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Genetic variation of traits related to salt stress response in Wheat (*Triticum aestivum* L.)

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GENERAL SUMMARY (English)

Salinity is one of the most severe abiotic stresses perceived by plants, and is continuously increasing due to climatic change and poor irrigation management practices. It is currently affecting ~800 million hectares of land worldwide, including over 20% of the world's irrigated arable land. Salinity causes significant growth reduction and crop yield losses. With the predicted geometric increase in the global population, improving the salt tolerance (ST) of crops has become an important challenge and target for plant breeders. Several approaches have been exhaustively exploited to ameliorate the impact of salinity on crop plants, but because of the complex nature of ST in crop plant, these approaches have not been optimally translated into the desired results. It is well known that ST is difficult to breed due to its interaction with many physiological processes that are controlled by many genes, plant growth stage and are influenced by environmental factors. Wheat is moderately salt tolerant which means that the grain yield is significantly affected under soil saline condition of ~10 dS m^{-1}. Therefore, improving wheat adaptation under high salinity is seen as the most efficient and economical approach to address the salinity problem and increase its grain yield especially in the poor resource wheat producing countries that are prone to soil salinity. This thesis applies several morphological and physiological evaluations, genetic and molecular approaches to elucidate the genetic and physiological mechanisms underlying natural variation for ST in wheat and to find ways to explore the inherent genetic variation, with the ultimate aim of finding new candidate genes that can be used to improve ST in wheat.

The performance of 150 genetically diverse wheat genotypes were evaluated under different salinity conditions at germination, seedling and adult plant field growth stages, to identify heritable variation for salt tolerance in the measured traits. In addition, the amount of Na^+, K^+ and K^+/Na^+ ratio in the different shoot parts such as third leaves, stem and remaining leaf parts were determined for each genotypes after 24 days of stress under 150 mM/L NaCl. Results revealed genotype and salt treatment effects across all the growth stages, and the salt stress applied caused 33%, 51% and 82% reductions in germination vigour, seedling biomass and grain yield, respectively. The ability of wheat to conserve water in both root and shoot tissues was positively correlated with the K^+ uptake under exposure to salinity. The wide-spectrum of responses to salt stress observed among the genotypes was exploited to identify genotypes with most consistent ST status across growth stages. Among the outstanding genotypes identified, four genotypes including *Altay2000, 14IWWYTIR-19* and *UZ-11CWA-8* (tolerant) and *Bobur* (sensitive*)* showed consistent ST status across the three growth stages

including germination, seedling and adult-plant field growth stages. Further evaluation of the identified genotypes using several physiological parameters showed that the tolerant genotypes possess better adaptation characteristics than the sensitive ones (*Bobur* and *UZ-11CWA-24)* which allowed them to sustain growth and reproduce under high salinity.

A high density molecular map with ~18,000 SNPs (average distance between markers of 0.49 cM cM) and all the morpho-physiological and seed quality data collected were used to map QTLs for ST in the studied population. The LD decayed moderately fast (10 cM, 11 cM and 14 cM ($r^2 > 0.1$) for the A, B and D-genome, respectively). By applying mixed linear modeling (MLM) while correcting for the effects of population structure and the kinship resulted in the detection of 302 SNPs (representing 50 distinct QTL regions) that were significantly associated with various ST traits. They explained between 2.00 and 63.45 % of the genetic variance. Most of the associated SNPs/loci showed pleiotropic effect on several traits and/or were detected across several independent experiments/growth stages. For instance, a single locus (at 90.04 cM) on 6AL was found to be strongly associated with ABS/RC, DIo/RC and shoot Na^+ traits. An important (about 1.8 cM interval) region on 2BL was also found to strongly contribute to the variation in ST in various salt stress related traits (ST_DRW, shoot Na^+, Fv/Fm, grain yield and seed crude protein). Five novel ST QTL regions were also detected on 1BS, 1DL, 5BS, 6AL and 5BL genomic regions. All the identified QTL have been discussed in this thesis.

By analyzing sequences of the associated SNPs, several key genes involved in salt and abiotic stress tolerance were identified. Among the categories of genes identified (Chapter 3 and 4), the genes involved in the stress response (24%), antiporter and transmembrane (18%), transcription and translation (14%), and redox homeostasis and detoxification (11%) related activities occurred predominantly. The transcriptome and RT-PCR expression analyses performed with the genes linked to the significant MTAs revealed differential expressions between the contrasting ST wheat genotypes. Moreover, the amino acid sequence analyses of the putative genes uncovered many sites of non-synonymous/missense mutation that may have contributed to the observed variable salt stress responses in the contrasting wheat genotypes. This study provides new insights towards understanding the traits and mechanisms related to ST. Thus, the underlying genetic and molecular response as presented in this thesis can be directly exploited by the breeders and scientists to improve salt tolerance in wheat.

ALLGEMEINE ZUSAMMENFASSUNG

Die Versalzung des Bodens zählt zu den größten abiotischen Stressfaktoren für Pflanzen, und steigt durch den Klimawandel und ein schlechtes Wassermanagement kontinuierlich. Zur Zeit sind etwa 800 Millionen Hektar weltweit und 20 % der künstlich bewässerten Flächen von Versalzung betroffen. Diese führt zu einer signifikanten Reduktion des Pflanzenwachstums und ist mitverantwortlich für Ertragseinbußen. Durch das weltweite Bevölkerungswachstum wird die Erhöhung der Salztoleranz (ST) von Nutzpflanzen eine immer wichtigere Aufgabe und ein anzustrebendes Ziel für die Pflanzenzüchtung. Verschiedene Forschungsansätze wurden verfolgt, um die Salztoleranz von Pflanzen zu verbessern, jedoch führten viele dieser Ansätze aufgrund der komplexen Natur der ST nicht zu verwertbaren Ergebnissen. Es ist bekannt, dass ST aufgrund der Interaktion zwischen vielen physiologischen Prozessen, den unterschiedliche Genen und der Umwelt, schwierig in die Züchtung zu integrieren ist. Weizen gilt als mäßig salztolerant und der Ertrag wird ab einem Bodensalzgehalt von ~10 dS m^{-1} signifikant beeinflusst. Gerade die landwirtschaftlich schwächer entwickelten Regionen sind für Bodenversalzung anfällig und eine Erhöhung der Salztoleranz wäre ein probates wirtschaftliches Mittel um den Weizenertrag zu steigern. Diese Dissertation nutzt mehrere morphologische und physiologische Auswertungen, genetische und molekulare Ansätze, um die genetischen und physiologischen Mechanismen zu erklären, die der ST des Weizens zugrunde liegen. Dabei soll die eigene genetische Variation des Weizens erklärt und schlussendlich neue Kandidatengene gefunden werden, welche die ST des Kulturweizens erhöhen.

Die Leistung von 150 genetisch verschiedenen Weizengenotypen wurde während der Keimung, dem Sämlingsstadium und an der adulten Pflanze unter unterschiedlichen Salzbedingungen geprüft, um die erbliche Variation des ST in unterschiedlichen Merkmalen oder Wachstumsstadien zu identifizieren. Nach 24 Stunden unter Stressbedingungen mit 150 mM/L NaCl wurde der Na^+-, K^+-Gehalt und des K^+/Na^+ - Verhältnis in verschiedenen Sprossteilen, wie dem dritten Blatt, dem Stängel und den übrigen Blättern für alle Genotypen bestimmt. Die Ergebnisse zeigten Interaktionen der Genotypen und der Salzbehandlung in allen Wachstumsstadien. Die Salzapplikation verursachte einen Rückgang von 33% bei der Keimfähigkeit, von 51 % der Sämlingsbiomasse und von 82% beim Kornertrag. Die Eigenschaft des Weizens, Wasser in Wurzel- und Sprossteilen zu speichern war positiv mit der K^+ -Aufnahme unter Stressbedingungen korreliert. Das beobachtete breite Spektrum der Pflanzenreaktionen auf die Salzstressapplikation wurde genutzt um die beständigsten,

beziehungsweise die salztolerantesten Genotypen über alle Wachstumsstadien zu identifizieren. Es wurden vier extreme Genotypen (Altay2000, 14IWWYTIR-19 und UZ-11CWA-8 (tolerant) und Bobur (sensitiv)) ausgewählt, die eine konstante ST über die untersuchten Wachstumsstadien zeigten. Weitere Tests der ausgewählten Genotypen mit verschiedenen physiologischen Parametern zeigten, dass die toleranten Genotypen über bessere Anpassungsmechanismen verfügen als die sensitiven (Bobur und UZ-11CWA-24). Dadurch ist es ihnen möglich, auch unter hohem Salzgehalt das Wachstum aufrecht zu erhalten und fertil zu bleiben.

Eine hochauflösende molekulare Karte mit ~18000 SNPs und einer durchschnittlichen Distanz zwischen den Markern von 0.49 cM wurde zusammen mit den gesammelten morphologischen-, physiologischen- und Saatgutqualitätsdaten genutzt um QTLs für die ST der untersuchten Population zu bestimmen. Das LD der Weizenpopulation liegt bei 10 cM auf dem A-, bei 11 cM auf dem B- und bei 14 cM auf dem D-Genom bei einem $r^2 > 0.1$. Mittels gemischten linearen Modellen (MLM) und deren Korrektur durch die Verwandtschaftsmatrix, wie auch die Populationsstruktur wurden 302 SNPs in 50 verschiedenen QTL Regionen detektiert, die signifikant mit verschiedenen Merkmalen für ST assoziiert sind. Diese SNPs erklären zwischen 2.00 und 63.45 % der genetischen Varianz in der Population. Die meisten assoziierten SNPs/Genorte zeigen pleiotrope Effekte mit mehreren Merkmalen und wurden außerdem in unabhängigen Experimenten und Wachstumsstadien nachgewiesen. Ein einziger Lokus bei 90.04 cM auf 6AL zeigte zum Beispiel eine starke Assoziation mit den Merkmalen: ABS/RC, DIo/RC und Spross Na^+. Eine weitere hervorzuhebende Region mit der Länge von 1.8 cM auf 2BL hatte eine starke Wirkung auf die Variation der ST in den Merkmalen: ST_DRW, Spross Na^+, Fv/Fm, Kornertrag und Rohproteingehalt im Samen. Weitere fünf neue ST-QTL Regionen auf 1BS, 1DL, 5BS, 6AL und 5BL wurden gefunden und in dieser Dissertation diskutiert.

Durch die Sequenzanalyse assoziierter SNPs wurden mehrere Schlüsselgene identifiziert, welche die Salz- und abiotische Stresstoleranz beeinflussen. Bei den Kategorien der identifizierten Gene (in den Kapiteln 3 und 4) handelt es sich um Gene die mit der: Stressantwort (24%), Antiporter und Transmembran (18%), Transkription und Translation (14%) und redox-gleichgewicht und Entgiftung (11%) verknüpft sind. Transkriptom und RT-PCR-Expressionsanalysen der Marker-Merkmal-Assoziierten (MTA) Gene zeigten, dass diese Gene in den unterschiedliche ST Weizengenotypen unterschiedlich exprimiert wurden. Darüber hinaus wurde die Aminosäuresequenz von einigen Genen überprüft, die wahrscheinlich zu den Salzstressreaktionen beitragen. Diese Studie zeigt neue Einsichten, die zum Verständnis der Merkmale und Mechanismen, die mit ST verbunden sind

beitragen. In dieser Dissertation werden genetische und molekulare Ergebnisse präsentiert, die direkt von Züchtern und Wissenschaftlern genutzt werden können, um die Salztoleranz in Weizen zu erhöhen.

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CHAPTER 1

General Introduction

Soil salinity

Salinization of arable land has continued to increase in recent times and, is particularly detrimental to irrigated agriculture, which provides one third of the global food supply. Soil salinity can be determined by measuring the electrical conductivity (EC) of the soil saturation extract. According to the standard definition, a soil is said to be saline if the EC ≥ of 4 dS m^{-1} (equivalent to about 40 mM NaCl), while soils with EC's exceeding 15 dS m^{-1} are considered strongly saline (FAO, 1996; SSSA, 1997). Traditionally, saline irrigation water is grouped into 4 categories (**Table 1**): *slightly saline* (EC< 2 dS m^{-1}); *moderately saline* (2–6 dS m^{-1}); *highly saline* (6–15 dS m^{-1}), and *extremely saline* (EC>15 dS m^{-1}) (FAO, 2008). The salinity of soils is associated with the excessive presence of primary cationic species (*i.e.,* Na^+, Ca^{2+}, and Mg^{2+}) and anionic (*i.e.,* Cl^-, SO_4^{2-}, HCO_3^-, and CO_3^-) species in the soil. However, Na^+ and Cl^- are the most important ions (Dubey, 1997; Hasegawa *et al*., 2000), because they not only cause degradation of soil physical structure but also impair plant growth and development. Thus, soils are said to be *saline*, *sodic* and/or *saline-sodic* based on the total concentration of salt and the ratio of Na^+ to Ca^{2+} and Mg^{2+} in the saturated extract of the soil (Dudley, 1994). The diverse ionic composition of salinized soils would result in a wide range of physiochemical properties.

Table 1. Approximate soil salinity classes

Salinity rating	**EC (dS m^{-1})**	**Impact on plants**
Slightly saline	1.5–2	Salinity effects usually minimal
Moderately saline	2–6	Yield of salt sensitive plants restricted
Highly saline	6–15	Only salt tolerant plants yield satisfactorily
Extremely saline	>15	Few salt tolerant plants yield satisfactorily

Source: FAO land and plant nutrition management service, 2008

Salinization of arable lands

Salinity is one of the most important abiotic stresses, limiting crop production in arid and semi-arid regions, where soil salt content is naturally high and precipitation can be insufficient for leaching (Zhao *et al*., 2007a). It may occur naturally in the top soil or may be introduced by man. The natural soil salinization is caused by either the shallow saline water table or weathering of parent rock materials which releases salts in the soil, while the human-induced soil salinity arises from human activities and improper irrigation/poor cultural practices, such as., the use of saline water for irrigation, deforestation, overgrazing and poor drainage of irrigated fields (Yadav *et al*., 2011). Salinity is becoming more extensive due to land clearing and unsustainable irrigation practices and through pressures for bringing marginal land into production (Munns and Gilliham, 2015). According to the FAO (2008), over 6% of the world's land is affected by salinity, accounting for more than 800 million ha of land. Salinity is already widespread in many regions and has continued to increase due to the changing climate. It has been estimated that 950 million ha of salt-affected lands occur in arid and semi-arid regions, which is about 33% of the arable land area of the world. Globally, 20% of irrigated land (450,000 km^2) is afflicted by salinity, with 2,500-5,000 km^2 of lost production lands every year as a result of salinity (UNEP, 2008). **Figure 1** shows the distribution of saline land world-wide, with the affected areas predominantly located in the wheat producing countries including Central and West Asia, Australia, Northern Africa and some parts of South and Northern America. Jamil *et al*. (2011) has predicted that more than 50% of the arable would be salinized by the year 2050. The global annual losses in agricultural production from salt-affected land are in excess of US$12 billion and rising (Qadir *et al.*, 2008; Flowers *et al.*, 2010). In view of this development, concerted efforts must be taken to manage the arable lands (especially those prone to salinity) to minimize the impact of salinity on crop yield by adopting practices that curtail further soil degradation. **Figure 2** shows a typical example of arable land affected by salinity.

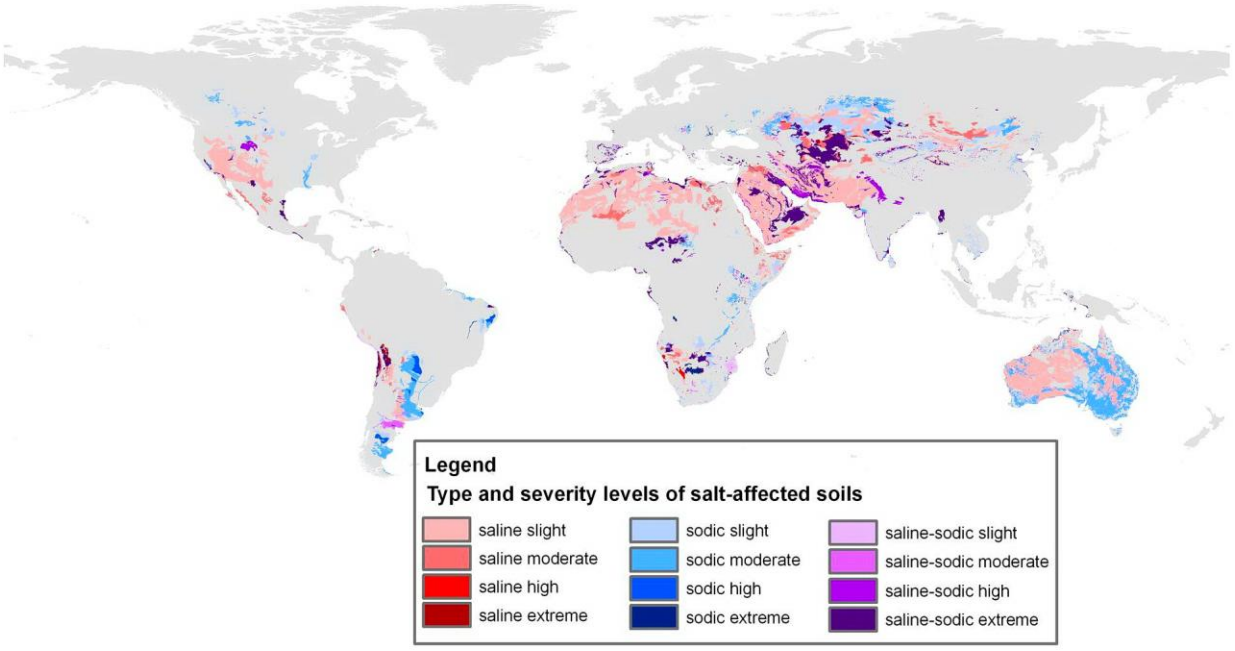

Figure 1 Losses of global lands due to salinity. (**Source:** Globusgreen, 2014)

Figure 2 Wheat farm (**in Karshi**, Uzbekistan) showing patches of salt deposit on the soil surface

Impacts of salinity on crop plants

Soil salinity reduces crop biological yield by affecting all aspects of plant physiology, growth and development, such as germination potential, vegetative growth and the reproductive growth stages, due to the complex interactions among morphological, physiological and biochemical processes (Akbarimoghaddam *et al*., 2011; Singh and Chatrath, 2001). The decrease in crop yield may be partly due to in ion (Na^+ and Cl^-) toxicity (Chinnusamy *et al.*, 2006; Serrano and Rodriguez-Navarro, 2001). Salinity can also upset the nutrient balance in the plant and/or interfere with the uptake of some nutrients (Blaylock, 1994). Reports have indicated that uptake of nutritive cations - potassium and calcium (Asch *et al.*, 2000; Glenn *et al.*, 1999; Maathuis and Amtmann, 1999; Niu *et al.*, 1995; Shabala, 2000) and anions - nitrate and phosphate (Song *et al.*, 2009; Cerezo *et al.*, 1999; García-Sánchez *et al.*, 2003; Glass and Siddiqi, 1985) by plants are significantly decreased under soil salinity conditions. The adverse effect of salt stress have also been observed on crop plant at physiological and biochemical levels (Munns and James, 2003), as well as at the molecular level (Tester and Davenport, 2003). Salt stress increases the formation of reactive oxygen species (ROS) in plant (Mittler, 2002; Miller *et al*., 2008). The ROS main primary production sites in plant are chloroplasts, mitochondria, and peroxisomes (Mittler *et al*., 2004; Asada, 2006). These important organs are very sensitive to ROS. Excessive ROS formation is often considered as the initial process that leads to cellular damage of these organs in plant under salt stress. ROS are toxic and damages the cellular membranes, membrane-bound structures, enzymes and DNA especially in mitochondria and chloroplasts, and can therefore severely impair plant growth and/or survival (Allen, 1995) and consequently, reduction crop yield. Reduction in crop yield of up to 76% has been reported due to salt stress (Rajpar *et al*., 2006).

An estimated 50% increase in grain yields of major cereals is needed to fulfill the food supply requirements for the projected population by 2050 (Godfray *et al*., 2010). However, most of the efforts geared towards achieving this target have more often than not hit a brick wall due to the continuous salinization of the agricultural soils. A number of agronomic and engineering solutions (such as, the use of salt-free water for irrigation, leaching of excess salts, soil pH adjustment and growing of salt loving plants to absorb large amount of the salts) have been exhaustively exploited. Therefore, the only way towards ameliorating the salt toxicity problems in crops is to use genetically improved salt tolerance crop plants to increase production in salt affected lands. The production of crops that can adapt favorably to saline conditions will increasingly be beneficial and profitable for farming most especially in the poor resource countries currently facing soil salinity problems.

Wheat (*Triticum aestivum* L.) is an allohexaploid (6x), having 21 pairs of chromosomes (2n = 42) that are sub-divided into 3 closely related (homologous) groups of chromosomes, the A-, B- and D-genomes. Each genome has 7 pairs of chromosomes. The size of the wheat genome is 17 Gbp (Bennett and Leitch, 1995) which is bigger than the genome size of barley (~5.3 Gbp in 7 chromosomes) and rice (~430 Mb in 12 chromosomes) due to high content of repeated sequences. This makes the genome study and complete sequencing of wheat a very daunting task. Wheat is the third most important cereals, is grown extensively across the globe, with global production and utilization now pegged at 732 and 759 million tons (FAO, 2016; Release date: 07/07/2016), respectively (**Figure 3**), a strong indication of the need to increase wheat production. It is moderately salt-tolerant crop and suffers significant grain yield losses when grown in moderately saline soil conditions (Quayyum and Malik, 1988; Shahbaz *et al*., 2011, 2012). The general assumption, as revealed by previous work, is that the effect of salinity varies among wheat genotypes. Thus, plants that are able to sustain their growth and reproduced under high salinity have higher salt tolerance. Kingsbury and Epstein (1984) have screened ~ 5000 wheat accessions for salt tolerance at 50% sea water and observed that only less than 7% survived up to maturity. The genetic variations for salt tolerance show the potential of the existing genetic resources for enhancing wheat salt tolerance. Meaningful progress in exploiting the genetic variation for development of salt tolerant genotypes will require a robust understanding of the plant agronomic, physiological, genetic and molecular response mechanisms of wheat under saline conditions.

Figure 3 Global wheat production and utilization (source: FAO, 2016)

Crop plant response to salinity

The ability of plants to survive under saline conditions varies among different species of halophytes and glycophytes. The halophytes adapted to live, support growth and reproduce in soils containing high concentration of salt (above 200 mM NaCl), by adapting various tolerance mechanisms (Bose *et al*., 2013). Unlike the halophytes, the glycophytes cannot tolerate more than 25% of the salinity levels of seawater without shortchanging their growth and yield. Unfortunately, most of the modern crops including wheat, rice and barley are glycophytes.

The growth response of glycophytes to salinity (>40 mM NaCl) occurs in two phases: (i) a rapid response to increase in external salt known as "osmotic phase" and (ii) slower response with accumulation of Na^+ ions in vacuoles refer to as "ionic phase". At both phases, the growth and yield of crops are significantly reduced (Munns and Tester 2008). The osmotic phase of growth reduction depends on the salt concentration outside the plant rather than the salt in the plant tissues and, growth inhibition is mostly due to a water deficit (drought stress) or osmotic stress, with little genotypic differences. However, the ionic phase of growth reduction takes time to develop (usually between 2-4 weeks) as results of an internal salt injury caused by excessive accumulation of toxic Na^+. At this

phase, salinity would cause the plants to close its stomatal apertures and consequently reduced the photosynthetic rate due to the negative effect of toxic Na^+ that accumulated in the thylakoid membranes of the chloroplasts. This would increase ROS formation and oxidative stress that would result in leaf injury and loss of photosynthetic capacity of the plants.

Leaf injury and death is associated with high salt loadings in the leaf to levels that exceeds the capacity of salt compartmentation in the vacuoles, causing salt to build up in the cytoplasm to toxic levels (Munns, 2002; 2005; Munns *et al*., 2006). The trade-off between the rate at which the leaves die and the rate at which new leaves are produced would determine the tolerance status of the plant under salt stress. Plants are unable to cope, tolerate and survive in saline conditions long enough to supply sufficient photosynthate to the reproductive organs and produce viable seeds, if the former process progresses faster than the latter. Based on this two-phase concept, the osmotic effect exerted by salts in the medium around the roots would cause the initial growth reduction in both salt tolerant and salt sensitive genotypes (i.e., *Osmotic Phase*) (**Figure 4a**). However, the salt-sensitive genotypes are much more affected at the ionic phase, because of their inability to prevent Na^+ build-up in transpiring leaves to toxic levels (Munns *et al*., 2006). Because of this development, crops have been classified into two categories: (i) salt-includers and, (ii) salt-excluders. Salt-includers take up Na^+ and translocate it to the shoot, where it is sequestered and used as vacuolar osmoticum (tissue tolerance), whereas the salt-excluders adapt to saline stress by avoiding Na^+ uptake (Mian *et al*. 2011). The salt-sensitive genotypes can be differentiated from the salt-tolerant ones at ionic phase (**Figure 4b**), and the effect of salinity on crops may also be as a result of the combination of osmotic and ionic salt effect (**Figure 4c**). The ionic phase has been associated with the reduction in the stomatal conductance, photosystem II efficiency, decrease in photosynthesis capacity, reduced biomass and poor yield in plants (Isla *et al*., 1998; Tester and Davenport, 2003; Netondo *et al*., 2004; Tavakkoli *et al*., 2011).

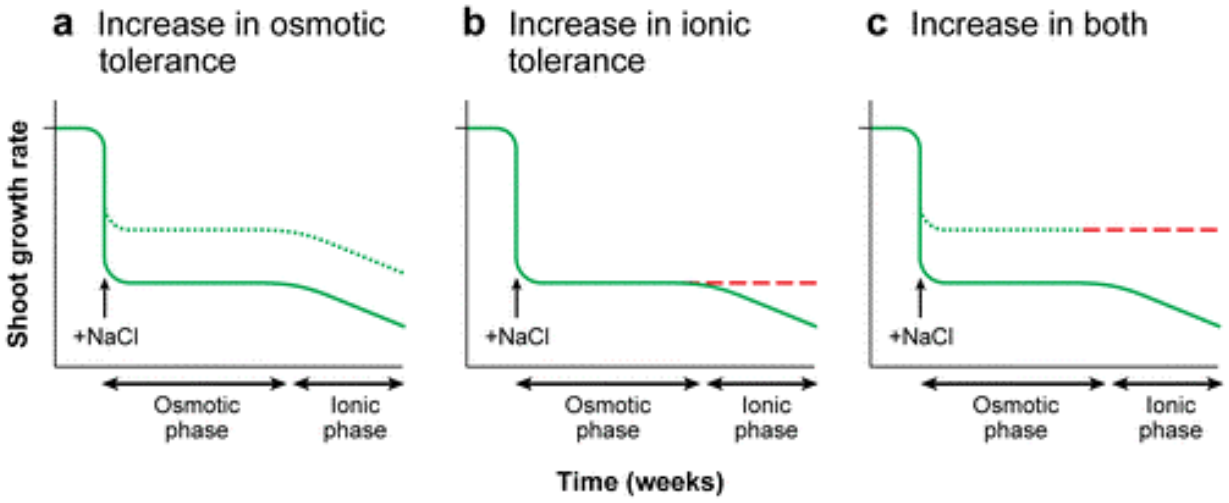

Figure 4 Model showing plant response phases to salt stress (**Source:** Munns and Tester, 2008)
The thick green line represents the change in the growth rate after salt application. (**a**) The broken green line represents the hypothetical response of a plant as a result of increased tolerance to the osmotic component of salt stress. (**b**) The broken red line represents the response of a plant with an increased in tolerance to the ionic component of salinity stress. (**c**) The green-and-red line represents the response of a plant with increased tolerance to both the osmotic and ionic components of salinity stress.

Mechanisms of salinity tolerance

Several reports have shown wide spectrum of responses to salinity in plants that warrant wide range of adaptations at the whole plant level (Wyn Jones and Gorham, 1983; Munns, 1993). Over the years, plants have evolved several mechanisms that allow them to adapt, grow and reproduce under high salinity conditions. According to Roy *et al.* (2014), these mechanisms are grouped into three main categories: (i) *osmotic stress tolerance*, which is controlled by long distance signals that reduce shoot growth and is triggered before shoot Na^+ accumulation; (ii) *Na^+ or Cl^- exclusion*, that tend to prevent Na^+ and Cl^- uptake and transport processes in roots in order to reduce the accumulation of these ions to a toxic concentrations within leaves and, (iii) *tolerance of tissue to accumulated Na^+ or Cl^-*, where *Na^+* or *Cl^-* that succeeded in getting into the plants are compartmentalized in the leaf vacuole (**Figure 5)** to prevent salt injury to the sensitive thylakoid membrane of the chloroplasts. These three mechanisms have also been reported by Munns (2002), Tester and Davenport (2003) and Kumari *et al.* (2014).

Although the information available for the plant tolerance to the 'osmotic phase' still remain vague, Mittler *et al*. (2011) have suggested that this process may be linked to the rapid, long-distance signaling via processes such as ROS waves, Ca^{2+} waves (Simon Gilroy, personal communication), or

the long distance electrical signaling (Maischak *et al*., 2010). This alludes to the fact that the differences of plants in osmotic tolerance may be due to the differences in the long-distance signaling and/or in the initial salt stress perception and/or in the response to the signals existing among plants (**Figure 5**). However, further studies are needed to gain a clearer understanding of osmotic tolerance in plants. The most researched aspect of salt tolerance mechanism is the 'ionic phase', which is due to Na^+ and Cl^- accumulation in the leaf blade. The ion toxicity in plants during the ionic phase can be minimized by reducing the accumulation of toxic ions (Na^+ and Cl^- exclusion) in the leaf blades and/or by increasing the ability of crops to cope with salts that succeeded in gaining entry into the shoot (tissue tolerance) via compartmentation in the vacuoles. Tissue tolerance, which entails Na^+ exclusion from the cytosol and compartmentalization in the vacuole before the ion has a detrimental effect on cellular processes (Roy *et al.*, 2014), may be essential in the synthesis of compatible solutes and higher level controls to coordinate transport and biochemical processes, thus plays a role in both osmo-protection and osmotic adjustment (Munns and Tester, 2008; Flowers and Colmer, 2008) in plants.

Munns *et al*. (2012) and Roy *et al.* (2014) have suggested that these three mechanisms of salt tolerance are not mutually exclusive. In other words, the occurrence of one does not prevent the other. However, it might be possible that each of these tolerance mechanisms is more effective in a particular circumstance and/or genotype and growth stage dependent. For instance, Na^+ exclusion may be more effective in higher salinity (Munns *et al*., 2012), while at moderately saline conditions, 'osmotic tolerance' may be much more pronounced. In view of this, salinity tolerance is considered a complex trait, being controlled by many genes and physiological factors. Thus, a probable reason why breeding for salt tolerance through introgression using traditional breeding has not been successful (as measured by the lack of commercial products), as has been usually been attributed to the multi-genic nature of salt stress tolerance in plants (Flowers and Yeo, 1995).

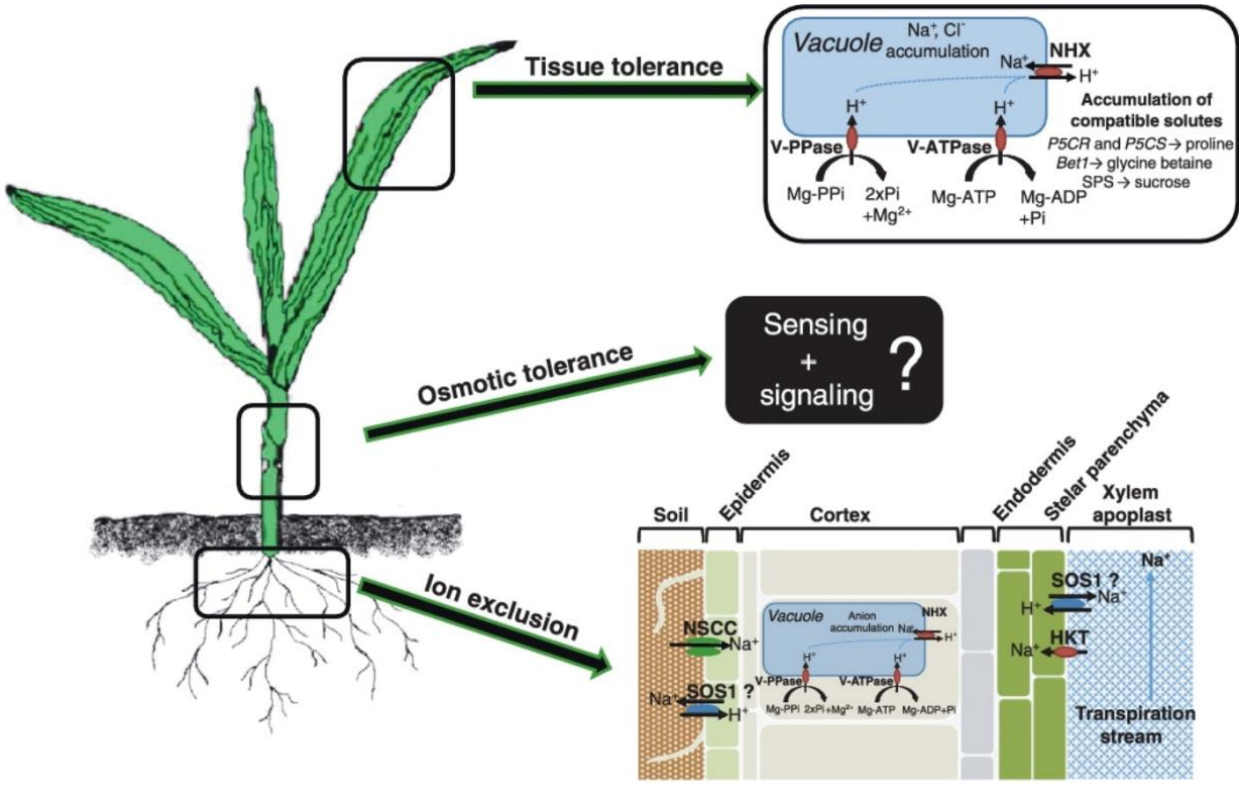

Figure 5 The three main mechanisms of salinity tolerance in a crop plant (Source: Roy *et al.*, 2014)
Tissue tolerance, where the accumulated toxic Na^+ in the leaves are compartmentalized at in the vacuole, a process involving ion transporters, proton pumps and synthesis of compatible solutes. *Osmotic tolerance*, associated with reduction of shoot growth and related to drought stress may be related to yet unknown sensing and signaling mechanisms. *Ion exclusion* is associated with the exclusion of toxic Na^+ and Cl^- from getting into the plant (predominantly from the roots). This mechanism may include retrieval of Na^+ from the xylem, compartmentation of Na^+ and Cl^- in vacuoles of cortical cells and/or efflux of Na^+ and Cl^- back to the soil.

The improvement of salt tolerance in glycophytic crops have been achieved by the development of cultivars with low Na^+ in shoot or high K^+/Na^+ ratio (Tester and Davenport, 2003; Ren *et al.*, 2005; Munns and Tester, 2008; Thomson *et al.*, 2010; Munns *et al.*, 2012). The ability of plants to maintain high K^+/Na^+ is a key feature of salt tolerance because high K^+/Na^+ is required for normal cellular functions and ion-homeostasis. When the plant roots are exposed to salinity, the K^+ /Na^+ ratio in the plant is reduced significantly (Tester and Davenport, 2003), because Na^+ competes antagonistically with K^+ uptake via K^+:Na^+ co-transporters, which may block the K^+-specific transporters of root cells under saline conditions (Zhu, 2003) and result in the accumulation of Na^+ to toxic levels in the plant tissues. This means that salt tolerance status of any plant mainly depend on its ability to exclude the Na^+ ions, through preferential absorption of K^+ over Na^+. Amtmann and

Sanders (1999) have demonstrated that glycophytes exhibit poor Na^+ exclusion potentials, which would disrupt the ion homeostasis and inhibit cellular growth and functions.

Na^+ transport in crop plants

AS has been described previously, the ability of plants to adapt under high salinity depend on the extent at which they can: prevent Na^+ initial entry, maximize Na^+ efflux transport, minimize loading to the xylem or maximize retrieval before reaching the shoot, maximize intracellular compartmentation or allocation to particular parts of the shoot (e.g. pith cells or old leaves), extrude, mobilize Na^+ ions and secrete salt onto the surface of the leaf (Tester and Davenport, 2003; Apse and Blumwald, 2007). The Na^+ transporter genes have been reported to perform these functions (Plett and Moller, 2010; Tester and Davenport, 2003). For instance, the overexpression of vacuolar Na^+/H^+ antiporter (*NHX1*) increased salinity tolerance of Arabidopsis (Apse *et al*., 1999). The Na^+/H^+ is involved in the intracellular compartmentation of Na^+ via pumping Na^+ into the vacuole and, its activity was increased upon Na^+ application in Barley (Gabarino and DuPont, 1989) and tomatoes (Wilson and Shannon, 1995) and, the Na^+/H^+ expression was significantly higher in salt-tolerant species, *Plantago maritima*, than in the salt-sensitive species, *P. media* (Staal *et al*., 1991).

The Na^+ transporters are members of the monovalent cation proton antiporter-1 (CPA1) family that were derived from bacteria, yeast, plants and animals (Kumari *et al*., 2014). They play a role in cytoplasmic pH regulation, pumping out H^+ generated by metabolism, K^+ homeostasis and salt tolerance due to Na^+ influx into vacuoles (Waditee *et al*., 2001; An *et al*., 2007). The ability of these transporters to prevent Na^+ entry into the plant root cells or facilitate the pumping out of Na^+ that have gained entry into the plant back to the soil solution are important adaptive features of plant under saline conditions. **Figure 6** illustrates the function and the control sites of two important Na^+ transporters - *Nax1* and *Nax2* genes in protecting plants from salinity stress. While, ***Nax1*** mediate Na^+ unloading from the xylem into the sheath under salinity stress; thus, preventing Na^+ over-accumulation in leaves, to protect the photosynthetic organs**,** the ***Nax2*** unloads Na^+ from the xylem in roots.

The influx of Na^+ is unidirectional at plasma membrane level and can be triggered and controlled by a complex set of signal molecules like Ca^{2+} and many nonselective cation channels (NSCCs) such as, cyclic nucleotide-gated channels (CNGCs) and glutamate receptors (Kumari *et al*., 2014). Four out of

the 20 known CNGCs including *AtCNGC1*, *AtCNGC3*, *AtCNGC4*, and *AtCNGC10* are involved in Na^+ uptake (Gobert *et al*., 2006; Guo *et al*., 2008), while *AtGLR2* and *AtGLR3* controls Na^+ and K^+ symport in plants. The exchange of K^+ or Na^+ for proton (H^+) are regulated by the Na^+/H^+ antiporters (NHXs) transporters family. In addition, Na^+ efflux from plasma membrane to the apoplast is regulated by the expression of salt overly sensitive (*SOS1*) gene, a sodium proton antiporter found in root epidermal cells. The loading of Na^+ into xylem is essential process for salt tolerance in plant. This process leads to increased Na^+ concentrations in leaves (Shi *et al*., 2002). The leaf blade appears to be more sensitive to salinity than the roots (Munns and Tester, 2008). Karley *et al*. (2000) have demonstrated that Na^+ accumulation is more in the older leaves than younger due of differential distribution of various nonselective cation channels in different cell types. High-affinity K^+ transporter1 (*AtHKT1)* is also associated with Na^+ transport from the shoot into the phloem and also in the unloading of Na^+ into stelar cells (Kumari *et al*., 2014).

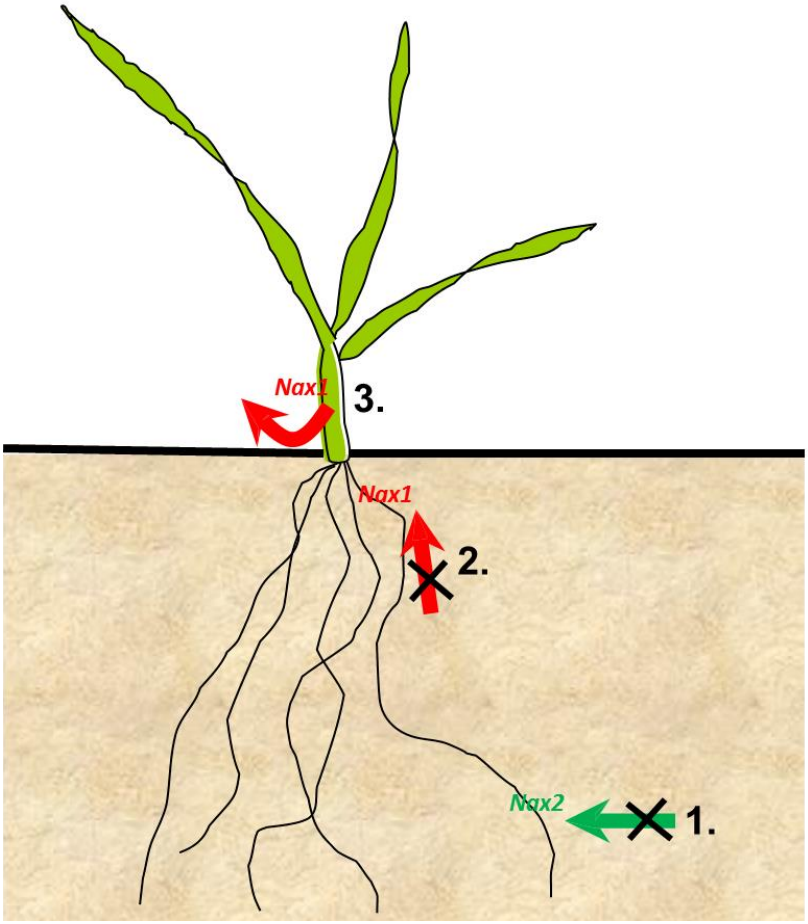

Figure 6 Na^+ transport control points in plants**. 1,** *Nax2*, unloads Na^+ from the xylem in roots; **2,** *Nax1*, controls loading of Na^+ into the xylem and; **3,** *Nax1*, removal of Na^+ from the xylem into sheath

K^+ transport in crop Plants

Optimal K^+ uptake is very crucial for salt tolerance in plants (Greenway and Munns, 1980). K^+ plays an important role in plant metabolism and functions including enzyme activation, protein synthesis, photosynthesis, osmoregulation, stomatal movement, energy transfer, phloem transport, cation-anion balance and stress resistance (Marschner *et al*., 2012; Wang *et al*., 2013a; Ahmad *et al*., 2014) and, K^+ influx in plant is inhibited under saline conditions (Blaylock *et al*., 1994; Ahanger *et al*., 2014). Low K^+ concentration at the binding sites would activate the hyperpolarization of membrane potential; but depolarization would occur when the K^+ concentration is high (Kumari *et al*., 2014). Several genes encoding K^+ channels/transporters have been linked to K^+ transport in plants. They include: *KUP/HAK/KT*, *HKT*, *Shakers*, *TPK*, *Kir-like* and *CPA* sub-families (Kumari *et al*., 2014).

KUP/HAK/KT transporters*:* This K^+ transporter gene family is homologous to bacterial *kup* (*K^+ up*take) and has been cloned from barley (Santa-Maria *et al*., 1997) and, it plays important roles in cell expansion and plant development (Davies *et al*., 2006; Grabov, 2007; Kumari *et al*., 2014) because K^+ is a major cellular solute. Reduction of K^+ uptake impairs K^+ homeostasis, leading to weak cell turgor and reduction in the rate of cell expansion. Elumalai *et al*. (2002) have shown reduction in the size of Arabidopsis shoot cells in the mutation *shy3-1* in the *AtKUP2* gene. In cotton fibers (*Gossypium hirsutum*), the expression of the *GhKT1* member of this gene family was positively correlated with build-up of turgor pressure (Ruan *et al*., 2001).

High-affinity K^+ (HKT) transporters: The HKT gene families regulate K^+ transport in plants (Rubio *et al*., 1995; Roy *et al.*, 2014) and, play vital role in salt tolerance (Mäser *et al*. 2002). Two classes of HKT transporters exits- the *HKT1* [which mediate relative Na^+ selective uniporters (Mäser *et al*., 2002; Horie and Schroeder, 2004; Garciadeblas *et al*., 2003)] and *HKT2* [which mediate Na^+/K^+ co-transport activity and homeostasis (Schachtman and Schroeder, 1994; Rubio *et al*., 1995)] transporters. Of the two classes, the *HKT1* group is perhaps of greatest potential for improving the salinity tolerance of crops, frequently appearing as the most likely candidate for quantitative trait loci when phenotyping for salt tolerance and/or Na^+ exclusion in mutant and mapping populations (Ren *et al*, 2005; James *et al*., 2006; Ahmadi *et al*., 2011) and, has been located on 2AL. Munns *et al*. (2012) and James *et al.* (2012) have demonstrated that the incorporation of novel *HKT1;5* gene from the salt-tolerant wheat relative *Triticum monococcum* into susceptible commercial durum wheat (*Triticum turgidum* ssp. durum) increased grain yield by 25% on saline soil. Moreover, the *HKT2* has

been reported to increase salinity tolerance, but not through Na^+ exclusion. Mian *et al.* (2011) indicated that the over-expression of *HvHKT2;1* would increase the Na^+ uptake, Na^+ concentrations in the xylem sap, and enhance translocation of Na^+ to leaves under saline conditions, suggesting that another way plant increase salt tolerance is rather not to translocate Na^+ to the shoot but rather to compartmentalize Na^+ in leaf tissues.

Shakers-type cation channels: The cation channels are mostly expressed in the plasma membrane and they possess high selectivity for K^+ ions (Kumari *et al*., 2014). The shakers-type cation channels are manipulated via gating which depend mainly on the voltage and/or changes in membrane potential and allosteric control and/or signals such as calcium-calmodulin mediated signals (Amtmann and Sanders, 1999; Maathuis and Amtmann, 1999; Kumari *et al*., 2014). This transporter is made up of three categories (based on their voltage dependence) (Wang and Wu, 2013): *K^+ inward rectifying channels (KIRC)*, *K^+ outward rectifying channels (KORC)* and *weakly rectifying channels*. *KIRC* is activated by hyperpolarization potential and mainly mediate K^+ uptake, while *KORC* activated by depolarization mediates the K^+ efflux (Wegner and Raschke, 1994; Maathuis and Sanders, 1997) into plant cells. The weakly rectifying channels, activated by hyperpolarization, mediate both K^+ uptake and K^+ release depending on the membrane potentials (Wang and Wu, 2013). The operation of shakers-type cation channels is dependent on external K^+ concentration (Zhu *et al.*, 1999). For example, at low K^+, the probability of openness of KORCs is very low so as to prevent leakage of K^+ (i.e. efflux) from root cells.

Two-pore K^+ (TPK) channel transporters: The two-pore K^+ (TPK) family plays an important role in maintaining vacuolar K^+ homeostasis (Maathuis, 2011; Isayenkov *et al.*, 2011). TPK is a non-voltage-gated K^+ channel (Gomez-Porras *et al*., 2012), which is regulated by the cytoplasmic calcium and 14-33 proteins (Maathuis, 2011). The TPK channels are not only mechanosensitive, but also osmo-sensitive, and functions as cellular osmo-sensors during rapid changes in external osmotic pressure (MacRobbie, 2006). They are activated under salt stress, resulting in the rapid release of K^+ from the vacuole, the main cellular depository of water and osmotica (Maathuis, 2011).

Monovalent Cation:Proton Antiporter (CPA): The CPA Superfamilies are named after one of its constituent members, the monovalent cation:proton antiporter-2 (CPA2), which happened to be the largest gene family of transporters belonging to CPA transporters. Members of the CPA2 family that have been functionally well-characterized include: KefB/KefC K^+ efflux proteins (Booth *et al*.,

1996), Na^+/H^+ antiporter (Waser *et al*., 1992) and K^+/H^+ antiporter (Reizer *et al*., 1992). These proteins functions essentially in the same mechanism (Reizer *et al*., 1992). For instance, *KefC* and *KefB* are responsible for glutathione-gated K^+ efflux in plants.

Improvement of salt tolerance in wheat

The use of wide range of genetic materials for comparative phenotype and physiology screening for salt stress tolerance and ion uptake in cereals have progressed steadily with the identification three mechanisms which may contribute to salt tolerance, such as *osmotic tolerance, ion exclusion* and *tissue tolerance*. This offers strong indication that salt tolerance in wheat can be improved via pyramiding and/or incorporation of useful alleles that are associated with the above mentioned mechanisms. Several breeding strategies have been adopted to achieve these objectives.

Conventional Breeding

Genetic variation in Na^+ for both exclusion and K^+/Na^+ discrimination exists amongst wheat genotypes, wheat progenitors, wild relatives (Gorham *et al*., 1987; Gorham, 1993), and in the halophytic species in the Triticeae (Gorham *et al*., 1985; Garthwaite *et al*., 2005). In the past, screening of a large collection of wheat germplasm for salt tolerance identified genotypes that can sustain growth and produce seeds under saline soil conditions. However, only few of the identified salt tolerant genotypes have been successfully released (**Table 1**). They include Indian *KRL1–4* and *KRL 19* (from Central Soil Salinity Research Institute at Karnal), *LU26S* and *SARC- 1* (released by the Saline Agriculture Research Centre at University of Agriculture, Faisalabad), *Sakha 8* (from the Agricultural Research Centre at Giza) and *Kharchia 65* (from India). Among them, *Kharchia 65* was the most widely and globally exploited salt tolerance "donor parent" that has been used to contribute positive alleles in many breeding programs (Munns *et al.*, 2006; Shahbaz and Ashraf, 2013). *Kharchia 65* was developed via selection by Indian farmers on sodic-saline soils of the Kharchi-Pali area of Rajasthan (Rana, 1986).

The tolerant genotype *KRL1-4*, derived from a cross between *Kharchia 65* and *WL711*, has performed well on the saline soils of northern India, but it was not successful in Pakistan due to the problem of water logging and soil texture (Hollington, 2000). Also, *KTDH 19* which was developed in UK by Quarrie and Mahmood from a cross between *Kharchia 65* and *TW161* (a line identified with exceptional Na^+ exclusion) performed well in Spain (Hollington *et al*., 1994); but in India and

Pakistan, it was found to be highly tolerant in terms of total dry matter but the grain yield was very low due to it maturing around 2 weeks later than local genotypes (Hollington, 2000). In addition, the cross of *LU26S, Kharchia-65* and two salt-tolerant genotypes, produced two salt-tolerant genotypes, *S24* and *S36* (selected from the F_3 populations) at salinity levels of 24 and 36 dS m^{-1}, respectively (Ashraf and O'Leary, 1996). *S-24* showed positive transgressive inheritance for salt tolerance when compared to its parents- *Kharchia-65*, *LU26S* and *SARC-1*, due to its low accumulation of Na^+ in leaves (Ashraf, 2002). It also exhibited higher grain yield potentials than most wheat cultivars (Arfan *et al.*, 2007; Shahbaz *et al.*, 2008; Perveen *et al.*, 2010, 2011, 2012).

Table 2. Improvement in salt tolerance of cereal crops using conventional breeding approach

Released cultivar	**Releasing source**	**Good performance**	**Reference**
KRL1-4 and KRL 19	Central Soil Salinity Research Institute (CSSRI) at Karnal, India	Saline soils of northern India	Hollington, 2000
LU26S and SARC-1	Saline Agriculture Research Centre (SARC) at University of Agriculture, Faisalabad-Pakistan	All saline soils	Munns *et al*., 2006
Sakha 8	Agricultural Research Centre, Giza, Egypt	All saline soils	Munns *et al*., 2006
Kharchia 65	Indian farmers through selection on sodic-saline soil	Kharchi-Pali area of Rajasthan, India	Rana, 1986
Line KTDH 19	Quarrie and Mahmood	Performed well in Spain only	Hollington *et al*., 1994
S-24	Department of Botany, University of Agriculture, Faisalabad-Pakistan	On all saline soils	Ashraf, 2002

Mutation breeding

Mutation breeding techniques have been used to generate a vast amount of genetic variability among genotypes for salinity tolerance. It has played a significant role in plant breeding and genetics and has been used to develop thousands of novel crop varieties which have been released to farmers for cultivation. It is cost effective, quick, proven, robust, non-hazardous and environmentally friendly. It is based on selfing mutants until the induced character has a stable expression in the advanced mutant generations. Mutation breeding has been used to reduce the maturity time by 3 weeks without adverse effects on yield at 150 mM NaCl (Mahar *et al*., 2003). Four salt tolerance mutant wheat

varieties have been officially released for commercial use as referenced in the mutant varieties database (https://mvd.iaea.org/). They include: *Jiaxuan 1* (released in 1974), *Changwei 19* (released in 1978), *Emai 9* (released in 1980), and *H6765* (released in 2004).

