Elucidating the role of osmotic, ionic and major salt responsive transcript components towards salinity tolerance in contrasting chickpea (*Cicer arietinum* L.) genotypes

Jogendra Singh¹ · Vijayata Singh¹ · P. C. Sharma¹

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Abstract The growth of chickpea (*Cicer arietinum* L.) is extremely hampered by salt stress. Understanding of physio-biochemical and molecular attributes along with morphological traits contributing to the salinity tolerance is important for developing salt tolerant chickpea varieties. To explore these facts, two genotypes CSG8962 and HC5 with contrasting salt tolerance were evaluated in the salinity stress (Control and 120 mM NaCl) conditions. CSG8962 maintained lower Na/K ratio in root and shoot, trammed Na translocation to the shoots from roots compared to HC5 which ascribed to better exclusion of salt from its roots and compartmentation in the shoot. In chickpea, salt stress specifically induced genes/sequences involved at several levels in the salt stress signaling pathway. Higher induction of trehalose 6 phosphate synthase and protein kinase genes pertaining to the osmotic and signaling modules, respectively, were evident in CSG8962 compared to HC5. Further transcripts of late embryogenesis abundant, non-specific lipid transfer protein, HI and 219 genes/sequences were also highly induced in CSG8962 compared to HC5 which emphasizes the better protection of cellular membranous network and membrane-bound macromolecules under salt stress. This further suppressed the stress enhanced electrolyte leakage, loss of turgidity, promoted the higher compatible solute accumulation and

maintained better cellular ion homoeostasis in CSG8962 compared to HC5. Our study further adds to the importance of these genes in salt tolerance by comparing their behavior in contrasting chickpea genotypes.

Keywords Gene expression · Glycinebetaine · Ionic homoeostasis · Membrane leakage · Real-time PCR

Introduction

Salinity stress is an alarming problem in many regions of the world. Approximately 1 billion ha land in the world is prone to salt stress (FAO 2015). The quantum of the saline area is further added by arid and semi-arid areas associated with saline underground water. This saline underground water has to be used for irrigation due to unavailability or utilization of good quality water to the non-agricultural purpose that ultimately makes the soil unfit for crop cultivation (Munns and Tester 2008; Pons et al. 2011; Singh et al. 2014).

Chickpea is one among the very important legume crops, known for its nutritional benefits, soil enrichment properties (Arnoldi et al. 2014; Araujo et al. 2015) and is a key component of South Asian and Mediterranean diets. Chickpea is particularly sensitive to salt stress which reduces its productivity by 0–100% depending upon the level of salinity (Turner et al. 2013; Flowers et al. 2015). Even after a lot of research done to improve salt tolerance in chickpea, till date, only few salt tolerant varieties have been released for commercial cultivation across the world (Malire et al. 2004). One major reason cited is the narrow genetic base of this crop which makes it difficult for breeders to produce new varieties with improved salt tolerance (Berger et al. 2003; Flowers et al. 2010).

✉ Jogendra Singh
jogendra.singh@icar.gov.in; jogendra82@gmail.com

Vijayata Singh
vijayatasingh.gpb@gmail.com

P. C. Sharma
pcsharma.knl@gmail.com

¹ ICAR-Central Soil Salinity Research Institute, Karnal, Haryana 132001, India

Salinity affects germination, initial seedling establishment, growth, nitrogen fixation, flowering, pod development and seed filling of chickpea (Toker et al. 2007; Al-Mutawa 2003). Salinity stress delayed flowering to a greater extent in the sensitive than tolerant genotypes due to higher concentrations of Na^+ in young leaves and the accumulations of Na^+ and K^+ in old green leaves, in the sensitive than in the tolerant chickpea genotypes (Pushpavalli et al. 2016). Further, the delay in flowering was much shorter in tolerant genotypes than sensitive one, and this difference in the delay of flowering may be cause of the higher reproductive failure of the sensitive genotypes in the late sown condition (Turner et al. 2013). Consequently, salinity tolerance was not associated with the accumulation of Na and Cl ions in seeds at maturity and in leaves (Kotula et al. 2015). Salinity tolerance is a complex trait that involves a multitude of physiological and biochemical responses by inducing multiple genes upon exposure to salt stress (Wu et al. 2013). Hence, experiments should comprises the screening of germplasm, characterization of genes, discovery of new quantitative trait loci (QTL) and ultimately, candidate genes, using modern genetics (Negrão et al. 2017).

An elevated intracellular NaCl concentration leads to an ionic and osmotic imbalance that disturbs the delicate cellular ion homeostasis and water potential, resulting in metabolic disruption, growth retardation and significant yield reduction (Pandit et al. 2011). Inter-varietal differences in salinity tolerance are largely ascribed to the potential of plants to control the amount of Na and Cl to reach its sensitive leaf tissue or to metabolic reprogramming when roots are subjected to high salt concentration (Flowers et al. 2015). Higher tolerance to increased Na is also often linked with these differences (Widodo et al. 2009). The concentration of numerous metabolites, including proline and glycinebetaine, also increases under salt stress, providing defence against osmotic challenge by serving as compatible solutes (Sanchez et al. 2008; Wu et al. 2013). Relative water content, electrolyte leakage, lipid peroxidation, and proline concentration can be used as direct indicators of salinity tolerance (Arefian and Sharfroudi 2015). This induction of osmolytes is one of the key physiological markers for salt tolerance traits in chickpea and is a part of a holistic strategy for multiple abiotic stress defence (Liao et al. 2016).

