

Differential transcript abundance of salt overly sensitive (SOS) pathway genes is a determinant of salinity stress tolerance of wheat

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Abstract Salt overly sensitive (SOS) pathway genes, *SOS1* (plasma membrane Na^+/H^+ antiporter), *SOS2* (CBL interacting protein kinase 24), and *SOS3* (calceinurin B like protein 4) are associated with active efflux of toxic sodium ions (Na^+) from cytosol and thus confer salinity tolerance in glycophytic plants such as *Arabidopsis*. The role of SOS pathway genes *SOS2* and *SOS3* in salinity tolerance of wheat is rarely studied. One-month-old seedlings of three bread wheat genotypes namely, HD 2009, HD2687 and Kharchia 65 were imposed with two levels of salinity stress (100 and 200 mM NaCl) for 30 days duration. Based on the physiological parameters, genotype Kharchia 65 was highly tolerant, HD 2009 was moderately tolerant and HD 2687 was sensitive to salinity stress. Tolerant genotypes accumulated lesser amount of Na^+ in root, stem and leaf tissues. Transcript abundance of *SOS1*, *SOS2* and *SOS3* genes was significantly higher in salt tolerant genotypes under long-term salinity and correlated with improved sodium exclusion, and higher potassium/sodium (K^+/Na^+) ratio. Expression levels of genes involved in vacuolar partitioning of Na^+ , *NHX1* (vacuolar Na^+/H^+ antiporter)

and *VPI* (Vacuolar pyrophosphatase) were also higher in salt tolerant wheat genotypes under 200 mM NaCl stress. Partial coding sequences of *SOS1*, *SOS2*, *SOS3*, *NHX1* and *VPI* genes were cloned and sequenced from the above mentioned three wheat genotypes. The results in the present study demonstrated that SOS pathway of ion homeostasis under salinity stress is conserved across species.

Keywords Potassium · Salinity stress · SOS · Sodium · Vacuolar antiporter · Wheat

Introduction

Salinity is a problem affecting approximately 20 % of the irrigated agricultural land and about 10 million ha arable land is abandoned yearly due to excessive salt accumulation (Szabolcs 1989). Development of salinity tolerant, high yielding genotypes requires thorough understanding of traits associated with salinity tolerance (Munns and Tester 2008). Most potent mechanism underlying the salinity tolerance is restriction of the entry of sodium ions (Na^+) into the cytosol by selective regulation of Na^+ uptake and efflux systems (Zhu 2003; Ji et al. 2013). Due to similarities in ionic features, Na^+ compete with and are taken up through potassium ions (K^+) uptake systems (Plett and Moller 2010). Due to the negative effect of Na^+ on K^+ nutrition, salinity stress leads to inhibition of photosynthesis, enzyme activities and growth of plants (Zhu 2003). To reduce cytosolic Na^+ levels, plants activate Na^+ efflux from roots and sequester Na^+ into vacuoles (Plett and Moller 2010). Bread wheat exhibits lower net Na^+ uptake possibly by counter-balancing the high Na^+ influx rate by an efficient Na^+ efflux mechanism (Davenport et al. 2005). The Salt Overly Sensitive 1 (SOS1), a plasma

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membrane Na^+/H^+ antiporter is the first well-characterized Na^+ efflux protein in plants (Shi et al. 2000). *SOS1* has been characterized in different plant species including *Arabidopsis thaliana* (Shi et al. 2000), *Thellungiella salsginea* (Oh et al. 2009), *Solanum lycopersicum* (Olías et al. 2009) and *Oryza sativa* (Martinez-Atienza et al. 2007). *SOS1* mediates Na^+ efflux at the root surface and regulates Na^+ transport from root to shoot (Tester and Davenport 2003) thereby maintaining favorable K^+/Na^+ ratio in leaves, where the major metabolic activities of plants take place. *SOS1* is activated through protein phosphorylation by the *SOS2/SOS3* kinase complex (Pardo et al. 2006). *SOS2* is a serine–threonine protein kinase, belonging to the sucrose non-fermenting 1-related kinase 3 (SnRK3) family (Liu et al. 2000). *SOS3* is a Ca^{2+} sensor belonging to calcineurin B-like (CBL) protein family, highly similar to neuronal calcium sensor of animals (Kolukisaoglu et al. 2004). *SOS3* upon sensing the salinity stress-induced calcium oscillations binds to FISL motif of *SOS2* and activates *SOS2*. The *SOS3/SOS2* complex controls the expression of *SOS1* gene and also activates the Na^+/H^+ antiport activity of *SOS1* (Sanchez-Barrena et al. 2005). Mutants deficient in both *SOS2* and *SOS3* exhibits salt-sensitive phenotype similar to *sos1* plants (Zhu 2001).

Na^+ sequestration into the vacuole depends on expression and activity of Na^+/H^+ antiporters (*NHX*), vacuolar type H^+ -ATPases and H^+ -pyrophosphatases (*VP*). The proton pumps generate the necessary proton motive force required for activity of *NHX* (Gaxiola et al. 2001). Transgenic *Arabidopsis* plants over-expressing a vacuolar H^+ -pyrophosphatase exhibited increased partitioning of Na^+ into the vacuole and maintained higher relative water content in leaves and improved salinity stress tolerance (Gaxiola et al. 1999). The *NHX1* gene is known to be induced, both by salinity and ABA in *Arabidopsis* (Shi and Zhu 2002) and rice (Fukuda et al. 2004).

