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Original Article

Allelic variations and differential expressions detected at quantitative trait loci for salt stress tolerance in wheat

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ABSTRACT

The increasing salinization of agricultural lands is a threat to global wheat production. Understanding of the mechanistic basis of salt tolerance (ST) is essential for developing breeding and selection strategies that would allow for increased wheat production under saline conditions to meet the increasing global demand. We used a set that consists of 150 internationally derived winter and facultative wheat cultivars genotyped with a 90K SNP chip and phenotyped for ST across three growth stages and for ionic (leaf K^+ and Na^+ contents) traits to dissect the genetic architecture regulating ST in wheat. Genome-wide association mapping revealed 187 Single Nucleotide Polymorphism (SNPs) (R^2 = 3.00–30.67%), representing 37 quantitative trait loci (QTL), significantly associated with the ST traits. Of these, four QTL on 1BS, 2AL, 2BS and 3AL were associated with ST across the three growth stages and with the ionic traits. Novel QTL were also detected on 1BS and 1DL. Candidate genes linked to these polymorphisms were uncovered, and expression analyses were performed and validated on them under saline and non-saline conditions using transcriptomics and qRT-PCR data. Expressed sequence comparisons in contrasting ST wheat genotypes identified several non-synonymous/missense mutation sites that are contributory to the ST trait variations, indicating the biological relevance of these polymorphisms that can be exploited in breeding for ST in wheat.

Key-words: developmental growth stages; genome-wide association study (GWAS); salt tolerance; transcription regulation.

INTRODUCTION

About 800 million hectares of global arable land are salt-affected (FAO, 2008). The extent and severity of salt-affected agricultural land are predicted to worsen as a result of inadequate drainage of irrigated land, rising water tables and global warming (Munns & Gilliham 2015). It has been estimated that 20% of irrigated land in the world is presently affected by salinity, excluding regions classified as arid and desert lands (Yamaguchi & Blumwald 2005). In rain-fed agriculture production systems where transient salinity occurs, yields can be well below theoretical for the rainfall received, when subsoil salinity is present, and unused water at harvest is one of its symptoms (Sadras *et al.* 2002). Wheat (*Triticum aestivum* L.) is the third most important cereal crop worldwide, with an estimated annual production of about 736 million metric tons (FAO 2015). An increase in wheat production is paramount to meet the need of the growing population, which has been predicted to reach nine billion by 2050. Agronomic and engineering solutions are being exhausted in an attempt to minimize the impact of salinity on global food production. The way forward is to breed greater salt tolerance (ST) into present crops and to introduce new species for cultivation (Munns & Gilliham 2015).

Under saline conditions, crops exhibit slower growth rates, increased leaf senenses and reduced tillering, and over months, the reproductive development is affected (Munns & Tester 2008), resulting in significant grain yield reduction. The effect of salinity on crops is due to osmotic stress caused by the Na^+ and Cl^- ions toxicities and their interference with the uptake of mineral nutrients (Mba *et al.* 2007). The mechanism of plant response to salt stress is a complex phenomenon that involves several genetic, physiological and environmental factors occurring at different levels including cellular, tissue and whole plant level. During long-term exposure to salinity, the plants' adaptive mechanisms related to salt stress tolerance would evolve and start to differentiate across growth stages, giving rise to the coordination of all cellular, tissue and organ responses that are needed for proper tolerance response. Reports indicated that ST is developmental growth stage dependent (Haq *et al.* 2010; Turki *et al.* 2014), but there may exist the possibility of salt stress response mechanisms that are active across all the different plant developmental growth stages. The discovery of key genetic switches controlling ST at various growth stages would facilitate breeding for improved ST.

Genetic diversity for salinity tolerance has been limited in bread wheat. One land race Kharcia 65 played a major role in salt-tolerant varietal development in India where the cultivars *KRL1-4* and later *KRL 19* emerged (Ogbonnaya *et al.* 2013). Dreccer *et al.* (2004) identified synthetic hexaploid wheat that possessed considerable variation for ST based on Na^+ exclusion. Similarly, Colmer *et al.* (2006) reviewed the

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potential of wild relatives to contribute towards improving salinity tolerance. The salinity tolerance of bread wheat is based on its ability to exclude Na^+ from the leaf blades and an overall maintenance of K^+/Na^+ ratio. Several studies have reported on the genetic variation for ST at various growth stages in wheat (Schachtman *et al.* 1992; Munns *et al.* 2000; Rahnama *et al.* 2011; Ahmad *et al.* 2013), providing great opportunity for ST improvement. However, the drawback of these studies is their inability to simultaneously analyse the genetic variation for ST at multiple key growth stages using the same population. In addition, most of the efforts towards exploring the genetic variation relied on the classical biparental linkage mapping that is characterized by poor resolution in QTL detection and costly, with considerable amount of time needed to develop appropriate mapping population and results in identifying limited number of alleles that can be studied simultaneously at any given locus (Flint-Garcia *et al.* 2003).

Genome-wide association studies (GWAS) have emerged as an alternative approach that is maximizing recent advances in genomic tools and statistical methods by exploiting cumulative recombination and mutation events available in a population to identify significant marker-trait associations (MTAs). The GWAS has proven to be a useful tool to dissect complex genetic mechanisms governing biotic (Jighly *et al.* 2015; Zegeye *et al.* 2014) and abiotic (Long *et al.* 2013; Turki *et al.* 2014) stress tolerance in many crops. In wheat, there has been little research into the identification of large-scale ST loci using GWAS for different stages of growth within the same germplasm simultaneously.

In this study, the genetic variations for ST across three growth stages (germination, seedling hydroponics and adult-field stages) were exploited to comprehensively evaluate and identify QTL conferring salt tolerance in 150 winter wheat cultivars using the GWAS approach. Probable putative genes controlling the observed trait variations were investigated and their gene expressions and amino acid sequences investigated in contrasting ST wheat genotypes at transcription regulational level.

MATERIALS AND METHODS

Plant material

The mapping panel consists of 150 internationally derived winter and facultative wheat genotypes previously described in Oyiga *et al.* (2016).

Phenotypic screening

The phenotypic screening for ST at three growth stages and the statistical analytical tools used have been described in Oyiga *et al.* (2016). Details of the traits measured and salt stress imposed are presented in the Supporting Information Table S1. Briefly, data on the germination vigour were collected under two salt types (NaCl and Na_2SO_4) in several concentrations: 100, 150 and 200 mM for NaCl and 75 and 100 mM for Na_2SO_4 plus control (without salt). At seedling stage, traits including fresh-shoot weight (FSW), fresh-root weight (FRW), dry-shoot weight (DSW) and dry-root weight (DRW) were collected in four independent hydroponic experiments, designated as E1, E2, E3 and E4, with three replications under saline and non-saline conditions. The adult-field grown plants (AFP) trials were conducted under saline and non-saline soil conditions in three different field locations: Urgench [Uzbekistan; 41°32′60′N, 60°37′60′E, 91 m above sea level (masl)], Karshi (Uzbekistan; 38°52′N, 65°48′E, 416 masl) and Dongying (China; 118°33′–119°20′E, 37°35′–38°12′N, 47 masl). Data collected include grain yield (GY), plant height (PHT), days to heading (DHD), days to maturity (DMT), days to grain filling (GFP) and one-thousand kernel weights (TKW). The ST indices of all the traits were calculated (Genc *et al.* 2010) and used for the GWAS studies.

Leaf Na^+ and K^+ content

The amounts of Na^+ and K^+ ions in the third leaf of the 150 genotypes were measured (after 25 days of growth under 150 mM NaCl) from pooled dried plants of three plants per genotype. The concentrations of Na^+ and K^+ were assessed by atomic absorption spectrometre (Perkin Elmer, Wellesley, MA, USA), and subsequently, K^+/Na^+ ratios were calculated.

SNP genotyping

The GWAS wheat panel was genotyped with the Illumina iSelect 90K SNP assay (Wang *et al.* 2014) at the TraitGenetics GmbH, Gatersleben, Germany.

Population structure (PS)

The population structure (PS) of the GWAS panel was examined with 582 selected SNPs that fulfilled the following criteria: Minor allele frequency (MAF) >5%; <2% missing data and spaced approximately 2 cM apart using the STRUCTURE V2.3.3 program based on an admixture model (Pritchard *et al.* 2000). The model was applied without the use of prior population information (i.e. USEPOPINFO was turned off) and population genetic clusters of K = 1 to 14 with 20 runs per K evaluated. The most likely number of sub-populations was determined using the delta K method (Evanno *et al.* 2005). Genetic relationships among the genotypes were graphically plotted via principal coordinates analysis (PCoA) using GenAlEx 6.5 (Peakall *et al.* 2012).

Linkage disequilibrium analysis

The linkage disequilibrium (LD) among SNP pairs was estimated for A, B and D genomes using the full-matrix option in TASSEL (available at: http://www.maizegenetics.net/tassel). Only SNPs with defined genetic positions and MAF 5% were used in this analysis. The LD decay was determined by plotting the pairwise squared correlation (r^2) values against the distance (centimorgan) between SNPs on the same chromosome. An LOESS curve (Breseghello & Sorrells 2006) was drawn to fit the data using second-degree locally weighted scatter plot smoothing in the SAS program (SAS Institute, Cary, NC; http://www.sas.com). The genetic distance corresponding to LD ≤0.1 was considered as the critical distance up to which a QTL extends.

Identification of MTAs using GWAS

The GWAS was performed by adopting the multilocus mixed-linear model (MMLM-P + K) that accounts for population structure (P-matrix) and kinship (I-matrix) (Zhoa *et al.* 2007). The association tests were performed using PROC MIXED in SAS version 9.3 (SAS Institute, Cary, NC; http://www.sas.com) and were verified with rrBLUP R package (Endelman 2011). To minimize false positives, only congruent significant MTAs in both analyses were reported. The P-matrix was estimated via principal component analysis (PCA). The kinship (K) matrix was considered as a 'random effect' and P-matrix as a 'fixed effect' by including five top principal components in the model. Both the P-matrix and K-matrix were generated with the TASSEL software (Henderson, 1975; Bradbury *et al.* 2007) and included in the following equation:

$$\boldsymbol{y} = \boldsymbol{X\beta} + \boldsymbol{Zu} + \boldsymbol{e}$$

where y is the phenotypes estimates, **X** and **Z** are the known design matrices, $\boldsymbol{\beta}$ is an unknown vector containing fixed effects including genetic marker and population structure (P), $\boldsymbol{u}$ is the vector of the random genetic effects from multiple background QTL for individuals or lines and e is the vector of the residuals. The genome LD decay values as described in Long *et al*. (2013) were used to calculate the threshold for accepting significant MTAs. All significant MTAs within the estimated LD block coverage were assigned to a single QTL region (Pasam *et al*. 2012).