Physio-biochemical mechanisms of salinity tolerance has been widely analyzed in various crop species; however, our present knowledge regarding molecular events regulating this trait is still limited. There is a further need to identify and characterize the major genes that form the base for salt stress regulatory networks (Liao et al. 2016). Numerous strategies, like subtractive hybridization, microarray, and serial analysis of gene expression (SAGE),

have been employed for identifying a large number of genes expressed under salt stress, which is deposited in public databases (Cotsaftis et al. 2011).

Salinity tolerance is the outcome of a complex series of molecular functions which comprise of specific gene activation and regulation of a wide range of salt stress-responsive genes (Zheng et al. 2004; Passaia et al. 2013). We concentrated on six major salt-responsive genes/sequences; late embryogenesis abundant 1 (*LEA1*), nonspecific lipid transfer protein precursor (*LTP*), trehalose 6 phosphate synthase (*TPS*), protein kinase (*PK*), H1 and 219 (cDNA sequences). These genes control the synthesis of compatible osmolytes, protection of cellular functions from high ion concentration damage, repair of stress-induced injuries in membranes and detoxification, regulation of cell growth and signal transduction. The earlier investigators (Deokar et al. 2011; Gao et al. 2008; Kavar et al. 2012; Medini et al. 2009; Tripathi et al. 2013) revealed that these genes are responsible for abiotic stress tolerance. The expression of most of these genes has not or little been reported for the defensive response of chickpea to salinity stress. Hence, we selected these six important genes for our study. LEA proteins are a group of hydrophilic proteins that play a vital role in protecting the native conformation of macromolecules under abiotic stresses by forming a hydration layer around them (Gu et al. 2012). Lipid transfer proteins (LTPs) are a group of low molecular weight cysteine-rich soluble proteins having the peculiar ability to transfer phospholipids between membranes and play a major role in repair of stress-induced injuries in membranes via ABA-dependent/independent signalling pathway (Pan et al. 2016). Trehalose 6 phosphate synthase is the enzyme which catalyzes the biosynthesis reaction of trehalose from UDP-glucose and glucose-6-phosphate that plays a vital role of energy source, compatible osmolyte and cellular membrane protectant in crop plants (Abdelgawad et al. 2014). Protein kinases are another group of key enzymes linking the sensor molecule with downstream candidates in the salt stress signalling pathway (Kulik et al. 2011). H1 is a cDNA sequence homologous to cold-regulated protein (COR) which is induced both by salt and drought (Kume et al. 2005). 219 is a lipid binding protein or lipid transfer protein family homologue which is also induced by drought and salinity in *Arabidopsis* (Shen et al. 2001).

We performed this study in order to assess the physio-biochemical alterations and expression pattern of major salt-responsive genes under high salt stress in two contrasting chickpea varieties. These two varieties are being utilized by our institute as parents for development and advancement of mapping population to map QTLs governing salt tolerance in chickpea.

Materials and methods

Plant material and experimental setup

Seeds of two popular chickpea varieties viz., CSG8962 (salt tolerant) and HC5 (salt susceptible) were obtained from ICAR-Central Soil Salinity Research Institute, Haryana, India (29° 41'N, 76° 59'E). Seeds were surface sterilized with 4% (w/v) sodium hypochlorite for 2 min, washed with distilled water three times and placed on a hydroponic systems consisting of perforated styrofoam floats filled with full-strength Yoshida solution (Yoshida et al. 1976) without NaCl as the control and another salinized with 120 mM NaCl (12 dS/m), considered as stressed. The mean air temperature in the greenhouse was maintained at 27 and 22 °C during day and night, respectively with 14 h photoperiod of intensity 270 $\mu\text{mol}/\text{m}^2/\text{s}$ and $50 \pm 5\%$ relative humidity. The nutrient solutions were renewed every 3 days. 15 days old seedlings were samples for physiological and biochemical analysis. Roots of salt-stressed plants were harvested; immediately frozen in liquid nitrogen and stored at -80°C before RNA isolation. The experiment was performed in a completely randomized block design with four replications. All physiological and molecular assays were done in triplicates.

Germination percent and germination index

The germination percent was calculated as per the formula suggested by Kader (2005):

$$\text{GP} (\%) = (\text{S}/\text{T}) \times 100$$

where 'S' = the number of seed germinated and 'T' = total seeds.

