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Genome-wide transcriptome analysis and physiological variation modulates gene regulatory networks acclimating salinity tolerance in chickpea

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ABSTRACT

Salinity is a major abiotic stress that is a global threat to crop production, including chickpea. This study focused on understanding the complex molecular mechanisms underlying salinity tolerance using comparative transcriptome analysis of tolerant (ICCV 10, JG 11) and sensitive (DCP 92-3, Pusa 256) chickpea genotypes in control and salt-stressed environments. A total of 530 million reads were generated from root samples of four genotypes using Illumina HiSeq-2500. A total of 21,698 differentially expressed genes (DEGs) were identified, of which 11,456 and 10,242 were up- and down-regulated, respectively, in comparative analysis. These DEGs were associated with crucial metabolic pathways, including hormone signaling, photosynthesis, lipid and carbohydrate metabolism, and cell wall biogenesis. Gene ontology (GO) examination revealed an enrichment of transcripts involved in salinity response. A total of 4257 differentially expressed GO terms were categorized into 64 functional groups; of which, GO terms like, integral component of membrane, organelle, and cellular anatomical entity were highly represented in tolerant genotypes under salt stress. Significant up-regulation of transcripts encoding potassium transporter family HAK/KUP proteins, MIP/aquaporin protein family, NADH dehydrogenase, pectinesterase, and PP2C family proteins occurred under salt stress. The tolerant lines (ICCV 10 and JG 11) engaged highly efficient machinery in response to elevated salt stress, especially for signal transduction, transport and influx of K^+ ions, and osmotic homeostasis. The overall study highlights the role of potential candidate genes and their regulatory networks which can be utilized in breeding salt tolerant chickpea cultivars.

1. Introduction

Salinity is a major abiotic stress that hampers crop production and productivity worldwide. More than 80 million hectares of land is degraded by salt globally (Flowers et al., 2010; Asif et al., 2018), including 45 million ha (20 %) of irrigated land and 32 million ha (2 %) of drylands (Machado and Serralheiro, 2017). Salinity is expected to intensify in the coming years due to unsustainable irrigation, traces of NaCl in irrigation water and rising water tables (Deinlein et al., 2014). Repercussion of changing climate, results in excessive evaporation and salt accumulation in the soil, which in turn increases soil salinity (Tester, 2003; Zhu et al., 2015). Soil salinity has deleterious consequences on soil health leading to osmotic stress and ion toxicity, which in turn affect agricultural productivity (Zhu, 2016). The injurious effects of salt stress

Abbreviations: ANOVA, analysis of variance; CRD, completely randomized block design; DEGs, differentially expressed genes; EL, electrolyte leakage; FPKM, fragment per kilo per million; GO, gene ontology; qRT-PCR, quantitative real time-PCR; RWC, relative water content; TFs, transcription factors.

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on plants includes osmotic and oxidative stress along with Na^+ toxicity and an imbalance in ionic homeostasis (Bartels and Sunkar, 2005; Zhu, 2002). Excess salinity induces ionic imbalance via shoot-independent and shoot-dependent ion stresses, affecting plant growth and development (Roy et al., 2014). A reduction in external osmotic potential restricts water uptake, which affects plant growth and reduces biomass yield (Puniran-Hartley et al., 2014; Turner et al., 2012). Ion toxicity mainly affects the Na^+ signal transduction pathway, which is critical for detoxification and balancing cellular ion homeostasis (Zhu, 2002; Quintero et al., 2002). The K^+ ion is essential for important metabolic processes in the cytoplasm, including protein synthesis, enzymatic reactions, and ribosome function (Shabala and Cuin, 2008). Thus, the Na^+/K^+ ratio is an important determinant of salt stress tolerance; maintaining cellular Na^+/K^+ is a basic feature of tolerant plants under salt stress (Zhu, 2003).

To deal with salt stress, plants reprogram their protective machinery from the physiological to molecular level, where genes involved in ion transport, transcription regulation, stress signaling, hormone signaling, and biosynthesis of specific metabolites respond to elevated salt stress (Abbas et al., 2010). Na^+ influx into root tissues occurs via different transporters, including the K^+/H^+ exchanger (NHX) and high-efficiency K^+ transporter (HKT) (Li et al., 2019), essential for transport and detoxification of Na^+ ions from plants (Wang et al., 2016a, b). Differentially expressed transcription factor (TF) genes—including the AP2/EREBP (Yang et al., 2018), MYB (Yang et al., 2012), WRKY (Jiang et al., 2017), bZIP (Banerjee and Roychoudhury, 2015), NAC (Song et al., 2011), and bHLH (Mao et al., 2017) families—respond to elevated external salinity (Golldack et al., 2011) by intensifying signals for gene regulation and protective mechanisms in plants.

Chickpea (*Cicer arietinum* L.) is one of the most important grain legume crops with worldwide production of 17.2 million tons, including 11.38 million tons in India (FAO, 2018). Chickpea provides a large fraction of protein and essential amino acids for populations in arid and sub-arid regions of the world (Jukanti et al., 2012). However, chickpea is a salt-sensitive legume, showing reduced growth parameters at EC levels >6 dS/m (Flowers et al., 2010). Excessive soil salinity reduces annual chickpea yields by 8–10 % globally (Flowers et al., 2010). Despite its salt sensitivity, chickpea has large morphological variation for salinity tolerance (Turner et al., 2012; Vadez et al., 2007). Salinity tolerance is governed by traits involved in complex physiological and biochemical responses by inducing multiple genes under stress (Vadez et al., 2011). Physio-biochemical markers, such as relative water content (RWC), electrolyte leakage (EL), chlorophyll, and proline content, are direct indicators of stress tolerance (Arefian and Malekzadeh Shafaroudi, 2015). Identification of genes associated with salt stress and physio-biochemical factors responding to salinity will help in the development of improved chickpea varieties with enhanced salinity tolerance. Several attempts have been made to understand the molecular and physiological basis of salt tolerance in crop species such as rice (Mansuri et al., 2019; Li et al., 2018), wheat (Mahajan et al., 2017), and eggplant (Li et al., 2019), but a comprehensive study involving transcriptome analysis and physiological profiling is lacking for chickpea under salt stress. Recent advances in high-throughput next-generation sequencing technologies have paved the way for studying expression profiles in chickpea (Kudapa et al., 2018; Jain, 2011; Varshney et al., 2013). However, limited knowledge is available on the physiological and molecular responses in chickpea for salinity tolerance, and only a few studies have generated functional genomic resources. Earlier studies used microarray analysis to identify differentially expressed genes under high salinity (Mantri et al., 2007) along with deepSuperSAGE method to identify salt related transcriptome in root and nodules (Molina et al., 2011). The recent study shows the application of RNA-seq to identify genes associated with root morphogenesis and cell wall modification under high salinity in chickpea (Kaashyap et al., 2018). While, our study advances the understanding of salinity tolerance mechanism in chickpea by integrating the physio-biochemical and ion (Na^+/K^+) dynamics results of both tolerant (ICCV 10, JG 11) and sensitive (DCP 92-3, Pusa 256) genotype along with the RNA-seq data to get in-depth and unique information about transcriptional reprogramming salt stress related pathways and regulatory networks

Comparative differential gene expression studies between contrasting genotypes should identify candidate gene(s) and molecular mechanisms underlying stress tolerance in chickpea (Cotsaftis et al., 2011; Lenka et al., 2010). Here, we used comparative transcriptomics with RNA-seq of root tissues to identify salt stress-responsive genes in chickpea. As roots are the first to get exposed towards elevated stress, which assists in signal transduction and reprogramming plants molecular machinery to adapt and acclimatize under salt stress environments. Studies in cotton and alfa-alfa, it has been reported that under salt stress mRNAs and other forms of RNAs are associated actively in roots as compared to leaves and shoot (Wang et al., 2015; Deng et al., 2018) therefore root tissue was selected for this study. The identification of the candidate gene(s) and mechanisms of salinity response in chickpea will support the development of salt-tolerant high-yielding chickpea varieties and contribute to increasing chickpea productivity, especially in saline regions.

2. Materials and methods

2.1. Plant material and experimental setup

Seeds of four chickpea cultivars—ICCV 10 and JG 11 (salt-tolerant) and DCP 92-3 and Pusa 256 (salt-sensitive)—were used to delineate the molecular mechanisms underlying salinity. A hydroponic experiment was conducted at the National Phytotron Facility, ICAR-Indian Agricultural Research Institute, New Delhi, India. The phytotron was set to 22/18 °C (±2 °C) day/night temperature; 10/14 h light/dark photoperiod, and 45 ± 5% relative humidity. Seeds were sterilized with 2% (w/v) sodium hypochlorite solution for 2 min, rinsed and washed with distilled water, and germinated on sterilized germination paper. After five days of germination, uniform seedlings from different genotypes were transplanted to the hydroponic system and grown in water for four days. On the fifth day, the water in the hydroponic systems was replaced with 0.5× (half-strength) modified Hoagland's nutrient medium for two days, and 1× (full-strength) modified Hoagland's solution (pH 6.5) on the seventh day, which was subsequently renewed every three days. The salt stress treatment was imposed on the 18th day by transferring half the seedlings to 1× modified Hoagland's solution with 150 mM NaCl (Kumar et al., 2020; Mansuri et al., 2019), while the remaining seedlings were kept as the control without NaCl. After 72 h of stress, root tissues from stressed and control plants were harvested using sterilized scalpel blade and preserved in *RNAlater*™ stabilization solution (Ambion) for RNA extraction. After five days of stress, plant samples (roots and shoots) were collected for various physio-biochemical analyses. The experiments were performed in triplicate (3 replications) in control (0 mM) and salt stress (150 mM) treatments using, completely randomized design (CRD) with separate three biological replications of samples prepared for various physio-biochemical and transcriptome related studies.

2.2. Seedling growth analysis

Fresh shoot and root weights and lengths were recorded for both treatments, control (0 mM, NaCl) and salt stressed (150 mM, NaCl). The shoots and roots were oven-dried at 65 °C for 72 h, and dry weights recorded. For each genotype three biological replicates were used for both the treatments (4 genotypes × 2 treatments × 3 replication).

2.3. Physio-biochemical responses and ion profiling

Relative water content (RWC) was estimated as per (Barrs and Weatherley, 1962): RWC= [(Fresh Weight – Dry Weight) / (Turgid

Weight – Dry Weight)] × 100. Electrolyte leakage (EL) was calculated as follows: EL = [C_1 / C_2] × 100, where C_1 is initial conductivity of 10 g sample in 10 mL distilled water at 45 °C for 30 min, and C_2 is final conductivity measured at 100 °C for 10 min. Total chlorophyll content was analyzed according to Arnon (1949) —finely chopped leaf tissue added to 10 mL dimethyl sulfoxide (DMSO) at 65 °C for 4 h, with absorbance recorded at 663 and 645 nm. Shoot and root Na^+ and K^+ concentrations were determined for each genotype after five days of salt stress. Plant tissues were washed carefully in distilled water, oven-dried at 65 °C for 72 h, before finely ground samples were digested in a 20 mL mixture of HNO_3 and $HClO_4$ (9:4 ratio) using a hot plate digestion system. The Na^+ and K^+ concentrations were determined using a flame photometer as per (Bhargava and Raghupathi, 1993). For every physio-biological analysis we used three biological replicates for each genotype for two treatments (4 physio-biochemical traits × 4 genotypes × 2 treatments × 3 replication).

2.4. RNA isolation & quantification

Total RNA was isolated from root samples using RNeasy® Plant Mini Kit (QIAGEN®, Cat. No. 74904) and purified with DNase I to obtain DNA-free RNA. The purified total RNA quality was estimated using Tapestation 2200, Qubit 3 Fluorometer and Nanodrop and quantified with an Agilent Technologies 2100 Bioanalyzer using the Agilent RNA chip (Agilent Technologies, CA, USA).

2.5. Library construction and Illumina sequencing

Equal amounts of RNA samples from three independent biological replicates (of each genotype/condition) having RIN value ≥8 (RNA Integrity Number) were pooled prior to library preparation. The RNA-seq library was prepared from ~4 ug of total RNA using a TruSeqRNA sample prep kit (Illumina). RiboZero was used to eliminate rRNA transcripts, and mRNA molecules containing poly-A were purified by poly T oligo attached magnetic beads. After purification, elevated temperature (94 °C for 5 min) was used to fragment mRNA using divalent cations. The first cDNA strand was synthesized using the mRNA fragments, random primers and reverse transcriptase, followed by the second cDNA strand using DNA polymerase I and RNaseH. The cDNA fragments were end-repaired by adding 'A' base and ligating adapters. After agarose gel electrophoresis, Bioanalyzer/Tapestation was used to assess mRNA quality. Appropriate size fragments (~200 bp) were selected for PCR amplification for the final cDNA library. After library construction, they were sequenced on Illumina HiSeq-2500 to obtain 2 × 100 bp paired-end reads in two sets with 60 million reads (85 % bases have > Q30) per sample.