Identification of ORFs at the chromosome regions harbouring identified quantitative trait loci

The DNA sequence information surrounding the detected SNPs (Wang *et al*. 2014) were used for *in silico* analysis. To expand the sequence information up and downstream of the short core SNP sequences (<80 bp), matches were searched in the CerealsDB database (http://www.cerealsdb.uk.net/). The sequence information obtained was used as queries to identify the open reading frames (ORFs) using BLASTn of the wheat URGI wheat database (https://urgi.versailles.inra.fr/blast/).

Gene expression analyses

Gene expression analyses of genes in the associated QTL regions were performed using the salt-tolerant (*Altay2000* and *UZ-11CWA-8*) and salt-sensitive (*UZ-11CWA-24* and *Bobur*) wheat genotypes identified in the studied panel in our previous study (Oyiga *et al*. 2016). The genotypes were grown in the growth chamber (temperature: 20/15 °C; day length: 14 day/10 night hours) using the modified Hoagland solution (Tavakkoli *et al*. 2010). Ten days after planting, salt stress [non-saline and saline (100 mM NaCl)] was imposed. The solution pH was monitored and adjusted daily to 6.0.

Transcriptome analysis of the identified candidate genes. The blades of the third leaf from five plant samples were harvested at 2 h, 11 d, and 24 d after salt application in both non-saline and saline conditions for next-generation sequencing (NGS)-based massive analysis of cDNA ends (MACE) transcriptome profiling. The harvested samples at each time point were pooled and the NGS libraries generated for each genotype and subsequently sequenced using *MACE* (80–100 bp paired ends) (performed at GenXPro GmbH, Frankfurt am Main, Germany). Reads were annotated and mapped to wheat genome using the Ensembl Genomes database (Kersey *et al.* 2015; http://plants.ensembl.org). Thereafter, the transcript counts of the candidate genes were extracted from the annotated NGS libraries and used for the expression pattern analysis in the ST contrasting wheat genotypes.

RNA extraction and qRT-PCR analyses. Total RNA was extracted from the harvested leaf samples after 30 d in saline and non-saline conditions using E.Z.N.A. Plant RNA Kit (Omega Bio-Tek, Norcross, GA, USA), followed by DNA removal step using DNA Digestion kit (Omega Bio-Tek, Norcross, GA, USA). The cDNA synthesis was performed with Thermo Scientific First Strand cDNA Synthesis Kit (Cat. #K1632) using three technical replicates. The quantification of the amplified product was carried out using real-time PCR on SDS-7500 Sequence Detection System (Applied Biosystems). The qRT-PCR reaction (20 μl) consisted of gene-specific primers (0.3 μl) (Table 1), DyNamo ColorFlash SYBR Green 2X-master mix with ROX (Cat.#F456L; Thermo Fisher Scientific) and the template (3 μl). The gene primers were designed around the associated SNPs using primer3

Table 1. Sequences of the primers used in the qRT-PCR

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product size (bp)
Target			
ZIP7	TCTCATTCCACCAGTTCTTCG	GATGCCTTCAACCACTAGAGC	191
KeFC	AGCAAAACTTCCAATGTCCG	ATCAATGGTGTCGCTCTCGT	175
AtABC8	CAACAAGACCACAATGCCTG	TCTCCCTCACATCCATACCA	177
6-SFT	CGTGGAGGAGATTGAGACCC	GCAGAAGCATCAAGGTGGA	141
Internal control			
TaEf-1a	CTGGTGTCATCAAGCCTGGT	TCCTTCACGGCAACATTC	151
TaEf-1a	CAGATTGGCAACGGCTACG	CGGACAGCAAAACGACCAAG	227

The sizes of the corresponding amplified fragment are shown.
ZIP7, putative zinc transporter; **KefC**, glutathione-regulated potassium-efflux system protein; At**ABC8**, putative ABC transporter B family member 8; 6-SFT, sucrose: sucrose 1-fructosytransferase.

(http://primer3.wi.mit.edu/). Thermal cycling conditions were 95 °C/7 min followed by 95 °C/10 s, 60 °C/30 s and 72 °C/30 s (fluorescence acquisition) for 40 cycles. The gene expression data were analysed with the standard methods of Livak & Schmittgen (2001), normalized with two internal control genes, *TaEf-1a* and *TaEf-1b* (Unigene accession: Ta659). The PCR reaction efficiencies of target and internal control genes are comparable (Supporting Information Fig. S1). Melting curves (Supporting Information Fig. S2) of the amplified PCR products were generated using the following programme: 95 °C for 10 s, 60 °C for 30 s and 95 °C for 15 s.

Comparisons of the expressed sequence tags between Altay2000 and Bobur

To examine the relationship between ST and the putative genes, expressed sequence tags (ESTs) of six identified candidate genes were compared in Altay2000 and Bobur by sequence alignment with their corresponding wheat gene draft sequences (Kersey *et al.* 2015). The candidate genes analysed include ZIP7 (gene ID: *Traes_1BS_D68F0BED6.1*), KeFc (gene ID: *Traes_2AL_A2CBDB5F7.1*), SAP8 (gene ID: *Traes_7AL_B88F6A3D3.1*), HAK18 (gene ID: *Traes_5BL_F112FA40E.2*), GST1 (gene ID: *Traes_3AL_F205FA0941.2*) and SWEET17 (gene ID: *Traes_5AS_9937DABBA.1*). Their amino acid sequences were inferred using the Sequence Manipulation Suite (Stothard 2000) and aligned with MAFFT version 7 (http://mafft.cbrc.jp/alignment/server/) to check for possible polymorphic sites that may cause structural changes of the associated genes in the contrasting wheat genotypes.

RESULTS

Phenotypic variations for salt tolerance

The association mapping panel was derived from 673 crosses involving 371 unique ancestral co-founders, which highlighted the inherent genetic diversity in the panel used for this study and thus a valuable genetic resource for QTL identification. The genetic variation among the constituent genotypes in response to salt stress treatment under six different salt concentrations at germination stage has been reported (Oyiga *et al.* 2016).

Several traits were scored to analyse the effect of salt stress and their genetic variation at seedling stage. Analysis of variance results indicate that genotype varied significantly for all the traits measured, except for FRW in E4 (Table 2). Salt treatment showed strong effect on the traits across the four experiments, but the interaction effects of genotype and salt treatment were significant on the traits only in E2 and E3 experiments. Application of salt stress had a negative effect on all the traits (Table 2), although the effect was stronger in the shoot than in the root traits (Fig. 1). The trait heritability (h^2) was moderate to high and varied from 0.44 in E2 to 0.79 in E4 for DRW, with the exception of FRW (Hb: 0.30) in E2. The magnitude of variations among the genotypes for the measured traits was ≥15%.

There was highly significant genotype effect on all AFP traits measured, except for PHT at Dongying (Table 3). Field soil salinity significantly affected all the AFP-related traits, except TKW and DHD at the Urgench and Karshi locations, respectively. The genotype-by-field salinity interactions were observed in most of the traits. The h^2 ST trait estimates at Urgench and Karshi ranged from 0.54 for DHD to 0.89 for TKW at Karshi. The lowest h^2 (0.08) was observed for PHT at Dongying. The magnitude of variation observed for the ST AFP-related traits was highest (44.3%) for PHT at Dongying and lowest (1.3%) for DMT at Karshi.

Third leaf ionic evaluations

The results revealed that K^+, Na^+ and K^+/Na^+ ratio were normally distributed among the 150 genotypes after 25 d of salt treatment (Fig. 2). The leaf K^+ showed the narrowest variation (CV = 8.84%, ranged from 4.14 to 6.90%), whereas leaf

Table 2. The effects of genotype (G), treatment (T) and results of genotype by treatment (G*T) on fresh and dry (DRW) and fresh (FRW) root weights and fresh shoot weight (FSW) based on analysis of variance tests are shown

Experiments	Trait	G	T	G*T	h2	CV	Effect (%)
E1	DRW	678.31**	136.23**	166.66 ns	0.52	18.71	−16.80
	FRW	786.87**	63.07**	145.26^{ns}	0.57	19.16	−12.20
	FSW	308.22**	325.69**	76.84^{ns}	0.59	14.99	−28.75
E2	DRW	567.49**	1065.14**	266.78**	0.44	19.42	−36.90
	FRW	434.29**	814.38**	235.46**	0.3	21.97	−38.19
	FSW	611.67**	5556.08**	387.70**	0.49	17.79	−60.36
E3	DRW	404.01**	565.08**	185.07*	0.67	15.93	−26.25
	FRW	345.58**	654.73**	206.63*	0.70	19.01	−30.83
	FSW	548.30**	4763.63**	313.94**	0.66	15.76	−57.85
E4	DRW	210.76*	332.79**	139.10^{ns}	0.79	17.18	−23.81
	FRW	165.65^{ns}	213.16**	124.86^{ns}	0.74	20.47	−23.05
	FSW	189.97*	2946.91**	127.29^{ns}	0.74	15.94	−63.45

Number of stars indicates significant level. E1, E2, E3 and E4 are four independent salt screening experiments. The DSW has been reported in Oyiga *et al.* (2016).
h^2, heritability estimates at seedling growth stage; **CV**, coefficient of variations; ns, non-significant.
*$P < 0.05$.
**$P < 0.01$.

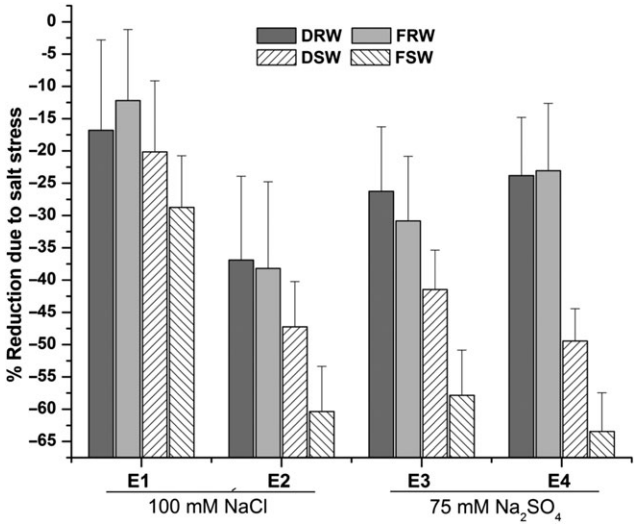

Figure 1. Histogram showing the effect of salt stress on the DRW (dry root weight), FRW (fresh root weight), DSW (dry shoot weight) and FSW (fresh root weight) of the genome-wide association study panel across the four experiments at seedling stage. E1, E2 (with 100 mM NaCl), E3 and E4 (with 75 mM Na_2SO_4) are four independent salt-screening experiments.

