Induced defence responses of contrasting bread wheat genotypes under differential salt stress imposition

Archana Singh, Bharat Bhushan*, Kishor Gaikwad#, O P Yadav, Suresh Kumar* and R D Rai
Division of Biochemistry, Indian Agricultural Research Institute, New Delhi-110012, India
#National Research Centre on Plant Biotechnology, New Delhi-110012, India

Received 28 August 2014; revised 05 December 2014

Plants, being sessile in nature, have developed mechanisms to cope with high salt concentrations in the soil. In this study, the effects of NaCl (50-200 mM) on expression of high-affinity potassium transporters (HKTs), antioxidant enzymes and their isozyme profiles were investigated in two contrasting bread wheat (Triticum aestivum L.) genotypes viz., HD2329 (salt-sensitive) and Kharchia65 (salt-tolerant). Kharchia65 can successfully grow in salt affected soils, while HD2329 cannot tolerate salt stress. Differential expression studies of two HKT genes (TaHKT2;1.1 and TaHKT2;3.1) revealed their up-regulated expression (~1.5-fold) in the salt-sensitive HD2329 and down-regulated (~5-fold) inducible expression in the salt-tolerant genotype. Specific activity of antioxidant enzymes, viz. superoxide dismutase (SOD), peroxidase (POX), ascorbate peroxidase (APX), catalase (CAT) and glutathione reductase (GR) was found to be higher in the salt-tolerant genotype. Isozyme profile of two (POX and GR) antioxidant enzymes showed polymorphism between salt-tolerant and salt-sensitive genotypes. A new gene TaHKT2;3.1 was also identified and its expression profile and role in salt stress tolerance in wheat was also studied. Partial sequences of the TaHKT2;1.1 and TaHKT2;3.1 genes from bread wheat were submitted to the EMBL GenBank database. Our findings indicated that defence responses to salt stress were induced differentially in contrasting bread wheat genotypes which provide evidences for functional correlation between salt stress tolerance and differential biochemical and molecular expression patterns in bread wheat.

Keywords: Antioxidant enzymes, Gene expression, High-affinity potassium transporter, Wheat, Salt tolerance, Triticum aestivum L.

Salinity limits crop productivity, mainly in arid and semi-arid areas. The problem is further exacerbated due to irrigation with saline water, rising water tables resulting from land clearing and natural subsoil salinity. Since most of the crops have reached yield plateau, a gain in agricultural production may be expected from cultivation on saline soils. Wheat is the second most important staple food crop of India. Salt stress is well-known to affect crop yield, and even a significant effect on grain-protein content and quality in durum wheat (Triticum durum) has been reported. In wheat, increased salinity leads to decreased average yields to the extent of 50%. In spite of several efforts, improvement in salt stress tolerance of crop plants remains elusive, mainly due to the fact that salt stress tolerance is a multigenic, complex trait affected by many of the biochemical and molecular processes at both whole-plant and cellular levels via ion homeostasis.

To overcome deleterious effect of salt stress, plants have evolved several combating/defence mechanisms (enzymatic and non-enzymatic), such as generation of reactive oxygen species (ROS) and their intermediates like superoxide radicals, hydrogen peroxide (H2O2) and hydroxyl radicals. Several antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT) peroxidase (POX), ascorbate peroxidase (APX) and glutathione reductase (GR) are involved in the removal of these toxic chemical species and providing tolerance to salt stress.

It is believed that maintenance of a low Na+ ion concentration in cytoplasm is one of the important strategies for salt tolerance in crop plants. Sodium is compartmentalized into vacuoles in preference to K+ ion resulting in maintenance of high K+/Na+ ratio in
the cytosol of shoot cells. K\(^+\) is preferred for uptake into roots from the soil and most plants show a high degree of K\(^+\)/Na\(^+\) discrimination for their uptake. High-affinity potassium membrane transporters (HKTs) are active at plasma membrane level and have been reported to function as Na\(^+/K\(^+\) symporters and as Na\(^+\) selective uniporters\(^9\). Phylogenetic analyses of the known HKTs have revealed their two major subfamilies viz., HKT1 and HKT2. It is suggested that the transporters of the subfamily HKT1:x are permeable to Na\(^+\) only, whereas transporters of the subfamily HKT2:yz are permeable to both Na\(^+\) and K\(^+\) ions\(^9\). HKTs have two major functions, first to take up Na\(^+\) from soil to reduce K\(^+\) requirement when K\(^+\) is a limiting factor, and second to reduce Na\(^+\) accumulation in leaf by both removing Na\(^+\) from the xylem sap and loading Na\(^+\) into the phloem sap\(^10\).

