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*Original Research*

# Physio-chemical and co-expression network analysis associated with salt stress in sorghum

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**Abstract**

**Background**: Abiotic stress can damage crops and reduce productivity. Among them, salt stress is related to water stress such as osmosis and ions, and like other abiotic stresses, it can affect the growth of plants by changing gene expressions. Investigating the profiles of gene expression under salt stress may help us understand molecular mechanisms of plants to cope with unfavorable conditions. **Results**: To study salt tolerance in sorghum, physiological and comparative transcriptomic studies were performed using a Korean sorghum cultivar 'Sodamchal' which is considered sensitive to soil salinity. In this study, the samples were treated with two concentrations of NaCl [0 (control) and 150 mM], and the leaves and roots were harvested at 0, 3, and 9 days after the treatment. For the physiological study, the levels of anthocyanin, proline, reducing sugar, and chlorophyll were evaluated in the control and the treatment group at each sampling point. The results show that the cultivar 'Sodamchal' has salt-susceptible profiles. We also analyzed the transcription profile in the presence of 0 and 150 mM NaCl to confirm the candidate genes under the saline stress condition. Between the control and salt treatment, we found a total of 1506 and 1510 differentially expressed genes (DEGs) in the leaves and roots, respectively. We also built a gene co-expression network to determine the association of the candidate genes in terms of biological pathways. **Conclusions**: Through the co-expression network, genes related to salt stress such as AP2/ERF and Dehydrin were identified. This study provides the physiological and genic markers that could be used during intense salt stress in sorghum. These markers could be used to lay the foundation for the distribution of high-quality seeds that are tolerant to salt in the future.

**Keywords:** *Sorghum bicolor*; salt stress; QuantSeq; DEG; co-expression network

## 1. Introduction

Plants can face multiple stress environments at the same time during a variety of developmental stages. Abiotic stresses such as salinity, heat, drought, and cold could do great damage to agriculture worldwide which could lead to a loss of production [1,2]. The effects of these abiotic stresses are expected to be exacerbated by global climate changes [3]. In order for plants to adapt to these specific abiotic stress conditions, they need specific reactions to the environment they face. Therefore, molecular, biochemical and physiological processes that are perturbed by specific conditions of stress may be activated differently based on the environment variables [4,5].

Salinity stress is one of the major abiotic stresses in global agriculture which has a major impact on the environment [6,7]. Approximately 20% of the arable land and 50% of all irrigated land are affected by salt stress [7,8]. The effects on plants when treated with high concentrations of salt can reduce productivity or cause death at the overall plant level. When plants are exposed to salinity, substances such as proteins are synthesized, and energy is generated, and lipid metabolism and photosynthesis are also affected [9]. Prolonged exposure to salts induces hyperosmotic stress, which leads to physiological problems. In other words, it severely impairs metabolic functions such as photosynthesis, ion homeostasis and hormones of plants and affects plant development and growth [10,11]. Because of this, many plant species have developed mechanisms to remove salt from cells and to tolerate the presence of salt in cells which sequestrate saline stress [12]. Plants maintain ion homeostasis and compartmentalize in order to adapt to such hyperosmolarity, and respond to salt stress in various ways and strategies that plants can cope with. This problem stabilizes cell structure, membrane, and distribution of ions when ROS is removed [13,14]. Stress-responsive genes are considered one method with which plants can tolerate saline stress [15,16].

Sorghum (*Sorghum bicolor* [L.] Moench) is an African-origin grain crop cultivated in the semi-arid or arid areas of the world. Sorghum, the so-called camel of the field, is commonly known to be a salt- and drought-tolerant plant compared to other crops [17]. Therefore, it grows well in areas where salt exists in soil, so it can be used in research as a model species related to osmotic stress such as drought

stress and salt stress given that the mechanisms for those two stresses have crosstalk. Several studies have screened sorghum varieties for salt tolerance genotypes [18,19].

The concept of gene co-expression networks was first introduced in 1999 by Butte and Kohane [20]. The co-expression network collects data from plant experiments and computes pairs of data that are highly correlated with the results from each test based on the Pearson correlation. Utilizing this approach, Butte and Kohane built the first gene co-expression network with gene expression data, using them as a measure of co-expression which can be applied to a variety of studies related to gene expression. Generally, gene expression networks are built in two steps. First, a measure of the co-expression is selected, and the values are used to calculate a score for the similarity of each pair of genes. Then, as the determined threshold, pairs of genes with scores that have a higher similarity than the selected threshold are considered to have a co-expression relationship, and finally, they are linked to an edge in the network. The main purpose of building co-expression networks is to infer the function of unknown genes and to generate related hypotheses and associations [21].

The purpose of this study was to evaluate the response to various physiological and transcriptomic changes in a salt-sensitive Korean sorghum cultivar 'Sodamchal' ('chal' means sticky in Korean, so this cultivar is a sticky sorghum). Plants develop a variety of physiological and biochemical mechanisms to survive in soils with high salt concentrations [22]. This paper characterizes plant susceptibility in a physiological context and discusses in detail the response of plants to the expected and predicted effects of salt stress. We measured anthocyanins, proline, reducing sugar, and chlorophyll under different conditions over a time period of about one week as the best physiological method for assessing the response to salt stress. We also profiled differentially expressed genes using QuantSeq technologies and analyzed gene co-expression networks, supported by those physiological parameters associated with salt stress.

## 2. Materials and methods

A salt-sensitive Korean sorghum cultivar, Sodamchal, was used as the plant material in this study. Sodamchal seeds were obtained from the Center of Agricultural Genetic Resources (Rural Development Administration, Jeonju, Korea). The seeds were sterilized overnight for 24 hours and planted in 32-hole pots in a greenhouse at Chungnam National University (CNU), Republic of Korea. The first leaves of the seedlings were grown and transferred to new pots filled with sterilized vermiculite. The plants were hydroponically grown using half-strength Hoagland solution.

Salt was present for 7 days after transplanting the seedlings by adding NaCl to a final concentration of 150 mM to the Hoagland solution, and the concentration was maintained throughout the experiment by measuring the electronic conductivity. The solution without NaCl was set as the control under the same growth conditions. All the treatments and controls were biologically replicated three times. The treated seedlings were sampled at intervals of 0, 3, and 9 days after the treatment (DAT). Samples from the leaves and roots were harvested and immediately frozen in liquid nitrogen and stored at –80 °C until use.

### *2.1 Physio-chemical analysis*

#### 2.1.1 Quantification of the anthocyanin contents

A 300 mg sample of sorghum leaves was ground using a mortar and pestle with three biological replicates. It was mixed with five volumes of extraction buffer (45% methanol and 5% acetic acid). The mixture was spun down at room temperature at 12,000 g for 5 min, and the supernatant was transferred to a new tube. Absorbance was measured at 530 and 657 nm with a nano-MD UV-Vis Bio Spectrophotometer (Scinco, Korea), and the anthocyanin contents were calculated based on the following formula [23]:

Anthocyanin contents (mg/g) = Abs530/g F.W. [Abs530 – (0.25 × Abs657)] × 5

#### 2.1.2 Quantification of the reducing sugar contents

First, a standard curve of sugar concentration using a glucose standard solution was generated based on a linear regression model. A total of 7 mL of distilled water and 2 mL of Dextrose Normal Saline (DNS) solution were used as the extraction buffer. First, 300 mg of frozen leaves were ground with a mortar and pestle in three biological replicates. The ground tissues were mixed with the extraction buffer and heated in a glass test tube in a 95 °C water bath for 5 min. The mixture was immediately cooled down in ice water for 10 min. The absorbance was measured at 570 nm using a nano-MD UV-Vis Bio Spectrophotometer (Scinco, Korea).

#### 2.1.3 Quantification of the proline contents

A total of 50 mg of frozen leaf tissue was ground using a mortar and pestle in three biological replicates. The powder was mixed with 1 mL of 40% ethanol, and it sat overnight. On the next day, the mixture was centrifuged at 12,000 g for 5 min, and 500 $\mu$L of the supernatant were transferred to a new tube. Then, 1 mL of the reaction mixture (1% ninhydrin in 60% acetic acid and 20% ethanol) was added to the mixture, and the tube was heated in a 95 °C water bath for 20 min [24]. The absorbance was measured at 530 nm using a nano-MD UV-Vis Bio Spectrophotometer (Scinco, Korea). The quantification of proline was done with a standard curve of proline standard solutions based on a linear regression model.