2.6. Sequencing data analysis and DEG estimation

Quality control of sequencing data was done by fasta QC, and filtered reads with a quality score (Phred score ≥ 20) were considered for analysis. Pre-processing of raw reads was undertaken, with adaptor sequences and low-quality bases removed via AdapterRemoval-v2 (version 2.2.0). From the processed reads, ribosomal RNA sequences were removed by aligning the reads with the Silva database using Bowtie2 (version 2.2.9) and subsequently SAMtools (version 0.1.19), Sambamba (version 0.6.7), and BamUtil (version 1.0.13). The clean reads were submitted to the sequence read archive of the National Center for Biotechnology Information (NCBI) under BioProject ID No: PRJNA579008 (https://dataview.ncbi.nlm.nih.gov/object/PRJNA579008?reviewer=p40jep3k2msuacdmgfm9ub88io) Trinity assembler was used to assemble the clean reads, and the reference-based analysis (Cufflinks assembles) undertaken with Tophat 2 (version 2.0.13) to identify novel transcripts/genes. The clean reads were aligned to the *Cicer arietinum* genome and gene model (http://cegsb.icrisat.org/gtbt/ICGGC/GenomeManuscript.html).

The assembled transcriptome was annotated using the NCBI-NR (viride plant) and UniProt databases. The aligned reads were used for expression estimation of genes and transcripts using Cuffdiff (version 2.2.1) in the Cufflinks package. Differential expression analysis was performed using P-value cut-offs <0.05 and Log2 fold-change up to (+2/–2) separately for up- and down-regulated genes. Expression values were recorded in fragment per kilo per million (FPKM) units for each of the genes and transcripts.

2.7. Annotation and pathway analysis

DEGs were annotated with transcription factors (TFs) based on the annotation file for *Cicer arietinum* (http://planttfdb.cbi.pku.edu.cn/download/TF_list/Car_TF_list.gz). DEGs were subjected to enrichment analysis of GO terms based on molecular function, biological processes, and cellular components using AgriGO (web tool). For biological pathway analysis of DEGs, MapMan (version 3.5.1; http://mapman.gabipd.org/mapman-version-3.5.1) was used, with the MapMan input file generated using Arabidopsis thaliana as a model organism, with a P-value cut-off ≤0.05 to visualize salt stress-related changes.

2.8. Networks analysis between DEGs

The protein-protein interaction plays important role in function, expression profiling as well as localization of particular protein. Here, we have studied the interaction and network analysis between putative candidate genes using STRING (Search Tool for Recurring Instances of Neighbouring Genes) database (http://string-db.org/) version 11.0 (Szklarczyk et al., 2019).

2.9. Validation of DEG by quantitative real-time PCR (RT-qPCR)

For DEGs validation using RT-qPCR, 11 genes responding to salinity stress were selected randomly from the panel of genes identified in the RNA-seq studies. Gene-specific primers were designed using Primer3-Plus software (Table S5). RNA extraction from root tissues was done using RNA-isolation reagent NucleoZOL™ (TAKARA®, Cat.740406.50) and quantified in a Nano drop Spectrophotometer. A standard Accu-Script High Fidelity cDNA Synthesis kit was used to convert RNA into cDNA. Normalization of the cDNA samples was done to equalize their concentration. The RT-qPCR reactions were performed using ultra-Fast SYBR Green- QPCR master mix in the Biorad CFX 96 Real-time PCR, with taking β-actin and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) as a reference gene to normalize the data. The relative transcriptional levels in terms of fold-change were determined using quantification method $2^{-(\Delta\Delta CT)}$ to calibrate the expression level of target genes.

3. Results

3.1. Morphological and seedling phenotypic variability under stress

Salt stress dramatically reduced the values for various measured traits, with significant differences observed between tolerant (ICCV 10 and JG 11) and sensitive (DCP 92-3 and Pusa 256) chickpea genotypes ($P < 0.05$) (Table S3). In general, the two tolerant genotypes maintained vigorous root and shoot growth, with minor reductions in shoot and root length, and fresh and dry weights under salt stress, relative to the reductions in the two sensitive genotypes (Fig. 1**A**–**E**, Table S4).

3.2. Trends in physiological traits under stress

Salt stress significantly reduced ($P < 0.05$) leaf RWC content in all the genotypes, but reduction was more prominent in sensitive than in tolerant genotypes (Tables S3, S4). A similar trend was observed for total chlorophyll content, with a greater reduction in DCP 92-3 (–0.736-fold)

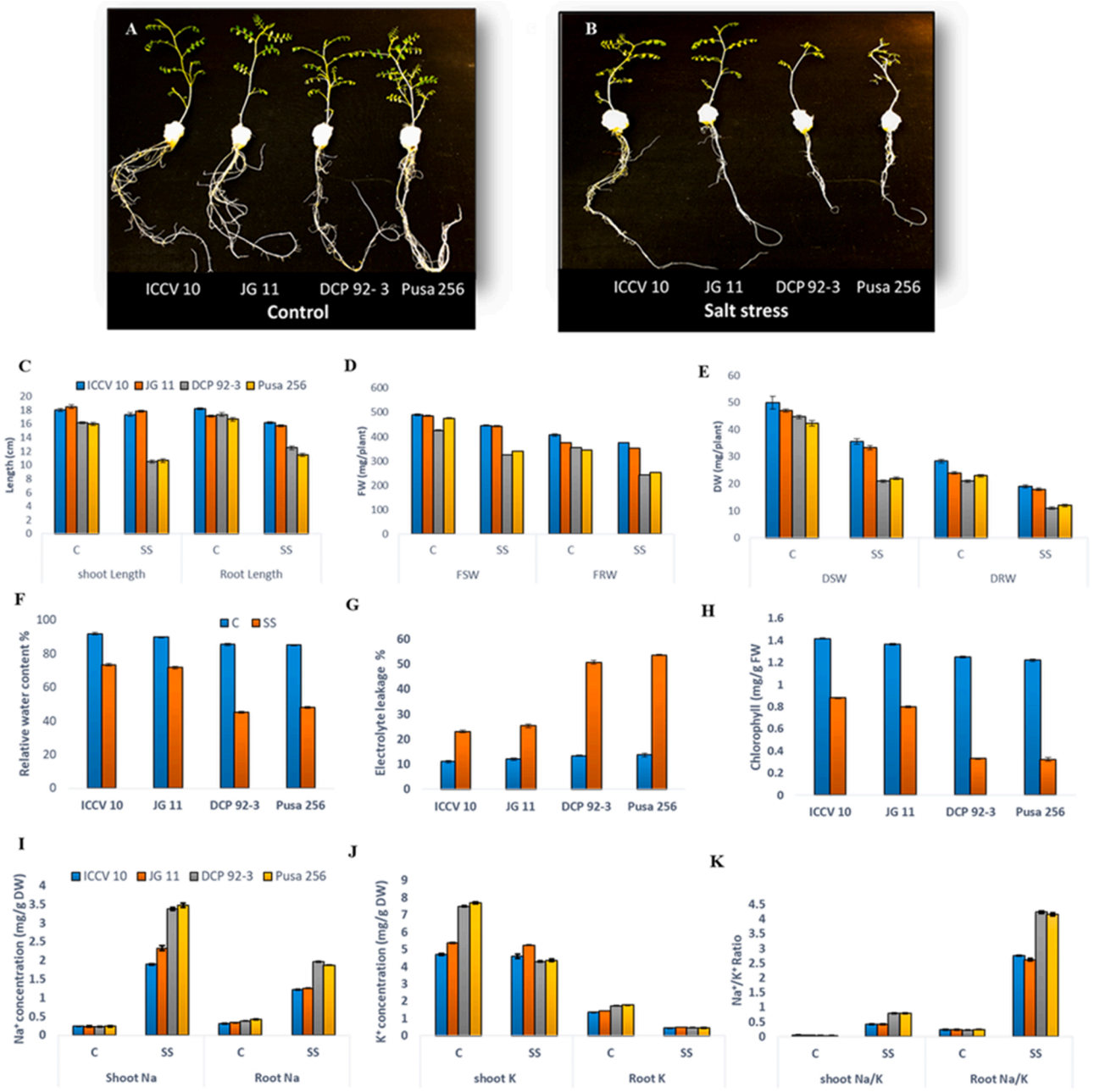

Fig. 1. Performance of four chickpea genotypes—ICCV 10, JG 11 (salt-tolerant) and DCP 92-3, Pusa 256 (salt-sensitive)—under normal (control) and salt stress conditions. (**A**) 21-day-old seedlings in the control; (**B**) 21-day-old seedlings after five days of exposure to salt stress (150 mM NaCl). Effect of the salt treatment on (**C**) root and shoot length, (**D**) fresh shoot weight (FSW) and fresh root weight (FRW), and (**E**) dry shoot weight (DSW) and dry root weight (DRW), (**F**) Relative water content (RWC), (**G**) electrolyte leakage (EL), (**H**) total chlorophyll content, (**I**) concentrations of Na^+, (J) K^+, and (**K**) shoot and root Na^+/K^+ ratios. Values for all traits are means ± SD of three biological replicates (C: control; SS: salt stress).

and Pusa 256 (–0.735) than the ICCV 10 (–0.379) and JG 11 (0.415) over the control condition (Table S4). Electrolyte leakage in response to salt stress varied between the genotypes ($P < 0.05$), with a 2.8- and 2.9-fold increase in the electrolyte leakage ratio of DCP 92-3 and Pusa 256, respectively, but less than half that in ICCV 10 (1.09-fold) and JG 11 (1.11) (Fig. 1**F**–**H**, Table S4), indicating that ICCV 10 and JG 11 possess better membranous network and protection ability from uncontrolled ion leakage under stress.

3.3. Ion dynamics under salt stress

The tolerant and sensitive genotypes had similar shoot and root Na^+ concentrations in the control in contrast, in the salt stress treatment, the sensitive genotypes, DCP 92-3 (3.37) and Pusa 256 (3.47) had significantly higher values of shoot Na^+ concentration than the tolerant genotypes, ICCV 10 (1.89) and JG 11 (2.32). Further, the tolerant genotypes maintained higher shoot K^+ concentrations under salt stress, while it drastically declined in the sensitive genotypes. Root K^+ concentrations declined in all four genotypes at 150 mM NaCl, more so in the two sensitive genotypes. Salt stress significantly affected the shoot and root Na^+/K^+ ratios in all four genotypes, more so in sensitive DCP 92-3 and Pusa 256 genotypes than the tolerant ICCV 10 and JG 11 one the sensitive genotypes (Fig. 1**I**–**K**, Table S4).

3.4. RNA sequencing and analysis

The Illumina paired-end sequencing generated about 530.5 million reads from four genotypes in two environmental conditions (control/salt-stress) with two biological replicates. About 237.1 million clean reads were obtained after filtration (Table S1). On average, about 93.26 % of the high-quality reads (Q ≥ 30) were mapped to the chickpea CDC frontier kabuli v2.6.3 reference genome (http://cegsb.icrisat.org/gtbt/ICGGC/GenomeManuscript.html), ranging from 92.11 % (DCP 92−3) to 94.46 % (Pusa 256) (Table S2). Details of the data generated, filtered and aligned reads mapped on the genome are in Tables S1; S2. For clarity, the samples were designated as 'Genotype_treatment-behavior,' e.g., ICCV 10_C-T represents genotype ICCV 10 in the control treatment (C), and it is salt-tolerant (T) and ICCV 10_S-T represents genotype ICCV 10 in the salt stress treatment (S), and it is salt-tolerant. Similarly DCP 92-3_C-S represents genotype DCP 92-3 in control treatment (C), and it is salt-sensitive (S) and DCP 92-3_S-S represents genotype DCP 92-3 in salt stress treatment (S), and it is salt-sensitive (S). Similar notation was used for JG 11 and Pusa 256 genotypes.