The germination index was calculated by the formula of Kader (2005):

$$\text{GI} = (\text{Germination percent in each treatment} / \text{Germination percent in control}) \times 100$$

Growth measurements

The fresh weight of roots and shoots under control and salt stress conditions were recorded. Further, roots and shoots were dried at 80°C for 48 h and dry weight was calculated.

Chlorophyll determination

Finely chopped fresh shoot tissue was placed into test tubes with 10 ml dimethyl sulfoxide (DMSO). The aluminium foil was used to cover the tubes and kept in an oven at 65°C for 4 h. Thereafter, the absorbance of the

chlorophyll solution was read at 663 and 645 nm. The chlorophyll was analyzed according to Arnon (1949).

Total chlorophyll (mg/g of fresh weight) = (chlorophyll *a* + chlorophyll *b*) and chlorophyll *a* and *b* were calculated by the following formulae:

$$\begin{aligned} \text{Chlorophyll } a (\text{mg/g of fresh weight}) \\ = [12.7 (A_{663}) - 2.69 (A_{645})] \times V/1000 \times W \end{aligned}$$

$$\begin{aligned} \text{Chlorophyll } b (\text{mg/g of fresh weight}) \\ = [22.9 (A_{645}) - 4.68 (A_{663})] \times V/1000 \times W \end{aligned}$$

where: A = absorbance (nm); V = volume of the extract (ml); W = weight of the fresh leaf tissue (g)

Relative water content

Relative water content (RWC) was estimated as per the protocol developed by Weatherly (1950). Six plants from each treatment were randomly selected and weighed to determine the fresh weight (FW). The samples were then soaked in fresh deionized water for 24 h under the light, and excess water was removed by blotting in between filter papers. The samples were then weighed to determine the turgid weight (TW). Samples were further oven-dried at 80°C for 3 days to obtain the dry weight (DW). The RWC was calculated using the formula:

$$\text{RWC} = (\text{FW} - \text{DW}) / (\text{TW} - \text{DW}) \times 100$$

Electrolyte leakage

To determine the electrolyte leakage ratio (ELR), fresh shoot samples were dissected and soaked in a bottle containing 10 ml of de-ionized water and the bottles were gently shaken overnight. The initial electrical conductivity of the solution (EC_1) was measured with an EC meter (LMCM-20, Labman Scientific Instruments Pvt. Ltd., Chennai, India). The bottles were then autoclaved at 121°C for 20 min to completely kill the tissues and release all electrolytes. It was then cooled to 25°C and the conductivity of the solution were again measured (EC_2). The ELR was calculated using the following formula:

$$\text{ELR} = (\text{EC}_1 / \text{EC}_2) \times 100.$$

Proline estimation

The proline concentration of shoot tissues under control and salt stress conditions was determined as described by Bates et al. (1973). Approximately 0.5 g of shoot tissue was ground in liquid nitrogen, homogenized in 10 ml 3% sulfosalicylic acid, and filtered through Whatman no. 2 filter paper. Then, filtrate (2 ml) was incubated with 2 ml

of each acid ninhydrin and glacial acetic acid for 1 h at 100 °C. The reaction was stopped by transferring the samples into an ice bath. After this, the reaction mixture was extracted with toluene (4 ml) and the absorbance of aqueous phase measured at 520 nm. Proline concentration ($\mu\text{g/g}$) was calculated on a fresh weight basis by using a standard graph of L-Proline with following formula:

$$\text{Proline } (\mu\text{moles/g of fresh weight}) = [(\mu\text{g proline/ml} \times \text{ml toluene})/115.5]/(\text{g sample})$$

Quantification of glycinebetaine

Levels of glycinebetaine in shoot tissues under control and salt-stressed condition were determined as described previously by Grieve and Grattan (1983) with minor modifications. Finely ground dry shoot tissue (0.5 g) was shaken with 20 ml of deionized water for 48 h at 25 °C in an orbital shaker. The samples were then filtered and the filtrate was stored in the freezer until analysis. Properly thawed extracts were diluted with 2 N sulphuric acid in 1:1 ratio. Further, 0.5 ml aliquot was taken into a test tube and cooled in ice water for 1 h. Thereafter, 0.2 ml cold potassium iodide–iodine reagent was added and the mixture was vortexed gently. The samples were stored at 0–4 °C for 16 h. After this, samples were transferred to centrifuge tubes and then centrifuged at 10,000 rpm for 15 min at 0 °C. The supernatant was carefully aspirated and the periodite crystals were dissolved in 9 ml of 1, 2-dichloro ethane. Vigorous vortex mixing was done to completely solubilize the crystals in developing solvent. After 2.0–2.5 h, the absorbance was measured at 365 nm using a UV–visible spectrophotometer. The glycinebetaine concentration was calculated using reference standards of glycinebetaine (50–200 $\mu\text{g/ml}$) and expressed as ($\mu\text{g/g DW}$) with following formula:

$$\text{Glycinebetaine } (\mu\text{g/g of dry weight}) = (A_{365} - 0.0121)/0.035 \times 1.5 \times 25/\text{g dry sample}$$

where A = absorbance (nm)