Modern Breeding for salt tolerance using molecular markers

The development of molecular markers for the exploitation of DNA polymorphisms in plant systems is one of the most significant developments in the field of molecular biology and biotechnology (Soto-Cerda and Cloutier, 2012). DNA marker is a portion of DNA situated on a chromosome and tightly linked to a known gene controlling trait variation in a given population. Because salt tolerance is polygenic in nature and is largely influenced by environment and genotype, it is difficult to breed using conventional methods. Thus, the use of DNA marker systems have gained prominent in plant breeding, because of the absence of genotype x environment interaction, epistatic effect, and also ease in the picking up of homozygous plants which can be greatly distinguished from the others at an early generation (Kumar *et al*., 2015). Once a marker is found to be linked to gene/QTL contributing to the trait (i.e., salt tolerance) variation in the crop species, such marker can be used "*as surrogate*" to incorporate the gene into the commercial crop varieties using either marker assisted selection (MAS) or transgenic approach. MAS has been successfully used to incorporate the Na^+ exclusion gene *HKT1;5* into the susceptible commercial durum wheat (Munns *et al*., 2012 and James *et al*., 2012). DNA marker systems have been used to tag/map several genes or QTL contributing to salt tolerance in cereals (**Table 3).** The association and application of the indirect selection markers which are genetically linked with the trait(s) of interest is a well-known approach for improvement of the crop having difficult complex traits such as salt stress tolerance (Im *et al*., 2014). This approach has contributed immensely on deciphering the genetic basis of salt tolerance in many crops.

Table 3. Salt tolerant QTL/genes that has been identified using DNA markers

Crop plants	Locus name	Associated traits	Reference
Wheat (*Triticum aestivum* L.)	*Kna1*	Controls the selectivity of Na^+ and K^+ transport from root to shoot and maintains high K^+/Na^+ ratio	Gorham *et al*. (1990); Dubcovsky *et al*. (1996)
	Nax1	Both are involved in decreasing Na^+ uptake and enhancing K^+ loading into the xylem	Lindsay *et al*. (2004); Huang et al. (2006)
Rice (*Oryza sativa* L.)	*qRL-7, qDWRO-9a* and *qDWRO-9b qBI-1a* and *qBI-1b*	Play important roles in root length and root dry weight at seedling stage under saline conditions	Sabouri and Sabouri (2008)
	QNa, QNa:K, SKC1/OsHKT8	Control K^+/Na^+ homoeostasis	Ren *et al*. (2005)
	qDM-3 and *qDM-8, qSTR-6*	Enhance Na^+/K^+ ratio under saline conditions	Sabouri (2009)
	qNAK-2 and *qNAK-6*	Enhance Na^+/K^+ ratio	Yao *et al*. (2005)
	Saltol	shoot Na^+/K^+ homoeostasis	Thomson *et al*. (2010)
	Saltol and *non-Saltol*	shoot Na^+/K^+ homoeostasis	Alam *et al*. (2011)
	QKr1.2	K^+ content in root	Ahmadi and Fotokian (2011)
Barley (*Hordeum vulgare*)	Five QTL for ST were identified on chromosomes 1H, 2H, 5H, 6H, and 7H, which accounted for more than 50% of the phenotypic variation	Improve vegetative growth under saline stress	Zhou *et al*. (2012)
	A locus *HvNax3* on the short arm of chromosome 7H in wild barley (*Hordeum vulgare* ssp. *spontaneum*) accession CPI-71284-48	Reduces shoot Na^+ content by 10–25% in plants grown under salt stress (150 mM NaCl)	Shavrukov *et al*. (2010)

The modern plant breeding approaches emphasize the importance of the location of the genes/QTL controlling the trait of interest for crop improvement. Molecular genetics which entails using either or both forward and reverse genetic approaches have been adopted to identify QTL and genes contributing to variability (Takeda and Matsuoka, 2008) in a population under study. The bi-parental linkage mapping approach has been extensively used for mapping quantitative traits, but not until recently that the use of the association mapping (AM), which has initially gained tremendous success in human and animal genetics, was adopted to study genetic architecture controlling important plant phenotypes. The QTL mapping populations are broadly divided into two types: (1) family-based linkage populations, and (2) natural populations that use linkage disequilibrium mapping approaches (Semagn *et al.,* 2010; Mackay and Powell, 2007). In contrast to the biparental mapping approaches, AM populations are carefully sampled diverse lines representing the diversity of natural or breeding populations of the crops (Zhao *et al*., 2007b; Zhu *et al*., 2008). Recently, more advanced mapping populations also known as next-generation populations (NGPs) been brought to the fore and adopted in various crops in other to overcome the limitations posed by both the bi-parental linkage and association mapping approaches. The NGP design entails crossing of multiple parents and/or advanced generation intercrosses with further advancement for generations to improve genetic resolution of mapping (Morrell *et al*., 2011). The NGP that have been used for QTL mapping in crop improvement include: *Nested association mapping (NAM) populations*, *Multi-parent advanced generation intercross (MAGIC) population* and *advanced intercross recombinant inbred lines (AI-RILs)*. For successful identification of QTL/genes underlying complex traits (salt tolerance), the investigator must make a decision on the type of population, DNA marker systems and QTL analysis method to be adopted. Examples of cloned genes that were initially identified through QTL analysis abound in the literature, which demonstrates the power of QTL analysis to discover genes controlling important agronomic traits.

Association Mapping

As described earlier, AM make use of collections of accessions with diverse genetic background and relies on the natural genetic variation in the studied germplasm. Thus, AM does not suffer from lack of variation that characterized several bi-parental mapping populations (Hall *et al*., 2010) and, it involves searching for significant genotype (DNA-marker) -phenotype correlations among unrelated germplasm collections using different statistical tools. AM relies on the degree of ancestral

recombination and mutation events that occurred within the population, taking into account all the alleles present in the studied population, to detect significant genotype-phenotype associations. By exploiting non-random associations of alleles at nearby loci (i.e., loci in linkage disequilibrium), it is possible to detect significantly associated QTL regions with a set of mapped markers (Breseghello and Sorrells, 2006; Pasam and Sharma, 2014) that are affecting the traits under investigation. Thus, the success of AM depends on the quality of the measured phenotypic data, size of the population and population LD (Flint-Garcia *et al.,* 2005; Mackay and Powell, 2007; Pasam *et al*., 2012).

AM broadly falls into two main categories: (i) *candidate gene association mapping*, in which candidate genes are selected, sequenced and their sequence polymorphism correlated with the measured traits of interest, and (ii) *genome-wide association mapping* (GWAM), that correlate the polymorphic markers across the genome with the measured traits. GWAM has become a powerful tool to identify novel loci involved in the genetic variation of complex traits in plant genetics, because it depends less on prior information about the candidate genes, in contrast with the candidate gene association mapping approach. An increasing number of association studies based on the GWAM have been successfully used to localize genes/QTL controlling complex traits in cereals, such as maize (Remington *et al*., 2001; Belo *et al.,* 2008), barley (Pasam *et al.,* 2012; Cockram *et al.,* 2010; Long *et al.,* 2013), wheat (Jighly *et al.,* 2015; Zegeye *et al*., 2014; Turki *et al*., 2014; Edae *et al.,* 2014) and rice (Huang *et al.,* 2010; Agrama *et al*., 2007; Zhao *et al.,* 2011). By exploiting broader genetic diversity, AM offers four advantages over the family-based linkage mapping: (i) generation of bi-parental populations is not needed, so it saves time (in years) for population construction, (ii) the constructed population can be used to study several important agronomic traits, (iii) can be used to discover new favorable alleles and (iv) has higher resolution mapping of putative QTL.

Implications of Linkage Disequilibrium in Association Mapping

Both Linkage equilibrium (LE) and linkage disequilibrium (LD) are used to define the linkage relationships in population genetics. While LE is defined as the random association of alleles at different loci, the LD is the non-random association of alleles at separate loci (Flint-Garcia *et al*., 2003; Hill and Robertson, 1968). Tightly linked loci are considered to be in LD due to limited recombination between these loci, which implies that the effect coming from these loci may be as a result of the influence of single/few genes. AM is dependent on LD because in a situation where the

functional polymorphism is not among the genotyped markers, it is expected that the functional polymorphism is in high LD with the genotyped marker and thus would be captured via the genotyped marker during the analysis. Morton *et al.* (2001) have shown that only closely linked loci remain associated and co-segregate for many generations. Closely linked loci would provide a great opportunity to map QTL with higher resolution mapping at the gene level (Ersoz *et al*., 2009). Moreover, the power of AM depends on the degree of LD between the genotyped markers and the functional polymorphisms (Pasam and Sharma, 2014) and, the LD decay is species, population and genome dependence (Caldwell *et al*., 2006; Gupta *et al*., 2005). In case of extensive LD decay, the associated QTL/markers are easily detected due to the high chance that many of the genotyped markers are in high LD with causal variant; however, it will be very difficult to detect the main causal variant. All the associated markers/QTL are resolved into independent genetic variants where the LD decays at short distances (also called, LD blocks). The resolution and power of association studies depend on the extent of LD, which in turn is influenced by several factors such as mutation across the whole genome, population history, sample size, genetic drift, regional variability in recombination patterns, chromosome region, diversity and population admixture and the pattern of mating within a population (Flint-Garcia *et al*., 2003; Ersoz *et al*., 2009; Zhang *et al*., 2009a; Chao *et al*., 2010). The estimates of LD (r^2) ranged between 0 and 1. The value $r^2 = 0$ means that the loci are in complete LE, $r^2 = 1$ means that the loci are in complete LD. The LD may decay over a long or short distance based on the population under study and the chromosomal region.

Population structure

AM results are influence by population structure (PS) if not accounted for. PS would often lead to spurious associations of unlinked loci (Sneller et al., 2009), due to genetic drift, domestication and/or background selection. In other words, loci could be identified as being associated with a trait of interest when in fact no real associations exist between the loci and the trait. In view of this, statistical models have been developed to account for PS during association analysis so as to improve the power of AM for QTL detection. Although several models has been developed to account for PS during association studies (Pritchard *et al*., 2000; Yu *et al*. (2006), the use of the unified mixed model (MLM) which accounts for both population structure (Q) and family relatedness (K) simultaneously as covariates in the model is considered to perform best. This model accommodates both fixed and random effects. According to Yu *et al.* (2006), the mixed model equation for Q+K is presented below:

$y = X\beta + S\alpha + Qv + Zu + e$

where, Xβ represents those fixed effects other than the SNP under testing and the population structure; **y** is a vector of phenotypic observation; β is a vector of fixed effects other than SNP or population group effects; α is a vector of SNP effects (QTN); **v** is a vector of population effects; **u** is a vector of polygene background effects; **e** is a vector of residual effects; *Q* is a matrix from STRUCTURE relating **y** to **v**; and *X*, *S* and *Z* are incidence matrices of 1s and 0s relating **y** to β, α and **u**, respectively. The variances of the random effects are assumed to be $\mathrm{Var}(\mathbf{u}) = 2KV_g$, and $\mathrm{Var}(\mathbf{e}) = RV_R$, where *K* is an n × n matrix of relative kinship coefficients that define the degree of genetic covariance between a pair of individuals; *R* is an n × n matrix in which the off-diagonal elements are 0 and the diagonal elements are the reciprocal of the number of observations for which each phenotypic data point was obtained; V_g is the genetic variance; and V_R is the residual variance. Best linear unbiased estimates (BLUE) of β, α and **v** (fixed effects) and best linear unbiased predictions (BLUP) of **u** (random effects) were obtained by solving the mixed-model equations (Henderson, 1984; Kennedy *et al*., 1992).

The schematic framework of steps involved in AM studies is presented in Figure 7 (below). The steps include: (1) construction of genetically diverse mapping population or panel; (2) Phenotypic data collection in a replicated and/or multi-environmental field trials; (3) genotyping the mapping population with available molecular markers (in this case, we used the 90K Illumina Chip); (4) Chromosomal and/or genome LD quantification using molecular marker data of the mapping panel; (5) population structure and kinship assessment (coefficient of relatedness between each pair of individuals) of the mapping population; (6) Association analysis using the phenotypic and genotypic data with the incorporation of information gained from LD and population structure using appropriate statistical methods (Abdurakhmonov and Abdukarimov, 2008). And finally, the specific gene(s) controlling a QTL of interest can be identified and cloned using the marker tags and annotated for an exact biological function. In the present study, we used the MLM- Q+K approach.

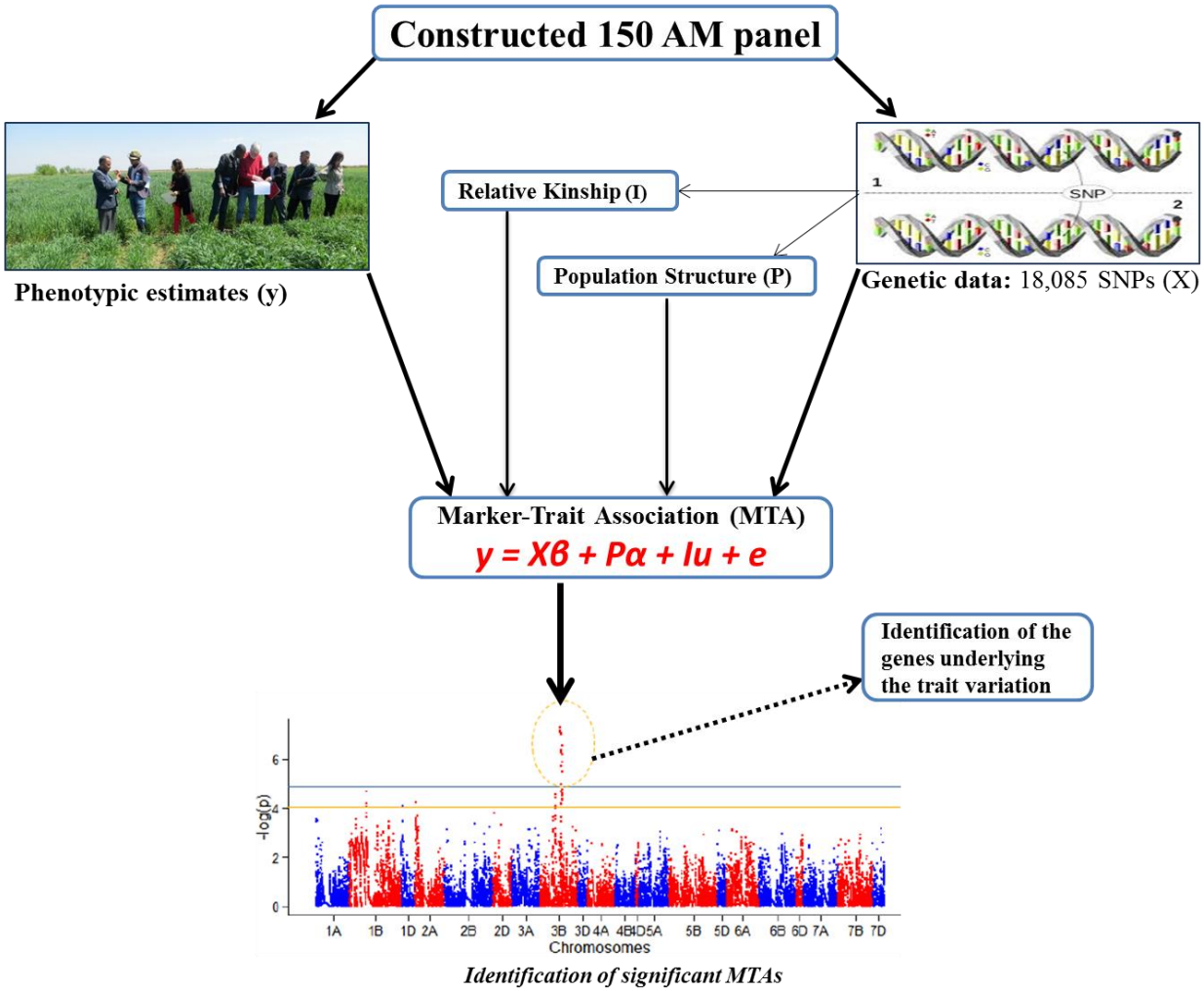

Figure 7 Schematic framework of the steps involved in genome-wide association studies for tagging a gene of interest using 150 wheat genotypes.

Hypothesis of this study

1. The response of wheat to salt stress is genotype specific.

2. Salt-stress tolerance is regulated at genetic, transcriptomic, and molecular levels and differs in the plant developmental growth stages.

3. The adaptation to salt stress can be improved by selection of relevant alleles of salt-responsive genes.

The objectives of the thesis

Given the amount of wheat needed to feed the growing population in an increasing climatic change, improving wheat salt tolerance status has become a herculean task for breeders. Gaining understanding of the agronomic, physiological, genetic, and molecular mechanisms underlying salt tolerance is of key importance to reach the desired breeding goal of developing high yielding wheat genotypes that can be cultivated in the regions prone to salinity where wheat is grown. Therefore, the overall goal of this study (thesis) was to use the genetic variations among the studied wheat germplasm to dissect its physiological and genetic mechanisms of salt tolerance by performing genome-wide association studies (GWAS). The genetic dissection of the quantitative traits controlling the salt tolerance in wheat is a prerequisite to allow for the application of the cost effective genomics-based approaches in breeding high yielding wheat genotypes for saline conditions. In particular, the objectives were to:

1. To screen 150 internationally derived wheat genotypes for salinity tolerance at germination, seedling and adult plant stages, with the aim of identifying new genetic resources that can be used to improve salt tolerance in wheat through breeding programs;

2. To evaluate the association of the physiological traits of wheat such as ion (K^+, Na^+ and K^+/Na^+ ratio) contents in leaves and stems, leaf chlorophyll fluorescence parameters and water relation parameters with the salt tolerance in terms of seedling biomass and grain yield, with a view of finding out the reliable physiological traits that can be used as a surrogate when screening for salt tolerance in wheat;

3. To unravel common QTL controlling several salt-stress related agronomic, physiological and seed quality parameters that could be exploited the breeding programs and in future research;

4. To identify plausible candidate genes underlying QTL mapped for all the measured traits;

5. To perform single gene expression analysis of some of identified causative genes in other to further our understanding on the molecular mechanism of salt tolerance in wheat.

CHAPTER 2

Identification and characterization of salt tolerance of wheat germplasm using a multivariable screening approach

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ABSTRACT

Salinity is one of the major limitations to wheat production worldwide. This study was designed to evaluate the level of genetic variation among 150 internationally-derived wheat genotypes for salinity tolerance at germination, seedling and adult plant stages, with the aim of identifying new genetic resources with desirable adaptation characteristics for breeding programs and further genetic studies. In all the growth stages, genotype and salt treatment effects were observed. Salt stress caused 33%, 51% and 82% reductions in germination, seedling shoot dry matter and grain yield, respectively. The rate of root and shoot water loss due to salt stress exhibited significant negative correlation with shoot K^+, but not with shoot Na^+ and shoot K^+/Na^+ ratio. The genotypes showed a wide spectrum of response to salt stress across the growth stages, however, four genotypes: *Altay2000*, *14IWWYTIR-19* and *UZ-11CWA-8* (tolerant) and *Bobur* (sensitive*)* exhibited consistent responses to salinity across the three growth stages. The tolerant genotypes possessed better ability to maintain stable osmotic potential, low Na^+ accumulation, higher shoot K^+ concentrations, higher rates of PSII activity, maximal photochemical efficiency and lower non-photochemical quenching (NPQ), resulting in the significantly higher dry matter production observed under salt stress. The identified genotypes could be used as parents in breeding for new varieties with improved salt tolerance as well as in further genetic studies to uncover the genetic mechanisms governing salt stress response in wheat.

INTRODUCTION

The continuous salinization of arable land is a threat to global food security. Over 800 Mha of land are affected by salinity, which equates to more than 6% of the world's total land area (FAO**,** 2010) and affects more than 20% of present-day agriculture (Mickelbart *et al*., 2015). Salinized soils extend over all the continents leading to annual losses of arable land to about 10 mha (Pessarakli and Szabolcs, 1999). About 27.3 billion US dollars is spent annually to combat irrigation induced salinity (Qadir *et al*., 2014).Salt stress, mainly due to accumulation of toxic Na^+ and Cl^- ions in plant tissues, causes osmotic and ionic stresses in plants. Wheat (*Triticum aestivum* L.) is one of most important crop plants worldwide with annual production of about 736 million metric tons (FAO, 2015), but suffers significant grain yield losses due to soil salinity. Although, there are several strategies to increase wheat production in the salt affected areas (such as leaching, drainage etc), the cultivation of tolerant genotypes is recognized as the most effective way to overcome the limitations. The

prerequisite is the identification of wheat genotypes with proven wide adaptation under saline conditions. The cultivar, *Kharchia 65* is one of the very few reputed donors of salt tolerance (ST) in wheat and has been extensively used in breeding for ST cultivars globally (Chatrath *et al*., 2007). Thus, there is an urgent need to identify new sources of ST to broaden the gene base and to provide donor parents in locally adapted genetic backgrounds.

An imminent task is the efficient characterization of wheat plants for tolerance towards salt stress. The most valuable agronomical traits might serve as good surrogates to discriminate among genotypes under salt stress conditions. Munns and James (2003) consider biomass yield as a useful criterion because it permits the direct estimation of economic return under saline conditions. Moreover, it has been reported that shoot growth is more sensitive to salt stress than the root growth firstly, because the reduction in leaf area development relative to the root growth leads to a decrease in water use by the plant, thus allowing it to conserve soil moisture and prevent an escalation of the salt concentration in the soil, and secondly, due to the accumulation of Na^+ and/or Cl^- at toxic concentration levels affects the photosynthetic capacity resulting in less supply of carbohydrates to the young leaves, that further reduces the shoot growth rate (Munns and Tester, 2008). The ST status of plants can be assessed as the percent biomass production in saline versus control conditions (Genc *et al*., 2007) over a prolonged period of time. Selection of plants with high ST values would allow breeders to identify genotypes better adapted to the salinized arable lands. Screening for chlorophyll fluorescence characteristics has also gained increasingly interest in plant abiotic stress research. Salinity stress has negative impact on photosynthesis by inhibiting photosystem II (PSII) activity and destruction of chlorophyll pigments due to the accumulation of toxic ions. The relationship between the PSII operating efficiency and CO_2 assimilation in leaves allows fluorescence to be used to detect differences in the response of plants to environmental challenges and, consequently, to screen for tolerance to environmental stresses (Baker and Rosenqvist, 2004).

Tolerance to salt stress is a complex biological phenomenon governed by several physiological and genetic factors and it is growth stage specific (Haq *et al*., 2010). Little effort has been made so far to simultaneously characterize the wheat germplasm across different growth stages. Experiments carried out under controlled conditions were not exposed to those conditions that prevail in salt-affected soil such as spatial and temporal heterogeneity of soil chemical and physical properties, high diurnal temperature variations, low humidity, and presence of drought stress (Munns and James, 2003). These could be one of the reasons why breeding for ST has not gained significant progress up

till now. To meaningfully characterize the ST status of wheat genotypes, it is necessary to evaluate wheat response to salt stress across several developmental growth stages, with a view of identifying genotypes with desirable ST across all the growth stages. Access to new wheat genotypes with contrasting response to salt stress would allow for further characterization of the genetic mechanisms controlling ST in wheat.

The response of wheat to salt stress is genetically and physiologically controlled and may differ from one growth stage to another. Thus, a better understanding of these mechanisms and processes would help in the breeding programs to enhance wheat production under salt stress. This study was designed to characterize salt tolerance in a set of winter and facultative wheat landraces, cultivars and elite breeding lines at the germination, seedling and mature plant field growth stages, with the aim to identify contrasting (salt tolerant and salt sensitive) genotypes for further genetic studies. The identified genotypes were evaluated for the effect of salinity on some key physiological traits including the cell membrane stability, osmotic potential, leaf chlorophyll fluorescence and dry matter production. The identified genotypes would be valuable resources for breeding programs and scientific research towards better understanding of plant tolerance to salt stress.

MATERIALS AND METHODS

Plant Materials

A total of 150 winter and facultative wheat genotypes consisting of advanced lines from the International Winter Wheat Improvement Program (IWWIP-Turkey/CIMMYT/ICARDA), cultivars from Turkey national wheat program (TNP) and cultivars from countries of the Central and Western Asia (CWA) region. To ensure that pure seeds were used and to minimize heterogeneity and contamination, multiplication step and cleaning were performed at the greenhouse of Crop Science and Resource conservation Institute (INRES), University of Bonn, Germany. The harvested seeds were then used for the ST evaluation at germination, seedling and mature growth stages.

Figure 1 The seed multiplication of the 150 association mapping panel

Salt stress test

Salt-water flooding method as described by the Association of Official Seed Analysts (AOSA, 2009) was adopted to evaluate the genotypes germination ability under two salt types (NaCl and Na_2SO_4) and several concentrations: 100, 150, 200 mM for NaCl and 75, 100 mM for Na_2SO_4 plus control (without salt). Twenty-five seeds of each genotype, in three repetitions, were sown in 29 x 22.5 cm plastic transparent boxes containing blotting paper (ALBET Lab Science, Germany) soaked in 75-ml of each salt treatment solution. Thereafter, the boxes were placed in a growth chamber with white fluorescent light (600 µmol m^{-2} s^{-1}; 14 h light/10 h dark) at 15±1°C, and relative humidity of 65±8%. Ten days after sowing, the germination potentials of each genotype were determined with the scale from 0 to 9 as described by Mano *et al*. (1996).

The seedling stage screening was performed in a supported hydroponic system using the modified Hoagland solution as described by Tavakkoli *et al.* (2010). Four independent experiments designated E1, E2, E3 and E4, with three replications each were conducted, in the greenhouse. In E1 (October - November, 2013) and E2 (February – March, 2014), the genotypes were screened with non-saline (control) and saline (100 mM NaCl) nutrient solution, while the solutions containing non-saline and saline (75 mM Na_2SO_4) were used to screen the genotypes during the E3 (April – May, 2014) and E4 (May to June, 2014) experiments. Supplementary Ca^{2+} as $CaCl_2$ was added to the saline nutrient solution in 20:1 molar ratio of NaCl or Na_2SO_4:$CaCl_2$ (Haq et al., 2010), to improve nutrient uptake

and ameliorate the effects of salinity on the plant growth. In each experiment, comparisons were made between saline and non-saline conditions. The electrical conductivity EC values for control, 100 mM NaCl (+5.0 mM $CaCl_2$) and 75 mM Na_2SO_4 (+3.75 mM $CaCl_2$) solutions ranged: 1.79 - 1.84, 11.89 12.54 and 12.44 -13.68 dS/m, respectively.

Figure 2 The designed supported hydroponic experiments used for the seedling screening under control (Right) and salt (Left) stress conditions.

156 cylindrical PVC tubes (4.5 cm diameter x 45 cm depth) were placed on each tub served by a separate tank containing 164 liters of nutrient solution at 75 minutes interval using EHEIM Universal-pump1046 (EHEIM GmbH and Co, Germany). Prior to the transfer into the hydroponic system, seeds were exposed to 45°C for 24 hours to remove the inherent differential dormancy. The seeds were sown and germinated *in situ* in the tubes filled with Aquagran filter quartz, 2-3.15 mm (Euroquarz GmbH) with tap water. Three days after planting (DAP), salt treatments were introduced together with the nutrient solution. The salt application was done in an equal incremental basis for 3 days to avoid osmotic shock. The stress was continued for 22 days after the final salt stress level was reached. The nutrient solutions were changed every 7 days accompanied by adjustment of the pH to 5.5. Thereafter, the solution pH were monitored daily and adjusted to 6.0. The nutrient solution temperature varied from 14.1 to 21.7 °C. At harvest (28 DAP), plant shoots were cut off from the base and weighed to obtain the fresh shoot weight (FW). The harvested samples were dried at 55 °C for 10 days and weighed to obtain the dry shoot weight (DW). The relative shoot water loss (WL) due to salt stress was calculated on the basis of FW and DW in stress conditions (S) *vis-a-vis* the control conditions (C): $WL = [(FW_C – DW_C) - (FW_S – DW_S)]$.

The field trials were conducted under saline and non-saline soil conditions in four locations: Urgench (Uzbekistan) (41° 32'60N and 60°37'60E, 91 meters above sea level (masl) in 2011-2012; Karshi, (Uzbekistan) (38°52'N and 65°48'E, 416 masl) in 2012-2013 and Dongying (China) (118°33'-119°20'E, 37°35'-38°12'N) in 2013 -2014. The field layout for the trials in Uzbekistan was α-lattice design with three replications. Each plot measured 2 m^2 with different number of rows in different locations. In Dongying, seeds were sown in 2 rows (20 seeds per row) with plant spacing of 10 cm and the width is 1 m for each genotype. The soil chemical properties of all the field locations are presented in **Table1.** At harvest, the grain yield (GY) was measured and recorded for both saline and non-saline fields.

Figure 3 The 150 AM panel growing in one of the multi-locational research fields (in Urgench, Uzbekistan).

Table 1 Soil chemical properties of Karshi, Urgench and Dongying field locations

Soil Chemical properties	Non-saline	saline	Non-saline	saline
	Karshi		Urgench	
Sodium concentration, dS/m	2.40-6.34	9.24-17.58	3.42-7.05	11.02-19.58
pH	7.67-8.00	7.59-7.81	6.76-8.03	7.54-7.83
***Total dissolved solids* (TDS), mg/L**	1100-8400	2200-11300	1200-1800	1400-10500
Ca^{2+}, me/L	10.0-42.4	17.5-82.3	7.4-14.9	9.9-64.8
Mg^{2+}, me/L	4.9-22.2	7.4-30.4	2.5-.5.0	2.5-40.1
Cl^-/SO_4^{2-}	0.14-1.55	0.16-0.58	0.20-2.13	0.07-1.48
Cl^-, me/L	n.a.	n.a.	2.9-13.8	3.9-66.1
Sodium Absorption Ratio (SAR)	n.a.	n.a.	0.95-5.62	0.48-13.82
Soil texture	silty clay	silty clay	silty clay	silty clay
	Dongying			
Sodium concentration, g/kg	1.9	4.3		
pH	7.58	8.06		
Organic, g/kg	17.86	9.96		
Phosphate, mg/kg	25.52	5.22		
Nitrate, mg/kg	72.02	34.04		
Potassium, mg/kg	258.04	693.15		
Water Content, %	16.56	19.16		
Soil texture	salic fluvisols	Salic fluvisols		

n.a.= not available (measured data were not consistent).

Shoot Na^+ and K^+ concentration (%) determination

The 3rd leaf, stem and the remaining leaves (RLP) of each genotype were analyzed for accumulated K^+ and Na^+ after 25 days of stress with 150 mM NaCl (+7.5 mM $CaCl_2$) in the hydroponics. Three replicates for each genotype were bulked and dried at 55°C for 10 days. The concentrations of K^+ and Na^+ in the respective shoot parts were determined from 2g grounded sample using atomic absorption spectrophotometer (type 2380; Perkin Elmer, Wellesley, MA, USA) and subsequently, the K^+/Na^+ ratios were calculated.

Salt tolerance estimation

The ST status of each genotype was determined for the measured traits across the growth stages as a ratio of trait mean value under salt stress to control condition (Genc *et al*., 2010a). Thereafter, the 150 genotypes were ranked for each trait from the highest down to the lowest trait ST values. The overall ST ranking for each genotype was calculated as:

$$\mathbf{ST}_{Overall} = \sum_i^M \boldsymbol{ST}_{rankings}$$

Where *i* is the ST estimates of genotypes for each measured traits; M is the number of measured traits across growth stages. Genotypes with extreme response to salt stress were identified: tolerant (ST > 75th percentile) and sensitive (STg < 25th percentile).

Physiological analyses of contrasting wheat genotypes

Two genotypes from each extreme were used to examine the effects of salt stress on some plant physiological and growth parameters such as leaf electrolyte leakage (EL), osmotic potential (ψ_π), chlorophyll a fluorescence (ChlF), shoot Cl^- accumulation and shoot biomass production. The genotypes were grown under saline (150 mM NaCl) and non-saline conditions in the controlled conditions (Temperature: 20/15°C; day length: 14 day/10 night hours) in the hydroponics.

Leaf electrolyte leakage (EL) was performed following the procedure outlined by Apostolova *et al.* (2008), with slight modifications. Freshly harvested leaf (0.4 g) were placed in tubes, containing 50 ml distilled water and kept for 4 h in a shaking water bath at 30 °C for measuring the initial conductivity (EC1). The final electrolyte conductivity (EC2) was measured after boiling the leaf samples for 20 min, upon equilibration at 30°C. The rate of EL per minutes (EL_R) for each of the identified genotype was calculated as: $EL_R = (EC2 - EC1)/(0.4 \times 20)$.

Leaf osmotic potential (ψ_π) was determined as outlined by Pérez-López *et al*. (2009). The four youngest leaves were detached from each genotype under non-saline and stress conditions and frozen in liquid nitrogen to break the cell walls. The samples were then thawed and sap was extracted by squeezing with garlic press and micro-centrifugation at 15000 rpm for 5 min. The ψ_π of the extracts were obtained using an OSMOMAT 3000 (Gonotec GmbH, Berlin, Germany). The ψ_π readings were taken from six different plants for each genotype.

Chlorophyll a fluorescence (ChlF) of the leaf samples of an 8-weeks old wheat plants under saline and non-saline conditions were measured using the FluorPen FP100 (Photon Systems Instruments, Brno, Czech Republic). The following OJIP parameters were analyzed: (i) fluorescence fast-transients (Fo = fluorescence intensity at 50 μs, Fj= fluorescence intensity at J-step (at 2 ms), Fi= fluorescence intensity at i-step (at 60 ms), Fm = maximal fluorescence intensity, Fv = maximal variable fluorescence); (ii) PSII efficiencies (Fo/Fm = non-photochemical loss in PSII, Fv/Fo = efficiency of the water-splitting complex on the donor side of PSII, Fv/Fm = quantum yield of PSII, PI(ABS) = performance index on absorption) and, (iii) specific energy fluxes (ABS/RCm= effective antenna size of an active reaction center (RC), TRo/RC,= maximal trapping rate of PSII, ETo/RC= electron transport in an active RC, DIo/RC = effective dissipation in an active RC). A total of 24 data

points were taken for each genotype. The light intensity reaching the leaf was 3000 mol (photons) m^{-2} s^{-1}, which was sufficient to generate maximal fluorescence.

Statistical analysis

Analysis of variance (ANOVA) was carried out for the traits values by adopting the restricted maximum likelihood (REML) model using the GENSTAT 16 program to account for both spatial and temporal differences in the seedling and field screening experiments. The GENSTAT procedure was used to estimate the un-biased estimates of variance components due to genotypic (σ^2_g) and environment (σ^2_e) effects (O'Neill, (2010). Thereafter, the heritability (h^2) estimates for the traits were calculated as described by O'Neill. (2010) and Gitonga et al. (2014) using the equation: $h^2 = (\sigma_g^2)/[\sigma_g^2 + \sigma_e^2/r]$; where r is the number of replications of each genotype.

RESULTS

Phenotypic analysis

Compared to control, all treatment with different salinity concentrations reduced seed germination significantly. These reductions amounted to 7, 19 and 33% for 100, 150 and 200 mM NaCl, respectively and, 14 and 24% for 75 and 100 mM Na_2SO_4, respectively (**Figure 4A**). The interactions of salt treatment and genotypes were significant in all the stress concentrations applied, except for 100 mM NaCl. The effect induced by NaCl stress was stronger than Na_2SO_4, when equal elemental Na^+ concentrations were considered. Significant genotype-by-treatment interactions were also observed in all salt treatments applied, except for 100 mM NaCl. The h^2 estimates were 0.58 under 200 mM NaCl and 0.85 under 100 mM NaCl, while the coefficient of variation (CV) increased from 3 to 8% with the increase in the salt concentrations. The genotypes responded similarly to salt stress of equal elemental sodium (Na^+), as indicated by their comparable values of h^2 and CVs (**Table 2**).

In DW, genotypes responded differently to salt stress as well as between the salt treatments across the four experiments at seedling stage (**Table 2**). Salt stress significantly decreased the DW by 51% in E2, 50.6% in E4, 39% in E3 and 18.6% in E1 (**Figure 4B**). Significant genotypes x treatment interactions were observed in E2 and E3. The h^2 estimates of DW in response to salt stress varied from 0.42 in E1 to 0.73 in E2 and the observed CV of $\geq$ 15%.

Highly significant ($p<0.01$) differences among genotypes, salt treatment and their interactions were detected at all the four field trials. Salt stress caused the highest yield reduction in Dongying (82.8%) and the lowest in Karshi (10.1%). The CV ranged from 16.25% Karshi to 71.6% Dongying, while the highest h^2 estimates were observed in Urgench with 0.76 (**Table 2**).

Table 2 Analysis of ST traits at germination, seedling and maturity growth stages. Shown are: MS-mean squares of 150 genotype (G) and treatment (T), CV - coefficient of variation and h^2- heritability. All the experiments were replicated three times and the number of stars indicates the significance level, one star = $p<0.05$ and two stars=$p<0.01$

Stage	Experiments	MS_G	MS_T	MS_{G*T}	CV_{ST}	h^2
	Germination score after 10 days of salt stress					
	100 mM NaCl	0.56**	48.61**	0.08^{ns}	2.87	0.85
	150 mM NaCl	0.55**	564.20**	0.20**	5.12	0.76
Germination	**200 mM NaCl**	0.49**	1862.09**	0.36**	7.94	0.58
	75 mM Na_2SO_4	0.44**	307.59**	23.5**	4.23	0.8
	100 mM Na_2SO_4	0.49**	1149.08**	0.40**	7.67	0.6
	Dry shoot weight (g/plant) after 25 days of salt stress					
	100 mM NaCl (E1)	716.74**	191.25**	91.01^{ns}	14.57	0.42
Seedling	**100 mM NaCl (E2)**	795.92**	3172.41**	357.04**	16.99	0.57
	75 mM Na_2SO_4 (E3)	583.50**	2104.01**	249.94**	14.74	0.63
	75 mM Na_2SO_4 (E4)	210.69*	1716.28**	125.23^{ns}	15.45	0.73
	Grain yield (t/ha)					
	Urgench	1054.07**	494.71**	281.33**	23.07	0.76
Mature plants	**Syrdarya**	288.18**	-	-	16.41	0.5
	Karshi	747.00**	188.77**	437.95**	16.25	0.57
	Dongying	217.13**	1791.53**	199.11*	71.6	0.23

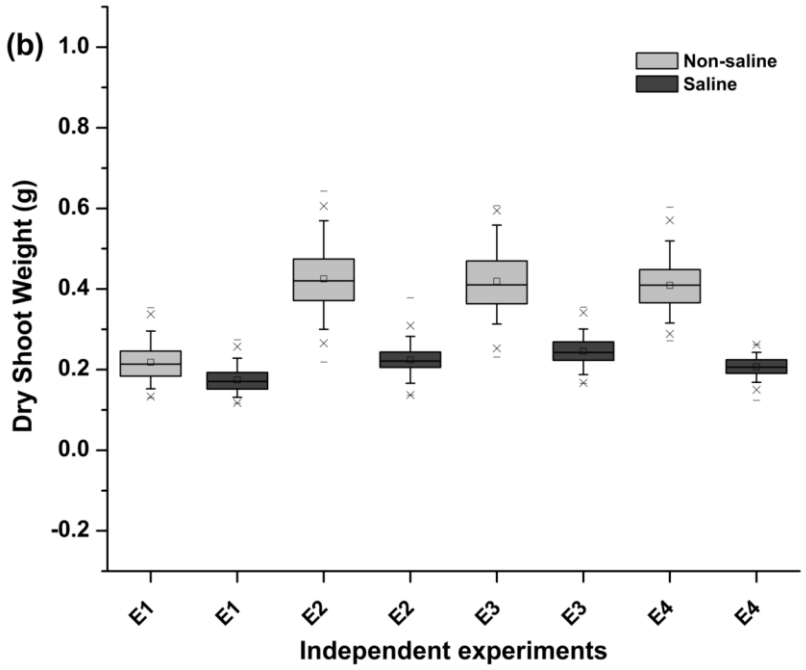


Figure 4 Boxplot showing the effect of salt stress on germination vigour **(a)** and shoot dry mass **(b)** at germination and seedling stages, respectively. E1, E2, E3 and E4 are the four independent screening experiments conducted at the seedling stage in both control and salt stress conditions.

Correlations between ST estimates across growth stages

Significant positive and negative correlations occurred between some pairs of ST traits, based on genotype means, across the growth stages (Table 3). There were significant positive correlations between ST estimates at the germination, and the seedling growth stages, but no apparent significant trend was detected between ST traits for GY at the mature growth stage. Across the growth stages, the DW response to Na_2SO_4 salt increased with the decrease in the germination vigor in response to 100 mM Na_2SO_4, 150 mM NaCl and 200 mM NaCl salt stress. All the significant correlations observed between traits at germination and adult plant stages were negative. However, ST for DW estimated under NaCl salt stress showed negative and positive correlation with the ST for GY in Urgench and Dongying field trials, respectively.

Analysis of the shoot K^+ and Na^+ concentration

Highest K^+ accumulation was found in the stem, and was significantly different from the amount in the 3rd leaf and/or RLP after 25 days of stress (Figure 5). The K^+/Na^+ ratios in the 3rd leaf and stem were similar to each other and varied significantly from the K^+/Na^+ ratio in the RLP. The K^+ and Na^+ concentrations in the 3rd leaf, stem and RLP after 22 days of salt stress were positively correlated with

each other. The shoot K^+/Na^+ ratio value was influenced stronger by the sodium than by potassium (Table 4). The shoot and root water loss due to the salt stresses applied were positively correlated with each other. Data indicated that the shoot K^+ was negatively correlated with root water loss, shoot water loss (NaCl) and shoot water loss (Na_2SO_4); however, shoot Na^+ concentration and shoot K^+/Na^+ ratio did not correlate with the root/shoot water loss.

Table 3 Pearson correlation coefficients among ST estimates of the genotype mean across the three growth stages

Traits	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
	G					S		AP		
$^1G_{75mM\ Na2SO4}$	1									
$^2G_{100mM\ Na2SO4}$	0.517**	1								
$^3G_{100mM\ NaCl}$	0.283**	0.188*	1							
$^4G_{150mM\ NaCl}$	0.495**	0.516**	0.426**	1						
$^5G_{200mM\ NaCl}$	0.563**	0.554**	0.242**	0.528**	1					
$^6DSW_{NaCl}$	-0.009	-0.013	0.04	0.038	0.006	1				
$^7DSW_{Na2SO4}$	-0.101	-0.163*	-0.024	-0.211**	-0.284**	0.171*	1			
$^8GY_{Urgench}$	0	-0.215**	-0.069	-0.071	-0.117	-0.178*	-0.081	1		
$^9GY_{Karshi}$	0.026	-0.025	0.015	0.027	-0.018	0.014	0.081	-0.071	1	
$^{10}GY_{Dongying}$	-0.245**	-0.455**	0.054	-0.026	-0.235**	0.214**	0.021	0.116	0.038	1

**. Correlation is significant at the 0.01 level (2-tailed); *. Correlation is significant at the 0.05 level (2-tailed); G (germination score), S and AP are germination, seedling and mature plant, respectively.

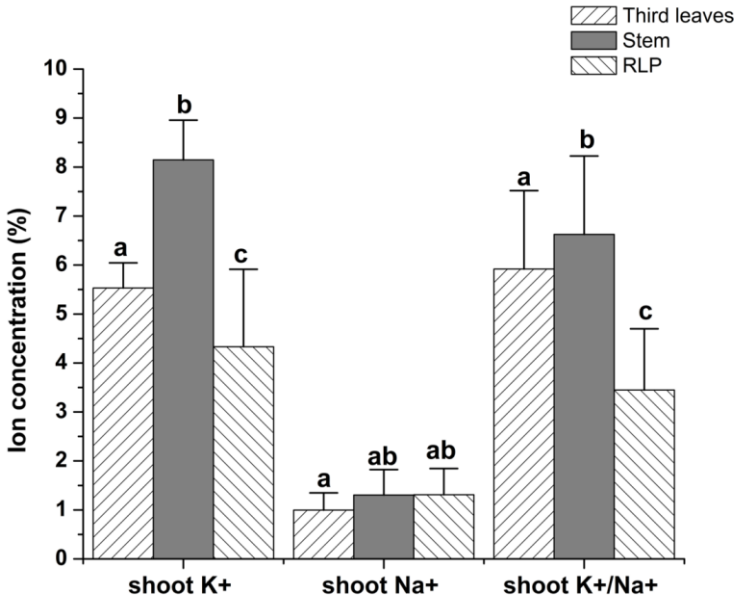


Figure 5 Comparison of the amount of K^+ (A), Na^+ (B) accumulations (in %) and the K^+/Na^+ ratio of the 150 genotypes at different shoot parts: 3rd leaves (blue), stem (red) and remaining leaf parts (RLP) (green), after 25 days under salt stress. Letters on top of the error bars for each shoot parts indicate comparison of the means. Means with the same letter are not significantly different from each other.

Table 4 Correlation coefficients of the genotype mean of root and shoot water losses caused by salt stress conditions and the shoot accumulated K^+ and Na^+ after 25 days under salt stress

Traits	RWL_{NaCl}	RWL_{Na2SO4}	SWL_{NaCl}	SWL_{Na2SO4}	Shoot K^+	Shoot Na^+	Shoot K^+/Na^+ ratio
RWL_{NaCl}	1						
RWL_{Na2SO4}	.348**	1					
SWL_{NaCl}	.705**	.317**	1				
SWL_{Na2SO4}	.311**	.650**	.586**	1			
Shoot K^+	-0.099	-.235**	-.198*	-.259**	1		
Shoot Na^+	0.111	0.036	0.004	-0.045	-0.015	1	
Shoot K^+/Na^+	-0.072	-0.089	-0.067	-0.046	.393**	-.817**	1

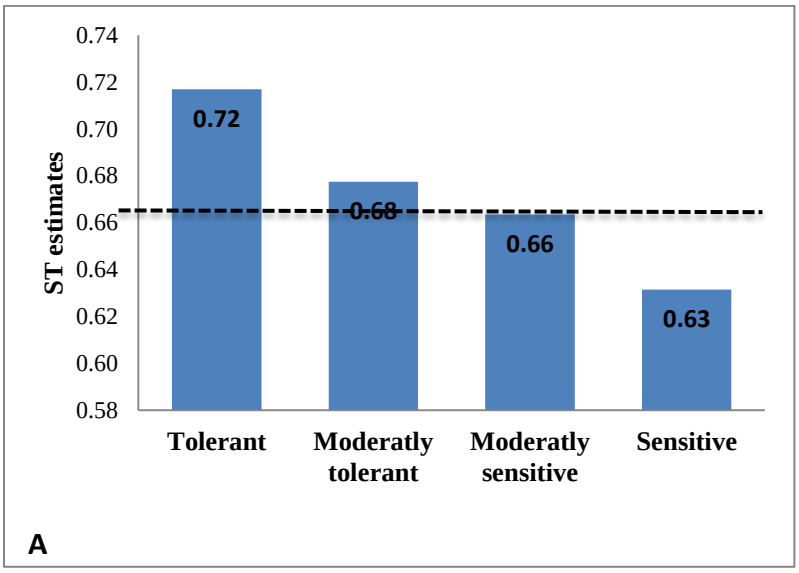
RWL and SWL are root and shoot water loss due to NaCl and Na_2SO_4 salt stress, respectively

ST rankings of the germplasm

Based on the overall ST rankings (Data not shown), 33, 39, 45 and 34 genotypes were considered as tolerant, moderately tolerant, moderately sensitive and sensitive to salt stress, respectively. The mean ST estimates ranged from 0.72 in tolerant genotypes to 0.63 in sensitive genotypes (Figure 6A), while the overall mean was 0.67. The PC1 which accounted for 75.49% of the observed variation in the cluster analysis plot clearly separated the 33 tolerant and 34 sensitive genotypes into two major groups (Figure 6B). While tolerant genotypes showed higher capacity for K^+ uptake in the 3rd leaf and stem (in comparison with the population average) than the sensitive genotypes (Figure 7A), the salt sensitive genotypes had higher accumulated Na^+ than the salt tolerant genotypes in the three shoot parts considered (Figure 7B). These results translated to the significantly higher shoot K^+/Na^+ ratio observed in the tolerant genotypes compared to the sensitive ones (Figure 7C). A total of 22 tolerant and 13 sensitive genotypes exhibited consistent response to salt stress in at least two growth stages (Table 5). Among them, three extreme tolerant (Altay2000, *14IWWYTIR-19* and *UZ-11CWA-8)* and one extreme sensitive (*Bobur)* genotypes were identified across the three growth stages.

Table 5 Salt tolerant and sensitive genotypes identified based on the ST values in more than one growth stages. √ and x - indicate the detected tolerant and sensitive genotypes in the corresponding stage, respectively.

Entry Name	Germination	Seedling	mature field plant
	Tolerant genotypes		
Altay2000	√	√	√
UZ-11CWA-8	√	√	√
14IWWYTIR-19	√	√	√
14IWWYTIR-10	√	√	
14IWWYTIR-20	√	√	
UZ-11CWA-17	√	√	
10AYTIR-9014	√	√	
Esaul	√	√	
KR10-015	√	√	
Demir2000		√	√
Gerek79		√	√
Esook3		√	√
Katia		√	√
14IWWYTIR-7		√	√
14IWWYTIR-8		√	√
14IWWYTIR-35		√	√
UZ-11CWA-5		√	√
UZ-11CWA-6		√	√
UZ-11CWA-11		√	√
14IWWYTIR-30	√		√
14IWWYTIR-38	√		√
169/2004	√		√
	Sensitive genotypes		
Bobur	x	x	x
İzgi2001	x	x	
Konya2002	x	x	
UZ-11CWA-4	x	x	
10AYTIR-9047	x	x	
Oktyabrina	x	x	
14IWWYTIR-14		x	x
UZ-11CWA-13		x	x
UZ-11CWA-24		x	x
10AYTIR-9074		x	x
Turkmen-basy		x	x
Elomon		x	x
KR10-028	x		x



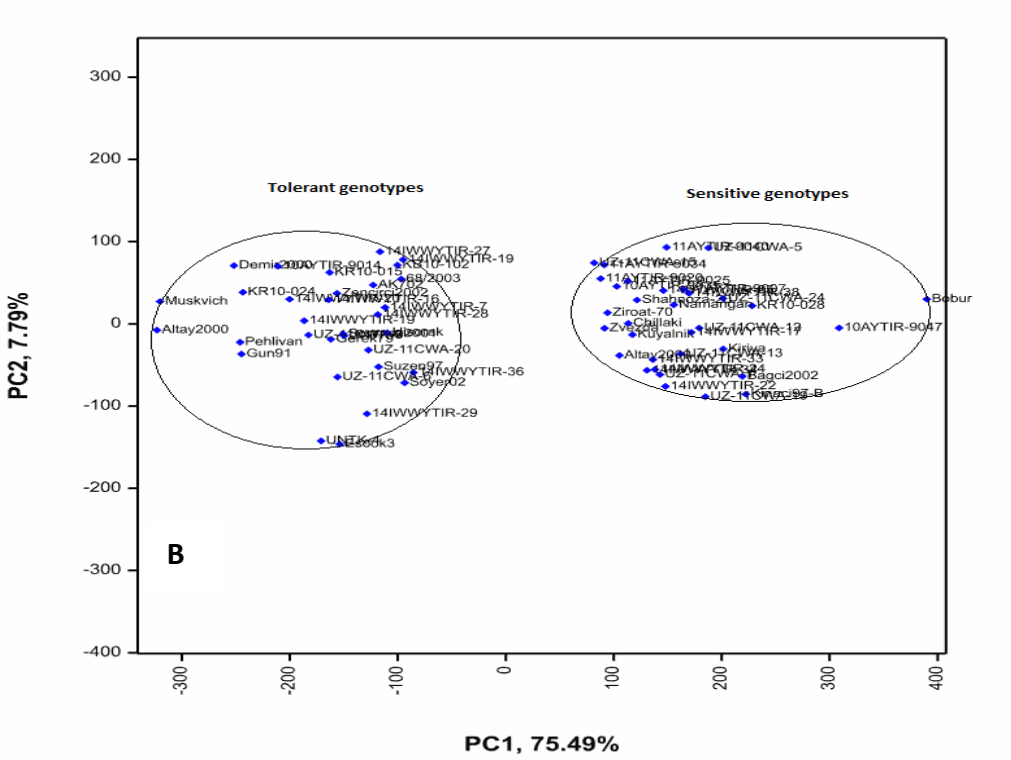


Figure 6 Illustrated the representation of the studied genotypes based on the ST rankings. **(A)** ST status of all the 150 genotypes. The dotted line represents the average ST value of the entire population. **(B)** Scatter plot showing clustering of the the tolerant and sensitive genotypes based on the genotype variance-covariance matrix of their ST rankings across the three growth stages.