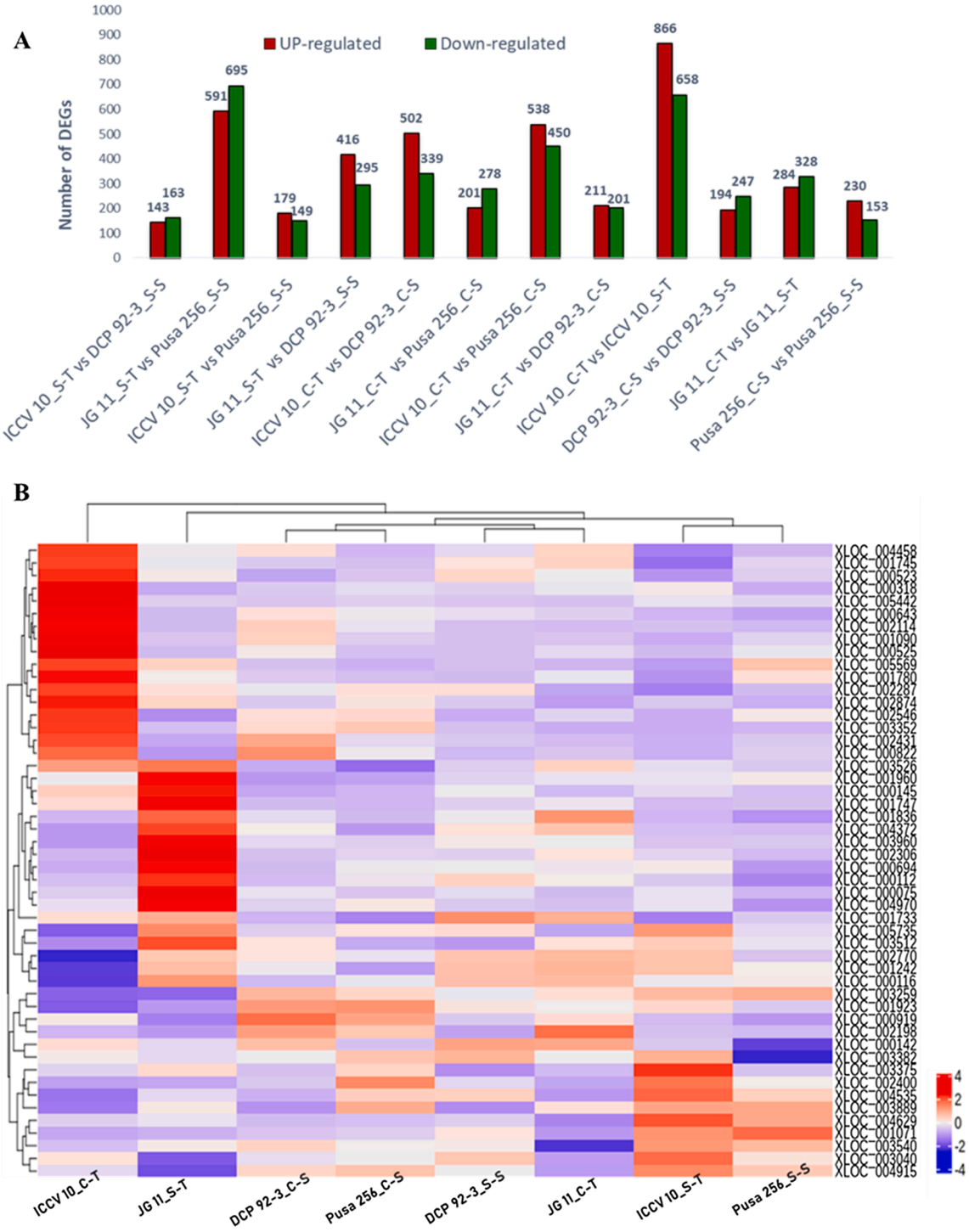

Fig. 2. Data (**A**) Overview of differentially expressed genes (DEGs) in various combinations; (**B**) Heat map and clustering of top 50 salt stress responsive DEGs in stress and control root tissues of tolerant (ICCV 10, JG11) and sensitive (DCP 92-3, Pusa 256) chickpea genotypes.

3.5. Differential gene expression analysis

In total, 21,698 DEGs were detected, of which 11,456 were up-regulated and 10,242 were down-regulated. The comparison between control and salt-stressed tissues of each chickpea genotype had the following number of DEGs: ICCV 10, 1524 (886 up-regulated; 658 down-regulated); JG 11, 612 (284 up-regulated; 328 down-regulated); DCP 92-3, 441 (194 up-regulated; 247 down-regulated); and Pusa 256, 383 (230 up-regulated; 153 down-regulated) (Fig. 2**A**). The distributions of all samples for the FPKM count were similar, and are shown as a violin plot in Fig. S1**A**. The principal component (PC) analysis revealed that the first two PCs explained most of the variance (59.02 %), and the genotypes were grouped to similar cluster as per the treatment (Fig. S1**B**). We further analyzed the expression profiles of the DEGs regulated under stress and control treatments across all genotypes. The heat map of the top 50 salt-responsive genes, scaled on FPKM expression values, revealed that all DEGs showed different expression patterns in the samples for salt stress and control treatments, indicating that the main response mechanism differed between the tolerant and sensitive genotypes. Based on their nature (tolerant or sensitive) and treatment, the genotypes were grouped into similar groups displaying different expression patterns for control and stress (Fig. 2**B**).

3.6. Comparative co-expression analysis of DEGs

Comparative transcriptome analysis was used to delineate the DEGs underlying the salt stress tolerance mechanism in chickpea by comparing each tolerant genotype with both sensitive genotypes under salt stress (Condition 1: ICCV 10_S-T vs DCP 92-3_S-S and Pusa 256_S-S; Condition 2: JG 11_S-T vs. DCP 92-3_S-S and Pusa 256_S-S; Condition 3: ICCV 10_C-T vs DCP 92-3_C-S and Pusa 256_C-S; Condition 4: JG 11_C-T vs DCP 92-3_C-S and Pusa 256_C-S) (Fig. 3**A**–**D**).

For Condition 1, 11 % (n = 33) of the up-regulated DEGs and 22.4 % (n = 57) of the down-regulated DEGs were co-expressed when root transcriptome of ICCV 10 was compared with DCP 92-3 and Pusa 256 under salt stress. In comparison, for Condition 3 (the control of Condition 1), 42.7 % (n = 311) of the up-regulated DEGs and 36 % (n = 209) of the down-regulated DEGs were co-expressed. For Condition 2, 0.01 % (n = 1) of the up-regulated DEGs and none (n = 0) of the down-regulated DEGs were co-expressed when root transcriptome of JG 11 was compared with DCP 92-3 and Pusa 256 under salt stress. In comparison, for Condition 4 (the control of Condition 2), 34 % (n = 124) of the up-regulated DEGs and none (n = 0) of the down-regulated DEGs were co-expressed. To delineate co-expressed exclusive DEGs, pooled comparisons were made among the four conditions. Interestingly, 33.6 % (n = 332) of the up-regulated DEGs and 31.6 % (n = 255) of the down-regulated DEGs were common for Condition 1 vs. Condition 3 (Fig. 3**E**, **F**). In contrast, 0.01 % (n = 1) of the up-regulated DEGs and none of the down-regulated DEGs were common for Condition 2 vs. Condition 4 (Fig. 3**G**, **H**). These analyses and results suggest that tolerant and sensitive genotypes have unique response mechanisms under salt stress.

3.7. Functional classification of DEGs and GO analysis

GO analysis of the differentially expressed transcripts indicated that a large fraction of the genes were involved in cellular processes, metabolic processes, response to stimulus, signaling receptor activity, transmembrane transporter activity, integral component of membrane, when comparison was done with tolerant (ICCV 10 and JG 11) and sensitive (DCP 92-3 and Pusa 256) (Fig. S2). Notably, in biological processes category GO terms like, cellular processes, metabolic processes, and response to stimulus constituted highly represented transcripts. Molecular function category terms including, catalytic activity, ATP binding, DNA-binding transcription factor activity, signaling receptor activity, and transmembrane transporter activity comprised several novel transcripts in both tolerant genotypes. Terms such as integral component of membrane, organelle, and cellular anatomical entity of cellular components category were highly represented in tolerant genotypes under salt stress (Fig. S2). Based on the DEG list, the GO enrichment analysis classified all DEGs in the 'biological processes,' 'cellular components' and 'molecular functions' categories. When both tolerant genotypes were compared with both sensitive genotypes under salt stress, 4257 differentially expressed GO terms were categorized into 64 functional

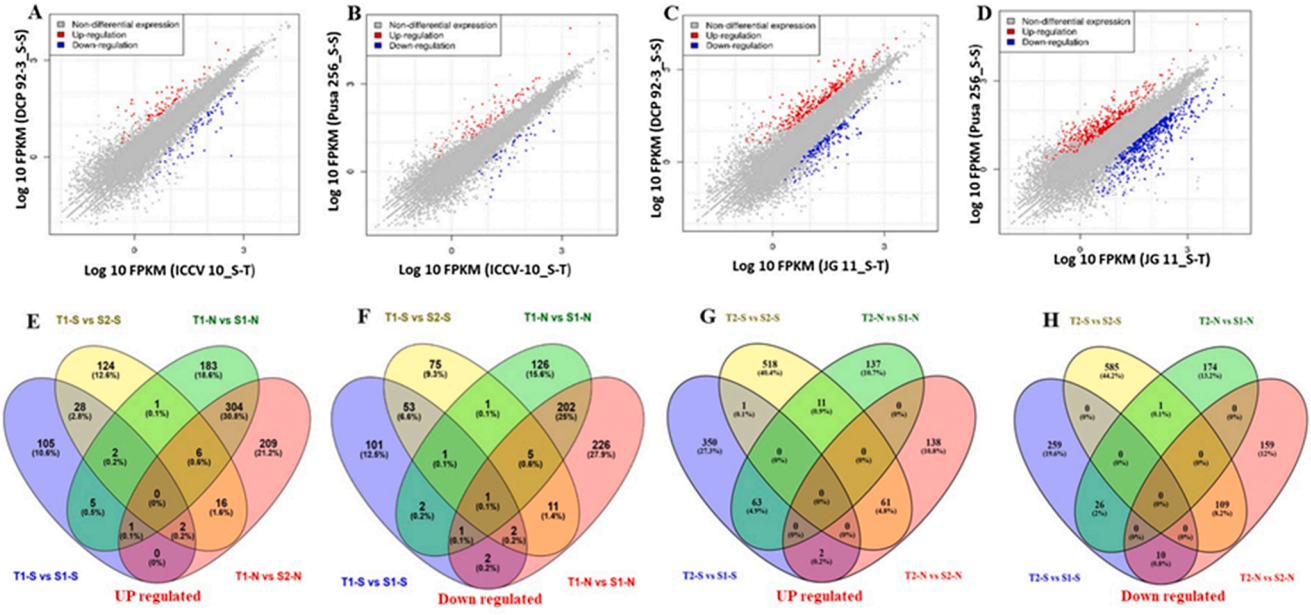

Fig. 3. Overview of DEGs in (**A**) ICCV 10 and DCP 92-3, (**B**) ICCV 10 and Pusa 256, (**C**) JG 11 and DCP 92-3, and (**D**) JG 11 and Pusa 256 genotypes in response to salt stress. Venn diagram of ICCV 10 (T1) vs DCP 92-3 and Pusa 256 for (**E**) up- regulated DEGs and (**F**) down-regulated DEGs (T1-S: ICCV 10_salt stress; T1-N: ICCV 10_normal condition; S1-S: DCP 92-3_salt stress; S1-N: DCP 92-3_normal condition; S2-S: Pusa 256_salt stress and S2-N: Pusa 256_normal condition). Venn diagram of JG 11 (T2) vs DCP 92-3 and Pusa 256 for (**G**) up- regulated DEGs and (**H**) down-regulated DEGs (T2-S: JG 11_salt stress; T2-N: JG 11_normal condition; S1-S: DCP 92-3_salt stress; S1-N: DCP 92-3_normal condition; S2-S: Pusa 256_salt stress, and S2-N: Pusa 256_normal condition).

groups; of these, the highest proportion had roles in various molecular functions (41.08 %) followed by cellular components (36.26 %) and biological processes (22.65 %). Most of the DEGs coding for molecular function were involved in binding (12.85 % Up- and 18.35 % Dn-), (Up- and Dn- for up-regulation and down-regulation) followed by catalytic activity (14.44 % Up- and 18.11 % Dn-) and transmembrane transporter activity (2.22 % Up- and 1.38 % Dn-). Under cellular components, cellular anatomical entity (12.41 % Up- and 15.72 % Dn-), integral component of membrane (9.30 % Up-), and intrinsic component of membrane (7.07 % Dn-) accounted for most of the unigenes. In biological processes, cellular processes (7.64 % Up- and 7.59 % Dn-), metabolic processes (7.395 % Up- and 7.06 % Dn-), and response to stimulus (2.06 % Up- and 1.45 % Dn-) were the most abundant (Fig. S3**A**, **B**). The GO enrichment revealed significantly enriched GO terms, including DNA binding (GO:0003677), DNA-binding transcription factor activity (GO:0003700), ATP binding (GO:0005524), protein kinase activity (GO:0004672), heme-binding (GO:0020037), and catalytic activity (GO:0003824) in the molecular function category; integral component of membrane (GO:0016021) and cytoplasm (GO:0005737) in the cellular component category; and regulation of transcription DNA-templated (GO:0006355), carbohydrate metabolic process (GO:0005975), ATP synthesis coupled electron transport (GO:0042773), and metabolic process (GO:0008152) in the biological process category.