#### 2.1.4 Quantification of the chlorophyll contents

First, 300 mg of frozen leaf tissue (partially dehydrated by soaking in 95% ethanol for 2–5 minutes if the wa-

ter content of the leaves was high) were ground in a mortar and pestle with three biological replicates. Then, 5 mL of 80% acetone were used for extracting the chlorophylls from the ground tissues in a 15 mL falcon tube in the dark with a Hula mixer (Thermo Fisher scientific, USA) for 15–30 min. The mixture was centrifuged at 4 °C for 15 min at 3000 rpm, and the supernatant was transferred to a new tube. Untra violet absorbance was measured at 645 and 663 nm using a nano-MD UV-Vis Bio Spectrophotometer (Scinco, Korea). The concentrations of the chlorophyll were calculated as follows [25]:

Chlorophyll a (mg/g) = [12.7 × A663 – 2.69 × A645] × V/1000 × W

Chlorophyll b (mg/g) = [22.9 × A645 – 4.86 × A663] × V/1000 × W

Chlorophyll total (mg/g) = [8.02 × A663 + 20.20 × A645] × V/1000 × W

V = volume of the extract (mL); W = Weight of fresh leaves (g)

### *2.2 Statistical analysis*

Statistical comparisons were performed based on one-way ANOVA analysis using Tukey's HSD. Variables in the physiological analysis were expressed using the means ± standard error of the means (SEM), and a *p*-value was considered significant if $<0.05$. The tests performed in all experiments were analyzed by SPSS version 26 for Windows (SPSS, Chicago. IL, USA).

### *2.3 RNA extraction*

Total RNA was isolated from the leaves and roots of the sorghum cultivar Sodamchal treated with 150 and 0 mM (control) of NaCl at 3 DAT using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. The RNA quality was evaluated using an Agilent 2100 bioanalyzer with an RNA 6000 Nano Chip (Agilent Technologies, Amstelveen, Netherlands), and the extracted RNA was quantified using an ND-2000 spectrophotometer (Thermo Inc., DE, USA).

### *2.4 QuantSeq library construction and sequencing*

QuantSeq is an economic RNA sequencing tool for quantitative analysis of expression bodies. QuantSeq generates high-strand-specific next-generation sequencing (NGS) libraries that appear close to the 3' end of polyadenylated RNA in less than 4 hours. It is advantageous for gene counting because it captures the 3'-end more efficiently. This reduces the data analysis time and enables a higher level of multiplexing per run, indicating that this method is suitable for the gene expression profiling among specific treatment groups in plants.

The library was constructed using the QuantSeq 3'mRNA-Seq Library Prep Kit (Lexogen, Inc., Austria) according to the manufacturer's instructions. Briefly, 500 ng of total RNA were hybridized with oligo-dT primers, and reverse transcription was done. The oligo-dT primer contains Illumina compatible sequences at the 5'-end. Synthesis of the second strand was initiated by random primers containing Illumina compatible linker sequences at the 5'-end after the RNA template was digested. Magnetic beads were used to remove all reactive components of the double-stranded library. The library was amplified to add the sequence of the entire adapters needed to create the cluster. High-throughput sequencing was performed with single-ended 75 cycle sequencing using NextSeq 500 (Illumina, Inc., USA). QuentSeq data for the untreated control and salt-treated samples were obtained from 2 biological replicates for each sample. RNA quality check was performed using the Bioanalyzer 2100 System. The cDNA library was made with the NEBNext Ultra II Directional RNA - Seq Kit (NEW ENGLAND BioLabs, Inc., UK) and sequencing was performed with Hiseq X10 (Illumina, Inc., USA) as paired-end 100. Trimmed to Average Q20 using BBDuk and mapped using TopHat (https://tophat.com/) to reference genome computation FPKM using Cufflink [26]. Quantile normalization was performed using EdgeR [27] and comparison was performed with DEG master file production using ExDEGA (https://www.e-biogen.com/product3.php).

### *2.5 Data analysis*

Bowtie2 [28] was used to align the QuantSeq reads. Bowtie2 indexes were created on representative transcript sequences to align with the reference genome. Alignment files were used for assembling the transcripts to estimate the abundance of the transcripts and to detect the differentially expressed values of the genes. The differentially expressed genes were used as a standard for a range of applications implemented in the Bed tool [29] and were determined based on the coefficients of multiple and unique alignments. The RC (Read Count) data were processed with the quantile normalization method using Edge R, which is found within the R (https://www.r-project.org/) software. Gene classification was performed by DAVID (https://david.ncifcrf.gov/) and based on the search within the program. The sequencing results for QuentSeq such as the numbers of processed reads, the numbers of mapped reads, and mapping percentages are presented in **Supplementary File**. As the reference genome, the genome of *Sorghum bicolor* of NCBI (https://www.ncbi.nlm.nih.gov/) was used. Gene expression sets are provided under the NCBI's accession numbers (SAMN24813607, SAMN24813608, SAMN24813609, SAMN24813610, SAMN24813611, SAMN24813612).

### *2.6 Functional annotation and pathway analysis of the DEGs*

Gene Ontology (GO) terms were determined based on AgriGO (http://bioinfo.cau.edu.cn/agriGO/) at a *p*-value $<$ 0.05, and duplicate GO terms were removed using REVIGO (http://revigo.irb.hr/). Three GO terms, categorized as biological processes, molecular functions, and cellular com-

ponents, were assigned to query sequences to generate an overview of the genes categorized by each transcript into groups [30].

The KEGG pathway was also searched by assigning DEGs on the online KEGG web server (http://www.genome.jp/kegg/) at a $p$-value $< 0.05$ and then find the related gene ID [31].

### *2.7 Construction of the gene co-expression network*

QuantSeq read counts were analyzed to construct a gene co-expression network and normalized using DESeq in R package [32]. Genes with QuantSeq read counts of 0 or no information in all replications were removed from the analysis. Gene co-expression networks of differentially expressed genes (DEG) were constructed for the leaves and roots, in both normal and salt stress conditions. The nodes represent genes, and the edges represent the co-expression level for a pair of genes at the expression level, which show the correlation value of the Pearson correlation coefficient. Genes with a Log2 value of 5 or more (for up-regulated DEGs) or –3 or less (for down-regulated DEGs) were selected, and genes associated with the surroundings nodes were further selected. The result was visualized as a graph with a positive or negative correlation score.

### *2.8 Quantitative real time PCR (qRT-PCR) validation*

Quantitative real-time PCR with four and two DEGs from the leaves and roots was done to validate the QuentSeq results. Six genes were selected, and total RNA was extracted using three biological replications from the samples of the Sodamchal leaf and root for the control and the NaCl treatment group at 3 DAT. Primer pairs were designed using the Primer3 (v.0.4.0) software (https://bioinfo.ut.ee/primer3-0.4.0/) available in the NCBI database (Table 1). cDNA synthesis was performed using the Compact cDNA Synthesis kit (Smart Gene, Korea) according to the manufacturer's instructions. The qRT-PCR analysis was performed using the SYBR green Q-PCR Master mix (Smart Gene, Korea) and the CFX Connect™ Real-Time PCR Detection System (BIO-RAD, USA). For the volume of qRT-PCR, a total of 20 $\mu$L including 2 $\mu$L of forward and reverse primers (100 nM), 2 $\mu$L of cDNA, 10 $\mu$L of 2 $\times$ SYBR Green qPCR master mix, and 4 $\mu$L of ddHO was used for the reaction. The PCR reaction was programmed at 95 °C for 10 min with a three-step cycle of template denaturation and enzyme activation. It was then programmed for 40 cycles with denaturation at 95 °C for 15 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C for 30 seconds. The PCR data values were used for analysis by normalizing the mean of three biological replication values using the mean Ct value of the Housekeeping gene. The representation values were measured using the delta dealt Ct (DDCT) method [33]. The values shown in Fig. 1 are calculated as follows:

Fold gene expression = $2^{-\Delta\Delta Ct}$

$\Delta\Delta Ct = \Delta Ct$ (Sample) – $\Delta Ct$ (Control)

**Table 1. Primer information for qRT-PCR analysis.**

| Gene Name | Primer sequences (5′-3′) | |
|---|---|---|
| | Forward primer | Reverse primer |
| LOC8060409 | tcgcttactacccgatggac | aagactgacctcgcattcgt |
| LOC8077913 | ccacaaggacaaccagcac | ccatgatgccttttcttctcg |
| LOC8074608 | tccatgttcggattcttcgt | cacgaagttggtggcgtag |
| LOC8072351 | tccatgttcggattcttcgt | cacgaagttggtggcgtag |
| LOC8079279 | ggaacgaggtgctcctacag | gtcgagtggtcttggctctc |
| LOC8075945 | catcatccgtcctcgtcttc | cgcagttcctcttgctcag |

$\Delta Ct = Ct$ (gene of interest) – Ct (housekeeping gene)

## 3. Results

### *3.1 Effects of salt stress on the growth parameters*

Sodamchal, known as a susceptible cultivar of sorghum, was grown in a greenhouse under a salt stress condition. At three and nine days after treatment (DAT), a visual comparison was conducted in the presence of 150 mM NaCl with the control which was not treated with NaCl (Fig. 2). In the presence of 150 mM NaCl, the growth of Sodamchal was affected and inhibited by salt stress: reduced growth and chlorosis were observed in the 150 mM NaCl treatment group compared to the control. In comparison with the control, we can see that the growth of the Sodamchal increased over time. Despite the severe salt stress (150 mM NaCl), the sorghum cultivar still gradually grew, indicating that the sorghum has a certain level of ability to tolerate salt stress even if Sodamchal is known as a salinesusceptible cultivar.

### *3.2 Effects of salt stress on the quantity of anthocyanin*

The anthocyanin contents were determined when exposed or not exposed to saline stress in the leaves at 0, 3, and 9 DAT (Fig. 3a). Anthocyanin tended to be increased significantly at 3 DAT in both the control and 150mM NaCl sample. However, it was significantly reduced at 9 DAT compared to 3 DAT. Interestingly, anthocyanin decreased to 41.4% at 9 DAT in the control whereas the salt stressed sorghum at 9 DAT showed almost the same amount as that of the 0 DAT. In fact, the decrease of the anthocyanin contents at 9 DAT in the 150 mM treatment group was slightly more than that of the control (by 44.6%), indicating that anthocyanin may not have positive effects on salt stress in this sorghum cultivar.

### *3.3 Effects of salt stress on the reducing sugar expressions*

Reducing sugars can act as osmoprotectants under salt stress conditions; thus, they are considered as a parameter that can evaluate the intensity of salt stress on plants. The effect of 150 mM NaCl on the reducing sugar contents of Sodamchal was also determined in this study (Fig. 4b). The reducing sugar contents of Sodamchal was not statistically changed in the control. On the other hand, in the 150

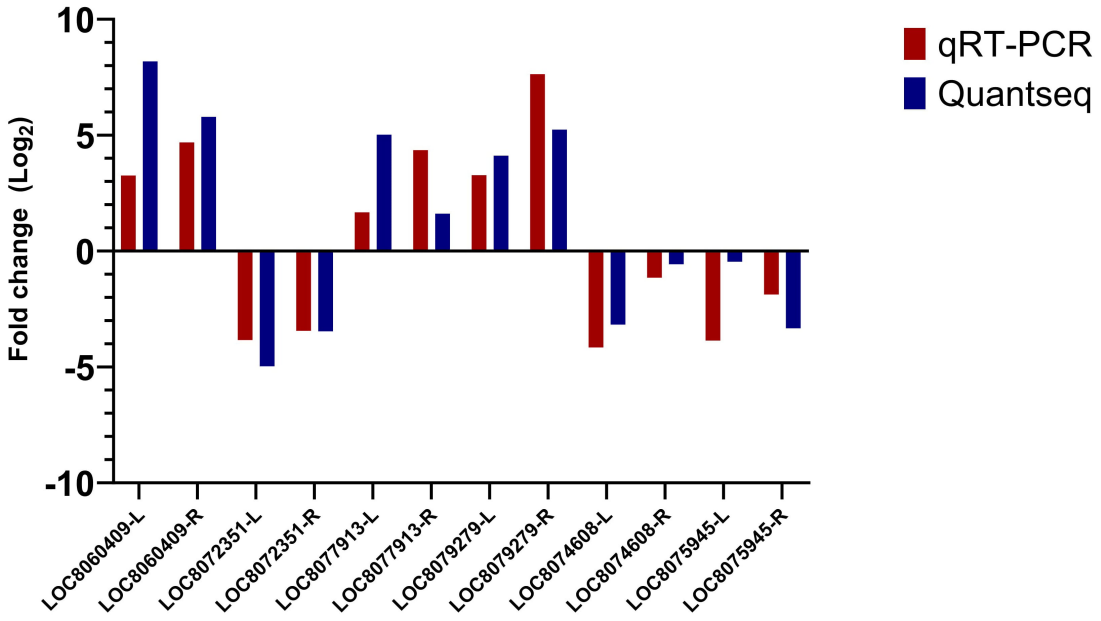


**Fig. 1. Validation of selected genes using qRT-PCR.** L and R represent the leaf and root of the plant. Histograms show the relative values for concordance of gene expression patterns between control and 150 mM NaCl.

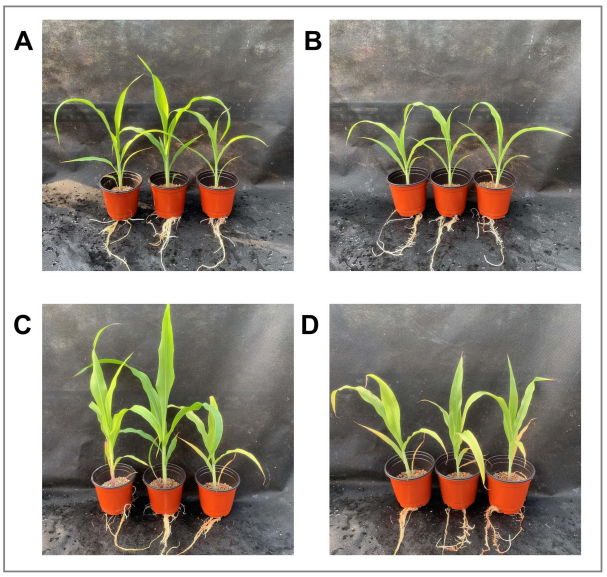


**Fig. 2. Phenotype with untreated control and 150 mM NaCl at three and nine days after treatment in Sodamchal.** (A) Day three control. (B) Day three treated with 150 mM. (C) Day nine control. (D) Day nine treated with 150 mM.

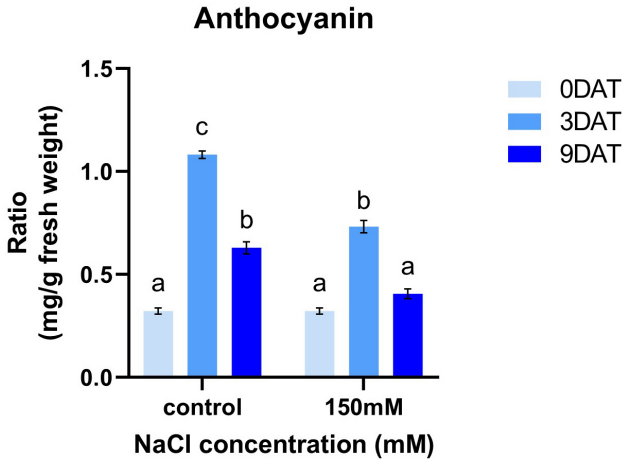


**Fig. 3. Anthocyanin expression in leaves between untreated control and 150 mM NaCl treatment of Sodamchal at 0, 3, and 9 DAT.** Values represent mean ± SEM of three replicates. Letters on top of bars indicate significant differences by a one-way analysis of variance followed by a test of Tukey HSD ($p < 0.05$). Bars with the same letter are not significantly different at $p = 0.05$.

mM treatment group, the contents were not significantly changed at 3 DAT, but they gradually decreased over time by 27.1% at 9 DAT.