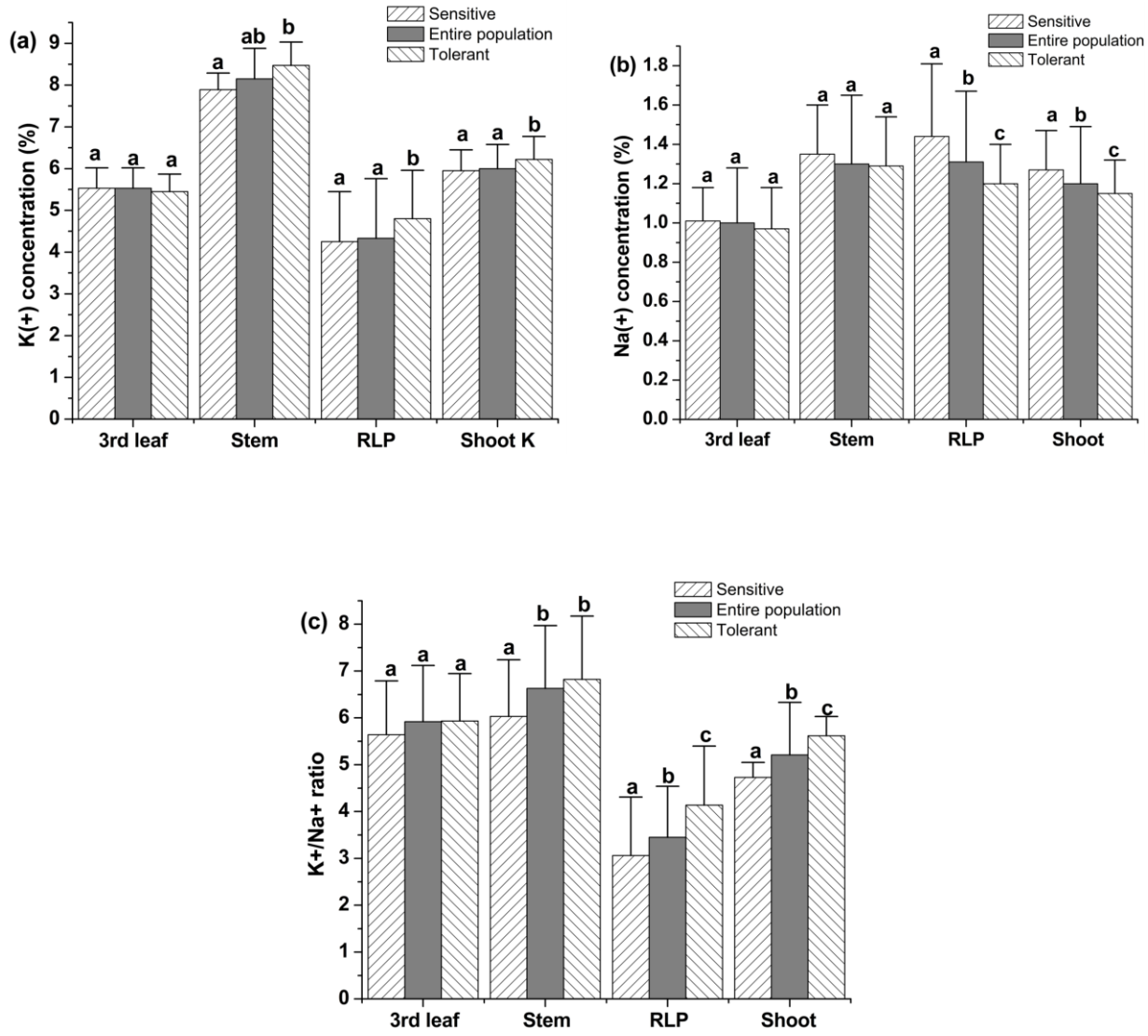


Figure 7 Comparison of elemental constitution of different shoot parts of the studied genotypes. (a–c) show the concentration (%) of K^+, Na^+ and K^+/Na^+ ratio, respectively, for the 34 sensitive, entire studied population and 33 tolerant wheat genotypes after 25 days under salt stress. RLP is the ion concentration in the bulked leaves without the 3rd leaf, whereas the shoot is the mean ion estimates of the three shoot parts. Letters on top of the error bars for each shoot parts indicate comparison of the means. Means with the same letter are not significantly different from each other

Analysis of contrasting genotypes for membrane stability and osmotic potential

The data obtained from the measurements indicate that salt stress affected both the EL and ψπ of the tolerant (*Altay2000* and *UZ-11CWA-8*) and sensitive (*UZ-11CWA-24* and *Bobur*) genotypes (Figure 8). The amount of electrolytes leaked from the membranes of the sensitive genotypes was much higher than that observed in the tolerant genotypes after 8 weeks of salt stress (Figure 8A). The rate of EL of up to 11% and 2% due to salt stress were calculated for the sensitive and tolerant genotypes, respectively. Application of salt stress induced an increase in the osmotic potential of both tolerant and sensitive genotypes, however, the increase was highest in the sensitive genotypes (654 and 660 Osmol/Kg for *UZ-11CWA-24* and *Bobur*, respectively) compared to the tolerant (610 and 575 Osmol/Kg for *Altay2000* and *UZ-11CWA-8*, respectively) genotypes (Figure 8B).

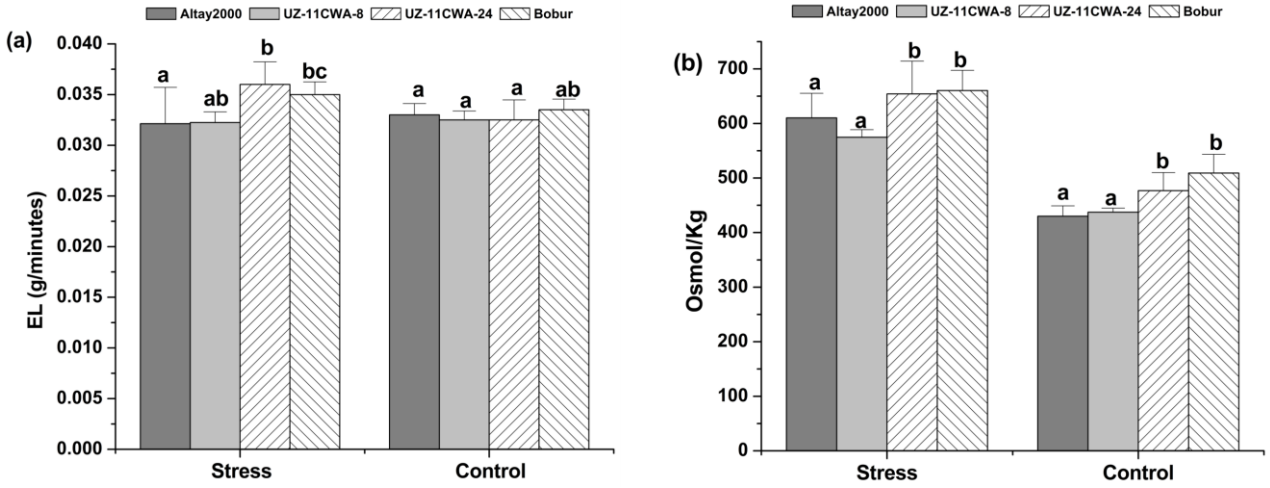


Figure 8 Rate of release of electrolytes into deionized water per-min intervals (a) and osmotic potentials (b) for the leaf segments of the contrasting ST genotypes: tolerant Altay2000 and UZ-11CWA-8 and sensitive UZ-11CWA-24 and Bobur under salt stress and control conditions. Letters on top of the error bars indicate comparison of the genotype means under control and salt stress conditions. Means with the same letter are not significantly different from each other

Analysis of contrasting genotypes for leaf chlorophyll fluorescence

The pattern of fluorescence transients (Fo, Fj, Fi, Fm and Fv) varied among the genotypes under salt stress (Figure 9A), but showed a similar trend under non-saline conditions. Salt stress significantly inhibited the fluorescence transients across all the OJIP phases; but the inhibition was more intense on the two extreme sensitive genotypes. A decrease in the *Fm/Fo* in tolerant genotypes (up to -2.95% and -1.24% for *Altay2000* and *UZ-11CWA-8*) and an increase in sensitive ones (up to +3.0% and +4.09% for *UZ-11CWA-24* and *Bobur*) were observed after application of salt stress (**Table 6**). The *Fv/Fo* and

Fv/Fm also showed similar trend between the two groups. The stress impact on the *PI(ABS)* was genotype dependent. It increased by 7.74% in *Altay2000* but decreased by 2.67%, 6.12% and 8.67% in *UZ-11CWA-8, UZ-11CWA-24* and *Bobur*, respectively. Salt stress also affected negatively all the energy fluxes, except ABS/RC and DIo/RC for Altay2000; however, the effect was more severe on the salt sensitive genotypes (Table 6). The fix area estimates increased in all the genotype under salt stress (Fig 9B), but the increase was much higher (up to +16%) in tolerant genotypes than in sensitive genotypes (up to +8%). The effects of salt stress on some of the physiological parameters described above resulted in the reduction of DW in both the tolerant and sensitive genotypes, although the reduction was much pronounced in the sensitive (79% for *UZ-11CWA-24* and 76% for *Bobur)* than in tolerant *(*21% for *Altay2000* and 24% for *UZ-11CWA-8)* ones (Figure 10).

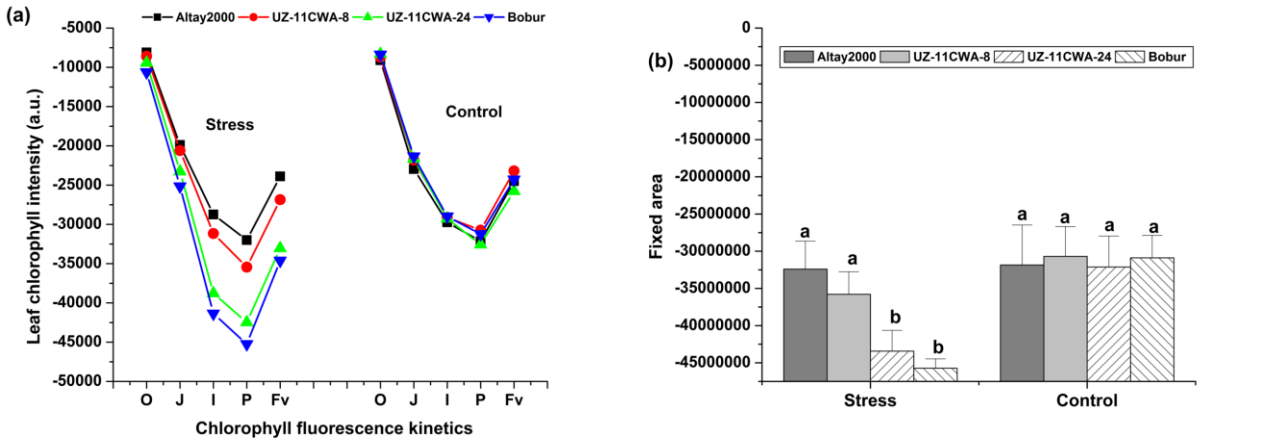


Figure 9 Effect of salt stress on the chlorophyll a fluorescence and OJIP test parameters of light-adapted leaves of two tolerant (Altay2000, UZ-11CWA-8) and two sensitive wheat genotypes (UZ-11CWA-24) identified in this study. (a) Chlorophyll ***a*** fluorescence kinetics curve (Fo, = fluorescence intensity at 50 µs; Fj = fluorescence intensity at J-step (at 2 ms); Fi = fluorescence intensity at i-step (at 60 ms); Fm = maximal fluorescence intensity; Fv = maximal variable fluorescence). **(b)**. Fix area representing the area above the chlorophyll fluorescence curve between Fo and Fm (size of the plastoquinone pool). Letters on the error bars indicate comparison of the genotype means under control and salt stress conditions. Means with the same letter are not significantly different from each other.

Table 6 Effect of salt stress on the energy fluxes of two salt tolerant (in asterisk) and two sensitive wheat genotypes. Fm/Fo, Non-photochemical loss in PSII; Fv/Fo, Efficiency of the water-splitting complex; Fv/Fm, Maximum quantum yield of PSII; PI(ABS), Performance index; ABS/RC, Effective antenna size of an active reaction centre (RC); TRo/RC, Maximal trapping rate of PSII; ETo/RC, Electron transport in an active RC; DIo/RC, Effective dissipation in an active RC.

Energy fluxes	Genotypes	control	stress	Effect of salt (%)
Fm/Fo	Altay2000*	-4.46	-4.33	+2.95
	UZ-11CWA-8*	-4.44	-4.38	+1.24
	UZ-11CWA-24	-4.37	-4.50	-3.01
	Bobur	-4.40	-4.58	-4.09
Fv/Fo	Altay2000*	-3.46	-3.33	+3.80
	UZ-11CWA-8*	-3.44	-3.38	+1.61
	UZ-11CWA-24	-3.37	-3.50	-3.90
	Bobur	-3.40	-3.58	-5.30
Fv/Fm	Altay2000*	-0.77	-0.77	+0.88
	UZ-11CWA-8*	-0.77	-0.77	+0.38
	UZ-11CWA-24	-0.77	-0.78	-0.89
	Bobur	-0.77	-0.78	-1.19
PI(ABS)	Altay2000*	1.55	1.43	+7.47
	UZ-11CWA-8*	1.50	1.54	-2.66
	UZ-11CWA-24	1.46	1.55	-6.12
	Bobur	1.50	1.63	-8.67
ABS/RC	Altay2000*	2.94	2.95	0.49
	UZ-11CWA-8*	2.99	2.92	-2.34
	UZ-11CWA-24	3.12	2.97	-4.83
	Bobur	3.13	2.94	-6.23
TRo/RC	Altay2000*	2.28	2.27	-0.34
	UZ-11CWA-8*	2.31	2.25	-2.7
	UZ-11CWA-24	2.41	2.31	-3.98
	Bobur	2.42	2.29	-5.04
ETo/RC	Altay2000*	1.28	1.26	-1.31
	UZ-11CWA-8*	1.31	1.28	-2.15
	UZ-11CWA-24	1.38	1.31	-5.15
	Bobur	1.40	1.31	-6.3
DIo/RC	Altay2000*	0.66	0.69	3.33
	UZ-11CWA-8*	0.67	0.67	-1.09
	UZ-11CWA-24	0.71	0.66	-7.59
	Bobur	0.72	0.64	-10.25

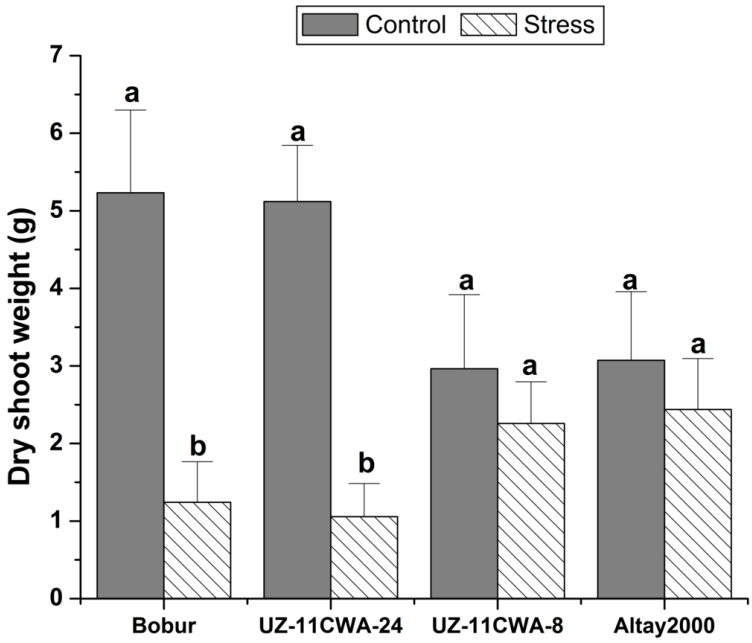


Figure 10 Salt stress intensity (SI) on the sensitive (*UZ-11CWA-24* and *Bobur*) and tolerant (*Altay2000* and *UZ-11CWA-8)* wheat genotypes grown for 6 weeks in hydroponics under 100 mM NaCl stress SI calculated as: $SI = 100[1 - (DW_{stress}/DW_{control})]$ using 14 plants for each genotype. Blue bars represent controls and red stress variants, while the letters on top of the error bars for each genotype indicate comparison of the means under control and salt stress conditions. Means with the same letter are not significantly different from each other.

DISCUSSION

Access to appropriate genetic diversity is critical to current and future breeding efforts to improve wheat yield in the areas affected by soil salinity. Considerable efforts have been made so far to identify salt tolerant wheat genotypes, but with few studies reporting on the simultaneous evaluations of salinity tolerance in more than one growth stages. In the present study, 150 winter and facultative wheat germplasm were evaluated for ST at germination, seedling stage and mature plants grown under field conditions to identify genotypes that can be used in breeding and development of new wheat varieties with improved and desirable level of salt tolerance and for further genetic studies. The studied germplasm showed significant genetic variation for the traits measured across the growth stages. The germination vigor, dry shoot weight and grain yield were negatively affected by salt stress as already reported (Gomes-Filho *et al*., 2008; Munns and Tester, 2008; Rasheed, 2009). However, the variation in the plant growth and development in response to the applied salt stress provided an opportunity to identify genotypes with contrasting attributes under stress amongst the germplasm used. Salt-tolerant genotypes would differ from salt-sensitive ones by allowing optimal growth under saline conditions. The response to the applied salt stress could partly be attributed to inherent different genotype superiority due to the moderate to high heritability estimates in the studied germplasm set.

The ST estimates for each salt concentration at germination stage correlated positively with each other, suggesting similar mechanisms controlling salt tolerance at the germination stage. The within growth stage correlation observed for ST traits at both germination and seedling stages in response to both NaCl and Na_2SO_4 applied stress provides evidence that both salt types are surrogate and, can be used for the evaluation of wheat response to salt stress at the early seedling growth stage. Most of the ST estimates at germination stage were significant and negatively correlated with ST estimates at seedling stage. The mechanisms of salt stress response are highly growth stage-specific and change during the plant life cycle (Walia *et al*., 2005).

Ion analysis revealed that the accumulated K^+ in the stem after stress was significantly higher than that accumulated in the 3rd leaf and RLP but, no significant difference was found between K^+ concentration in the 3rd leaf and RLP. This was in line with the findings in maize (Kobaissi *et al*., 2014) and barley (Booltink and Verhagen, 1997). In contrast, there was no significant difference among the accumulated Na^+ in 3rd leaf, stem and RLP, although highest and lowest amounts were found in the stem and 3rd leaf, respectively. The high K^+ observed in the stem indicates that the ion is transported preferentially through the stem channels to other plant parts under salt stress conditions. The K^+ accumulation in the

3rd leaf, stem and RLP were positively correlated among each other, an indication that K^+ is mobile within the plant and, can be transported from the stem to the other shoot parts. The increase in the shoot K^+ was accompanied by a significant decline in the shoot Na^+, showing antagonism between K^+ and Na^+ (Elhamid *et al*., 2014). Antagonism exits between K^+ and Na^+ in the site of ion uptake due to direct competition of both ions for absorption in the plants (Epstein, 1966).

The rate of root and shoot water loss due to salt stress correlated positively with each other, suggesting that shoot water loss is a direct consequent of the decreased water absorption capacity of root systems due to high osmotic potential exerted by salt stress around the plant rooting zone. The shoot K^+ concentrations increased with the decrease in the rate of root and shoot water loss, an indication that maintaining optimum K^+ status is favorable for water conservation in plant and would ultimately improve the plant growth and survival under salt stress. Reports have also indicated that sufficient K^+ status would contribute to greater water retention in plant tissues, due to its vital role in the osmotic adjustment and turgor regulation during stomatal movement that affects transpiration and photosynthetic rates and xylem hydraulic conductance (Guo *et al*., 2007, Tuna *et al*., 2010; Wang *et al*., 2013b; Sá *et al*., 2014).

Some of the genotypes analyzed in this study have been previously reported to be resilient to different abiotic and biotic stresses. Four genotypes with high ST estimates, have been shown to be resistant to different stresses: Gerek-*79* and *Altay-2000* to drought, salt and cold resistant genotypes (Kara and Kara, 2010; Mutlu *et al*., 2009; Akfirat and Uncuoglu, 2013), *Katia* to zinc and drought tolerance (ICARDA, 2005) and *Demir2000* to lodging, cold, stripe and leaf rust resistant (Mazid *et al*., 2009), have shown to be resistant to different stresses. However, the salt stress sensitive genotype *Bobur* is susceptible to stripe rust at seedling and mature stages (Ziyaev *et al*., 2013). These findings may suggest cross-tolerance among these stress factors in wheat. Mantri *et al.* (2010) reported that plant responses to fungal infection (*Ascochyta blight*) are similar to high-salinity stress.

Among the genotypes identified in this study showing contrasting response to salt stress (Table 1), *Atlay2000, 14IWWYTIR-19* and *UZ-11CWA-8* were tolerant, while *Bobur* was sensitive, across the three growth stages. These genotypes could serve as additional sources of ST for exploitation in breeding programs and genetic studies. The ionomics revealed that the tolerant genotypes had lower shoot Na^+ and higher shoot K^+ concentration than the sensitive ones. Salt tolerant crops are characterized with higher affinity of K^+ over Na^+ uptake (Teakle and Tyerman, 2010, Kausar *et al.*, 2014). The significantly higher shoot K^+/Na^+ ratio compared to the sensitive ones is a consequence of the high

shoot K^+ and low shoot Na^+ concentration. Optimum K^+/Na^+ ratio plays a vital role in maintaining an ideal osmotic and membrane potential for cell volume regulation in plant under salt stress and, has contributed to salt tolerance in wheat (El-Hendawy *et al.,* 2009). Thus the difference in ST among the two extreme genotypes could be attributed to their K^+/Na^+ discrimination ability associated with the machinery of water flow in plant under salt stress. The presented data showed increased levels of EL in sensitive genotypes caused by salt stress, whereas the EL was low in the tolerant genotypes. This suggests a negative impact of the salt stress on the cell membrane integrity. Salt stress would increase reactive oxygen species that often results in programmed cell death in plant (Demidchik *et al.,* 2014). The rate of EL which measures the amount of membranes leaked over a given time period due to membrane injury can be considered useful screening protocol for discriminating among wheat genotypes for ST. Salt stress induced an increase in the leaf osmotic potential in both groups, but the impact was less in *Atlay2000* and *UZ-11CWA*, which could be attributed to efficient osmotic adjustment in the tolerant genotypes due to the higher shoot Na^+/K^+ ratio.

The chlorophyll fluorescence transients (*Fo*, *Fj*, *Fi*, *Fm* and *Fv*) in both tolerant and sensitive genotypes declined **(Figure 9A)** under saline conditions but the sensitive genotypes were more severely affected. The decrease in Fo due to salt stress indicates an increased thermal dissipation (Guidi et al. 2002, Bussotti et al. 2011), while the decrease in Fv may be attributed to the pigment losses due to salt injury. Salinity stress reduces photosynthesis by inhibiting photosystem II complex (PSII) at both acceptor [QA] and donor side (oxygen evolving complex OEC) and destruction of chlorophyll pigments by accumulation of toxic ions (Chen and Murata 2011). However, the higher fluorescence transients observed in the tolerant genotypes can be attributed to higher number of deactivating PSII and PSI associated with increase in the excitation energy (increased energy trapping capacity of PSII) and decrease in the photochemical quenching coefficient (Krause and Weis, 1991, Guidi et al., 2002). Baker (2008) suggested the use of fluorescence induction parameters to detect metabolic perturbations by abiotic stresses. Under salt stress, the *Fv/Fm*, *Fo/Fm* and *Fv/Fo* declined in tolerant genotype and increased in the sensitive genotypes, suggesting different mechanisms controlling these salt-responsive traits in wheat, making them useful parameters for distinguishing salt stress tolerant from sensitive genotypes. However, the quantum yield of PSII as measured by *Fv/Fm* was found to be insensitive to salt stress. The *PI(ABS)* was also affected by salt stress (increased by +7.47% in Atlay2000 and decreased by -2.66%, -6.12% and -8.67% in UZ-11CWA-8, UZ-11CWA-24 and Bobur, respectively), but no noticeable pattern was observed between the tolerant and sensitive genotypes and could be considered genotype specific. The fix Area was twice higher in the tolerant genotypes compared to the

sensitive ones. Salt stress also affected the energy fluxes including *ABS/RC*, *TRo/RC*, *ETo/RC* and *DIo/RC* were mostly negative among the genotypes but the effect was more severe on the sensitive genotypes. From these results, it can be anticipated that salt stress reduced energy absorption, energy trapping efficiency and conversion of excitation energy into electron flow by damaging oxygen evolving complex, over reduction of QA resulting in occurrence of chronic photo-inhibition.

In conclusion, the ST index can be utilized to discriminate against genotypes response to salt stress in wheat. The identified contrasting wheat genotypes clearly showed differential physiological responses mechanisms to salt stress. The tolerant genotypes (*Atlay2000* and *UZ-11CWA-8)* exhibited higher shoot K^+/Na^+ ratio, higher membrane stability, lower osmotic potential and higher rates of PSII photochemical activities than sensitive (*UZ-11CWA-24* and *Bobur*) genotypes which resulted in the significantly higher dry matter observed under salt stress condition. These parameters might be routinely used to screen for salt tolerance in plants and the identified genotypes could be considered for inclusion in wheat breeding program and in future genetic studies for salt tolerance.

CHAPTER 3

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

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ABSTRACT

The increasing salinization of arable lands is a continuing threat to maintaining crop productivity. This study aimed to identify genes conferring salt tolerance (ST) in order to understand the mechanistic basis of salt stress tolerance and to develop breeding and selection strategies in wheat. A genome-wide association study and gene expression analyses were performed on 150 winter wheat germplasm characterised for ST agronomic traits at three growth stages and for ionic (K^+, Na^+ and K^+/Na^+ ratio) parameters to dissect the genetic architectures for ST. A total of 187 significant marker-trait associations (MTAs), representing 37 distinct quantitative trait loci (QTL) regions, were identified for the measured traits using multilocus mixed linear model (MMLM-P+K). Of these, four QTL on chromosomes 1BS, 2AL, 2BS and 3AL were linked to ST-related traits across the three growth stages. Novel ST QTL loci were identified on chromosomes 1BS, 1DL, 5BS and 5BL. Allelic variations were detected in the expressed sequence tags (ESTs) of the identified candidate genes. Comparative gene expression analyses performed using salt-tolerant versus salt-sensitive wheat genotypes under non-saline and saline conditions identified transcriptionally regulated genes in the contrasting panel. These genes were differentially expressed in the contrasting wheat genotypes, suggesting that they contribute to ST in wheat. The identified loci or genes can serve as direct targets for both genetic engineering and selection for wheat trait improvement.

INTRODUCTION

About 800 million hectares of global arable land are salt-affected (FAO, 2008). The extent and severity of salt-affected agricultural land is predicted to worsen as a result of inadequate drainage of irrigated land, rising water tables and global warming (Munns and Gilliham, 2015). It has been estimated that 20% of the irrigated land in the world is presently affected by salinity excluding the regions classified as arid and desert lands (Yamaguchi and Blumwald, 2005). Recent estimates indicated that efforts to combat soil salinity are annually gulping about 27.3 billion US dollars (Qadir *et al*., 2014). 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). With the world's population expected to reach 9 billion by 2050 coupled with the growing salinization in arable lands, an increase in wheat production would be paramount to meet the global demand. Agronomic and engineering solutions are being exhausted in the attempt to minimize the impact of saline land on global

food production. The way forward is to breed greater salt tolerance into present crops and to introduce new species for cultivation (Munns and Gilliham, 2015). Consequently, access to new genetic resources is important in identifying valuable genetic diversity that could be deployed in breeding for ST in wheat.

Under saline conditions, crops exhibit slower growth rates, increased leaf senenses, reduced tillering and, over months, the reproductive development is affected (Munns and Tester, 2008), resulting in significant grain yield reduction. The effect of salinity on crops is due to osmotic stress caused by the accumulation of Na^+ and Cl^- ions to toxic levels within the plant cells and its 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. The cell-based synthesis of osmo-protectants and the mechanisms of ion-homeostasis are essential determinants for salt tolerance (Borsani *et al*., 2003). As the specialization of plant cell progress during ontogeny, the adaptive mechanisms to tolerate salt stress start to differentiate, giving rise to the coordination of all the cellular, tissue and organ responses which are needed for proper tolerance response. It has been suggested that salt tolerance (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 growth stages. The discovery of key genetic switches associated with genes controlling ST at various growth stages would allow not only for characterization of the genetic architectures of salt stress responses, but would also 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 potential of wild relatives to contribute towards improving salinity tolerance. The salinity tolerance of bread wheat is based on a relatively high ability to exclude Na^+ from the leaf blades and an overall increase in the K^+/Na^+ ratio, in some cases associated with increased K^+ uptake. Several studies have reported on the genetic variation for ST at various growth stages in wheat (Schachtman *et al*., 1992; Munns *et al*., 2000; El-Hendawy *et al*. 2005; Rahnama *et al*., 2011; Ahmad *et al*., 2013; Sardouie-Nasab *et al*., 2014), providing great opportunity for ST improvement. However, the drawback of these studies is their inability to simultaneously analyze the genetic variation for salt tolerance at three key growth stages using the same population. In addition, most of the efforts towards exploring the genetic

variation to identify loci associated with salinity tolerance relied on the classical biparental linkage-mapping that are characterized by poor resolution in QTL detection, 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). But, once mapping is developed, it is then necessary to validate the results in different breeding populations.

Genome-wide association studies (GWAS) has emerged as an alternative approach that is maximizing recent advances in genomic tools and statistical methods by exploiting cumulative recombination and mutation events that occurred in a population and taking into account numerous alleles present in the population to identify significant marker-trait associations (MTAs). GWAS has proven to be useful tool to dissect the complex genetic mechanisms governing biotic (Jighly *et al*., 2015; Zegeye *et al*., 2014) and abiotic (Long *et al*., 2013; Turki *et al*., 2014; Edae *et al*., 2014) stress tolerance in many crops. The inclusion of population structure and kinship matrixes in GWAS model during analysis accounts for false positives and thus, improves its effectiveness and power to detect genetic variants for the trait of interest. In wheat, there has been little research into the identification of large-scale salt tolerance loci using GWAS for different stages of growth within the same germplasm simultaneously. It is well known that several genes are differentially expressed in response to a range of biotic and abiotic stresses including drought, heat and salinity (Mukhopadhyay *et al*., 2004; Kang *et al*., 2011; Yarra *et al*., 2012; Li *et al*., 2015; He *et al*., 2015).

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

Materials and Method

Plant Material

The association panel consists of 150 internationally-derived wheat genotypes previously described in Oyiga et al. (2016).

Phenotypic screening

The phenotypic screening for salt stress tolerance at three growth stages and the statistical analyses of the traits have been described in Oyiga *et al*. (2016). Details of all the traits measured are presented in **Table 1.** Briefly, data on the germination potential were collected under two salt types (NaCl and Na_2SO_4) in several concentrations: 100, 150, 200 mM for NaCl and 75, 100 mM for Na_2SO_4 plus control (without salt). At seedling growth 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 for each experiment under saline and non-saline conditions. The amount of biomass (for short and root) accumulation due to salt stress for individual genotype was calculated as difference between DSW and/or DRW values in non-saline and 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° 32'60N and 60°37'60E, 91 meters above sea level (masl)), Karshi (Uzbekistan; 38°52'N and 65°48'E, 416 masl) and Dongying (China; 118°33'-119°20'E, 37°35'-38°12'N, 47 masl). The soil properties of each location have been described (Oyiga *et al*., 2016). Data collected include: grain yield (GY), plant height (PHT), days to maturity (DMT), days to heading (DHD), days to grain filling (GFP) and thousand kernel weights (TKW). The salt tolerance indices of all the traits measured were also calculated according to Genc *et al*. (2010), and were also used in the GWAS studies.

Leaf Na^+ and K^+ content

The amounts of Na^+ and K^+ ions in the third leaf of all the genotypes were measured, after 25 days of growth under saline stress (150 mM NaCl) conditions, from three pooled dried plants of each genotype. The concentration of each ion (Na^+ and K^+) was assessed by Atomic Absorption spectrometer (type 2380; Perkin Elmer, Wellesley, MA, USA) as described in Oyiga *et al*. (2016) and subsequently, the K^+/Na^+ ratios were calculated.

Table 1 Description of the taits studied on the diversity panel of 150 wheat genotypes

Traits	Abbreviation	Descriptions
Germination stage (GS) across five different salt concentrations		
0 mM NaCl	-	Control
100 mM NaCl	-	Screening with 100 mM NaCl salt
150 mM NaCl	-	Screening with 150 mM NaCl salt
200 mM NaCl	-	Screening with 200 mM NaCl salt
75 mM Na_2SO_4	-	Screening with 75 mM Na_2SO_4 salt
100 mM Na_2SO_4	-	Screening with 100 mM Na_2SO_4 salt
***Seedling Stage (SS) in four independent experiments* - E1, E2, E3 and E4**		
Fresh shoot weight	FSW	Fresh shoot weight (g), measured after 24d in both control and saline conditions
Fresh root weight	FRW	Fresh root weight (g), measured after 24d in both control and saline conditions
Dry shoot weight	DSW	Dry shoot weight (g), dried shoot sample in oven set at 65°C for days
Dry root weight	DRW	Dry root weight (g), dried root sample in oven set at 65°C for days
Adult field grown plants (AFP) across three distinct Field trial locations		
Grain yield,	GY	The plots were harvested and the grain cleaned. Cleaned samples were weighed and the grain yield expressed in Kg/ha calculated.
Plant height	PHT	Plant height was measured at physiological maturity from the soil surface to the tip of the head, excluding awns (cm)
Days to maturity	DMT	Days to physiological maturity of 50% of the plants
Days to heading	DHD	Days to heading of 50% of the plants
Days to grain filling	GFP	Days to grain filling 50% of the plant grains
Thousand kernel weights	TKW	Weight of a thousand well developed whole grain dried sample (g)
Ion accumulation in third leaf after 25 days of stress (%)		
Potassium content	K^+	Amount of potassium in the third leaf after 25 days of salt stress
Sodium content	N^{a+}	Amount of sodium in the third leaf after 25 days of salt stress
Ion ratio	K^+/Na^+	-

SNP Genotyping and data curation

The genomic DNA of each genotype was extracted from 12 bulked leaf samples of 2-weeks old seedlings using the Qiagen DNA extraction kit (Qiagen GmbH, , Hilden, Germany), and was assayed with the Illumina iSelect 90K SNP Assay (Wang *et al*., 2014) at the TraitGenetics GmbH, Gatersleben, Germany. The SNP data analyses were performed using the raw intensity data from the Illumina Beadchip and Genome Studio ver. 2011.1 (Illumina) following the methods outlined in Bowers *et al*. (2012).

Population structure

The population structure of the GWAS panel was examined with 582 SNPs (MAF>5%; SNPs with <2% missing data and spaced approximately 2cM apart) using the STRUCTURE V2.3.3 program based on 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 value evaluated. For each run, the initial burn-in period was set to 50,000 followed by 50,000 MCMC iterations. The most likely number of sub-populations was determined using the Delta K method (Evanno *et al*., 2005). All genotypes were subsequently plotted according to their origin, and the genetic relationships among the genotypes were shown graphically via principal coordinates analysis (PCoA) as implemented in GenAlEx 6.5 (Peakall *et al*., 2012).

Linkage disequilibrium (LD) analysis

The LDs among SNP pairs were estimated for A-, B- and D-genomes using the full-matrix option as implemented in TASSEL (Available at: http://www.maizegenetics.net/tassel). Only SNPs with defined genetic map positions and MAF > 5% were included in this analysis. The extent at which LD decay over genetic distance was determined by plotting the pair-wise squared correlation (r^2) values against the distance (cM) between SNPs on same chromosome. The *P*-values for each r^2 estimate were calculated using 1000 permutations. The percentage of SNP-marker pairs above and below the critical LD for each genome was compared. LOESS curve (Breseghello and Sorrells, 2006) was drawn to fit the data using second-degree locally weighted scatter plot smoothing in SAS program (SAS Institute, Cary, NC; http://www.sas.com). The genetic distance corresponding to LD $\leq$0.1 was considered as the critical distance up to which a QTL extends.

Genome-Wide Association Studies

GWAS was performed by adopting the multilocus mixed linear model (MMLM-P+K) that accounted for population structure (P-matrix) and kinship (K-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 principle component analysis (PCA). The K-matrix was considered as random effect and, P-matrix as fixed effect by including five top principal components in the model. Both the P- and I-matrixes were

generated with the TASSEL software (Henderson, 1975; Bradbury *et al*., 2007) and included in the equation below. The vector of the phenotypes estimates as "y" was modeled as:

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

Where **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 the significant MTAs identified within an LD block for each genome were assigned to single QTL region (Pasam *et al*., 2012; Pasam and Sharma, 2014).

Structure analysis of the chromosome region harboring identified QTL

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 first searched in CerealsDB database (http://www.cerealsdb.uk.net/). The obtained sequence information were used as queries for identification of ORFs using BLASTn of the wheat URGI wheat database (https://urgi.versailles.inra.fr/blast/).

Single gene expression analysis

Gene expression analyses of candidate salt-stress responsive genes (identified through the *in silico* analysis) were performed using the salt-tolerant (*Altay2000* and *UZ-11CWA-8*) and salt-sensitive *(UZ-11CWA-24* and *Bobur*) wheat genotypes. These genotypes exhibited contrasting salt stress tolerance phenotypes across growth stages (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 (control) and saline (100 mM NaCl)] was imposed and the pH monitored daily and adjusted to 6.0. At harvest, pooled third leaf samples of 5 representative plants were harvested and immediately frozen in liquid nitrogen. Samples were preserved at -80 ^{o}C until the expression analyses were carried out.

Using the genome-wide gene expression profiling by *MACE* (Massive *Analysis* of cDNA Ends) (GenXPro GmbH, Frankfurt am Main, Germany), the expression levels of the putative genes linked to the significant MTAs were investigated in the 3rd leaves of salt tolerant (*Atlay2000*) and sensitive (*Bobur*) genotypes under saline and non-saline conditions after 2hr, 11d and 24 days of stress

application, in other to have foreknowledge about the transcription of the associated genes. The comparative expression at day 24 was performed to analyse the genes identified to be genetically associated with scored traits at this time point. Thereafter, we performed reverse transcription polymerase chain reaction (RT-PCR) in other to validate the expressions of four of the associated putative candidate genes.

RNA extraction and RT-PCR: Total RNA was extracted from the harvested leaf samples after 30d in saline and non-saline conditions using E.Z.N.A. Plant RNA Kit (Omega Bio-Tek, Norcross, GA, USA) following the manufacturer instructions followed by DNA removal step using DNA Digestion kit (Cat.#F1091). Three microgram of total RNA was used for cDNA synthesis with Thermo Scientific First strand cDNA Synthesis kit (Cat.#K1632) using the oligo $(dT)_{18}$ primers in a 20 µL reaction. The gene quantification was done using real-time PCR on on SDS-7500 Sequence Detection System (Applied Biosystems). The qRT-PCR reaction (20 µl) consisted of gene specific primers (**Table 2)**, DyNamo Color Flash SYBR Green 2X-master mix with ROX (Cat.#F456L) and the template. Thermal cycling conditions were 95^{o}C/7 min followed by 95^{o}C/10s, 60^{o}C/30s, 72^{o}C/30s (fluorescence acquisition) for 40 cycles. The target gene primers were designed around the associated SNP using the primer3 online program (http://primer3.wi.mit.edu/). The expression data were analyzed with the standard methods of Livak and Schmittgen (2001), normalized with the internal control genes, *TaEf-1a* and *TaEf-1b* (Unigene accession Ta659). The PCR reaction efficiencies of both target genes and internal control are comparable (**Figure 1).** The melting curve of the amplified targets is presented in **Figure 2.**

Table 2 Sequences of the PCR primers used in the qRT-PCR and the size of the amplified fragment are shown

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

ZIP7-putative *zinc transporter*; **KefC**-*Glutathione-regulated potassium-efflux system protein*; At**ABC8**-*Putative ABC transporter B family member 8* and; **6-SFT-***sucrose: sucrose 1-fructosytransferase*.

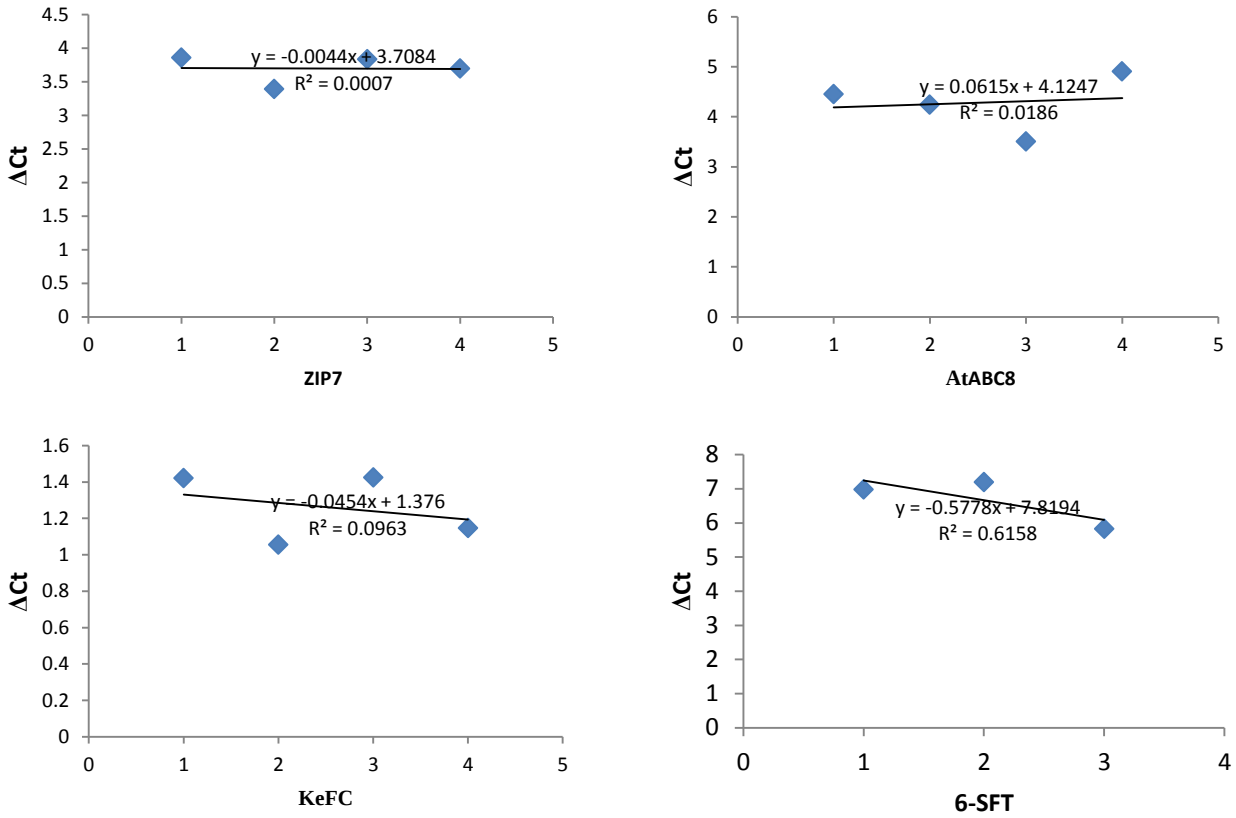


Figure 1 PCR efficiency comparison. CT values were determined for the reference genes and the target genes using pooled DNase treated RNA samples of all the genotypes extracted from treated and untreated leaves. Real-time RT-PCR was performed using DyNamo ColorFlash Probe qPCR Kit. The CT values of target 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.

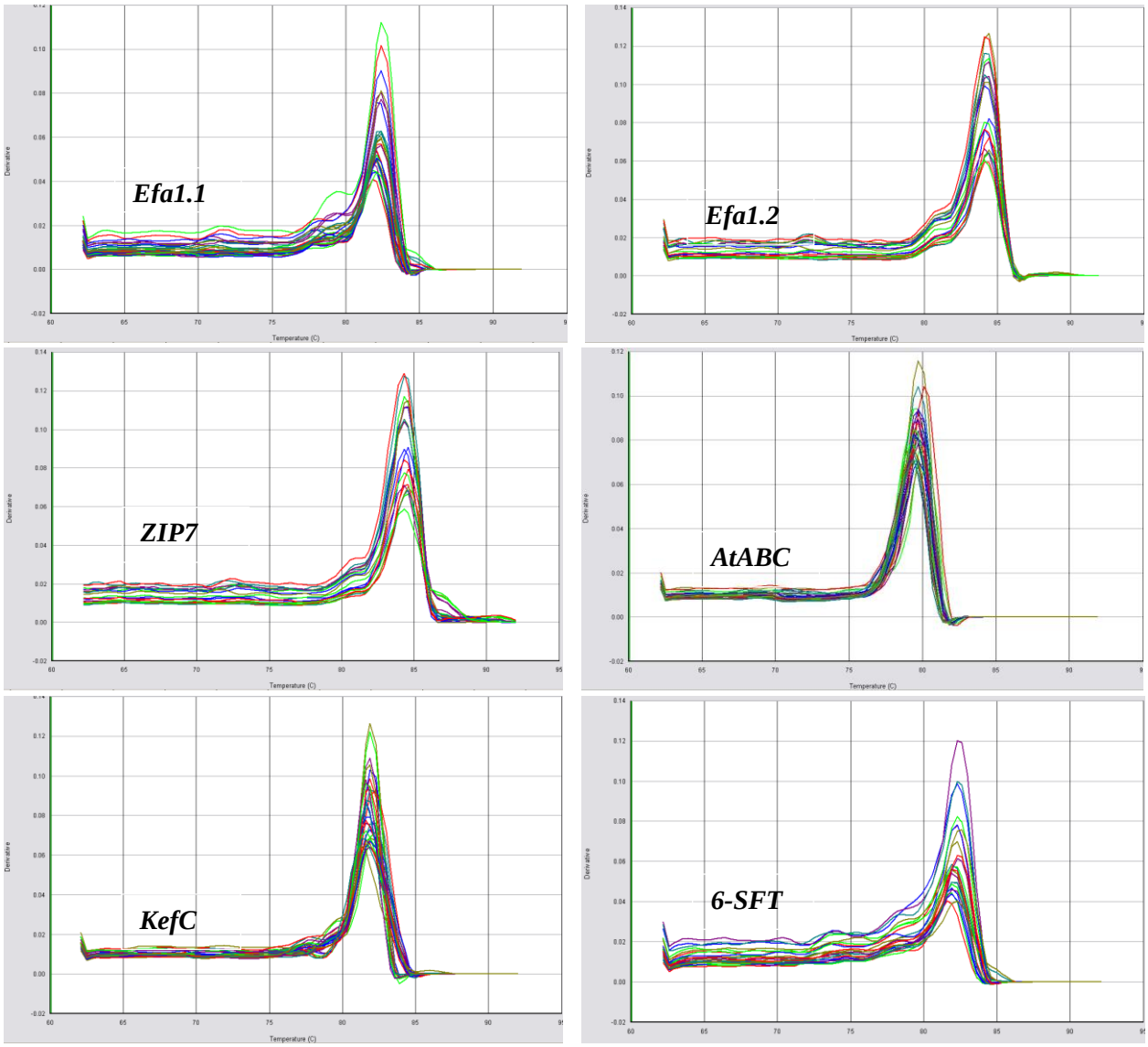


Figure 2 The dissociation curves showing single peaks for endogenous reference genes and four target genes. **ZIP7-**putative *zinc transporter*; **KefC**-*Glutathione-regulated potassium-efflux system protein*; At**ABC8**-*Putative ABC transporter B family member 8* and; **6-SFT-***sucrose: sucrose 1-fructosytransferase*

Comparism of the Expressed Sequence Tags (ESTs) between Altay2000 and Bobur

To examine the relationship between salt tolerance and the putative genes, EST sequences of 6 putative genes in Atlay2000 and Bobur were aligned with their corresponding wheat gene draft sequences available at Ensembl Genomes database (Kersey *et al.*, 2015). The analyzed gene sequences 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*). All the ESTs amino acid sequences were inferred using the Sequence Manipulation Suite (Stothard, 2000) and, aligned using MAFFT version 7 online (http://mafft.cbrc.jp/alignment/server/) to check for possible mutation that are responsible for the observed trait variation.

RESULTS

Genotypic variation of traits for ST

The effect of salt stress on the germination and DSW among the studied panel has been reported (Oyiga *et al*., 2016). At seedling stage, ANOVA results indicate that genotype varied significantly for all the traits measured, except for FRW in E4 (**Table 3**). Salt treatment also showed strong effect on all the remaining three seedling traits across the four experiments, but the interaction effect of genotype x treatment was significant on the traits only in E2 and E3 experiments. The application of salt stress had negative effect on all the seedling stage traits (**Table 3**); however, the reduction was more on the shoot traits (FSW and DSW) than the root traits (FRW and DRW) across the four independent experiments (**Figure 3)**. The trait heritability (h^2) was moderate to high and varied from 0.44 for DRW in E2 to 0.79 for DRW in E4 with the exception of FRW (H_b: 0.30) in E2. Results also indicate that the magnitude of variations among the genotypes in response to the applied salt stress was $\geq$ 15%.

Table 3 ANOVA, heritability estimates (h^2) for ST- traits at seedling growth stage. DRW, dry root weights, FRW, fresh root weight; FSW; fresh shoot weight. Number of stars indicates the significant level, one star = p<0.05, two stars=p<0.01 and ns= non-significant.

Experiments	Trait	G	T	G*T	h^2	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

G= genotype effect; **T**= treatment effect; **G*T**= interaction effect of G and T; **CV**= coefficient of variations. E1, E2, E3 and E4 are four independent salt screening experiments

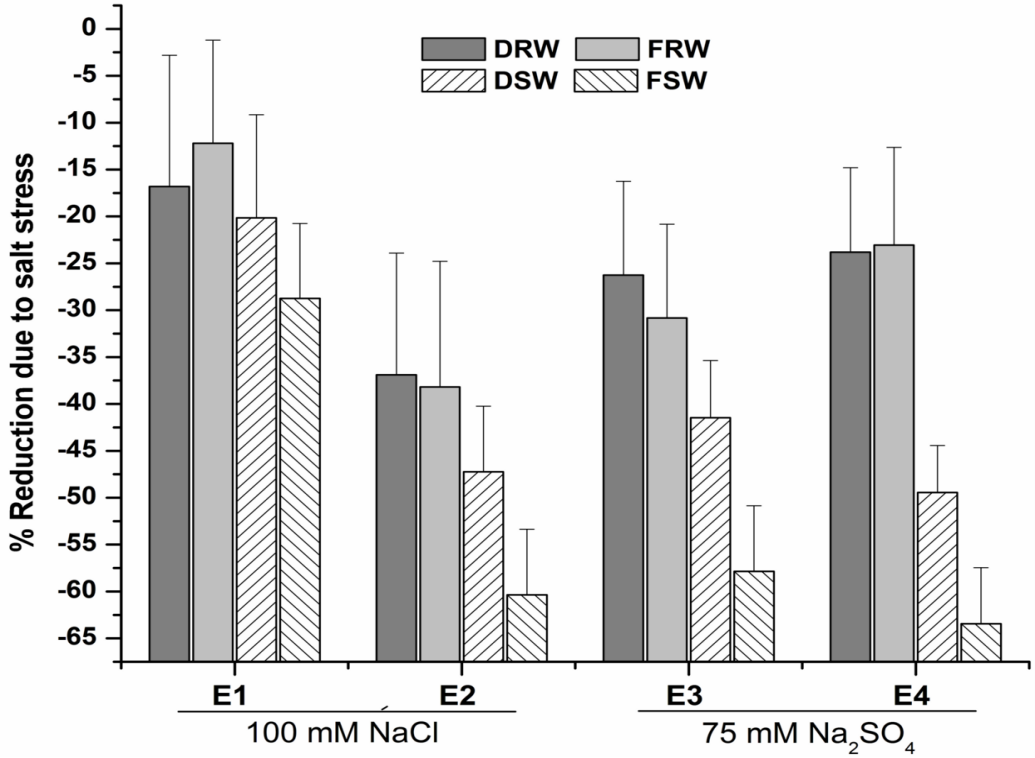


Figure 3 Histogram showing the effect of salt stress on the DRW (dry root weight), FRW (fresh root weight, DSW (dry shoot weight) and FSW (fresh root weight) across the four experiments at seedling stage. **E1**, **E2**, **E3** and **E4** are four independent salt screening experiments

For all the AFP traits (TKW, PH, DHD, DMT and GY) evaluated, there was highly significant genotype effect, except for PH at Dongying (**Table 4**). Soil salinity impacted negatively on all the AFP traits except for TKW and DHD at Urgench and Karshi locations, respectively. The genotype-by-saline soil interactions were also observed in most of the traits measured. The h^2 estimates of the measured traits at Urgench and Karshi locations ranged from 0.54 for DHD to 0.89 for TKW at Karshi. The lowest h^2 (0.08) was observed for PH at Dongying location. The magnitudes of variation observed for the ST-traits was highest (44.3%) for PH at Dongying) and lowest (1.3%) for DMT at Karshi.

Table 4 ANOVA, heritability estimates (h^2) for ST- traits at adult field grown plants. Shown is the effect of genotype (G), treatment (T) and their interactions (G*T), CV_{ST} - coefficient of variation. Number of stars indicates the significance level, one star = p<0.05, two stars=p<0.01 and ns= non-significant.

Field Locations	Traits	G	T	G*T	h^2	CV_{ST}
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

TKW= thousand kernel weight; PHT= plant height; DHD= days to heading; GFP= days to grain filling; DMT= days to maturity; GY= grain yield.

Figure 4 shows the frequency distribution of ions accumulated in the third leaves among the 150 genotypes after 25 days of salt treatment. The leaf K^+, Na^+ and K^+/Na^+ ratio were normally distributed. Among them, the leaf K^+ concentrations showed comparatively narrowest variation (CV=8.84%, ranged from 4.14 to 6.90%; **Figure 4A**), whereas Na^+ concentration (CV= 28.14%; **Figure 4B**) varied from 0.59 to 3.11% and K^+/Na^+ ratio (CV= 26.80%; **Figure 4C**) from 2.07 to 10.67%. The relationships between the root biomass production under salt stress and the leaf ion concentrations are shown in **Figure 5**. There was no significant pattern observed between the K^+ concentration and the root biomass production in response to salt treatment (**Figure 5A**). However, the Na^+ concentration (r^2= 0.47**; **Figure 5B**) and K^+/Na^+ ratio (r^2 = - 0.24**; **Figure 5C**) in the third leaf showed an observable pattern with the root biomass production in response to salt treatment after 25 days of stress.