Measurement of ion concentration

Chickpea plants selected from control and saline treatment were separated into roots and shoots and washed repeatedly with tap water and finally with double distilled water and then oven dried at 80 °C. Six replicates were sampled from each treatment for determining Na^+ and K^+ concentration in the root and shoot tissues under control and imposed salt stress condition. Dry root and shoot tissues were ground and 0.5 g samples were digested in diacid mixture (20 ml) containing HNO_3 and HClO_4 acid (9:4 ratio) using a hot plate digestion system. After proper cooling, the digest was

diluted with double distilled water; filtered and final volume was made up to 50 ml and stored in polypropylene bottles. Elemental analysis of Na^+ and K^+ ions was performed using inductively coupled plasma emission spectroscopy (ICPE-9000, Shimadzu Europa GmbH, Germany).

RNA extraction, quality analysis, and cDNA preparation

Total RNA was extracted from root tissues of control and salt-stressed plants using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA (1 μg) was further digested with DNaseI of *Sigma-Aldrich* following the instruction manual. RNA was treated with 1U of RNase inhibitor (RNase K; Sigma) and stored at – 80 °C till further use. To verify quality and integrity of the RNA samples, it was run in 1% agarose gel and the 28S and 18S bands were confirmed. Furthermore, the absorbance ratio at 260/280 and 260/230 nm were determined by Nanodrop (Spectrophotometer ND-2000, Thermo Fischer, USA).

The double-stranded cDNA was synthesized from the mRNA by gene-specific primers (Arefian and Shafaroudi 2015) (Table 1) by using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) as follows: 6 μl of nuclease free water, 2 μl of 5X Reaction mix, 1 μl of 1000 ng/ μl RNA and 1 μl of enzyme. The mixture was incubated for 10 min at 25 °C, 1 h at 42 °C, 5 min at 95 °C followed by 10 min at 4 °C. The cDNA was immediately stored at –20 °C till further use.

Quantitative real-time PCR analysis

In the present study, expression analysis of six salt-responsive genes/sequences (Table 1) was carried out using qRT-PCR. The qRT-PCR was performed using Fast SYBR Green Master Mix (Sigma-Aldrich, USA) on ABI Step One Plus real-time amplification thermal cycling system (Applied Biosystems, USA). Amplification reactions were conducted as above in triplicate and normalized using *-Actin* gene as an internal control. The total volume of the reaction mixture was 25 μl , which consisted of 2 μl cDNA, 1 μl of forward and reverse primers (1 nmol each), 10 μl of SYBR Green and 11 μl of nuclease-free water. The relative expression level of the transcripts was calculated using the $2^{-\Delta\Delta\text{CT}}$ method (Livak and Schmittgen 2001).

Statistical methods

Data were subjected to statistical analysis using factorial analysis of variance (ANOVA) and the means were tested by the Least Significant Difference (LSD) at $P \leq 0.05$

Table 1 Gene-specific primers used in real-time PCR analysis (Reproduced with permission from Arefian and Shafaroudi 2015)

Gene/EST	Primer pair	Sequence (5′–3′)	Amplicon (bp)	T _a (°C)	Gene bank accession no.
Trehalose-6-phosphate synthase	TPS-F	ACAGGGTGTGAGCAAGGGATTAG	190	59.4	CD051305
	TPS-R	CCGACAAACGGTGCAAGCGAAC			
Late embryogenesis abundant-1	LEA-F	ACAGACAACCGAAGCAAC	164	57.3	XM_004506840
	LEA-R	GGGCCATACCCTTAACCT			
Protein kinase	PK-F	AGGTCAGGATACGGCATGTCACAA	168	58.4	HO062281
	PK-R	GCGAGTGAAGCATCAAATCCCAA			
Nonspecific-LTP precursor	LTP-F	GCTGGTTCTATTTCTCGTTTG	418	62.3	NM_001309682
	LTP-R	AAGATGAGCCTCCATCACTC			
H1	H1-F	CTATGCCTTGATGATGAGC	79	54.3	EG359333
	H1-R	CACATGCCCTTCTTTAGCC			
219	219-F	GCTGTGACTATCCCTAAACG	96	54.8	EG359330
	219-R	GCAATAACCTCAACTCTATCAAG			
Actin	Actin-F	TGTCTTGAGTGGTGGTTCTAC	202	59.2	XM_004493535.1
	Actin-R	TTCATCATATTCTGCCTTTG			

using the SAS 9.3 software (SAS Institute Inc., Cary, USA).

Results

Effect of NaCl on germination percentage and germination index

The germination and germination index differed significantly for genotype, treatment and genotype × treatment interaction at the $P \leq 0.05$. The higher salt stress (120 mM NaCl) reduced the germination potential of the genotypes compared with the control. The reduction in germination percent under stress was more pronounced in HC5 (80%) than CSG8962 (32%). Similarly, germination index was found higher in CSG8962 (67.79%) than HC5 (19.60%) under salt stress (Table 2).