3.8. DEGs playing role under salinity

Many DEGs were discovered with significant role and associations under salt stress (Table 1), including K^+ transporter-like protein HAK/KUP transporter, MIP/aquaporin, NADH dehydrogenase, cysteine-rich knottin fold-containing protein, pectinesterase, PP2C family protein, pectinesterase family, PMEI family proteins. These DEGs are involved in providing cellular membrane stability, signal transduction, stress response, and transporter roles, and associated with rendering key functions under salt stress conditions. The overall schematic representation of common differentially expressed genes between tolerant (ICCV 10, JG 11) and sensitive (DCP 92-3, Pusa 256) genotype was investigated to explain the role of these DEGs under salt stress response in chickpea (Fig. 5). Under elevated salinity condition initial stress signal perception is mediated by calcium signaling regulated by calcium-transporting- ATPase gene (XLOC_024020/Ca8:3558556-3564115) which was 3.4–fold up regulated on an average in both the tolerant genotypes. Calcium dependent protein kinases (CDPK) (XLOC_011390/Ca4:10748618-10751862) which help in signal relay mechanism was 2.8–fold more induced in tolerant genotypes. Potassium transporter gene (XLOC_020154/Ca6:56052452-56054038) and potassium channel SKOR gene (XLOC_004326/Ca2:29180453-29194331) was upregulated 6.27 and 3.4–folds respectively in tolerant genotypes which are crucial for Na^+/K^+ transport and maintaining ion homeostasis. Up regulation of sulfate transporter (XLOC_000699/ Ca1:9629010-9636351) by average 3.2–folds in tolerant genotypes was also observed in our study. DEGs regulating classical hormones and altering physiological responses under salinity like, auxin family genes (XLOC_023226/ Ca7:29857368-29858094) along with the gibberellin pathway genes (XLOC_002022/Ca1:4155478-4156812; XLOC_004387/Ca2:30891255-30894270) were up-regulated 2.8 and 3.3–folds in tolerant genotypes during the stress. One of the important ABA-responsive protein is LEA which was 4.65–folds more induced in the tolerant genotype (XLOC_019999/Ca6:50609352-50610607). Genes coding for proline rich protein (XLOC_020334/ Ca7:115237-117244) involved in cell wall reshaping was upregulated 3– folds in tolerant genotypes, along with HSP family gene (XLOC_018435/ Ca6:4438826-4442354) induced 3.2–folds under stress. Important TFs like, NAC transcripts (XLOC_001827/Ca1:885079-897343; XLOC_002698/Ca1:16634934-16637079; XLOC_002699/Ca1:16679036-16680994) were significantly induced 3–folds on average among tolerant genotype. Along with MYB TFs (XLOC_006905/Ca3:31295435-31297967; XLOC_024671/Ca8:1441983-1443055) which are upregulated 3.2–folds. WRKY TFs (XLOC_020071/Ca6:53172650-53175146) was highly induced by 2.5–folds in the tolerant genotype. While in our study ERF gene (XLOC_008940/Ca4:1226609-1229348; XLOC_009021/Ca4:2797715-2798126) were repressed 2.7 –folds in tolerant genotypes under salt stress with respect to sensitive genotypes (Fig. 5).

3.9. Differentially expressed (D) genes encoding TFs

A total of 173 TFs encoding transcripts were differentially regulated; of which, 85 were up-regulated and 88 were down-regulated when root tissue of 1. ICCV 10_S-T (T1) vs. DCP 92-3_S-S (S1) and Pusa 256_S-S (S2), 2. JG 11_S-T (T2) vs. DCP 92-3_S-S (S1) and Pusa 256_S-S (S2) were compared under salt (Fig. 4**A**). These TFs belong to 25 different families that are important regulators of salinity stress responses, including the ERF, NAC, WRKY, MYB, bZIP, HD-ZIP, C2H2, and HSF families, with 54 % belonging to the ERF, MYB, WRKY, and NAC families (Fig. 4**B**). Most of the TFs encoded by DEGs were regulated in JG 11_S-T vs. Pusa 256_S-S (89) and JG 11_S-T vs. DCP 92-3_S-S (39), when compared to ICCV 10_S-T vs. DCP 92-3_S-S (28) and ICCV 10_S-T vs. Pusa 256_S-S (17).

3.10. Analysis of metabolic and phytohormonal pathways triggered under stress

A comprehensive analysis of DEGs revealed enrichment of DETs and enzymes involved in UDP-glucose biosynthesis (EC-3.1.3.11) and trehalose (EC-2.4.1.15), a non-reducing disaccharide, under salt stress. Transcripts for cell wall metabolites biosynthesis such as xyloglucan (EC-2.4.2.39) and cellulose (EC-2.4.1.12) were also enriched under salt stress. Transcripts essential for lipid metabolism (EC-2.3.1.199), starch/sucrose metabolism (EC-2.7.7.27), and photosynthesis (EC-1.10.3.9) (Fig. S4**A**) and secondary metabolites such as, phenylpropanoids (EC-2.3.1.133) and isoflavonoids (EC-4.2.1.105) were significantly upregulated in tolerant genotypes in response to salt stress (Fig. S4**B**) suggesting differential response of DETs acclimatizing salinity tolerance in ICCV 10 and JG 11 genotypes under stress.

Numerous DEGs investigated for various phytohormonal metabolic pathways were regulated differentially in tolerant and sensitive genotypes. Under salt stress, most of pathways and enzymes involved in biosynthesis and signaling of ethylene, GA, ABA and JA were altered differentially. Enzymes regulating the JA pathway such as lipoxygenase (EC-1.13.11.12), allene oxide cyclase (EC-5.3.99.6), GA pathway such as gibberellin3 and beta-dioxygenase (EC-1.14.11.15), ABA pathway such as xanthoxin dehydrogenase (EC-1.1.1.288), epoxy carotenoid dioxygenase (EC-1.13.11.51), and ethylene biosynthesis such as amino cyclopropane carboxylate oxidase (EC-1.14.17.4) were mostly expressed and enriched under salt stress. Pathway details and annotation, along with corresponding gene information, are provided in Table S6. The overall results suggest a crucial role of DETs regulating pathways and enzymes essential for signaling, metabolism, and biosynthesis of several metabolites under salt stress (Fig. S5).

3.11. STRING –Based interaction network analysis of DEGs

Out of 11 genes validated through qRT-PCR playing vital role in salinity response, significant interaction was observed among 8 DEGs studied using STRING network analysis database with a confidence score of >0.5 (Fig. S5; Table S7). In our study ABR-18 (XP_004508176.1), an important ABA signaling pathway gene in response to abiotic stress interacted with proteins like, thaumatin-like protein and CEVI57-like proteinase inhibitor, which also plays crucial role in signaling and transduction. Similarly genes having role in cell wall remodeling and epigenetic response under abiotic stress like, methyltransferase (XP_004512403.1) and pectinesterase

Table 1
List of some differentially expressed genes (DEGs) playing important roles under salinity stress.

Sr. No.	Gene-id	Locus-id	Sample	log2 (fold change)	q value	FDR	Protein names	Length	Function
1	XLOC_020154	Ca6:56052452-56054038	ICCV 10_S-T vs DCP 92-3_S-S	2.95	0.01736	5.88235E-05	Potassium transporter	794	Integral component of membrane ; potassium ion transmembrane transporter activity
2	XLOC_023784	Ca7:48288085-48292841	JG 11_S-T vs Pusa 256_S-S	–2.82	0.01736	5.88235E-05	Tyrosine aminotransferase-like	544	Pyridoxal phosphate binding; transaminase activity
3	XLOC_027724	scaffold2356:136286-140082	Pusa 256_C-S vs Pusa 256_S-S	–3.40	0.01736	5.88235E-05	Alcohol dehydrogenase-like 1 (EC 1.1.1.284)	552	S-(hydroxymethyl) glutathione dehydrogenase activity ; zinc ion binding
4	XLOC_021634	Ca7:32902184-32903830	ICCV 10_C-T vs ICCV 10_S-T	5.31	0.01736	5.88235E-05	NADH dehydrogenase	760	Membrane ; iron-sulfur cluster binding
5	XLOC_014232	Ca5:44845013-44847201	ICCV 10_C-T vs ICCV 10_S-T	3.33	0.01736	5.88235E-05	Alcohol dehydrogenase 1	380	Oxidoreductase activity ; zinc ion binding
6	XLOC_006244	Ca3:15776984-15781198	ICCV 10_C-T vs ICCV 10_S-T	–4.88	0.01736	5.88235E-05	Pectinesterase (EC 3.1.1.11)	555	Cell wall; extracellular region; integral component of membrane
7	XLOC_007200	Ca3:37193403-37195384	JG 11_S-T vs Pusa 256_S-S	–4.03	0.01736	5.88235E-05	Chitinase 10	278	Carbohydrate metabolic process ; cell wall macromolecule catabolic process
8	XLOC_006398	Ca3:20964462-20966491	ICCV 10_C-T vs ICCV 10_S-T	–4.22	0.01736	5.88235E-05	Peroxidase (EC 1.11.1.7)	316	Heme binding ; metal ion binding ; response to oxidative stress
9	XLOC_022896	Ca7:17530986-17531919	Pusa 256_C-S vs Pusa 256_S-S	4.31	0.01736	5.88235E-05	Defensin Ec-AMP-D2-like	74	Defense response
10	XLOC_008327	Ca3:30235311-30235884	JG 11_S-T vs Pusa 256_S-S	–4.83	0.01736	5.88235E-05	Dirigent protein	190	Apoplast
11	XLOC_007428	Ca3:2898890-2901870	ICCV 10_C-T vs ICCV 10_S-T	–3.36	0.01736	5.88235E-05	Proline dehydrogenase (EC 1.5.5.2)	492	Proline dehydrogenase activity
12	XLOC_020081	Ca6:53477910-53479566	ICCV 10_C-T vs ICCV 10_S-T	–3.86	0.01736	5.88235E-05	Extensin-2-like isoform X1	551	Structural constituent of cell wall ; plant-type cell wall organization
13	XLOC_015801	Ca5:41567328-41568967	JG 11_S-T vs Pusa 256_S-S	–3.45	0.01736	5.88235E-05	NAD(P)H-dependent 6′-deoxychalcone synthase	314	Oxidoreductase activity
14	XLOC_003283	Ca1:41795277-41797707	JG 11_S-T vs Pusa 256_S-S	3.31	0.01736	5.88235E-05	Proline-rich extensin-like protein EPR1	779	Electron transfer activity
15	XLOC_013297	Ca5:26574618-26577422	DCP 92-3_S-S vs JG 11_S-T	4.91	0.04120	0.00015	Peroxidase (EC 1.11.1.7)	318	Extracellular region ; heme binding; metal ion binding; response to oxidative stress
16	XLOC_020593	Ca7:4658459-4659243	ICCV 10_S-T vs DCP 92-3_S-S	4.43	0.01736	5.88235E-05	ABA-responsive protein ABR18-like	158	Defence response
17	XLOC_000695	Ca1:9494514-9501538	JG 11_S-T vs Pusa 256_S-S	−4.77	0.01736	5.88235E-05	Pleiotropic drug resistance protein 1-like	1423	Integral component of membrane; ATP binding
18	XLOC_014700	Ca5:12454217-12456602	JG 11_S-T vs Pusa 256_S-S	−5.76	0.01736	5.88235E-05	NAD(P)H-dependent 6′-deoxychalcone synthase-like	315	Oxidoreductase activity
19	XLOC_011849	Ca4:19831460-19835276	JG 11_S-T vs Pusa 256_S-S	−4.32	0.01736	5.88235E-05	Peroxidase (EC 1.11.1.7)	326	Extracellular region; heme binding ; metal ion binding; response to oxidative stress
20	XLOC_019130	Ca6:18298741-18299470	ICCV 10_C-T vs ICCV 10_S-T	6.95	0.01736	5.88235E-05	Ethylene-responsive transcription factor ERF109-like	242	DNA binding ; DNA-binding transcription factor activity; transcription, DNA-templated

(*continued on next page*)

Table 1 (*continued*)