Even though active efflux of Na^+ is one of the important mechanisms of salinity tolerance, *SOS* pathway has not yet been experimentally established in wheat. Although *SOS1* homologues have been identified and functionally characterized in durum (Xu et al. 2008) and bread wheat (Cuin et al. 2011), none of these studies examined the whole *SOS* pathway including *SOS2* and *SOS3* genes. Ramezani et al. (2013) studied the expression of *SOS1* and *SOS4* genes in cultivated and wild wheat plants and concluded that all the three alleles of *SOS1* gene are active in their function. In present study we aimed to examine the expression of all the genes of *SOS* pathway in bread wheat genotypes with contrasting salinity tolerance and to analyze their association with salinity tolerance.

Materials and methods

Plant growth conditions and stress treatments

Three bread wheat (*Triticum aestivum* L.) genotypes viz., Kharchia 65, HD 2009 and HD 2687 were sown in 30 cm earthen pots filled with clay loam soil and farmyard manure in 3:1 ratio, pots were lined with 2-layers of 400 gauge polythene sheets to avoid leakage of the salt solution. Pots were fertilized with recommended dose of N, P, and K. Two different levels of salinity treatments i.e. 100 and 200 mM NaCl (2.5 liter of NaCl solution per pot containing 10 kg soil) were given to the plants at 25 days after sowing (DAS), while control plants were supplied with 2.5 liter of water. To avoid sudden osmotic shock to plants, salt solutions were supplied as batches of 0.5 liter per pot per day, finally reaching the desired concentration at 30 DAS. Scheduled routine of irrigation was practiced for both control and treated pots throughout the crop growth period. Each treatment was replicated 20 times. Soil samples were collected at three stages viz. 30DAS, 45 DAS and 60 DAS, and mean electrical conductivity of extract (ECe) of three stages were taken as the actual salinity level, viz. 1.21, 8.43 and 13.04 dS m^{-1} for control (C), 100 mM NaCl (S1) and 200 mM NaCl (S2), respectively.

Physiological and growth parameters

Physiological observations were taken at 60DAS. Upper most expanded leaf (third leaf from top) sampled in duplicate from 3 pots were used for recording observations. Relative water content (RWC) and membrane stability index (MSI), were estimated using standard protocols (Sairam et al. 1997; Weatherley 1950). For estimating leaf area, all the green leaves were sampled from two plants each from three pots and area was determined using leaf area meter, LICOR-3000 (Li-COR Ltd., Lincoln, Nebraska, USA). Roots were thoroughly washed and used for 2D root image analysis using Win Rhizo (Regent Instruments Inc., Canada).

Determination of potassium and sodium contents

For ion estimations, two plants each were harvested from 3 different pots and separated into leaves, stem and roots. The plant samples were dried and a representative ground plant sample (0.5 g) was digested in a di-acid mixture (20 ml) containing HNO_3 and HClO_4 acid (9:4) on digestion unit (Turbotherm, Gerhardt analytical systems, Germany) following the established procedure (Tandon 1995). The digested material was cooled, diluted with distilled water and filtered through Whatman No. 42 filter paper. The volume was made up to 25 ml and stored in a

polypropylene container. The K^+ and Na^+ content in the standard solutions, leaf, stem, and root samples were estimated using flame photometer (Systronics FF128).

RNA extraction and semi quantitative RT-PCR

For gene expression analysis, leaf and root samples were collected 24 h after the desired level of salinity concentration was achieved, i.e., on 31DAS. Third leaf from top was collected from two plants each of 4 different pots. Similarly roots were collected after thoroughly washing the roots in running tap water. Total RNA was extracted from leaf and root tissues using RNeasy plant minikit (Qiagen Inc., Chatsworth CA 91311, USA, Cat No: 749040). DNA contamination from the RNA samples was removed using on-column digestion with DNase I (Qiagen Science, Maryland, USA). For RT-PCR expression analysis and cloning of cDNAs, wheat homologues of the SOS pathway genes identified based on the sequence similarity to *O. sativa* SOS genes. Homologs in wheat genome database were identified by tBLASTn (wheat-urgi.versailles.inra.fr/Seq-Repository/BLAST) with default parameters, and further verified by updated expressed sequence tags (ESTs) or/and full-length cDNAs (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and tentative consensus sequences (TC) at DFCI gene index (<http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=wheat>). The chromosomal localization and physical position in bacterial artificial chromosome (BAC) were analyzed on the basis of wheat high throughput genomic sequences (urgi.versailles.inra.fr/Seq-Repository/BLAST). Due to the hexaploid genome of wheat all of the genes are having copies in A, B and D genomes. Primers were designed from highly conserved regions of the sequence to amplify all the possible alleles of the genes. Primer sequences of the genes are given in Online Resource 1. One μg of total RNA was reverse transcribed using Qiagen one-step RT-PCR kit on QN 96 Thermal cycler (Quanta biotech, England). Linear amplification for RT-PCR was obtained with 27 cycles and products were electrophoresed on 1.2 % agarose gel at 120 volts in TBE buffer (0.4 M Tris-borate, 0.001 M EDTA, pH 8.0) along with known concentration DNA ladders. Gels were stained with ethidium bromide and visualized on AlphaImager[®] imaging system (Protein simple, USA). *Actin* was used as internal standard. The electrophoretic bands were quantified using AlphaView[®] Software of AlphaImager[®] imaging system, and the *gene/actin* ratio was calculated by dividing the value of candidate gene bands by that of *actin* in each sample.