Growth dynamics under salt stress

Morphological attributes like root length, shoot length, fresh weight and dry weight varied significantly ($P \leq 0.05$) among the two genotypes and with salinity treatment. In general, CSG8962 maintained a much vigorous root and shoot architecture under salt stress as compared to HC5 (Fig. 1). CSG8962 exhibited a minor reduction in root length (5.04%) and shoot length (6.06%) under salt stress as compared to non-saline treatments. However, there was a significantly higher reduction in the root (30.23%) and shoot length (32.40%) of HC5 under salt stress (Fig. 1a).

Table 2 Germination percentage and germination index of chickpea varieties under control and saline condition

Trait	Germination %		Germination index	
	Control	120 mM	Control	120 mM
CSG8962	97.80a	66.30a	100.00a	67.80a
HC5	97.95a	19.20b	100.00a	19.60b
LSD:				
Genotype (G)	2.71**		2.91**	
Salinity (S)	2.70**		2.92**	
Interaction (GXS)	3.83**		4.14**	

Growth reduction was observed in both genotypes under NaCl treatment, but it was much more severe in HC5 compared to CSG8962. There was a drastic reduction in root and shoot fresh weight of HC5 (39 and 46%, respectively) compared to CSG8962 which showed only 19% and 9% reduction in root and shoot fresh weight respectively (Fig. 1b). The dry mass of root and shoot also showed a similar trend under NaCl treatment. The reduction in root and shoot dry mass was much more conspicuous in HC5 (47 and 78%, respectively) compared to CSG8962 which displayed a significantly lower reduction in its root and shoot dry mass (17 and 38%, respectively) (Fig. 1c).

Ion profiling in root and shoot under salt stress

The tissue Na^+ concentration increased with a simultaneous reduction in cellular K^+ concentration in root and shoot tissues of both genotypes under salinity (120 mM NaCl) as compared to control. The salinity increased the

Fig. 1 Effect of salt treatment on **a** roots and shoots length, **b** fresh weight (FW) and **c** dry weight (DW) of contrasting chickpea genotypes. Plotted values are the average of 10 independent biological replicates with standard error

Na^+ concentration of CSG8962 by 45 fold in roots with a simultaneous fourfold decrease in its K^+ concentration. However, HC5 displayed a 22 fold increase in Na^+ concentration in its root with a simultaneous sevenfold decrease in K^+ concentration (Fig. 2a, b).

The shoot tissues of CSG8962 exhibited 23 fold increases in Na^+ concentration under salt stress with a simultaneous 1.8 fold decrease in its K^+ concentration. However, salinity increases Na^+ concentration in the shoot of HC5 by 100 fold with a simultaneous 2.6 fold decrease

in its K^+ concentration (Fig. 2a, b). This clearly shows the altered cellular ion balance under stress which is highly detrimental to normal growth and development of the crop. The Na^+/K^+ ratio was significantly affected by the saline treatment in shoot and root of both chickpea genotypes. CSG8962 marked a lower Na^+/K^+ ratio in the root (2.84) and shoot (0.60) as compared to HC5 which showed a

higher Na/K ratio in the root (3.63) and shoot (1.03) under salt stress (Fig. 2c).

Physiological and biochemical modulations under salt stress

It was observed that salinity decreased significantly the total chlorophyll in both chickpea genotypes. The decrease

Fig. 2 Effect of salt stress on cellular ion accumulation and physiological/biochemical parameters; root and shoot; **a** Na^+ concentration, **b** K^+ concentration, **c** Na^+/K^+ ratio, **d** total chlorophyll, **e** relative water

content (RWC), **f** electrolyte leakage (EL), **g** proline, and **h** glycine-betaine (GB) of contrasting chickpea genotypes. Plotted values are the average of 10 independent biological replicates with standard error

in chlorophyll was more in HC5 (60%) as compared to CSG8962 (38%) at 120 mM NaCl stress (Fig. 2d). Relative water content (RWC) of leaves in both genotypes decreased significantly ($P \leq 0.05$) in response to saline treatment. There was 66% reduction in relative leaf water content of HC5 which was higher than CSG8962 (42%) in response to higher salinity (Fig. 2e). Electrolyte leakage ratio varied significantly ($P \leq 0.05$) among the two genotypes in response to salinity treatment. As seen in Fig. 2f, membrane stability decreased rapidly in both genotypes under saline treatment. There was a 45 fold increase in the electrolyte leakage ratio of HC5 as compared to a lower leakage ratio in CSG8962 (20 fold). This clearly suggests the better ability of CSG8962 to protect its membranous network from uncontrolled leakage under stress.

Proline and glycinebetaine concentration of both genotypes increased significantly ($P \leq 0.05$) in response to salt stress. CSG8962 accumulated a greater amount of proline (134 $\mu\text{g/g}$ FW) and glycinebetaine (24 $\mu\text{mol/g}$ DW) at the higher salt stress which was 75 and 97%, respectively more than non-saline condition (Fig. 2g, h). On the other hand, HC5 exhibited lower concentration of proline (58.40 $\mu\text{g/g}$ FW) and glycinebetaine (16.29 $\mu\text{mol/g}$ DW) at 120 mM NaCl stress.