Physiological and molecular analyses at seedling stage in two Tunisian durum wheat varieties have indicated repression of plasma membrane Na\(^+/\)H\(^+\) antiporter (TaSOS1) in the tolerant variety, resulting into salt stress tolerance due to the reduced loading of Na\(^+\) in shoot\(^11\). Deploying Na\(^+\) transporter in the plasma membrane of root cells surrounding xylem vessels, grain yield of durum wheat on saline soils could be significantly increased\(^12\). Single nucleotide polymorphism in HKT genes of durum wheat has been observed to cause change in amino acid sequence of the corresponding protein\(^13\).

Characterization of differentially synthesized stress-associated proteins during salt stress may help in identification of the stress responsive genes and marker(s) for salt stress tolerance, which may provide valuable insights to plant breeders for crop improvement and development of salt-tolerant varieties. Bread wheat (Triticum aestivum L.) varieties, namely Kharchia65 (salt-tolerant) and HD2329 (salt-sensitive) may be efficiently utilized for such studies\(^7\). Understanding the molecular basis of salt stress tolerance in wheat has become necessary for screening the local wheat genotypes and engineering elite lines for increased tolerance to salt stress. Although some reports challenge the assumption that Na\(^+\) exclusion leads to better salt tolerance, HKT alleles have emerged as an important component of salt stress tolerance\(^15\). The paradox may be resolved by characterizing natural variants of different components for salt tolerance, such as the ability to tolerate the effects of salt accumulation in shoot or to cope with the osmotic components of salinity\(^15\). This is an important area which should be investigated, as it may turn out to be the most effective way of modifying HKT function in plants with respect to modifying their interacting partners.

Here, we report differential expression of two HKT genes (TaHKT2;1.1 and TaHKT2;3.1) and activity of antioxidant enzymes in two contrasting bread wheat genotypes, revealing inducible defence responses to salt stress. The expression profile of TaHKT2;3.1, a newly identified gene from bread wheat and its possible role in salt stress tolerance has been investigated. Our findings provide evidences for functional correlation between salt stress tolerance and differential biochemical and molecular expression patterns in bread wheat, which is scanty in the available literature.

Materials and Methods

Plant material and salt treatment

Seeds of two bread wheat (Triticum aestivum L.) genotypes, viz. HD2329 (one of the most salt-sensitive varieties) and Kharchia65 (the most salt-tolerant variety) were surface-sterilized with 0.1% HgCl\(_2\) for 2 min, followed by washing three-times with sterile water. Ten seeds of each cultivar were sown in Agro-coir peat in 15 cm pots and grown at 25°C inside a growth chamber under 16 h photoperiod and 60 ± 10% relative humidity. Two weeks old seedlings were exposed to salt stress by irrigating them at alternate days with 0.5X Hoagland solution containing varying concentration (0, 50, 100, 150 and 200 mM) of NaCl. Shoot from the control and treated plants collected after 0, 3, 6, 9 and 12 days of treatment were used for protein/enzyme and RNA isolation.

RNA isolation and cDNA synthesis

Total RNA was isolated in triplicate from the shoot tissues using RNA isolation Nucleospin plant RNA kit (Machery-Nagel) following the manufacturer’s instructions. The isolated RNA (10 µg) was treated with RNase-free DNase I (Fermentas) to remove contaminating DNA. Integrity of RNA was checked on denaturing agarose (1.0%) gel and concentration was determined spectrophotometrically (Bioline-SPECORD 200, Analytikjena). The RNA samples showing A\(_{260/280}\) between 1.8-2.0 and A\(_{260/230}\) >2.0 were used for cDNA synthesis. First-strand cDNA was synthesized from 0.5 µg of the total RNA using Revert Aid Premium first strand cDNA synthesis kit (Fermentas) as per the manufacturer’s instructions.
**PCR amplification of HKT genes**

The first-strand cDNA (2.0 µl) was used as template for amplification of HKT genes using primer pairs designed from conserved region of HKT genes in barley, oat, maize and rice. Primers for five different HKT genes were tested, of which only two (forward: 5'-TGGGATGCCCACAAAGAC-3'; and reverse: 5'-TGATACGCAATGTTGAGC-3' for HKT2;1; forward: 5'-TGGGATGCCCACAAAGAC-3', and reverse: 5'-TGATACGCAATGTTGAGC-3' for HKT2;3) could give amplification. PCR conditions were: initial denaturation at 94°C for 5 min, followed by 36 cycles each at 94°C for 30 s, 58°C for 45 s, 72°C for 1 min, and final extension at 72°C for 5 min. The PCR products were visualized on 1.5% agarose gel and the products from salt-tolerant genotype Kharchia65 were got sequenced commercially.