Sr. No.	Gene-id	Locus-id	Sample	log2 (fold change)	q value	FDR	Protein names	Length	Function
21	XLOC_016328	Ca6:3542179-3545755	JG 11_C-T vs JG 11_S-T	−2.91	0.04120	0.00015	Auxin-induced protein	302	Auxin-activated signaling pathway; regulation of transcription
22	XLOC_024846	Ca8:4375531-4378017	JG 11_S-T vs Pusa 256_S-S	−3.36	0.03003	0.000105263	Peroxidase (EC 1.11.1.7)	326	Integral component of membrane; heme binding ; metal ion binding; response to oxidative stress
23	XLOC_003845	Ca2:10018106-10020979	JG 11_S-T vs Pusa 256_S-S	5.44	0.01736	5.88235E-05	Pectinesterase (EC 3.1.1.11)	330	Cell wall; pectinesterase activity; cell wall modification
24	XLOC_012457	Ca4:43346544-43349396	JG 11_S-T vs Pusa 256_S-S	−3.16	0.01736	5.88235E-05	G-type lectin S-receptor-like serine/threonine-protein kinase At1g34300	796	Integral component of membrane ; ATP binding; carbohydrate binding ; protein serine/threonine kinase activity
25	XLOC_024671	Ca8:1441983-1443055	JG 11_S-T vs Pusa 256_S-S	−3.91	0.03003	0.000105263	MYB-like transcription factor ETC3	89	DNA binding
26	XLOC_019433	Ca6:26818667-26820154	ICCV 10_C-T vs ICCV 10_S-T	−3.46	0.03003	0.000105263	Aquaporin TIP1-3-like	252	Integral component of membrane ; channel activity
27	XLOC_007185	Ca3:37018986-37024185	ICCV 10_C-T vs ICCV 10_S-T	−3.07	0.01736	5.88235E-05	Probable boron transporter 2	724	Integral component of membrane ; inorganic anion exchanger activity
28	XLOC_003743	Ca2:6725516-6730244	JG 11_S-T vs Pusa 256_S-S	−4.74	0.01736	5.88235E-05	Glutamate dehydrogenase	411	Oxidoreductase activity, acting on the CH-NH2 group of donors, NAD or NADP as acceptor
29	XLOC_022294	Ca7:4661750-4662413	JG 11_S-T vs Pusa 256_S-S	−3.56	0.01736	5.88235E-05	ABA-responsive protein ABR18-like	158	Defense response ; response to biotic stimulus
30	XLOC_013038	Ca5:16885219-16890174	JG 11_S-T vs Pusa 256_S-S	5.87	0.01736	5.88235E-05	Polygalacturonase QRT2	422	Carbohydrate metabolic process ; cell wall organization
31	XLOC_003099	Ca1:33648586-33651426	JG 11_S-T vs Pusa 256_S-S	−3.51	0.01736	5.88235E-05	UDP-arabinopyranose mutase 3-like	358	Intramolecular transferase activity ; plant-type cell wall organization or biogenesis
32	XLOC_024279	Ca8:7753681-7758420	JG 11_S-T vs Pusa 256_S-S	−3.23	0.01736	5.88235E-05	Probable methyltransferase PMT21	600	Integral component of membrane ; methyltransferase activity
33	XLOC_002699	Ca1:16679036-16680994	ICCV 10_S-T vs JG 11_S-T	3.73	0.01736	5.88235E-05	NAC domain-containing protein 72	339	DNA binding; Regulation of transcription
34	XLOC_024238	Ca8:7123235-7125975	JG 11_C-T vs JG 11_S-T	−4.17	0.01736	5.88235E-05	ABC transporter G family member 14	661	Integral component of membrane; ATP binding ; ATPase activity
35	XLOC_015822	Ca5:42022191-42023501	JG 11_S-T vs Pusa 256_S-S	−4.98	0.01736	5.88235E-05	Chalcone synthase 1B	389	Transferase activity ; biosynthetic process
36	XLOC_005865	Ca2:36172245-36185403	JG 11_S-T vs Pusa 256_S-S	−3.59	0.01736	5.88235E-05	NAD(P)H-dependent 6′-deoxychalcone synthase-like	315	Oxidoreductase activity
37	XLOC_011424	Ca4:11325147-11326559	DCP 92-3_S-S vs JG 11_S-T	4.17	0.01736	5.88235E-05	Peroxidase (EC 1.11.1.7)	357	Extracellular region; peroxidase activity; response to oxidative stress
38	XLOC_020894	Ca7:10137353-10138151	ICCV 10_S-T vs JG 11_S-T	2.98	0.01736	5.88235E-05	ethylene-responsive transcription factor 1A	265	DNA-binding transcription factor activity
39	XLOC_007155	Ca3:36524384-36524773	ICCV 10_C-T vs ICCV 10_S-T	3.48	0.01736	5.88235E-05	Indole-3-acetic acid-induced protein ARG2-like	96	Response to stress
40	XLOC_027343	scaffold1981:249921-251361	JG 11_S-T vs Pusa 256_S-S	−2.734	0.03003	0.000105263	Glycosyltransferase (EC 2.4.1.-)	479	Transferase activity, transferring hexosyl groups; metabolic process

(XP_004489925.1) were also interacting with other proteins having similar role under stress (Fig. S5). Network analysis of important TFs like, NAC (XP_004487680.1), MYB (XP_004511522.1) and ERF (XP_004508948.1) showed interactions with other proteins expressed under elevated salinity, including WRKY, zinc finger protein, LEA, E3 ubiquitin-protein ligase MBR2, and bHLH92. In our study gene having role in protection against nitro-oxidative stress induced by adverse environmental conditions NADPH (XP_004491606.1) have been also found to be interacting with other proteins like, chalcone synthase, chalcone-flavonone and trans-cinnamate 4-monooxygenase-like

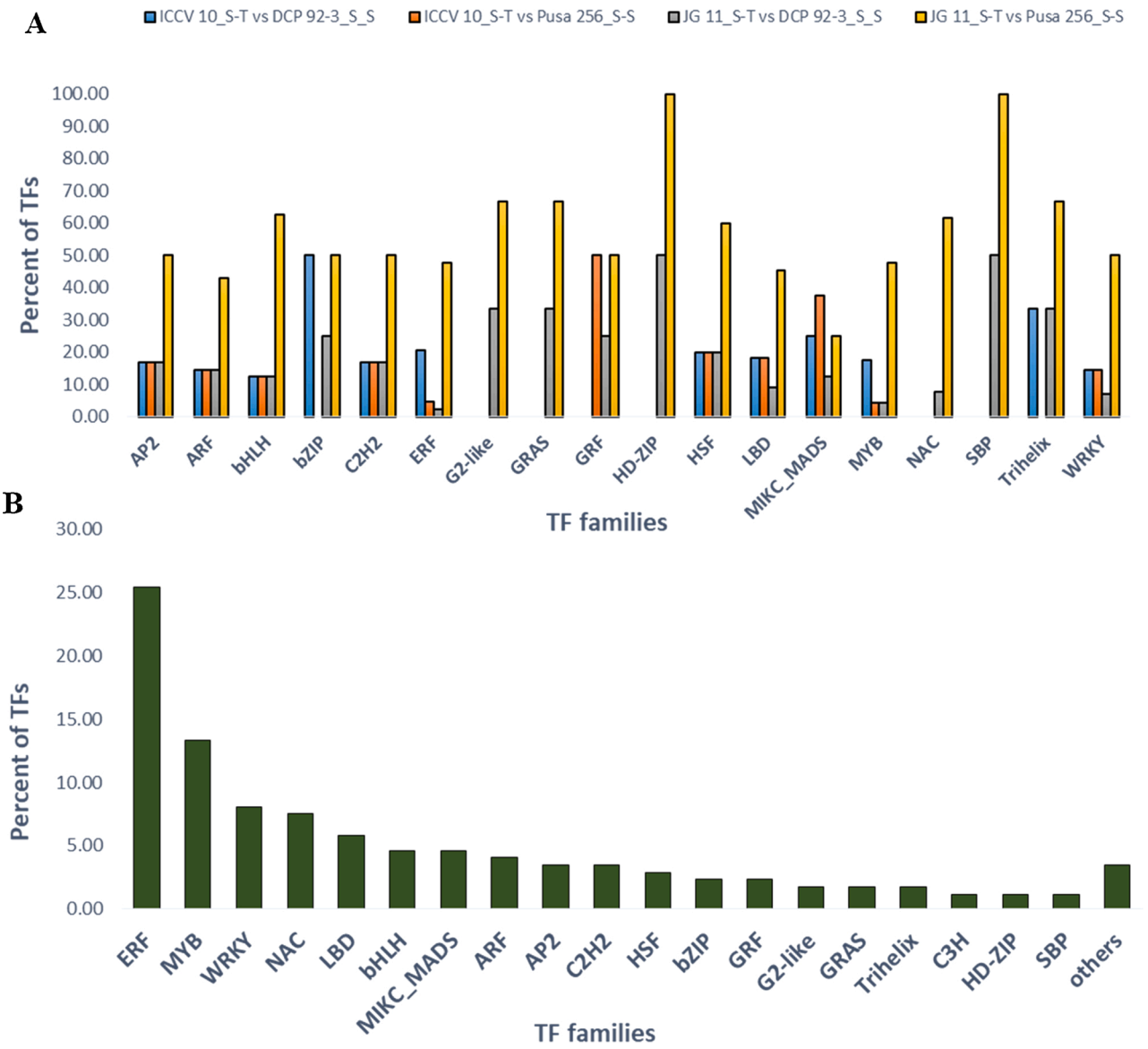

Fig. 4. (**A**) Comparisons of differentially expressed transcription factors in both tolerant with sensitive genotypes under salt stress; (**B**) differentially expressed transcription factor families in chickpea genotypes under salt stress.

proteins. Another important gene regulating signaling and structural component of cell wall under stress EPR1 (XP_004488730.1) (proline-rich extensin-like protein), was interacting with other proteins like, L-allo-threonine aldolase-like, dihydroflavonol-4-reductase, calcium-binding protein CML18-like (Fig. S5; Table S7).

3.12. Validation of differential gene expression

The reliability of sequencing results was confirmed by qRT-PCR studies. A separate set of samples were prepared by mimicking the RNA-seq experimental conditions and 11 DEGs selected based on their differential roles under salt stress were subjected to reverse transcription-PCR. Of these genes, XLOC_005865 (NADPH-dependent 6′-deoxychalcone synthase-like), XLOC_020154 (potassium transporter), XLOC_024671 (MYB-like transcription factor ETC3), XLOC_024279 (methyltransferase PMT21), XLOC_003845 (pectinesterase), XLOC_027724 (alcohol dehydrogenase-like 1), and XLOC_001747 (epidermis-specific secreted glycoprotein EP1) were up-regulated in tolerant (ICCV10 and JG 11) and down-regulated in sensitive (DCP 92-3 and Pusa 256) genotypes under salt stress. Genes such as XLOC_020593 (ABA-responsive protein ABR18-like) and XLOC_020894 (ethylene-responsive transcription factor 1A) were down regulated in tolerant and up-regulated in sensitive genotypes. All 11genes followed similar trends of expression (up- or down-regulation) in both the qRT-PCR and RNA-seq analysis. For most of the samples, the results obtained in qRT-PCR studies were highly consistent with the RNA-seq data ($r^2 = 0.89$). However, the detected fold-changes in qRT-PCR of the four genes (XLOC_001747, XLOC_005865, XLOC_020593, and XLOC_024671) were less than those obtained in RNA-seq (Fig. 6).

4. Discussion

To investigate the mechanism of salt stress tolerance in chickpea, phenotypic and physiological comparisons were studied under hydroponic conditions. This study aimed to delineate the molecular mechanism underlying salt tolerance in well-characterized chickpea genotypes, ICCV 10 and JG 11 (tolerant) and DCP 92-3 and Pusa 256 (sensitive) (Kaur et al., 2014). Phenotypic and physiological attributes, including seedling growth attributes, physio-biological traits such as RWC, EL, chlorophyll content, and ionic distribution of Na^+ and K^+ in

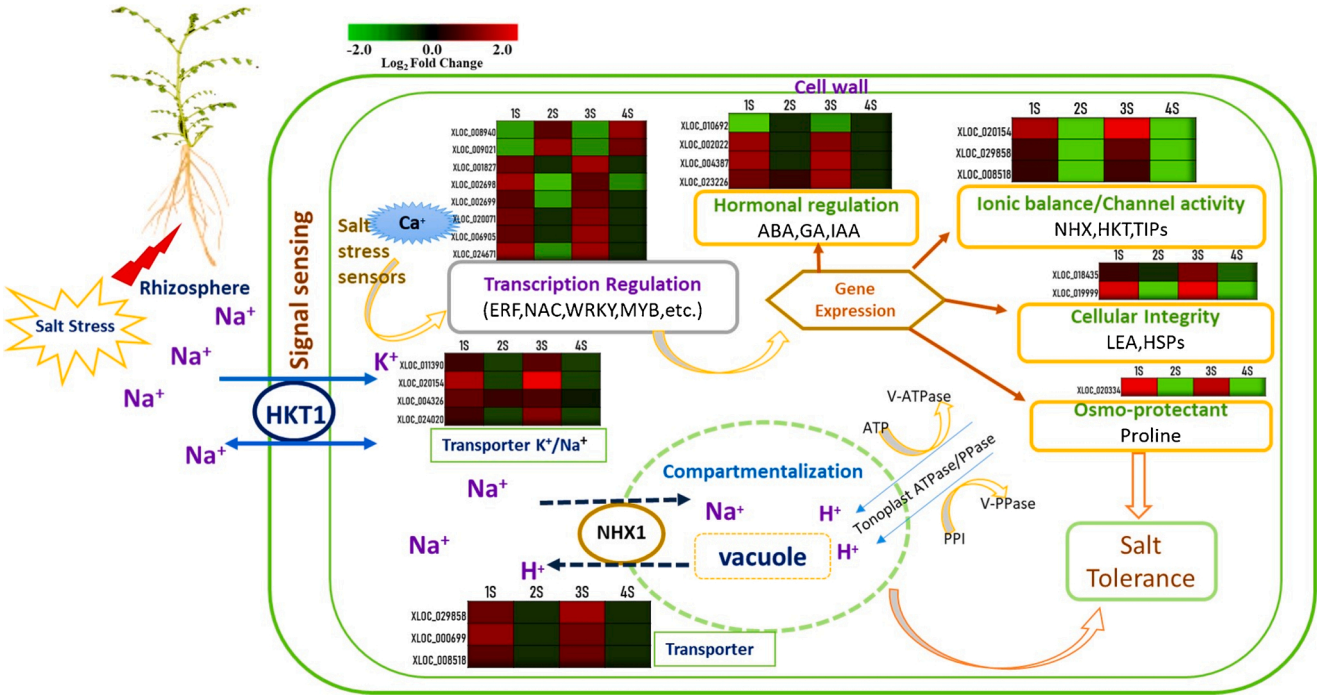

Fig. 5. Schematic representation of the molecular response and mechanisms underlying tolerance to salinity stress (1S: ICCV 10_S-T; 2S: DCP 92-3_S-S; 3S: JG 11_S-T; 4S: Pusa 256_S-S) *Each Sample name combines the genotype name followed by the treatment (S: salt stress) and tolerance to salinity (T, salt-tolerant; S, salt-sensitive).