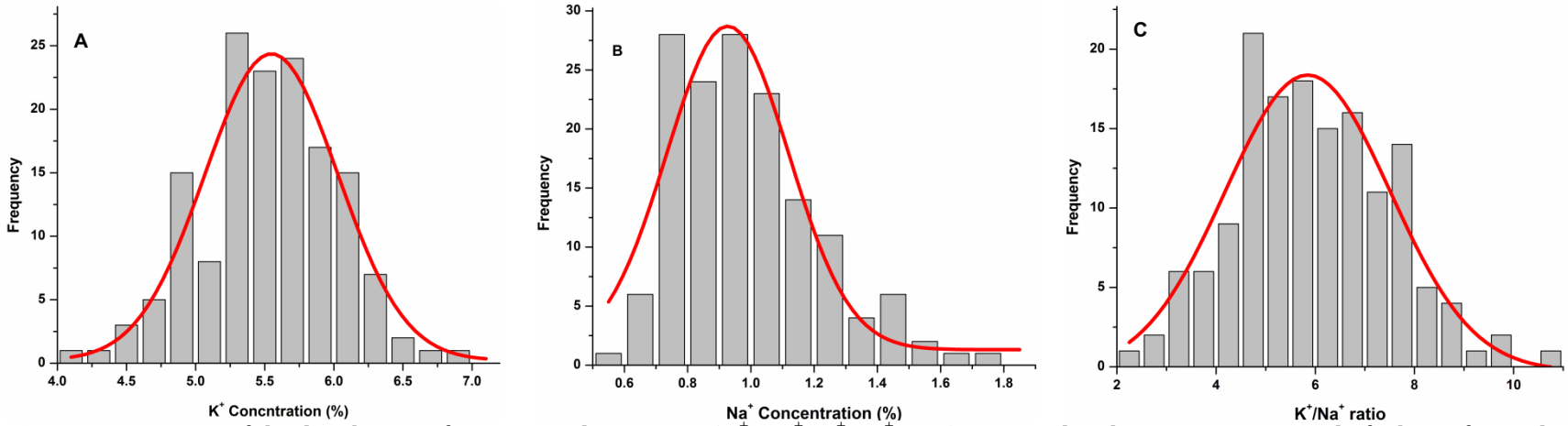


Figure 4 Histogram of the distribution of ion accumulation traits (K^+, Na^+, K^+/Na^+ ratio) measured in the 150 GWAM panel of wheat after 24d under stress.

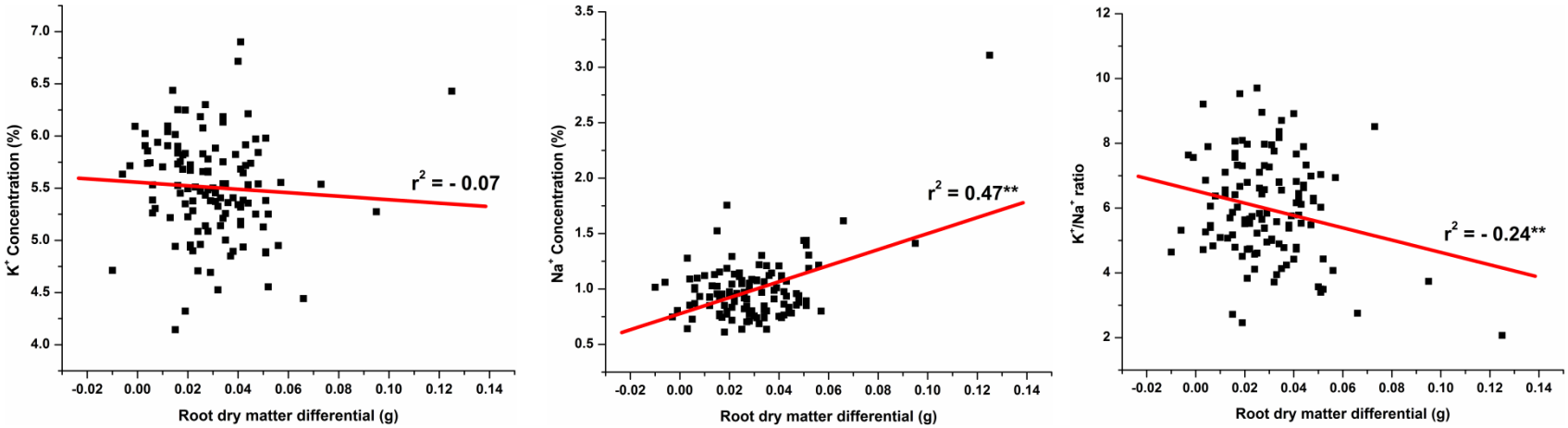


Figure 5 Relationship of root dry weight differential, calculated as a difference between dry root weight in non-saline and saline conditions, with leaf K^+ and Na^+ concentration and the estimated K^+/Na^+ ratio in the third leaves of the 150 GWAM panel grown in the hydroponics and treated with 150 mM NaCl. Concentrations of K^+ and Na^+ were estimated using Atomic Absorption Spectrometer after 24 day of stress.

SNP marker analysis

After applying several filters (SNPs with MAF<5%; missing data < 5% were excluded), a total of 18,085 SNPs with known genetic positions were found to be polymorphic, but were not evenly distributed among the three wheat genome (Table 5; Figure 6). 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. The SNP-map spanned a total genetic distance of 3644.10 cM with an average SNP-marker density of 0.49 cM. The longest genetic distance between SNPs was 242 cM.

Table 5 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.

Chromosomes	**Length (cM)**	**Number of mapped SNP**	**SNP densitiy (cM)**	**Number of tests**	**Number of tests revealing LD (r^2>0.1)**	**Marker pairs in LD (%)**
A-genome	1252.28	7,001	0.18	7063880	964558	13.65
B-genome	1139.62	9,246	0.13	6057777	1044383	17.24
D-genome	1252.2	1,567	1.16	201713	48031	23.81
1A	156.3	959	0.16	459361	58828	12.81
1B	173.62	1,760	0.10	1549680	311087	20.07
1D	181.52	482	0.38	115921	22567	19.47
2A	185.46	1,087	0.17	589155	113623	19.29
2B	185.67	1,615	0.11	587815	96483	16.41
2D	152.84	524	0.29	27375	16585	60.58
3A	193.78	892	0.22	397386	53380	13.43
3B	144.74	1,206	0.12	799480	108179	13.53
3D	156.06	197	0.79	19306	3371	17.46
4A	164.13	830	0.20	344035	69007	20.06
4B	115.45	537	0.21	143916	32610	22.66
4D	170.43	60	2.84	1770	316	17.85
5A	148.3	1,058	0.14	559153	72074	12.89
5B	219.77	1,785	0.12	1595791	185217	11.61
5D	207.33	171	1.21	14535	2390	16.44
6A	162.91	1,101	0.15	606651	65422	10.78
6B	122.92	1,276	0.10	813450	217376	26.72
6D	160.5	1,67	0.96	14028	2098	14.96
7A	241.4	1,074	0.22	576201	49945	8.67
7B	177.45	1,067	0.17	567645	93431	16.46
7D	223.52	133	1.68	8778	704	8.02
Genome-wide	**3644.1**	**17,814**	**0.49**	**13323375**	**2056972**	**15.44**

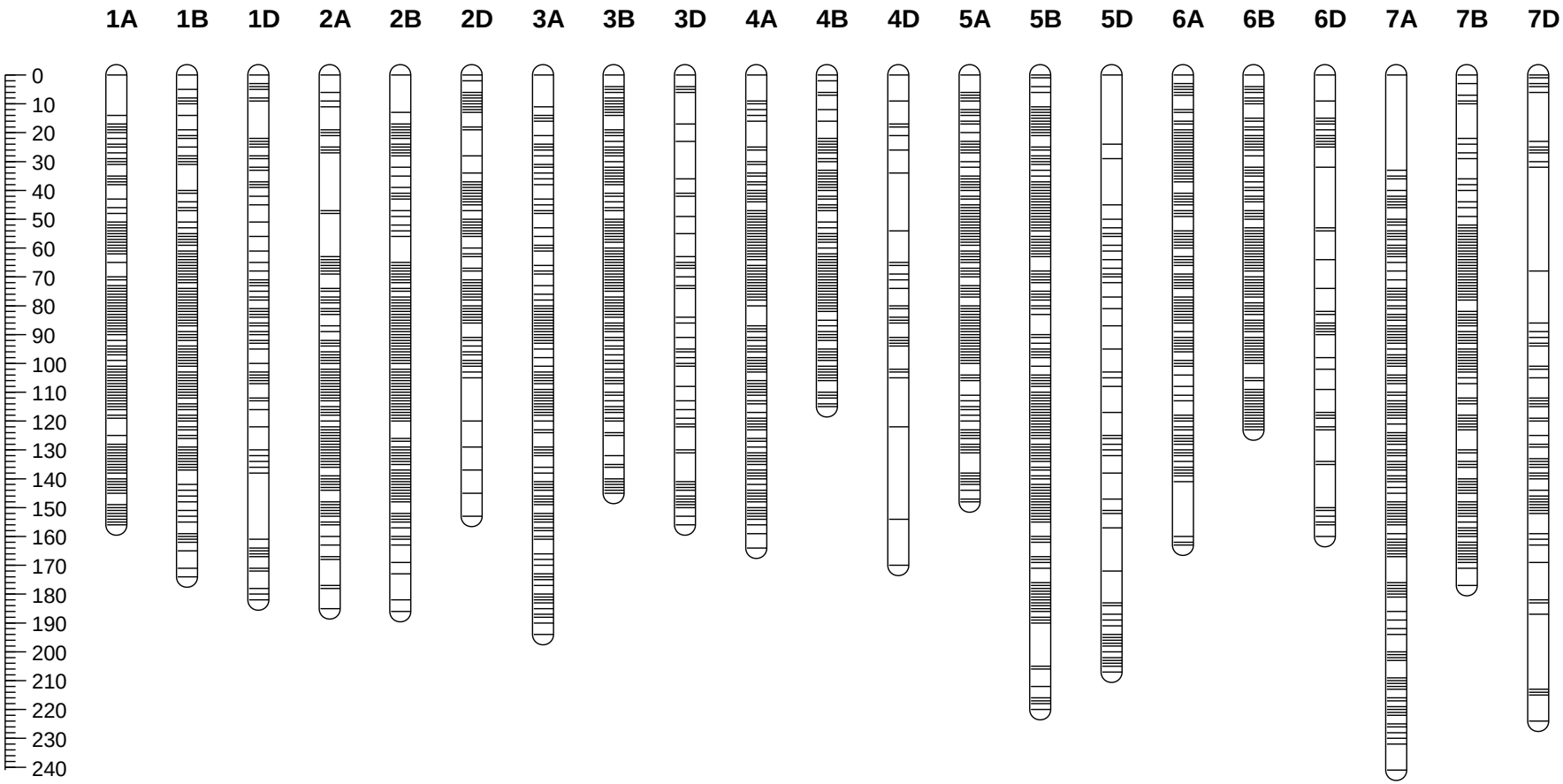


Figure 6 Genetic linkage maps of wheat containing 18,027 SNP markers spanning 3643.10 cM over 21 chromosomes based on GWAS panel. The scale in centi-Morgans (cM) is given at the y-axes and chromosomes at the x-axes. Horizontal lines represent the positions of SNPs on each of the corresponding chromosome.

Population structure

Population structure analysis of 150 GWAM panel indicated that the most likely number of subpopulations (K) was two. The maximum value of ΔK occurred at K=2 (Figure 7), confirming that two sub-populations provided the optimal structure. Principal coordinate analysis (PCoA) of the panel also revealed two major sub-groupings (Figure 8); however, there was no evidence of clear groupings among genotypes based on the origin of the germplasm. The estimates of genetic diversity in the panel based on neighbor joining (NJ) trees revealed similar results (data not shown), which confirmed the results obtained using the STRUCTURE and PCoA analyses.

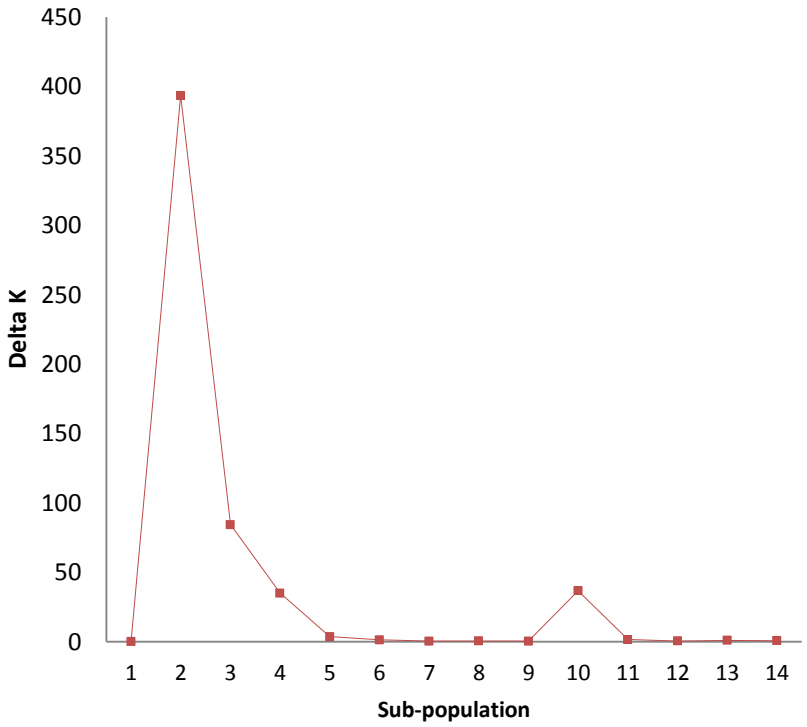


Figure 7 Magnitude of delta K as a function of K-values= 1 to 14 (x-axes) in the GWAM panel. A distinct peak at K=2 was indicative that model with 2 sub-groups was optimal.

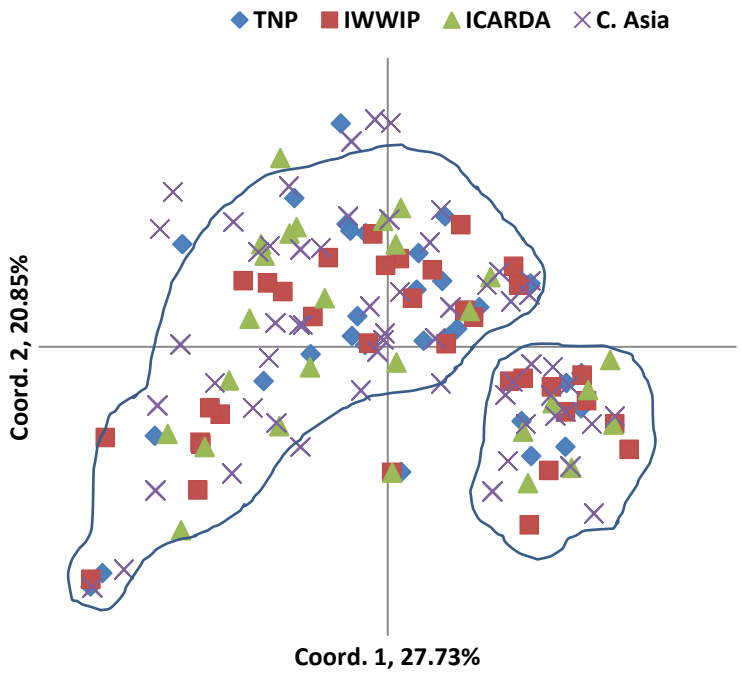


Figure 8 Principal coordinates analysis (PCoA) of the association panel based on genetic distance estimates. The colored figures in the plot represent the core collection centers: blue-TNP (Turkey National breeding program); red-IWWIP, green-ICARDA-CYMMYT and the cross the Central Asia.

Linkage Disequilibrium Decay

Figure 9 show that LD decays with increase in the genetic distance. The lowest LD decay of 14 cM was found in the D-genome and at approximately 10 and 11 cM in the A- and B-genomes, respectively, with the genome-wide LD decay of about 13 cM. 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 (Table 5). Individually, chromosome 2D (60.58%), 6B (26.722%), 4B (22.66%), 1B (20.07%) and 4A (20.06%) chromosomes had the highest number of SNPs in significant LD. Since the average inter-SNP-marker distance is 0.49 cM, indicates that the marker coverage used for this study was appropriate for detecting QTL using a GWAS approach.

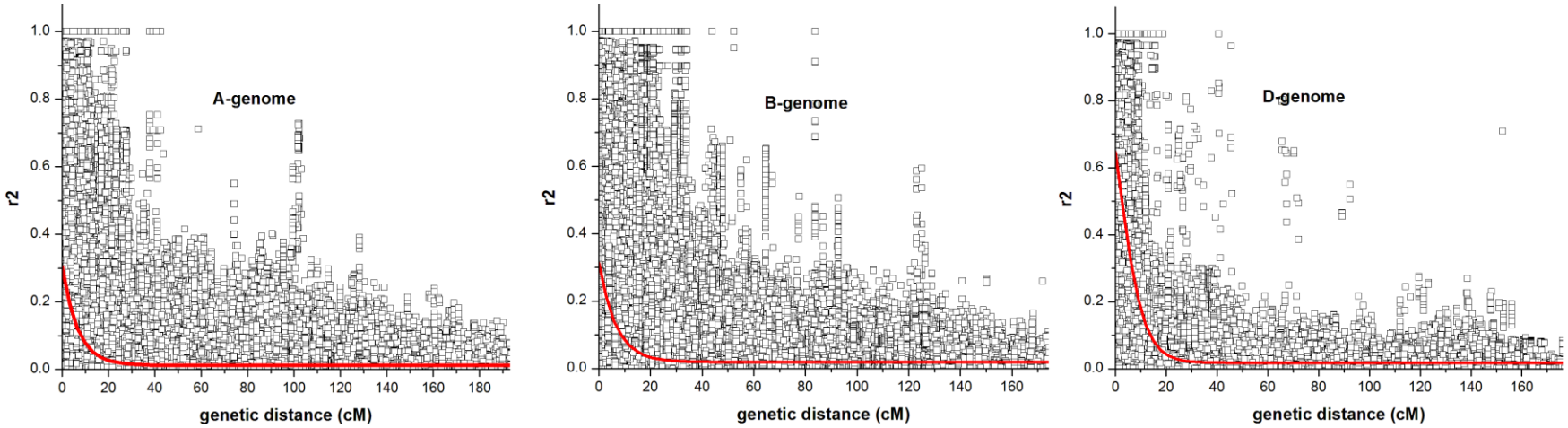


Figure 9 LD decay analysis of A-, B- and D-genome. Inner fitted trend line is a non-linear logarithmic regression curve of r^2 on genetic distance. LD decay is considered below $r^2 = 0.1$ threshold.

MTAs for the phenotypic traits across growth stages

A total of 172 significant MTAs were detected for ST with all the measured traits using MLM + PK at $p<0.01$, each explain 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-, seedling- and AFP-growth stages, respectively. Highest number of MTAs were detected on the A- genome (77) followed by B-genome (68), and the D-genome (8) in that order. Several of the detected 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 at different growth stages are presented in **Table 6.**

Chromosomal regions harboring multiple MTAs for the phenotypic traits

Several SNPs were significantly associated with salt-stress related traits in more than one growth stage. For example, SNP *GENE_3156_152* at 68.36 cM on 5BL had remarkable effect on the traits at germination (germination vigor under 75 mM Na_2SO_4) and seedling (FSW, FRW, DSW and DRW) growth stages. This SNP accounted for 24.20% of the observed R^2. Similarly, SNP marker *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 phenotypic variance at both 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 with germination vigor (under 100 mM $Na_2SO_{4)}$ and ST_DSW, respectively.

SNPs with pleiotropic and growth stage specific effects were identified on five chromosomes - 1DS, 2AL, 2DS, 3AL and 7BL. The 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* showed 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 associated with FSW and DRW, while the later was linked with FSW and DSW. On chromosome 3AL, two SNPs affecting ST_FSW (E1+E2) and ST_DSW (E3+E4) were detected in a 0.1-cM interval (*Jagger_c765_61* - *wsnp_RFL_Contig2011_121680*).

Table 6 Summary of significant SNP marker-trait associations at germination, seedling and adult field growth stages

Experiment	mM	SNP	Chr	Pos	Alleles	MAF	-log$_{10}$ (p)	R^2	Effect
		Germination growth stage							
	75	Kukri_c18230_1633	1BL	137	C/A	0.38	7.14	11.04	
	75	RAC875_c30507_292	3AL	123.01	G/A	0.37	4.06	5.87	-
	75	tplb0031e09_1763	3BS	67.45	T/C	0.29	4.34	12.47	-
	75	RAC875_c34981_294	4AL	164.13	C/T	0.14	4.11	11.85	+
	75	Kukri_c35140_75	4BL	64.26	A/G	0.35	4.49	12.88	+
	75	IAAV8258	5AL	86.91	C/T	0.19	13.04	16.22	
	75	GENE_3156_152	5BL	68.36	C/T	0.14	21.51	21.22	
	75	Excalibur_c4699_215	5BL	151.62	C/T	0.38	5.30	7.71	
Na_2SO_4	**75**	BS00003852_51	Unk	-	G/A	0.08	12.06	10.93	
	100	BobWhite_c8218_162	1BS	62.32	C/T	0.15	3.95	9.17	-
	100	Ku_c6019_806	**2AL**	103.62	A/G	0.33	4.21	12.12	-
	100	Kukri_c38852_100	2AL	103.62	G/A	0.34	4.15	11.96	+
	100	RAC875_rep_c109658_382	5BL	131.79	T/C	0.22	4.57	9.14	-
	100	BobWhite_c10954_467	5BS	38.5	T/G	0.23	4.05	9.15	-
	100	Ra_c7974_1192	7BS	71.33	C/T	0.4	4.06	11.72	-
	100	IAAV565	1BL	122.52	C/T	0.27	5.44	15.38	
	100	wsnp_Ku_c32477_42086760	5BS	40.6	G/A	0.24	4.11	11.85	
	100	Kukri_c41157_433	6BL	122.92	A/G	0.32	4.08	11.77	
	100	BS00063365_51	Unkn	-	C/T	0.42	4.17	12.02	
	100	RAC875_c4682_646	Unkn	-	G/A	0.09	4.04	11.66	
	150	wsnp_Ex_rep_c68117_66883366	5AL	43.27	G/A	0.31	4.43	12.72	-
NaCl	**150**	Excalibur_c2978_667	6AS	25.53	G/T	0.31	5.18	**7.31**	-
	200	Kukri_c29039_315	1AL	83.7	T/C	0.22	4.21	12.12	+
	200	wsnp_JD_c12088_12411845	2AL	106.3	G/A	0.37	4.15	11.96	-
	200	Ex_c12563_1279	2A	103.62	A/G	0.32	4.49	12.88	-
	200	wsnp_Ku_c4042_7375053	2BS	88.93	C/T	0.1	4.01	11.58	+
	200	wsnp_RFL_Contig4814_5829093	3AL	89.36	C/T	0.38	4.08	11.77	-
	200	RAC875_c20785_1219	5BL	100.64	T/C	0.34	4.79	13.68	+
	200	Kukri_rep_c109463_264	5BL	154.54	T/C	0.39	4.95	14.1	+
	200	BS00022758_51	Unkn	-	C/T	0.11	4.28	12.31	
		Seedling growth stages							
E1	ST_FSW	RFL_Contig7_380	1BL	90.26	C/T	0.13	4.21	12.12	-
E1	DSW	IAAV5776	1BL	159.87	A/G	0.09	5.53	7.45	-
E1	DRW	GENE_1353_136	2AL	101.97	C/T	0.10	14.62	16.93	-
E1	DSW	GENE_1353_136	2AL	101.97	C/T	0.10	16.6	19.57	-
E1	FRW	GENE_1353_136	2AL	101.97	C/T	0.10	20.27	22.09	-
E1	ST_FSW	RAC875_c38018_278	2AL	110.13	CT	0.41	4.53	12.98	+
E1	DSW	Excalibur_c91176_326	2AL	150.29	G/A	0.42	8.82	10.76	+
E1	ST_DSW	BS00091763_51	2AL	166.66	A/G	0.26	9.03	14.02	-
E1	ST_DRW	Excalibur_c25921_230	2BS	96.99	C/T	0.19	4.21	12.12	+
E1	ST_DRW	Ex_c18484_2026	3AL	88.02	C/T	0.34	4.11	11.85	-
E1	ST_FSW	tplb0033c09_1345	4AL	91.19	C/T	0.15	4.79	13.68	-
E1	DSW	Excalibur_c6314_91	5AL	53.11	G/A	0.34	9.07	14.56	-
E1	ST_FSW	wsnp_Ex_c6314_10992814	5AL	53.47	A/G	0.37	3.96	11.45	-
E1	ST_DRW	BS00029412_51	5AL	75.96	C/A	0.17	5.20	14.76	-
E1	DRW	GENE_3156_152	5BL	68.36	C/T	0.14	20.09	20.15	-
E1	DSW	GENE_3156_152	5BL	68.36	C/T	0.14	23.75	24.2	-
E1	FRW	GENE_3156_152	5BL	68.36	C/T	0.14	24.46	23.04	-
E1	FSW	GENE_3156_152	5BL	68.36	C/T)	0.14	19.3	21.81	-
E1	ST_FRW	wsnp_Ex_c18965_27868480	6AL	79.08	A/G	0.25	4.01	11.58	-
E1	ST_FRW	wsnp_Ex_c19770_28768859	6AS	77.14	T/C	0.08	4.42	12.69	-
E1	ST_DSW	BS00068032_51	7AL	212.66	G/A	0.45	4.05	11.69	-
E1	ST_DSW	IACX5996	7AL	226.07	C/T	0.12	4.34	12.47	
E1	ST_DSW	RAC875_c14173_207	7AS	33.45	C/T	0.08	5.03	14.31	+
E1	FSW	BS00032623_51	7AS	59.07	C/T	0.06	3.61	4.68	+
E1	DRW	Excalibur_c60598_158	Un	-	T/C	0.09	13.16	12.64	+
E1	DSW	Excalibur_c28592_377	Un	-	C/T	0.11	3.26	4.05	+
E1	DSW	Excalibur_c60598_158	Un	-	T/C	0.09	11.55	14.6	+

E1	FRW	Excalibur_c60598_158	Un	-	T/C	0.09	8.16	7.64	+
E2	DSW	CAP7_c4879_249	1AL	101.64	C/A	0.45	5.35	7.17	-
E2	ST_FRW	wsnp_Ku_c66585_65967792	1BS	65.42	T/C	0.22	4.27	12.29	-
E2	ST_DRW	BobWhite_c11044_322	1BL	83.47	T/C	0.35	5.64	15.9	+
E2	ST_DRW	BobWhite_c8293_236	1BL	137	C/T	0.06	3.95	11.42	-
E2	DRW	BS00063512_51	1BL	160.9	T/C	0.09	8.66	11.52	-
E2	ST_DRW	BS00002178_51	1DS	67.72	A/G	0.48	6.64	10.64	-
E2	DRW	Excalibur_c91176_326	2AL	150.29	G/A	0.42	7.88	9.77	+
E2	FRW	BobWhite_c5756_532	2BL	181.92	C/A	0.34	7.32	12.87	+
E2	FRW	IAAV790	2DL	97.14	T/G	0.42	10.13	5.92	-
E2	FSW	D_GBUVHFX02GV41H_67	2DS	36.54	A/G	0.07	5.25	7.5	-
E2	DSW	Kukri_c5579_466	2DS	50.83	G/A	0.13	11.43	13.17	+
E2	ST_DSW	BS00063300_51	3AL	109.95	G/A	0.10	4.50	12.9	+
E2	ST_DSW	BS00073732_51	3BS	61.64	C/T	0.29	4.31	12.39	+
E2	ST_FSW	tplb0031i04_452	5AL	45.32	C/T	0.34	4.4	12.64	+
E2	DRW	GENE_3156_152	5BL	68.36	C/T	0.14	7.34	8.81	-
E2	DSW	GENE_3156_152	5BL	68.36	C/T	0.14	9.91	10.85	-
E2	ST_DRW	Tdurum_contig25513_123	5BL	90.35	A/G	0.38	4.35	12.5	+
E2	DRW	BS00032003_51	5BS	0.43	C/T	0.15	9.34	11.53	+
E2	FRW	BS00032003_51	5BS	0.43	C/T	0.15	8.36	8.36	+
E2	FRW	Kukri_c8500_521	6AS	3.86	T/C	0.10	6.93	4.6	-
E2	ST_FRW	Kukri_c42622_369	7AS	35.31	C/T	0.20	4.01	11.58	-
E2	ST_FRW	Tdurum_contig85217_286	7AS	61.88	T/C	0.21	3.84	11.12	+
E2	DSW	BS00004171_51	7BL	171.11	T/C	0.23			-
E2	FSW	BS00004171_51	7BL	171.11	T/C	0.23	8.92	10.62	-
E2	FSW	BS00085688_51	Un	-	C/T	0.36	6.79	8.74	+
E3	ST_FRW	RAC875_c53725_217	1AL	137.12	G/A	0.46	4.17	12.02	-
E3	ST_DRW	BS00034450_51	1BL	103.98	A/G	0.12	4.57	13.09	-
E3	ST_DRW	RAC875_c62_1546	1DS	67.72	A/G	0.44	4.66	13.33	+
E3	DRW	Excalibur_c65341_303	2BL	114.09	A/G	0.40	9.27	12.11	
E3	ST_FSW	Ku_c56370_1155	3AL	87.78	G/A	0.11	3.94	11.39	-
E3	ST_DSW	BobWhite_rep_c63943_76	5AL	73.45	T/G	0.21	4.00	11.56	-
E3	ST_DRW	wsnp_Ku_c12211_19780409	5AL	74.76	G/A	0.17	4.45	12.77	-
E3	ST_FSW	RAC875_c23775_406	5AL	84.13	A/G	0.42	4.54	13.01	
E3	ST_DSW	BobWhite_c27193_217	5AL	114.97	G/A	0.20	4.18	12.04	+
E3	DRW	GENE_3156_152	5BL	68.36	C/T	0.14	**11.69**	13.73	
E3	FRW	GENE_3156_152	5BL	68.36	C/T	0.14	**9.14**	12.21	
E3	ST_DSW	Tdurum_contig44181_311	5BL	106.16	T/C	0.08	4.84	13.81	+
E3	ST_DRW	Tdurum_contig65330_190	5BL	167.71	A/G	0.22	4.10	11.83	
E3	ST_FRW	tplb0024k14_1812	6AS	48.09	T/C	0.15	4.31	12.39	-
E3	ST_DSW	RAC875_rep_c105182_460	7AL	135.54	C/A	0.07			-
E3	ST_FRW	Kukri_c1831_1243	7AS	51.36	T/C	0.43	4.12	11.88	+
E3	ST_DSW	Excalibur_rep_c67190_638	7BS	71.33	G/T	0.41	4.25	12.23	-
E4	ST_FSW	wsnp_Ex_rep_c67747_66422078	1BL	114.13	G/A	0.42	4.01	11.58	
E4	FSW	Excalibur_c11392_1193	2BL	107.87	A/G	0.15	7.41	9.56	+
E4	DSW	BS00009882_51	2BL	134.46	G/A	0.24	7.97	9.62	+
E4	DRW	RAC875_c11609_62	2BS	86.45	G/A	0.35	6.96	8.44	-
E4	DRW	D_GBUVHFX02GV41H_67	2DS	36.54	A/G	0.07	7.65	9.62	+
E4	FSW	Excalibur_rep_c116587_84	3AL	188.38	T/C	0.09	6.6	8.33	-
E4	ST_DRW	Ra_c45147_1600	4AS	48.98	A/G	0.44	4.64	13.28	+
E4	DRW	RAC875_c16405_84	4AS	37.82	C/T	0.35	7.09	8.57	
E4	FSW	BobWhite_c47456_121	5BL	76.94	T/C	0.16	**6.41**	**8.41**	
E4	DSW	Kukri_c54078_114	5BL	82.36	G/T	0.17	6.03	9.84	-
E4	ST_FRW	Kukri_c3973_101	5BL	122.64	C/T	0.23	4.53	12.98	+
E4	ST_DRW	BS00003655_51	5BL	126.02	C/T	0.16	4.08	11.77	+
E4	ST_DSW	wsnp_Ex_c11348_18327861	6AL	85.07	G/A	0.26	4.34	12.47	-
E4	FSW	BobWhite_c1635_691	7AL	219.59	A/G	0.08	5.13	3.95	
E4	ST_DRW	BS00095826_51	7AS	33.24	A/G	0.43	4.00	3	+
E4	FSW	BS00011330_51	7AS	89.21	T/C	0.28	**6.79**	**9.41**	
E4	DSW	BS00004171_51	7BL	171.11	T/C	0.23	10.41	11.46	+
E4	FSW	Excalibur_c64418_447	Un	-	G/A	0.13	10.68	13.66	+
E4	DRW	Kukri_c19784_441	Un	-	T/C	0.12	17.04	10.9	+
E1+E2	DSW	**IAAV7086**	2AL	162.89	A/G	0.35	**4.95**	14.02	

E1+E2	DSW	Ex_c2725_1442	1BS	62.54			31.10	30.67	
E1+E2	ST_FSW	Jagger_c765_61	3AL	89.48	A/G	0.05	4.17	12.02	-
E1+E2	ST_DSW	BS00097930_51	5AL	53.47	T/C	0.36	3.94	11.39	+
E1+E2	ST_DSW	BS00036211_51	6AL	79.08	A/C	0.17	3.90	11.28	-
E3+E4	ST_DRW	wsnp_Ex_c45_98113	2AL	139.35	G/A	0.18	4.08	11.77	+
E3+E4	ST_DSW	wsnp_RFL_Contig2011_1216801	3AL	89.47	G/A	0.12	4.11	11.85	-
E3+E4	ST_FSW	BobWhite_c15582_253	3AL	173.58	T/C	0.17	3.97	11.47	+
E3+E4	ST_DRW	IAAV8683	4AL	100.38	C/T	0.16	4.43	12.72	-
E3+E4	ST_DSW	BS00067983_51	6BL	91.5	C/A	0.35	4.38	12.58	+
E3+E4	ST_FSW	Kukri_rep_c68381_911	7AL	216.36	A/G	0.15	5.00	14.23	-
		Adult field grown plant stage							
Urgenchi	ST_GY	JD_c3173_947	1BS	64.1	T/C	0.15	4.26	12.26	-
Urgenchi	ST_GY	BS00084895_51	1BL	115.88	G/A	0.33	4.16	11.99	+
Urgenchi	ST_GY	RAC875_rep_c71760_57	2BS	96.99	G/T	0.05	4.42	12.69	-
Urgenchi	ST_GY	IAAV3173	2DS	40.05	A/G	0.26	4.09	11.8	+
Urgenchi	ST_TKW	Kukri_s117946_404	3AL	177.24	T/C	0.47	4.75	13.57	-
Urgenchi	Germ	BobWhite_rep_c49102_169	3AL	90.55	C/T	0.06	7.11	8.96	
Urgenchi	ST_TKW	Jagger_c791_62	3AS	81.82	T/C	0.39	4.10	11.83	-
Urgenchi	ST_TKW	wsnp_Ex_c4501_8092034	5AS	15.53	T/C	0.49	4.13	11.91	+
Urgenchi	ST_TKW	RFL_Contig3674_847	5AS	19.68	C/T	0.46	4.23	12.18	-
Urgenchi	ST_TKW	BS00081120_51	5AS	39.26	T/C	0.15	4.08	11.77	-
Urgenchi	ST_GY	wsnp_Ra_c5634_9952011	5BL	49.01	C/T	0.12	4.47	12.82	+
Urgenchi	ST_TKW	BS00064272_51	5BL	139.4	G/A	0.11	4.92	14.02	-
Urgenchi	ST_TKW	wsnp_Ex_c3175_5864335	5BL	176.61	A/G	0.21	4.10	11.83	-
Urgenchi	PH	BS00039561_51	7AL	126.8	C/T	0.22	7.39	9.53	
Urgenchi	Germ	BS00055665_51	Unkn	-	T/C	0.46	7.92	10	
Urgenchi	ST_TKW	Ku_c30943_843	Unkn	-	T/C	0.49	4.13	11.91	+
Urgenchi	PH	RAC875_c64603_663	Unkn	-	T/C	0.09	13.76	15.95	
Karshi	ST_GY	CAP12_c8163_118	1AL	131.5	G/A	0.24	4.42	12.69	+
Karshi	ST_TKW	TA003773_0807	1AS	62.04	G/T	0.12	5.06	14.39	-
Karshi	ST_TKW	Kukri_c4951_503	1BS	70.71	G/A	0.29	4.63	13.25	-
Karshi	ST_TKW	RAC875_c24895_311	1BL	79.77	C/A	0.22	4.05	11.69	+
Karshi	ST_GY	GENE_0543_201	1BL	159.02	G/A	0.24	4.23	12.18	-
Karshi	DHD	RAC875_c16752_283	2BL	129.08	C/T	0.13	11.91	15.01	
Karshi	ST_GY	Kukri_c2454_59	3AL	86.66	A/G	0.27	3.83	11.09	-
Karshi	ST_GY	BS00094770_51	4DL	80.43	C/A	0.32	4.43	12.72	+
Karshi	DMD	BobWhite_c1387_798	5AL	67.64	C/A	0.26	10.02	12.07	
Karshi	ST_GY	wsnp_Ku_c6977_12078791	5AL	92.35	G/A	0.11	4.11	11.85	-
Karshi	ST_TKW	BS00075525_51	7AL	135.62	G/A	0.46	4.49	12.88	-
Karshi	DHD	BobWhite_c32883_84	7AL	228.37	A/G	0.28	7.17	9.29	
Karshi	ST_TKW	Jagger_c7242_85	Unkn	-	C/T	0.47	4.67	13.36	+
Karshi	ST_TKW	RAC875_c39204_91	Unkn	-	C/T	0.32	4.40	12.64	-
Karshi	ST_GY	Kukri_c10254_95	Unkn	-	A/G	0.05	4.17	7.95	-
Dongying	ST_PH	Excalibur_c34697_831	1AL	79.19	A/C	0.37	4.27	12.29	
Dongying	PH	GENE_1353_136	2AL	101.97	C/T	0.1	7.73	10.1	
Dongying	ST_GY	Tdurum_contig30569_579	2AL	101.97	G/A	0.29	3.82	11.07	-
Dongying	ST_GY	Tdurum_contig82393_484	2BL	118.43	C/A	0.06	4.59	13.14	-
Dongying	ST_GY	Tdurum_contig59566_4435	3BL	143.29	A/C	0.29	3.81	11.04	+
Dongying	ST_GY	wsnp_Ex_rep_c101323_86702546	5AL	131.42	C/A	0.37	3.83	11.09	+
Dongying	ST_PH	Ex_c8134_363	6BL	109.86	C/T	0.09	4.60	13.17	+
Dongying	GY	RAC875_rep_c105937_467	6BL	113.28	T/C	0.44	7.25	11.79	
Dongying	ST_GY	IACX9024	6BS	39.24	C/G	0.08	4.52	12.96	+

SNPs associated with leaf K^+, Na^+ and K^+/Na^+ ratio

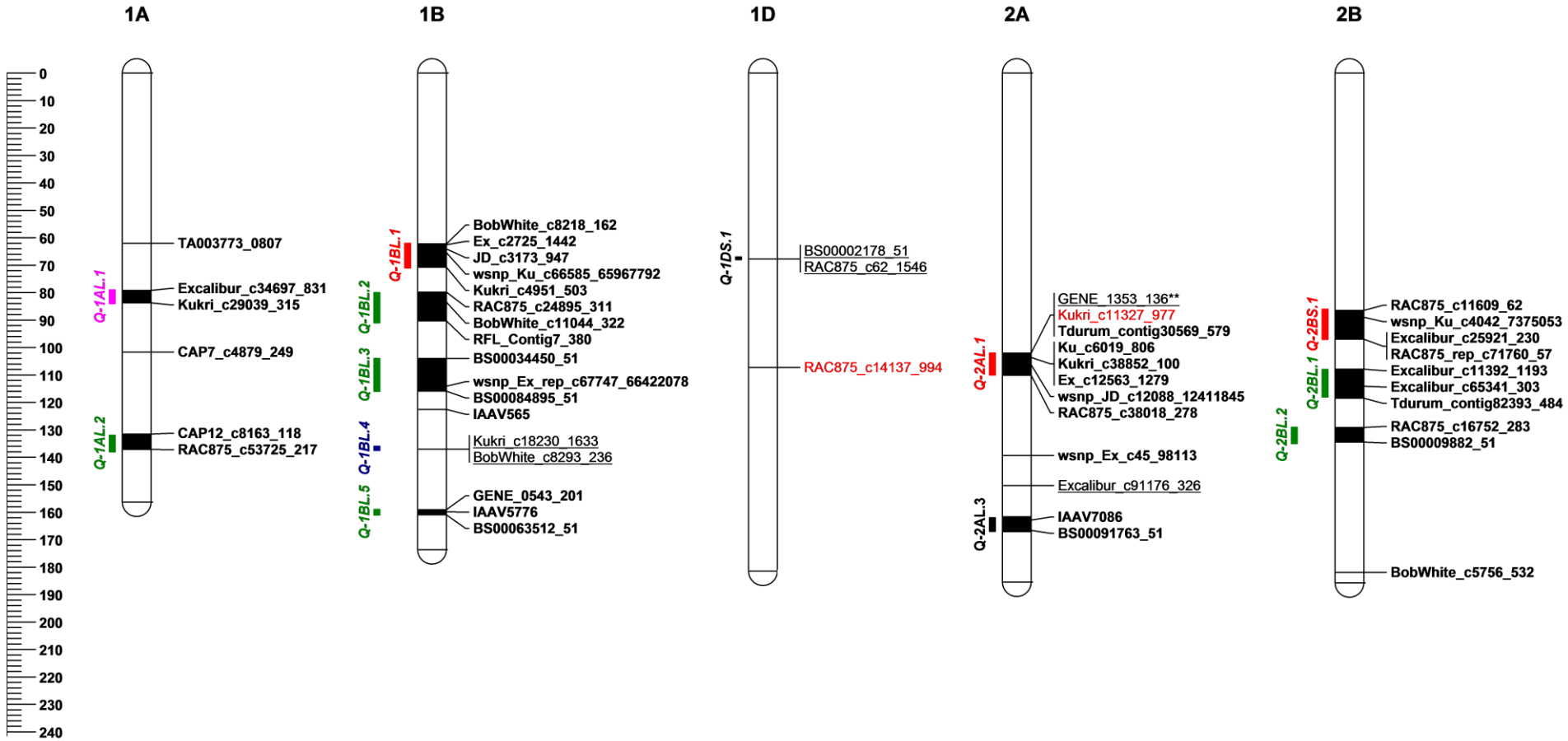
Fifteen SNPs were significantly associated with the concentration of K^+, Na^+ and the K^+/Na^+ ratio of the third leaf after 25 days of salt application, with R^2 which ranged from 6.96% for leaf Na^+ to 10.13% for leaf K^+ (**Table 7)**. Five SNP-loci on 2AL, 3AL, 4AS, 5AL and 6BL that showed associations with leaf ionic traits were also found to influence measured salt-related phenotypic traits (**Table 7; Figure 6**). SNP locus (*Kukri_c11327_977*) at 101.97 cM on 2AL (R^2 = 7.45%) detected for K^+/Na^+ ratio, coincided with the locus that was detected for the 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 vigor that was influenced by both 200 mM NaCl and 100 mM Na_2SO_4 salt stress conditions. The SNP *wsnp_Ex_rep_c106152_90334299* located on chromosome 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.

Table 7 Summary of SNP markers significantly associated the accumulated Na^+ and K^+ and K^+/Na^+ ratio in the third leaf after 25 days of salt stress

Ions	SNP	Chr.	Pos.	P.value	MAF	R^2 (%)
	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
K^+ content	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
	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
Na^+ content	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
	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
K^+/Na^+ ratio	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

Congruent QTL regions

Using genome LD-decay of 10, 11 and 14 cM for A-, B- and D-genomes, respectively, all associated SNPs were delineated into 37 distinct major QTL regions (**Table 8; Figure 10).** Four QTL regions including *Q-1BS.1* (R^2≥30.67%), *QTL_2AL.1 (*R^2≥ 16.93%), *QTL_2BS.1* (R^2≥12.69%) and *QTL_3AL.1* (R^2≥12.02%) are most significant because individually, they were significantly associated with ST-traits across the three growth states – germination, seedling and field evaluated trials and thus confer all-stage ST. Of these, *QTL_2AL.1* and *QTL_3AL.*1 were also associated with leaf K^+/Na^+ ratio and Na^+ exclusion, respectively. Eleven QTL regions exhibited significant genome-wide association with ST-traits at both seedling and AFP stages, while six QTL regions had effect on germination and seedling stage ST-traits. Two loci at 68.4 cM on 5BL (*GENE_3156_152*; R^2≥24.20%) and 71.32 cM on 7BS (*Q-7BS;* R^2≥12.23%) were pleiotropic and had multiple effects on ST-traits at both germination and seedling growth stages. A summary of the detected QTL regions, the associated traits and the previously reported QTL is presented in **Table 8.**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1B
1D
2A
2B
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30
40
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60
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150
160
170
180
190
200
210
220
230
240
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Q-1AL.2
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Kukri_c29039_315
CAP7_c4879_249
CAP12_c8163_118
RAC875_c53725_217
Q-1BL.1
Q-1BL.2
Q-1BL.3
Q-1BL.4
Q-1BL.5
BobWhite_c8218_162
Ex_c2725_1442
JD_c3173_947
wsnp_Ku_c66585_65967792
Kukri_c4951_503
RAC875_c24895_311
BobWhite_c11044_322
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BS00034450_51
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BS00084895_51
IAAV565
Kukri_c18230_1633
BobWhite_c8293_236
GENE_0543_201
IAAV5776
BS00063512_51
Q-1DS.1
BS00002178_51
RAC875_c62_1546
RAC875_c14137_994
Q-2AL.1
Q-2AL.3
GENE_1353_136**
Kukri_c11327_977
Tdurum_contig30569_579
Ku_c6019_806
Kukri_c38852_100
Ex_c12563_1279
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RAC875_c38018_278
wsnp_Ex_c45_98113
Excalibur_c91176_326
IAAV7086
BS00091763_51
Q-2BS.1
Q-2BL.1
Q-2BL.2
RAC875_c11609_62
wsnp_Ku_c4042_7375053
Excalibur_c25921_230
RAC875_rep_c71760_57
Excalibur_c11392_1193
Excalibur_c65341_303
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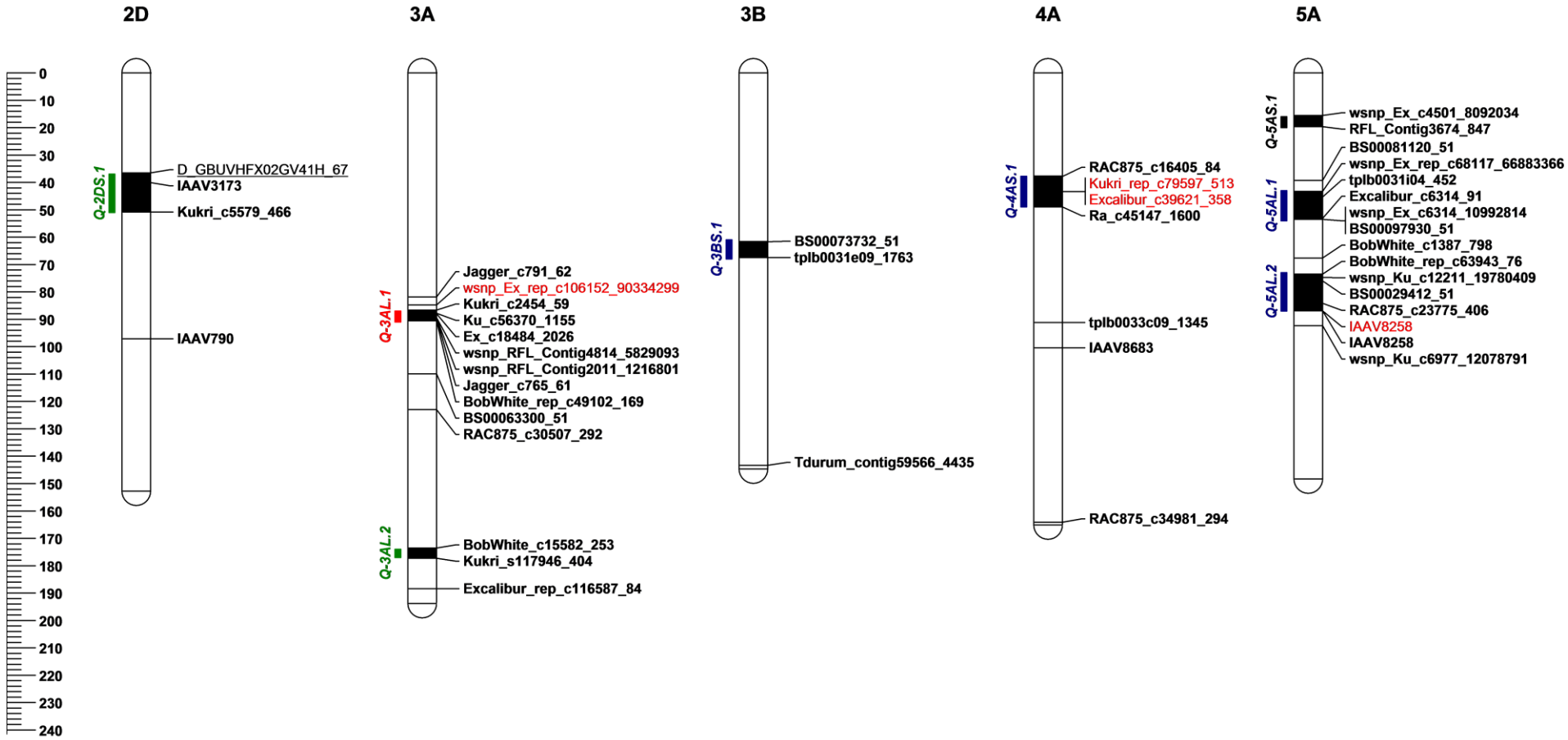
RAC875_c16752_283
BS00009882_51
BobWhite_c5756_532


2D
3A
3B
4A
5A
0
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90
100
110
120
130
140
150
160
170
180
190
200
210
220
230
240
Q-2DS.1
D_GBUVHFX02GV41H_67
IAAV3173
Kukri_c5579_466
IAAV790
Q-3AL.1
Jagger_c791_62
wsnp_Ex_rep_c106152_90334299
Kukri_c2454_59
Ku_c56370_1155
Ex_c18484_2026
wsnp_RFL_Contig4814_5829093
wsnp_RFL_Contig2011_1216801
Jagger_c765_61
BobWhite_rep_c49102_169
BS00063300_51
RAC875_c30507_292
Q-3AL.2
BobWhite_c15582_253
Kukri_s117946_404
Excalibur_rep_c116587_84
Q-3BS.1
BS00073732_51
tplb0031e09_1763
Tdurum_contig59566_4435
Q-4AS.1
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Kukri_rep_c79597_513
Excalibur_c39621_358
Ra_c45147_1600
tplb0033c09_1345
IAAV8683
RAC875_c34981_294
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RFL_Contig3674_847
Q-5AL.1
BS00081120_51
wsnp_Ex_rep_c68117_66883366
tplb0031i04_452
Excalibur_c6314_91
wsnp_Ex_c6314_10992814
BS00097930_51
Q-5AL.2
BobWhite_c1387_798
BobWhite_rep_c63943_76
wsnp_Ku_c12211_19780409
BS00029412_51
RAC875_c23775_406
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IAAV8258
wsnp_Ku_c6977_12078791

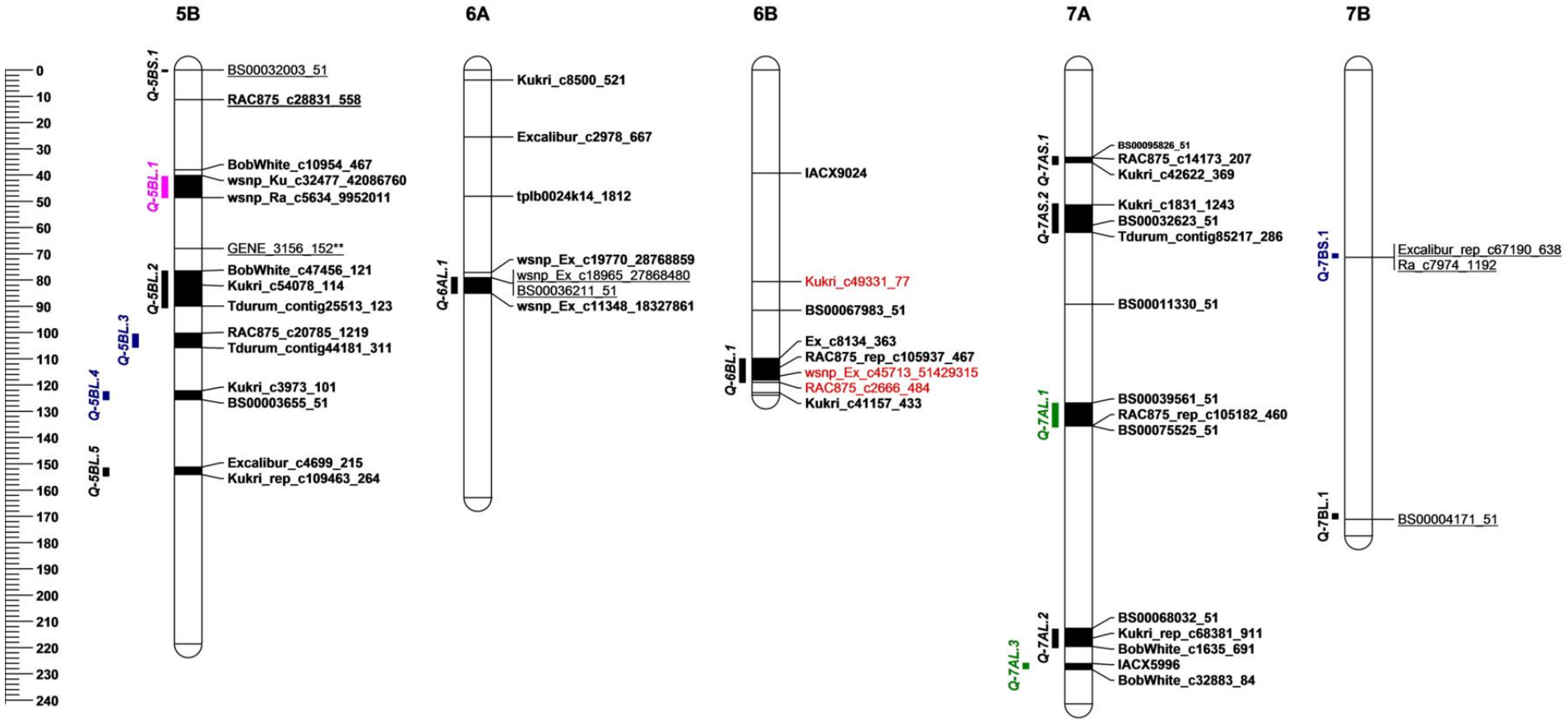


Figure 10 Map positions of all the SNPs associated with ST- traits. Map distance (in centiMorgan) is shown on the left. **"Underlined"** SNPs are pleiotropic; SNPs in **"red color"** were associated with leaf ions traits such as K^+, Na^+, K^+/Na^+; number of asterisk (*) indicates the number of growth stages the SNP was detected, while the colored bar in each chromosome designate QTL regions in significant LD. The QTL names are shown in the left with a solid bar. The bars are color coded to represent the growth stages at which the QTL regions conferred 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).