**RT-PCR analysis**

Reverse transcription was performed with the Revert Aid Premium first-strand cDNA synthesis kit using equal amount of total RNA as template and 2.0 µM oligo-dT primer at 37°C for 1 h on a Triple Master PCR system (Eppendorf). The first-strand cDNA (2.0 µl) was used for expression analysis of HKT2;1 and HKT2;3 genes using the gene-specific primers. PCR conditions were as mentioned above, and the number of PCR cycle for semi-quantitative analysis was optimized by checking amplification product after 28, 32 and 35 cycles on 1.5% agarose gel. Actin gene (UniGene cluster Ta54825; Primers, forward: 5'-TGGGATGCCCACAAAGAC-3', and reverse: 5'-TGATACGCAATGTTGAGC-3') was used as reference gene for the analysis. The experiment was repeated three-times.

**RT-qPCR analysis**

Quantitative gene expression analysis of TaHKT2;1.1 and TaHKT2;3.1 genes was carried out using RT-qPCR following the MIQE guidelines. Three biological replications (three different RNA isolations, RT and qPCR reactions) and three technical replications for each biological replication were used for the quantitative expression analysis. The first-strand cDNA (1.0 µl) was used as template for qPCR based expression analysis of the candidate genes using 10 picomoles of the gene-specific forward and reverse primers. The quantitative PCR was performed using CFX-96 Real Time PCR system (BioRad Platform) with SYBR Green qPCR Master Mix (BioRad). PCR conditions were: initial denaturation at 95°C for 3 min, followed by 40 cycles each of 30 s denaturation at 94°C, 30 s annealing at 58°C and 45 s extension at 72°C. Amplification data collection was set at the end of each extension step.

Preliminary qPCR assay was carried out with 10 picomole of primers using three different concentrations of cDNAs to generate lowest Ct value, a sharp peak in melting curve analysis and no unspecific amplicon or primer-dimer artifacts. No template and RT-minus controls were included to detect contamination, primer-dimer formation and presence of genomic DNA. To estimate the relative gene expression, Ct values (inversely related to the initial DNA concentration) for both target and reference genes were calculated based on the mean value of three replications. Actin gene (UniGene Ta54825) was used as reference gene. The Pfaff formula was used to calculate the relative expression of TaHKT2;1.1 and TaHKT2;3.1 genes under 150 mM NaCl salt stress after 12 days of treatment, where ∆∆Ct = (∆Ct sample−∆Ct control); ∆Ct sample = (∆Ct target−∆Ct reference) for all sampling times and NaCl concentrations; and ∆Ct control = (∆Ct target−∆Ct reference).

**Enzyme extraction**

SOD and POX were extracted using extraction medium consisting of 0.1 M Tris-HCl buffer (pH 7.5), 3% (w/v) polyvinylpyrrolidone, 1.0 mM EDTA and 1.0 mM CaCl₂. Extraction medium used for CAT, APX and GR consisted of 0.1 M potassium phosphate buffer (pH 7.5) in place of Tris-HCl buffer. Extraction was carried out at 4°C by macerating 1.0 g fresh shoot tissues in 10 ml ice-cold extraction medium using pre-chilled pestle and mortar. The homogenate was filtered through cheese-cloth and the filtrate was centrifuged (Sorval Rc5c) at 10,000 g for 20 min at 4°C. Supernatant was used as crude enzyme extract for enzyme activity assay and protein content in the extracts was estimation by the Lowry method.

**Enzyme activity assay**

The effect of salt stress on antioxidant enzyme activity was determined by in vitro assays of the enzymes involved in removal of reactive oxygen species viz. SOD, POX, APX, CAT and GR.