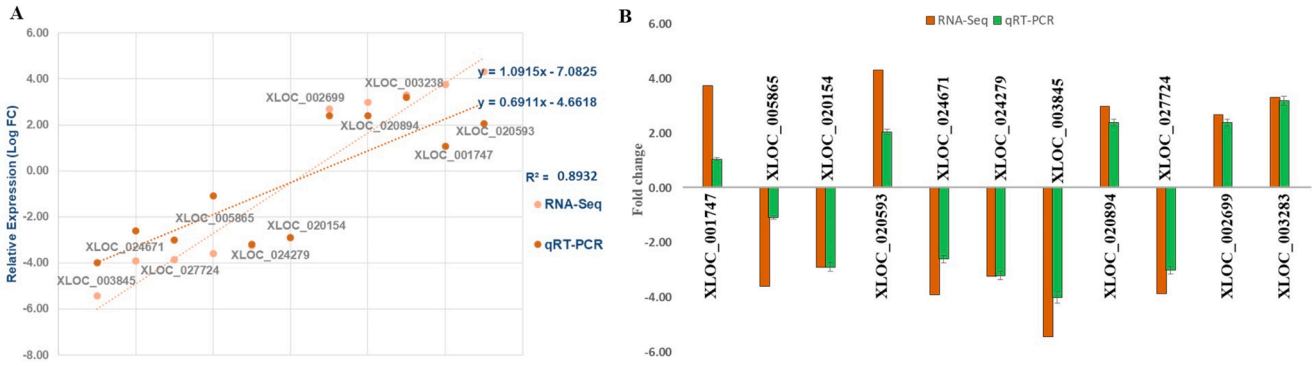

Fig. 6. qRT-PCR validation of the RNA-seq data of 11 DEGs, representing relative fold-change of expression data under salt stress.

shoot and root tissues, of sensitive genotypes were more affected than tolerant genotypes under salt stress (Fig. 1; Table S4). These results confirmed the contrasting response of selected chickpea genotypes under salt stress (Kaur et al., 2014; Singh et al., 2018).

Seedling growth parameters (length, fresh and dry weight of roots and shoots) followed a decreasing trend under salt stress in all genotypes, more reduction in the sensitive genotypes, DCP 92-3 and Pusa 256, than the tolerant genotypes, ICCV 10 and JG 11 was observed. These results may be attributed to ion toxicity, oxidative stress, osmotic imbalance, and distortion of metabolic activity under salt stress (Munns and Tester, 2008; Singh et al., 2018).

Physiological changes in genotypes were evident under salt stress. Tolerant genotypes (ICCV 10 and JG 11) had higher chlorophyll contents and RWCs but lower ELs than sensitive genotypes (DCP 92-3 and Pusa 256). Previous studies have suggested that these physiological traits can be used as biomarkers for salt tolerance screening (Arefian and Malekzadeh Shafaroudi, 2015; Negrão et al., 2016). This is due to the greater ability and efficiency of water absorption to avoid tissue dehydration in tolerant plants under stress (Hu et al., 2013). The lower EL ratio in tolerant genotypes indicates their ability to protect the membranous cellular network from uncontrolled EL under salt stress (Singh et al., 2018). A gradual reduction in chlorophyll content was noticed in all genotypes under salt stress, more so in sensitive genotypes, which can be attributed to the replacement of Mg^{2+} ions and disruption of the cellular membranous network by excess Na^+ and Cl– in sensitive genotypes (Molazem et al., 2010).

The ion profiling results showed that the sensitive genotypes had higher Na^+/K^+ ratios than the tolerant genotypes, and the distribution of Na^+ and K^+ in plant tissue is critical for tolerance (Shabala and Cuin, 2008). Na^+ and K^+ concentrations and their ratio serve as key parameters for studies related to the salt stress response. The shoot Na^+/K^+ ratios in the tolerant genotypes (ICCV 10 and JG 11) was maintained under salt stress by accumulating less Na^+ and more K^+ than the sensitive genotypes. The Na^+/K^+ ratios under salt stress was significantly higher in shoot tissue and lower in root tissue of sensitive genotypes (DCP 92-3 and Pusa 256) than tolerant genotypes (ICCV 10 and JG 11) (Fig. 1**I**–**K**, Table S4), which is consistent with previous studies (Cotsaftis et al., 2011; Walia et al., 2006). Salt-tolerant genotypes balance ion

homeostasis through carrier proteins, antiporters, and ionic pumps present on cellular membranes (Reddy et al., 2017), along with toxic ion exclusion and a compartmentation mechanism that avoids toxicity of shoot Na^+ under excessive salt concentrations (Flowers et al., 2010; Munns and Tester, 2008).

Understanding salinity tolerance at the molecular level can facilitate the development of salt-tolerant varieties through molecular breeding approaches in chickpea. We performed a comparative transcriptome analysis among tolerant and sensitive genotypes to delineate transcriptional differences under salt stress (Fig. 5), in line with previous studies in other crops (Li et al., 2019; Mansuri et al., 2019; Shinde et al., 2018). This study identified several DEGs/transcripts in chickpea, and their expression patterns in roots showed a genotype/treatment-specific response. The transcripts involved in various cellular, molecular, and biological processes, and metabolic pathways changed in tolerant and sensitive genotypes under salt stress.

DEGs regulating transmembrane transporters provides salt stress resilience by influencing genes regulating transporter pathways. Initial stress perception of stress signal is mediated by calcium signaling regulated via calcium-transporting ATPase gene and CDPKs. The calcium ions in turn activate the protein kinase genes which help in signal relay mechanism and efflux of Ca^{2+} is crucial for sensory pathways under elevated salinity (Soren et al., 2020; Kudla et al., 2010), which is also earlier reported to confer salinity tolerance in Arabidopsis and chickpea (Schulz et al., 2013; Kaashyap et al., 2018). Transcripts coding for ion transporter HAK/KUP (K^+ transporter) and potassium channel SKOR gene were significantly up-regulated in tolerant genotypes with possible involvement in salinity tolerance. These genes are related to the extrusion of Na^+ to improve the Na^+/K^+ ratio in plants maintaining Na^+ and K^+ homeostasis under salt stress (Li et al., 2019; Duan et al., 2015). Unique sulfate transporter gene was upregulated in tolerant genotypes which is crucial for uptake and distribution of sulphur, important for maintenance of the cellular redox balance and mitigates damage caused by reactive oxygen species under abiotic stress (Chan et al., 2013). DEGs regulating classical hormones signaling network and altering physiological responses under salinity like ARF family genes (auxin response factor) and GAs (Gibberellins) were up-regulated which are reported as major hormone for cell differentiation, cell elongation and inducing genes for stress tolerance aiding plant to cope up with elevated stress (Su et al., 2015). One of the important ABA-responsive protein is LEA (late embryonic abundant) protein which affects the accumulation of osmo protectant and stomatal closer to deal with water stress (Cramer et al., 2011; Battaglia and Covarrubias, 2013). Genes affecting these hormonal pathways are crucial to understand the roles of these endogenous molecules regulating several plant developmental processes in acclimating chickpea adaptation to salt stress. Mostly transcripts related to the transporter and signaling pathway were regulated under salt stress in the contrasting genotypes, suggesting their possible role in salt stress-adaptive traits in tolerant (ICCV 10 and JG 11) genotypes, consistent with previous reports (Garg et al., 2016; Shankar et al., 2016).

We identified several DEGs encoding NADPH, Cytochrome P450 and PRA1 family proteins, known for their role in membrane maintenance, oxidoreductase activity, and iron ion binding properties were highly induced under salinity in plants (Liu et al., 2020; Mao et al., 2013; Molina et al., 2011). The ABC transporter superfamily was also significantly regulated under salt stress in this study. Genes coding TIPs (tonoplast-expressed aquaporin), known for their role in osmotic and turgor homeostasis in Arabidopsis and rice under salt stress condition (Maathuis et al., 2003), were also differentially expressed in the contrasting chickpea genotypes. Proline rich protein gene involved in cell wall reshaping (Knoch et al., 2014) and HSP family gene involve in high-temperature-inducible chaperones that regulate normal plant growth processes by helping with protein folding and preventing protein aggregation under adverse abiotic stress conditions were upregulated in tolerant genotypes under elevated salinity (Arefian et al., 2019).

The expression of genes encoding AP2-EREBP, MYB, NACs, and WRKY TFs were differentially regulated in the chickpea genotypes (Fig. 4). Studies have reported their involvement in response to high salinity (Li et al., 2019; Mansuri et al., 2019) via gene regulatory networks (Jain and Chattopadhyay, 2010). Transcription factor families involved in hormone signaling, such as auxins, gibberellins, abscisic acid, and cytokinin were also differentially expressed, highlighting the important function of plant hormones in salt stress responses, as reported for other plants (Kumar, 2013; Peleg and Blumwald, 2011). Upregulation of DEGs regulating NACs, MYB and WRKY was observed among tolerant genotypes in this study. NACs TFs have been reported to impart salt tolerance in *Cicer arietinum*, *Oryza sativa* and *Glycine max* (Kaashyap et al., 2018; Fang et al., 2015; Hussain et al., 2017). The WRKY TFs were highly induced in the tolerant genotypes and they are reported to be distributed in plant genome which regulate the gene response to abiotic stress and affects, root development, leaf senescence and phytohormone signaling (Wang et al., 2016a, b; Yu et al., 2012). MYB TFs were also are upregulated which are important class of TFs playing role in cell cycle and primary/secondary metabolism. The MYB TFs can regulate both, positive and negative gene expression by silencing the transcription. This is accomplished by the expression of MYB-repressor genes that control the synthesis of lignin and modulation of secondary cell wall formation (Ambawat et al., 2013). Whereas, as in our study AP2/ERF transcription factors has been reported to have differential response to high salinity, osmotic stress (Xu et al., 2011). The previous investigations have confirmed that ethylene positively or negatively regulates salt tolerance. They can regulate the expression of downstream stress-related genes and act either as activators or repressors (Cao et al., 2007). In our study ERF gene were repressed in tolerant genotypes and upregulated in sensitive genotypes. Suggesting its role as repressor of Na^+/H^+ antiporter activity via downregulating NHX genes (Xu et al., 2013) which are vital for vascular compartmentalization of excess toxic Na^+ helping to confer salinity tolerance in various crop plants (Fukuda et al., 2011; Wang et al., 2016a, b). Based on the PPI network analysis, TFs under salinity were interacting with each other and influencing common target pathways (Fig. S5), suggests overlapping among regulatory mechanism between candidate genes associated under salt stress, which are in line to earlier reports in *Arabidopsis thaliana* and *Cicer arietinum* for salt and drought stress (Ghorbani et al., 2019; Sachdeva et al., 2020; Lindemose et al., 2013). These DEGs belonging to different transcriptional families and expressed specifically in the tolerant genotypes can be further used to identify important candidate genes for further functional analysis.

Our study observed comprehensive transcriptional reprogramming along with understanding the role of differentially expressed transcripts involved in several biological processes and metabolic pathways suggesting their role and regulatory mechanics under salt tolerance in chickpea as discussed in earlier transcriptome studies (Garg et al., 2016; Kaashyap et al., 2018). This study also provides useful information and understanding of phenotypic and physio-biochemical response of contrasting chickpea genotypes to decipher the genetics underlying the salinity tolerance. These results can eventually pave the path for genetic improvement of salinity tolerance via harnessing the candidate genes and their regulatory networks to formulate effective genomic-assisted breeding strategies for chickpea breeding programs.

5. Conclusion

The comprehensive comparison of RNA –seq data between salt tolerant (ICCV 10, JG 11) and sensitive (DCP 92-3, Pusa 256) genotypes via integrating physiological analysis has provided a better understanding of salt tolerance mechanism in chickpea. It gives a crucial understanding of molecular mechanics underlying metabolites, phytohormonal and gene regulatory pathways under stress. We found that DEGs encoding for various transcription factors, ion transporters, aquaporin genes were upregulated in response to elevated salt stress, especially for signal transduction, transport and influx of K^+ ions, and

osmotic homeostasis. Results also depicted that both tolerant genotypes deployed more sophisticated and efficient mechanisms of stress tolerance via actively upregulating candidate genes under stress. This study provides useful information to discern the genetics of salinity tolerance, which could ultimately pave the way to harness molecular breeding tactics to elevate salinity tolerance in chickpea breeding programs.

Author contributions

BC and SKR conceived the study. NK, SKR, SPPR and MP performed the experiments. AKS, SPPR, MR, BC, PBS, KS, NMS and AS analyzed the data. BC, NK, MR, KHMS, RKV wrote the paper with help from all co-authors.

