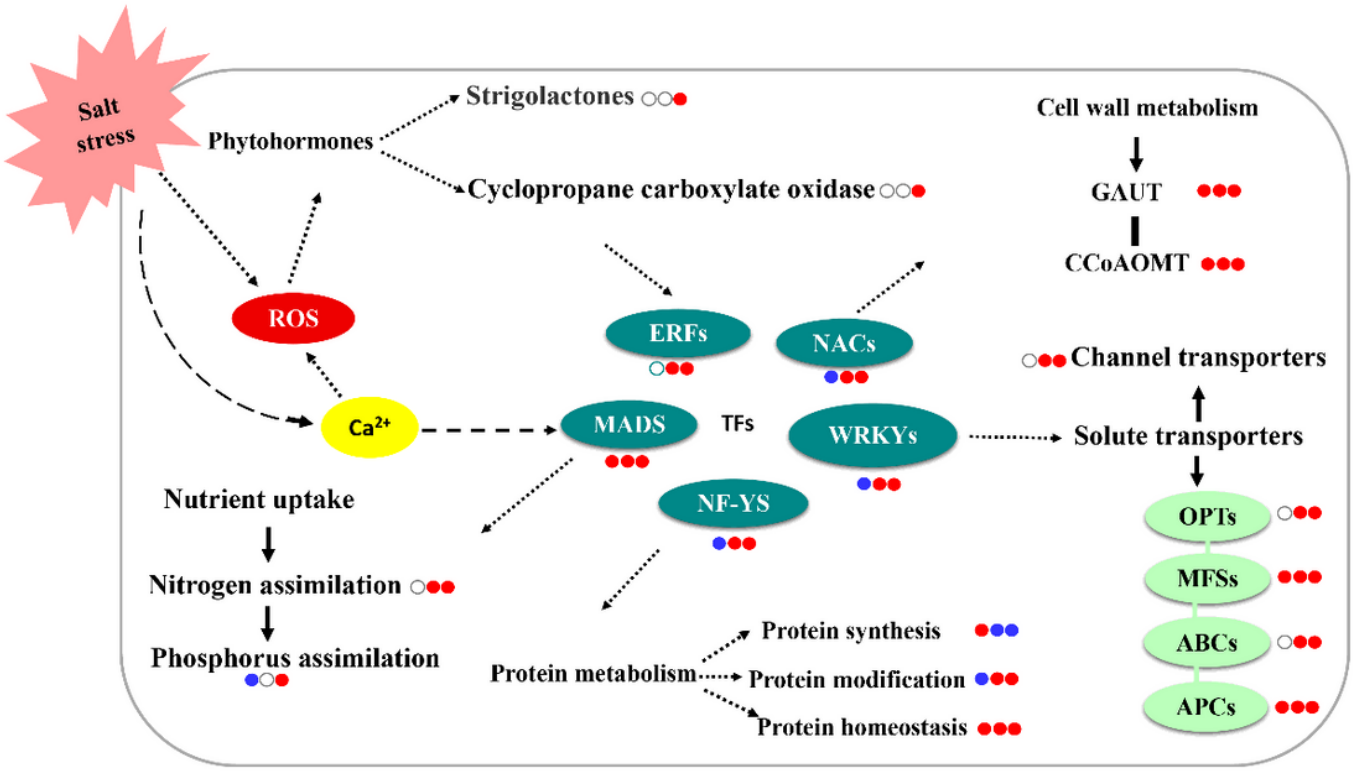


Figure 7

A proposed model of salt-stress response in *S. salsa* at cellular level. A proposed model of salt-stress response in *Suaeda salsa* for 30 days at different salinity levels. Red and blue circles indicate significant up-regulations and down-regulations respectively. White circle shows non-significant regulations. Left to right circles are 200 mM, 400 mM and 800 mM Na Cl salinity levels

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.xlsx
- TableS2.xlsx