Na^+ (CV = 28.14%) varied from 0.59 to 3.11% and K^+/Na^+ ratio (CV = 26.80%) from 2.07 to 10.67% (Fig. 2a,b,c). The relationships between root biomass produced under salt stress and leaf ion concentrations are shown in Fig. 3. No significant pattern was observed between the leaf K^+ and root biomass production under salinity stress (Fig. 3a). However, Na^+ ($r^2 = 0.47$**; Fig. 3b) and K^+/Na^+ ratios ($r^2 = -0.24$**; Fig. 3c) showed positive and negative trends with the root biomass, respectively.

SNP marker analysis

After data curation (MAF >5%; <5% missing data), a total of 18 085 SNPs with known genetic positions were found to be polymorphic. Seven thousand (32.66%), 9243 (43.04%) and 1734 (0.08%) SNPs were mapped to A, B and D genomes, respectively, with corresponding map lengths of 1252.3, 1139.6 and 1251.2 cM (Supporting Information Table S2; Supporting Information Fig. S3). The SNP map spanned a total genetic distance of 3644.10 cM with an average SNP density of 0.49 cM. The longest genetic distance between SNPs was 242 cM.

Population structure

Population structure analysis indicated that maximum value of △K occurred at K = 2 (Fig. 4), confirming that two sub-populations provided the optimal structure. The PCoA revealed two major sub-groupings (Supporting Information Fig. S4); however, the groupings did not reflect the four breeding centres where the genotypes originated from. The breeders may have exchanged germplasm in their breeding programmes, and recombination and mutation events in the panel may have resulted in diverse germplasm.

Linkage disequilibrium decay

The lowest LD decay of 14 cM was found in the D genome and about 10 and 11 cM in the A and B genomes, respectively (Fig. 5). The D genome had the highest number of SNPs (23.81%) in significant LD (r^2 0.1) followed by B genome (17.24%) and A genome (13.65%), with 15.44% recorded for the entire genome (Supporting Information Table S2). Individually, chromosome 2D (60.58%), 6B (26.722%), 4B (22.66%), 1B (20.07%) and 4A (20.06%) had the highest number of SNPs in significant LD. The SNP density of 0.49 cM obtained, indicates that the marker coverage used for this study was appropriate for detecting QTL using a GWAS approach.

Marker-trait associations for the phenotypic traits across growth stages

A total of 172 significant MTAs were detected for ST with all the traits, each explaining phenotypic variation (R^2) ranging from 3.0% for ST_DRW in E4 to 30.67% for DSW at E1 + E2. Of these, 30, 99 and 42 were detected at germination,

Table 3. The effects of genotype (G), treatment (T) and genotype by treatment (G*T) on traits measured in adult field-grown plants at three locations based on analysis of variance tests are shown

Field locations	Traits	G	T	G*T	h2	CV
Urgench, Uzbekistan (2011–2012)	TKW	1673.50**	0.26^{ns}	213.45*	0.84	6.53
	PH	1921.58**	447.28**	287.62**	0.85	8.4
	GY	1054.07**	494.71**	281.33**	0.76	23.07
Karshi, Uzbekistan (2012–2013)	TKW	2799.12**	21.48**	206.50*	0.89	4.44
	DHD	464.10**	5.04^{ns}	132.20^{ns}	0.54	2.06
	DMT	502.46**	24.29**	110.84^{ns}	0.59	1.28
	GY	747.00**	188.77**	437.95**	0.57	16.25
Dongying, China (2013–2014)	PHT	156.51^{ns}	814.77**	134.62^{ns}	0.08	44.31
	GY	217.13**	1791.53**	199.11*	0.23	71.6

h^2, trait heritability estimates; CV, coefficient of variation; TKW, thousand kernel weight; PHT, plant height; DHD, days to heading; GFP, days to grain filling; DMT, days to maturity; GY, grain yield; ns = non-significant.
Number of stars indicates significant level.
*$P < 0.05$.
**$P < 0.01$.

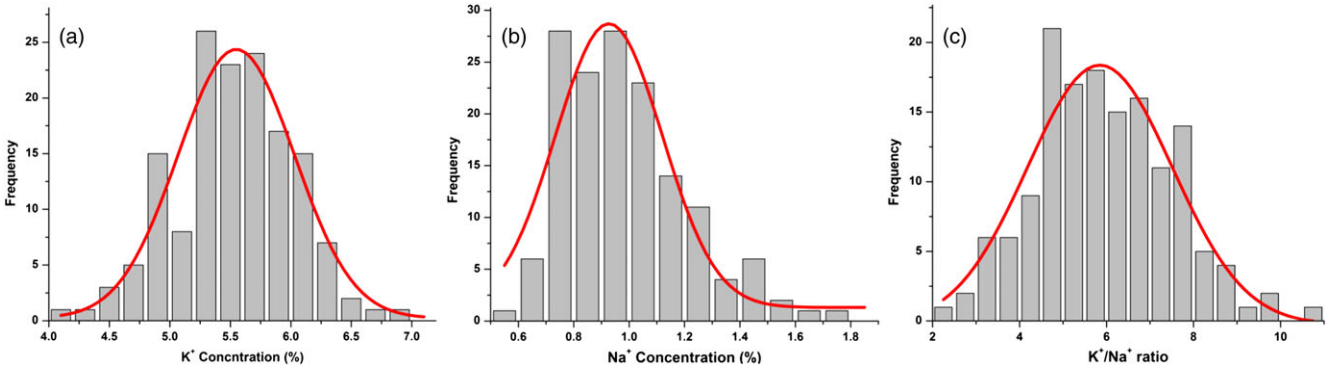

Figure 2. Histogram of the distribution of ion accumulation traits (K^+, Na^+, K^+/Na^+ ratio) measured in the association mapping panel of wheat after 24 d under 150 mM NaCl stress. [Colour figure can be viewed at wileyonlinelibrary.com]

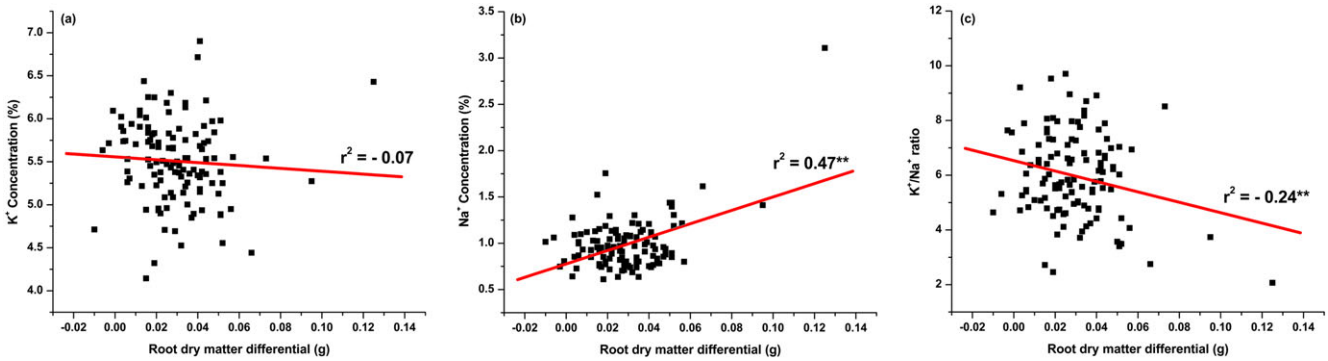

Figure 3. Relationship of root dry weight differential, calculated as a difference between dry root weight in non-saline and saline conditions, with leaf K^+ and Na^+ concentration and the estimated K^+/Na^+ ratio in the third leaves of the association mapping panel grown in the hydroponics and treated with 150 mM NaCl. Concentrations of K^+ and Na^+ were estimated using atomic absorption spectrometre after 24 d of salt stress. [Colour figure can be viewed at wileyonlinelibrary.com]

seedling and AFP growth stages, respectively. The highest number of MTAs was detected on the A genome (77) followed by the B genome (68) and D genome (8) in that order. Several associated SNPs/loci showed pleiotropic properties across growth stages. Novel QTL were detected on 1BS, 1DL, 5BS and 5BL chromosomes. Details and description of the associated SNPs are presented in Supporting Information Table S3.

Chromosomal regions harbouring multiple marker-trait associations for the phenotypic traits

Several SNPs were significantly associated with the ST traits in more than one growth stage. For example, SNP *GENE_3156_152* at 68.36 cM on 5BL had a remarkable effect on the traits at germination (germination vigour under 75 mM Na_2SO_4) and seedling (FSW, FRW, DSW and DRW) growth stages. It accounted for 24.20% of the observed R^2. Similarly, SNP *GENE_1353_136* (at 101.97 cM: $R^2 \geq 22.09\%$) on 2AL was associated with FRW, DSW and DRW at seedling stage and PHT at AFP with R^2 of 22.09%. The locus at 137 cM on 1BL with two coincident SNPs, *Kukri_c18230_1633* and *BobWhite_c8293_236*, accounted for 11.42% of the R^2 at germination (75 mM Na_2SO_4) and seedling during E2 (ST_DRW) growth stages, respectively. The locus on 2BS at 96.99 cM was significantly linked to ST_DRW at seedling and GY at AFP growth stages, with $R^2 \geq 12.69\%$. Moreover, the locus at 71.33 cM ($R^2 \geq 12.23\%$) on 7BS detected with *Ra_c7974_1192* and *Excalibur_rep_c67190_638* was associated