Real-time quantitative PCR analysis

Crop plants respond to any kind of stress by initiating a signalling cascade which results in induction or repression of a network of genes to re-programme the cellular physiology and thereby acclimatize to the new cellular conditions. In order to understand the basis for the physiological and ionic differences in the two genotypes under imposed salt stress, we studied the transcript abundance of six major genes/sequences (*TPS*, *LEA1*, *LTP*, H1, 219 and *PK*) playing significant roles at different levels in the salt stress signalling network. The expression of most of the genes/sequences were found to be significantly higher under salt stress ($P \leq 0.05$) in CSG8962 than HC5 as compared to the constitutively expressed housekeeping gene (Fig. 3). However, gene H1 had a relatively low expression in root tissues, therefore, the gene remained undetermined in our real-time analysis. This led us to show only four genes (out of five) in root tissues and all five genes in shoot tissues.

The differential expression pattern of *TPS* in the shoot tissue was quite evident in between the two genotypes. This candidate transcript showed higher induction (eightfold) under saline treatment in the CSG8962 shoot, while its expression level was very low under stress in HC5 (Fig. 3d). *LEA1* showed sevenfold induction in the shoot tissues of CSG8962 under imposed salt stress as compared to a threefold induction in HC5 (Fig. 3b). As shown in Fig. 3a, saline treatment induced the transcript

accumulation of another major gene i.e., *LTP* in the shoot tissues of both genotypes. Another interesting aspect that we found was the higher initial presence of this gene in CSG8962 under non-saline conditions. Further, there was a 1.6 fold induction of *LTP* transcript in the shoot tissues of CSG8962 under stress compared to very low induction in HC5 (Fig. 3a). Expression pattern ($P \leq 0.05$) of two important sequences (H1 and 219) was not significantly different under salt stress in the two genotypes. But, interestingly, there was a higher constitutive expression of both these transcripts under control conditions (Fig. 3c, e). The relative expression level of *LEA1*, *LTP*, 219 and *PK* in the root tissues was also found to be significantly higher under salt stress ($P \leq 0.05$) in CSG8962 than HC5 (Fig. 3). There was a sevenfold increase in the transcript levels of *LEA1* under imposed salt stress in the root tissues of CSG8962 as compared to a threefold induction in HC5 (Fig. 3g). Similarly, the increase in relative expression level of *LTP*, 219 and *PK* under saline treatment (Fig. 3f, h, i) was significantly higher in CSG8962 roots which clearly correlate with its improved cellular ion homeostasis under salt stress.

Discussion

The germination stage of the crop is exposed firstly to salinity conditions (Passam and Kakouriotis 1994). The reduced germination and seedling growth result in poor establishment and subsequently the crop failure (Soltani et al. 2006). Salt stress reduced the germination percent and germination index in both CSG8962 and HC5. The reduction in germination percent under stress was more in HC5 (80%) than CSG8962 (32%). It may be due to higher concentration of salt which caused the toxic effects and reduces the water potential in the medium. These impede the water imbibition and seed turgescence stages and hampered the germination (Jamil et al. 2006; Al-Mutawa 2003). Shoot and root parameters (length, fresh and dry weight) showed a decreasing trend under saline treatment in both the genotypes. The salt sensitive genotype (HC5) exhibited a higher reduction in shoot and root length with 120 mM NaCl treatment than CSG8962. This may be due to excess intracellular Na^+ accumulation, which results in osmotic and oxidative stresses, which restrained cell elongation of roots and shoots in HC5 than CSG8962 genotype (Munns and Tester 2008). Similarly, there was a considerable reduction in fresh and dry weight of root and shoot tissues in both the cultivars, but the extent of reduction was higher in salt sensitive genotype HC5 compared to CSG8962. This reduction in fresh and dry weight might be due to poor CO_2 fixation efficiency, the toxic effect of Na^+ and Cl^- ions, osmotic imbalance or

Fig. 3 Transcript abundance of salt-responsive genes/sequences; in the shoot (a–e) Non-specific LTP precursor (*LTP*), Late embryogenesis abundant-1 (*LEA1*), H1, Trehalose-6-phosphate synthase (*TPS*)

and 219; in root (f–i) *LTP*, *LEA1*, Protein Kinase (*PK*) and 219 of chickpea cultivars CSG8962 and HC5 under imposed salt stress

disturbed metabolic activity under saline conditions (Wang et al. 2016).