Table 8 Colocation of SNP clusters with QTL/genes already identified or published

Chr	QTL	R^2 (%)	LD region	Associated ST traits	Reference
All stage ST QTL					
1BS	Q-1BS.1	≥30.67	62.3-70.7	ST_GY, ST_TKW, ST_FRW, 100 mM Na_2SO_4	
2AL	Q-2AL.1	≥16.93	101.97- 110	DRW, DSW, FRW, PH, ST_GY, ST_FSW, 100 mM Na_2SO_4, 200 mM NaCl, K^+/Na^+ ratio	Nax1, QTL ^{SB} (Lindsay *et al*., 2004; Ma *et al*., 2007; Genc et al., 2010)
2BS	Q-2BS.1	≥12.69	86.5-97	DRW, FRW, ST_GY, ST_DRW, 200 mM NaCl	$QTL^{GY, SB}$ Quarrie *et al*., 2005; Ma *et al*., 2007; Genc *et al*., 2010
3AL	Q-3AL.1	≥12.02	86.7-90.6	ST_GY, ST_FSW, ST_DRW, ST_DSW, 200 mM NaCl, field germination, Na^+ content	QTL^{GY} (Quarrie *et al*., 2005)
Seedling + AFP ST QTL					
1AL	Q-1AL.2	≥12.69	131.5-137.12	ST_GY, ST_FRW	
1BL	Q-1BL.2	≥12.12	79.8-90.26	ST_TKW, ST_DRW, ST_FSW	
1BL	Q-1BL.3	≥13.09	104-115.9	ST_GY, ST_DRW, ST_FSW	
1BL	Q-1BL.5	≥12.18	159-160.9	ST_GY, DSW, DRW	
2BL	Q-2BL.1	≥13.14	107.9-118	ST_GY, FSW, DRW	
2BL	Q-2BL.2	≥15.01	129.1-134.5	DHD, DSW	Q.Na2B2 (et al., 2010)
2DS	Q-2DS.1	≥13.17	36.5-50.8	FSW, DRW, DSW, ST_GY	QSdw-*2D* (Xu *et al*., 2012) and *QSlc.ipk-2D* (Landjeva *et al*., 2008)
3AL	Q-3AL.2	≥15.57	173.6-177.2	ST_TKW, ST_FSW	
7AL	Q-7AL.1	≥12.88	126.8-135.6	PH, ST_TKW, ST_DSW	Q.sb7A (Genc et al., 2010)
7AL	Q-7AL.3	≥14.23	226.1-228.4	DHD, ST_DSW	$QTL^{GY, SB}$ (Quarrie et al., 2005; Genc et al., 2010)
5BL	GENE_3156_152	≥24.20	68.4	DRW, DSW, FRW, FSW, 75 mM Na_2SO_4	
Germination + Seedling ST QTL					
1BL	Q-1BL.4	≥11.42	137	75 mM Na_2SO_4, ST_DRW	
3BS	Q-3BS.1	≥12.47	61.6-67.5	75 mM Na_2SO_4, DSW	$QTL^{SSI, GST}$ (Ma *et al*., 2007)
5AL	Q-5AL.1	≥14.56	43.3-53.5	100 mM NaCl, DSW, ST_FSW, ST_DSW	$QTL^{TN, SB, CC, SKC}$ (Genc *et al*. , 2010)
5AL	Q-5AL.2	≥16.22	73.5-86.9	75 mM Na_2SO_4, ST_FSW, ST_DSW, ST_DRW, K^+ content	Nax2, Q.ls5A (James *et al*., 2006; Byrt *et al*., 2007; Genc *et al*., 2010; Munns *et al*., 2012)
5BL	Q-5BL.3	≥13.81	100.6-106.2	200 mM NaCl, ST_DSW	
5BL	Q-5BL.4	≥12.98	122.6-131	100 mM Na_2SO_4, ST_FRW, ST_DRW	
7BS	Q-7BS.1	≥12.23	71.3	100 mM Na_2SO_4, ST_DSW (E3)	
Germination + AFP ST QTL					
1AL	Q-1AL.1	≥12.29	79.19-83.7	200 mM NaCl, ST_PH	QTL^{TN} (Genc *et al*., 2010)
5BL	Q-5BL.1	≥21.22	41-49	100 mM NaCl, ST_GY	QTL^{GY} (Quarrie *et al*., 2005)
Germination ST QTL					
5BL	Q-5BL.5	≥14.10	151.6-154.5	75 mM Na_2SO_4, 200 mM NaCl	
Seedling stage ST QTL					
1DS	Q-1DS.1	≥13.33	67.72	ST_DRW in E2 and E3	$QTL^{RKC, SB}$ (Xu *et al*., 2012)
2AL	Q-2AL.2	≥14.02	162-167	ST_DSW, DSW	Nax1 (Huang *et al*., 2006; Genc *et al*., 2010)
4AS	Q-4AS.1	≥13.28	37.82- 48.98	DRW, ST_DRW, K^+/Na^+ ratio	
5BL	Q-5BL.2	≥9.84	76.9-90.35	FSW, DSW, ST_DRW	QTL^{GY} (Quarrie *et al*. 2005), ***Qsii-5B.2*** (Ma *et al*., 2007); Q.sb5B and ***Q.mat5B***, ***Q.K5B*** (Genc *et al*., 2010), ***Vrn-1B*** (Genc *et al*., 2010)
6AL	Q-6AL.1	≥12.47	79.1-85.1	ST_FRW, ST_DSW	
7AS	Q-7AS.1	≥14.31	33.2-35.31	ST_DRW, ST_DSW, STI_FRW	$QTL^{SB,GST}$ (Ma *et al*., 2007; Shavrukov et *al*., 2011)
7AS	Q-7AS.2	≥11.88	51.4-61.9	FSW, ST_FRW	QTL^{OR} (Morgan, 1991; Morgan and Tan, 1996)
7AL	Q-7AL.2*	≥14.23	212.7-219.6	FSW, ST_ FSW, ST_DSW	$QTL^{GY, SB}$ (Quarrie *et al*., 2005; Genc *et al*., 2010)
5BS	Q-5BS.1	≥11.53	0.43	DRW, FRW	
7BL	Q-7BL.1	≥11.46	171.1	FSW (in E2), DSW (in E2), DSW (in E4)	
AFP ST QTL					
5AS	Q-5AS.1	≥12.18	15.5-19.7	ST_TKW	Ma *et al*. (2007)
6BL	Q-6BL.1	≥13.17	109.9-113.3	GY, ST_PH	

where the traits controlled by the **QTL** are shown as superscripts: **GST**= germination salt tolerance; **SSI**= seedling salt injury; **SB**= seedling biomass; **TN**= tiller number; **GY**= grain yield; **LS**= leaf symptoms; **CC**= chlorophyll content and **SKC**= Shoot K^+ concentration, **RKC**= Root K^+ concentration, **OR**= osmo-regulation.

Principal Coordinates Analysis (PCoA) 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 identified in the studied panel **(Figure 11)**. The first three axes explained 28.57% of the total variation. Notwithstanding the relatively low contribution of the first three PCos to the total genetic variance, the PCoA mostly depicted the relationships that are consistent with the ST status of the individual genotypes. This is because, it was able to group the genotypes based on their salt tolerance status as was previously reported in Oyiga *et al*. (2016). The salt-tolerant genotypes (in green) were mostly distributed at the right side of the plot, while the salt-sensitive genotypes were distributed to the left side of the plot. The largest eigenvectors were associated with *Tdurum_contig30569_579* (101.77 cM) and *Tdurum_contig30569_579* (110.13 cM) on 2AL, *IAAV3173* (40.05 cM) on 2DS, *Ra_c45147_1600* (48.98 cM) on 4AS, *wsnp_Ex_c11348_18327861* (85.07 cM) on 6AL, *Excalibur_rep_c67190_638* and *Ra_c7974_1192* (71.33 cM) on 7BS (data not shown).

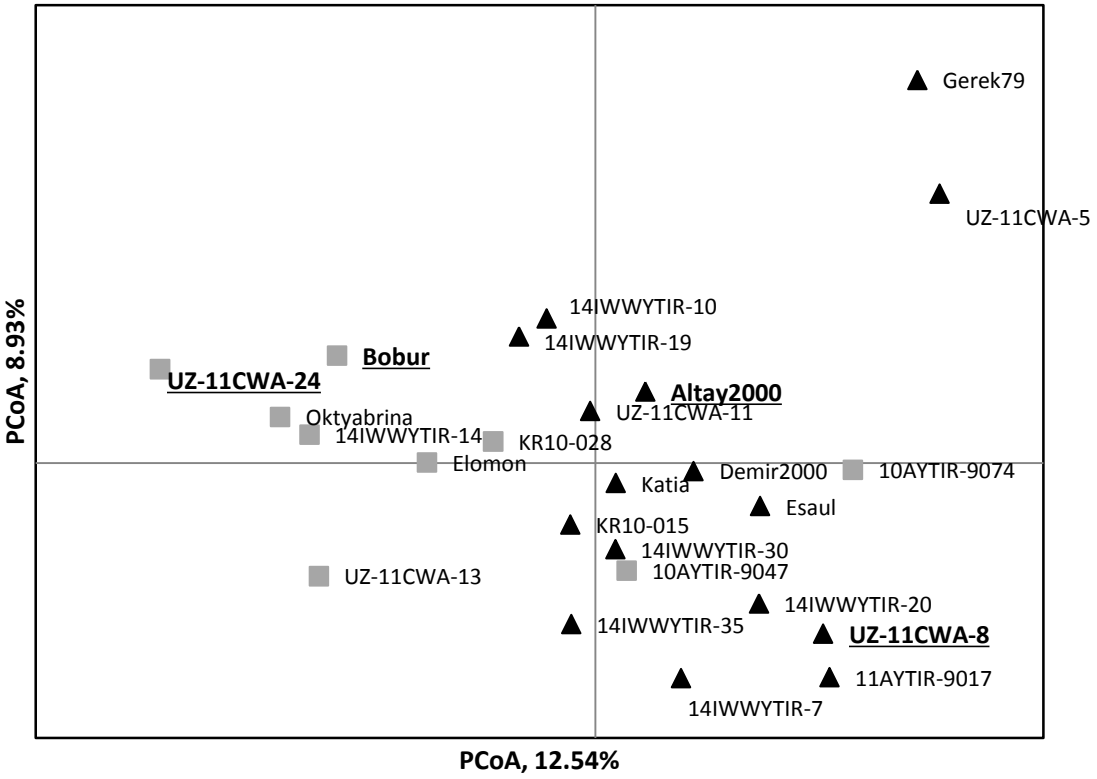


Figure 11 Principal coordinates analysis (PCoA) plot using a genetic distance matrix (GenAlEx 6.5) estimated with data from 187 associated polymorphisms of the salt tolerant (Black colour/triangular shaped) and salt sensitive (Gray colour/squared shaped) wheat genotypes previously identified in the GWAS analysis. The underlined genotypes (in bold) were used to perform the gene expression analysis.

Ontology classification of the associated DNA sequences

The goal was to identify ORFs in the chromosome segments harboring the QTL identified. The *in silico* analysis of the sequences surrounding 74 of the associated SNP sequences revealed high sequence homologies to genes known to be involved in salt-stress response (**Table S5**). The largest categories of genes identified were those involved in stress and defense (23%), antiporter/transport (22%), ion homeostasis/detoxification (18%), transcription/translation (11%), osmo-protectant (9%), 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 (**Figure 8**).

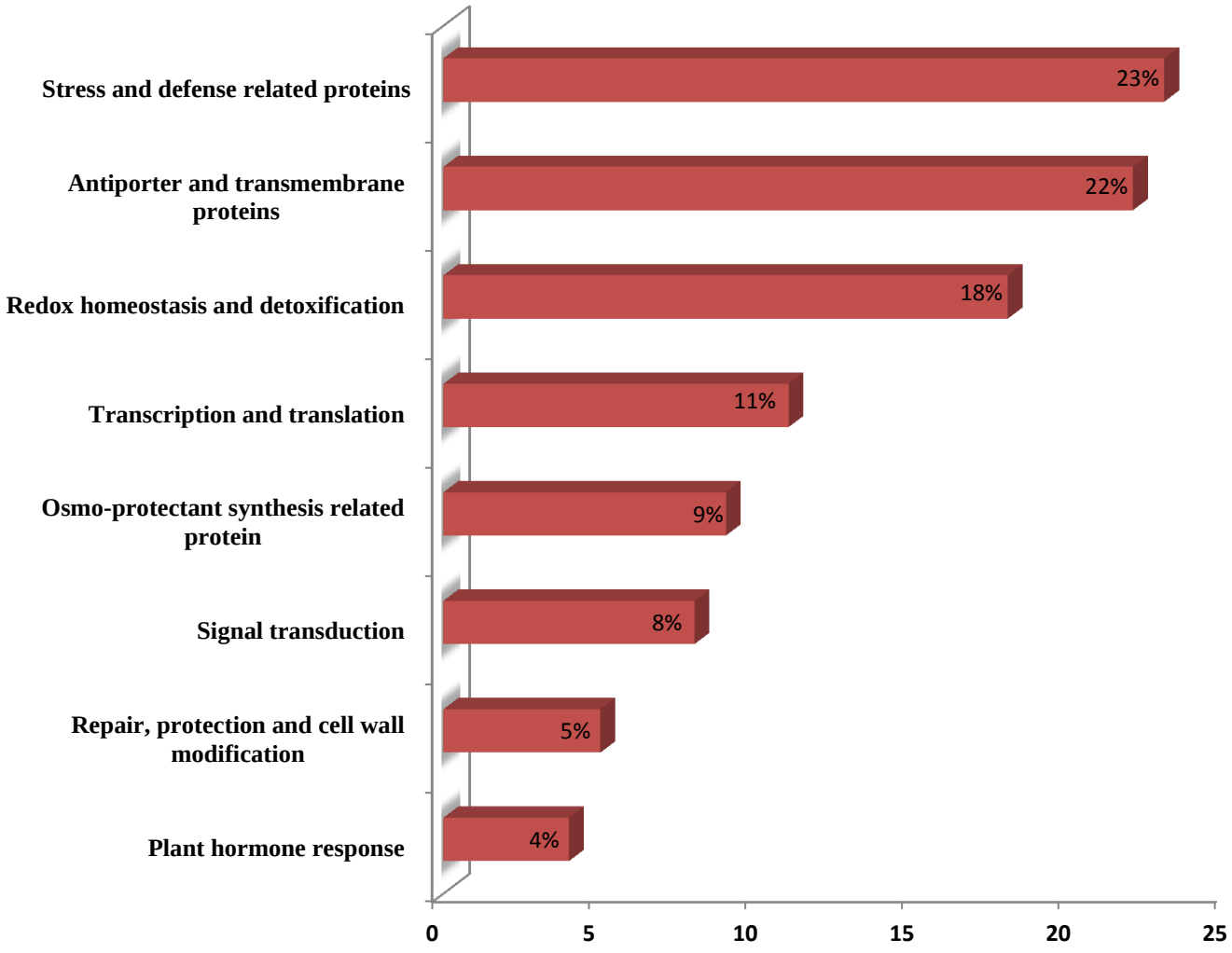


Figure 12 Ontology classifications of the associated SNP loci

Table 9 Ontology classification of the associated DNA sequences detected using the GWAS and the associated traits

Traits	Associated SNP	Chr	Functional Annotation	bp	Expected
			Redox homeostasis and detoxification (18.2%)		
ST_DRW	BobWhite_c11044_322	1BL	thioredoxin H [Triticum aestivum]	NC	1.00E-45
ST_GY	Tdurum_contig30569_579	2AL	Secologanin synthase [Triticum urartu]	C	2.00E-43
200 mM NaCl	wsnp_JD_c12088_12411845	2AL	ferredoxin-dependent glutamate synthase [Secale cereale x Triticum durum]	NC	2.00E-46
ST_GY	Tdurum_contig82393_484	2BL	NADPH-cytochrome P450 reductase [Triticum aestivum]	NC	8.00E-44
Germination	BobWhite_rep_c49102_169	3AL	Glutathione S-transferase 1 [Triticum urartu]	C	1.00E-45
Leaf K^+	IAAV8258	5AL	Pyruvate dehydrogenase E1 component subunit beta [Triticum urartu]	C	0.0
75 mM Na_2SO_4	IAAV8258	5AL	Pyruvate dehydrogenase E1 component subunit beta [Triticum urartu]	C	8.00E-57
ST_GY	wsnp_Ex_rep_c101323_86702546	5AL	Respiratory burst oxidase-like protein B [Triticum urartu]	C	2.00E-53
ST_TKW	RFL_Contig3674_847	5AS	2-aminoethanethiol dioxygenase [Aegilops tauschii]	C	1.00E-43
100 mM NaCl	wsnp_Ku_c32477_42086760	5B	4-hydroxyphenylacetaldehyde oxime monooxygenase	C	5e-97
GY	RAC875_rep_c105937_467	6BL	Lysine-specific histone demethylase 1-3-like protein [Aegilops tauschii]	C	2.00E-42
FSW	BobWhite_c1635_691	7AL	RecName: Full=Catalase [Triticum aestivum]	C	2.00E-35
DHD	BobWhite_c32883_84	7AL	Cell elongation protein DIMINUTO [Aegilops tauschii]	NC	2.00E-18
			Antiporter and transmembrane proteins (19.7%)		
100 mM NaCl	IAAV565	1BL	Ras-related protein Rab11B	C	1.00E-74
Leaf K^+	RAC875_c14137_994	1DL	Uncharacterized Na^+/H^+ antiporter [Triticum aestivum]	C	
ST_FSW	wsnp_Ex_rep_c67747_66422078	1BL	putative ubiquitin-conjugating enzyme E2 24 [Triticum urartu]	C/NC	2.00E-25
100 mM Na_2SO_4	BobWhite_c8218_162	1BS	Zinc transporter 7, chloroplastic [Aegilops tauschii]	C	5.00E-43
Leaf K^+/Na^+	Kukri_c11327_977	2AL	Protein transport protein Sec24B [Aegilops tauschii]	C	0.0
ST_DSW	BS00091763_51	2AL	Glutathione-regulated potassium-efflux system protein kefC [Aegilops tauschii]	NC	6.00E-44
DHD	RAC875_c16752_283	2BL	Glutamyl-tRNA(Gln) amidotransferase subunit A [Triticum urartu]	C	6.00E-26
200 mM NaCl	wsnp_RFL_Contig4814_5829093	3AL	Putative ABC transporter B family member 8 [Triticum urartu]	C	7.00E-42
ST_DRW	Ra_c45147_1600	4AL	Protein transport protein Sec24-like CEF [Aegilops tauschii]	C	7.00E-44
ST_TKW	BS00081120_51	5AS	Bidirectional sugar transporter SWEET17 [Triticum urartu]	C	1.00E-25
200 mM NaCl	Kukri_rep_c109463_264	5BL	boron transporter [Triticum aestivum]	C	3.00E-43
DSW	Kukri_c54078_114	5BL	Oligopeptide transporter 7 [Triticum urartu]	C	3.00E-43
ST_DRW	Tdurum_contig25513_123	5BL	Potassium transporter 18 [Triticum urartu]	C/NC	3.00E-45
Leaf Na^+	wsnp_Ex_c45713_51429315	6BL	ABC transporter F family member 3 [Triticum urartu]	C	3.00E-84
ST_DSW	Excalibur_rep_c67190_638	7BS	Transmembrane 9 superfamily member 4 [Aegilops tauschii]	C	1.00E-43
ST_GY	Kukri_c10254_95	Unkn	ATP synthase subunit beta, mitochondrial [Aegilops tauschii]		
			Plant hormone response (0.05%)		
ST_FRW	RAC875_c53725_217	1AL	Asparagine synthetase (glutamine-hydrolyzing) [Aegilops tauschii]	C	5.00E-31
ST_FSW	BobWhite_c15582_253	3AL	Abscisic stress-ripening protein 1 [Triticum urartu]	C/NC	1.00E-45
FSW	BobWhite_c47456_121	5BL	Nudix hydrolase 23, chloroplastic [Triticum urartu]	NC	4.00E-29
			Stress and defense related proteins/ Chaperone (24.2%)		
FSW	Excalibur_rep_c116587_84	3AL	DnaJ homolog subfamily B member 4 [Triticum urartu]	C	2.00E-43
Leaf Na^+	wsnp_Ex_rep_c106152_90334299	3AL	IAA-alanine resistance protein 1 [Aegilops tauschii]	C	3.00E-90
ST_DSW	BS00073732_51	3BS	Heat shock 70 kDa protein, mitochondrial [Triticum urartu]	C	1.00E-43
DMD	BobWhite_c1387_798	5AL	Putative salt tolerance-like protein [Aegilops tauschii]	C	2.00E-43
ST_DSW	BobWhite_c27193_217	5AL	hypersensitive response protein [Triticum aestivum]	NC	1.00E-26
ST_FRW	wsnp_Ex_c18965_27868480	6AL	Two-component response regulator-like PRR1 [Triticum urartu]	C	2.00E-97
ST_DSW	BS00067983_51	6BL	Two-component response regulator-like APRR2 [Aegilops tauschii]	C	6.00E-30
ST_FRW	Tdurum_contig85217_286	7AS	T-complex protein 1 subunit alpha [Aegilops tauschii]	C	1.00E-43

PH	BS00039561_51	7AL	Chaperone protein dnaJ 1, mitochondrial [Triticum urartu]	C	2.00E-19
ST_DSW	RAC875_rep_c105182_460	7AL	Zinc finger A20 and AN1 domain-containing stress-associated protein 8 [Triticum urartu]	C	2.00E-43
ST_GY	CAP12_c8163_118	1AL	Prohibitin-2 [Triticum urartu]	C	2.00E-43
ST_DRW	BS00002178_51	1DS	Defensin-like protein 2 [Aegilops tauschii]	C	6.00E-46
ST_FSW	tplb0033c09_1345	4AL	Disease resistance protein RPM1 [Aegilops tauschii]	C	2.00E-43
ST_DRW	BS00029412_51	5AL	Putative disease resistance RPP13-like protein 2 [Aegilops tauschii]	C/NC	2.00E-43
100 mM Na_2SO_4	BobWhite_c10954_467	5BS	Putative disease resistance protein RGA3 [Aegilops tauschii]	NC	2.00E-34
ST_TKW	BS00075525_51	7AL	Disease resistance protein RPM1 [Triticum urartu]	NC	2.00E-43
ST_DSW	IACX5996	7AL	Putative disease resistance RPP13-like protein 1 [Triticum urartu]	NC	1.00E-38
			Osmo-protectant synthesis related protein (0.08%)		
Leaf K^+/Na^+	Kukri_rep_c79597_513	4AS	putative methionyl-tRNA synthetase [Triticum urartu]	C	0.0
ST_FRW	Kukri_c3973_101	5BL	40S ribosomal protein S12 [Triticum urartu]	C/NC	3.00E-43
ST_DRW	BS00095826_51	7AS	sucrose:sucrose 1-fructosytransferase [Triticum aestivum]	C	1.00E-43
ST_DSW	RAC875_c14173_207	7AS	sucrose:fructan 6-fructosyltransferase [Triticum aestivum]	NC	1.00E-43
ST_FRW	Kukri_c42622_369	7AS	sucrose:fructan 6-fructosyltransferase [Triticum durum]	C	4.00E-38
ST_FRW	Kukri_c1831_1243	7AS	Sucrose synthase 1 [Triticum urartu]	C	1.00E-31
Leaf K^+/Na^+	Excalibur_c13094_523	7DL	Uridine-cytidine kinase-like protein 1 [Aegilops tauschii]	C	3.00E-47
			Transcription and translation (12.1%)		
ST_DRW	BobWhite_c8293_236	1BL	MYB-related protein [Aegilops speltoides]	C/NC	7.00E-43
ST_GY	JD_c3173_947	1BS	Transcription regulatory protein SNF2 [Triticum urartu]	C	2.00E-22
DRW, FRW	BS00032003_51	5BS	Ethylene receptor 1 [Aegilops tauschii]	C	1.00E-43
ST_FRW	tplb0024k14_1812	6AS	PHD finger protein 3 [Aegilops tauschii]	C	1.00E-43
ST_DSW	wsnp_Ex_c11348_18327861	6AL	BAH and coiled-coil domain-containing protein 1 [Triticum urartu]	C	2.00E-97
ST_PH	Ex_c8134_363	6BL	B3 domain-containing protein [Aegilops tauschii]	NC	2.00E-43
100 mM NaCl	Kukri_c41157_433	6BL	Protein furry homolog-like protein [Triticum urartu]	C	2.00E-43
100 mM NaCl	RAC875_c4682_646	unkn	putative transcription factor X1 [Triticum monococcum]	C	5.00E-40
			Repair, protection and cell wall modification (0.06%)		
200 mM NaCl	Kukri_c29039_315	1AL	Structural maintenance of chromosomes protein 4 [Triticum urartu]	C	1.00E-45
ST_FRW	wsnp_Ku_c66585_65967792	1BL	Vesicle-associated protein 1-1 [Triticum urartu]	C/NC	7.00E-98
DSW	IAAV5776	1BL	Structural maintenance of chromosomes protein 3 [Aegilops tauschii	C	1.00E-69
75 mM Na_2SO_4	Excalibur_c4699_215	5BL	Putative polygalacturonase [Aegilops tauschii]	C	2.00E-39
			Signal transduction (0.09%)		
ST_PH	Excalibur_c34697_831	1AL	Putative LRR receptor-like serine/threonine-protein kinase [Aegilops tauschii]	NC	2.00E-41
ST_DRW	RAC875_c62_1546	1DS	Serine/arginine-rich splicing factor 4 [Triticum urartu]	C/NC	1.00E-42
ST_DRW	wsnp_Ex_c45_98113	2AL	Glycerol kinase [Triticum urartu]	C	4.00E-76
ST_DSW	BS00097930_51	5AL	Protein kinase G11A [Aegilops tauschii]	C	2.00E-43
ST_GY	wsnp_Ku_c6977_12078791	5AL	Serine/threonine-protein kinase CTR1 [Aegilops tauschii]	C	9.00E-97
ST_DRW	Excalibur_c25921_230	2BS	Putative serine/threonine-protein kinase receptor [Aegilops tauschii]	C	1.00E-45

Analyses of associated gene transcripts

The transcript abundance of 22 of the identified candidate genes from QTL regions were investigated in the leaves of salt tolerant (*Atlay2000*) and sensitive (*Bobur*) genotypes under saline and non-saline conditions. The comparative expression at day 24 was performed to analyse the genes identified to be genetically associated with scored the measured traits at this time point. All the analyzed genes revealed differential expressions when compared to time zero or control and, are mostly up-regulated in the leaves of *Atlay2000* and down-regulated in *Bobur* (**Figure 13**), with the exception of *Protein kinase G11A*. The *ZIP-7* (located in the QTL region that influenced ST across all the three growth stages) exhibited strongest differential expression; it increased by 713.98% in *Atlay2000*, 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 **Table 10.**

The expression patterns of the four putative genes identified were further analyzed to monitor their accumulation after salt stress application using RT-PCR. At day 30, the expression of *ZIP7*, *KeFc*, *AtABC8* and *6-SFT* revealed similar pattern as was observed in day 24 (**Figure 14**), which were further substantiated by high correlations (r^2 = 0.63-0.98, *P* = 0.01) existing between the TranSNiPtomic data and RT-PCR data (**Data not shown**). They are up-regulated in tolerant genotypes in contrast to the sensitive genotypes.

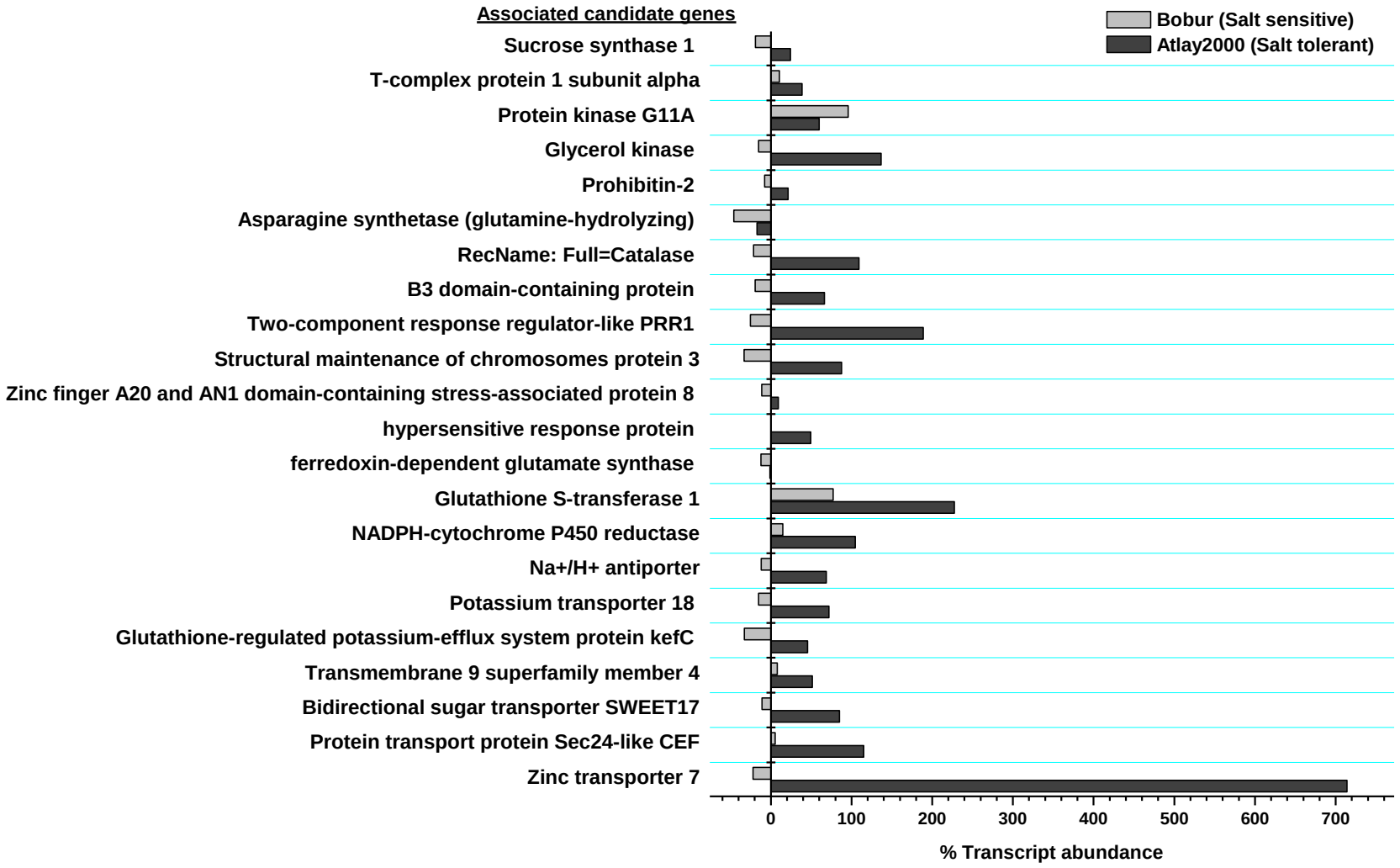


Figure 13 Effect of salt stress on some of identified gene transcript abundance (% change to control) between salt-tolerant genotype (*Atlay2000*, in Black) versus salt-sensitive genotype (*Bobur*, in grey) after 24 days of stress

Table 10 Illustrates the key biological functions associated with the 21 predicted gene proteins found to be differentially expressed in the tolerant and sensitive wheat genotypes. Their functions were adapted from the UniProt (www.uniprot.org) database.

Traits	Chr.	Gene Annotation	Gene ID	Biological process
ST_FRW	1AL	Asparagine synthetase (glutamine-hydrolyzing) [Aegilops tauschii]	P49094	glutamine metabolic process, L-asparagine biosynthetic process
ST_GY	1AL	Prohibitin-2 [Triticum urartu]	O94550	protein folding
100 mM Na_2SO_4	1BS	Zinc transporter 7, chloroplastic [Aegilops tauschii]	Q5Z653	zinc II ion transmembrane transport
200 mM NaCl, DSW	1BL	Structural maintenance of chromosomes protein 3 [Aegilops tauschii	O42649	cell division, DNA repair, positive regulation of maintenance of mitotic sister, chromatid cohesion, centromeric, mitotic sister chromatid cohesion
Leaf K^+	1DL	Uncharacterized Na+/H+ antiporter [Triticum aestivum]	O13726	cation transport, sodium ion transport, regulation of pH, transmembrane transport
200 mM NaCl	2AL	ferredoxin-dependent glutamate synthase [Secale cereale x Triticum durum]	KF521800.1	glutamate biosynthetic process, response to sucrose, ammonia assimilation cycle, photorespiration, response to light stimulus
ST_DRW	2AL	Glycerol kinase [Triticum urartu]	A9WJ21	phosphorylation, glycerol metabolic process, glycerol-3-phosphate metabolic process
ST_DSW	2AL	Glutathione-regulated potassium-efflux system protein kefC [Aegilops tauschii]	A4W6F3	potassium ion transmembrane transport, potassium ion transport, regulation of pH, response to toxic substance
ST_GY	2BL	NADPH-cytochrome P450 reductase [Triticum aestivum]	AF123610.1	Oxidoreductase
Field Germination	3AL	Glutathione S-transferase 1 [Triticum urartu]	P12653	Oxidoreductase
ST_DRW	4AL	Protein transport protein Sec24-like CEF [Aegilops tauschii]	Q2HH63	ER to Golgi vesicle-mediated transport, intracellular protein transport
ST_TKW	5AS	Bidirectional sugar transporter SWEET17 [Triticum urartu]	B8AYH1	carbohydrate transmembrane transport
ST_DSW	5AL	hypersensitive response protein [Triticum aestivum]	A5HE90	
ST_DSW	5AL	Protein kinase G11A [Aegilops tauschii]	M7YES1	-
ST_DRW	5BL	Potassium transporter 18 [Triticum urartu]	Q8VXB1-2	Ion transport, Potassium transport, Transport
ST_DSW, ST_DSW	6AL	Two-component response regulator-like PRR1 [Triticum urartu]	A2YQ93	phosphorelay signal transduction system, regulation of transcription, DNA-templated, rhythmic process
ST_PH	6BL	B3 domain-containing protein [Aegilops tauschii]	Q2QMT6	regulation of transcription, DNA-templated
FSW	7AL	RecName: Full=Catalase [Triticum aestivum]	A2YH64	response to oxidative stress, hydrogen peroxide catabolic process
ST_FRW	7AS	T-complex protein 1 subunit alpha [Aegilops tauschii]	O94501	protein folding, tubulin complex assembly
ST_FRW	7AS	Sucrose synthase 1 [Triticum urartu]	P04712	sucrose metabolic process
ST_DSW	7AL	Zinc finger A20 and AN1 domain-containing stress-associated protein 8 [Triticum urartu]	A2YEZ6	Stress response
ST_DSW	7BS	Transmembrane 9 superfamily member 4 [Aegilops tauschii]	Q9Y819	endosomal transport, vesicle-mediated transport

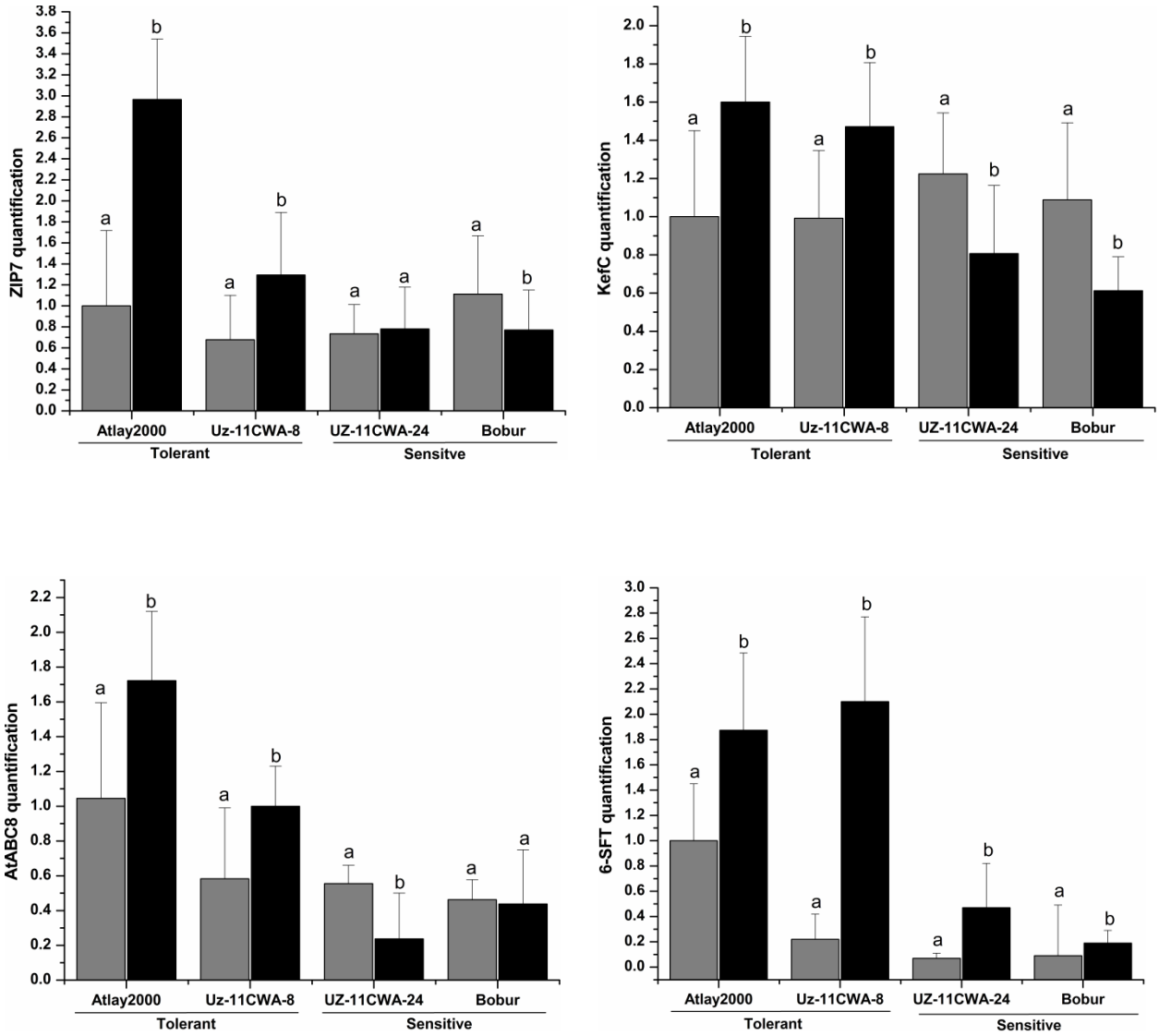
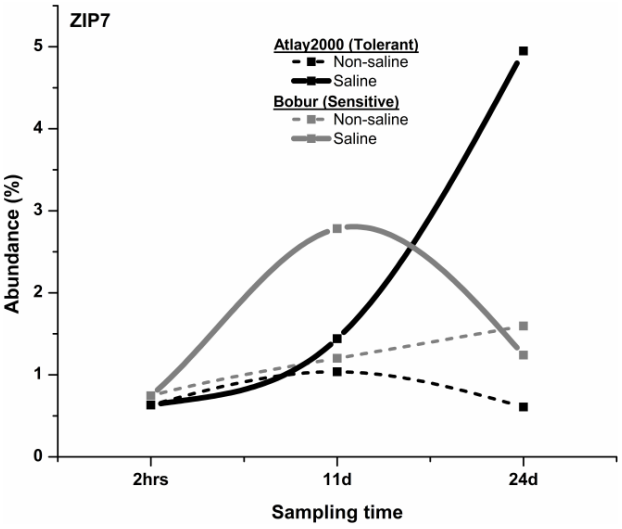
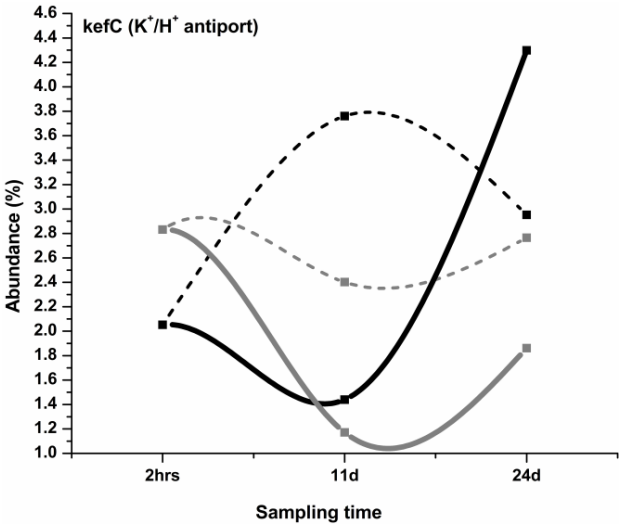


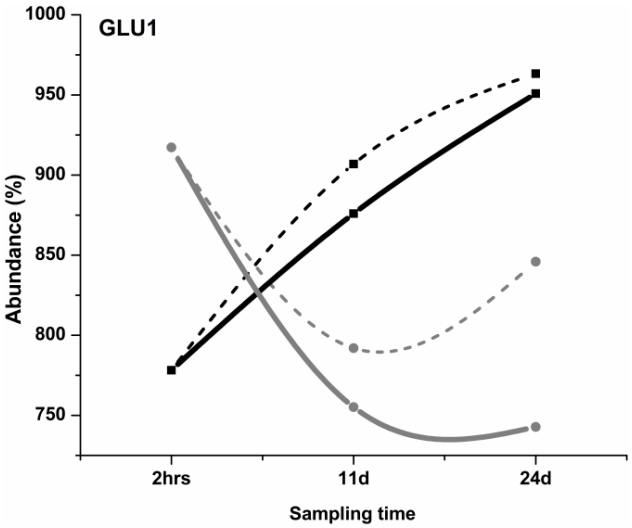
Figure 14 Expression levels of ***ZIP7***- putative Zi*nc transporter*; ***KefC***- *Glutathione-regulated potassium-efflux system protein*; ***AtABC8***- *Putative ABC transporter B family member 8* and *6-SFT-sucrose*: fructan-6-*fructosyltransferase* in leaves of two salt tolerant (*Atlay2000* and *UZ-11CWA-8*) and salt sensitive (*Uz-11CWA-24* and *Bobur*) after 30 days in non-saline (Grey) and saline (Black) conditions, determined by $2^{-\Delta CT}$ method. *Efa1.1* and *Efa1.2* genes were used as internal control genes. Bars are the means (n = 3) ± standard error.

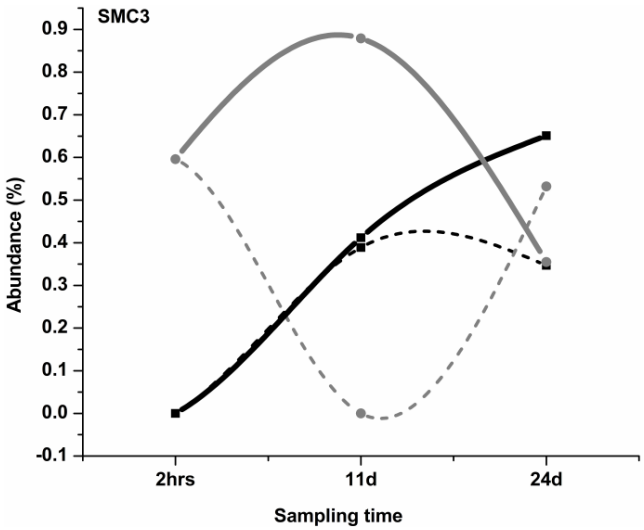
Time effect and kinetics of some of the associated genes

The expression-kinetics of the identified genes were compared in the contrasting genotypes over time-course of 2 hours, 11 days and 24 days in saline and non-saline conditions. Using the sigmoidal function revealed that the putative candidate genes had distinct but partially overlapping expression patterns at the onset of salt treatment *(***Figure 15**). The transcript amounts were higher in the sensitive genotypes at the early phase of salt treatment; but the trend was gradually altered over time, favoring the tolerant genotype. In general, there was an increase in the level of transcript expressions in both *Atlay2000* and *Bobur* as the salt treatment progressed. However, marked differences in the expression signatures between the two genotypes started to manifest at about 11 days after stress. From this time-point onward, the expression levels of the genes increased exponentially in *Atlay2000* but less so in *Bobur*. The *ZIP7*, *Structural maintenance of chromosomes protein 3* (SMC3) and Na^+/H^+ *antiporter* transcripts increased and decreased in *Atlay2000* and *Bobur*, respectively, after 11days of salt treatment; but *ferredoxin-dependent glutamate synthase* **(**GLU) was differentially expressed in both genotypes much earlier after 5 days of salt treatment. The *Zinc finger A20 and AN1 domain-containing stress-associated protein* 8 (SAP8) showed late differential response (beyond 24 days of stress). The gene–gene correlation matrix constructed using the expression patterns revealed mostly positive correlations and few negative correlations (***P*≤0.05, Figure 16**).


ZIP7
Atlay2000 (Tolerant)
Non-saline
Saline
Bobur (Sensitive)
Non-saline
Saline
Abundance (%)
0
1
2
3
4
5
2hrs
11d
24d
Sampling time


kefC (K+/H+ antiport)
Abundance (%)
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
3.0
3.2
3.4
3.6
3.8
4.0
4.2
4.4
4.6
2hrs
11d
24d
Sampling time


GLU1
Abundance (%)
750
800
850
900
950
1000
2hrs
11d
24d
Sampling time


SMC3
Abundance (%)
-0.1
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
2hrs
11d
24d
Sampling time

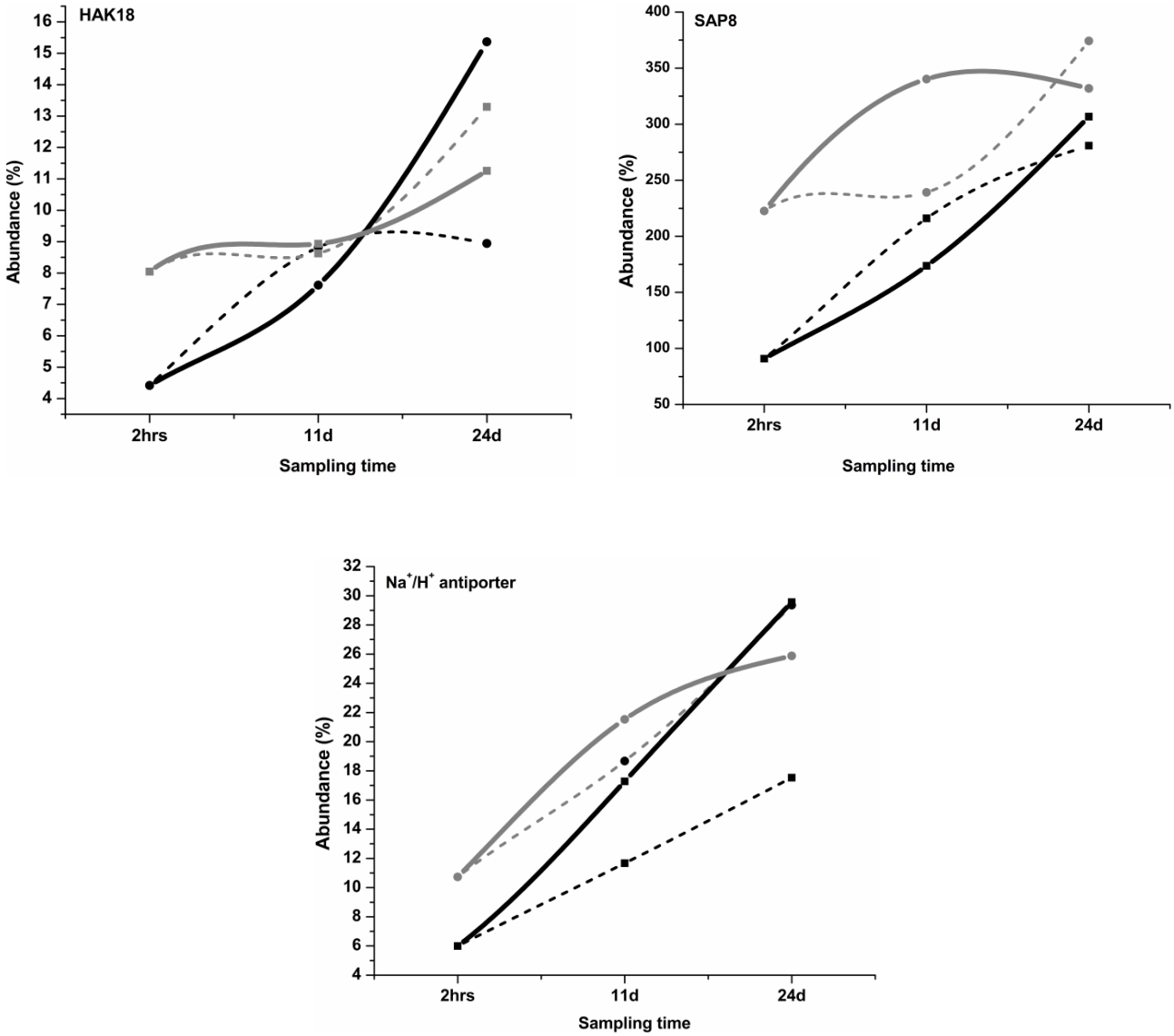


Figure 15 The expression kinetics of the associated salt tolerance genes over a period of 24d in salt tolerant (in black colour) and salt sensitive (in gray colour) genotypes. The "thick" and "dotted" lines indicate the gene expression kinetics over-time in saline and non-saline conditions, respectively. **ZIP7** = *Zinc transporter 7*; **kefC** = *Glutathione-regulated potassium-efflux system protein kefC*; **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- and y-axes are time of data collection and the amount of expressed transcripts, respectively.