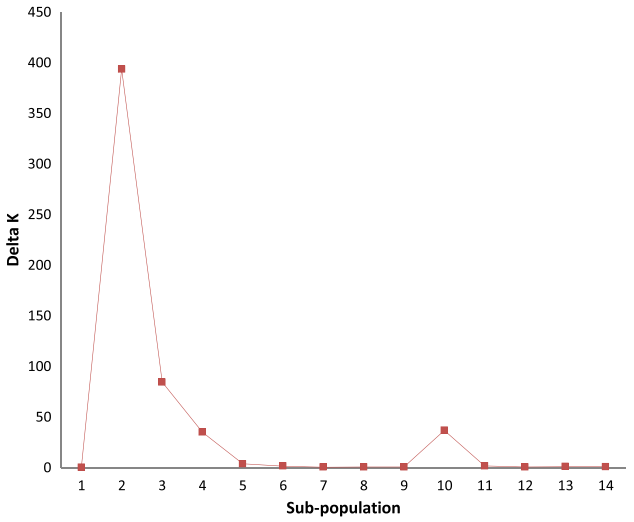

Figure 4. Magnitude of delta K as a function of K-values = 1 to 14 (*x*-axes) in the association mapping panel. A distinct peak at K = 2 was indicative that model with two sub-groups was optimal. [Colour figure can be viewed at wileyonlinelibrary.com]

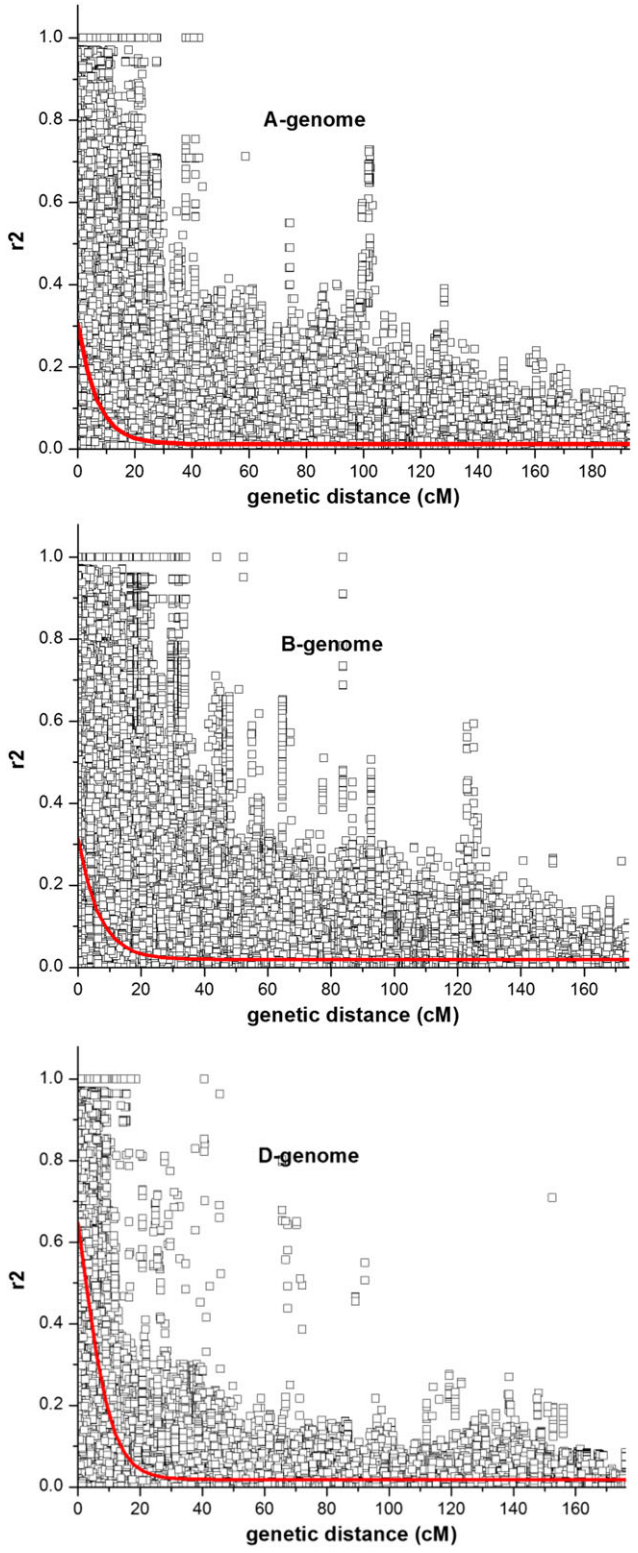

Figure 5. Linkage distribution decay of A, B and D genomes. Inner fitted red trend line is a non-linear logarithmic regression curve of r^2 on genetic distance. Linkage distribution decay is considered below $r^2 = 0.1$ threshold. [Colour figure can be viewed at wileyonlinelibrary.com]

with germination vigour (under 100 mM Na_2SO_4) and ST_DSW, respectively.

The SNPs with pleiotropic and growth stage specific effects were identified on 1DS, 2AL, 2DS, 3AL and 7BL chromosomes. SNP *Excalibur_c91176_326* (150.29 cM, $R^2 \geq 10.76\%$.) on 2AL was strongly associated with DSW and DRW ST traits in E1 and E2, respectively. Two SNPs on 1DS locus (at 67.72 cM, $R^2 \geq 13.33\%$), BS00002178_*51* and *RAC875_c62_1546* had strong effect on ST_DRW in both E2 and E3. SNPs on 2DS (*D_GBUVHFX02GV41H_67*; $R^2 \geq 9.62\%$) and 7BL (*BS00004171_51*; $R^2 \geq 11.46\%$) also affected multiple ST traits at seedling stage. The former was associated with FSW and DRW, while the latter was linked with FSW and DSW. On 3AL, two SNPs affecting ST_FSW (E1 + E2) and ST_DSW (E3 + E4) were detected in 0.1 cM interval using *Jagger_c765_61* and *wsnp_RFL_Contig2011_121680*, respectively.

Marker-trait associations for leaf ionic (K^+, Na^+ and K^+/Na^+ ratio) traits

Fifteen SNPs were significantly associated with the leaf ionic traits measured after 25 d of salt stress, with R^2 of between 6.96% for leaf Na^+ and 10.13% for leaf K^+ (Table 4). Five SNPs on 2AL, 3AL, 4AS, 5AL and 6BL showing associations with the leaf ionic traits were also found to have strong effect on the ST traits measured (Fig. 6). A locus (*Kukri_c11327_977*) at 101.97 cM on 2AL ($R^2 = 7.45\%$) detected for K^+/Na^+ ratio coincided with the locus affecting salt-related DRW, DSW and FRW traits at seedling and PHT and ST_GY at AFP growth stages. This locus is also 1.65 cM away from a locus identified for germination vigour that influenced ST under 200 mM NaCl and 100 mM Na_2SO_4 salt stress conditions. The SNP *wsnp_Ex_rep_c106152_90334299* located on 3AL at 84.78 cM was associated with Na^+ and accounted for 7.81% of the R^2. It lies less than 4.69 cM away from other SNPs that influenced ST traits at germination (under 200 mM NaCl), seedling (ST_FSW, ST_DSW and ST_DRW) and AFP (ST_GY) growth stages.

Congruent quantitative trait loci regions

Using genome LD decay, all the detected MTAs were delineated into 37 QTL regions (Fig. 6; Supporting Information Table S4). Four QTL regions including *Q-1BS.1* ($R^2 \geq 30.67\%$), *QTL_2AL.1* ($R^2 \geq 16.93\%$), *QTL_2BS.1* ($R^2 \geq 12.69\%$) and *QTL_3AL.1* ($R^2 \geq 12.02\%$) are most significant because individually, they were associated with ST traits across the three growth states – germination, seedling and AFP. Of these, *QTL_2AL.1* and *QTL_3AL*.1 were also linked with leaf K^+/Na^+ and leaf Na^+, respectively. Eleven QTL regions exhibited significant genome-wide association with ST traits at seedling and AFP stages, while six QTL regions had an effect on germination and seedling stages. Two loci at 68.4 cM on 5BL (*GENE_3156_152*; $R^2 \geq 24.20\%$) and 71.32 cM on 7BS (*Q-7BS*; $R^2 \geq 12.23\%$) were pleiotropic and had multiple effects on ST traits at germination and seedling growth stages. Summary of the detected QTL regions, the associated traits and the reported QTL are presented in Supporting Information Table S4.

Table 4. Summary of SNP markers significantly associated with the accumulated Na^+ and K^+ and K^+/Na^+ ratio in the third leaf after 24 d of salt stress

Ions	SNP	Chr	Position	P.value	MAF	R^2 (%)
K^+ content	Excalibur_c13094_523	7DL	134.69	5.10E-06	0.27	10.13
	RAC875_rep_c70595_321	5D	67.49	0.0000278	0.43	8.06
	IAAV8258	5AL	86.91	0.0000318	0.19	7.90
	RAC875_c14137_994	1DL	107.25	0.0000652	0.10	7.05
	Kukri_c49331_77	6BL	80.61	0.0000713	0.18	6.95
Na^+ content	wsnp_Ex_rep_c106152_90334299	3AL	84.78	0.0000308	0.38	7.81
	wsnp_Ex_c45713_51429315	6BL	116.55	0.0000333	0.33	7.72
	RAC875_c2666_484	6BL	118.99	0.0000353	0.29	7.65
	RAC875_c28831_558	5BS	11.73	0.0000448	0.40	7.37
	Jagger_c4026_328	2AL	124.81	0.0000638	0.28	6.96
K^+/Na^+ ratio	Excalibur_c13094_523	7DL	134.69	0.0000117	0.27	10.01
	Kukri_rep_c79597_513	4AS	43.39	0.0000289	0.13	8.81
	Excalibur_c39621_358	4AS	43.39	0.0000298	0.15	8.77
	Kukri_c11327_977	2AL	101.97	0.0000404	0.36	8.37
	wsnp_Ex_c59095_60108185	2AL	122.83	0.0000822	0.29	7.45

Principal coordinates analysis based on the identified polymorphisms

PCoA with the 187 identified SNPs were used to assess the genetic relatedness among the most consistent salt-tolerant and salt-sensitive genotypes in the studied panel (Fig. 7). The first three axes explained 28.57% of the total variation. The first three PCoAs mostly depicted the relationships that are consistent with the ST status of the individual genotypes, by grouping the genotypes based on their ST status as was previously reported in Oyiga *et al.* (2016). The salt-tolerant genotypes (in black/triangular-shaped) were mostly distributed at the right side of the plot, whereas the salt-sensitive genotypes (grey colour/squared-shaped) were distributed to the left side.

Ontology classification of the DNA sequences of chromosomes containing the identified quantitative trait loci

The *in silico* analysis of the sequences surrounding 74 of the associated SNP sequences revealed high sequence homologies to genes involved in salt stress response (Supporting Information Table S5). The largest categories of genes identified are those involved in stress and defence (23%), antiporter/transport (22%), ion homeostasis/detoxification (18%), transcription/translation (11%), osmo-protectant (9%) and signal transduction (8%) activities, while the genes involved in chromosomal repair, protection/cell wall modification (5%) and plant hormone synthesis (4%) accounted for relatively small portion (Fig. 8).