The two chickpea genotypes responded to saline treatment with a prominent decrease in chlorophyll. The much more conspicuous reduction in total chlorophyll under salt stress in salt sensitive genotype HC5 than the tolerant

CSG8962 may be due to the replacement of Mg^{++} with Na^{+} in this sensitive genotype (Demiroğlu et al. 2001; Katsuhara et al. 1990). Salt stress-mediated reduction in total chlorophyll may be due to the disruption of the cellular membranous network by excess Na^{+} and Cl^{-} ions (Molazem et al. 2010). The early maturity of leaves as

escape mechanism can be another cause for the reduction of chlorophyll under salt stress (Schippers et al. 2015; Hanumantharao et al. 2016). High relative leaf water content (RWC) and a lower electrolyte leakage can be used as biomarkers of membrane integrity and stability under salt stress (Arefian and Shafaroudi 2015; Negrão et al. 2017). Physiological analysis revealed a much higher reduction in RWC of HC5 compared to CSG8962 in the salt stress. This is because of a greater ability of salt tolerant crops to absorb and hold water under salt stress (El-Bassiouny and Bekheta 2005; Hu et al. 2013). The higher maintenance of leaf RWC in CSG8962 at 120 mM salt stress shows its greater efficiency of water absorption and avoiding tissue dehydration. It may also acclimatize the crop growth under salt stress, either downplaying water loss by reducing above-ground transpiration or enhancing water uptake by partitioning more assimilates towards the root system (Nunes et al. 2008). Reduced stomatal conductance is also a response to dehydration avoidance which upshots the maintenance of leaf water content (Hu et al. 2013). The 45 fold increase in electrolyte leakage ratio of HC5 as compared to a lower leakage ratio in CSG8962 (20 fold) clearly suggests the better ability of CSG8962 to protect its membranous network from uncontrolled leakage under stress.

Osmolytes like proline and GB play a major role in protecting the membrane bound proteins and enzymes apart from its basic role of osmoprotection. These compounds lower the osmotic potential of the cell sap, thereby regaining the water potential gradient. This leads to uptake of more water from the saline root zone, which may buffer the immediate effect of water deficiency within the crop so that the crop can perform its metabolic activities more efficiently during the stress (Ashraf and Foolad 2007; Giri 2011). Proline and glycinebetaine (GB), two very important osmoregulators increased significantly ($P \leq 0.05$) in response to saline treatment. CSG8962 showed increased proline and GB at the end of stress by 134 $\mu\text{g/g}$ FW and 24 $\mu\text{mol/g}$ DW respectively. On the contrary, HC5 accumulated lower proline and GB by 58.4 $\mu\text{g/g}$ FW and 16.29 $\mu\text{mol/g}$ DW respectively. The rapid activation and high levels of proline accumulation may be assumed to improve salt tolerance of chickpea genotypes (Arefian et al. 2014; Fang et al. 2010).

Another striking difference between the two genotypes under salt stress was of the ion composition of their root and shoot. The Na^+ and Cl^- concentrations have been increased in chickpea upon excess salts exposure in the root zone (Dua 1998). The Na^+ or ion exclusion is an important mechanism that avoids toxicity to photosynthetic tissues and retains minimum leaf Na^+ concentrations in the substrates (Munns and Tester 2008). In our study, saline treatment (120 mM NaCl) increased a 45 and 23 fold

increase in the Na^+ concentration of CSG8962 roots and shoots respectively, with a simultaneous 4 and twofold decrease in its K^+ concentration. However, HC5 displayed a 22 and 100 fold increase in Na^+ concentration in its root and shoot respectively, with a simultaneous 7 and threefold decrease in K^+ concentration. Hence, ion exclusion capacity of the roots was unable to decipher the differences in salt tolerance in these chickpea genotypes. Salinity tolerance plants maintain net CO_2 assimilation at high leaf Na^+ concentration due to avoiding excessive Na^+ accumulation in the photosynthetic mesophyll cells as a result of the efficient compartmentalization of Na^+ into vacuoles (Munns and Tester 2008; Flowers et al. 2015). The excess salts affected metabolic pathway that is a consequence of the activity of ions in the cytoplasm and/or vacuole and also influenced by compartmentation and cellular water content (Flowers et al. 2010; Shabala et al. 2016). The ability to maintain tissue water content under saline conditions may be considered as an index of salt tolerance in chickpea genotypes that helps in mitigating the changes in ion concentration (Dua 1998; Flowers et al. 2010).

In our study, the lower Na^+/K^+ ratio in CSG8962 than HC5 under salinity depicts a controlled uptake mechanism of Na in CSG8962. This also clearly suggests a higher specificity index of K^+ channels together with better Na^+ partitioning ability in CSG8962. The maintenance of a lower cytosolic Na^+/K^+ ratio and the high K^+ concentrations in shoots could have contributed to salt tolerance (Maathuis and Amtmann 1999; Kronzucker and Britto 2011). The Na^+ can also enter the plant through the apoplastic pathway. The differences in this bypass conductance among the genotypes may also lead to the genotypic variations in salt tolerance (Krishnamurthy et al. 2011). Previous researches have also suggested the toxic effect of salinity on chickpea due to the accumulation of Na^+ in shoot (Turner et al. 2013; Khan et al. 2015).