Superoxide dismutase (SOD; EC 1.15.1.1) activity was estimated by recording decrease in absorbance of formazan produced by superoxide-nitroblue tetrazolium (NBT) complex by the enzyme. Absorbance was recorded at 560 nm.
The enzyme extract containing 50 mg protein was used for in-gel enzyme assay on 10% non-denaturing polyacrylamide gel run at 30 mA for 2-4 h at 4°C and stained as per the procedure described by Mittler and Zilinskas. 

**POX isozyme activity staining** was performed as per the method of Guikema and Shermen. The gel was stained in a solution of 25% acetic acid containing 0.3% benzidine and 0.5% H₂O₂. Within 2 min, blue coloured bands appeared which turned brown after 10-15 min.

**APX isozyme activity staining** was carried out as per the procedure described by Mittler and Zilinskas. Achromatic bands against bluish background represented APX isoenzyme activity. The gel was washed with distilled water to remove excessive stain and photographed.

**CAT isozyme activity staining** was performed as per the method of Woodbury et al. Once white coloured bands appeared against the bluish-green background, the reaction was terminated by adding 1.0% HCl and the gel was photographed. GR isozyme activity staining was carried out as per the procedure described previously. Duplicate gels were assayed for GR activity: one with and one without GSSG. When brown coloured bands appeared on yellow background, the gels were photographed.

**Results and Discussion**

Accumulation of undesirable ions (e.g. Na⁺, CI⁻ etc.) and lower concentration of beneficial ions (e.g. K⁺, Ca²⁺ etc.) in the cytoplasm cause oxidative stress and adversely affect cellular metabolism, resulting into plant growth inhibition. Higher concentrations of Na⁺ in leaf adversely affect its growth and functions. Variability does exist in the wheat germplasm, and a genotype that can cope with the high Na⁺ concentration in soil may be utilized towards the development of salt-tolerant crop varieties. KHT genes/transporters have been reported to play important role in salt tolerance in plants. In this study, we investigated differential expression of TaHKT2;1.1 and TaHKT2;3.1 genes and induced defence responses in two contrasting bread wheat genotypes.

**Gene amplification, sequencing and in silico analyses**

Using homologous, gene-specific primers for 5 different HKT genes, only two HKT genes could be amplified with total RNA isolated from shoot of the salt-tolerant genotype. PCR products amplified with HKT2;1 and HKT2;3 gene-specific primers were ~0.3 Kb and ~0.4 Kb in size, respectively. Sequencing of the PCR products revealed their size to be 292 bp (HKT2;1) and 367 bp (HKT2;3). BLAST analysis of the partial HKT2;1 gene sequence showed 89-98% homology with HKT genes from oat, barley and wheat.
(DQ009003.1, U16709.1, DQ915706.1), and the gene was named \textit{TaHKT2;1.1} as per the nomenclature system.

BLAST search for the \textit{HKT2;3} gene sequence showed 94\% homology with a gene (AK370026.1) from barley and 89\% homology with a probable cation transporter \textit{HKT9-like} gene (XM_003563466.1) from \textit{Brachypodium distacyn}. Since this EST did not show significant homology with any of the \textit{HKTs} reported so far from wheat, the gene was named \textit{TaHKT2;3.1} as per the nomenclature system. This gene is considered to be a novel \textit{HKT} gene being reported in bread wheat. Partial cds of these \textit{HKT} genes of bread wheat were submitted to the EMBL GenBank database with the accession no. HF937364 (\textit{TaHKT2;1.1}) and HF937363 (\textit{TaHKT2;3.1}).

Expression of \textit{HKT} genes in contrasting genotypes

Semi-quantitative gene expression analysis revealed that the \textit{HKT} genes were differentially expressed in the two contrasting bread wheat genotypes. Up-regulation of \textit{TaHKT2;1.1} and \textit{TaHKT2;3.1} was observed in the salt-sensitive genotype, while the genes were down-regulated in salt-tolerant genotype with reference to the duration of salt stress imposition (Fig. 1). Initially (3 days of salt stress), the difference in gene expression between control and treatment was less in both the genotypes. However, with increasing duration (6, 9 and 12 days) of salt stress, a significant difference in expression level for both the \textit{HKT} genes was observed. The difference in expression level became more prominent after 9 days of salt treatment. This indicated that expression of these genes was induced by salt stress. Expression level of the reference (actin) gene was observed to be consistent throughout the salt stress treatment in both the genotypes.