Sequence analysis of SOS pathway genes

To confirm the specificity of amplification, RT-PCR products were fractionated on agarose gel and purified. The

purified cDNAs for each gene were cloned into pDrive vector and transformed into *Escherichia coli* (strain DH5 α) cells. Colonies with recombinant plasmids were identified by blue-white screening and the presence of recombinant plasmids were confirmed by restriction analysis. Recombinant plasmids were isolated, purified and sequenced (Chromous Biotech Pvt. Ltd., Bengaluru, India). All the partial ESTs were submitted to GenBank. To study the conservation of genes across genera, ClustalW2.1 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) multiple sequence alignment of wheat ESTs were done along with corresponding sequences from model organisms. Deduced protein sequences were analyzed for the presence of conserved domains using protein blast (<http://blast.ncbi.nlm.nih.gov/>) with default parameters.

Statistical analyses

The experiment was conducted following factorial CRD design. Data presented are means of 6 observations ($n = 6$) for physiological estimations. *F* test was carried out to test the significance of the treatment differences, and the least significant differences (LSD) were computed to test the significance of the different treatments at 5 % level of probability by using SPSS 16.0. Mean separation by Duncan's multiple range tests (DMRT) at $P < 0.01$ was done using SAS[®] vers. 9.3 (SAS Institute, Cary, NC, USA).

Results

Physiological parameters

Wheat genotypes utilized in the present study were selected from a set of 100 genotypes (data not shown) based on germination and seedling growth assay under salinity stress (100 mM NaCl). The contrasting genotypes, tolerant (Kharchia 65), moderately tolerant (HD 2009) and sensitive (HD 2687) were selected and further evaluated for their response to salinity stress in pot culture growth conditions during *rabi* season in New Delhi, India. RWC of leaves were measured after 30 days of stress imposition. Salinity significantly reduced RWC of plants (Table 1). Kharchia 65 (salinity tolerant genotype) showed lowest decline in RWC as compared with control. It maintained significantly higher RWC as compared to HD 2687 (salinity sensitive genotype). MSI values of all the three genotypes also showed significant decrease in response to salinity stress (Table 1). The mean percentage reduction over control was 19 and 23.8 % under S1 and S2 salinity levels, respectively. Salinity tolerant and moderately tolerant genotypes exhibited significantly higher MSI values than the sensitive genotype (Table 1).

Table 1 Effect of salinity stress on leaf relative water content (%) and membrane stability index of wheat genotypes Kharchia 65, HD 2009 and HD 2687

Salinity levels	Relative water content (RWC %)			Membrane stability index (MSI %)		
	C	S1	S2	C	S1	S2
Kharchia 65	83 ± 1.43 ^c	79 ± 1.60 ^d	76 ± 1.40 ^c	89 ± 1.72 ^a	79 ± 1.63 ^b	75 ± 0.65 ^d
HD 2009	85.4 ± 1.53 ^b	77 ± 1.21 ^d	73.44 ± 1.23 ^f	88 ± 1.47 ^a	76 ± 2.07 ^c	72 ± 1.21 ^e
HD 2687	88 ± 2.12 ^a	74 ± 0.84 ^f	70 ± 0.79 ^e	88 ± 0.38 ^a	66 ± 1.82 ^f	62 ± 1.57 ^e
CD at 5 %						
<i>V</i>	1.08			1.21		
<i>T</i>	0.75			0.65		
<i>V</i> × <i>T</i>	1.68			1.71		

Plants were subjected to three treatments: normal irrigation (C); treatment with 100 mM NaCl solution (S1); treatment with 200 mM NaCl solution (S2). Means followed by different letters are significantly different at $P \leq 0.05$. Values are mean ± SE ($n = 6$)

Salinity stress drastically reduced leaf area of all the genotypes (Table 2) as indicated by the mean reduction (58 %) in leaf area of all the genotypes under S2 treatment. Salinity tolerant genotype exhibited lowest reduction in leaf area (36 %), whereas sensitive genotype showed severe reduction in total leaf area (74 %) under S2 level. Severe reduction in root surface area was observed under S2 stress level in all the genotypes (Table 2). Salinity tolerant genotype maintained significantly higher root surface area under both S1 and S2 level of stresses as compared to other genotypes. Similarly maximum root length was also observed in salinity tolerant genotype followed by moderately tolerant genotype (Table 2).

Sodium and potassium contents and K^+/Na^+ ratio in different plant parts

Salinity stress resulted in excessive accumulation of Na^+ in different plant parts such as leaves, stem and roots of all the wheat genotypes (Fig. 1a). In stem and roots, Na^+ content increased under both S1 and S2 salinity treatments. In stem, highest level of Na^+ content was found in sensitive genotype both under S1 and S2 treatments. Among the plant parts, Na^+ accumulation was highest in roots in all the genotypes. In roots, 0.7- to 2-fold increase in Na^+ content was observed in tolerant genotype, but other genotypes showed 1.0- to 3.4-fold increase in Na^+ content.