Analyses of expression regulation of candidate genes

The transcript abundance of associated 22 candidate genes was investigated in the leaves of salt-tolerant (Altay2000) and salt-sensitive (*Bobur*) genotypes under saline and non-saline conditions after 24 d of stress. Day 24 was adopted to analyse the genes identified to be genetically associated with the measured traits. All the genes (except for protein kinase *G11A*) revealed differential expressions when compared with time zero or control and are mostly up-regulated in Altay2000 and down-regulated in *Bobur* (Fig. 9). The *ZIP-7* (located in the QTL region that influenced ST across the three growth stages) exhibited strongest differential expression; it increased by 713.98% in Altay2000 but declined by 22.19% in *Bobur* vis-à-vis the control. The gene ontology and their biological and molecular functions of the analysed genes are shown in Supporting Information Table S6.

The expression patterns of the four candidate genes identified were further analysed to monitor their accumulation after salt stress application using qRT-PCR. At Day 30, the expression of *ZIP7*, *KeFc*, *AtABC8* and *6-SFT* revealed similar pattern as was observed in Day 24 (Fig. 10), which were further substantiated by high correlations ($r^2 = 0.63$–0.98, $P = 0.01$) existing between the TranSNiPtomic data and qRT-PCR data (data not shown). They are up-regulated in tolerant genotypes in contrast with the sensitive genotypes.

Kinetics of the candidate genes expression following salt stress application

Expression kinetics of the genes were compared in the contrasting genotypes over a time course of 2 h, 11 d and 24 d in saline and non-saline conditions. The kinetics of the genes revealed differential transcript signatures across the three time points (2 h, 11 d and 24 d) after salt application. The genes showed distinct but partially overlapping expression patterns at the onset of salt treatment (Fig. 11). In most of the analyzed genes, the transcript amounts were higher in *Bobur* at the early phase of salt treatment; but the trend was gradually altered over time. There was an increase in the transcript amount in both Altay2000 and *Bobur* as the salt treatment progressed. However, marked differences in the expression signatures between the two genotypes started to manifest at about 11 d after stress. From this time point onward, the expression levels of the genes increased exponentially in Altay2000 but less so in *Bobur*. The transcripts of *ZIP7*, structural maintenance of chromosomes protein 3 (SMC3) and Na^+/H^+ antiporter increased and decreased in Altay2000 and *Bobur*, respectively,

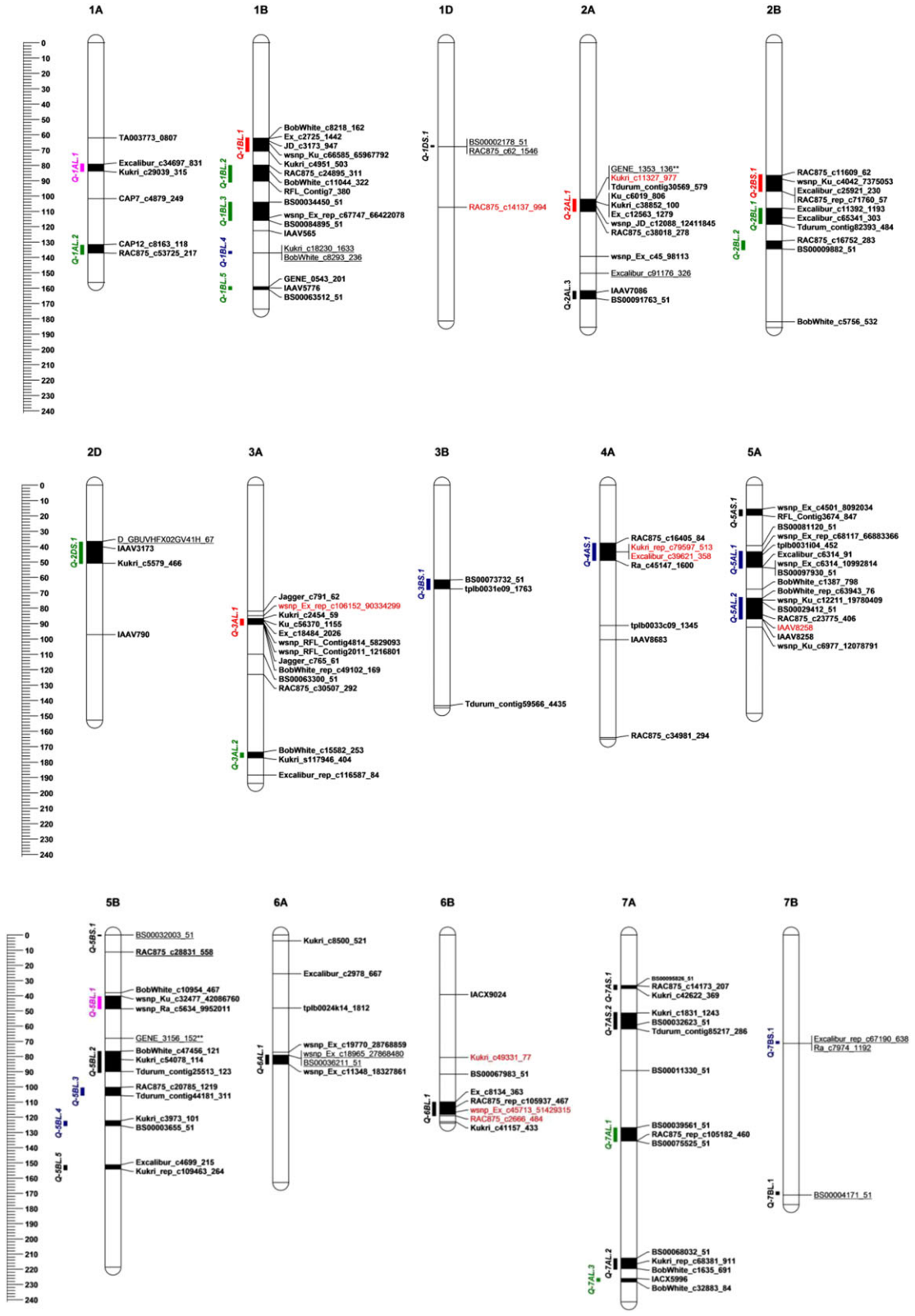

Figure 6. Map positions of all the SNPs associated with ST traits. Map distance (in centiMorgan) is shown on the left. 'Underlined' SNPs are pleiotropic; SNPs in 'red color' were associated with leaf ions traits such as K^+, Na^+, K^+/Na^+; number of asterisk (*) indicates the number of growth stages the SNP was detected, while the coloured bar in each chromosome designates quantitative trait locus (QTL) regions in significant linkage distribution. The QTL names are shown at the left with a solid bar. The bars are colour-coded to represent the growth stages at which the QTL regions conferred salt tolerance (ST) ('Red' = all-stage ST; 'Green' = seedling + adult field-grown plant (AFP) ST; 'Blue' = germination + seedling stage ST; 'Pink' = germination + AFP ST and 'Black' = growth-specific ST). [Colour figure can be viewed at wileyonlinelibrary.com]

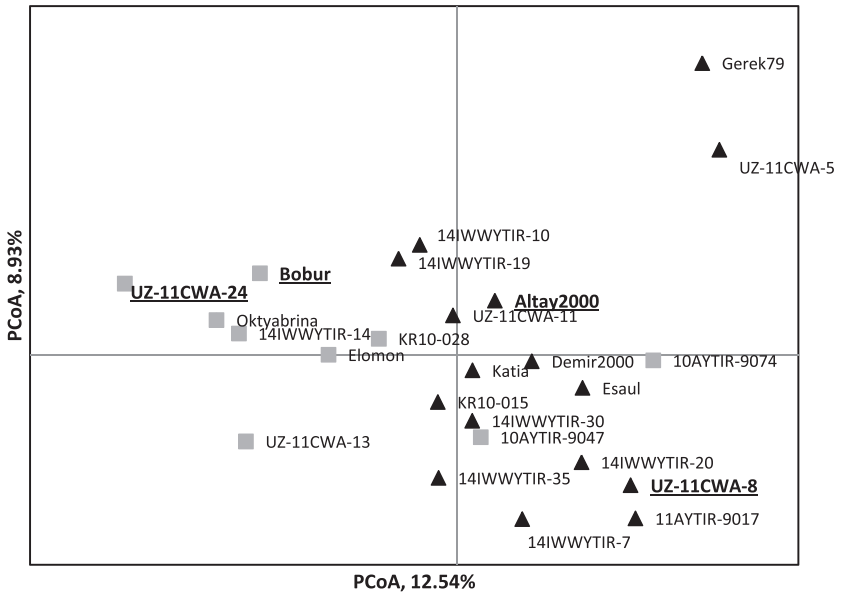

Figure 7. Principal coordinates analysis (PCoA) plot using a genetic distance matrix (GenAlEx 6.5) estimated with data from 187 associated polymorphisms of the salt-tolerant (black colour/triangular-shaped) and salt-sensitive (grey colour/squared-shaped) wheat genotypes previously identified in the genome-wide association study analysis. The underlined genotypes (in bold) were used to perform the gene expression analysis.

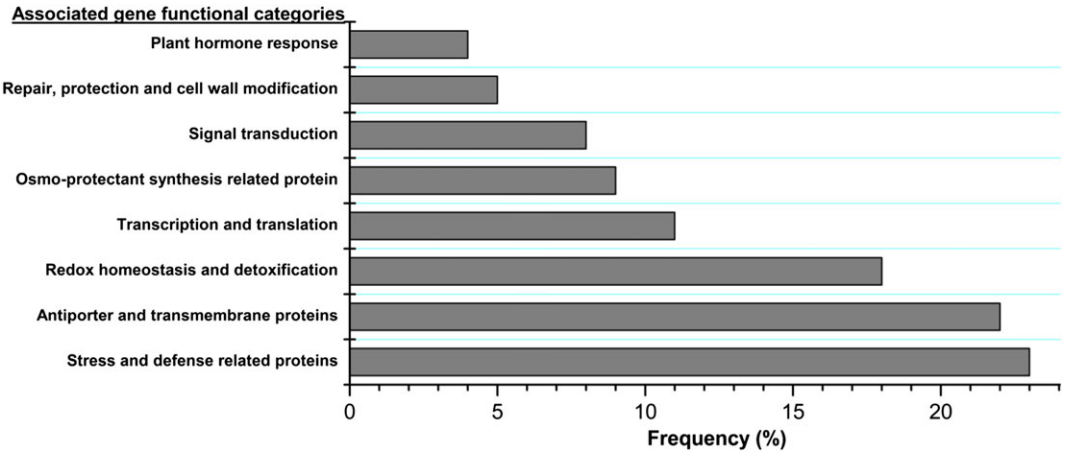

Figure 8. Ontology classification of the sequence flanking the SNP loci revealing association to the analysed traits. [Colour figure can be viewed at wileyonlinelibrary.com]

after 11 d of salt treatment; but ferredoxin-dependent glutamate synthase (GLU) became differentially expressed much earlier after 5 d of salt treatment. The zinc finger A20 *and* AN1 domain-containing stress-associated protein 8 (SAP8) showed late differential expression (beyond 24 d of stress).