Differential expression of the genes was further validated by RT-qPCR, which clearly indicated that both the genes were up-regulated in salt-sensitive genotype (HD2329), while their expression was down-regulated in salt-tolerant genotype (Kharchia65). Up-regulation of \textit{TaHKT2;3.1} gene was about 1.5-folds higher than that of \textit{TaHKT2;1.1} gene in the salt-sensitive genotype. Similarly, down-regulation of \textit{TaHKT2;3.1} gene was five-fold compared to that of the \textit{TaHKT2;1.1} gene in the salt-tolerant genotype, as estimated after 12 days of salt stress treatment (Fig. 2).

The down-regulated expression of these \textit{HKT} genes in salt-tolerant genotype might be responsible for exclusion of Na\(^+\). A major component of tolerance to long-term exposure to Na\(^+\) can be the ability of plants to exclude Na\(^+\) from shoot. Munns \textit{et al.}\(^{12}\) reported identification of an ancestral Na\(^+\) transporter from durum wheat responsible for Na\(^+\) exclusion which helps maintaining low Na\(^+\) concentration and high K\(^+\)/Na\(^+\) ratio in leaf. This ability of plants to maintain low Na\(^+\) concentration or a high K\(^+\)/Na\(^+\) ratio in shoot is considered a potential mechanism of salinity tolerance\(^{30}\). If we consider overall difference in fold expression of \textit{TaHKT2;1.1} gene between salt-sensitive and salt-tolerant genotypes, it was ~1.5-fold. But, the difference in fold expression between salt-sensitive and salt-tolerant genotypes was found to be ~3-fold.
for *TaHKT2;3.1* gene. This indicated that *TaHKT2;3.1* gene was more inducible and might play a major role in Na⁺ homeostasis. Although a direct correlation may not be correct, the difference in expression level of the *HKT* genes in the two contrasting genotypes supported the hypothesis that transporters contributed to the observed difference in salt stress tolerance.

Transports present in plasma membrane of plant cells are believed to be involved in Na⁺ homeostasis by extruding Na⁺ from root epidermal cells at the root-soil interface and by regulating the root-shoot distribution of Na⁺ in coordination with HKT proteins. In wheat, one of the mechanisms conferring salt tolerance is Na⁺ exclusion from leaves. Na⁺ exclusion is achieved by its low net uptake by cells in root cortex and tight control of net unloading of xylem by parenchyma cells in the stele. This may be the possible mechanism in bread wheat to exhibit slow rate of Na⁺ transport to shoot, and to maintain a high K⁺/Na⁺ ratio in leaf. This K⁺/Na⁺ discrimination may confer some degree of salt tolerance.

*TaHKT2;1* is an example of a gene that regulates Na⁺ transport and is expressed in root epidermal cells at the root-soil interface and by regulating the root-shoot distribution of Na⁺ in coordination with HKT proteins. This enables shoots to maintain a high K⁺/Na⁺ ratio in leaf and to exclude Na⁺ from the xylem. This is one of the mechanisms that contribute to salt tolerance in plants.

In this study, differential expression of the *HKT* genes might be responsible for Na⁺ retention in the shoot of salt-sensitive and its depletion/efflux in salt-tolerant genotype. A similar observation on differential expression of *TaSOS1* associated with Na⁺ flux from roots to shoots has been reported in contrasting durum wheat varieties. Knock-down of *TaHKT2;1* in wheat has been reported to increase shoot fresh weight under 200 mM NaCl stress and K⁺ deficiency which indicates that the gene mediates Na⁺ influx. Another report on root uptake system also supports that *TaHKT2;1* functions as a Na⁺ uniporter. Thus, down-regulated expression of *TaHKT2;1.1* and *TaHKT2;3.1* genes in salt-tolerant genotype in this study might be responsible for influx of Na⁺ into vacuoles and maintaining the high K⁺/Na⁺ ratio in the cytosol of shoot. However, further studies are required to analyze plant’s salt stress response at molecular level due to the complexity of events associated with sensing of salt stress and inducibility of the relevant pathways.

In amphiploid lines of *Lophopyrum elongatum* (a wild relative of wheat), salt tolerance is found to be associated with improved Na⁺ exclusion, a trait that is reported to be linked with chromosome 3E from *L. elongatum* that also contains *SOS1* gene. Thus, net Na⁺ accumulation in bread wheat may be controlled by the interplay of HKT and SOS proteins, and polymorphism in these genes may be responsible for the observed natural variation for salt tolerance.