*3.4 Effects of salt stress on the proline expressions*

Proline showed a clear difference between the control and the treatment group. Fig. 5c shows the proline contents during the saline stress treatment. A noticeable point is that the saline stress significantly changed the level of proline in Sodamchal (increased up to 46.5% from 3 DAT to 9 DAT) while the control did not show any statistical changes. This result will be further discussed later in this article, but the level of proline tends to increase under abiotic stress conditions regardless of the susceptibility/tolerance of a plant [34], indirectly indicating that the proline contents can also be used to evaluate the intensity of salt stress.

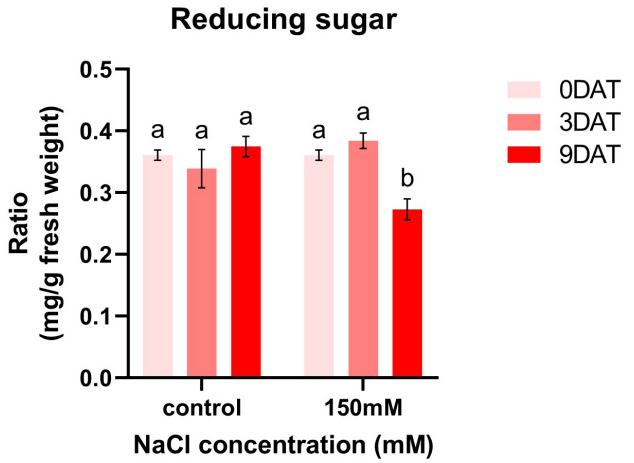


**Fig. 4. Reducing sugar expression in leaves between untreated control and 150 mM NaCl treatment of Sodamchal at 0, 3, and 9 DAT.** Values represent mean ± SEM of three replicates. Letters on top of bars indicate significant differences by a one-way analysis of variance followed by a test of Tukey HSD ($p < 0.05$). Bars with the same letter are not significantly different at $p = 0.05$.

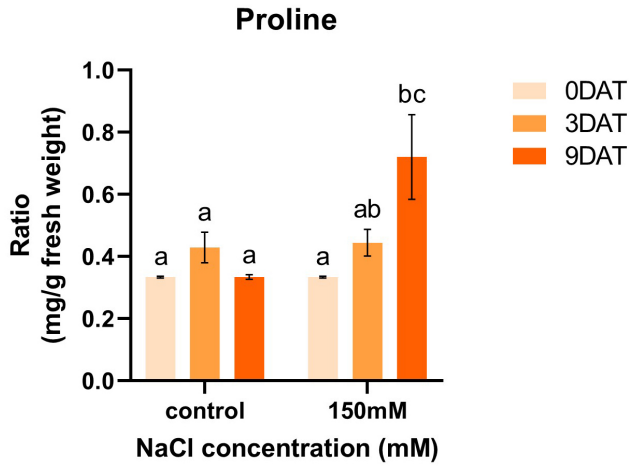


**Fig. 5. Proline expression in leaves between untreated control and 150 mM NaCl treatment of Sodamchal at 0, 3, and 9 DAT.** Values represent mean ± SEM of three replicates. Letters on top of bars indicate significant differences by a one-way analysis of variance followed by a test of Tukey HSD ($p < 0.05$). Bars with the same letter are not significantly different at $p = 0.05$.

### *3.5 Effects of salt stress on the chlorophyll expressions*

Abiotic stresses on plants generally causes the disruption of chlorophyll, resulting in chlorosis or necrosis. The effect on the chlorophyll contents of Sodamchal was observed for 9 days after treatment with or without 150 mM NaCl. The following chlorophyll contents were checked in the leaves: chlorophyll a and b and total chlorophyll (Fig. 6). All three chlorophyll contents remained unchanged in the control. However, in the presence of 150 mM NaCl, the graph shows a significant decrease over time from 3 to 9 DAT. For chlorophyll a and b, the decrease was similar to 50.7% and 50.1%, respectively. Total chlorophyll decreased by 29.4%. Despite the decrease in the chlorophyll contents from 3 to 9 DAT in the salt treatment group, the fluctuations were not statistically different.

### *3.6 Identification of DEGs in response to salt stress*

Differentially expressed genes were identified through Venn diagrams at 3 DAT by comparing the control to the 150 mM NaCl treatment group in the leaves and roots (Fig. 7). A total of 1506 DEGs was found in the leaves, and 1510 genes were differentially expressed in the roots between the plants with and without the salt treatment at 3 DAT. Among them, 898 genes were up-regulated, and 589 genes were down-regulated in the leaves. In the roots, 788 and 643 genes were up- and down-regulated, respectively. Among them, 288 genes differentially expressed in both the leaves and roots between the affected and unaffected individuals were up-regulated, and 87 were down-regulated. Contra-regulated genes are down-regulated in the roots by the same value when up-regulated in the leaves. The same applies to the opposite, and a total of 19 genes were identified.

### *3.7 Functional classification by GO*

We used Agrigo (http://bioinfo.cau.edu.cn/agriGO/) to search for GO annotations and extracted GO terms to categorize the function of the DEGs from the leaves and roots (Fig. 8). First, the biological processes in the leaves were classified into the order of cellular process, metabolic process, response to stimulus, and biological regulation in both the up- and down-regulated DEGs. However, a single-organism process was additionally included among the up-regulated DEGs. In the cellular component category, cells, cell parts and organelles were commonly found. In addition, membrane and membrane part appeared in the following additional terms in the up-regulated ones. In molecular function, binding and catalytic activity were the highest terms in common. It can be seen that the three categories of root gene ontology are also almost identical except for the difference in the order of some terms with the gene ontology of the leaves described above.

### *3.8 KEGG functional classification of the DEGs*

The KEGG (Kyoto Encyclopedia of Genes and Genomes database) [35] was used to understand and identify the biological pathways found in the DEGs of Sodamchal under a salt stress condition. A total of 754 genes from 1506 DEGs in the leaves was classified into 169 KEGG pathways. Of the identified genes, 439 up-regulated and 315 down-regulated genes were classified into 86 and 83 pathways, respectively (Tables 2 and 3). In the roots, 744 genes out of 1510 DEGs were classified and mapped into 151 KEGG pathways. Of the identified genes, 593 up-regulated and 151 down-regulated genes were classi-

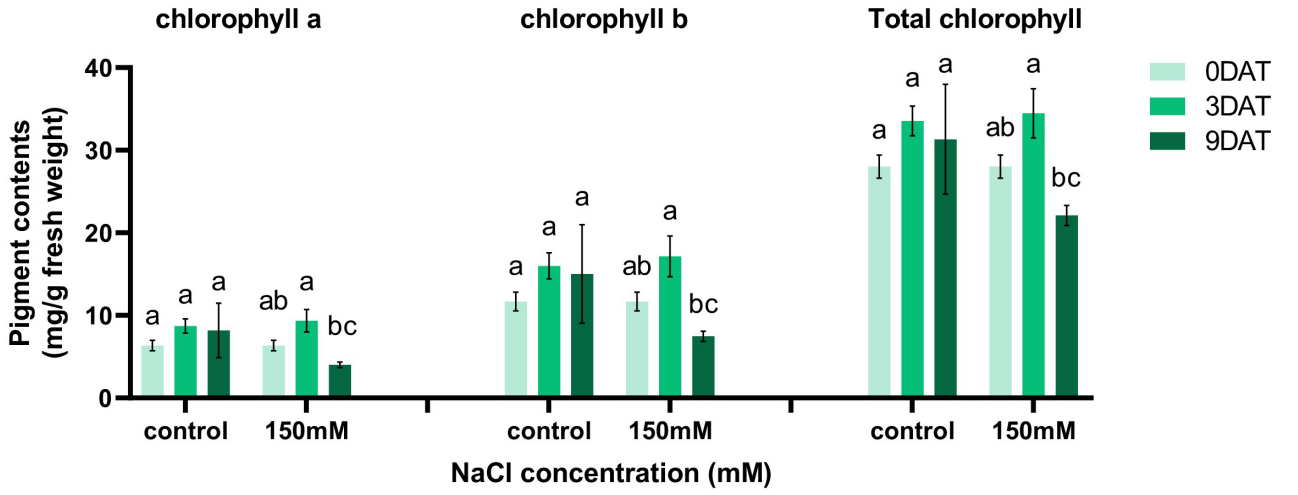


**Fig. 6. The level of chlorophyll expression in leaves between Sodamchal's untreated control and 150mM NaCl treatment at 0, 3, and 9 DAT.** Values represent mean ± SEM of three replicates. Letters on top of bars indicate significant differences by a one-way analysis of variance followed by a test of Tukey HSD ($p < 0.05$). Bars with the same letter are not significantly different at $p = 0.05$.