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Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.envexpbot.2021.104478.

References

Abbas, W., Ashraf, M., Akram, N.A., 2010. Alleviation of salt-induced adverse effects in eggplant (*Solanum melongena* L.) by glycinebetaine and sugarbeet extracts. Sci. Hortic. 125 (3), 188–195. https://doi.org/10.1016/j.scienta.2010.04.008.

Ambawat, S., Sharma, P., Yadav, N.R., Yadav, R.C., 2013. MYB transcription factor genes as regulators for plant responses: an overview. Physiol. Mol. Biol. Plants 19, 307–321. https://doi.org/10.1007/s12298-013-0179-1.

Arefian, M., Malekzadeh Shafaroudi, S., 2015. Physiological and gene expression analysis of extreme chickpea (*Cicer arietinum* L.) genotypes in response to salinity stress. Acta Physiol. Plant. 37 (9) https://doi.org/10.1007/s11738-015-1945-1.

Arefian, M., Vessal, S., Malekzadeh-Shafaroudi, S., Siddique, K.H.M., Bagheri, A., 2019. Comparative proteomics and gene expression analyses revealed responsive proteins and mechanisms for salt tolerance in chickpea genotypes. BMC Plant Biol. 19 (1), 1–26.

Arnon, D.I., 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta* vulgaris. Plant Physiol. 24 (1), 1.

Asif, M.A., Schilling, R.K., Tilbrook, J., Brien, C., Dowling, K., Rabie, H., Short, L., Trittermann, C., Garcia, A., Barrett-Lennard, E.G., Berger, B., 2018. Mapping of novel salt tolerance QTL in an Excalibur× Kukri doubled haploid wheat population. Theor. Appl. Genet. 131 (10), 2179–2196. https://doi.org/10.1007/s00122-018-3146-y.

Banerjee, A., Roychoudhury, A., 2015. Abscisic-acid-dependent basic leucine zipper (bZIP) transcription factors in plant abiotic stress. Protoplasma 254 (1), 3–16. https://doi.org/10.1007/s00709-015-0920-4.

Barrs, H.D., Weatherley, P.E., 1962. A re-examination of the relative turgidity technique for estimating water deficits in leaves. Aust. J. Biol. Sci. 15 (3), 413–428.

Bartels, D., Sunkar, R., 2005. Drought and salt tolerance in plants. Crit. Rev. Plant Sci. 24 (1), 23–58. https://doi.org/10.1080/07352680590910410.

Battaglia, M., Covarrubias, A.A., 2013. Late Embryogenesis Abundant (LEA) proteins in legumes. Front. Plant Sci. 4, 190. https://doi.org/10.3389/fpls.2013.00190.

Bhargava, B.S., Raghupathi, H.B., 1993. Analysis of Plant Materials for Macro and Micronutrients, Methods of Analysis of Soil, Plants, Water and Fertilizers. Development and Conservation Organization, New Delhi, India.

Cao, W.H., Liu, J., He, X.J., Mu, R.L., Zhou, H.L., Chen, S.Y., Zhang, J.S., 2007. Modulation of ethylene responses affects plant salt-stress responses. Plant Physiol. 143 (2), 707–719. https://doi.org/10.1104/pp.106.094292.

Chan, K.X., Wirtz, M., Phua, S.Y., Estavillo, G.M., Pogson, B.J., 2013. Balancing metabolites in drought: the sulfur assimilation conundrum. Trends Plant Sci. 18 (1), 18–29. https://doi.org/10.1016/j.tplants.2012.07.005.

Cotsaftis, O., Plett, D., Johnson, A.A., Walia, H., Wilson, C., Ismail, A.M., Close, T.J., Tester, M., Baumann, U., 2011. Root-specific transcript profiling of contrasting rice genotypes in response to salinity stress. Mol. Plant 4 (1), 25–41. https://doi.org/10.1093/mp/ssq056.

Cramer, G.R., Urano, K., Delrot, S., Pezzotti, M., Shinozaki, K., 2011. Effects of abiotic stress on plants: a systems biology perspective. BMC Plant Biol. 11 (1), 1–14. https://doi.org/10.1186/1471-2229-11-163.

Deinlein, U., Stephan, A.B., Horie, T., Luo, W., Xu, G., Schroeder, J.I., 2014. Plant salt-tolerance mechanisms. Trends Plant Sci. 19 (6), 371–379. https://doi.org/10.1016/j.tplants.2014.02.001.

Deng, F., Zhang, X., Wang, W., Yuan, R., Shen, F., 2018. Identification of *Gossypium hirsutum* long non-coding RNAs (lncRNAs) under salt stress. BMC Plant Biol. 18, 23. https://doi.org/10.1186/s12870-018-1238-0.

Duan, H.R., Ma, Q., Zhang, J.L., Hu, J., Bao, A.K., Wei, L., Wang, Q., Luan, S., Wang, S. M., 2015. The inward-rectifying K^+ channel SsAKT1 is a candidate involved in K^+ uptake in the halophyte Suaeda salsa under saline condition. Plant Soil 395 (1–2), 173–187. https://doi.org/10.1007/s11104-015-2539-9.

Fang, Y., Liao, K., Du, H., Xu, Y., Song, H., Li, X., Xiong, L., 2015. A stress-responsive NAC transcription factor SNAC3 confers heat and drought tolerance through modulation of reactive oxygen species in rice. J. Exp. Bot. 66 (21), 6803–6817. https://doi.org/10.1093/jxb/erv386.

FAO, 2018. FAOSTAT Food and Agriculture Organization of the United Nations. Available online at: http://faostat3.fao.org. (Accessed 4 September 2020).

Flowers, T.J., Gaur, P.M., Gowda, C.L., Krishnamurthy, L., Samineni, S., Siddique, K.H., Turner, N.C., Vadez, V., Varshney, R.K., Colmer, T.D., 2010. Salt sensitivity in chickpea. Plant Cell Environ. 33 (4), 490–509. https://doi.org/10.1111/j.1365-3040.2009.02051.x.

Fukuda, A., Nakamura, A., Hara, N., Toki, S., Tanaka, Y., 2011. Molecular and functional analyses of rice NHX-type Na^+/H^+ antiporter genes. Planta 233 (1), 175–188. https://doi.org/10.1007/s00425-010-1289-4.

Garg, R., Shankar, R., Thakkar, B., Kudapa, H., Krishnamurthy, L., Mantri, N., Varshney, R.K., Bhatia, S., Jain, M., 2016. Transcriptome analyses reveal genotype- and developmental stage-specific molecular responses to drought and salinity stresses in chickpea. Sci. Rep. 6 (1) https://doi.org/10.1038/srep19228.

Ghorbani, R., Alemzadeh, A., Razi, H., 2019. Microarray analysis of transcriptional responses to salt and drought stress in *Arabidopsis thaliana*. Heliyon 5 (11), e02614. https://doi.org/10.1016/j.heliyon.2019.e02614.

Golldack, D., Lüking, I., Yang, O., 2011. Plant tolerance to drought and salinity: stress regulating transcription factors and their functional significance in the cellular transcriptional network. Plant Cell Rep. 30 (8), 1383–1391. https://doi.org/10.1007/s00299-011-1068-0.

Hu, T., Yi, H., Hu, L., Fu, J., 2013. Stomatal and metabolic limitations to photosynthesis resulting from NaCl stress in perennial ryegrass genotypes differing in salt tolerance. J. Am. Soc. Hortic. Sci. 138 (5), 350–357. https://doi.org/10.21273/jashs.138.5.350.

Hussain, R.M., Ali, M., Feng, X., Li, X., 2017. The essence of NAC gene family to the cultivation of drought-resistant soybean (*Glycine max* L. Merr.) cultivars. BMC Plant Biol. 17, 55. https://doi.org/10.1186/s12870-017-1001-y.

Jain, M., 2011. Next-generation sequencing technologies for gene expression profiling in plants. Brief. Funct. Genomics 11 (1), 63–70. https://doi.org/10.1093/bfgp/elr038.

Jain, D., Chattopadhyay, D., 2010. Analysis of gene expression in response to water deficit of chickpea (*Cicer arietinum* L.) varieties differing in drought tolerance. BMC Plant Biol. 10 (1), 24. https://doi.org/10.1186/1471-2229-10-24.

Jiang, J., Ma, S., Ye, N., Jiang, M., Cao, J., Zhang, J., 2017. WRKY transcription factors in plant responses to stresses. J. Integr. Plant Biol. 59 (2), 86–101. https://doi.org/10.1111/jipb.12513.

Jukanti, A.K., Gaur, P.M., Gowda, C.L.L., Chibbar, R.N., 2012. Nutritional quality and health benefits of chickpea (*Cicer arietinum* L.): a review. Br. J. Nutr. 108 (S1), S11–S26. https://doi.org/10.1017/s0007114512000797.

Kaashyap, M., Ford, R., Kudapa, H., Jain, M., Edwards, D., Varshney, R., Mantri, N., 2018. Differential regulation of genes involved in root morphogenesis and cell wall modification is associated with salinity tolerance in chickpea. Sci. Rep. 8 (1) https://doi.org/10.1038/s41598-018-23116-9.

Kaur, P., Kaur, J., Kaur, S., Singh, S., Singh, I., 2014. Salinity induced physiological and biochemical changes in chickpea (*Cicer arietinum* L.) genotypes. J. Appl. Nat. Sci. 6 (2), 578–588. https://doi.org/10.31018/jans.v6i2.500.

Knoch, E., Dilokpimol, A., Geshi, N., 2014. Arabinogalactan proteins: focus on carbohydrate active enzymes. Front. Plant Sci. 5, 198. https://doi.org/10.3389/fpls.2014.00198.

Kudapa, H., Garg, V., Chitikineni, A., Varshney, R.K., 2018. The RNA-Seq-based high resolution gene expression atlas of chickpea (*Cicer arietinum* L.) reveals dynamic spatio-temporal changes associated with growth and development. Plant Cell Environ. 41 (9), 2209–2225. https://doi.org/10.1111/pce.13210.

Kudla, J., Batistič, O., Hashimoto, K., 2010. Calcium signals: the lead currency of plant information processing. Plant Cell 22 (3), 541–563. https://doi.org/10.1105/tpc.109.072686.

Kumar, P.P., 2013. Regulation of biotic and abiotic stress responses by plant hormones. Plant Cell Rep. 32 (7) https://doi.org/10.1007/s00299-013-1460-z, 943–943.

Kumar, N., Bharadwaj, C., Soni, A., Sachdeva, S., Yadav, M.C., Madan, Pal., Soren, K.R., Meena, M.C., Roorkiwal, M., Varshney, R.K., Rana, M., 2020. Physio-morphological