### Effect of salt stress on activity of antioxidant enzymes and their isozymes

Activity of antioxidant enzymes (POX, APX, CAT and GR) was found to increase in both the genotypes with increasing concentration (50-200 mM) of NaCl used for stress imposition, except for the SOD. For most of the enzymes, the maximum activity was observed 6 days after treatment (DAT), particularly in the salt-tolerant genotype. However, SOD activity was observed to be higher in control (0 mM NaCl) plants of salt-sensitive genotype than that of the salt-tolerant genotype. Significant reduction in activity of SOD was observed with increasing salt concentration in the salt-tolerant genotype. On the contrary, activity of SOD was observed to increase with the increasing concentration of NaCl up to 100 mM in case of the salt-tolerant genotype.

SOD showed three isomeric forms in both salt-sensitive and salt-tolerant genotypes. However, SOD activity in untreated control was found to be higher in salt-sensitive genotype than that in the salt-tolerant genotype. These observations suggested that the salt-tolerant genotype possessed a better O₂⁻ radical scavenging ability. Earlier, severe reduction in SOD activity has also been reported by Neto *et al.* in a salt-sensitive maize cultivar. They also reported that salt stress induces SOD activity in both shoot and root of salt-tolerant cultivar, while reduces it in salt-sensitive cultivar. Thus, our observations were in agreement with the findings of Neto *et al.* and supported the hypothesis that SOD plays important role in salt stress tolerance in plants.

POX activity was significantly higher in the salt-tolerant genotype compared to that in the salt-sensitive genotype at all the concentrations and duration of salt stress (Fig. 4A-C). POX activity increased with the increasing salt concentration in both the genotypes, but over the stress period (3-9 DAT), decrease in enzyme activity was observed. Isozyme profile of POX showed significant variation with only two isoforms in the salt-tolerant genotype, but three isoforms in the salt-sensitive genotype.
Transient increase in POX activity and isozyme polymorphism has been reported to play role in antioxidant defence mechanism by detoxification of ROS. APX activity also increased with the increasing salt concentration in both the genotypes and over the stress period, the enzyme activity decreased. The salt-tolerant genotype showed significantly higher APX activity, compared to that in the salt-sensitive genotype up to the 6th day of stress (Fig. 5A-C). APX showed the same four isoforms in both the genotypes (Fig. 5D).

CAT showed two-times higher activity in salt-tolerant genotype compared to that in the salt-sensitive genotype. Only a minor variation in CAT activity was observed with the increase in salt concentration and the duration of salt treatment (Fig. 6A-C). CAT showed five isoforms in both the genotypes (Fig. 6D).

An increase in GR activity was observed with increasing concentration of NaCl in both the genotypes (Fig. 7A-C). Salt-tolerant genotype showed a significant increase in the GR activity by 6th day of salt treatment (Fig. 7B), and maintained the higher enzyme activity subsequently. In contrast, the salt-sensitive genotype showed significant decrease in GR activity just after 3 days of salt treatment. Though two isoforms of GR were observed in both the genotypes, one of them was polymorphic in nature with higher molecular weight in salt-tolerant genotype (Fig. 7D). Earlier, investigations on antioxidant enzyme activity in salt-tolerant and salt-sensitive cultivars of different crops have suggested a direct relationship between salt tolerance and GR activity. A distinct isoform of GR observed in salt-tolerant genotype may be responsible for the increased GR activity.

Among the ROS, superoxide is converted by SOD enzyme to $\text{H}_2\text{O}_2$, which is further scavenged by CAT and POX. CAT and POX have been reported to have increased activity under various environmental stresses, including salt stress. Related studies suggest that tolerance to salt stress can be correlated with a more efficient antioxidant system, with high enzymatic activities and better biomass production in salt-tolerant genotype. Thus, the higher antioxidant enzyme activities observed in Kharchia65 under salt stress indicated its tolerance to salt stress, as compared to HD2329.