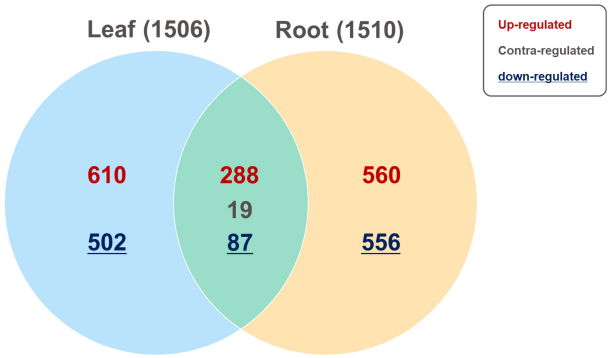


**Fig. 7. Venn diagram showing differentially expressed genes (DEGs) up-regulated or down-regulated between control and 150mM NaCl treatment after three days in the leaves and roots of Sodamchal.** Contra-regulated genes indicate down-regulated in the roots by the same value when up-regulated in the leaves and vice versa. Only DEGs with >2 folds and >4 LOG2 changes in three replicates were included.

fied into 87 and 64 pathways, respectively (Tables 4 and 5). The KEGG pathways were selected by including the top 20 major categories. Through this KEGG pathway, the "metabolic pathways" and "biosynthesis of secondary metabolites" categories of all the DEGs are commonly displayed in descending order, indicating that they somehow affect the response to the saline stress.

*3.9 Co-expression analysis of the DEGs in normal and salt stress conditions*

A gene co-expression network is a graph where each node has no direction corresponding to a gene. If there is an important co-expression step between a pair of nodes, then the pair of nodes is connected to the edge. Gene co-expression networks can be constructed by searching for pairs of genes that exhibit similar expression patterns throughout the sample because the transcriptional levels of the two co-expressed genes show similar expression profiles throughout the sample [36].

A co-expression network was constructed using the DEGs of Sodamchal (Fig. 9). It was constructed using strings in the Cytoscape software (http://www.cytoscape.org/) based on 1506 and 1510 DEGs from the leaves and roots identified using a Venn diagram, respectively. Genes from the constructed network were selected based on the log2 values of the up-regulated (≥5) and down-regulated (≥–3) genes. In addition to those selected genes, we selected more genes that were close to each other in the network. There was a total of 15 genes (9 up-regulated and 6 down-regulated ones) found throughout the gene co-expression networks in the leaves (Table 6). The identified genes were annotated with the UniProt databases to search for related transcription factors. Transcription factors related to the up-regulated genes were AP2/ERF, CBF5/6, dehyrin (DHN), and xyloglucan endotransglucosylase/hydrolase. For the down-regulated genes, chlorophyll a-b binding protein, NADPH, and C2H2-type domain were found as transcription factors.

A total of 9 up-regulated and 3 down-regulated genes were identified in the roots (Table 7). AP2/ERF and CBF5/6 and DHN transcription factors were also present for the up-regulated genes. In addition, the BHLH domain transcription factor could be further identified. In the down-regulated genes, new transcription factors different from those in the leaves were identified as expansin, lysin histidine transporter (LHT) 2, and defensin.

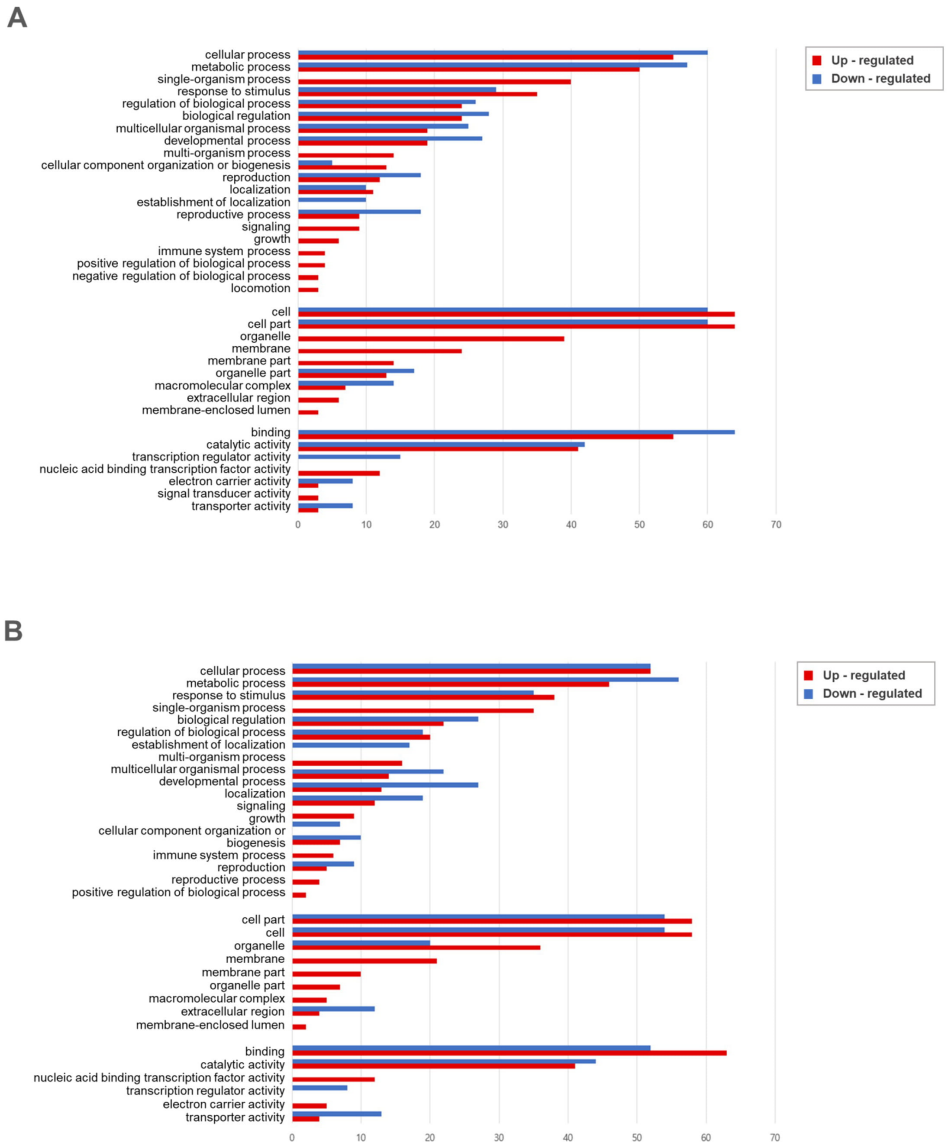


**Fig. 8. Gene Ontology (GO) analysis of the set of DEGs in response to salt stress and untreated controls in Sodamchal.** (A) Leaves. (B) Roots.

### *3.10 Verification of the QuantSeq data*

To validate the QuantSeq gene expression profiling, quantitative real-time PCR was performed on six randomly selected genes that have a certain role in regulating salt stress in this study. When the gene expression patterns were compared between the control and the 150 mM NaCl treatment in the Sodamchal leaves and roots, the expression levels from the QuantSeq data were consistent with those from the qRT-PCR results (Fig. 7), indicating that our DEG analyses are suitable for identifying genes associated with salt stress conditions.