Salinity responsive gene regulation is a regular adaptive strategy of plants to cope with the salt induced metabolic changes (Mantri et al. 2007). Under salt stress, transcript modulation leads to eventual re-establishment of cellular ion homeostasis, water status, membrane integrity and recovery of growth (Xiong and Zhu 2002). Evaluating transcript abundance under saline treatment can also give a reliable estimate of specific gene activation or down-regulation (Liao et al. 2016). We examined the transcript abundance of six major genes/sequences viz., *TPS*, *LEA1*, *LTP*, *H1*, *219* and *PK* in the shoot and root tissues of CSG8962 and HC5 under control and 120 mM saline treatment. The relative expression of genes was given in terms of 'fold change' which was calculated from the $2^{-\Delta\Delta\text{CT}}$ method for a gene expressed in experimental v/s control condition. *TPS* and *LEA1* transcripts showed sevenfold higher expressions in the shoot of CSG8962 under

saline treatment, while its expression level was very low under stress in HC5. *TPS* gene family may contribute to enhanced salt tolerance via osmoprotection by increasing the synthesis of the non-reducing disaccharide sugar trehalose and regulating stress related gene expression (Li et al. 2011). The higher abundance of *TPS* transcript and biosynthesis of trehalose in CSG8962 in the salt stress might lead to increase the accumulation of proline and higher leaf water content (Jang et al. 2003; Hackel et al. 2012). LEA proteins are related to osmotic tolerance and response of the plant to drought and salinity stress (Gu et al. 2012). Based on structural characteristics and homology in amino acid sequence, LEA proteins have been classified into six different groups (Hong-Bo et al. 2005). Our gene of interest is *LEA1*, belonging to Group 3 which is reported to be highly induced by salt stress (Liu et al. 2009). Previous studies in different crops also clearly indicate that over-expression of *LEA* from various groups confers tolerance to osmotic stress (Duan and Cai 2012).

Further, saline treatment induced the expression of another major gene *LTP* in both genotypes. But interestingly, its expression was higher in the shoot of CSG8962 than HC5 under stress. The higher expression of *LTP* under salt stress in CSG8962 suggests its possible role in repair of stress-induced membrane damage by altering the lipid composition and fluidity level of membranes vis-a-vis restricting the permeability of membranes to toxic ions (Pan et al. 2016). Increasing level of *LTP* gene in root and shoot of CSG8962 may have ultimately culminated in reduced leakage from cellular membranes (Wang et al. 2008). Protein kinases are other key players in the control of signal transduction, and different stress or ABA-regulated protein kinases have been studied at various levels in the salt stress signalling pathway (Mantri et al. 2007; Kulik et al. 2011; Gollack et al. 2014). The higher expression of this gene was evident only in the root tissues of CSG8962 upon exposure to 120 mM NaCl in our study. The up-regulation of protein kinase family under salt stress in tolerant genotypes has been reported earlier (Kulik et al. 2011; Gollack et al. 2014). H1(EG359333) and 219 (EG359330) are differentially expressing drought-inducible genes encoding for “lipid binding protein” and “Cold responsive (COR)” protein, respectively (Medini et al. 2009). Both the genes expressed at par under saline treatment in the shoot tissues of both the chickpea varieties. However, in the root tissues, 219 displayed higher induction in CSG8962 compared to HC5. H1 is highly induced under any kind of osmotic stress specifically in the tolerant cultivars under osmotic stress (Medini et al. 2009) for the maintenance of membrane integrity and thereby protects the membrane bound macromolecules (Kobayashi et al. 2004).

Conclusions

Overall CSG8962 performed better in terms of morpho-physiological traits over HC5 under saline condition which might be attributed to higher RWC, cell membrane stability accompanied by increased accumulation of osmolyte molecules and lower intracellular Na/K ratio by virtue of a better exclusion and compartmentation ability of toxic ions. The osmotic, ionic and major salt responsive transcript components analysis might help in the further understanding of the molecular and morpho-physiological mechanisms inherent to the various critical biological pathways towards the chickpea salt tolerance. Further, our research has also facilitated studying the role of genes like *TPS* and *PK* (genes pertaining to the osmotic and signaling modules of salt stress tolerance) and *LEA1*, *LTP*, H1 and 219 (genes/sequences pertaining to protection of cellular membranous network and membrane bound macromolecules under salt stress) which showed differential expression under salt stress. These genes could be the best targets to understand functional genomics/molecular mechanisms for salt tolerance in CSG8962. The utilization of the knowledge these genes/sequences in the breeding programmes for mutant screening, modifying the expression of small numbers of genes, developing transgenic plants and combining natural alleles with knowledge of pedigrees and phenotypes can be leveraged to optimize selection response under salt stress. Further, we can utilize the gene-specific markers to identify salinity tolerant accessions/donors among chickpea germplasm too.

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Authors Contribution JS conceived the project, analyzed the data and wrote the first draft and the final version of the paper. VS performed the experiments and PCS performed the critical revision of the data.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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