Sequence analysis in the putative genes

Amino-acid sequence analyses revealed several non-synonymous substitution sites between Altay2000 and *Bobur* in the coding regions anchoring the significant MTAs of all the genes analysed (Fig. 12; Supporting Information Fig. S5). For instance, the 496st amino acid EST of *Traes_1BS_D68F0-BED6.1.mrna1-E4* coding for zinc transporter (*ZIP7*) changed from C (cysteine) in Altay2000 to S (serine) in Bobur; and at 503 and 504 positions, 'Threonine (T)' and '– (an amino acid deletion)' were observed in *Bobur* (Fig. 12a) instead of alanine '(A)' and 'Leucine (L)', respectively. Three non-synonymous changes were found within the associated coding sequence of Glutathione-regulated potassium-efflux system protein (kefC) (*Traes_2AL_A2CBDB5F7.1.mrna1-E2*) (Fig. 12b). The first change is from L (leucine) in Altay2000 to G (glutamine) in Bobur, while the second is from S (serine) to T (threonine). The third change is from P (proline) in Altay2000 to A (alanine) in Bobur. There were five non-synonymous substitutions sites that may have contributed to the alteration of the gene functional capacity and structure of Potassium transporter 18 (HAK18) (*Traes_5BL_F112FA40E.2*) at exon 10 (Fig. 12c). Amino-acid sequence variations were also detected in the coding regions of the remaining three genes including *SAP8*, *GST1* and SWEET17 (Supporting Information Fig. S5).

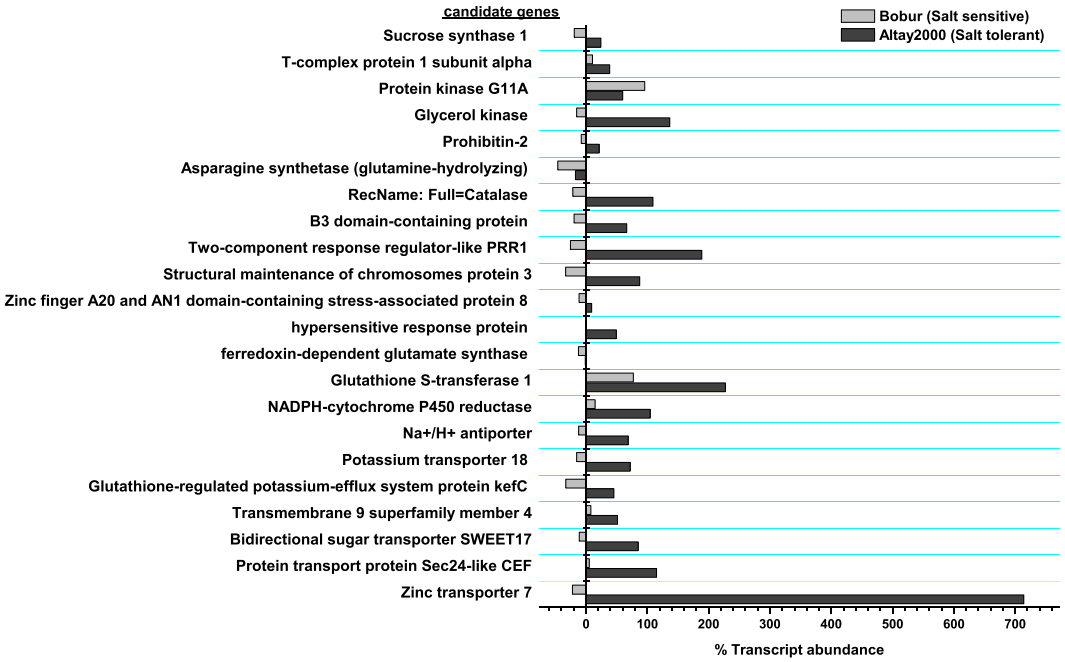

Figure 9. Effect of salt stress on some of identified gene transcript abundance (% change to control) between salt-tolerant genotype (Altay2000, in black) versus salt-sensitive genotype (*Bobur*, in grey) after 24 d of stress. [Colour figure can be viewed at wileyonlinelibrary.com]

DISCUSSION

The genotypes responded differently to salt stress across the three growth stages, and the CV ranged from 2.87 to 7.95% for germination, 15–22% for seedling and 1.28–44% for AFP. The variations observed are within the range of 5.4 to 22.8% that have been reported and exploited to uncover QTL controlling ST in wheat (Xu *et al.* 2012; Xu *et al.* 2013, Turki *et al.* 2014). Salt stress impacted negatively on germination, seedling biomass and yield-related traits, as was reported in Oyiga *et al.* (2016). A similar effect on plant growth has been reported previously (Munns & Tester 2008; Gomes-Filho *et al.* 2008). Genetic variation in leaf Na^+ and K^+/Na^+ ratio indicates the possibility of genetic improvement of salt tolerance (Karan & Subudhi 2012). Sufficient genetic variations for leaf Na^+ contents and K^+/Na^+ ratio were found in this panel. The leaf Na^+ and K^+/Na^+ ratio correlated positively and negatively, respectively, with the root biomass under salt stress, suggesting that root plays an important role in Na^+ transport and ion homeostasis (K^+/Na^+ ratio) (Lacan & Durand 1996; Krishnamurthy *et al.* 2009). Munns *et al.* (2006) reported that increase in root biomass may be associated with excessive amounts of salt entering the transpiration stream, which will cause injury to the cells in the transpiring leaves and may reduce growth.

The panel LD decayed after 10, 11 and 14 cM in the A, B and D genomes, respectively, suggesting that large numbers of SNPs are required to define the recombination profiles as a means to achieve high resolution. With the SNP density of 0.49 cM in the GWAS panel, it is expected that sufficient SNP density for high resolution was achieved. LD decay of <14 cM has been reported in breeding populations such as maize (Stich *et al.* 2005), barley (Kraakman *et al.* 2004) and wheat (Chao *et al.* 2007; Emebiri *et al.* 2010), although LD decay of over 40–50 cM has also been reported in wheat (Joukhadar *et al.* 2013; Turki *et al.* 2014). The LD decayed less rapidly in the D genome owing to the introduction of new haplotypes from *Aegilops tauschii* (D genome donor) into the genome of hexaploid wheat germplasm through synthetic wheat (Edae *et al.* 2014).

Most of the identified ST QTL loci in this study correspond to regions carrying published QTL/genes linked to ST in wheat (Table 6). Notable are *Q-1BS.1*, *Q-2AL.1*, *Q-2BS.1* and *Q-3AL.1*, which were linked to the ST traits across the three growth stages. The *Q-2AL.1* and *Q-3AL.1* regions were also associated with leaf K^+/Na^+ ratio and leaf Na^+ concentrations, respectively. Using the recently developed consensus map framework of different marker types, such as SSR, DArT and SNP (Maccaferri *et al.* 2015), we found out that *Q-2AL.1* at the ST QTL locus for seedling biomass (Ma *et al.* 2007; Genc *et al.* 2010) is proximal to the codominant SSR marker *gwm312* that is closely linked to the *Nax1* gene (Lindsay *et al.* 2004; James *et al.* 2006; Huang *et al.* 2006). The *Q-2BS.1* is coincident with the ST QTL for yield and seedling biomass (Quarrie *et al.*, 2005; Genc *et al.* 2010) and the *Ppd-B1* locus (Mohler *et al.* 2004). The *Q-3AL.1* was found in the ST QTL region for grain yield (Quarrie *et al.* 2005). To the best of our knowledge, the *Q-1BS.1* has not been previously reported. The identified QTL regions could be of value in future efforts to a better understanding of ST mechanisms in wheat. The *QTL_2DS.1* on 2DS showing a pleiotropic effect in both seedling and AFP traits was located proximal to ST QTL *QSdw-2D* (Xu *et al.*, 2012) and *QSlc.ipk-2D* (Landjeva *et al.* 2008) as well as the *Ppd-1* gene reported to exert a strong pleiotropic effect on many traits (Beales *et al.* 2007; Bennet *et al.* 2012), suggesting that *QTL_2DS.1* may be operating in multiple pathways regulating stress responses and adaptation in wheat. The