## 4. Discussion

In the current study, salt stress had an adverse effect on the growth of Sodamchal when compared to the control. These results also provide some evidence that Sodamchal may be susceptible to salt stress, consistent with a previous report [37].

In general, the effect of salt stress includes ion accumulation and osmotic stress. The accumulation of ions under abiotic stress in the cytoplasm has an important role as signals to induce osmoprotectants or regulators [7,13].

Anthocyanins are flavonoids that occur in any tissue of a plant but are water-soluble pigments found in most flowers and fruits [38]. They also protect plants affected by UV rays and induce antioxidant activity [39,40]. This substance accumulates in various ways in different plant tissues

**Table 2. The top 20 KEGG categories of up regulated genes in leaves under salt stress compared to the control condition.**

| KEGG accession | Pathway | No. of genes |
|---|---|---|
| sbi01100 | Metabolic pathways | 93 |
| sbi01110 | Biosynthesis of secondary metabolites | 59 |
| sbi04626 | Plant pathogen interaction | 22 |
| sbi04075 | Plant hormone signal transduction | 16 |
| sbi00520 | Amino sugar and nucleotide sugar metabolism | 12 |
| sbi00940 | Phenylpropanoid biosynthesis | 11 |
| sbi04016 | MAPK signaling pathway | 11 |
| sbi01230 | Biosynthesis of amino acids | 10 |
| sbi00270 | Cysteine and methionine metabolism | 8 |
| sbi00053 | Ascorbate and aldarate metabolism | 7 |
| sbi00250 | Alanine, aspartate and glutamate metabolism | 7 |
| sbi00500 | Starch and sucrose metabolism | 7 |
| sbi01200 | Carbon metabolism | 7 |
| sbi00010 | Glycolysis/Gluconeogenesis | 6 |
| sbi00330 | Arginine and proline metabolism | 6 |
| sbi01240 | Biosynthesis of cofactors | 6 |
| sbi00052 | Galactose metabolism | 5 |
| sbi00460 | Cyanoamino acid metabolism | 5 |
| sbi00561 | Glycerolipid metabolism | 5 |
| sbi00564 | Glycerophospholipid metabolism | 5 |

**Table 3. The top 20 KEGG categories of down regulated genes in leaves under salt stress compared to the control condition.**

| KEGG accession | Pathway | No. of genes |
|---|---|---|
| sbi01100 | Metabolic pathways | 72 |
| sbi01110 | Biosynthesis of secondary metabolites | 51 |
| sbi04075 | Plant hormone signal transduction | 13 |
| sbi00196 | Photosynthesis-antenna proteins | 8 |
| sbi00460 | Cyanoamino acid metabolism | 8 |
| sbi00940 | Phenylpropanoid biosynthesis | 7 |
| sbi01200 | Carbon metabolism | 7 |
| sbi00592 | alpha Linolenic acid metabolism | 6 |
| sbi00030 | Pentose phosphate pathway | 5 |
| sbi00250 | Alanine, aspartate and glutamate metabolism | 5 |
| sbi00270 | Cysteine and methionine metabolism | 5 |
| sbi00500 | Starch and sucrose metabolism | 5 |
| sbi01230 | Biosynthesis of amino acids | 5 |
| sbi01240 | Biosynthesis of cofactors | 5 |
| sbi03008 | Ribosome biogenesis in eukaryotes | 5 |
| sbi04016 | MAPK signaling pathway | 5 |
| sbi00941 | Flavonoid biosynthesis | 4 |
| sbi04712 | Circadian rhythm | 4 |
| sbi00230 | Purine metabolism | 3 |
| sbi00260 | Glycine, serine and threonine metabolism | 3 |

and is affected by different environments [41]. The anthocyanin contents changed significantly in both the control and the NaCl treatment group (Fig. 2A), presumably indicating that the change is not solely caused by salt stress. Anthocyanin accumulation is also triggered by some nutrients such as phosphorous and nitrogen [42]. Previous studies using *Raphanus sativus* (radish) showed a tendency for the anthocyanin contents to change in plants grown in Hoagland

**Table 4. The top 20 KEGG categories of up regulated genes in roots under salt stress compared to the control condition.**

| KEGG accession | Pathway | No. of genes |
|---|---|---|
| sbi01100 | Metabolic pathways | 71 |
| sbi01110 | Biosynthesis of secondary metabolites | 47 |
| sbi04626 | Plant pathogen interaction | 18 |
| sbi01200 | Carbon metabolism | 11 |
| sbi04075 | Plant hormone signal transduction | 11 |
| sbi00520 | Amino sugar and nucleotide sugar metabolism | 8 |
| sbi00940 | Phenylpropanoid biosynthesis | 8 |
| sbi04016 | MAPK signaling pathway | 8 |
| sbi00010 | Glycolysis/Gluconeogenesis | 7 |
| sbi00500 | Starch and sucrose metabolism | 7 |
| sbi00270 | Cysteine and methionine metabolism | 6 |
| sbi00350 | Tyrosine metabolism | 5 |
| sbi00480 | Glutathione metabolism | 5 |
| sbi04141 | Protein processing in endoplasmic reticulum | 5 |
| sbi04144 | Endocytosis | 5 |
| sbi00071 | Fatty acid degradation | 4 |
| sbi00250 | Alanine, aspartate and glutamate metabolism | 4 |
| sbi00592 | alpha Linolenic acid metabolism | 4 |
| sbi00710 | Carbon fixation in photosynthetic organisms | 4 |
| sbi00908 | Zeatin biosynthesis | 4 |

**Table 5. The top 20 KEGG categories of up regulated genes in roots under salt stress compared to the control condition.**

| KEGG accession | Pathway | No. of genes |
|---|---|---|
| sbi01100 | Metabolic pathways | 53 |
| sbi01110 | Biosynthesis of secondary metabolites | 41 |
| sbi00940 | Phenylpropanoid biosynthesis | 13 |
| sbi04075 | Plant hormone signal transduction | 12 |
| sbi00460 | Cyanoamino acid metabolism | 8 |
| sbi01230 | Biosynthesis of amino acids | 6 |
| sbi00250 | Alanine, aspartate and glutamate metabolism | 5 |
| sbi00260 | Glycine, serine and threonine metabolism | 5 |
| sbi01200 | Carbon metabolism | 5 |
| sbi00230 | Purine metabolism | 4 |
| sbi00270 | Cysteine and methionine metabolism | 4 |
| sbi00480 | Glutathione metabolism | 4 |
| sbi00500 | Starch and sucrose metabolism | 4 |
| sbi00592 | alpha Linolenic acid metabolism | 4 |
| sbi00910 | Nitrogen metabolism | 4 |
| sbi01240 | Biosynthesis of cofactors | 4 |
| sbi04626 | Plant pathogen interaction | 4 |
| sbi00010 | Glycolysis/Gluconeogenesis | 3 |
| sbi00053 | Ascorbate and aldarate metabolism | 3 |
| sbi00062 | Fatty acid elongation | 3 |

solution containing nutrients [43]. Therefore, it might be affected by other external factors rather than the salt stress.

Reducing sugar regulates the water and osmotic potential of plant cells [44]. The reducing sugars of Sodamchal, a susceptible variety of sorghum, tended to decrease over time when salt stress was applied, particularly at 9 DAT (Fig. 2B). Previous research revealed that the sugar contents decreased when salt stress was introduced in barley and wheat [45]. These results suggest that sugar metabolites did not contribute positively to maintain osmotic regulations in the leaves in the condition of salt stress due to the susceptibility of Sodamchal to salt stress.