Salinity stress lead to decrease in K^+ content, irrespective of type of tissues and genotypes (Fig. 1b). The average decrease in salinity tolerant genotype was much lesser than other genotypes. While moderately tolerant genotype showed medium levels of K^+ content, sensitive genotype showed very low levels of K^+ under salinity stress. In stem and roots also K^+ content decreased under both S1 and S2 salinity treatments. Salinity tolerant

genotype maintained the highest level of K^+ content in stem and roots under both S1 and S2 salinity treatments.

Salinity stress decreased potassium/sodium (K^+/Na^+) ratio in leaves, stem and roots in all the genotypes (Fig. 1c). Salinity tolerant genotype maintained highest K^+/Na^+ ratio in leaves under salinity treatment. Similar trend was observed in stem and root tissues also. Salinity tolerant genotype was able to reduce net Na^+ uptake and sustain K^+ uptake, and thus exhibited favorable K^+/Na^+ ratio in all the tissues.

Effect of salinity stress on expression of SOS pathway genes

To elucidate the role of SOS pathway, expression of *SOS1*, *SOS2* and *SOS3* genes were studied in leaves and roots of salinity tolerant and sensitive genotypes. Both constitutive and salinity induced expression of *SOS1* gene was approximately 2 fold higher in leaf tissues of salinity tolerant genotype (Fig. 2a). Both moderately tolerant and sensitive genotypes also showed salinity induced expression *SOS1* gene in leaf tissue, but the level of expression was significantly lower than tolerant genotype. Up-regulation of SOS pathway genes in root tissue was very high, 100- to 750-fold higher in tolerant genotype as compared to 10- to 50-fold increase in leaf tissue. In root tissues, salinity induced up regulation of *SOS1* was observed only in tolerant genotype and expression increased with the increase in salinity levels (Fig. 2b), while other genotypes showed little or undetectable expression of *SOS1* under control and salinity stress conditions.

In leaves, stress-induced *SOS2* gene expression was observed in tolerant and moderately tolerant genotypes, while significantly lower expression was detected in sensitive genotype (Fig. 2c). In root tissues, tolerant genotype showed significant salinity induced up regulation of *SOS2*

Table 2 Effect of salinity stress on leaf area ($\text{cm}^2 \text{ plant}^{-1}$), root length (cm plant^{-1}) and root surface area ($\text{cm}^2 \text{ plant}^{-1}$) of wheat genotypes Kharchia 65, HD 2009 and HD 2687

Salinity levels	Leaf area ($\text{cm}^2 \text{ plant}^{-1}$)			Root length (cm plant^{-1})			Root surface area ($\text{cm}^2 \text{ plant}^{-1}$)		
	C	S1	S2	C	S1	S2	C	S1	S2
Kharchia 65	445.85 ± 7.4 ^a	341.69 ± 4.3 ^b	295.75 ± 4.13 ^c	5717.60 ± 72 ^a	5107.31 ± 21.2 ^b	4579.85 ± 36.52 ^d	755.19 ± 3.34 ^a	668.24 ± 6.59 ^a	401.19 ± 2.27 ^c
HD 2009	214.00 ± 4.2 ^d	94.05 ± 1.2 ^c	71.70 ± 2.2 ^f	4721.45 ± 27 ^c	3842.14 ± 36.3 ^e	1416.92 ± 14.56 ^e	583.54 ± 4.27 ^b	371.16 ± 9.44 ^d	313.00 ± 5.23 ^e
HD 2687	210.6 ± 2.8 ^d	52.7 ± 1.3 ^e	52.03 ± 2.5 ^e	4734.25 ± 17 ^c	3472.64 ± 27.37 ^f	1178.46 ± 18.9 ^b	591.14 ± 7.02 ^b	320.69 ± 7.14 ^c	316.50 ± 3.24 ^e
CD at 5 %									
V	7.28			28.25			10.1		
T	8.91			17.48			9.84		
V × T	12.61			48.93			12.5		

Plants were subjected to three treatments: normal irrigation (C); treatment with 100 mM NaCl solution (S1); treatment with 200 mM NaCl solution (S2). Means followed by different letters are significantly different at $P \leq 0.05$. Values are mean ± SE ($n = 6$)

gene at both salinity levels, while moderately tolerant genotype showed expression only at S2 level. Similar to *SOS1* gene expression, transcript abundance of the *SOS2* gene increased with increase in salinity levels. Sensitive genotype showed minimal expression under control and stress conditions (Fig. 2c).

Constitutive as well as salinity stress-induced expression of *SOS3* gene in leaf tissue was highest in tolerant genotype. Moderately tolerant and sensitive genotypes also exhibited stress induced over expression of *SOS3* gene in leaves (Fig. 2c). Similar to *SOS1* gene expression, only tolerant genotype showed expression of *SOS3* gene in of root tissues (Fig. 2c).