Salt stress is detrimental to normal functioning of plant and may cause membrane damage, leading to the formation of different ROS, such as superoxide...
Fig. 4—Effect of salt stress on POX activity in shoot of the contrasting wheat genotypes HD2329 (salt-sensitive) and Kharchia65 (salt-tolerant) after 3 days (A), 6 days (B), 9 days (C), and isozyme pattern of POX after 6 days (D) of salt treatment [C = control (0 mM), T1 = 50 mM NaCl treatment, T2 = 100 mM NaCl treatment, T3 = 150 mM NaCl treatment, T4 = 200 mM NaCl treatment, DAT = days after treatment. Each value represents the mean of three replications and vertical bars indicate ± SD. Horizontal arrow indicates a distinct isoform of POX observed in salt-sensitive (HD2329)]

Fig. 5—Effect of salt stress on APX activity in shoot of the contrasting wheat genotypes HD2329 (salt-sensitive) and Kharchia65 (salt-tolerant) after 3 days (A), 6 days (B), 9 days (C), and isozyme pattern of APX after 6 days (D) of salt treatment [C = control (0 mM), T1 = 50 mM NaCl treatment, T2 = 100 mM NaCl treatment, T3 = 150 mM NaCl treatment, T4 = 200 mM NaCl treatment, DAT = days after treatment. Each value represents the mean of three replications and vertical bars indicate ± SD]
Fig. 6—Effect of salt stress on CAT activity in shoot of the contrasting wheat genotypes HD2329 (salt-sensitive) and Kharchia65 (salt-tolerant) after 3 days (A), 6 days (B), 9 days (C), and isozyme pattern of CAT after 6 days (D) of salt treatment [C = control (0 mM), T1 = 50 mM NaCl treatment, T2 = 100 mM NaCl treatment, T3 = 150 mM NaCl treatment, T4 = 200 mM NaCl treatment, DAT = days after treatment. Each value represents the mean of three replications and vertical bars indicate ± SD]

Fig. 7—Effect of salt stress on GR activity in shoot of the contrasting wheat genotypes HD2329 (salt-sensitive) and Kharchia65 (salt-tolerant) after 3 days (A), 6 days (B), 9 days (C), and isozyme pattern of GR after 6 days (D) of salt treatment [C = control (0 mM), T1 = 50 mM NaCl treatment, T2 = 100 mM NaCl treatment, T3 = 150 mM NaCl treatment, T4 = 200 mM NaCl treatment, DAT = days after treatment. Each value represents the mean of three replications and vertical bars indicate ± SD. Horizontal arrows indicate polymorphic isoforms of GR]
(O$_2^-$), H$_2$O$_2$ and hydroxyl radical (•OH). Antioxidant enzymes play important role in cellular defence mechanisms against ROS. Their activities modulate the relative amount of O$_2^-$ and H$_2$O$_2$, the two Haber-Weiss reaction substrates. They also decrease the risk of hydroxyl radical formation, which is highly reactive and may cause severe damage to membranes, proteins and DNA.$^{37}$

Maximum activity of the antioxidant enzymes was observed 6 DAT, particularly in the salt-tolerant genotype, indicating induced defence responses against salt stress. The observed variations in isoforms of certain antioxidant enzymes in the salt-tolerant genotype might be explained on the basis of alternate splicing. Two-staged model for Na$^+$ exclusion in rice, supported by 3D modelling and alternate splicing for HKT (O$_2$HKT1;4) transporter was proposed by Cotsaftis et al.$^{39}$ Our findings of differential regulation of the HKT genes and induced defence response by antioxidant machinery supported the two-staged Na$^+$ exclusion hypothesis, thus indicating a similar mechanism might be operative in bread wheat also.

In conclusion, activity of antioxidant enzymes, viz. SOD, POX, APX, CAT and GR was found to be higher in salt-tolerant genotype. Isozyme profile of two antioxidant enzymes (POX and GR) showed clear polymorphism between salt-tolerant and salt-sensitive genotypes. The increased antioxidant enzyme activity in salt-tolerant genotype helps fighting against the ROS generated during salt stress. A new HKT gene (TaHKT2; 3.1) was identified, cloned and characterized for its role in salt stress tolerance in wheat. TaHKT2;1.1 and TaHKT2;3.1 genes were found to be up-regulated (~1.5-fold) in the salt-sensitive genotype (HD2329), while they were down-regulated (~5-fold) in the salt-tolerant genotype (Kharchia65). The differential expression of HKT genes might help in avoiding accumulation of toxic level of Na$^+$ and maintaining adequate level of K$^+$. However, further studies on differential protein expression may help in better understanding the molecular mechanism of salt stress tolerance in wheat.

Acknowledgements
We acknowledge the facilities provided by the Director, Indian Agricultural Research Institute, New Delhi, India, to undertake the present study.

References