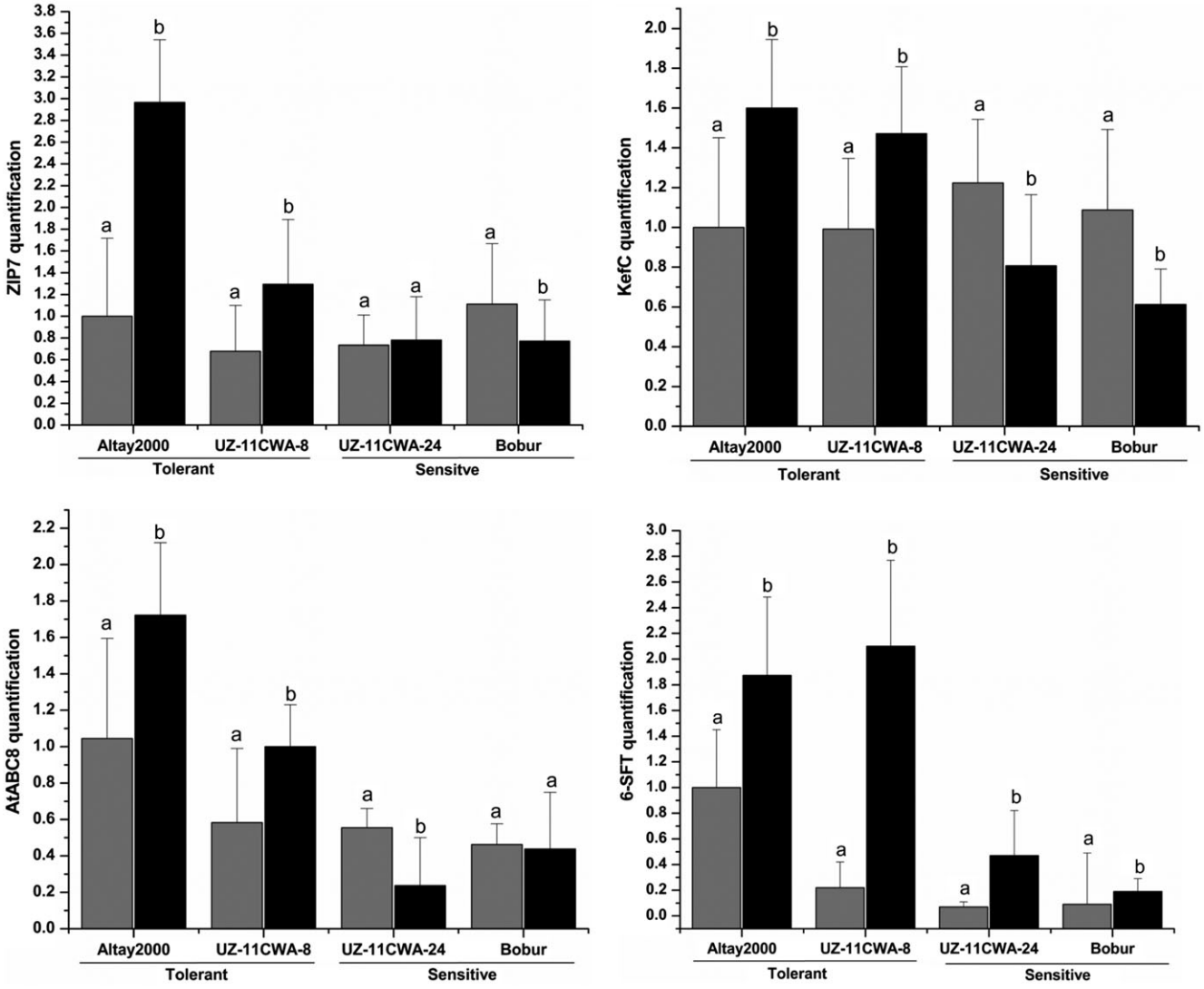

Figure 10. Expression levels of zinc transporter (*ZIP7*), glutathione-regulated potassium-efflux system protein (*KefC*), ABC transporter B family member 8 (*AtABC8*) and sucrose*: fructan-6*-fructosyltransferase (*6-SFT*) in leaves of two salt-tolerant (Altay2000 and *UZ-11CWA-8*) and salt-sensitive (*Uz-11CWA-24* and *Bobur*) after 30 d in non-saline (grey) and saline (black) conditions, determined by 2-ΔCT method. *Efa1.1* and *Efa1.2* genes were used as internal control genes. Bars are the means ($n = 3$) ± standard error. Differential expression significant (ab) and non-significant (aa) based on statistic analyses.

QTL_5AL.1 overlapped with QTL controlling frost (Baga *et al.* 2007) and copper (*QCut.ipk-5A1*; Bálint *et al.* 2007) tolerance, while *QTL_5AL.2* on 5AL, detected for 75 mM Na_2SO_4, ST_FSW, ST_DSW, ST_DRW and leaf K^+, corresponds to the Na^+ exclusion *TmHKT1*;*5* locus identified as a candidate for *Nax2* (James *et al.* 2006; Byrt *et al.* 2007; Munns *et al.* 2012). SNP *GENE_3156_152* at 68.4 cM on 5BL linked with ST traits at germination and seedling growth stages was domiciled at the *Vrn-1B* region known to have pleiotropic effects on genes controlling frost, salt, drought and osmotic stress tolerance (Yan *et al.* 2003; Limin & Fowler 2006; Dhillon *et al.* 2010). Novel QTL regions were found in *Q.5BS.1*, *QTL.5BL.3* and *QTL.5BL.4* regions.

The PCoA with SNPs showing significant MTAs discriminated the most consistent contrasting wheat genotypes (Fig. 7), based on their ST status (Oyiga *et al.* 2016). Singh *et al.* (2013) reported that genetic information based on marker information is very informative and would enable accurate groupings of genotypes sharing common lineage and/or genotypes with similar adaptive features. Our result not only reflected on the genetic diversity among the genotypes but also demonstrated that the detected SNPs are linked to genes involved in ST mechanisms. Thus, annotation of the associated loci can uncover the genes controlling genetic variants for salt stress response in the GWAS panel. Based on genetic relationships from cluster analysis, salinity tolerance might be improved by selecting parental genotypes from different clusters.

The SNP density of 0.49 cM means that QTL mapping can be resolved into a single gene. The *ZIP7*, identified in the novel *Q-1BS.1* region, controls Zn uptake (van der Zaal *et al.* 1999) and confers salinity and drought tolerance in rice (Liu *et al.* 2014). The uptake of Zn increases ST status by improving the

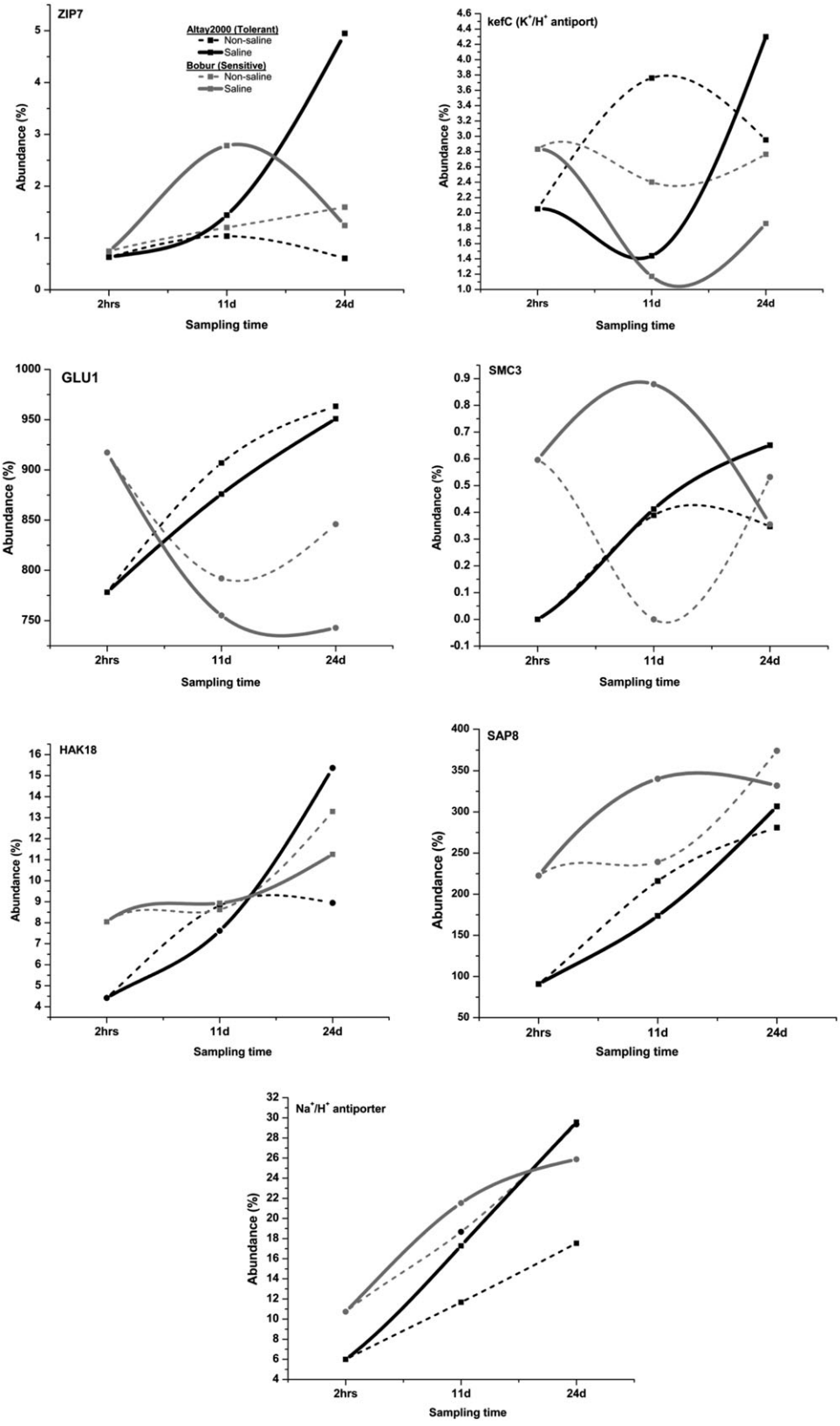

Figure 11. The expression kinetics of the associated salt tolerance genes over a period of 24 d in salt-tolerant (in black colour) and salt-sensitive (in grey colour) genotypes. The 'thick' and 'dotted' lines indicate the gene expression kinetics over time in saline and non-saline conditions, respectively. ZIP7 , zinc transporter 7; *kefC*, glutathione-regulated potassium-efflux system protein; GLU1**,** ferredoxin-dependent glutamate synthase; SMC3**,** structural maintenance of chromosomes protein 3; HAK18, potassium transporter 18; SAP8, zinc finger A20 and AN1 domain-containing stress-associated protein 8 and the Na^+/H^+ antiporter. The *x*-axes and *y*-axes are time of data collection and the amount of expressed transcripts, respectively.