VPI gene expression in leaf tissue was down-regulated by salinity stress in all the three genotypes; however, the reduction in gene expression was lowest in salinity tolerant and moderately tolerant wheat genotypes (Fig. 3). *NHX1* gene expression was induced by salinity stress in all the wheat genotypes. Salinity tolerant and moderately tolerant wheat genotypes showed very high salinity induced expression of *NHX1*, while comparatively low gene expression was observed in sensitive genotype.

Partial coding sequences of *SOS1*, *SOS2*, *SOS3*, *VPI* and *NHX1* were cloned and sequenced from all the 3 genotypes and were submitted to GenBank (Online Resource 2). Comparison of deduced partial protein sequences of these genes revealed high levels of similarity to their homologs in rice and *A. thaliana* (Online Resource 3). The partial protein sequence showed a conserved domain: sodium/hydrogen exchanger 3 (amino acid residues 1–126), Monovalent Cation: Proton Antiporter-1 (CPA1) Family (TC 2.A.36). Transmembrane domain prediction revealed the presence of 2 transmembrane (TM) spans in the partial protein sequences (Online Resource 2). The partial sequence showed two conserved domains: (i) cd05123 (amino acid residues 1–78), catalytic domain of serine/threonine kinases (STKs) (ii) pfam03822 (amino acid residues 120–178)—NAF domain—a conserved protein–protein interaction module found in Ca^{2+} -regulated kinases. Blast analysis indicated the presence of a conserved domain: cd00051: amino acid residues 19–89: EFh, the calcium binding EF-hand. The *VPI* partial protein sequence showed a conserved domain: (amino acid residues 1–218) H^+ -translocating inorganic pyrophosphatase. Topology prediction using TMHMM program predicted the presence of 3 TM spans in the partial protein sequence. The *NHX1* deduced partial protein sequences of wheat genotypes showed a conserved domain: (amino acid residues 1–173) TIGR00840: b_cpa1 sodium/hydrogen exchanger 3, the Monovalent Cation: Proton Antiporter-1 (CPA1) Family (TC 2.A.36). Topology prediction using TMHMM program predicted presence of 4 TM spans in the partial protein sequences (Online Resource 3).