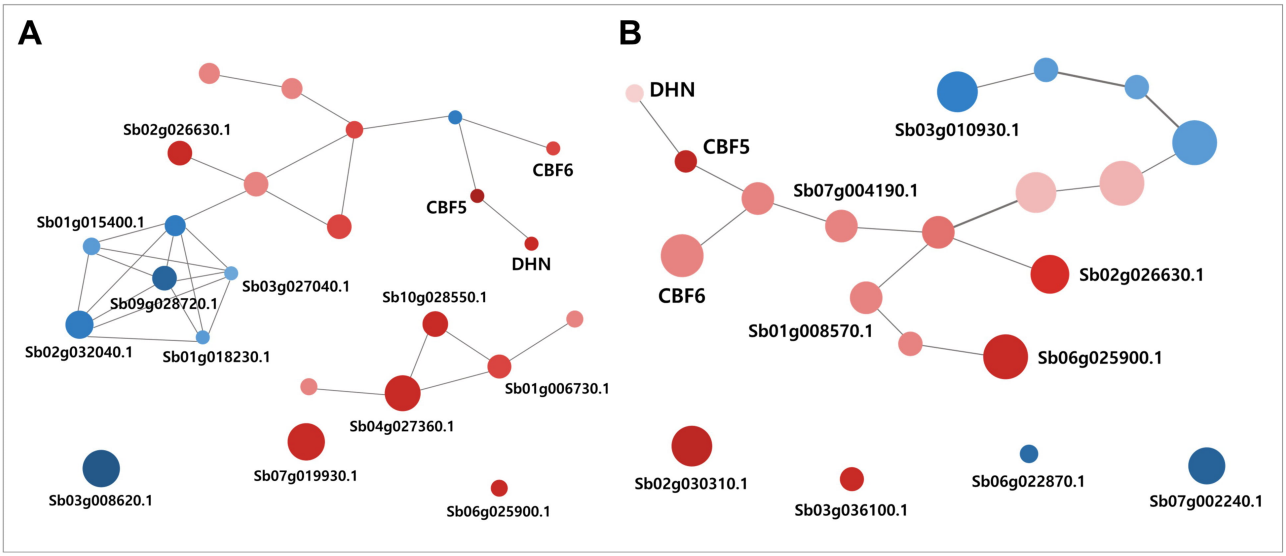


**Fig. 9. Co-expression network in DEGs of Sodamchal at $p < 0.05$, and The size of circle increases as the *p*-value approaches 0.** (A) Leaves. (B) Roots. Red nodes are up-regulated genes and blue nodes are down-regulated genes. Genes starting with sb are sorghum IDs from Uniprot, some of which have known functions and some are not.

**Table 6. Transcription factors corresponding to genes in gene co-expression networks in leaves.**

| Gene ID | Locus ID | FC (log2) | *p*-value | regulation | Annotation |
|---|---|---|---|---|---|
| LOC8060409 | Sb06g025900.1 | 8.183 | 0.024 | up | AP2/ERF domain-containing protein |
| LOC8069036 | CBF5 | 7.624 | 0.002 | up | SbCBF5 |
| LOC8072968 | Sb06g015590.1 | 6.938 | 0.019 | up | Uncharacterized protein |
| LOC8074815 | Sb04g027360.1 | 6.261 | 0.001 | up | Uncharacterized protein |
| LOC8083217 | Sb02g026630.1 | 6.221 | 0.016 | up | AP2/ERF domain-containing protein |
| LOC8065132 | Sb10g028550.1 | 6.117 | 0.015 | up | Xyloglucan endotransglucosylase/hydrolase |
| LOC8077913 | DHN | 5.022 | 0 | up | Dehydrin |
| LOC8083956 | Sb01g006730.1 | 4.91 | 0.023 | up | Uncharacterized protein |
| LOC8054870 | CBF6 | 3.597 | 0.002 | up | SbCBF6 |
| LOC8082204 | Sb03g027040.1 | –1.614 | 0.004 | down | Chlorophyll a-b binding protein, chloroplastic |
| LOC8062994 | Sb01g015400.1 | –1.835 | 0.009 | down | Chlorophyll a-b binding protein, chloroplastic |
| LOC8065810 | Sb01g018230.1 | –1.913 | 0.003 | down | NADPH-protochlorophyllide oxidoreductase |
| LOC8077219 | Sb02g032040.1 | –2.317 | 0.031 | down | Chlorophyll a-b binding protein, chloroplastic |
| LOC8074608 | Sb09g028720.1 | –3.169 | 0.021 | down | Chlorophyll a-b binding protein, chloroplastic |
| LOC8072351 | Sb03g008620.1 | –4.971 | 0 | down | C2H2-type domain-containing protein |

One of the most important mechanisms in plants under salt stress is the accumulation of substances like proline. The accumulation of proline is the primary defense reaction to maintain the osmotic pressure of cells in plants, and in addition, it contributes to the removal of ROS, regulation of redox homeostasis in cells, and stabilization of structures within cells [46–48]. ROS is also induced by abiotic stress and when excessively accumulated can cause cellular damage to plants and, in severe cases, death. In sorghum, SbNAC2 enhances the ability of ROS to scavenge, and stress response genes make plants such as Arabidopsis thaliana and rice resistant to abiotic stress [49]. The proline accumulation of Sodamchal also increased over time to maintain the osmotic pressure by the 150 mM NaCl treatment (Fig. 2C). These results lead to gene induction and accumulation when the biosynthesis of proline is affected by salt. The content of proline in tomato(*Solanum lycopersicum* L.) was significantly increased according to salt stress regardless of the genotype of the cultivar [50]. This confirmed that during plant development, proline is accumulated in a small amount even when subjected to slight stress and regulates osmotic pressure. This suggests that proline may be an optimal indicator of the intensity of saline stress [51,52].

Previous studies have allowed us to identify responses to high salinity through cellular and gene regulatory net-

**Table 7. Transcription factors corresponding to genes in gene co-expression networks in root.**

| Gene ID | Locus ID | FC (log2) | *p*-value | Regulation | Annotation |
|---|---|---|---|---|---|
| LOC8060409 | Sb06g025900.1 | 5.791 | 0 | up | AP2/ERF domain-containing protein |
| LOC8069036 | CBF5 | 5.28 | 0.021 | up | CBF5 |
| LOC8079279 | Sb03g036100.1 | 5.243 | 0.019 | up | BHLH domain-containing protein |
| LOC8054868 | Sb02g030310.1 | 5.223 | 0 | up | AP2/ERF domain-containing protein |
| LOC8083217 | Sb02g026630.1 | 4.283 | 0.005 | up | AP2/ERF domain-containing protein |
| LOC8069641 | Sb07g004190.1 | 3.672 | 0.013 | up | BHLH domain-containing protein |
| LOC8060491 | Sb01g008570.1 | 3.036 | 0.012 | up | BHLH domain-containing protein |
| LOC8054870 | CBF6 | 2.451 | 0.002 | up | CBF6 |
| LOC8077913 | DHN | 1.615 | 0.004 | up | Dehydrin |
| LOC8074527 | Sb03g010930.1 | –2.268 | 0.001 | down | Expansin |
| LOC8066129 | Sb07g002240.1 | –3.158 | 0.002 | down | Lysine histidine transporter 2 |
| LOC8075945 | Sb06g022870.1 | –3.334 | 0.024 | down | Defensin |

works [53]. Among them, hormones such as abscisic acid (ABA), zilevelin, and auxin were involved in signal transduction [54]. It belongs to the TF families, and we importantly highlight its role as a plant regulator in response to salinity and we observed upregulation of DEG regulating NAC, WRKY and MYB in the resistance gene SSG 59-3 in sorghum [55]. It has also been reported that TF of NAC confer salt tolerance in plants such as Glycine max [56]. In the current study, we identified genes for transcriptional changes in the leaves and roots treated with NaCl compared to untreated controls. To compare the response to salt stress at the transcriptome level, QuantSeq was conducted for the leaves and roots treated without (control) and with 150 mM NaCl at 3 DAT. Consequently, we identified 898 up-regulated and 589 down-regulated DEGs in the leaves under saline stress. In the roots, 788 and 643 genes were up- and down-regulated. The analysis of the GO enrichment for each of the DEGs identified in the leaves and roots suggested a variety of biological functions under salt stress. In many of the DEGs, GO terms were abundant including "metabolic process", "response to stimulus", and "binding and catalytic activity". Results such as these will be useful for studying the mechanisms and genes associated with salt stress. The KEGG analysis also identified abundant pathways containing DEGs. KEGG pathways such as "metabolic pathways" and "biosynthesis of secondary metabolites" were commonly found in the leaf and root DEGs. To further investigate those DEGs based on the level of gene expression, we tried co-expression gene network analysis and found some useful resources that may be able to help to understand salt-tolerance mechanisms in sorghum plants. Under salinity stress, a co-expressed gene network is an effective way to identify candidate genes and target transcription factors together with their biological pathways. We identified up- and down-regulated transcription factors associated with these genes in the leaves and roots of Sodamchal (Fig. 5).