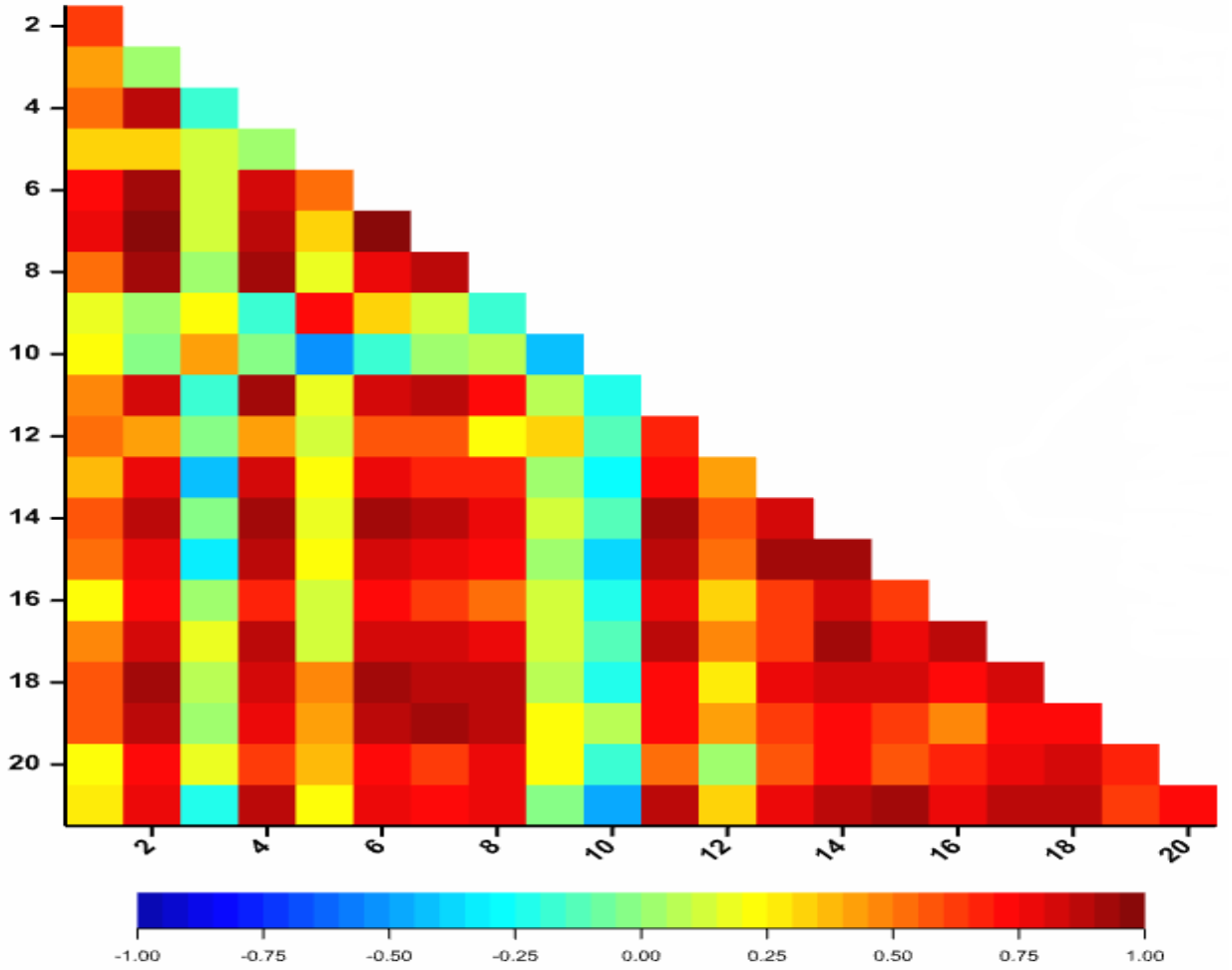


Figure 16 Correlation matrixes of 21 expressed genes identified in the GWAS analysis, based on the comparison of transcript abundance among these genes at three time-points after salt stress application. Blue colors indicates negative and red the positive correlations, whereas, the brightness is proportional to the strength of the correlation. **1**= *Zinc transporter 7*; **2**= *Protein transport protein Sec24-like CEF*; **3**= *Bidirectional sugar transporter SWEET17*; **4**= *Transmembrane 9 superfamily member 4*; **5**= *Glutathione-regulated potassium-efflux system protein kefC*; **6**= *Potassium transporter 18*; **7**= *NADPH-cytochrome P450 reductase*; **8**= *Glutathione S-transferase* 1; **9**= *ferredoxin-dependent glutamate synthase*; **10**= *hypersensitive response protein*; **11**= *Zinc finger A20 and AN1 domain-containing stress-associated protein* 8; **12**= *Structural maintenance of chromosomes protein 3*; **13**= *Two-component response regulator-like PRR1*; **14**= *B3 domain-containing protein*; **15**= *RecName: Full=Catalase*; **16**= *Asparagine synthetase (glutamine-hydrolyzing)*; **17**= *Prohibitin-2*; **18**= *Glycerol kinase*; **19**= *Protein kinase G11A*; **20**= *T-complex protein 1 subunit alpha* and, **21**= *Sucrose synthase 1*.

Sequence analysis in the putative candidate genes

The amino acid sequence analysis revealed several non-synonymous substitutions between Atlay2000 and Bobur at the coding regions anchoring the associated SNPs for all the putative genes analyzed (**Figure 17**). Most of the substitutions detected showed dissimilar phyisco-chemical properties. For instance, the 496st amino acid in *Traes_1BS_D68F0BED6.1.mrna1-E4* coding region of the *ZIP-7* changed from C (Cysteine) in Atlay2000 to S (Serine) in Bobur; and at 503rd and 504th positions, "Threonine (T)" and "– (an amino acid deletion)" were observed in *Bobur* (**Figure 17A**) instead of Alanine (A)" and "Leucine (L)", respectively. There were three amino acid changes within the coding sequence (*Traes_2AL_A2CBDB5F7.1.mrna1-E2)* anchoring the associated SNP at second exonic region of KefC **(Figure 17B**). The first change is from L (leucine) in Atlay2000 to G (glutamine) in Bobur, while the second is from S (serine) to T (threonine). The third change is from P (proline) in Atlay2000 to A (alanine) in Bobur. In associated exon 10 coding region of HAK18 (*Traes_5BL_F112FA40E.2.mrna1-E10),* we detected five non-synonymous substitutions sites that may have contributed to the alteration of the gene functional capacity and structure (**Figure 17C**). Sequences variations were also observed between Atlay2000 and Bobur in the remaining three genes including *SAP8, GST1* and SWEET17 (**Figure 17D-F).**

A. Zinc 7 transporters (ZIP7)

```
Gene ID    --SMSSLAVWATGLMRRRMTPTSQHTACDTCTLMHVPVFILHTYIRRALVFDGHERTAPVETPEHCLCIYMNPRALLRDILC-- 510
Atlay2000  --SMSSLAVWATGLMRRRMTPTSQHTACDTCTLMHVPVFILHTYIRRALVFDGHERTAPVETPEHCLCIYMNPRALLRDILC-- 510
Bobur      --SMSSLAVWATGLMRRRMTPTSQHTACDTCTLMHVPVFILHTYIRRALVFDGHERTAPVETPEHCLSIYMNPRT-LRDILC-- 510
```

B. Glutathione-regulated potassium-efflux system protein (kefC)

```
Gene ID      --LCSTSGSSLGYGFSRVMSKTKPVVSDDESDTIDGALAIAHYANVKGSASLVLPGLYSRCVRVK-- 1141
Atlay2000    --LCSTSGSSLGYGFSRVMSKTKPVVSDDESDTIDGALAIPHYANVKGSASLVLLPGLYSRCVRV-- 1141
Bobur        --LCSTSGSSQGYGFTRVMSKTKPVVSDDESDTIDGALAIAHYANVKGSASLVLLPGLYSRCVRV-- 1141
```

C. Potassium transporter 18 (HAK18)

```
Gene ID      --PGFETVGDEVTFLNSCRDAGVVHILGNTVIRARRDSGPLKKLAIDYLYAFLRKICRENSAIFNVPHESLLNVGQVFYV 875
Atlay2000    --PGFETVGDEVAFLNSCRDAGVVHILGNTVIRARRASGPLKKLAIDYLYAFLRKICRENSAIFNVPHESLLNVGQVFYV 875
Bobur        --PGFETVGDEVAFLNSCRDAGVVHILGNTVIRARRDSGPLKKLAIDYLYAFLRKICRENSAIFNVPHESLLNVGQVFYV 875

Gene ID      LKWMITVFVCRLFYRRTLQKLIDFTYLEHVDFSTNKMAHVNFSNKMANFSAV-- 927
Atlay2000    LKWMITVFVCRLFYRRTLQKLIDFTYLENVDFSTNKMAHVNFSNKMANFSAV-- 927
Bobur        LKWMITVFVCTLFYRRTLQKLIDFTYMEHVDFSTNKMAHVNFSNKMANFSAV-- 927
```

D. Zinc finger A20 and AN1 domain-containing stress-associated protein 8 (SAP8)

```
Gene ID      --AGPSEAAMENPKGPSRCSTCRKRVGLTGFNCRCGNLYCATHRYSDKHECKFDYRAAAMDAIAKANPVVKAEKLDKIEG 258
Atlay2000    --AGPSEGAMENPKGPSRCSTCRKRVGLTGFNCRCGNLYCATHRYSDKHECKFDYRAAAMDAIAKANPVVKAEKLDKIKG 258
Bobur        --AGPSEGAMENPKGPSRCSTCRKRVGLTGFNCRCGTLYCATHRYSDKHECKFDYRAAAMDAIAKANPVVKAEKLDKIEG 258

Gene ID      PPYGRQNIRSQIKSALPRHRASLLHYPFIMLASAILVVVGQSCTAHAPRRQPQVRTVSLLAMLCKLYLWSSL--330
Atlay2000    PPYGRQNIRSQIKSALPRHRASLLHYPFIMLASAILVVVGQSCTAHAPRRQPQVRTVSLLAMLCKLYLWSSL--330
Bobur        PPYGRQNIRSQIKSALPRHRASLLHYPFIMLASASLVVVGQSCMAHAPRRQPQVLTVSLLAMLCKLYLWSSL--330
```

E. Glutathione S-transferase 1 (GST1)

```
Gene ID      --VAALMKPSFLALVRREISRGVFPPMCACPSVCSCDVSSCMFRLVLAILSRSCTEMSIPMLWFNNKLCPEIISASIFS--372
Atlay2000    --VAALMTPSFLALVRREISRGVCPPMCACPSVCSCDVSSCMFRLVLAILSTSCTEMSIPMLWFNNKLCPDIISASIFS--372
Bobur        --VAALMRPSFLALVRREISRGVFPPMCACPSVCSCDVSSCMFRLVLAILSQSFTEMSVPMLWFNNKLCLDIIPASIFS--372
```

E. Bidirectional sugar transporter (SWEET17)

```
Gene ID      --AYDASASLLSSKASRHGQDDVATRVLCKIMFSMSKVQFLSHALIFFKASWTIFLSRDRP--272
Bobur        --AYDASASLLSSKASRHGQDDVATRVLCKIMFSMSKVQFLSHALIFFKASWTIFLSRDRP--272
Atlay2000    --AYDASASLLSSKASRHGQDDVATRVLCKIMFSMSKVQFLSHALIFFKASCTIFLSRDRL--272
```

Figure 17 Comparison of the deduced EST amino acid sequence of the associated: **A- ZIP7** (*Traes_1BS_D68F0BED6.1.mrna1-E4)*; **B- KefC** (*Traes_2AL_A2CBDB5F7.1.mrna1-E2)*; **C- HAK18** (*Traes_5BL_F112FA40E.2.mrna1-E10);* ***D*- SAP8** (*Traes_7AL_B88F6A3D3.1*)**; E- GST1** (*Traes_3AL_F205FA0941.2.mrna1-E1),* and **F- SWEET17 (**Traes_5AS_9937DABBA.1.mrna1-E5) in Atlay2000 (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 analyzed sequences are the identical and polymorphic sites found between the contrasting wheat genotypes, respectively, while gray colour represent region anchoring the associated SNP marker identified in the GWAS analysis.

DISCUSSION

Genetic variation among the studied panel under salt stress

The availability of genetic resources, knowledge about the genetic diversity for the desired traits including genes and the population structure is paramount for effective use of genetic resources for gene discovery, germplasm development and deployment in breeding. Kulwal *et al.* (2012) have shown that the success of GWAS to detect putative QTL/genes depends greatly on the sample size, choice of germplasm, marker density and the heritability of the traits. In this study, 150 ICARDA-CIMMYT-IWWIP elite wheat germplasm were used to map the QTLs controlling salt tolerance across three different growth stages. The association mapping panel was derived from 673 crosses that involved 371 unique ancestral co-founders; thus, highlighting the potential genetic diversity inherent in the panel used for this study which makes it a valuable genetic resource for QTL identification and characterization of the genetic loci contributing to salinity tolerance.

The genotypes responded differently to the salt stress across the three growth stages and, the CV ranged from 2.87-7.95% for germination, 15-22% for seedling and 1.28–44% for AFP (full details previously reported in Oyiga et al 2016). The variations observed across the growth stages 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). However, unlike the present study, the previous studies evaluated the genetic architectures controlling ST based on single growth stage experiments. The results reported here are to our knowledge the first which simultaneously evaluated salt tolerance mechanisms in wheat based on three growth stages. All the traits analyzed showed moderate to high h^2, lending credence in the quality of data collected for various traits to allow for the identification of useful QTL linked to such salt stress tolerance traits. Salt stress impacted negatively on germination vigor, root/shoot seedling biomass production and yield related traits, as was reported in Oyiga et al. (2016). Similar effect in the decrease of plant growth and development due to salinity has been reported in wheat (Munns and Tester 2008; Gomes-Filho *et al*., 2008).

Genetic variation in the two key physiological traits, leaf Na^+ and K^+/Na^+ ratio indicates the possibility of genetic improvement of salt tolerance (Karan and Subudhi, 2012). In the mapping panel, sufficient genetic variations were observed for Na^+ concentrations (CV= 28.14%; **Figure 2B**) and K^+/Na^+ ratio (26.80%; **Figure 2C**) in the third leaves among the germplasm, after 25 days of salt stress thus, making the panel amenable for the dissection of genetic mechanisms controlling ST. The leaf Na^+ concentration and the K^+/Na^+ ratio correlated positively and negatively, respectively, with the accumulated root biomass under salt stress, which is consistent with previous studies that indicated that root plays an important role in Na^+ transport to the shoot as well as in ion-homeostasis (K^+/Na^+ ratio) (Lacan and Durand, 1996; Krishnamurthy *et al.,* 2009). Munns *et al*. (2006) reported that increase in root biomass under salt stress would encourage excessive amounts of salt entering the transpiration stream which will cause injury to the cells in the transpiring leaves and may reduce growth.

Population structure and LD

Population structure can result in spurious associations between markers and traits and necessitates consideration in GWAS studies to minimise its confounding effects (Flint-Garcia *et al*., 2003; Yu *et al*., 2006). The population structure of the mapping panel was examined using three approaches (STRUCTURE and PCoA) that produced similar results. Two sub-populations were observed, which is in line with Wingen *et al*. (2014), but the groupings did not reflect the four breeding centres where the

genotypes originated from, due to the intermixing of several genotypes. A likely explanation for the intermixed population could be that the breeders exchanged germplasm in their breeding programs and, with the established long history of recombination and mutation events in the panel gave rise to a highly diverse germplasm, thus making it suitable for association studies.

The resolution of association mapping depends on the extent of LD across the genome and the rate of LD decay with genetic distance (Stracke *et al*., 2007). The LD of the GWAS panel decayed after 10, 11 and 14 cM in A-, B- and D-genome, respectively, suggesting that large number of SNPs are required to define the recombination profiles in the panel as a means to achieve high resolution. This implies that we can expect to detect significant LD due to linkage between SNPs separated by less than 10, 11 and 14 cM in A-, B- and D-genome, respectively. With the average SNP interval of 0.49 cM in the GWAM panel, it is expected that sufficient SNP-marker density for high resolution was achieved to detect QTL controlling ST. LD decay of <14 cM has also 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 been reported in wheat (Joukhadar *et al*., 2013; Turki *et al*., 2014). The LD decayed less rapidly in D-genome compared to A- and B-genomes, due 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), thus making the D-genome less genetically diverse.

Association mapping

GWAS identified 172 and 15 SNPs associated with the phenotypic and ionic traits, respectively; representing 37 LD defined QTL regions and, explained between 3.0% and 30.67% of the R^2. Most of the associations identified correspond with the chromosomal regions carrying published salt tolerance QTL/genes (**Table 8**). Notable, are *Q-1BS.1*, *Q-2AL.1*, *Q-2BS.1* and *Q-3AL.1* that affected the salt-stress related 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, an indication that they could be involved in ion-homeostasis (ROS scavenging) and solute transport in wheat. The *Q-2AL.1* found in the region of ST QTL for seedling biomass (Ma *et al*., 2007; Genc et al., 2010) coincided with the previously reported Na^+ exclusion locus in durum wheat that hosts HKT1;4 (Nax1) that is closely linked with gwm312 marker (Lindsay *et al*., 2004; James et al., 2006; Huang *et al*., 2006), while the *Q-2BS.1* is co-localized with a ST QTL controlling yield and seedling biomass (Quarrie *et al*., 2005; Genc *et al*., 2010) and *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* on 1BS at 8.4 cM interval has not been previously

reported. These QTL regions could be of value for future efforts to better understanding of salt tolerance mechanisms in wheat.

The *QTL_2DS.1* on 2DS showing pleiotropic effect in both seedling- and AFP-traits was located proximal to ST QTL *QSdw-2D* (Xu *et al*., 2012), *QSlc.ipk-2D* (Landjeva *et al*., 2008) as well as *Ppd-1* gene reported to exert strong pleiotropic effect on many traits (Kumar *et al.*, 2007; Beales *et al*., 2007; Bennet *et al*., 2012). It could be hypothesized that *QTL_2DS.1* operates in multiple pathways controlling plant responses to stress and plant adaptation. Ma *et al.* (2007) reported a QTL for leaf symptoms due to Na^+ effect close to *QTL_5AS.1* identified in the present study, while *QTL_5AL.1* overlapped with QTL controlling frost (Baga *et al*., 2007) and copper (*QCut.ipk-5A1*; Bálint *et al*., 2007) tolerance, suggesting that this locus might be linked to genes conferring multiple-tolerance to abiotic stresses. The *QTL_5AL.2* ($R^2 \geq 16.22\%$) on 5AL, detected for multiple ST-traits such as 75 mM Na_2SO_4, ST_FSW, ST_DSW, ST_DRW and leaf K^+, corresponds to the genomic region of gene for Na^+ exclusion *TmHKT1* identified as a candidate for *Nax2* (James *et al*., 2006; Byrt *et al*. 2007; Munns *et al*., 2012). SNP *GENE_3156_152* on 5BL at 68.4 cM, associated with ST-traits at germination and seedling growth stages, was found within the *Vrn-1B* locus reported to have pleiotropic effects on several genes controlling frost, salt, drought and osmotic stress tolerance (Yan *et al*., 2003; Limin and Fowler 2006; Dhillon *et al*., 2010). The QTL regions including *Q.5BS.1, QTL.5BL.3* and *QTL.5BL.4* did not overlap with any reported QTL, suggesting that they may be potentially novel ST QTL regions

PCoA based on the associated polymorphisms

The PCoA using all the SNPs showing significant MTAs in our GWAM studies correctly discriminated the most consistent contrasting wheat genotypes, identified in the studied panel (Oyiga *et al*., 2016), based on their salt tolerance status (**Figure 11**), as the genotypes were broadly separated into two genetically distinct groups. The salt-tolerant genotypes were mostly grouped on the right side of the PCoA plot, whereas the salt-sensitive genotypes were clustered at the left side of the plot. Singh *et al*. (2013) posited 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. This result not only reflected on the genetic diversity among the genotypes but also showed that the identified polymorphisms are involved in salt stress response. This means that the identified SNPs are linked to genes regulating ST, suggesting that sequence annotation of the associated loci can uncover the genetic variants. Based on genetic relationships from cluster analysis, salinity tolerance might be improved by selecting parental genotypes from different clusters.

Gene annotations and interpretation of genetic mechanisms

The map used in this study had a resolution of 0.49 cM, which means that the GWAS results can be resolved into a single gene. Among the genes that were identified are genes involved in ST (**Table 9**). The *ZIP7* transporter, identified in the novel *Q-1BS.1* region that was detected across the three growth stages, controls Zn uptake (van der Zaal *et al*., 1999) and has been shown to confer salinity and drought tolerance in rice (Liu *et al*., 2014). The uptake of Zn in plant increases ST status by improving the expression of Na^+/H^+ antiporter genes, *TaSOS1* and *TaNHX1*, while decreasing the Na^+ accumulation (Abou Hossein *et al*., 2002; Xu *et al*., 2014) as well as the ROS accumulation and homeostasis (Chen *et al*. 2011; Sinclair and Kramer, 2012). The *QTL.2A.3* on 2AL is homologous to KefC, which confer protection against electrophiles catalyzes K^+/H^+ antiporter and, export rubidium, lithium and sodium (Fujisawa *et al*., 2007). The SNP *RAC875_c14137_994* at 107.25 cM on 1DL (with R^2=7.05%) significantly linked to a new QTL detected for leaf K^+ showed high sequence homology with an uncharacterized *Na^+/H^+* antiporter. The SWEET 17 transporters mediate sucrose, fructose and glucose transport across tonoplast of roots and leaves (Schroeder *et al*., 2013; Chen, 2014; Guo et al., 2014). This gene is associated with pathogen resistance (Schroeder *et al*., 2013) and, it´s sequence is associated with TKW at 39.26 cM on 5AS.

The *Response to ABA and Salt 1B* that encode ABA- and salt stress-inducible (Rab11B) genes was homologous with the SNP *IAAV565* that is associated with germination vigor on 1BL. Ren *et al*. (2010) reported that Rab11B is a negative regulator of salt tolerance during seed germination and early seedling growth by enhancing ABA sensitivity. The homologous *Transmembrane 9 superfamily* member 4 on 7BS has been reported to be involved in the adaptation to NaCl toxicity in ryegrass (Li *et al*., 2012) and rice (Senadheera *et al*. 2009). SAP8 was identified on the *QTL_7AL.1* region. This gene is known to confer salt, cold and dehydration stress tolerance in transgenic tobacco (Mukhopadhyay *et al*., 2004), tea (Paul and Kumar, 2015), *Arabidopsis* (Giri *et al*., 2011) and rice (Kanneganti and Gupta, 2008) by exhibiting multiple responses. Two associated SNPs *RAC875_c14173_207* (33.45 cM; R^2= 14.31%) and *Kukri_c42622_369* (35.31 cM; R^2= 11.58%) on *Q-7AS.1* region were both coding for sucrose: *fructan 6-fructosyltransferase* (6-SFT), a key enzyme for fructan synthesis (Müller *et al*., 2000). Fructans supports osmo-protectants synthesis, anti-oxidation and membrane stability in plants (Valluru and Van den Ende, 2008; He *et al*., 2015). The *Q-7AS.1* might be similar to the osmoregulation genes regions previously described by Morgan (1991) and Morgan and Tan (1996). This further confirmed the earlier study by Ogbonnaya *et al*. (2013) in which they reported the identification of minor gene for Na^+ exclusion in

synthetic derived population 'AUS29639//Yitpi' on chromosome 7A though they did not characterize the underlying gene.

Transcriptomic and single gene expression analysis

All the putative genes analyzed (except *Protein kinase* G11A) were significantly up-regulated in the *Atlay2000* but down-regulated in *Bobur* after 24 days of salt stress (**Figure 13)**, suggesting that these genes are salt-responsive and their expressions may contribute to ST in wheat. Among the genes, *ZIP7* showed strongest differential response to salt stress. It increased by 713.98% in *Atlay2000* but declined by 22.19% in *Bobur*, an indication of a positive link existing between Zn transport and salt stress tolerance. Lonergan *et al.* (2009) have shown that candidate locus *HvNax4* controls shoot Na^+ accumulation in barley and is also associated with Zn^{2+} accumulation. Available reports indicated that *ATP-binding cassette (ABC)* transporters identified on 3AL are involved in a diverse range of processes, including hormones, lipids, metals, secondary metabolites and modulators of ion channels (Perlin *et al*., 2014; Hellsberg *et al.*, 2015) and its up-regulation enhances salt and drought resistance (Kim *et al*., 2010; Li *et al*., 2015). Moreover, the over-expression of *KefC* in *Arabidopsis thaliana* (Shi *et al*., 2000; Shi *et al*., 2003) and *6-SFT* in tobacco (He *et al*., 2015) and wheat (Kerepesi *et al*., 2002), similar to results of the present study, have been demonstrated to confer ST. The RT-PCR results of putative genes including *ZIP7*, *KefC*, *AtABC8* and *6-SFT* showed similar expression patterns in the leaves of two tolerant (up-regulation) and two sensitive (down-regulation) genotypes after 30 days of salt treatment, thus confirming the role of these genes in ST. Thus, transcriptome and RT-PCR results suggested that the ST-status of *Atlay2000* can partly be attributed to the increased activities of the candidate genes identified in the present study. The results presented here were data obtained from the shoot parts, since the analyzed 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 which is in close contact with the solution.

The kinetics of the putative genes under saline and non-saline conditions revealed differential transcript signatures across the three time points (2hour, 11 days and 24 days) after salt application. Prior to salt stress initiation, *Bobur* exhibited higher tendency toward transcript accumulation than *Atlay2000*, suggesting that both genotypes are genetically different. Over-time, the accumulated transcripts in *Atlay2000* increased exponentially surpassing that of *Bobur*, confirming the former to possess better adaptation mechanisms to deal with salt stress. The differential expressions of ZIP7, *SMC3* and

uncharacterized *Na^+/H^+ antiporter* between the genotypes became conspicuously obvious after 11d of salt treatment. This time period may coincides with the ionic phase earlier described by Munns and Tester (2008) when the accumulation of salts is becoming deleterious 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. Much earlier (about 4 days) and late (after 24 days) differential response were observed in GLU and *SAP*8, respectively, between *Atlay2000* and *Bobur*. These findings provide probable reasons and support our earlier results report (Oyiga *et al.*, 2016) that *Atlay2000* is a widely adapted salt tolerant genotype. Further, judging by the mostly positive correlations of the expressions observed among the genes analyzed, we might infer that most of the investigated putative genes are co-expressed, although further studies are needed to validate this claim.

Sequence variations at candidate gene loci

Several amino acid changes that resulted in non-synonymous substitutions were detected between *Atlay2000 and Bobur* in the gene coding regions anchoring the SNPs identified by GWAS (**Figure 17**). These substitutions belonged to different physico-chemical properties, suggesting that the detected mutation sites may have affected the gene structure and function differently in both genotypes during salt stress. Three non-synonymous substitutions were detected sites 496, 503 and 504 of the EST amino acid sequence of ZIP7 **(Figure 17A**). From *Atlay2000* to *Bobur*, the protein sequences were altered from C (Cysteine) to S (Serine), from A (Alanine) to T (Threonine) and from L (Leucine) to – (a SNP deletion) at the discriminating amino acid sequence sites, respectively. The aaccumulations of A and L have been reported in salt-tolerant plants (Mansour, 2000). In addition, C is more hydrophobic than S and, Hessels (2015) has shown that C-to-S substitution decreases the Zn^{2+} affinity. Thus, the up-and down-regulations of ZIP7 observed in Atlay2000 and Bobur, respectively, during salt stress may have been largely contributed by the C496S substitutions at the exon 4 of ZIP7. Amino acid substitution detected at exon 2 in of putative *KefC* changed from L (Leucine) in Atlay2000 to Q (Glutamine) in Bobur, from S (Serine, in Atlay2000) to T (Threonine, in Bobur) and, from P (Proline, in Atlay2000) to A (Alanine, in Bobur) at position 1087, 1092 and 1117, respectively (**Figure 17B**); offering the probable reason for the differential response of this gene in both contrasting genotypes. L and P, which were substituted by G and A in Bobur, respectively, have been reported to play osmoprotective role (Arbona *et al.*, 2013) in plants and may have contributed to increased salt sensitivity of Bobur to salt stress. The results of this study provide new strategy to increase ST in wheat.

CHAPTER 4

Genetic variability and identification of salt tolerance QTL affecting ion uptake, leaf chlorophyll fluorescence and seed quality traits in association mapping panel of wheat

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ABSTRACT

Soil salinity is one of the most important environmental factors that affect wheat productivity worldwide. The dissection of the genetic architectures of salt tolerance (ST) is invaluable towards improvement of salt tolerance. We conducted a genome-wide association study using 18,000 single nucleotide polymorphism (SNP) markers across 150 diversity panel of wheat that were phenotyped for some key physiological and seed quality traits, in order to assess the genetic diversity and to identify marker-trait associations and candidate genes involved in ST. Using the mixed-linear model, we identified a total of 54, 44 and 17 SNPs significantly associated with the salt-related chlorophyll fluorescence (ChlF), shoot ionic (SI) and seed quality (SQ) parameters, respectively. They explained between 2% and 63.45 % of the observed phenotypic variance. All the significant MTAs were located in 21 LD-defined clusters in the wheat genome. Among them, a locus on 6AL were strongly associated with ChlF and shoot Na^+ content, while another locus on 2DS affected ChlF and SQ traits. We also found a region <1.55 cM on 2BL to be influencing *Fv/Fm*, shoot Na^+, shoot K^+/Na^+ and seed crude fiber content. *In-silico* analysis of the flanking sequences of trait-associated SNPs in wheat databases uncovered several putative genes that may be regulating variations in the measured traits. Expressed sequence tags allelic variations and expressions analyses performed provided useful information for understanding the genetic mechanisms of ST. Thus, this study establishes a fundamental research platform for developing salt-stress responsive functional genetic markers that can be utilized in breeding programs in wheat.

INTRODUCTION

Wheat is one of the world´s most important strategic food crops, with an estimated annual production of about 736 million metric tons (MMT) (FAO, 2015). With the projected increase in the world population to 9.1 billion by 2050 coupled with the climatic change, there is need to the improve the wheat resilient to environmental stresses so as to increase its production to meet the global demand, which has been predicted to surpass 880 MMT by 2050 (Weigand, 2011). Salinity is largely limiting the wheat production globally with more than 6% of the world's total land area affected by salinity (FAO 2010) and, is expected to increase in the coming years (Munns and Tester, 2008). Salt stress leads to ionic imbalance, hyperosmotic stress and nutrient deficiency (Flowers, 2004) and, would decrease the seedling growth and survival (Lutts *et al*., 1995), damage the structure of chloroplasts and photosynthesis capabilities (Parida and Das 2005; Yamane *et al*. 2008; Abbaspour *et al*. 2012), consequently resulting in poor seed set and seed quality (Asch *et al*., 2000; Nayidu *et al*., 2013) and yield (Atkinson and Urwin, 2012). Plant response to salinity is a complex phenomenon involving contributions from several

physiological, genetic, molecular and cellular mechanisms, in addition to the interactions between the plant and it's continually changing environmental conditions. Thus, understanding of how these factors interact and contribute to ST would be necessary in designing an efficient breeding strategy.

Several strategies have been adopted to minimize the effect of salt stress on plants, but the use of salt tolerant wheat cultivars is considered as the most economical and efficient strategy to increase grain yield. Salinity tolerance depends on how the plant deals with the process of salt uptake and accumulation patterns into different organs (Paranychianakis and Angelakis, 2008, Munns *et al*., 2012; Guan *et al*., 2014), elimination of reactive oxygen species (ROS) (Suzuki *et al*., 2012; Peng *et al*., 2014), organic compound accumulations (Ashraf and Foolad, 2007) and hormone regulation (Jiang *et al*., 2013; Osakabe *et al*., 2014). Recent progress in plant molecular biology have shown that adaptation of plant to salt stress is achieved via ion homeostasis (Misra *et al*., 2011; Yang *et al*., 2012), associated with high K^+ uptake, restriction Na^+ uptake, activating Na^+ exclusion or cellular compartmentalization of excessive Na^+ into the vacuole and ROS-homeostasis (Meyer *et al*., 2012; Noctor *et al*., 2014; You and Chan., 2015). The expressions of enzymes linked to the genes involved in these processes play vital roles in plants adaptation under salt stress conditions.

Khayyat *et al*. (2014) have reported that plants response to high salinity stress via non-stomatal and stomatal levels. While the former inhibits photosynthetic by destroying the chloroplast structure (Rouhi *et al*., 2006), the latter reduces photosynthesis by lowering stomatal conductance (photon flux energy) and transpiration rate in leaves (Cornic, 1994; Wang *et al*., 2013b). The use of the high throughput leaf chlorophyll fluorescence (ChlF) measurements allows for quick detection of the stress or damage to the photosynthetic apparatus (Glynn *et al.* 2003). It is a non-invasive means for direct assessment of plant photosynthetic performance and capacity and, has been exploited to detect genetic variations for ST in plant (Ranjbarfordoei *et al*. 2006; Kalaji *et al*. 2011). In spite of the several studies reporting on the impact of salt stress on the photosynthetic apparatus using the ChlF techniques, none have reported on how the plant photosynthetic performance would impact on the accumulation of shoot ionic (K^+, Na^+ and K^+/Na^+) components and seed quality under salt stress conditions. The establishment of the genetic relationships among these traits would help in the identification of reliable, fast, easy and economical criteria that would serve as surrogates to assess the genetic variation for salt tolerance in wheat. Thus, reduces large investments (*i.e*., time, money and labour) that are associated with screening for ionic and seed-quality traits, especially in a large population. Identification of common QTL domain regulating

these traits would benefit efforts toward developing salt-stress resilient wheat genotypes through targeted selection strategies.

In the present study, we used the genome-wide association study (GWAS) approach employing >18,000 SNP markers in a diversity wheat panel of 150 genotypes in order to: i) investigate marker-trait associations (MTAs) for chlorophyll fluorescence (ChlF), shoot ionic (SI) and seed quality (SQ) ST-traits and ii) scan for putative candidate genes that control genetic variations in the measured traits. Furthermore, we aimed to gain insight into the genetic mechanisms and features regulating salt tolerance in the associated ST loci. This study was designed to provide useful information for understanding the genetic mechanism of salt stress tolerance in wheat and further unlock common regulatory networks of complex physiological traits under salt-stress conditions in wheat.

MATERIALS AND METHODS

Plant material and growth conditions

The association mapping panel and hydroponic screening experiments have been described in Oyiga *et al* (2016). In the present study, the 150 diversity wheat panel were grown under non-saline and saline (150 mM NaCl) conditions in the greenhouse. The salt treatment was introduced three days after planting (DAP) in an incremental basis of 50 mM daily. Thereafter, salt-stress was sustained for 24 days when the final concentration was reached. At harvest, all the genotypes were evaluated for ChlF and shoot ionic (K^+, Na^+ and K^+/Na^+) traits.

Leaf chlorophyll fluorescence: The ChlF-traits were measured in both saline and non-saline conditions at three positions in the 3rd leaf (about 5 cm from the stem, in the middle and about 5 cm from the end) using the FluorPen FP100 (Photon Systems Instruments, Brno, Czech Republic). All the readings were taken on the light-adapted leaves of 10 plants per genotypes, totaling 30 data points for each genotype. The light intensity reaching the leaf during measurement was 3000 mol (photons) m^{-2} s^{-1}, which was sufficient to generate maximal fluorescence. The ChlF-traits collected are described in **Table 1.**

Table 1 List of OJIP test parameters with explanations and formulae used for calculation.

Parameters	Formula explanation	Description
Extracted and technical fluorescence		
Fo	Fo= F50μs, fluorescence intensity at 50 μs	Fluorescence intensity when all reaction center (RC) are open
Fm	Maximal fluorescence intensity	Fluorescence intensity when all RCs are closed
Fv	Fv = Fm-Fo	maximal variable fluorescence
Vi	Vi = (Fi - Fo) / (Fm - Fo)	
Fv/Fo	(Fm −Fo)/Fo	Efficiency of the water-splitting complex on the donor side of PSII
Fv /Fm		Maximum quantum yield of PSII within light-adapted
Fm/Fo		Non-photochemical loss in PSII
Quantum efficiencies or flux ratios		
PI(ABS)		Performance index on absorption basis where
Specific fluxes or specific activities **(per Q_A-reducing PSII reaction center—RC)**		
ABS/RC	ABS/RC = Mo×(1/Vj)×(1/Phi Po)	Effective antenna size of an active reaction center (RC). Total number of photons absorbed by Chl molecules of all RC divided by the total number of active RCs
TRo/RC	TRo/RC = Mo×(1/Vj)	Maximal trapping rate of PSII. Maximal rate by which an excitation is trapped by the RC resulting in the reduction of QA to QA−
ETo/RC	ETo/RC = Mo×(1/Vj)×Psi o	Electron transport in an active RC. Re-oxidation of reduced QA via electron transport in an active RC. It reflects the activity of only the active RCs
DIo/RC	DIo/RC = (ABS/RC)−(TRo/RC)	Effective dissipation in an active RC. Ratio of the total dissipation of un-trapped excitation energy from all RCs with respect to the number of active RCs

Shoot ion contents: The amount of Na^+ and K^+ that were accumulated in the different shoot parts including 3rd leaf, stem and RLP (remaining leaf parts) under salt-stress were determined using atomic absorption spectrophotometer (type 2380; Perkin Elmer, Wellesley, MA, USA), following the procedure described in Oyiga *et al*. (2016). Thereafter, the K^+/Na^+ ratios were calculated.

Seed grain quality measurements: The SQ parameters including protein content (PC), starch content (SC), neutral detergent fiber (NDF), crude fiber (CFC), seed moisture content (SMC), seed hardness (SH) and seed sedimentation value (SSV) were measured using the DA 7250 NIR analyzer (Perkin Elmer, Wellesley, MA, USA), following the manufacturer's instructions. The analyzed seed samples were obtained from the replicated field evaluation trials in Karshi, Uzbekistan. The analyzed seeds are field grown grain harvests in both saline and non-saline conditions. The soil chemical properties and the geographical locations of the field trials are published in Oyiga *et al*. (2016).

Statistical analyses of the phenotype data

All the data collected were analyzed using the REML procedure as implemented in GENSTAT 16 edition to determine the genotype, salt treatment and their interactions effects. The significant differences were determined with the Wald statistics. The GENSTAT procedure was used to estimate the un-biased

estimates of variance components due to genotypic (σ^2_g) and environment (σ^2_e) effects. The heritabilities (h^2) of the traits were calculated as (O'Neill, 2010): $h^2 = [\text{genetic variance}/\text{Phenotypic variance} = \sigma_G^2/(\sigma_G^2 + \sigma_R^2/r)]$; where r and σ_R^2 correspond to the number of replicates and the residual variance, respectively. The best linear unbiased predictor (BLUP) was estimated taking into account the genotype by environment interaction (Piepho et al., 2008) and was used to perform correlation analysis and GWAS analysis.

Genetic analysis of the GWAS panel

Detailed information on SNP genotyping and analysis, population structure and linkage disequilibrium (LD) of the studied panel has been described in Chapter 4.

Marker-trait associations: MTAs of 18,085 SNP markers with minor allele frequency (MAF) > 0.05 were evaluated based on the BLUP values of all the measured traits using two software programs: TASSEL 5.2.13 standalone version (Bradbury *et al*., 2007) and SAS programs (SAS Institute Inc., Cary, NC). Only congruent QTL loci identified by both programs were reported. The multi-locus mixed linear model (MLM) adjusted using both population structure (*Q* matrix, as the fixed covariate) and kinship (K-matrix, as random effect) matrixes were employed to reduce errors from population structure. The cut-offs for accepting significant MTAs were calculated according to Long *et al*. (2013): -log10 (α/#tests), where α= 0.05 and #tests = the number of effective tests calculated as the total genome coverage divided by genome LD. We considered the effects of several associated SNPs to be coming from a single QTL, if the SNPs are within the genetic interval defined by the LD (Breseghello and Sorrells, 2006; Pasam and Sharma, 2014).

Putative candidate genes analysis

Putative candidate genes were proposed for each significant MTA by BLASTn searches of the extended associated SNP sequences from the CerealsDB database (http://www.cerealsdb.uk.net/). The searches were performed in the NCBI (http://www.ncbi.nlm.nih.gov/) GenBank non-redundant database.

Gene expression analysis

The transcript abundances of some putative candidate genes, obtained from the Massive Analysis of 3′-cDNA Ends (MACE) analysis (Unpublished) data, were analyzed in the leaves of salt-tolerant (Atlay2000) and salt-sensitive (Bobur) wheat genotypes over time points of 2hour, 11 days and 24 days

under non-saline and saline conditions. The RT-PCR of three putative genes including *Myo* (*myosin-J heavy chain*) on 6AL, At**ABC3** (ABC *transporter F family member* 3) on 2DS and *NAD(P)H* (*NAD(P)H-quinone oxidoreductase subunit* L, chloroplastic) on 5AL were performed to validate their expression pattern in two salt tolerant (*Altay2000* and *UZ-11CWA-8*) and two salt-sensitive (*UZ-11CWA-24* and *Bobur*) after 30 days under non-saline and saline conditions. The RT-PCR procedure has already been described in **Chapter 3.** The target gene primers and endogenous control genes are presented in the **Table 1.**

Table 1 RT-PCR primer pairs used for the endogenous control gene and amplification of selected wheat transcripts.

	Forward primer	**Reverse primer**	**Product size (bp)**
Target genes			
Myo	GCCCAACGCCAGCAAAATA	GGATTCAAAAGCACGCCAGT	175
AtABC	ATTCCCAACCCCAGATGAC	ACTGTTCCCGATGTTGGTTG	210
NAD(P)H	GGATGAGGCAGAGGTGGTT	GCGGGTATCTGTCCTTGAAC	195
Internal control genes			
TaEf-1a	CTGGTGTCATCAAGCCTGGT	TCCTTCACGGCAACATTC	151
TaEf-1a	CAGATTGGCAACGGCTACG	CGGACAGCAAAACGACCAAG	227

Details of the primers used for the gene amplification and their corresponding product size. At**ABC3,** ABC *transporter F family member* 3; **Myo**, *myosin-J heavy chain* and **NAD(P)H**, NAD(P)H-quinone oxidoreductase subunit L (chloroplastic).

RESULTS

Analysis of the phenotypic traits

Chlorophyll a fluorescence: Analysis of variance (ANOVA) revealed significant ($P<0.05$) variation among genotypes for all the ChlF-traits (except, *Fo* and *Fm*) (**Table 2**), which was also reflected by their standard deviation (SD) and coefficient of variation (CV) of traits in saline and non-saline conditions. Under non-saline conditions, the CV ranged from 0.9 in *Fv/Fm* to 13.67% in *PI(ABS)*; but, varied from 1.37 in *Fv/Fm* to 16.40% in *PI(ABS)* in saline condition. Significant differences ($P < 0.01$) were observed between saline and non-saline conditions for all traits. However, interaction effects were non-significant for all the ChlF-traits. Among the trait h^2 estimates, *ETo/RC* (63%), *Vi* (59%), *Fv/Fm* (33%) and *PI(ABS)* (30%) had the highest estimates, while Fo had the lowest h^2 estimates (8%). Salt stress decreased mean values of all the ChlF-traits, except for *DIo/RC* and *ETo/RC* (**Table 2**).

The genetic variations among the 150 genotypes obtained for the ionic parameters after 24 days of salt stress have been reported in our previous publications (Oyiga *et al*., 2016).

Seed grain quality traits: ANOVA revealed significant variation among genotypes for all the 7 seed quality traits (Table 2). Moreover, all the SQ traits showed significant ($P < 0.01$) salt treatment and genotype x salt treatment interactions effects. Results revealed that seeds from saline fields contain higher SSV (+42.17%), PC (+13.05%), SH (+4.39%), SMC (+3.28%) contents than the seeds obtained from non-saline fields. However, seed CF and SC of the saline field decreased by -1.27% and -1.17%, respectively in contrast to values obtained from non-saline field. The h^2 estimates for all the seed quality traits was high and ranged from 89% (PC) to 97% (SH).

Traits correlations

Pearson's correlation was used to investigate the relationships among the traits (**Table 3**). Highly significant correlations were mostly observed between the ChlF traits. Shoot K^+ and several ChlF traits including *Fv*, *Fv/Fo*, *Fv/Fm*, *TRo/RC*, *ETo/RC* and *PI(ABS)* were positively correlated (r^2= 0.25 – 0.52; p= 0.01). Moreover, the seed grain starch showed positively correlation with *Fo*, *Vi*, *ABS/RC*, *TRo/RC* and *DIo/RC* and; negatively correlated with *Fm/Fo*, *Fv/Fm*, *ETo/RC* and PI(ABS). Seed grain protein and starch contents were highly and negatively correlated (r= -0.80, p<0.01) with each other.

Table 2: Statistics of leaf fluorescence and seed quality traits of the mapping panel under control and saline conditions. **SD**, standard deviation; **CV**, coefficient of variation and; **E**, effect of salt stress on the traits

					Non-saline					Saline					
Traits	**G**	**T**	**G*T**	**h^2**	**Mean**	**SD**	**CV**	**Skewness**	**Kurtosis**	**Mean**	**SD**	**CV**	**Skewness**	**Kurtosis**	**E (%)**
									Leaf chlorophyll fluorescence						
Fo	-	**	-	0.08	-13311.0	736.6	5.5	0.2	0.5	-12316.0	868.6	7.10	-0.1	-0.4	-7.23
Fm	-	**	-	0.16	-48301	2409	5.00	0.16	1.02	-47654	3185	6.68	-0.12	-0.21	-0.96
Fv	**	**	-	0.19	-34990	1770	5.06	0.24	1.16	-35338	2409	6.82	-0.05	-0.07	-1.43
Vi	*	*	-	0.59	0.90	0.01	1.28	-0.28	-0.42	0.88	0.01	1.51	-0.22	-0.22	-2.00
Fm/Fo	**	**	-	0.42	-3.64	0.08	2.30	-0.19	0.12	-3.89	0.11	2.76	-0.01	-0.39	-6.98
Fv/Fm	**	**	-	0.33	-0.72	0.01	0.90	-0.33	0.29	-0.74	0.01	1.37	-4.14	33.00	-2.51
Fv/Fo	**	**	-	0.42	-2.64	0.08	3.18	-0.19	0.12	-2.89	0.11	3.72	-0.01	-0.39	-9.63
ABS/RC	**	**	-	0.11	3.99	0.08	2.09	0.37	0.50	3.83	0.09	2.47	1.25	6.75	-3.89
DIo/RC	**	**	-	0.10	1.00	0.05	4.57	-0.47	4.50	1.10	0.08	8.46	6.68	64.79	+9.71
ETo/RC	**	**	-	0.63	1.25	0.08	6.45	0.12	0.27	1.36	0.09	6.58	0.20	-0.22	+8.98
TRo/RC	**	**	-	0.12	2.88	0.05	1.59	-0.02	0.02	2.84	0.05	1.59	-0.28	0.17	-1.60
PI(ABS)	**	**	-	0.30	-0.54	0.07	13.67	0.30	0.40	-0.74	0.12	16.40	0.66	-0.10	-37.04
									Seed grain quality						
SMC	**	**	**	0.92	8.83	0.28	3.19	0.64	2.01	9.12	0.20	2.15	0.40	0.25	+3.28
SH	**	**	**	0.97	55.52	3.23	5.82	-0.48	-0.41	57.96	2.88	4.97	-1.01	0.75	+4.39
NDF	**	-	-	0.87	16.84	0.90	5.33	-0.10	-0.12	17.01	1.05	6.19	-0.13	0.11	+1.01
PC	**	**	**	0.89	12.18	1.10	9.00	0.16	-0.40	13.77	0.99	7.21	-0.03	0.35	+13.05
CFC	**	**	**	0.98	2.37	0.19	8.03	-0.25	-0.27	2.34	0.20	8.44	0.23	0.29	-1.27
SSV	**	**	**	0.93	28.62	7.86	27.45	0.42	-0.27	40.69	7.47	18.35	-0.11	0.08	+42.17
SC	**	**	**	0.93	72.10	1.09	1.52	-0.42	0.16	71.26	1.11	1.56	-0.18	0.05	-1.17

. Correlation is significant at the 0.01 level (2-tailed), -. Non-significant difference; **Fo, fluorescence intensity at 50 μs; ***Fm, m***aximal fluorescence***;*** ***Fv,*** maximal variable fluorescence; ***Fm/Fo,*** non-photochemical loss in PSII; ***Fv/Fm,*** maximum quantum yield of PSII within light-adapted; ***Fv/Fo,*** efficiency of the water-splitting complex on the donor side of PSII; ***ABS/RC,*** effective antenna size of an active reaction center (RC); ***DIo/RC,*** effective dissipation in an active RC; ***ETo/RC,*** electron transport in an active RC; ***TRo/RC,*** maximal trapping rate of PSII; ***PI(ABS),*** performance index on absorption basis; **SMC**, seed moisture content; **SH**, seed hardness; **NDF**, neutral detergent fiber; **PC**, protein content; **CFC**, crude fiber; **SSV**, sedimentation value; **SC**, starch content.

Table 3 Shows the correlation coefficients based on the genotype mean among the leaf chlorophyll fluorescence shoot ions contents and seed quality traits

	Fo	**Fv**	**Fm/Fo**	**Fv/Fm**	**ETo/RC**	**ABS/RC**	**TRo/RC**	**Dio/RC**	**PI/(ABS)**	**Leaf Na^+**	**Leaf K^+**	**SMC**	**CFC**	**SSV**	**PC**	**SC**
Fo	1															
Fv	.667**	1														
Fm/Fo	-.573**	.214**	1													
Fv/Fm	-.508**	.205**	.943**	1												
ETo/RC	-.256**	.339**	.704**	.618**	1											
ABS/RC	.701**	0.085	-.829**	-.785**	-.389**	1										
TRo/RC	.746**	.358**	-.574**	-.508**	-0.053	.825**	1									
Dio/RC	.504**	-0.079	-.762**	-.752**	-.469**	.897**	.527**	1								
PI/(ABS)	-.506**	.219**	.905**	.798**	.896**	-.701**	-.445**	-.665**	1							
Shoot Na^+	-.139*	-0.098	0.064	0.04	0.02	-0.093	-.127*	-0.035	0.077	1						
Shoot K^+	0.074	.252**	.471**	.453**	.288**	-0.016	.253**	-0.1	.518**	-0.015	1					
SMC	-.364**	0.047	.534**	.468**	.414**	-.463**	-.320**	-.410**	.519**	-0.017	.120*	1				
CFC	0.011	0.009	-0.019	-0.034	0.091	0.013	0.026	-0.006	0.068	0.051	.305**	.398**	1			
SSV	-.393**	-0.031	.493**	.437**	.345**	-.430**	-.304**	-.378**	.439**	-0.029	-0.09	.172**	-.453**	1		
PC	-.422**	-0.041	.520**	.450**	.413**	-.444**	-.305**	-.393**	.504**	-0.026	-0.038	.204**	-.303**	.919**	1	
SC	.299**	0.078	-.306**	-.260**	-.260**	.259**	.182**	.228**	-.306**	0.047	-0.056	0.048	0.082	-.658**	-.798**	1

*. Correlation is significant at the 0.05 level (2-tailed); **. Correlation is significant at the 0.01 level (2-tailed); **Fo**, fluorescence intensity at 50 µs; ***Fm, m***aximal fluorescence***; Fv,*** maximal variable fluorescence; ***Fm/Fo,*** non-photochemical loss in PSII; ***Fv/Fm,*** maximum quantum yield of PSII within light-adapted; ***Fv/Fo,*** efficiency of the water-splitting complex on the donor side of PSII; ***ABS/RC,*** effective antenna size of an active reaction center (RC); ***DIo/RC,*** effective dissipation in an active RC; ***ETo/RC,*** electron transport in an active RC; ***TRo/RC,*** maximal trapping rate of PSII; ***PI(ABS),*** performance index on absorption basis; **SMC**, seed moisture content; **PC**, protein content; **CFC**, crude fiber; **SSV**, sedimentation value; **SC**, starch content.

Genetic properties of the association panel

The population structure and the genome LD of the population under study have been described in **Chapter 3**. In brief, the genome LD decay estimates showed a clear decay in each genome between SNPs spaced up to about 10, 11 and 14 cM for A-, B- and D-genome (Chapter 3), respectively. Thus, the genome LD-decay at 10, 11 and 14 cM for A-, B- and D-genome, respectively were used to calculate the threshold for marker-trait associations, as has been described in ‘‘Materials and methods’’. All significant SNP-trait association that are within the genetic distance defined by each genome LD was grouped into one SNP-cluster and their effects were considered to be coming from a single/few QTL.

Marker-trait association analysis

By applying MLM, a total of 115 SNPs revealed significant MTAs with the ST-related traits measured (**Table 3**). Of these, 54, 17 and 44 SNPs were associated with ChlF, SI and SQ traits, respectively. Each associated SNP explained between 2% (*BobWhite_c28819_787* for *Fm/Fo* on 2AL) and 63.45% (*wsnp_Ex_c1146_2200823* for *Fv* on 7AL) of the observed phenotypic variation explained (R^2). Using the genome LD, all the SNPs that showed significant ST effect on the traits were resolved into 21 QTL regions (**Table 4, Figure 1**). Additionally, 10 out of the 21 QTL regions were detected previously in our GWAS using the phenotypic and yield related ST-traits (**see Chapter 3**).

Pleiotropy and multigenic effect revealed by GWAS

A single locus showing significant association with multiple traits might be due pleiotropy. Three QTL loci were independently associated with the ChlF- and SI-traits on 6AL. The first QTL, Q.chl*I(6AL) (R^2 = 4.40 - 14.7%) located at 99.04 cM was associated with *ABS/RC*, *DIo/RC* and shoot Na^+; while the second QTL. Q.chl*I(6AL) (R^2 ≤12%) which at 0.17 cM genetic interval had significant effect on Fv and shoot Na^+. We assumed that the third QTL (R^2 = 4.10 to 14.90%) spanning from 78.64 to 85.07 cM on 6AL is novel, since no ST QTL has been reported on this region (**Table 4**). This region is linked with *ABS/RC*, *DIo/RC*, *Vj* and shoot Na^+ traits. Of interest is the QTL, *Q.chl*Qu*I(2BL)* (R^2 ≤8.3%) that spanned 1.55 cM on 2BL. This QTL was significantly associated with *Fv/Fm*, grain crude fiber content, shoot Na^+ and shoot K^+ /Na^+.