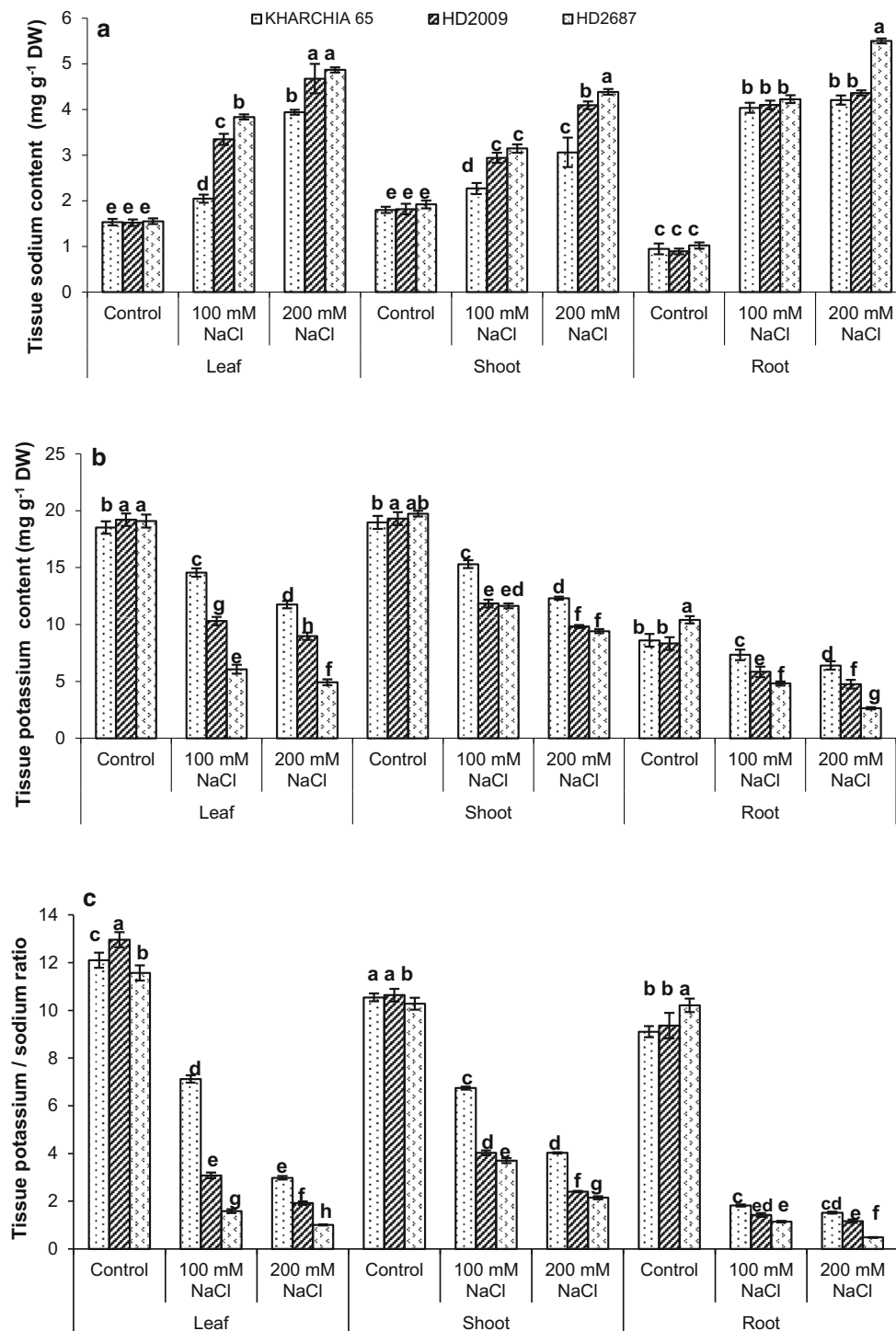


Fig. 1 Effect of salinity stress on sodium (a) and potassium (b) contents and potassium sodium ratio (c) of leaf, stem, and root tissues of wheat genotypes. Plants were subjected to three treatments: normal irrigation (control); treatment with 100 mM NaCl solution;

treatment with 200 mM NaCl solution. Vertical bars represent standard error. Within each tissue type, different letters indicate significant differences between the means ($P \leq 0.01$)

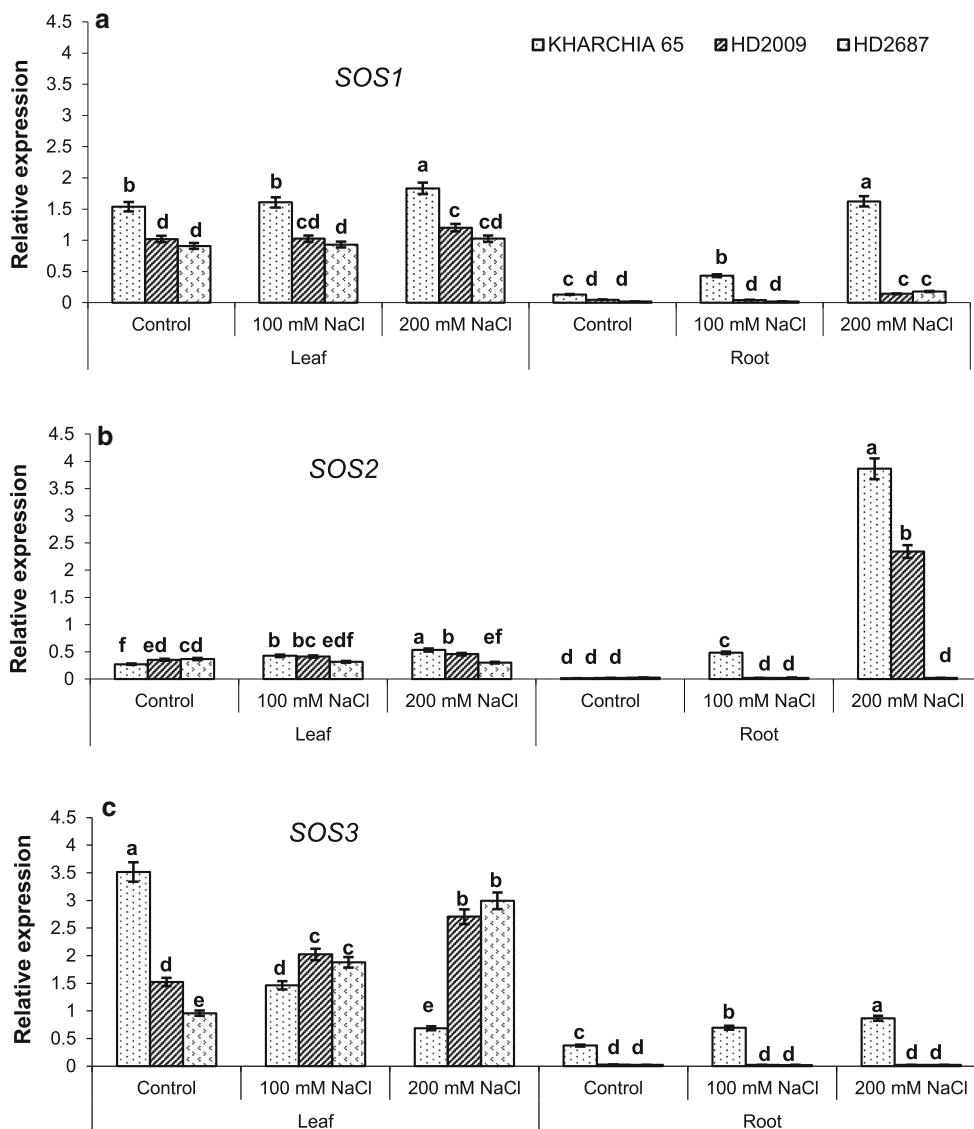


Fig. 2 Effect of salinity stress on transcript abundance of *SOS1* (a) *SOS2* (b) and *SOS3* (c) genes in leaf and root tissues of wheat genotypes. Plants were subjected to three treatments: normal irrigation (control); treatment with 100 mM NaCl solution; treatment with

200 mM NaCl solution. Vertical bars represent standard error. Within each tissue type, different letters indicate significant differences between the means ($P \leq 0.01$)

Discussion

High genetic variability exists for salinity tolerance in wheat gene pool. Kharchia 65 is well-known salinity tolerant bread wheat genotype developed in India (Hollington 2000). However, the role of SOS pathway in salinity tolerance of Kharchia 65 has not been examined before. Further, the relationship between SOS pathway expression and Na^+ exclusion also has not been established in wheat. Hence in this study, expression levels of SOS pathway genes and salinity tolerance were examined in wheat genotypes with contrasting salinity tolerance.