We confirmed that the AP2/ERF domain-containing protein gene was expressed in response to salt stress among DEGs commonly upregulated in leaves and roots, suggesting that it may play an important role as follows. Among the transcription factors involved in the regulation of abiotic and biotic stress responses, some of the up-regulated transcription factors were members of the AP2/ERF (APETAL2/ethylene response factor) superfamily [57]. Many AP2/ERF genes have been proposed to form regulatory networks related to abiotic stress, but in this network, AP2/ERF responds to different stimuli and stresses in different patterns [58]. APETALA2/ethylene responsive factor (AP2/ERF) contributes significantly to resistance to abiotic stress in plants. In Arabidopsis, overexpression of the peanut gene AhERF019 was shown to be more resistant to stress such as drought and salt stress than the WT line [59]. It was also shown that a transgenic line of sweet potato IbRAP2-12 in Arabidopsis thaliana improved salinity and drought tolerance [60].

Our results also identified dehydrin (DHN), a gene that is up regulated in both the leaves and roots. Dehydrin is commonly known as Group II of the Late Embryogenesis Abundant (LEA) proteins [61,62]. Like common LEA proteins, dehydrin is very hydrophilic and heat resistant. Dehydrin accumulates in the late stages of embryonic development in response to various environmental stresses [63]. Dehydrins fall into five categories: $Y_nK_n$, $Y_nSK_n$, $SK_n$, $K_n$ and $K_nS$ [64]. Every type of function is different. For example, $Y_nSK_n$ is a neutral protein that is upregulated under cold stress [65]. $SK_n$ Dehydrin can bind phospholipids and prevent denaturation under the influence of heat, and is resistant to plant development, growth and cold stress [66]. As described above, many studies say that the expression of dehydrin is resistant to abiotic stress and interacts positively. Overexpression of CaDHN5 in pepper showed that plants had increased tolerance to salinity and osmotic stress and increased expression of related genes compared to wild-type [67]. Some dehydrins also react to abscisic

acid (ABA), also known as Response to ABA (RAB). For example, the expression of Dehydrin WZY2 in wheat increases salt and drought tolerance by the induction of ABA [68,69]. It also supports the fact that Dhn-5 in wheat contributes to improving tolerance to salt and drought stress through its role in protecting plants through osmotic regulation [70].

We also identified photosynthetic-antenna proteins that account for most of the down-regulated transcription factors of genes identified in the leaf network. The four related transcription factors were mapped to the database using KEGG to attach and identify functional annotations. The gene functions of the co-expression network were consistent with the results of KEGG, and we confirmed light-harvesting chlorophyll (LHC) from the results. In higher plants, LHC proteins are expressed by 14 (Lhca1-Lhca6 and Lhcb1-Lhcb8) or 12 (Lhca1-Lhca5 and Lhcb1-Lhcb7). These LHC proteins appear as two types of proteins (Lhca and Lhcb). The Lhca type protein is an external antenna present in the PSI and is composed of two heterodimer domains. The response of PSII consists of the Lhcb type protein [71]. Among these, the light-harvesting chlorophyll (LHCB) protein is commonly associated with chlorophyll and xanthophyll. First, it acts as an antenna complex that induces photosynthetic electron transport by absorbing light and transferring the energy to the core complex of PSII [72,73]. Light energy is converted into chemical energy in photosynthesis. Chlorophyll and carotenoid pigments make up most of the light and absorb PSI and PSII and the light harvesting complex (LHC) externally [74,75]. In both the NaCl-treated and control plants, DEGs encoding LHC, which are required to regulate PSII activation, were down-regulated. These results suggest that salt stress reduced the sub-binding of several units of PSII.

In the roots, we identified Expansins, a transcription factor that is typically downregulated. Expansins regulate cell wall expansion as cells grow or directly regulate the process of cell wall loosening [76,77]. Cell wall-unwinding proteins from expansin include groups of $\alpha$, $\beta$-expansin and expansin-like A,B subfamily [78]. Some of the expressions of the expansin isoforms are involved in the process of growth and development, and external actions can stimulate various metabolic systems and alter the cell wall [79,80]. Expansin has been shown to have an essential role in root growth [81] and leaf [82] and floral [83] development and wall expansion with low changes in water potential at the transcript level [84]. The regulation of expansin is likely to make the cell wall loosening of the maize cultivar proceed rapidly in water deficit conditions [85]. $\beta$-expansin tended to decrease in salt-sensitive maize [86]. Correspondingly to these results, we also observed a decrease in expansin when exposed to salt in sorghum.

In order to examine whether transcription factors obtained through co-expression analysis are related to salt stress, we compared them with previous studies and confirmed them. Therefore, it can be seen that the results of our tests were affected by the salt and the expression was confirmed. Regulating plant development and growth processes under salinity stress is very complex. Depending on the species of the crop, it depends on the growing type, growth stage and environmental conditions. As previously confirmed [87], comparative studies of the overall association of sorghum genome in salt-affected processes are still in their infancy, so we aimed to find possible regulatory networks in salt tolerance in sorghum through genomic and transcriptomic studies. In addition to genomics, omics technologies including transcriptomics, proteomics, and metabolomics are gradually increasing, which will be used importantly for regulating the transcription and metabolism of plants subjected to other abiotic stresses as well as salinity.

## 5. Conclusions

In the current study, we outlined the physiological responses and transcriptomic changes to understand the molecular basis of the sorghum cultivar sensitive to salt stress. In the physiological analysis, changes in the salt treatment duration were investigated to see if some physicochemical changes in plants can be used to evaluate the intensity of abiotic stresses like saline or drought. For the analysis of the transcriptome, DEGs were identified from salt-treated leaves and roots and compared to their untreated controls. As a result, 1506 and 1510 DEGs were found in the leaves and roots, respectively, which were annotated based on the reference genome of sorghum. The identified genes were examined further to infer the associations in terms of the level of expression through a co-expression network. The analyses found that several transcription factors and their pathways might be related to salt stress. As for the up-regulated transcription factors, AP2/ERF and DHN were identified which are associated with salt stress. In addition, chlorophyll a-b binding proteins identified in several down-regulated transcription factors were found to be closely related to each other on the network, and it could be related to the decrease in chlorophyll in the physiological analysis; however, it may need some further study in the future with salt-tolerant varieties. In fact, we are conducting research on the response of salt stress for a sorghum variety, ‘Nampungchal’, which is considered salt-tolerant. To summarize some of the salt-tolerant sorghum studies we have conducted so far, the expression of anthocyanin, proline, and reducing sugar in salt-tolerant sorghum increased significantly under the salt stress. In addition, the expression of genes reported to confer salt tolerance to plants, such as WRKY, UDP-glycosyltransferase, and zinc finger was identified under the salt stress condition. Comparing the salt stress responses of two plant genotypes in future studies may provide an opportunity for better understanding of the molecular mechanisms underlying salt stress in sorghum. Our findings may serve as a cornerstone for understanding

the mechanisms by which plants respond to salt stress. In particular, it may provide an opportunity to elucidate the resistance mechanism of sorghum to salt stress. Based on these results, it will be an excellent resource for future studies related to salt tolerance and will achieve development in plant genome research.

## Abbreviations

DEGs, differentially expressed genes; NGS, next-generation sequencing; DAT, days after the treatment.

## Author contributions

CK and SC designed the research study. SC, SL, and DHJ performed the research. THL provided help and advice on bioinformatics analyses. SC, YK, and SS analyzed the data. SC and CK wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

Not applicable.


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## Conflict of interest

The authors declare no conflict of interest. CK is serving as one of the Editorial Board members of this journal. We declare that CK had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to GP.

## Supplementary material

Supplementary material associated with this article can be found, in the online version, at https://www.imrpress.com/journal/FBL/27/2/10.31083/j.fbl2702055.

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