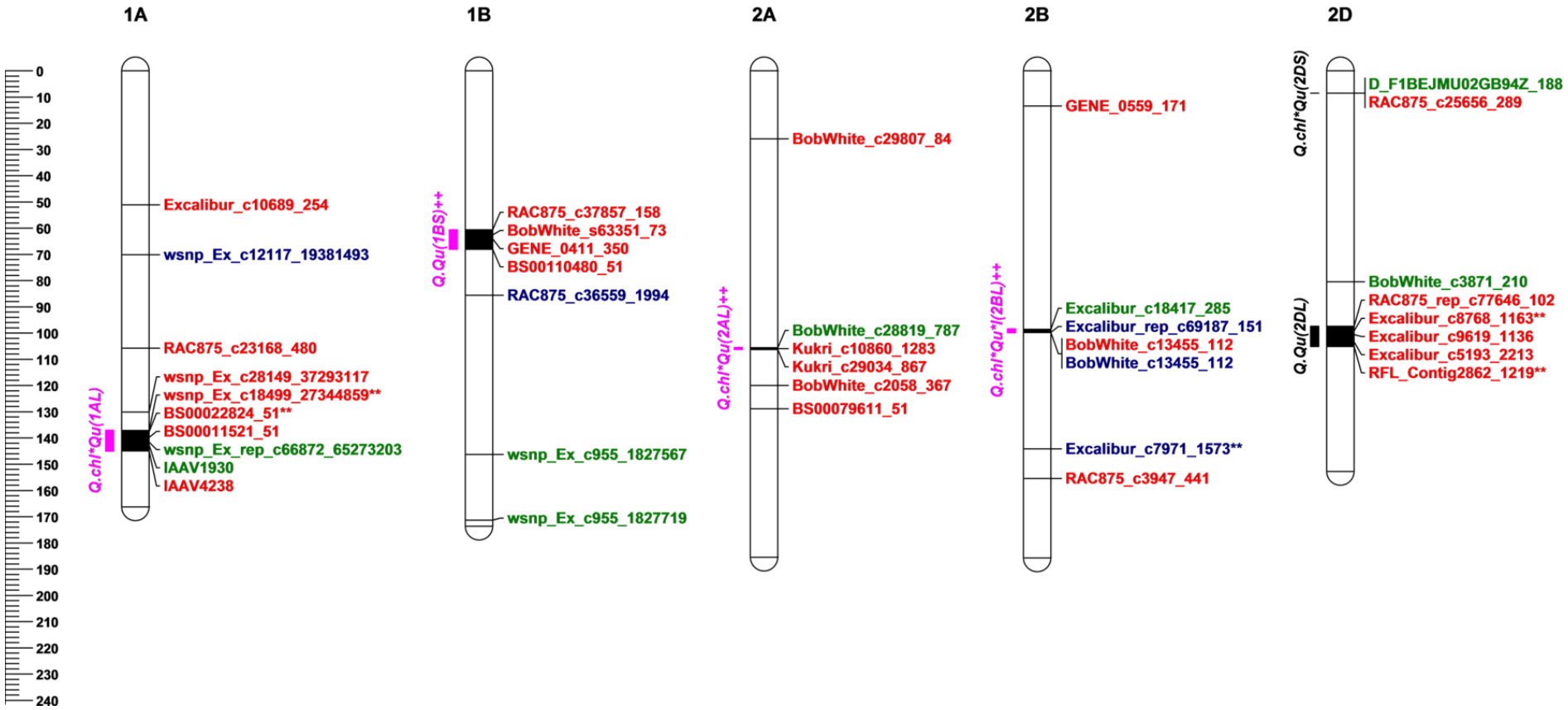
Table 3 Summary of significant SNP marker-trait associations for leaf chlorophyll fluorescence, shoot ionic, and seed quality traits

Trait	SNP	Chr	cM	p	Alleles	SNP R^2
Leaf fluorescence traits						
ABS/RC	wsnp_Ex_rep_c66872_65273203	1A	141.53	2.63E-07	C/T	3.2
ABS/RC	wsnp_RFL_Contig1984_1169021	1D	91.53	4.95E-07	A/G	2.9
ABS/RC	BobWhite_c3871_210	2D	80.41	2.66E-07	C/T	3.2
ABS/RC	BS00060391_51	3A	111.62	7.74E-07	G/A	2.8
ABS/RC	BS00109052_51	5A	49.73	2.05E-07	T/C	3.3
ABS/RC	IACX5753	6A	82.38	7.49E-11	T/C	4.8
ABS/RC	Kukri_rep_c107624_603	6A	99.04	4.05E-10	T/C	4.4
ABS/RC	Ra_c106775_711	6D	82.14	2.46E-07	C/T	3
DIo/RC	IAAV1930	1A	142.62	1.76E-06	C/T	6.2
DIo/RC	BS00021955_51	5A	81.96	1.35E-13	T/C	7.3
DIo/RC	BS00003616_51	6A	82.38	1.09E-25	T/C	14.9
DIo/RC	wsnp_Ex_c11348_18327861	6A	85.07	2.74E-09	A/G	4.8
DIo/RC	Kukri_c15096_4206	6A	99.04	2.02E-25	T/C	14.7
DIo/RC	RAC875_rep_c105906_124	6B	23.32	4.76E-12	A/G	6.4
DIo/RC	Kukri_c9424_195	6B	46.96	4.86E-12	A/G	6.4
DIo/RC	wsnp_CV776265A_Ta_2_1	6B	76.2	5.30E-12	A/G	6.4
ETo/RC	wsnp_Ex_c955_1827567	1B	146.25	1.48E-09	A/G	5.4
ETo/RC	wsnp_Ex_c955_1827719	1B	171.31	1.42E-08	G/A	4.8
ETo/RC	wsnp_Ex_rep_c66331_64502558	3B	11.56	1.58E-08	G/A	4.7
ETo/RC	Kukri_c22602_791	4A	154.3	4.50E-09	C/T	5
ETo/RC	BS00062617_51	5B	5.7	1.19E-08	C/T	4.8
ETo/RC	wsnp_CAP8_c2589_1356390	5D	67.49	6.01E-09	A/C	4.9
Fm/Fo	BobWhite_c28819_787	2A	105.53	1.13E-07	A/G	2
Fm/Fo	D_F1BEJMU02GB94Z_188	2D	8.52	4.93E-08	G/A	2.2
Fm/Fo	CAP7_c3950_160	7B	155.41	9.53E-13	C/T	3.6
Fm/Fo	Excalibur_rep_c110429_536	7B	166.24	8.27E-12	C/T	3.5
Fm/Fo	Kukri_c45404_121	7B	171.11	4.18E-11	C/T	3.1
Fv	RAC875_c27986_1460	3B	4.54	2.44E-86	A/G	1.602
Fv	Excalibur_c6782_253	3B	5.86	1.55E-59	C/T	0.945
Fv	Kukri_rep_c79597_513	4A	43.39	8.60E-237	T/C	9.246
Fv	wsnp_BE591195A_Ta_1_1	4A	47.53	9.30E-156	T/C	3.902
Fv	Kukri_rep_c103857_458	5A	62.72	2.00E-127	A/G	2.825
Fv	BS00062617_51	5B	5.7	4.50E-136	C/T	3.085
Fv	wsnp_Ku_c1045_2115866	5B	143.55	8.79E-97	T/C	1.799
Fv	wsnp_Ex_rep_c76495_73453891	6A	140.7036	3.15E-56	C/T	0.91461
Fv	wsnp_CAP11_c651_429263	7A	127.75	1.50E-201	G/A	6.23
Fv	wsnp_Ex_c1146_2200823	7A	131.11	2.60E-253	A/G	63.445
Fv	RAC875_rep_c72959_187	7B	156.54	1.00E-189	T/C	21.959
Fv/Fm	Excalibur_c18417_285	2B	98.53	5.35E-11	T/C	4
Fv/Fm	CAP7_c3950_160	7B	155.41	8.43E-08	C/T	3.4
Fv/Fo	wsnp_Ku_c35386_44598937	5A	60.61	2.96E-09	G/A	2.5
Fv/Fo	BS00003861_51	6A	48.09	6.80E-09	T/C	2.4
Fv/Fo	CAP7_c3950_160	7B	155.41	9.53E-13	C/T	3.6
Fv/Fo	Tdurum_contig8448_363	7B	164.24	3.49E-11	A/C	3.2
Fv/Fo	Excalibur_rep_c110429_536	7B	166.24	8.27E-12	C/T	3.5
Fv/Fo	Kukri_c45404_121	7B	171.11	4.18E-11	C/T	3.1
TRo/RC	GENE_4252_246	3A	77.57	5.85E-08	G/A	4.6

TRo/RC	CAP8_c1393_327	3A	90.55	1.57E-09	T/C	5.8
TRo/RC	CAP7_rep_c12537_81	3A	177.24	2.34E-08	A/G	5
TRo/RC	IACX11112	7A	74.25	9.29E-09	G/C	5.1
Vi	RAC875_c3947_441	2B	155.41	5.15E-13	C/T	7.2
Vi	Tdurum_contig49608_1185	4B	26	1.20E-11	A/G	5.5
Vi	Kukri_c5685_1066	5B	115.69	1.60E-12	C/T	6.9
Vi	wsnp_Ra_c31052_40235870	7B	67.47	1.14E-11	C/T	6.5
Shoot ion contents after 25 day of salt treatment						
Shoot K^+/Na^+	RAC875_c36559_1994	1B	85.57	8.69E-06	G/A	3.2
Shoot K^+/Na^+	Excalibur_c7971_1573	2B	144.16	3.73E-07	A/G	4.1
Shoot K^+/Na^+	Excalibur_c39621_358	4A	43.39	1.18E-05	G/A	3.1
Shoot K^+/Na^+	Kukri_c59051_579	5B	146.48	2.60E-06	G/A	3.6
Shoot K^+/Na^+	BS00099804_51	7A	119.11	3.20E-06	C/T	3.5
Shoot Na^+	wsnp_Ex_c12117_19381493	1A	70.1	1.26E-08	C/A	8
Shoot Na^+	Excalibur_rep_c69187_151	2B	99.73	2.19E-08	A/G	8.1
Shoot Na^+	BobWhite_c13455_112	2B	99.8	1.46E-08	A/G	8.3
Shoot Na^+	Excalibur_c7971_1573	2B	144.16	1.27E-17	A/G	18.3
Shoot Na^+	BS00084096_51	5B	107.37	2.60E-08	A/G	7.9
Shoot Na^+	Tdurum_contig8171_1602	5B	140.17	5.91E-13	T/C	13.1
Shoot Na^+	Kukri_c59051_579	5B	146.48	2.16E-13	G/A	13.3
Shoot Na^+	Kukri_c21443_827	6A	28.46	1.01E-08	C/T	8.3
Shoot Na^+	BS00040124_51	6A	82.38	9.95E-09	G/A	8.1
Shoot Na^+	IAAV5585	6A	99.04	1.19E-08	G/T	8.1
Shoot Na^+	Jagger_c1134_353	6A	140.87	2.69E-12	A/G	12
Shoot Na^+	RAC875_c25194_55	7A	35.31	2.71E-08	G/A	7.7
Seed quality traits						
SMC	Excalibur_c10689_254	1A	51.09	1.13E-09	A/G	4.3
SMC	wsnp_Ex_c28149_37293117	1A	130.09	5.87E-10	T/G	4.2
SMC	RAC875_rep_c77646_102	2D	97.42	1.84E-09	G/A	4.2
SMC	Excalibur_c8768_1163	2D	99.19	8.57E-10	A/C	4.2
SMC	Excalibur_c5193_2213	2D	103.33	3.19E-10	C	3.9
SMC	wsnp_Ex_c24215_33462239	5A	67	1.17E-09	T/C	4.1
SMC	GENE_3601_145	5A	89.02	3.09E-10	T/C	3.8
SMC	Ku_c19745_1093	7A	211	5.10E-11	T/C	4.8
SH	RAC875_c37857_158	1B	60.62	1.39E-26	G/T	10.2
SH	BobWhite_s63351_73	1B	62.58	2.49E-19	A/G	7.5
SH	GENE_0411_350	1B	64.1	5.46E-27	C/A	10.6
SH	BobWhite_c29807_84	2A	25.97	2.46E-20	C/T	7.7
SH	Kukri_c10860_1283	2A	105.89	4.74E-20	G/A	7.5
SH	Kukri_c29034_867	2A	106.3	7.33E-21	C/T	7.8
SH	BS00079611_51	2A	128.89	2.13E-19	G/T	7.2
SH	GENE_0559_171	2B	13.44	6.24E-21	T/C	7.9
SH	RFL_Contig2862_1219	2D	105.13	4.98E-21	A/G	7.9
SH	GENE_0559_171	3B	5.86	6.24E-21	T/C	7.9
SH	wsnp_Ku_c15531_24168235	4A	121.67	9.91E-20	C/T	7.3
SH	Kukri_c52257_991	5B	109.53	1.49E-26	A/G	10.3
SH	wsnp_Ex_c11265_18216936	5B	116.11	1.67E-24	C/T	9.7
SH	Excalibur_c827_666	6D	117.58	5.01E-21	C/T	8.2
SH	wsnp_JD_c14118_13933380	7A	126.8	3.35E-21	T/C	8.9
SH	tplb0045p11_893	7A	148.43	3.78E-21	T/C	8.3
NDF	RAC875_c23168_480	1A	105.74	6.47E-11	C/T	4.6

NDF	Excalibur_rep_c101324_1680	5A	26.51	9.24E-10	C/T	4.1
NDF	wsnp_Ex_rep_c68117_66883366	5A	43.27	1.00E-09	G/A	4.1
NDF	wsnp_Ex_rep_c68269_67060931	5A	43.44	2.21E-10	G/T	4.5
NDF	Kukri_c15761_1634	6B	71.76	7.40E-10	C/T	4.1
PC	wsnp_Ex_c18499_27344859	1A	137.12	7.84E-13	C/T	4.6
PC	BS00022824_51	1A	137.69	1.84E-12	T/C	4.4
PC	BS00011521_51	1A	139.74	6.73E-12	A/G	4.1
PC	wsnp_Ex_c742_1458743	3A	81.82	1.43E-11	G/T	4.2
PC	Excalibur_c60683_908	5B	49.01	7.19E-11	T/C	3.7
PC	RFL_Contig4251_851	6B	71.9	3.04E-12	A/G	4.4
CFC	IAAV4238	1A	144.94	6.18E-14	G/A	5.6
CFC	BS00110480_51	1B	68.04	3.27E-12	G/A	5.2
CFC	BobWhite_c2058_367	2A	119.93	6.65E-10	C/T	4
CFC	BobWhite_c13455_112	2B	99.8	8.11E-09	A/G	3.5
CFC	RAC875_c25656_289	2D	8.52	5.85E-09	C/T	3.5
CFC	Excalibur_c8768_1163	2D	99.19	1.44E-10	A/G	4.2
CFC	Excalibur_c9619_1136	2D	100.58	2.02E-09	G/A	3.8
CFC	RFL_Contig2862_1219	2D	105.13	3.39E-11	A/G	4.6
CFC	RAC875_c19099_434	5B	68.93	1.40E-12	C/T	5.2

SMC, seed moisture content; **SH**, seed hardness; **NDF**, neutral detergent fiber; **PC**, protein content; **CFC**, crude fiber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2D
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Q.chl*Qu(1AL)
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RAC875_c23168_480
wsnp_Ex_c28149_37293117
wsnp_Ex_c18499_27344859**
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BS00011521_51
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IAAV1930
IAAV4238
Q.Qu(1BS)++
RAC875_c37857_158
BobWhite_s63351_73
GENE_0411_350
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RAC875_c36559_1994
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wsnp_Ex_c955_1827719
Q.chl*Qu(2AL)++
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BobWhite_c28819_787
Kukri_c10860_1283
Kukri_c29034_867
BobWhite_c2058_367
BS00079611_51
Q.chl*Qu*(2BL)++
GENE_0559_171
Excalibur_c18417_285
Excalibur_rep_c69187_151
BobWhite_c13455_112
BobWhite_c13455_112
Excalibur_c7971_1573**
RAC875_c3947_441
Q.chl*Qu(2DS)
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Q.Qu(2DL)
BobWhite_c3871_210
RAC875_rep_c77646_102
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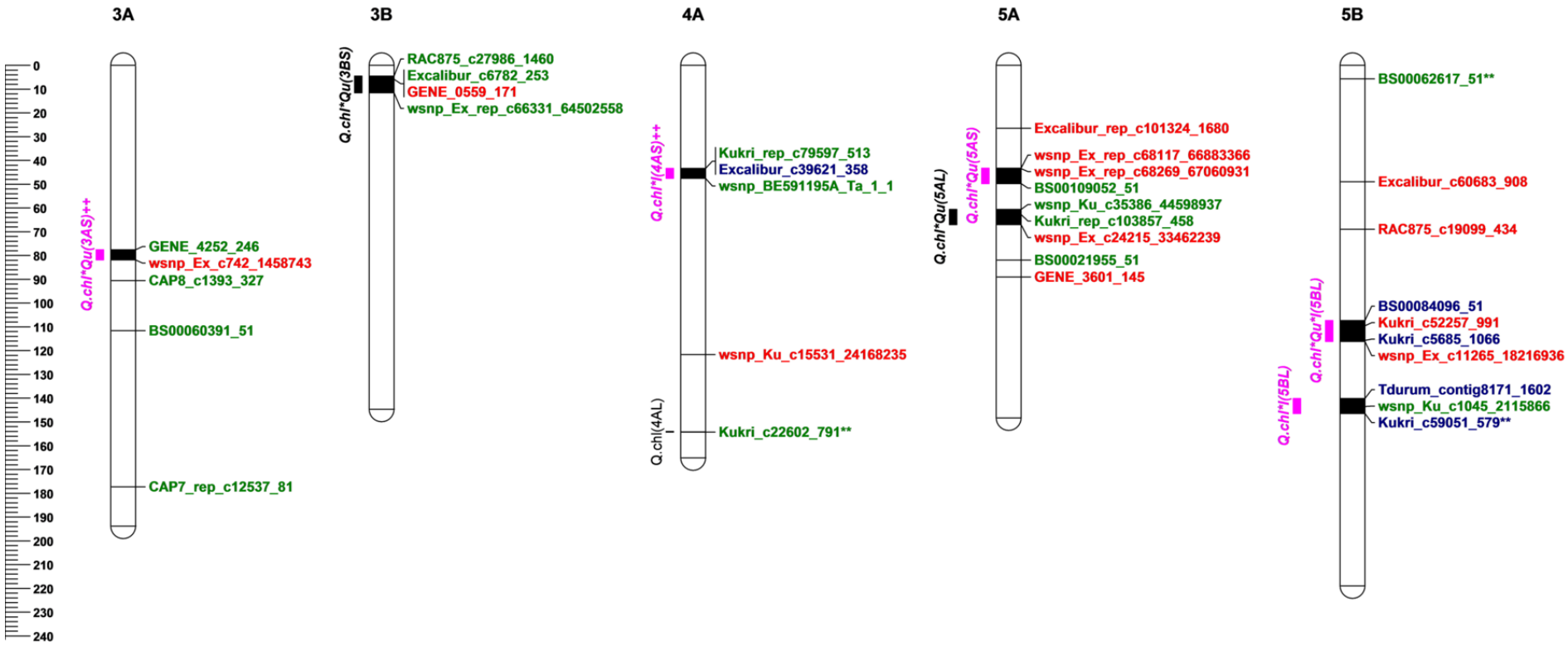
Excalibur_c9619_1136
Excalibur_c5193_2213
RFL_Contig2862_1219**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200
210
220
230
240
Q.chl*Qu(3AS)++
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wsnp_Ex_c742_1458743
CAP8_c1393_327
BS00060391_51
CAP7_rep_c12537_81
Q.chl*Qu(3BS)
RAC875_c27986_1460
Excalibur_c6782_253
GENE_0559_171
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Q.chl*(4AS)++
Kukri_rep_c79597_513
Excalibur_c39621_358
wsnp_BE591195A_Ta_1_1
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Q.chl(4AL)
Kukri_c22602_791**
Q.chl*Qu(5AL)
Q.chl*Qu(5AS)
Excalibur_rep_c101324_1680
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wsnp_Ex_rep_c68269_67060931
BS00109052_51
wsnp_Ku_c35386_44598937
Kukri_rep_c103857_458
wsnp_Ex_c24215_33462239
BS00021955_51
GENE_3601_145
Q.chl*(5BL)
Q.chl*Qu*(5BL)
BS00062617_51**
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Kukri_c5685_1066
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Tdurum_contig8171_1602
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Kukri_c59051_579**

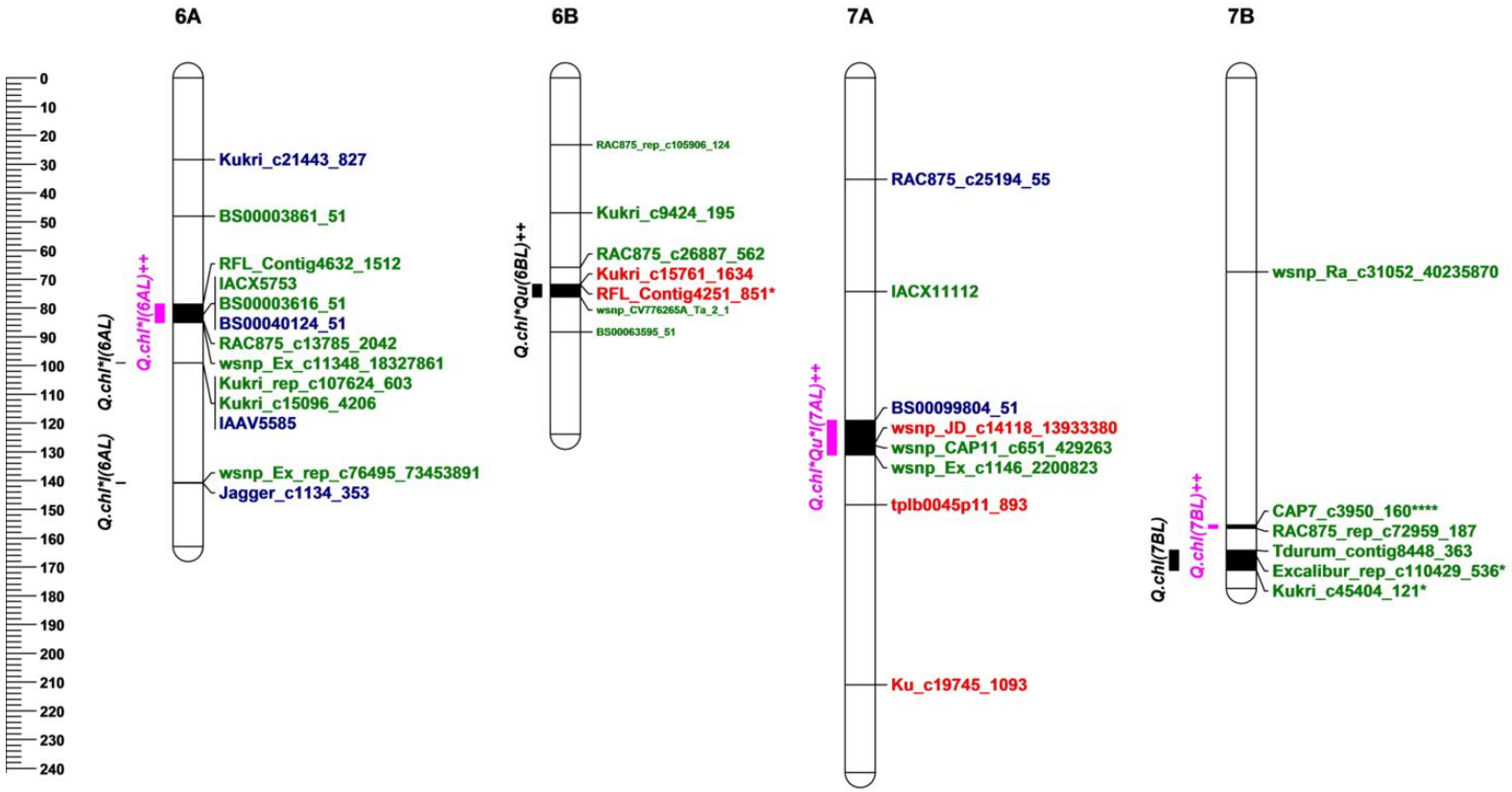


Figure 1 Genetic map of wheat showing the location of the SNP markers associated with the chlorophyll fluorescence (in green), ionomics (in blue) and the seed quality (in red) traits in the studied wheat germplasm. Number of *Asterisks in each SNP* indicates number of traits it affected, Pink QTL region in Asterisks (∗) indicates previously reported QTL region in Chapter 3, ++ on QTL names indicates congruent regions detected with rrBLUP program.

Table 4 Colocation of SNP clusters with QTL/genes already identified or published

QTL	cM	Associated traits	References		
			ChlF QTL	**Shoot ions QTL**	**Seed Quality QTL**
Leaf fluorescence					
Q.chl(4AL)	154-154.3	ETo/RC, Vj	Chl a (Zhang et al., 2009b)		
Q.chl(7BL)	155.41-156.54	Fm/Fo, Fv/Fm, Fv			
Q.chl(7BL)	164.24-171.11	Fm/Fo, Fv/Fo			
Seed grain quality					
Q.Qu(1BS)	60.6-68.04	CFC, SH			QBwa.mna-1B, QMpv.mna-1B.1, QMpt.mna-1B.1, QMpi.mna-1B.1, QMixopa.mna-1B (Tsilo et al., 2011)
Q.Qu(2DL)	97.42-105.13	CFC, SWC, SH			QAlc.sdau-2D and QDgc.sdau-2D (Sun et al 2008)
Leaf fluorescence + ionomics					
Q.chl*I(4AS)	43.39-47.53	FV and K^+/Na^+	qChlN-4A (Li et al., 2010); Tm4A (Zhang et al., 2010)		Grain strength (Nelson et al 2006)
Q.chl*I(5BL)	140.17-146.48	Fv, Na^+, K^+/Na^+			
Q.chl*I($6AL_c$)	78.64-85.07	ABS/RC, DIo/RC, Vj and Na^+			
Q.chl*I(6AL)	99.04	ABS/RC, DIo/RC and Na^+	Fm, Fv/Fm (Li et al., 2012b)	Shoot Na^+ (Genc et al., 2010)	
Q.chl*I(6AL)	140.7-140.87	Fv and Na^+	Fo (Li et al., 2012b)		
Leaf fluorescence + Seed grain quality					
Q.chl*Qu(1AL)	137-145	ABS/RC, DIo/RC, PC, CFC	Fo (Zheng et al., 2013)		
Q.chl*Qu(2AL)	105.5-106.3	Fm/Fo, SH	Chl a, chl b (Li et al., 2012b); qFv/FmN-2A (Li et al., 2010, Czyczyło-Mysza et al., 2013)	TmHKT1;4-A (Huang et al., 2006); Shoot Na^+ (Lindsay et al., 2004)	QSkhard.mna-2A (Tsilo et al., 2011)
Q.chl*Qu(2DS)	8.52	Fm/Fo, CFC	Fm (Zheng et al., 2013)	*Tg-D1* (Okamoto et al. 2012), SD (Tan et al., 2006)	GY: qGY2Da (Zhang et al., 2009b), Rht8 and Ppd-D1 (Pestsova and Röder, 2002; Gasperini et al., 2012)
Q.chl*Qu(3AS)	77.57-81.82	TRo/RC, PC			Viscosity (Nelson et al 2006)
Q.chl*Qu(3BS)	4.54-11.56	ETo/RC, Fv, SH	Chl a+b, Chl a (Li et al., 2012b)		QMxT.upm-3BS (Kerfa et al., 2010)
Q.chl*Qu(5AL)	43.26-49.73	NDF and ABS/RC	qChlN-5A (Li et al., 2010)		Dough extensibility-QPext.upm5AS (Kerfa et al., 2010); QSkhard.mna-5A.1 (Tsilo et al., 2011)
Q.chl*Qu(5AL)	60.61-67.00	Fv/Fo, Fv, SWC	Chl:wPt-1370-Vrn1A (Genc et al., 2010b)	TaSRO1 (Liu et al., 2014), shoot K^+ (Genc et al., 2010b)	grain protein (Nelson et al 2006)
Q.chl*Qu(6BL)	71.76-76.2	NDF, PC, Dio/RC	Fv/Fo, Fv/Fm, Chl a, Chl a+b (Li et al., 2012b)		grain protein content (Prasad et al., 2003); QFn.sdau-6B (Sun et al., 2008)
Leaf Fluorescence + ionomis + Seed grain quality					
Q.chl*Qu*I(2BL)	98.35-99.9	Fv/Fm, shoot Na^+, CFC, shoot K^+/Na^+	Fm, Fv/Fm, Fv/Fo (Li et al, 2012b; Zheng et al., 2013); qChlN-2B (Li et al., 2010); Tm2Bb (Zhang et al., 2010)	Shoot Na^+ (Genc et al., 2010b)	seed dormancy and PHS loci (Chao et al., 2015)
Q.chl*Qu*I(5BL)	107.37-116.11	Vi, Na^+, SH	Fv/Fmi5B (Zhang et al., 2010)	Cu-tolerance (Bálint et al., 2007)	SD and PHS QTL (Tan et al., 2006)
Q.chl*Qu*I(7AL)	119.11-131.11	K^+/Na^+, Fv, SH	Chl a, Chl b, Chl a+b, Fm (Zhang et al., 2009b; Li et al., 2012b; Zheng et al., 2013)	Shoot Na^+ (Genc et al., 2010b)	PHS: Qsd.sau-7A (Jiang et al., 2015)

PHS-preharvest sprouting; **PHS-** preharvest sprouting; **SB-** seedling biomass; **SSI-**Seedling salt injury; **LS-** Leaf symptoms; **TN-** Tiller number; **QDgc.sdau-2D-** QTL for dry gluten content (protein trait); **QAlc.sdau-2D**- QTL for Amylose (Starch trait); **QFn.sdau-6B-** QTL for falling number (starch trait); **QSkhard.mna-2A** (Xgwm339–Xbarc311)-QTL for endosperm texture (seed hardness); **QMixopa.mna-1B**- Micrograph pattern; **QMpi.mna-1B.1**- Midline peak integral (MPI); **QMpt.mna-1B.1**- Midline peak time (MPT); **QMpv.mna-1B.1**-Midline peak value (MPV); **SD-** seed dormancy; **QBwa.mna-1B**- Bake water absorption; ***Tg-D1*** - tenacious glume locus; **PC-** seed protein content, **CFC-** crude fibre, **SWC-** seed water content, **SH-** seed hardness, **NDF-** neutral detergent fiber.

The PCoA indicated that the most consistent contrasting ST wheat genotypes can be distinguished using the 115 SNPs that showed significant MTAs in the GWAS analysis (Figure 2). Most of the salt-tolerant genotypes were mostly found on the left side of the plot, whereas, the salt-sensitive ones were distributed at right side of the plot (Figure 2). The first three PCos accounted for 28.97% of the variation for salt tolerance observed among the genotypes. Highest eigen-values for the associated SNPs were detected on 1AL (141.53 to144.94 cM), 2BL (*RAC875_c3947_441*) (155.41 cM), 2DL (97.42 to105.13 cM), 4AS (43.39 cM), 6AL (99.04 cM and 140 cM) and 7AL (126.8 cM and 148.43 cM) (data not shown).

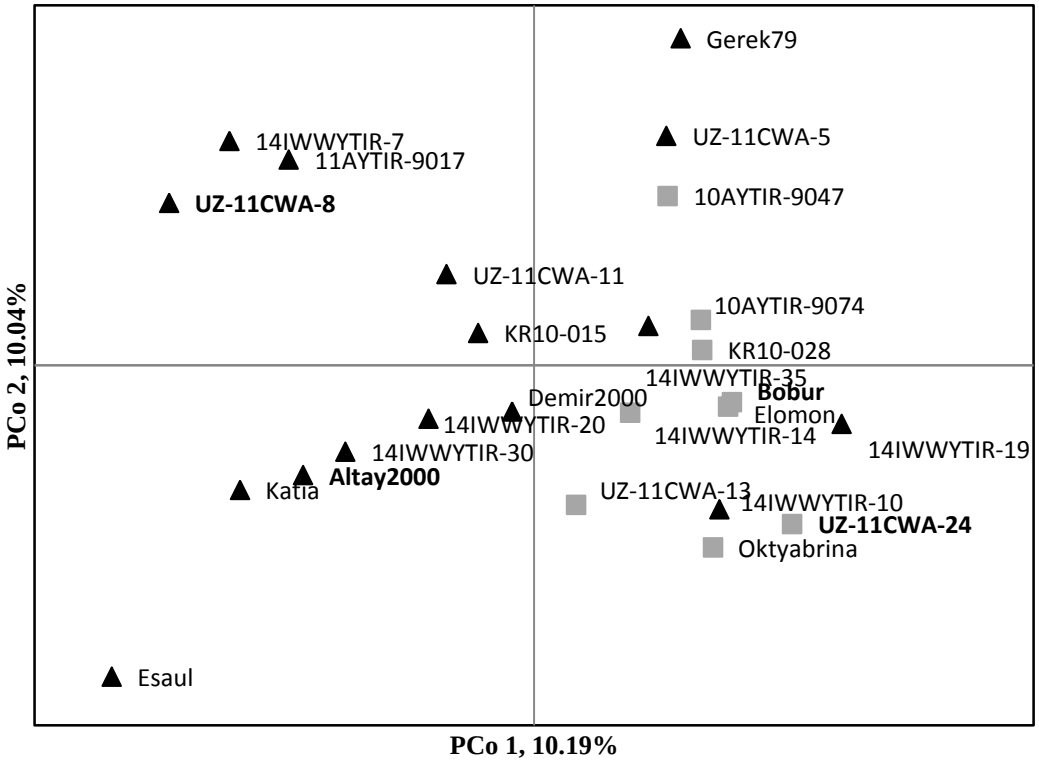


Figure 2 Principal coordinates analysis (PCoA) plot using a genetic distance matrix (GenAlEx 6.5) estimated with data from 115 associated polymorphisms of the salt tolerant (Black colour/triangular shaped) and salt sensitive (Gray colour/squared shape) wheat genotypes previously identified among the studied population. The genotypes (in bold) were used to perform the gene expression analysis.

Candidate genes linked with the associated polymorphisms

Probable candidate genes responsible for the genetic variations in the salt stress related traits are shown in **Table 5.** The blast search revealed that the sequences of the associated SNP markers are homologous with genes known to regulate salt and stress responses. Among categories of genes identified, genes involved in the stress response (25%), transport (15%), transcription (13%) and carbohydrate/sucrose metabolisms (11%) activities were highly abundant **(Figure 3**). Moreover, 9% and 6% of the associated SNPs showed high sequence homology with genes functioning in the oxidoreductase and photosynthesis/photo-morphogenesis pathways, respectively, while few associated SNP sequences coded for genes involved in translation (3%) and disease resistance (3%) activities.

Table 5 Ontology classification of the associated DNA sequences detected using the GWAS in this study

Trait	SNP	Chr	Hits
Carbohydrate/sucrose metabolism - 12%			
DIo/RC	IAAV1930	1AL	starch catabolic process
SH	BobWhite_s63351_73	1BS	Phosphorylated carbohydrates phosphatase [Aegilops tauschii]
ABS/RC	BS00060391_51	3AL	xylanase inhibitor 602OS [Triticum aestivum]
ETo/RC	wsnp_Ex_rep_c66331_64502558	3BS	sucrose-phosphate synthase 2 [Triticum aestivum]
SMC	wsnp_Ex_c24215_33462239	5AL	Putative 6-phosphogluconolactonase 4, chloroplastic [Aegilops tauschii]
Na^+	BS00084096_51	5BL	UDP-glucose 6-dehydrogenase [Aegilops tauschii]
ETo/RC	wsnp_CAP8_c2589_1356390	5DL	Pyruvate kinase, cytosolic isozyme [Triticum urartu]
Fv	wsnp_CAP11_c651_429263	7AL	Beta-glucosidase 28 [Triticum urartu]
Fv	wsnp_Ex_c1146_2200823	7AL	UDP-sugar pyrophosphorylase [Triticum urartu]
Fv	RAC875_rep_c72959_187	7BL	UDP-sugar pyrophosphorylase [Triticum urartu]
Fm/Fo	Excalibur_rep_c110429_536	7BL	Sucrose synthase 2 [Triticum urartu]
Fv/Fo	Excalibur_rep_c110429_536	7BL	Sucrose synthase 2 [Triticum urartu]
Vi	wsnp_Ra_c31052_40235870	7BS	Fructose-bisphosphate aldolase [Aegilops tauschii]
Disease resistance protein - 3%			
TRo/RC	CAP8_c1393_327	3AL	Putative disease resistance protein [Aegilops tauschii]
Na^+	BS00040124_51	6AL	Nephrocystin-3 [Triticum urartu]
DIo/RC	Kukri_c9424_195	6BS	Disease resistance protein RGA2 [Aegilops tauschii]
Oxidoreductase - 8%			
SH	Kukri_c10860_1283	2AL	Cytochrome P450 78A3 [Aegilops tauschii]
K^+/Na^+	Excalibur_c7971_1573	2BL	Brachypodium distachyon dihydroorotate dehydrogenase (quinone), mitochondrial (LOC100837635), mRNA
Na^+	Excalibur_c7971_1573	2BL	Dihydroorotate dehydrogenase (quinone), mitochondrial [Triticum urartu]
Fv	Excalibur_c6782_253	3BS	1-deoxy-D-xylulose 5-phosphate reductoisomerase, chloroplastic [Aegilops tauschii]
SH	wsnp_Ku_c15531_24168235	4AL	Cytochrome P450 704C1 [Aegilops tauschii]
Vi	Tdurum_contig49608_1185	4BS	Monodehydroascorbate reductase [Triticum urartu]
Vi	Kukri_c5685_1066	5BL	Malate dehydrogenase, glyoxysomal [Triticum urartu]
DIo/RC	wsnp_CV776265A_Ta_2_1	6BL	Alpha-aminoadipic semialdehyde synthase [Aegilops tauschii]
Photosynthesis/photomorphogensis -6%			
K^+/Na^+	RAC875_c36559_1994	1BL	Lipoyl synthase, mitochondrial [Aegilops tauschii]
Fv	Kukri_rep_c79597_513	4AS	putative methionyl-tRNA synthetase [Triticum urartu]
K^+/Na^+	Excalibur_c39621_358	4AS	Neutral alpha-glucosidase AB [Triticum urartu]
Fv	wsnp_BE591195A_Ta_1_1	4AS	Translocase of chloroplast 34, chloroplastic [Triticum urartu]
ABS/RC	BS00109052_51	5AS	NADH dehydrogenase complex (plastoquinone) assembly, photosynthetic electron transport in photosystem I and transport
DIo/RC	BS00021955_51	5AL	Cysteinyl-tRNA synthetase [Triticum urartu]
Na^+	Kukri_c21443_827	6AS	pentatricopeptide repeat-containing protein At2g37230 [Brachypodium

			distachyon]
ABS/RC	Ra_c106775_711	6DL	37 kDa inner envelope membrane protein, chloroplastic [Triticum urartu]
Protein repair and maintenance - 8%			
NDF	Excalibur_rep_c101324_1680	5AS	putative galacturonosyltransferase 13 [Triticum urartu]
Protein	RFL_Contig4251_851	6BL	Galactoside 2-alpha-L-fucosyltransferase [Aegilops tauschii]
CP	RFL_Contig4251_851	6BL	galactoside 2-alpha-L-fucosyltransferase-like, transcript variant X2, mRNA
Fv/Fm	Excalibur_c18417_285	2BL	Putative mixed-linked glucan synthase 3 [Aegilops tauschii]
Na^+	Excalibur_rep_c69187_151	2BL	Nipped-B-like protein [Aegilops tauschii]
SH	Kukri_c29034_867	2AL	Pre-mRNA-splicing factor SYF1 [Triticum urartu]
ABS/RC	wsnp_Ex_rep_c66872_65273203	1AL	Structural maintenance of chromosomes protein 3 [Triticum urartu]
Fm/Fo	Kukri_c45404_121	7BL	Callose synthase 3 [Aegilops tauschii]
Fv/Fo	Kukri_c45404_121	7BL	Callose synthase 3 [Aegilops tauschii]
Stress response - 25%			
Fv	RAC875_c27986_1460	3BS	protein EXECUTER 1, chloroplastic isoform X1 [Brachypodium distachyon]
SH	GENE_0559_171	3BS	Protein tumorous imaginal discs, mitochondrial [Triticum urartu]
SH	GENE_0559_171	2BS	Protein tumorous imaginal discs, mitochondrial [Triticum urartu]
Fv/Fo	BS00003861_51	6AS	WD40 protein [Triticum aestivum]
PC	Excalibur_c60683_908	5BL	Annexin D5 [Triticum urartu]
SMC	Excalibur_c10689_254	1AS	Heat stress transcription factor A-2a [Triticum urartu]
SH	GENE_0411_350	1BS	Tubulin-specific chaperone E [Triticum urartu]
CFC	BS00110480_51	1BL	Metallothionein-like protein 1B [Aegilops tauschii]
CFC	RAC875_c19099_434	5BL	Phospholipase D delta [Triticum urartu]
ABS/RC	BobWhite_c3871_210	2DL	Nucleoredoxin [Aegilops tauschii]
ABS/RC	IACX5753	6AL	universal stress protein A-like protein [Brachypodium distachyon]
DIo/RC	BS00003616_51	6AL	Universal stress protein A-like protein [Triticum urartu]
SMC	GENE_3601_145	5AL	Auxin-responsive protein IAA13 [Triticum urartu]
ABS/RC	wsnp_RFL_Contig1984_1169021	1DL	Acylamino-acid-releasing enzyme [Triticum urartu]
SMC	RAC875_rep_c77646_102	2DL	DDB1- and CUL4-associated factor-like protein 1 [Triticum urartu]
CFC	BobWhite_c13455_112	2BL	Alanine aminotransferase 2 [Aegilops tauschii]
Na^+	BobWhite_c13455_112	2BL	Alanine aminotransferase 2 [Aegilops tauschii]
Fm/Fo	BobWhite_c28819_787	2AL	Auxin-induced protein [Aegilops tauschii]
NDF	RAC875_c23168_480	1AL	Dual specificity protein phosphatase 4 [Triticum urartu]
SH	Kukri_c52257_991	5BL	Molybdenum cofactor sulfurase [Triticum urartu]
SH	wsnp_Ex_c11265_18216936	5BL	Receptor protein kinase CLAVATA1 [Aegilops tauschii]
SH	BS00079611_51	2AL	Adenosylhomocysteinase [Aegilops tauschii]
PC	wsnp_Ex_c18499_27344859	1AL	DnaJ homolog subfamily C member 2 [Triticum urartu]
CP	wsnp_Ex_c18499_27344859	1AL	DnaJ homolog subfamily C member 2 [Triticum urartu]
Na^+	Tdurum_contig8171_1602	5BL	Leukotriene A-4 hydrolase [Aegilops tauschii]
Fv	wsnp_Ku_c1045_2115866	5BL	Auxin-induced protein 5NG4 [Triticum urartu]
TRo/RC	CAP7_rep_c12537_81	3AL	Abscisic stress-ripening protein 1 [Triticum urartu]
SMC	Ku_c19745_1093	7AL	Dual specificity protein phosphatase 4 [Triticum urartu]
Transcription factor - 13%			
PC	BS00022824_51	1AL	Mitogen-activated protein kinase 9 [Aegilops tauschii]
CP	BS00022824_51	1AL	Brachypodium distachyon mitogen-activated protein kinase 9 (LOC100835396), mRNA
Na^+	wsnp_Ex_c12117_19381493	1AS	Nuclear receptor corepressor 1 [Aegilops tauschii]
SH	RAC875_c37857_158	1BS	Lysine-specific demethylase 5A [Aegilops tauschii]
CFC	BobWhite_c2058_367	2AL	Wall-associated receptor kinase 3 [Aegilops tauschii]
Vi	RAC875_c3947_441	2BL	putative serine/threonine-protein kinase Cx32, chloroplastic [Triticum urartu]
Fv/Fo	wsnp_Ku_c35386_44598937	5AL	Brachypodium distachyon MADS-box transcription factor 8 (LOC100843405), mRNA
Fv	Kukri_rep_c103857_458	5AL	putative NOT transcription complex subunit VIP2 [Triticum urartu]
ETo/RC	BS00062617_51	5BS	High affinity cationic amino acid transporter 1 [Triticum urartu]
DIo/RC	wsnp_Ex_c11348_18327861	6AL	BAH and coiled-coil domain-containing protein 1 [Triticum urartu]
Fv	wsnp_Ex_rep_c76495_73453891	6AL	NAC domain-containing protein 78 [Triticum urartu]
Fm/Fo	CAP7_c3950_160	7BL	putative serine/threonine-protein kinase Cx32, chloroplastic [Triticum

			urartu]
Fv/Fm	CAP7_c3950_160	7BL	putative serine/threonine-protein kinase Cx32, chloroplastic [Triticum urartu]
Fv/Fo	CAP7_c3950_160	7BL	putative serine/threonine-protein kinase Cx32, chloroplastic [Triticum urartu]
Translation regulation - 3%			
CFC	IAAV4238	1AL	Lysine-specific demethylase 5B [Triticum urartu]
SH	Excalibur_c827_666	6DL	diphthine--ammonia ligase [Brachypodium distachyon]
Fv/Fo	Tdurum_contig8448_363	7BL	Chloroplastic group IIA intron splicing facilitator CRS1, chloroplastic [Triticum urartu]
Transporters -15%			
ETo/RC	wsnp_Ex_c955_1827567	1BL	Mitochondrial outer membrane porin [Aegilops tauschii]
ETo/RC	wsnp_Ex_c955_1827719	1BL	Mitochondrial outer membrane porin [Aegilops tauschii]
CFC	Excalibur_c9619_1136	2DL	K(+) efflux antiporter 2, chloroplastic [Triticum urartu]
SMC	Excalibur_c5193_2213	2DL	Multiple C2 and transmembrane domain-containing protein 1 [Triticum urartu]
SH	RFL_Contig2862_1219	2DL	ABC transporter E family member 2 [Aegilops tauschii]
CFC	RFL_Contig2862_1219	2DL	ABC transporter E family member 2 [Aegilops tauschii]
Fm/Fo	D_F1BEJMU02GB94Z_188	2DS	Bidirectional sugar transporter SWEET6b [Triticum urartu]
CFC	RAC875_c25656_289	2DS	ABC transporter C family member 3 [Aegilops tauschii]
PC	wsnp_Ex_c742_1458743	3AS	PREDICTED: myosin heavy chain, muscle [Brachypodium distachyon]
NDF	wsnp_Ex_rep_c68117_66883366	5AS	boron transporter [Triticum aestivum]
Fv	BS00062617_51	5BS	High affinity cationic amino acid transporter 1 [Triticum urartu]
ABS/RC	Kukri_rep_c107624_603	6AL	Myosin-J heavy chain [Aegilops tauschii]
DIo/RC	Kukri_c15096_4206	6AL	Myosin-J heavy chain [Triticum urartu]
Na^+	IAAV5585	6AL	Myosin-J heavy chain [Aegilops tauschii]
Na^+	Jagger_c1134_353	6AL	ABC transporter F family member 3 [Aegilops tauschii]
NDF	Kukri_c15761_1634	6BL	Potassium transporter 25 [Aegilops tauschii]
K^+/Na^+	BS00099804_51	7AL	protochlorophyllide-dependent translocon component 52, chloroplastic (LOC100825042), mRNA
Unknown protein -7%			
SMC	wsnp_Ex_c28149_37293117	1AL	putative galacturonosyltransferase 4 [Triticum urartu]
ETo/RC	Kukri_c22602_791	4AL	U-box domain-containing protein 12 [Triticum urartu]
NDF	wsnp_Ex_rep_c68269_67060931	5AS	Putative Xaa-Pro aminopeptidase 3 [Aegilops tauschii]
DIo/RC	RAC875_rep_c105906_124	6BS	Putative U5 small nuclear ribonucleoprotein 200 kDa helicase [Aegilops tauschii]
SH	wsnp_JD_c14118_13933380	7AL	PREDICTED: protein DJ-1 homolog B-like [Brachypodium distachyon]
SH	tplb0045p11_893	7AL	PREDICTED: formin-like protein 3 [Brachypodium distachyon]
Na^+	RAC875_c25194_55	7AS	Cycloartenol synthase [Aegilops tauschii]
TRo/RC	IACX11112	7AS	transposon protein, putative, CACTA, En/Spm sub-class [Oryza sativa Japonica Group]

SMC, seed moisture content; **SH**, seed hardness; **NDF**, neutral detergent fiber; **PC**, protein content; **CP**, crude protein; **CFC**, crude fiber

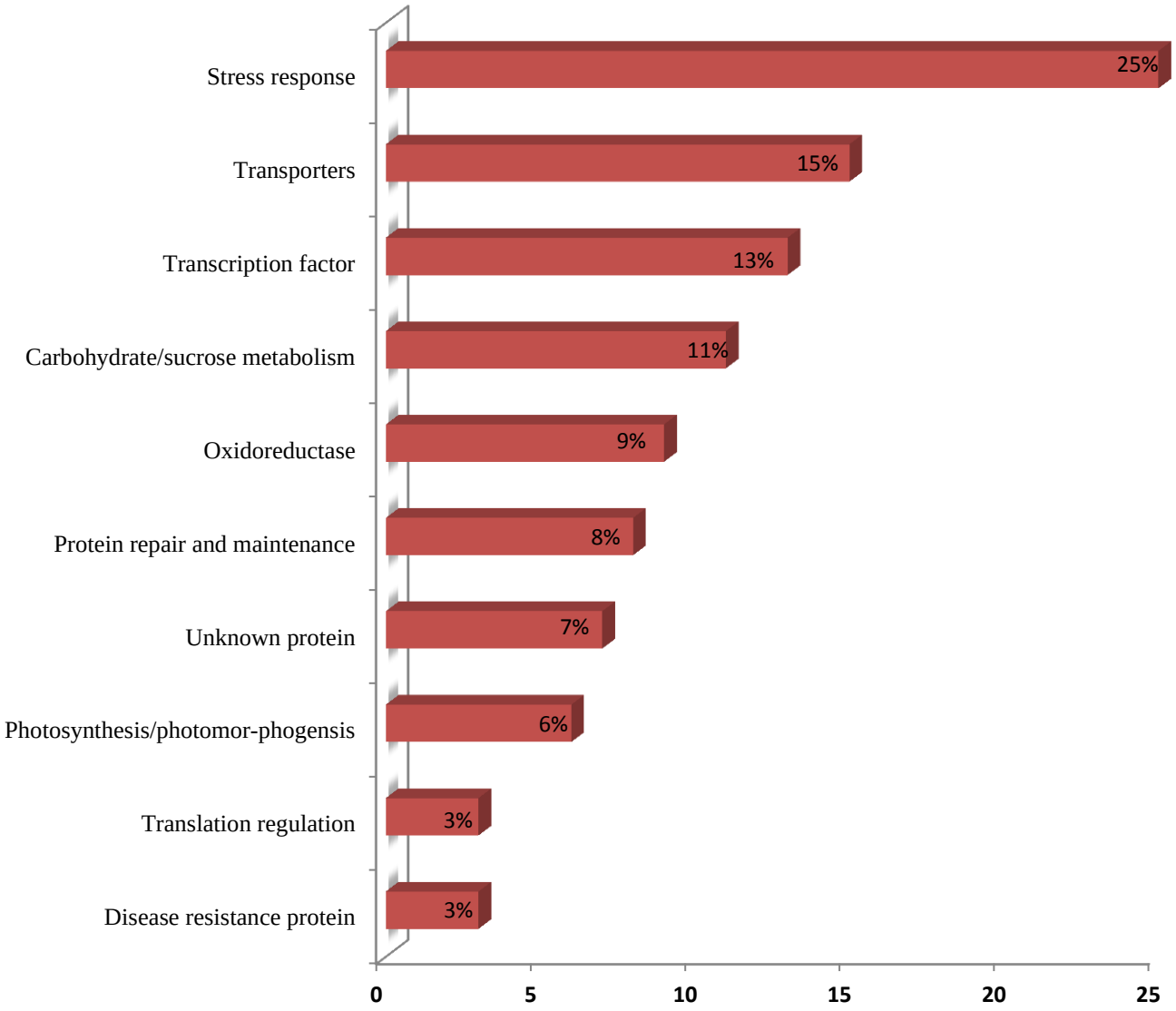


Figure 3 Functional analyses of the associated SNPs.

Functional analysis of the identified genes in this study

The transcript profiles of 28 putative candidate genes were investigated in leaves of salt-tolerant (*Atlay2000*) and salt-sensitive (*Bobur*) genotypes after 24 days in saline (100 mM NaCl) and non-saline conditions (F**igure 4A-C)**. The expressed transcript amount of each candidate gene was obtained from the genome-wide gene expression profiling (unpublished) we recently conducted using the quantitative next generation sequencing (NGS) by Massive Analysis of 3′-cDNA Ends (MACE). The gene transcript abundances were visualized in bar charts with colors red, representing the expressed transcripts of *Atlay2000* and green bars, for that of *Bobur*. All the investigated putative candidate genes (except for *NAD(P)H-quinone oxidoreductase subunit L*, *Malate dehydrogenase* and *putative alanine aminotransferase*) were differentially expressed between the two contrasting ST genotypes (**Figure 4; see also Table 6**). They showed up- and down-regulation in the salt-tolerant and salt-sensitive genotypes, respectively.