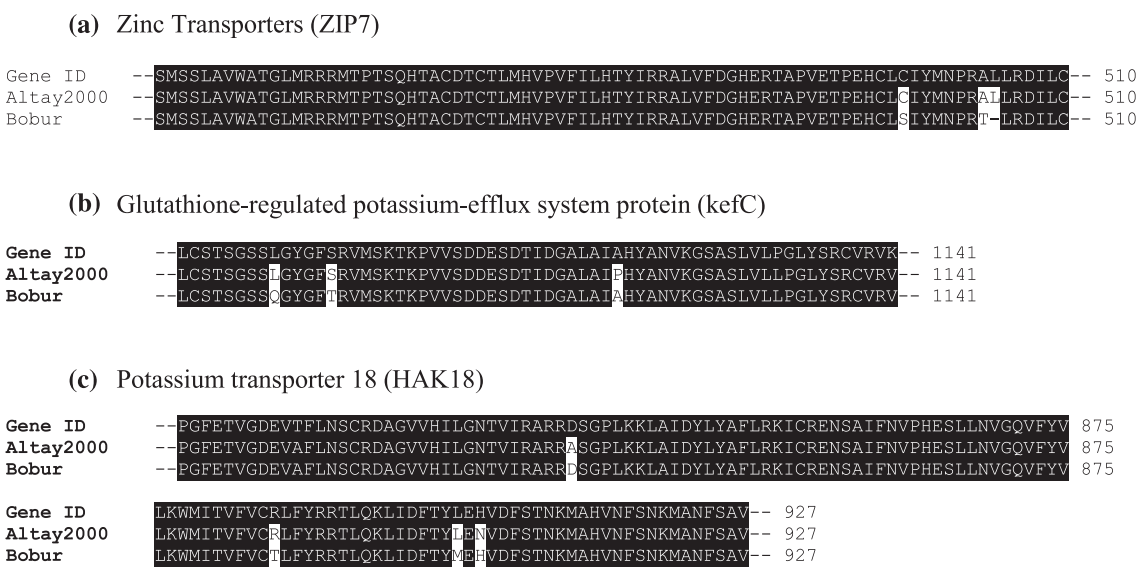

Figure 12. Comparison of the deduced EST amino acid sequence of the associated (a**)** ZIP7 transporters and gene ID: *Traes_1BS_D68F0BED6.1.mrna1-E4*; (b**)** KefC (K^+/H^+ antiporter) and gene ID: *Traes_2AL_A2CBDB5F7.1.mrna1-E2*; and (c**)** potassium transporter 18 and gene ID: *Traes_5BL_FI12FA40E.2.mrna1-E10* in Altay2000 (salt-tolerant) and Bobur (salt-sensitive) genotypes with their corresponding draft sequence obtained from Ensembl Genomes database (http://www.ensemblgenomes.org). The 'black' and 'white' colours in the analysed sequences are the identical and polymorphic sites found between the contrasting wheat genotypes, respectively.

expression of Na^+/H^+ antiporter genes, *TaSOS1* and *TaNHX1*, while decreasing the Na^+ accumulation (Abou-Hossein *et al.* 2002; Xu *et al.* 2014) and ROS accumulation (Chen *et al.* 2011; Sinclair & Kramer 2012). The SNP *RAC875_c14137_994* at 107.25 cM on 1DL significantly linked to a new QTL detected for leaf K^+ showed high sequence homology with an uncharacterized Na^+/H^+ antiporter. SNP BS00081120_51 at 39.26 cM on 5AS linked to TKW coded for SWEET 17. This gene mediate sucrose, fructose and glucose transport across tonoplast of roots and leaves (Schroeder *et al.* 2013; Chen 2014; Guo *et al.* 2014) and is associated with pathogen resistance (Schroeder *et al.* 2013).

Response to *ABA* and *Salt 1B* that encode ABA-inducible and salt stress-inducible (Rab11B) genes was identified with SNP *IAAV565* on 1BL at germination stage. Rab11B is a negative regulator of ST during seed germination and early seedling growth (Ren *et al.* 2010). The transmembrane 9 superfamily member 4 on 7BS is involved in the adaptation to NaCl toxicity in ryegrass (Li *et al.* 2012) and rice (Senadheera *et al.* 2009). SAP8 identified on the *QTL_7AL.1* is known to confer salt, cold and dehydration stress tolerance in transgenic tobacco (Mukhopadhyay *et al.*, 2004), tea (Paul & Kumar, 2015), Arabidopsis (Giri *et al.* 2011) and rice (Kanneganti & Gupta 2008). Two SNPs on 33.45 and 35.31 cM (*Q-7AS.1*) coded for sucrose: fructan 6-fructosyltransferase (6-SFT), an enzyme involved in fructan synthesis. Fructans support osmoprotectants synthesis, anti-oxidation and membrane stability in plants (Valluru & Van den Ende 2008; He *et al.* 2015). The *Q-7AS.1* region might be similar to the osmoregulation gene regions previously described by Morgan (1991) and Morgan & Tan (1996) on 7AS. This further confirmed the earlier study by Ogbonnaya *et al.* (2013) in which they reported the identification of a minor gene for Na^+ exclusion in a synthetic derived population 'AUS29639//Yitpi' on chromosome 7A, although they did not characterize the underlying gene.

All the putative genes (except protein kinase G11A) were up-regulated in Altay2000 (salt tolerant) but down-regulated in *Bobur* (salt-tolerant) after 24 d of salt stress (Fig. 8). Among them, *ZIP7* showed the strongest differential response to salt stress. It has been revealed that the candidate locus *HvNax4* that controls shoot Na^+ accumulation in barley is also associated with Zn^{2+} accumulation (Lonergan *et al.* 2009). Reports have shown that the overexpression of *KefC* (Shi *et al.* 2000; Shi *et al.* 2003), ATP-binding cassette (ABC) transporters (Kim *et al.* 2010; Li *et al.* 2015) and 6-SFT (He *et al.* 2015; Kerepesi *et al.* 2002), similar to results of the present study, improves ST in plants. The qRT-PCR results of *ZIP7*, *KefC*, *AtABC8* and 6-SFT genes showed similar expression patterns in two tolerant (up-regulation) and two sensitive (down-regulation) genotypes after 30 d of salt treatment, demonstrating that they are involved in ST. The results presented here were data from the shoot parts, because the analysed genes including *ZIP7* (Milner *et al.* 2013), *KefC* (Han et al. 2015), *AtABC8* (Ma *et al.* 2016), 6-SFT (Nagaraj *et al.* 2004) and *Nax1* (James *et al.* 2006; Munns *et al.* 2012) are expressed in the shoot. Further analyses of the transcription of these genes in the root cells are essential, as the organ that is in close contact with the solution.

Prior to salt stress initiation, *Bobur* exhibited higher transcript abundance than Altay2000. Over time, the transcript amounts in Altay2000 increased exponentially surpassing that of *Bobur*, indicating Altay2000 possesses better ST adaptation mechanisms than Bobur. The differential expressions of *ZIP7*, *SMC3* and the uncharacterized Na^+/H^+ antiporter between both genotypes was obvious after 11 d of salt treatment. This time period may coincide with the ionic phase described by

Munns & Tester (2008) when the accumulation of salts is becoming detrimental to the plant, resulting in increased leaf senescence, reduced photosynthetic capacity and reduced growth rate. At this time, only plants that can tolerate the accumulated Na^+ and/or exclude Na^+ would have a sustained growth rate under salt stress.

Three non-synonymous substitutions (Fig. 12a), C496S, A503T and L504– (an SNP deletion) detected on the associated exon 4 coding region may have contributed to the differential expression of *ZIP7* in the contrasting genotypes. The 'C' is more hydrophobic than 'S', and C-to-S substitution has been found to decrease the Zn^{2+} affinity (Hessels 2015), thus providing genetic and molecular evidence for the sensitivity of Bobur to salt stress. Three polymorphic sites (Fig. 11b): L1087Q, S1092T and P1117A were detected at exon 2 of *KefC*. L and P, which were substituted by Q and A in Bobur at sites 1087 and 1117, respectively, have been reported to play an osmoprotective role (Arbona *et al*. 2013) in plants and may have contributed to the increased ST observed in Altay2000 relative to Bobur. Our results indicated that the detected SNP markers are linked to salt-responsive genes and can serve as direct targets for selection of ST and for genetic studies in wheat.

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AUTHORS CONTRIBUTIONS

B. C. O. performed the experiments, data analyses and drafted the manuscript. B. C. O. and R. C. S. contributed to the data collection. A. B., B. C. O., F. C. O. and J. L. designed the experiments and interpreted the results. A. B., F. C. O., M. B. and J. L. were responsible for the correction and critical revision of the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. PCR efficiency comparison. CT values were determined for the reference genes and the target genes using pooled DNase treated RNA samples of all the genotypes extracted from treated and untreated leaves. Real-time RT-PCR was performed using DyNamo ColorFlash Probe qPCR Kit. The CT values of target genes were subtracted from the average CT values of the reference genes. The difference in CT values was plotted against template amount and the difference in PCR efficiency determined by calculating the slope of the line. The resulting slope for each target gene is < 0.1, except 6-SFT.
Figure S2. The dissociation curves showing single peaks for PCR amplicons for the two endogenous reference genes and four target genes.
Figure S3. **Genetic linkage maps of wheat containing** 18,027 **SNP markers spanning** 3643.10 cM **over 21 chromosomes based on association mapping panel**. The scale in centi-Morgans (cM) is given at the y-axes and chromosomes at the x-axes. Horizontal lines represent the positions of SNPs on each of the corresponding chromosome.
Figure S4. Principal coordinates analysis (PCoA) of the association panel elite wheat germplasm based on genetic distance estimates. The colored figures in the plot represent the core collection centers: blue-TNP (Turkey National breeding program); red-IWWIP, green-ICARDA-CYMMYT and the cross the Central Asia.
Figure S5. Comparison of the deduced EST amino acid sequence of: **SAP8**- Zinc finger A20 and AN1 domain-containing stress-associated protein 8 (Gene ID: Traes_7AL_B88F6A3D3.1), **GST1** - Glutathione S-transferase 1 (Gene ID: Traes_3AL_F205FA0941.2.mrna1-E1) and **SWEET17**- Bidirectional sugar transporter SWEET17 (Gene ID: Traes_5AS_9937DABBA.1.mrna1-E5) in Altay2000 (salt tolerant) and Bobur (salt sensitive) genotypes with their corresponding draft sequence obtained from Ensembl Genomes database (http://www.ensemblgenomes.org). The "black" and "green" colours indicate identical and polymorphic sites in the associated coding sequences between the contrasting wheat genotypes, respectively, while "pink" colour represent regions anchoring the associated SNP marker identified in the GWAS analysis.
Table S1. Description of the taits studied on the diversity panel of 150 wheat genotypes
Table S2. shows the analysis of the polymorphic SNPs used for the GWAM analysis and the significant LD statistics in each chromosome and across the wheat genomes
Table S3. Summary of significant SNP marker-trait associations at germination, seedling and adult field growth stages
Table S4. Colocation of SNP clusters with QTL/genes already identified or published
Table S5. Ontology classification of the associated DNA sequences detected using the GWAS
Table S6. The key biological functions of the 21 predicted proteins found to be differentially expressed in the tolerant and sensitive wheat genotypes. Their fuctions were adapted from the UniProt (www.uniprot.org) database.