and molecular analysis for salt tolerance in chickpea (*Cicer arietinum*). Indian J. Agric. Sci. 90 (4), 804–808.
Lenka, S.K., Katiyar, A., Chinnusamy, V., Bansal, K.C., 2010. Comparative analysis of drought-responsive transcriptome in Indica rice genotypes with contrasting drought tolerance. Plant Biotechnol. J. 9 (3), 315–327. https://doi.org/10.1111/j.1467-7652.2010.00560.x.
Li, Y.F., Zheng, Y., Vemireddy, L.R., Panda, S.K., Jose, S., Ranjan, A., Yaish, M.W., 2018. Comparative transcriptome and translatome analysis in contrasting rice genotypes reveals differential mRNA translation in salt-tolerant Pokkali under salt stress. BMC Genomics 19 (10), 935. https://doi.org/10.1186/s12864-018-5279-4.
Li, J., Gao, Z., Zhou, L., Li, L., Zhang, J., Liu, Y., Chen, H., 2019. Comparative transcriptome analysis reveals K^+ transporter gene contributing to salt tolerance in eggplant. BMC Plant Biol. 19 (1), 67. https://doi.org/10.1186/s12870-019-1663-8.
Lindemose, S., O'Shea, C., Jensen, M.K., Skriver, K., 2013. Structure, function and networks of transcription factors involved in abiotic stress responses. Int. J. Mol. Sci. 14 (3), 5842–5878. https://doi.org/10.3390/ijms14035842.
Liu, M., Yu, H., Ouyang, B., Shi, C., Demidchik, V., Hao, Z., Shabala, S., 2020. NADPH oxidases and the evolution of plant salinity tolerance. Plant Cell Environ. 43 (12), 2957–2968. https://doi.org/10.1111/pce.13907.
Maathuis, F.J., Filatov, V., Herzyk, P.C., Krijger, G.B., Axelsen, K., Chen, S., Green, B.J., Li, Y., Madagan, K.L., Sánchez-Fernández, R., Forde, B.G., 2003. Transcriptome analysis of root transporters reveals participation of multiple gene families in the response to cation stress. Plant J. 35 (6), 675–692. https://doi.org/10.1046/j.1365-313x.2003.01839.x.
Machado, R., Serralheiro, R., 2017. Soil salinity: effect on vegetable crop growth. management practices to prevent and mitigate soil salinization. Horticulturae 3 (2), 30. https://doi.org/10.3390/horticulturae3020030.
Mahajan, M.M., Goyal, E., Singh, A.K., Gaikwad, K., Kanika, K., 2017. Transcriptome dynamics provide insights into long-term salinity stress tolerance in *Triticum aestivum* cv. Kharchia Local. Plant Physiol. Biochem. 121, 128–139. https://doi.org/10.1016/j.plaphy.2017.10.021.
Mansuri, R., Shobbar, Z.-S., Babaeian Jelodar, N., Ghaffari, M.R., Nematzadeh, G.-A., Asari, S., 2019. Dissecting molecular mechanisms underlying salt tolerance in rice: a comparative transcriptional profiling of the contrasting genotypes. Rice 12 (1). https://doi.org/10.1186/s12284-019-0273-2.
Mantri, N.L., Ford, R., Coram, T.E., Pang, E.C., 2007. Transcriptional profiling of chickpea genes differentially regulated in response to high-salinity, cold and drought. BMC Genomics 8 (1), 303. https://doi.org/10.1186/1471-2164-8-303.
Mao, G., Seebeck, T., Schrenker, D., Yu, O., 2013. CYP709B3, a cytochrome P450 monooxygenase gene involved in salt tolerance in *Arabidopsis thaliana*. BMC Plant Biol. 13 (1), 1–13. https://doi.org/10.1186/1471-2229-13-169.
Mao, K., Dong, Q., Li, C., Liu, C., Ma, F., 2017. Genome wide identification and characterization of apple bHLH transcription factors and expression analysis in response to drought and salt stress. Front. Plant Sci. 8 https://doi.org/10.3389/fpls.2017.00480.
Molazem, D., Qurbanov, E.M., Dunyamaliyev, S.A., 2010. Role of proline, Na and chlorophyll content in salt tolerance of corn (*Zea mays* L.). Am.-Eur. J. Agric. Environ. Sci. 9 (3), 319–324.
Molina, C., Zaman-Allah, M., Khan, F., Fatnassi, N., Horres, R., Rotter, B., Steinhauer, D., Amenc, L., Drevon, J.J., Winter, P., Kahl, G., 2011. The salt-responsive transcriptome of chickpea roots and nodules via deep Super SAGE. BMC Plant Biol. 11 (1), 1–26. https://doi.org/10.1186/1471-2229-11-31.
Munns, R., Tester, M., 2008. Mechanisms of salinity tolerance. Annu. Rev. Plant Biol. 59 (1), 651–681. https://doi.org/10.1146/annurev.arplant.59.032607.092911.
Negrão, S., Schmöckel, S.M., Tester, M., 2016. Evaluating physiological responses of plants to salinity stress. Ann. Bot. 119 (1), 1–11. https://doi.org/10.1093/aob/mcw191.
Peleg, Z., Blumwald, E., 2011. Hormone balance and abiotic stress tolerance in crop plants. Curr. Opin. Plant Biol. 14 (3), 290–295. https://doi.org/10.1016/j.pbi.2011.02.001.
Puniran-Hartley, N., Hartley, J., Shabala, L., Shabala, S., 2014. Salinity-induced accumulation of organic osmolytes in barley and wheat leaves correlates with increased oxidative stress tolerance: in planta evidence for cross-tolerance. Plant Physiol. Biochem. 83, 32–39. https://doi.org/10.1016/j.plaphy.2014.07.005.
Quintero, F.J., Ohta, M., Shi, H., Zhu, J.-K., Pardo, J.M., 2002. Reconstitution in yeast of the Arabidopsis SOS signalling pathway for Na^+ homeostasis. Proc. Natl. Acad. Sci. 99 (13), 9061–9066. https://doi.org/10.1073/pnas.132092099.
Reddy, I.N.B.L., Kim, S.-M., Kim, B.-K., Yoon, I.-S., Kwon, T.-R., 2017. Identification of rice accessions associated with K^+/Na^+ ratio and salt tolerance based on physiological and molecular responses. Rice Sci. 24 (6), 360–364. https://doi.org/10.1016/j.rsci.2017.10.002.
Roy, S.J., Negrão, S., Tester, M., 2014. Salt resistant crop plants. Curr. Opin. Biotechnol. 26, 115–124. https://doi.org/10.1016/j.copbio.2013.12.004.
Sachdeva, S., Bharadwaj, C., Singh, R.K., Jain, P.K., Patil, B.S., Roorkiwal, M., Varshney, R., 2020. Characterization of ASR gene and its role in drought tolerance in chickpea (*Cicer arietinum* L.). PLoS One 15 (7), e0234550. https://doi.org/10.1371/journal.pone.0234550.
Schulz, P., Herde, M., Romeis, T., 2013. Calcium-dependent protein kinases: hubs in plant stress signaling and development. Plant Physiol. 163 (2), 523–530. https://doi.org/10.1104/pp.113.222539.
Shabala, S., Cuin, T.A., 2008. Potassium transport and plant salt tolerance. Physiol. Plant. 133 (4), 651–669. https://doi.org/10.1111/j.1399-3054.2007.01008.x.
Shankar, R., Bhattacharjee, A., Jain, M., 2016. Transcriptome analysis in different rice cultivars provides novel insights into desiccation and salinity stress responses. Sci. Rep. 6 (1) https://doi.org/10.1038/srep23719.
Shinde, H., Tanaka, K., Dudhate, A., Tsugama, D., Mine, Y., Kamiya, T., Takano, T., 2018. Comparative de novo transcriptomic profiling of the salinity stress responsiveness in contrasting pearl millet lines. Environ. Exp. Bot. 155, 619–627. https://doi.org/10.1016/j.envexpbot.2018.07.008.
Singh, J., Singh, V., Sharma, P.C., 2018. Elucidating the role of osmotic, ionic and major salt responsive transcript components towards salinity tolerance in contrasting chickpea (*Cicer arietinum* L.) genotypes. Physiol. Mol. Biol. Plants 24 (3), 441–453. https://doi.org/10.1007/s12298-018-0517-4.
Song, S.-Y., Chen, Y., Chen, J., Dai, X.-Y., Zhang, W.-H., 2011. Physiological mechanisms underlying OsNAC5-dependent tolerance of rice plants to abiotic stress. Planta 234 (2), 331–345. https://doi.org/10.1007/s00425-011-1403-2.
Soren, K.R., Madugula, P., Kumar, N., Barmukh, R., Sengar, M.S., Bharadwaj, C., Sharma, P.C., Singh, S., Bhandari, A., Singh, J., Sanwal, S.K., Pal, M., P.R, SP, Mann, A., Sagurthi, S.R., PS, S, Siddique, K.H.M., Singh, N.P., Roorkiwal, M., Varshney, R.K., 2020. Genetic dissection and identification of candidate genes for salinity tolerance using Axiom® *Cicer* SNP array in chickpea. Int. J. Mol. Sci. 21 (14), 5058. https://doi.org/10.3390/ijms21145058.
Su, Y.H., Liu, Y.B., Bai, B., Zhang, X.S., 2015. Establishment of embryonic shoot–root axis is involved in auxin and cytokinin response during *Arabidopsis* somatic embryogenesis. Front. Plant Sci. 5, 792. https://doi.org/10.3389/fpls.2014.00792.
Szklarczyk, D., Gable, A.L., Lyon, D., Junge, A., Wyder, S., Huerta-Cepas, J., Simonovic, M., Doncheva, N.T., Morris, J.H., Bork, P., Jensen, L.J., 2019. STRING v11: protein–protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Res. 47 (D1), D607–D613.
Tester, M., 2003. Na^+ tolerance and Na^+ transport in higher plants. Ann. Bot. 91 (5), 503–527. https://doi.org/10.1093/aob/mcg058.
Turner, N.C., Colmer, T.D., Quealy, J., Pushpavalli, R., Krishnamurthy, L., Kaur, J., Singh, G., Siddique, K.H., Vadez, V., 2012. Salinity tolerance and ion accumulation in chickpea (*Cicer arietinum* L.) subjected to salt stress. Plant Soil 365 (1–2), 347–361. https://doi.org/10.1007/s11104-012-1387-0.
Vadez, V., Krishnamurthy, L., Serraj, R., Gaur, P.M., Upadhyaya, H.D., Hoisington, D.A., Varshney, R.K., Turner, N.C., Siddique, K.H.M., 2007. Large variation in salinity tolerance in chickpea is explained by differences in sensitivity at the reproductive stage. Field Crops Res. 104 (1–3), 123–129. https://doi.org/10.1016/j.fcr.2007.05.014.
Vadez, V., Krishnamurthy, L., Thudi, M., Anuradha, C., Colmer, T.D., Turner, N.C., Siddique, K.H., Gaur, P.M., Varshney, R.K., 2011. Assessment of ICCV 2 × JG 62 chickpea progenies shows sensitivity of reproduction to salt stress and reveals QTL for seed yield and yield components. Mol. Breed. 30 (1), 9–21. https://doi.org/10.1007/s11032-011-9594-6.
Varshney, R.K., Song, C., Saxena, R.K., Azam, S., Yu, S., Sharpe, A.G., Cannon, S., Baek, J., Rosen, B.D., Tar'an, B., Millan, T., 2013. Draft genome sequence of chickpea (*Cicer arietinum*) provides a resource for trait improvement. Nat. Biotechnol. 31 (3), 240–246. https://doi.org/10.1038/nbt.2491.
Walia, H., Wilson, C., Zeng, L., Ismail, A.M., Condamine, P., Close, T.J., 2006. Genome-wide transcriptional analysis of salinity stressed japonica and indica rice genotypes during panicle initiation stage. Plant Mol. Biol. 63 (5), 609–623. https://doi.org/10.1007/s11103-006-9112-0.
Wang, T.Z., Liu, M., Zhao, M.G., Chen, R., Zhang, W.H., 2015. Identification and characterization of long non-coding RNAs involved in osmotic and salt stress in *Medicago truncatula* using genome-wide high-throughput sequencing. BMC Plant Biol. 15 (1), 131. https://doi.org/10.1186/s12870-015-0530-0535.
Wang, N., Qi, H., Su, G., Yang, J., Zhou, H., Xu, Q., Yan, G., 2016a. Genotypic variations in ion homeostasis, photochemical efficiency and antioxidant capacity adjustment to salinity in cotton (*Gossypium hirsutum* L.). Soil Sci. Plant Nutr. 62 (3), 240–246. https://doi.org/10.1080/00380768.2016.1172022.
Wang, H., Wang, H., Shao, H., Tang, X., 2016b. Recent advances in utilizing transcription factors to improve plant abiotic stress tolerance by transgenic technology. Front. Plant Sci. 7 https://doi.org/10.3389/fpls.2016.00067.
Xu, Z.-S., Chen, M., Li, L.-C., Ma, Y.-Z., 2011. Functions and application of the AP2/ERF transcription factor family in crop improvement. J. Integr. Plant Biol. 53, 570–585. https://doi.org/10.1111/j.1744-7909.2011.01062.x.
Xu, Y., Zhou, Y., Hong, S., Xia, Z., Cui, D., Guo, J., Xu, H., Jiang, X., 2013. Functional characterization of a wheat NHX antiporter gene TaNHX2 that encodes a K^+/H^+ exchanger. PLoS One 8 (11), 78098.
Yang, A., Dai, X., Zhang, W.-H., 2012. A R2R3-type MYB gene, OsMYB2, is involved in salt, cold, and dehydration tolerance in rice. J. Exp. Bot. 63 (7), 2541–2556. https://doi.org/10.1093/jxb/err431.
Yang, R., Liu, J., Lin, Z., Sun, W., Wu, Z., Hu, H., Zhang, Y., 2018. ERF transcription factors involved in salt response in tomato. Plant Growth Regul. 84 (3), 573–582. https://doi.org/10.1007/s10725-017-0362-4.
Yu, F., Huaxia, Y., Lu, W., Wu, C., Cao, X., Guo, X., 2012. GhWRKY15, a member of the WRKY transcription factor family identified from cotton (*Gossypium hirsutum* L.), is involved in disease resistance and plant development. BMC Plant Biol. 12 (1), 1–18. https://doi.org/10.1186/1471-2229-12-144.
Zhu, J.-K., 2002. Salt and drought stress signal transduction in plants. Annu. Rev. Plant Biol. 53 (1), 247–273. https://doi.org/10.1146/annurev.arplant.53.091401.143329.
Zhu, J.-K., 2003. Regulation of ion homeostasis under salt stress. Curr. Opin. Plant Biol. 6 (5), 441–445. https://doi.org/10.1016/s1369-5266(03)00085-2.
Zhu, J.-K., 2016. Abiotic stress signalling and responses in plants. Cell 167 (2), 313–324. https://doi.org/10.1016/j.cell.2016.08.029.
Zhu, N., Cheng, S., Liu, X., Du, H., Dai, M., Zhou, D.X., Yang, W., Zhao, Y., 2015. The R2R3-type MYB gene OsMYB91 has a function in coordinating plant growth and salt stress tolerance in rice. Plant Sci. 236, 146–156. https://doi.org/10.1016/j.plantsci.2015.03.023.