The presence of salt in the root zone reduces soil water potential, which leads to osmotic stress, affects water uptake and retards growth of crop plants (Chinnusamy et al. 2005). Salinity induced decline in RWC was significantly lesser in tolerant genotype as compared to moderately tolerant and sensitive genotypes (Table 1). Maintenance of higher RWC (%) by tolerant genotype indicates either sustenance of water uptake and/or minimization of transpirational water keeping optimum leaf turgor. It is deduced from the present results that, better root growth of salinity stressed plants of tolerant genotype (Table 2) might have resulted in better water relations.

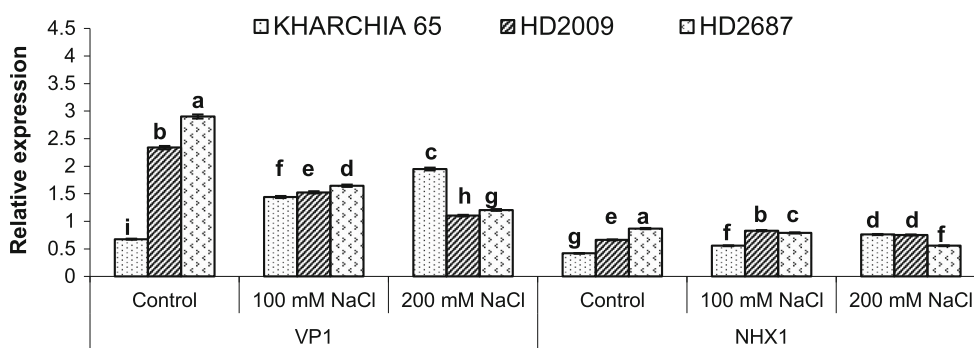


Fig. 3 Effect of salinity stress on transcript abundance of *VPI* and *NHX1* genes in leaf tissue of wheat genotypes. Plants were subjected to three treatments: normal irrigation (control); treatment with 100 mM NaCl solution; treatment with 200 mM NaCl solution.

Vertical bars represent standard error. Within each tissue type, different letters indicate significant differences between the means ($P \leq 0.01$)

Membrane stability index (MSI) is one of the important physiological traits suitable for screening of drought tolerance in various crops including wheat (Sairam et al. 2001, Iseki et al. 2014). Sairam et al. (2002) have used MSI and RWC as key parameters to differentiate wheat genotypes differing in salinity tolerance. MSI exhibited a positive correlation with osmotic potential, K^+ concentration, osmotic adjustment, and/or RWC under salinity stress (Munns 2002). Salinity induced decline in MSI was significantly less in tolerant genotype followed by moderately tolerant genotypes under salinity stress as compared to sensitive genotypes (Table 1). Comparatively better maintenance of RWC and MSI in salinity tolerant genotype might be due to the higher tissue tolerance, resulting in lesser salinity induced decline of leaf area (Tables 1, 2).

Maintenance of K^+ uptake and/or reduction in Na^+ entry, efflux of Na^+ from the cytosol, and sequestration of Na^+ for osmotic adjustment are strategies commonly used by plants to maintain the desirable K^+/Na^+ ratios in the cytosol (Zhu 2003). In this study, tolerant genotype accumulated lower amount of Na^+ and higher amount of K^+ and thus maintained higher K^+/Na^+ ratio in different plant parts as compared with sensitive genotype under both salinity stress levels (Fig. 1). Though salinity stress increased the Na^+ content in all the genotypes irrespective of the tissue, leaf Na^+ content was significantly higher in sensitive genotypes as compared with tolerant genotype of wheat. Interestingly, the tolerant genotype Kharchia 65 accumulated greater proportion of Na^+ in the root and stem tissues, and lesser amount in the leaf. Thus, tolerant genotypes excluded Na^+ ions selectively from leaves, the source tissue that supplies photosynthates for grain development.

Molecular basis of ion homeostasis under salinity stress was elucidated by the discovery of *Arabidopsis* salt overly sensitive (*sos*) mutants (Zhu 2003). SOS pathway regulates Na^+ and K^+ homeostasis at the root–soil interface and also

regulates long-distance Na^+ transport from roots to shoots. Salinity stress invokes cytosolic calcium oscillations that are sensed by a specialized calcium binding protein SOS3, which further transduces the signal by binding to and activating SOS2, a serine/threonine protein kinase (Qiu et al. 2002). The SOS2 kinase phosphorylates and activates SOS1, the plasma membrane Na^+/H^+ antiporter (Quintero et al. 2002). Activated SOS1 pumps out Na^+ from cytosol to apoplast of root tissues. SOS1 also helps in exporting Na^+ from the leaves into the phloem and then unloading it into the roots for efflux (Munns and Tester 2008). Physiological analysis clearly showed that salinity tolerant Kharchia 65 effectively excluded Na^+ away from leaves and also maintained lower levels of Na^+ in all the plant parts. This suggests that Kharchia 65 may have low net uptake of Na^+ at the root level and able to exclude the absorbed Na^+ from entering xylem stream and reaching leaves. Both of these processes are mediated by plasma membrane Na^+/H^+ exchanger encoded by the *SOS1* gene. Hence, we analyzed the expression of *SOS1*. The expression of *SOS1* was up-regulated under salinity in tolerant genotype in both root and leaf tissues, which had low tissue Na^+ levels and maintained better leaf K^+/Na^+ ratio (Figs. 1, 2a). Higher expression of *SOS1* in tolerant genotypes as compared with sensitive genotype has probably facilitated exclusion of toxic Na^+ into root apoplast and thus resulted in higher K^+/Na^+ ratio of leaves. A putative Na^+/H^+ antiporter from bread wheat enhanced Na^+/H^+ exchange activity and salinity tolerance of yeast (Xu et al. 2008). Similarly, durum wheat SOS1 protein effectively arbitrated Na^+/H^+ antiport in yeast and *Arabidopsis* (Feki et al. 2011, 2014).

The *SOS2* gene expression was induced in roots of tolerant genotype under salinity stress as compared with control. However, in roots of sensitive genotypes, *SOS2* was not induced and expression levels were lowest as compared with tolerant genotype. In leaf tissue also, *SOS2*

expression was highest in tolerant genotype. Greater expression of *SOS2* in tolerant genotype (Kharchia 65) thus might have helped sustained activity of *SOS1* under salinity stress (Fig. 2b). The *SOS3* is required for the activation of *SOS2* (Halfter et al. 2000). The *SOS3* gene expression was up-regulated in the roots of tolerant genotype under salinity stress, while no induction was observed in sensitive genotypes. Similar to *SOS1*, constitutive expression of *SOS3* gene was high in both leaf and root tissues of Kharchia 65 (Fig. 2c). Comparatively less expression of *SOS3* in sensitive genotype HD 2687 under salinity stress was probably responsible for susceptibility to salinity stress. Thus, transcript abundance of SOS pathway genes is correlated with salinity tolerance in wheat. Constitutive as well as stress induced expression of SOS pathway genes correlated with salinity tolerance of *Brassica* genotypes (Kumar et al. 2009).

The expression levels of genes involved in Na^+ sequestration namely *VPI* and *NHX1* were also higher in tolerant genotype at S2 level of salinity as compared with sensitive genotypes (Fig. 3). Results showed that salinity treatment sharply increased gene expression of *VPI* in tolerant and moderately tolerant genotypes, which might have created favorable proton gradient leading to increased activity of *NHX1*, which sequesters Na^+ into vacuole (Apse et al. 1999). In case of sensitive genotypes, the *VPI* and *NHX1* gene expressions were impaired under severe salinity stress.

ClustalW (2.1) multiple sequence alignment and comparison of deduced partial protein sequences of wheat SOS pathway genes with corresponding genes of *Arabidopsis thaliana* and *Oryza sativa* revealed the inter-generic similarity among the sequences (Online Resource 3). Apart from sequence homology, presence of unique conserved domains and TM spans (in *SOS1*, *VPI* and *NHX1*) unequivocally prove that the genes studied here, actually belong to wheat SOS pathway. Transgenic over expression of SOS pathway genes imparted favorable Na^+ and K^+ contents in *Arabidopsis* plants (Yang et al. 2009; Nie et al. 2015). Martinez-Atienza et al. (2007) concluded that in rice, higher expression of *SOS1*, *SOS2* and *SOS3*, correlated with better sodium exclusion from shoot. Cuin et al. (2011) reported that Na^+ efflux mediated probably by a *SOS1* like protein was highest in Kharchia 65 genotype, which also exhibited better Na^+ sequestration into the vacuoles of root cells as revealed by confocal microscopy using sodium binding fluorescent dye. Similarly, Ramezani et al. (2013) studied the expression of *SOS1* and *SOS4* genes in cultivated and wild wheat genotypes and concluded that all the three alleles of *SOS1* gene are active in salinity tolerance function.

It can thus be concluded that significantly higher transcript abundance of *SOS1*, *SOS2* and *SOS3* in tolerant genotype, Kharchia 65 as compared with sensitive

genotype HD 2687 in roots and leaves might have restricted the entry of Na^+ into the leaves and resulted in higher K^+/Na^+ ratio. Expression of *VPI* and *NHX1* genes were also high in leaves of tolerant genotypes, resulting in efficient sequestration of Na^+ into vacuoles. The results showed that the salinity tolerant varieties maintained proper growth by restricting the absorption of toxic Na^+ and lesser reduction in tissue K^+ content. Calcium ions which are the major signaling molecules in SOS pathway was maintained at significantly higher level in salinity tolerant genotypes exposed to long term salinity stress (Lekshmy et al. 2013). Our results provide evidence of the importance SOS pathway genes (*SOS1*, *SOS2*, and *SOS3*), *VPI* and *NHX1* in maintaining ion homeostasis in the well-known salinity tolerant wheat cv. Kharchia 65.

Author contribution statement Lekshmy Sathee conducted the experiments and contributed to the manuscript writing. R. K. Sairam and Chinnusamy V designed the experiment and wrote the manuscript. S. K. Jha did the cloning, bioinformatics and statistical analyses.

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