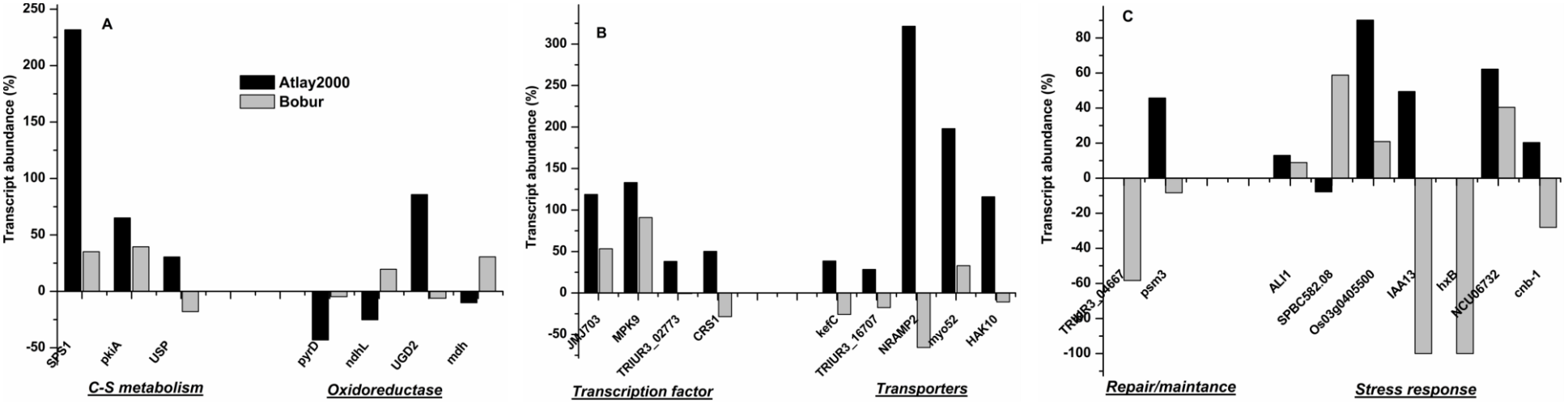


Figure 4 Effect of salt stress on some of identified gene transcript abundance (% change to control) between salt-tolerant genotype (*Atlay2000*, in Black) versus salt-sensitive genotype (*Bobur*, in gray) after 24 d of stress. **SPS1**= Probable sucrose-phosphate synthase 1; **pkiA**= Pyruvate kinase; **USP**= UDP-sugar pyrophosphorylase OS=Oryza sativa subsp. Indica; **pyrD**= Dihydroorotate dehydrogenase (quinone); **ndhL**= NAD(P)H-quinone oxidoreductase subunit L; **UGD2**= UDP-glucose 6-dehydrogenase 2; **mdh**= Malate dehydrogenase; **JMJ703**= Lysine-specific demethylase; **MPK9**= Mitogen-activated protein kinase 9; **TRIUR3_02773**= Putative serine/threonine-protein kinase Cx32, chloroplastic; **CRS1**= Chloroplastic group IIA intron splicing facilitator CRS1, chloroplastic; **kefC**= Glutathione-regulated potassium-efflux system protein; **TRIUR3_16707**= Multiple C2 and transmembrane domain-containing protein 1; **NRAMP2**= NADH dehydrogenase complex (plastoquinone) assembly (Metal transporter Nramp2); **myo**= Myosin-J heavy chain; **HAK10**= Potassium transporter 10; **TRIUR3_04667**= Callose synthase 2; **psm3**= Structural maintenance of chromosomes protein 3; **ALI1**= Metallothionein-like protein 1; **SPBC582.08**= Putative alanine aminotransferase; **Os03g0405500**= Probable nucleoredoxin 1-1; **IAA13**= Auxin-responsive protein; **hxB**= Molybdenum cofactor sulfurase; **NCU06732**= Leukotriene A-4 hydrolase homolog; **cnb-1**= Calcineurin subunit B.

Table 6 Relative transcript abundance of 28 candidate genes identified in our GWAS study and previous reports on them

Associated traits	gene_symbol	description	UniProt	Abundance (%)			Reference
				Atlay2000	Bobur	RE	
Fv/Fo	TRIUR3_04667	Callose synthase 2 OS=Triticum urartu	M7YGW	0	-58.35	+,-	Kosová et al. (2013); Sengupta and Majumder (2009)
Crude fiber	JMJ703	Lysine-specific demethylase JMJ703 OS=Oryza sativa subsp. japonica	Q53WJ1	118.71	53.33	+,+	Shen et al. (2014)
Protein	MPK9	Mitogen-activated protein kinase 9 OS=Oryza sativa subsp. japonica	Q6L5D4	133.11	90.94	+,+	Kumar and Sinha, (2013)
ABS/RC	psm3	Structural maintenance of chromosomes protein 3 OS=Schizosaccharomyces pombe	O42649	45.74	-8.30	+,-	
Crude fiber	ALI1	Metallothionein-like protein 1 OS=Triticum aestivum	P43400	13.00	8.90	+,+	Sekhar et al. (2011); Yang et al. (2015)
Shoot Na^+	pyrD	Dihydroorotate dehydrogenase (quinone) OS=Azorhizobium caulinodans	A8HZX8	-43.09	-4.77	-,-	Liu et al. (2009)
Shoot Na^+, Rohfaser	SPBC582.08	Putative alanine aminotransferase OS=Schizosaccharomyces pombe	Q10334	-7.76	58.78	-,+	
Crude fiber	kefC	Glutathione-regulated potassium-efflux system protein KefC OS=Enterobacter sp.	A4W6F3	38.61	-25.89	+,-	
ABS/RC	Os03g0405500	Probable nucleoredoxin 1-1 OS=Oryza sativa subsp. japonica	Q7Y0E8	90.16	20.87	+,+	
Moisture content	TRIUR3_16707	Multiple C2 and transmembrane domain-containing protein 1 OS=Triticum urartu	M7YGD3	28.42	-17.64	+,-	
ETo/RC	SPS1	Probable sucrose-phosphate synthase 1 OS=Oryza sativa subsp. indica	A2WYE9	231.83	35.15	+,+	Strand et al. (2003); Chen et al. (2005)
ABS/RC2	NRAMP2	NADH dehydrogenase complex (plastoquinone) assembly (Metal transporter Nramp2)	Q10Q65	321.52	-65.78	+,-	Rumeau et al. (2005)
Moisture content	IAA13	Auxin-responsive protein IAA13 OS=Oryza sativa subsp. indica	A2XLV9	49.43	-100.00	+,-	Jiang and Guo (2010)
ABS/RC	ndhL	NAD(P)H-quinone oxidoreductase subunit L OS=Nostoc sp.	Q8YMW5	-25.21	19.65	-,+	
Seed hardness	hxB	Molybdenum cofactor sulfurase OS=Neosartorya fischeri	A1CX75	0	-100.00	+,-	Xiong *et al*. (2001)
Shoot Na^+	NCU06732	Leukotriene A-4 hydrolase homolog OS=Neurospora crassa	Q7S785	62.17	40.43	+,+	
Crude fiber	PLD1	Phospholipase D alpha 1 OS=Zea mays	Q43270	65.10	316.94	+,+	
Shoot Na^+	UGD2	UDP-glucose 6-dehydrogenase 2 OS=Oryza sativa subsp. japonica	B7F958	85.74	-6.07	+,-	
Vi	mdh	Malate dehydrogenase OS=Proteus mirabilis	B4F2A1	-10.08	30.56	-,+	
ETo/RC	pkiA	Pyruvate kinase OS=Emericella nidulans	P22360	65.09	39.44	+,+	
Shoot Na+, Dio/RC, ABC/RC	myo	Myosin-J heavy chain [Aegilops tauschii]	O94477	198.03	32.94	+,+	
Vj	cnb-1	Calcineurin subunit B OS=Neurospora crassa	P87072	20.35	-28.04	+,-	
NDF	HAK10	Potassium transporter 10 OS=Oryza sativa subsp. japonica	Q67VS5	115.92	-10.822	+,-	
Fv	USP	UDP-sugar pyrophosphorylase OS=Oryza sativa subsp. indica	A2YGP6	30.51	-17.96	+,-	Juan et al. (2005)
Fm/Fo	TRIUR3_02773	Putative serine/threonine-protein kinase Cx32, chloroplastic OS=Triticum urartu	M7ZVA6	38.05	-0.69	+,-	Diédhiou et al. (2008)
Fv/Fo	CRS1	Chloroplastic group IIA intron splicing facilitator CRS1, chloroplastic OS=Zea mays	Q9FYT6	50.14	-28.49	+,-	
Fm/Fo	SUS2	Sucrose synthase 2 OS=Oryza sativa subsp. japonica	P30298	-50.00	819.81	-,+	
Crude protein	MPK9	Mitogen-activated protein kinase 9	Q6L5D4	133.11	90.94	+,+	

RE, effect of salt on the gene expression in relation to non-saline condition; **+,-** = gene transcript abundance was up-regulated in Atlay2000 but down-regulated in Bobur; **- ,+** = gene transcript abundance was down-regulated in Atlay2000 but up-regulated in Bobur; **+,+** = gene transcript abundance was up-regulated in both Atlay2000 and Bobur; -- = gene transcript abundance was up-regulated in both Atlay2000 and Bobur

The RT-qPCR was used to verify the expression patterns of *Myosin-J heavy chain* (myo), *ABC transporter F family member 3* (ABC) and *NAD(P)H-quinone oxidoreductase subunit L, chloroplastic* (NAD(P)H) after 30 days of salt stress (**Figure 5A, B** and **C**, respectively). The SNP locus (99.04 cM) on 6AL coding for *Myosin-J heavy chain* is of significant important because it was independently associated with *ABS/RC, DIo/RC* and shoot Na^+ content. *Myosin-J heavy chain* was up-regulated in the two salts-tolerant genotypes (*Atlay2000* and *UZ-11CWA-8)* after 30 days of salt treatment, in contrast with the salt-sensitive genotypes (*UZ-11CWA-24* and *Bobur*). The expressions of *AtABC3* and NAD(P)H were both up-regulation the tolerant and sensitive genotypes, although the fold-changes were much higher in the salt tolerant wheat genotypes**.**

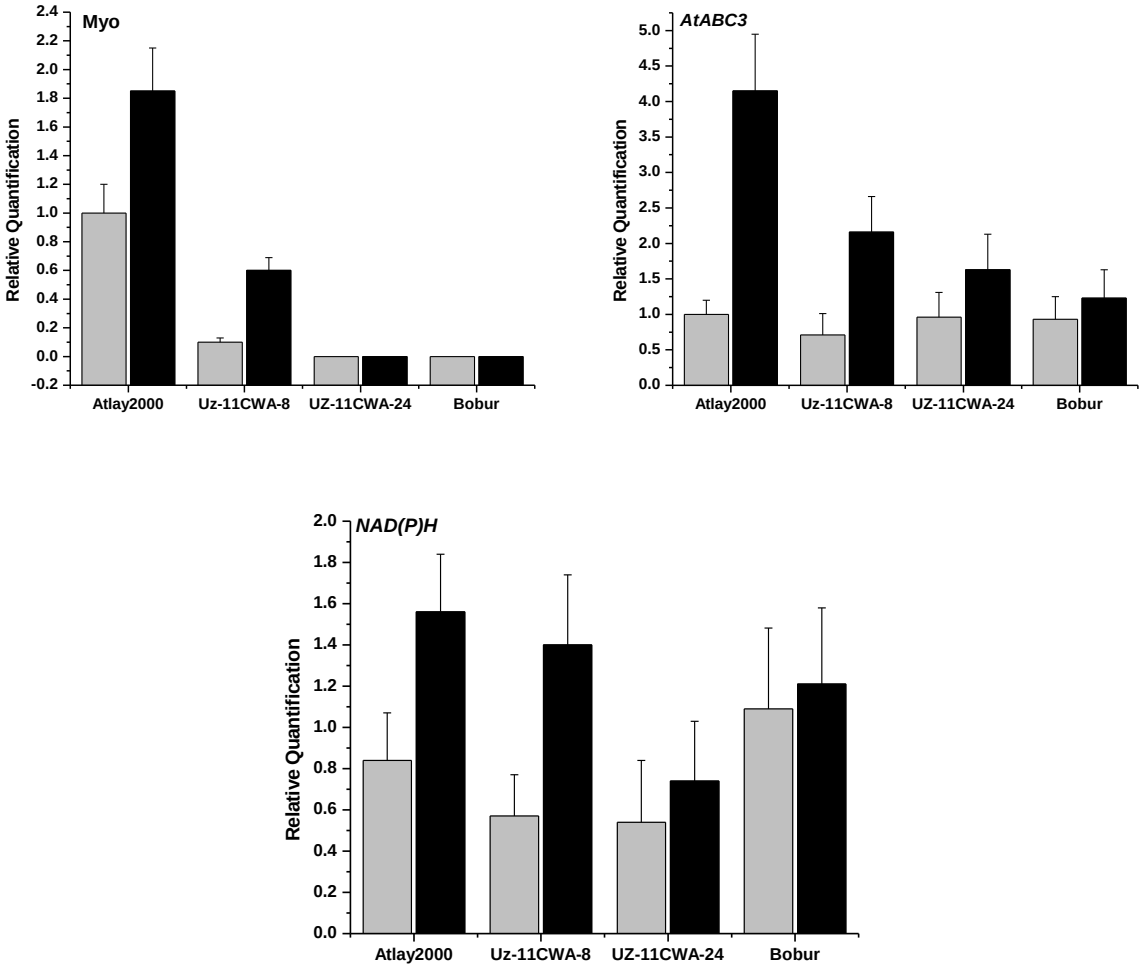
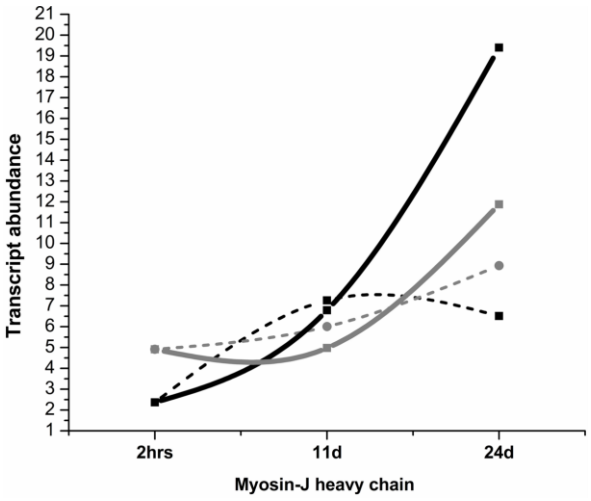
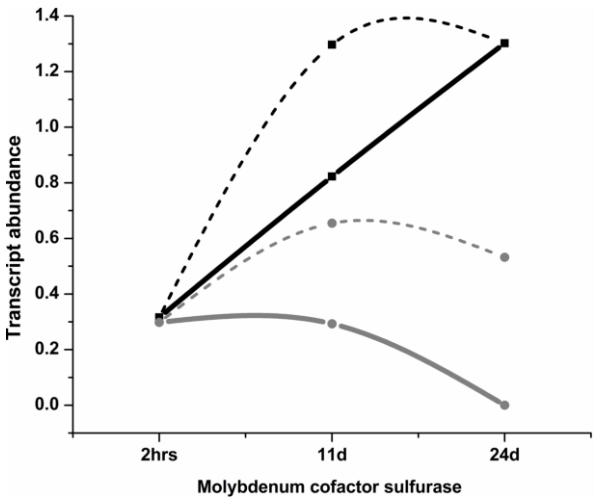
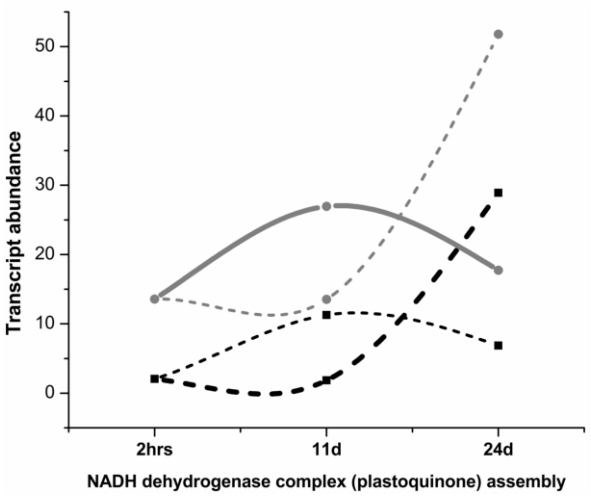


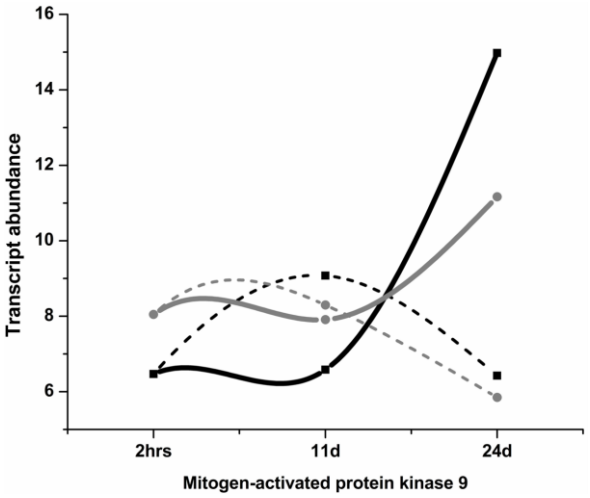
Figure 5 Expression levels of **Myo**: *Myosin-J heavy chain*, **AtABC3**: *ABC transporter F family member 3* and ***NAD(P)H***: NAD(P)H-quinone oxidoreductase subunit L, chloroplastic in leaves of two salt tolerant (*Atlay2000* and *UZ-11CWA-8*) and salt sensitive (*UZ-11CWA-24* and *Bobur*) after 30 days in non-saline (Grey) and saline (Black) conditions, determined by $2^{-\Delta\Delta CT}$ method. *Efa1.1* and *Efa1.2* genes were used as internal control genes. Bars are the means (n = 3) ± standard error.

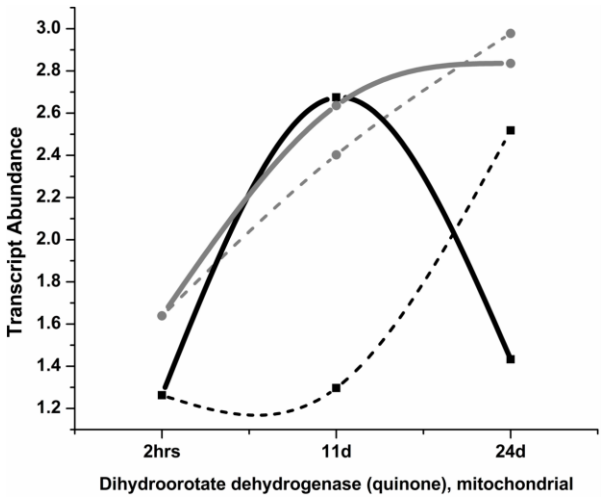
The sigmoidal curves indicated that the kinetics of the putative candidate genes induced by 0 and 100 mM NaCl in *Atlay2000* and *Bobur* were investigated after 2hrs, 11d and 24 days of salt application (**Figure 6)**. Both genotypes showed differential expression signatures under saline and non-saline conditions after 2hrs of stress, with *Bobur* showing higher transcripts amount than in *Atlay2000* in most of the genes. This trend was maintained for few (0-5) days. Distinct transcriptional changes were observed between the two genotypes in most genes after 11 days of salt stress. At this period, the expressed transcripts in *Atlay2000* increased steadily and exponentially under salt-stress, but decreased in *Bobur*. Three genes including *myo*, *hxB* and *NRAMP2* coding for *Myosin-J heavy chain*, *Molybdenum cofactor sulfurase* and *NADH dehydrogenase complex* (plastoquinone), respectively, exhibited an early response, while *Mitogen-activated protein kinase 9* was differentially expressed after 15 days of salt stress.

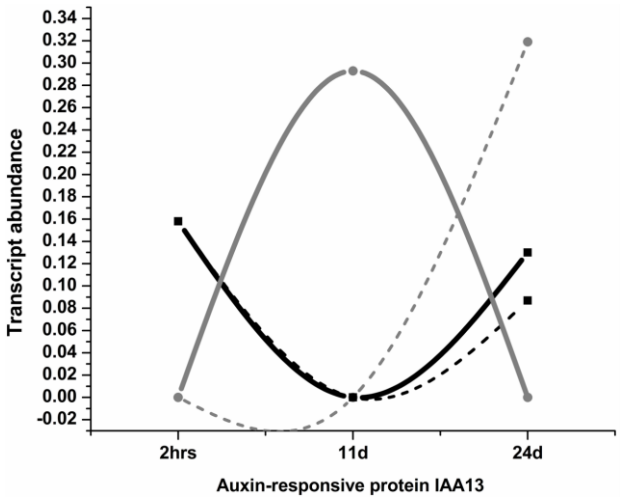


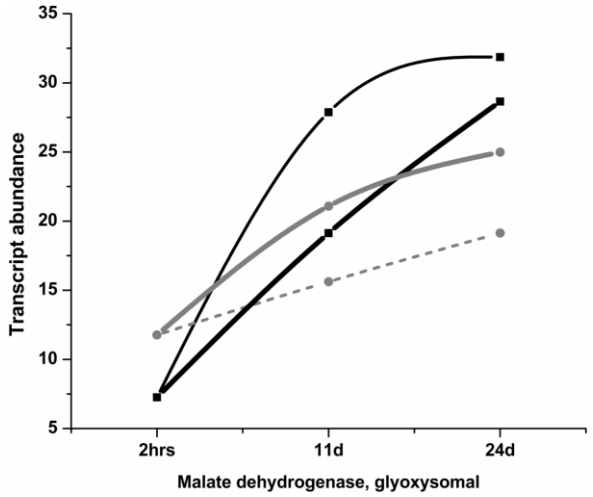


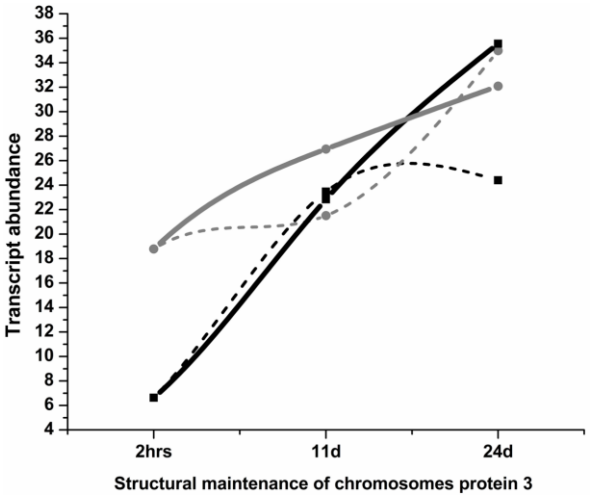





Transcript Abundance
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
2hrs
11d
24d
Dihydroorotate dehydrogenase (quinone), mitochondrial


Transcript abundance
0.34
0.32
0.30
0.28
0.26
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
2hrs
11d
24d
Auxin-responsive protein IAA13


Transcript abundance
35
30
25
20
15
10
5
2hrs
11d
24d
Malate dehydrogenase, glyoxysomal


Transcript abundance
38
36
34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
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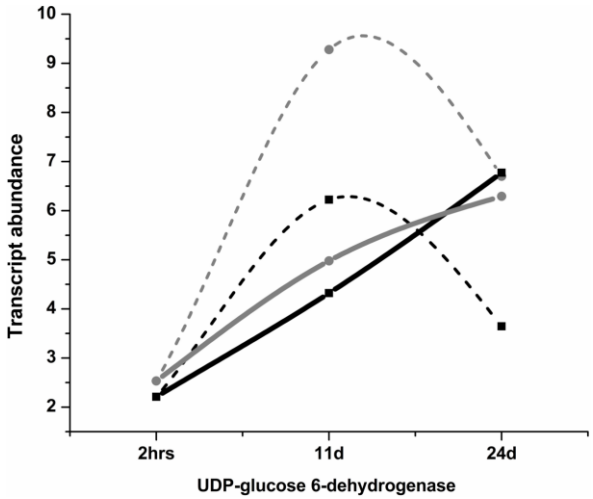
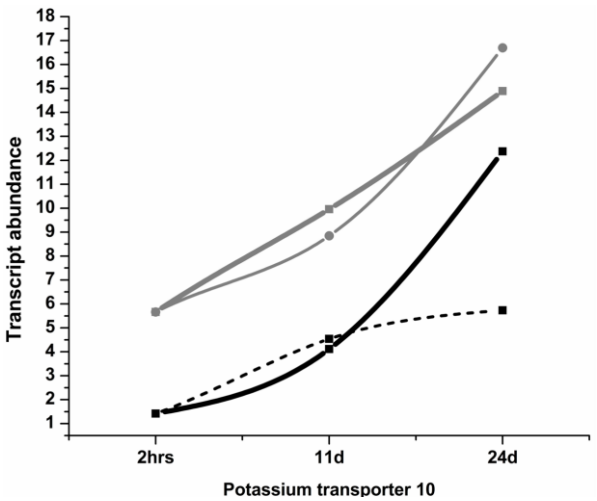
2hrs
11d
24d
Structural maintenance of chromosomes protein 3


Transcript abundance
10
9
8
7
6
5
4
3
2
2hrs
11d
24d
UDP-glucose 6-dehydrogenase


Transcript abundance
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
2hrs
11d
24d
Potassium transporter 10

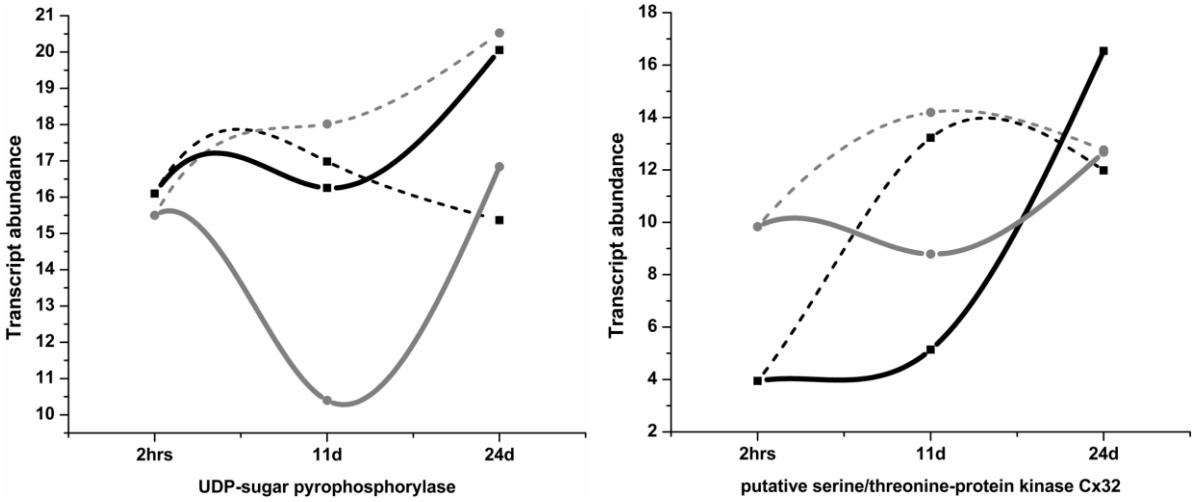


Figure 6 MACE analysis showing the expression kinetics of the associated salt tolerance genes over a period of 24d in salt tolerant (in black) and salt sensitive (in gray) genotypes. The "**thick**" and "**dotted**" lines indicate the gene expression kinetics over-time in saline and non-saline conditions, respectively.

Sequence analysis in the putative candidate genes

The EST sequence variations in the coding regions of the putative candidate genes anchoring the significant MTAs are shown in Figure 7. Four missense amino acid substitutions: C (Cysteine) to G (Glycine), A (Alanine) to V (Valine), R (Arginine) to G (Glycine) and, C (Cysteine) to W (Tryptophan) were detected at 1529, 1549, 1626 and 1628 sites on exon 37 of *Myosin-J heavy chain (Traes_6AL_891456790.1*), respectively (**Figure 7A**). The Dihydroorotate dehydrogenase (quinone) (*Traes_2BL_3A44C99D2.1*) contains four non-synonymous substitutions at position 361 [V-to-L], 413 [L-to-C], 412 [K-to-P] and 411 [-(a SNP deletion)-to-C] of exon 10 (**Figure 7B).** Two non-synonymous substitutions which might have altered the functionality of UDP-glucose 6-dehydrogenase 2 (*Traes_5BL_7F59B65A3.1)* were found on exon 1 at 599 [R-to-P] and 662 [P-toT] positions (**Figure 7C).** The ESTs of SPS1 (*Traes_3B_35D6F6CE7.1)* and NRAMP2 (*Traes_4BL_C6A3F5C8A.1)* also showed allele variations between the two contrasting genotypes (**Figure 7D and E**, respectively); although the regions anchoring their corresponding MTAs were located in the introns.

A. Myosin-J heavy chain (Traes_6AL_891456790.1.mrna1-E37)

```
Gene_ID    --ESLHHYPSAQFLLTASPTPCVIEQQGGLGLKISSPDFYGVAPLQQCCTVVFSRANLIGGRGWVCSSKATIVARRRTALAS 1589
Atlay2000  --ESLHHYPSAQFLLTASPTPCVIEQQGGLGLKISSPDFYGAAPLQQCCTVVFSRANLIGGRGWVCSSKATIVARRRTALAS 1589
Bobur      --ESLHHYPSAQFLLTASPTPGVIEQQGGLGLKISSPDFYGVAPLQQCCTVVFSRANLIGGRGWVCSSKATIVARRRTALAS 1589

Gene_ID      WVSSCLGYLCCTKNTVEMRSSCPKLITSVAKCPVWCRTCFA--1630
Atlay2000    WVSSCLGYLCCTKNTVEMRSSCPKLITSVAKCPVWCRTCFA--1630
Bobur        WVSSCLGYLCCTKNTVEMRSSCPKLITSVAKCPVWCGTWFA--1630
```

B. Dihydroorotate dehydrogenase (quinone) (Traes_2BL_3A44C99D2.1.mrna1-E10)

```
Gene_ID    --IIQPLVSTYWSTCHRCIHGWHLVLLSLASCTRNFSLTCISPGFLPCGETMAGLRIVLGYSEAAAFCLCRSLHYAKVICPC 416
Atlay2000  --IIQPLVSTYWSTCHRCIHGWHLVLVSLASCTRNFSLTCISPGFLPCGETMAGLRIVLGYSEAAAFCLCRSLHYAKVILK- 416
Bobur      --IIQPLVSTYWSTCHRCIHGWHLVLLSLASCTRNFSLTCISPGFLPCGETMAGLRIVLGYSEAAAFCLCRSLHYAKVICPC 416

Gene_ID      FLVYALNWSLLVGLNLVP--434
Atlay2000    FLVYALNWSLLVGLNLVP--434
Bobur        FLVYALNWSLLVGLNLVP--434
```

C. UDP-glucose 6-dehydrogenase 2 (Traes_5BL_7F59B65A3.1.mrna1-E1)

```
Gene_ID    --VHLVGRPDRRKSKKWFDRDSFFIVCILQEKHPHFLSRPEFSKSSCLVKFPIHVCATPSVDLTYNQILGPLCCLRRFFRC--672
Atlay2000  --VHLVARPDRRKSKKWFDRDSFFIVCILQEKHPHFLSRPEFSKSSCLVKFPIHVCATPSVDLTYNQILGPLCCLRRFFRC--672
Bobur      --VHLVAPPDRRKSKKWFDRDSFFIVCILQEKHPHFLSRPEFSKSSCLVKFPIHVCATPSVDLTYNQILGTLCCLRRFFRC--672
```

D. Sucrose-phosphate synthase 1 (Traes_3B_35D6F6CE7.1.mrna1-E2)

```
Gene_ID    --LRILPTFHFHSHGKIVHRFQINYRTIVIKCVLWGMSTLLCGNHIYTQQCACISTNNHLQGAILILLGYICTVSPP--617
Atlay2000  --LRILPTFHFHSHGKIVHRFQINYRTIVIKCVLWGMSTLLCGNHIYTQQCACISTNNHLQGAILILLGFLCTVSPP--617
Bobur      --LRILPTFHFHSHGKIVHRFQINYRTIVIKCVLWGMSTLLCGNHIYTQQCACISTNNHLQGAILILLGYICTVSPP--617
```

Figure 7 Comparison of the deduced EST amino acid sequence in Atlay2000 (salt tolerant) and Bobur (salt sensitive) genotypes with their corresponding draft sequence obtained from Ensembl Genomes database (http://www.ensemblgenomes.org). Black and white colours indicate identical and polymorphic sites, respectively, while gray colour represent region anchoring the associated SNP marker identified in the GWAS analysis.

DISCUSSION

Phenotypic variation and correlations among traits

This study combined the high throughput ChlF traits with SI and SQ parameters to gain insight into underlying trait-by-trait associations and genetic architectures controlling ST in 150 diversity wheat panel. The genotypes showed significant ($P < 0.001$) level of genetic diversity, as revealed by ANOVA and distribution statistics. Majority of the traits analyzed showed relatively high heritability estimates, indicating that genetic factors also contributed to the observed phenotypic variations. Thus, uncovering the genetic architectures controlling ST using the measured traits is possible.

It is well-known that the genotype, salinity and their interaction determines plant phenotypes. Results showed that the means of some measured traits were significantly reduced under salinity stress compared to the non-saline stress, suggesting that the traits were affected strongly by salt stress. In line with the present study, Chen and Murata (2011) and Shu *et al*. (2012) have demonstrated that maximal efficiency of PSII

photochemistry (*Fv/Fm*) is inhibited under salt stress. The non- significant-interaction effect of genotype x salt treatment observed in all the ChlF traits suggests that the genotypes are insensitivities to both saline and non-saline treatments. This means that either of the treatment conditions can be relevant for studying genetic variations in ChlF traits under salt stress conditions. Lutts *et al*. (1996) reported non-significant interaction of genotypes and salt treatment for *Fv/Fm* in rice.

Seed protein content (PC) of grains harvested from the saline fields were higher than that harvested from the non-saline field, whereas the starch contents (SC) of the seed from saline soil decreased, suggesting that salt stress has contrasting impact on both SQ traits. Reports abound on the effects of major abiotic stresses on SQ traits in wheat, but none had reported on salinity. For instance, Ozone (O_3) (Piikki *et al*., 2008; Zheng *et al*., 2013) and Heat stresses (Farooq *et al*., 2011) is known to increase PC in wheat. Fuhrer (1990) has also shown that SC is decreased under O_3 stress. Salinity stress is associated with decreased SC and increase PC in rice (Baxter *et al*., 2011; Thitisaksakul *et al*., 2015). The reduction in SC under salt stress might be due to the combination of slower growth and development as a consequence of limitation in the photosynthesis in salt-stressed plant and, which might have indirectly resulted in the reduction in the sink capacity and less starch deposition in the seed grains. Unlike N-metabolism (which results in increase in the PC) (Sastry and Gupta, 2009), Stitt et al. (2002) have demonstrated that carbohydrate metabolism is negatively affected by salinity. These findings revealed that salt stress may have increased the plants ability to re-mobilize N to the active photosynthetic shoot parts, thus, depleting the carbon sink and source.

Shoot K^+ was positively correlated with several ChlF traits (*Fv*, *Fv/Fo*, *Fv/Fm*, *TRo/RC*, *ETo/RC* and *PI/ABS*). This suggests that shoot K^+ amount is critical during photosynthesis (Marschner, 2012). Using the correlated ChlF traits as a 'physiological marker' for indirect measurement K^+ status salt-stress leaves may present an indirect approach for rapid evaluation for salt tolerance in a large population. In tomatoes (Albacete *et al*., 2009), *Fm/Fv* also exhibited high positive correlation (r = 0.76; $P \leq 0.001$) with leaf K^+ under salt stress. The PC correlated negatively (r^2= -0.80; P=0.01) with SC. Burešová *et al.* (2010) and Hucl and Chibbar (1996) have also reported a negative correlation (r= –0.83, -0.83, respectively) between protein and starch contents in wheat. Base on the correlations observed among the ChlF, shoot ionic and seed quality parameters (**Table 3**), it may be possible that genes controlling some of these traits are related, either through linkage or pleiotropy.

Association mapping of salt tolerance

By employing the GWAS approach, a total of 115 significant MTAs using the ST-traits, representing 21 LD-defined QTL regions, were identified. Of these, 54, 17 and 44 SNPs had effect on ChlF, SI and SQ traits, respectively. Some of the associated SNP loci/QTL regions are pleiotropic and/or, were located in genomic regions that have been reported for QTL/genes regulating salt tolerance in wheat **(see Table 4).** In addition, three QTL regions on 5BL, 6AL and 7BL have not been previously reported for ST in wheat.

A ST locus at 99.04 cM on 6AL influenced *ABS/RC*, *DIo/RC* and shoot Na^+. This region is known to habour QTL for Fm, Fv/Fm (Li *et al*., 2012b) as well as a QTL for shoot Na^+ (Genc *et al*., 2010). The locus- *Q.chl*Qu(2DS)* at 8.52 cM on 2DS controlling *Fm/Fo* and CF traits coincides with several QTL controlling *Fm* (Zheng *et al*., 2013), tenacious glume locus *Tg-D1* (Okamoto *et al*., 2012) and grain dormancy (Tan *et al*., 2006), ST QTL for grain yield, qGY2Da (Zhang *et al*., 2009b) and the *Rht8* and *Ppd-D1* genes (Pestsova and Röder, 2002; Gasperini *et al*., 2012). The *Q.chl*Qu*I(2BL)* region on 2BL spanning genetic interval of 1.55 cM had strong effect on *Fv/Fm*, CFC, shoot Na^+ and shoot K^+/Na^+ in this study. ST QTL for traits such as *Fm, Fv/Fm, Fv/Fo* and qChlN-2B (Li *et al.*, 2010; Zhang *et al*., 2010; Li *et al*, 2012b; Zheng *et al*., 2013), shoot Na^+ (Genc *et al*., 2010), seed dormancy and pre-harvest sprouting (PHS) (Chao *et al*., 2015) have also been reported in this region. The coincidences and co-location of the QTL controlling most of the measured parameter (**Table 4**), is an indication that the identified QTL loci have the potential to be exploited for breeding programs and basic genetic research.

Identification of candidate genes contributing to the genetic variance for ST

The putative candidate genes linked with the detected MTAs are presented in (**Table 5**). Result showed that associated homologous genes are involved in ST and, belong to different functional categories (**Figure 3**). Worthy to mention is the locus (99.04 cM) on 6AL [that influenced *ABS/RC, DIo/RC,* shoot Na^+ and shoot K^+/Na^+ traits]. It showed high sequence homology to *Myosin-J heavy* chain protein (Myo) that is involved in stress response to heavy metal (Ahsan et al., 2007) and cold stress (Yan *et al.*, 2006) in tomatoes and rice plants, respectively. Sottosanto *et al*. (2007) revealed that Myo is salt-responsive and is directly influenced by the vacuolar Na^+/H^+ antiporter. SNP locus (*Jagger_c1134_353*) at 140.87 cM on 6AL [associated with shoot Na^+] also coded for *ABC transporter F family member 3*, an indicating that it may be involved in Na^+ transport. Recent reports have also indicated that 6AL harbors three wheat plasma membrane transporters including *TaYSL10, TaYSL14* and *TaYSL15* (Pearce *et al*., 2014). The 2DS SNP locus (8.52 cM), associated with *Fm/Fo* and grain crude fiber content, was found to be highly homologous to *ABC transporter C family*

member 3. The activity of this gene has been linked to seed grain formation and myco-toxin tolerance in wheat (Walter *et al*., 2015).

Single gene expression analysis using contrasting ST wheat genotypes

We have analyzed 28 associated gene transcripts expression pattern in the leaves of *Atlay2000* (salt-tolerant) and Bobur (salt sensitive). Both genotypes showed contrasting phenotypes for ST in our previous study with the entire studied 150 germplasm (**Oyiga *et al*., 2016;** also see **Figure 2).** They showed general trend of up- and down-regulation in the salt-tolerant and salt-sensitive genotypes, respectively after 24 days of stress (**Figure 4**), suggesting that their activities contribute to the salt tolerance variation in wheat. Several reports have demonstrated that the activities of the candidate genes analyzed confer ST in plants (**Table 4**). The RT-PCR analysis of *Myosin-J heavy chain* on 6AL revealed that this gene is also up-regulated in *Atlay2000,* with no observable change in the expressed transcript in Bobur under saline and non-saline conditions; thus validating the expressions of this gene in response to salt stress. In addition, At*ABC3* and *NAD(P)H* show that salt stress induced higher expressions of both genotypes, although highly significant fold change was observed in the salt tolerant ones. The expressions of profile of *NAD(P)H* observed in the MACE data and RT-PCR data were found contradictory, with the available reports so far supporting the latter (Zhou et al., 2009; Wang et al.,2015).

Carbohydrate, sucrose and energy metabolism are rapidly adjusted under salt stress, because large amounts of ATPase are required to provide energy for the growth and development of plants under salt stress conditions (Wang *et al*. 2009). Three of the associated SNPs coded for *sucrose-phosphate synthase 1* (SPS1), *Pyruvate kinase (pkiA)* and *UDP-sugar pyrophosphorylase (USP)* genes that are involved in carbohydrate and energy metabolism. These genes showed higher transcript abundance in *Atlay2000* than in *Bobur*, a strong indication that their up-regulation enhances salt tolerance via increase in the carbon metabolism and ATP production. In addition, the *USP* gene is one of the well-documented protein markers for salinity tolerance and, is differentially expressed in the salt tolerant and sensitive barley cultivars (Mostek et al., 2015). Over-expressions of transporters are known to regulate and/or prevent build-up of toxic ions in plant cell (Ohta *et al*., 2002); thus lowering rate of ROS formation in the leaves of stressed plants. All the transporter encoding genes (including *Glutathione-regulated potassium-efflux system protein (KefC)*, *Multiple C2 and transmembrane domain-containing protein 1*, *NADH dehydrogenase complex (plastoquinone) assembly*, *Myosin-J heavy chain* and *Potassium transporter 10*) were up-regulated in *Atlay2000* and down-regulated in Bobur (**Table 4**), a probable explanation for the enhanced growth

observed in Atlay2000 relative to Bobur under saline conditions (Oyiga *et al*., 2016). The expression of *Myosin-J heavy chain* was also validated by RT-PCR in the present study.

Sequence variations at candidate gene loci between Atlay2000 and Bobur

Salt tolerance may arise from a target-site-based mechanism involving mutations in the genes that are contributing to adaptation under saline conditions. Non-synonymous substitutions associated with trait variation for ST were detected at 1529, 1549, 1626 and 1628 mutation sites in exon 37 of myo (*Traes_6AL_891456790.1*) (**Figure 7A**). Among the identified substitutions, the R (Arginine) to G (Glycine) substitution at 1626 position showed non-conservative modified variation (where alterations result in the substitution of an amino acid with biochemically dissimilar amino acid), indication that it may have contributed majorly to the differential response of this gene in the two contrasting genotypes. Although the effect of R to G mutation has not been reported for ST, available report indicates that such mutation is linked to *quinol oxydation inhibitors* (fungicide) resistance (Sierotzki *et al*., 2006).

Four amino acid substitutions, from valine to leucine at position 361 (V361L), from Leucine to Cysteine at 413 (L413C), from Lysine to Proline at 412 (K412P) and, from – (a SNP deletion site) to Cysteine at 411 (-411C), were detected in exon 10 of Dihydroorotate dehydrogenase (quinone) (*Traes_2BL_3A44C99D2.1*) coding region (**Figure 7B**). The V361L and L413C substitutions seem not to have significantly affected the functionality of this protein because they have similar aliphatic and hydrophobic properties, respectively. However, it is highly likely that structural variation in this gene between the contrasting ST wheat genotypes may come from K412P and -411C polymorphic sites, given that both amino acids do not have similar physio-chemical properties. The cysteine residue, which we found to be deleted in Atlay2000 but present in Bobur, has been implicated as active site base residues that promotes substrate oxidation in *pyrD* and its absent would resulted in extremely low activity of the gene (Björnberg et al., 2001). Thus, the higher activities' of pyrD observed in Bobur may be partly connected to the present of cysteine residue in the 411 substitution site. These findings provide essential knowledge for further unlocking of the genetic mechanism and cloning of genes related to salt tolerance in wheat.

CHAPTER 5

General Discussion

Soil salinity is a great threat to global food security in the face of dwindling arable lands and increasing human population (Tester and Langridge 2010; Mainuddin *et al*. 2011; Bansal *et al*. 2014). Thus, the continuous salinization of arable land either by natural or by human induced processes is forcing plant breeders to look for new sources of salt tolerance, to identify crop traits and candidate genes that confer the tolerance to salt stress that can be exploited through the conventional breeding or molecular biotechnological manipulations (Ashraf and Akram 2009; Ford-Lloyd *et al*. 2011; Rajalakshmi and Parida 2012; Kumar *et al*. 2012). The improvement of salt tolerance in crop plants is often challenged by lack of effective salt screening and evaluation methods among the crop plants (Zeng et al., 2003). The screening of genotypes of diverse genetic background is perceived as a prerequisite step in identifying the new sources of salt tolerance. Salt tolerance is a complex phenomenon and depends not only on the plant physiology, genetics and molecular mechanisms but also on the stage of development during which the stress occurs (Epstein and Rains, 1987; Shannon, 1985; Mano and Takeda, 1997; Bayuelo Jiménez *et al*., 2002; Haq *et al*., 2010). This means that for successful identification and development of elite salt tolerant wheat genotypes, salt-tolerance status of genotypes at each growth stage should be evaluated separately.

Screening of germplasm across growth stages

In Chapter 2, a total of 150 wheat genotypes of diverse genetic background were screened simultaneously for salt tolerance at germination, seedling and field adult growth, together with the K^+, Na^+ and K^+/Na^+ constituents of their different shoot parts including third leaf, stem and remaining leaf parts. The morpho-physiological assessment revealed substantial genetic variation and trait heritabilities that can be exploited to characterize the salt tolerance status of all the studied genotypes. The response of the genotypes to salt stress varied across growth stages, with a total of 33, 39, 45 and 34 genotypes being identified as being tolerant, moderately tolerant, moderately sensitive and sensitive, respectively. The identified tolerant genotypes showed higher "K^+ inclusion" and "Na^+ exclusion" mechanisms than the sensitive genotypes. We found *Altay2000*, *14IWWYTIR-19* and *UZ-11CWA-8* (salt-tolerant) and Bobur (salt-sensitive) genotypes very outstanding because they were consistently identified across the three growth stages. Interestingly, *Altay2000* has been previously found to be resilient to salt, drought and cold stress (Kara and Kara, 2010; Mutlu *et al*., 2009; Akfirat and Uncuoglu, 2013). Further physiological evaluations performed revealed that the

tolerant (*Altay2000* and *UZ-11CWA-8*) genotypes were better equipped with higher membrane stability, lower osmotic potential and higher rates of PSII photochemical activities and higher K^+/Na^+ ratio under salt stress. The identified genotypes can be considered as new genetic resources for the conventional breeding programs, although studying the genetics and molecular mechanisms of salt tolerance in these genotypes would be helpful in confirming their ST status because the conclusions reached in the present study were only based on comparing the agronomic and some physiological data.

The conventional breeding as a means of genetic improvement for crop yield has been exploited to develop salt tolerance genotypes, but till now this has not yielded the desire goals (Flowers 2004; Munns *et al*., 2006), due to complex nature of salt tolerance in plant. The development of inexpensive high-throughput genotyping platforms such as the Illumina wheat SNP 90K Beadchip (Wang *et al*., 2014) and/or genotyping-by-sequencing (Elshire *et al.,* 2011) has made the use of functional DNA markers for selection very useful tools for identification of wheat genotypes that are salt tolerant. Development of functional markers for salt tolerance and deploying them through marker-aided selection in breeding program would fasten the process of developing tolerance genotypes, thus, circumventing the limitations of conventional breeding. One of our objectives in Chapter 3 and 4 was to identify the genetic architectures underlying salt tolerance using the genome-wide association studies (GWAS) approach that could be applied in the cost effective genomics-based approaches when breeding high yielding wheat genotypes for saline conditions. GWAS link genetic variants to complex traits at high precision by exploiting ancestral linkage disequilibrium (LD) between genetic markers and causal variants in diverse population (Rafalski, 2010; Stich and Melchinger, 2010). This approach has led to the discovery of important genes controlling complex traits such as biotic and abiotic stress tolerance in many plants.

The GWAS depends on the quality of phenotypic data, population structure, rare marker alleles and level of LD between the causal factor and the SNPs. Thus, in Chapter 3, we studied the structure and LD of the studied population in other to improve our mapping strategies. Since rare alleles are more likely to result in the declaration of false positive in GWAS (Lamet *et al*., 2007), we excluded from our dataset all the genotypes and alleles that have less than 5% allele frequency. The population analysis using three statistical programs reveal that the studied panel is made up of two sub-groups and their distribution in the plots did not reflect germplasm collection centers. The LD analyses revealed that the whole genome LD value (r^2) rapidly decreased when the genetic distance was less

than 13 cM, but the LD was not distributed uniformly across the genome due to the irregular distribution of recombination along the chromosome (Cericola *et al*., 2014). This phenomenon has been reported in several studies (Breseghello and Sorrells, 2006; Robbins *et al*., 2011; Ranc *et al*., 2012). In addition, the speed at which LD decay ($r^2 > 0.1$) was 10, 11 and 14 cM in A-, B- and D-genomes, respectively, which was similar to that previously described in association analysis studies (Chao *et al*., 2007; Emebiri *et al*., 2010), but faster than that reported by Joukhader *et al*. (2013) and Turki *et al*. (2014) in wheat. In line with the report by Pasam *et al*. (2012), we considered SNPs that are ≤ 10, ≤ 11 and ≤ 14 cM from each other for A-, B- and D-genomes, respectively, to belong to similar LD block, and thus are linked to one/few genes. Rapid LD decay is an indication of high genetic diversity within a population. The LD decay of ≤14 cM in the studied panel may be due to large genetic diversity. This also demonstrated the availability of genetic variability in the population that could be exploited in the identification of QTL contributing to salt tolerance.

An association mapping (MLM, K + Q-model) approach that take into account both the *STRUCTURE* output and the kinship matrix (Yu et al., 2006; Price *et al*., 2006; 2010) was applied on all the morpho-physiological trait data collected to identify promising alleles of QTL/genes contributing to salt tolerance to facilitate future breeding for salinity tolerance. Several important QTLs were identified for most of the traits and were discussed in Chapter 3 and 4. The coexisting chromosomal regions/loci governing different traits offers a great opportunity for breeders to introgress such regions together as a unit into high-yielding but salt sensitive cultivars through MAS/MAB and to develop cultivars possessing increased adaptation to saline conditions. In this section, I will briefly discuss the prospects of some of these QTL regions that were consistently identified in both Chapter 3 and 4, the associated traits in the identified regions, predicted genes and their implications for the future genetic studies and breeding. Some of these coincident QTL regions have been represented in the **Figure 1** (below).

Chromosome 1AL

The *QTL* region that spanned from 137.12 to 142.62 cM located on 1AL seems to be novel, although it was proximal to a QTL for chlorophyll fluorescence, *Fo* (Zheng *et al*., 2013) and was strongly associated with ST_FRW, Seed protein, Crude fiber, ABS/RC and DIo/RC and was located ~ 6 cM away from ST QTL detected for seed grain yield. One of the important genes identified in this region was *mitogen-activated protein kinase* 9 (MAPK9). The overexpression of constitutively active

MAPK6 gene family enhances tolerance to salt stress in rice (Kumar and Sinha, 2013). The MACE analysis showed that this gene was also constitutively expressed in extreme salt tolerant genotypes, but was significantly higher in the tolerant genotype. It has been reported that MARK9 confer resistance to *Verticillium dahliae* pathogen in cotton (Zhang *et al*., 2014), suggesting that the associated 1AL region found in this study may be involved in both biotic and abiotic stress tolerance.

Chromosome 1BS

Although the *QTL* region on 1BS (60.62-68.04 cM) has been reported to harbor several QTL for endosperm proteins and end-use quality traits - *QBwa.mna-1B, QMpv.mna-1B.1, QMpt.mna-1B.1, QMpi.mna-1B.1, QMixopa.mna-1B* (Tsilo *et al*., 2011) and Grain filling duration (Zhang *et al*., 2009b), to the best of our knowledge, no salt tolerant QTL has been reported on this region. In this study, this region strongly affected ST traits at germination (100 mM Na2SO4), seedling (DSW and ST_FRW), and field adult plant (ST_TKW and ST GY) stages as well as ST traits for seed hardness and crude fiber contents. Based on the *in silico* analyses performed on this QTL region, the most causal gene detected were the ZIP7 transporters. The relevance of this gene to salt tolerance has been discussed in Chapter 3. The MACE analysis revealed up-regulation (+713.98%) of ZIP7 transporter in *Atlay200* (salt tolerant) and down-regulation *(*-22.19%) in *Bobur (*salt sensitive) genotypes after 24 day of salt treatment. RT-qPCR performed for ZIP7 validated the MACE results, suggesting the potential of using this gene as early biomarkers of salt stress tolerance in cereals.

Chromosome 2AL

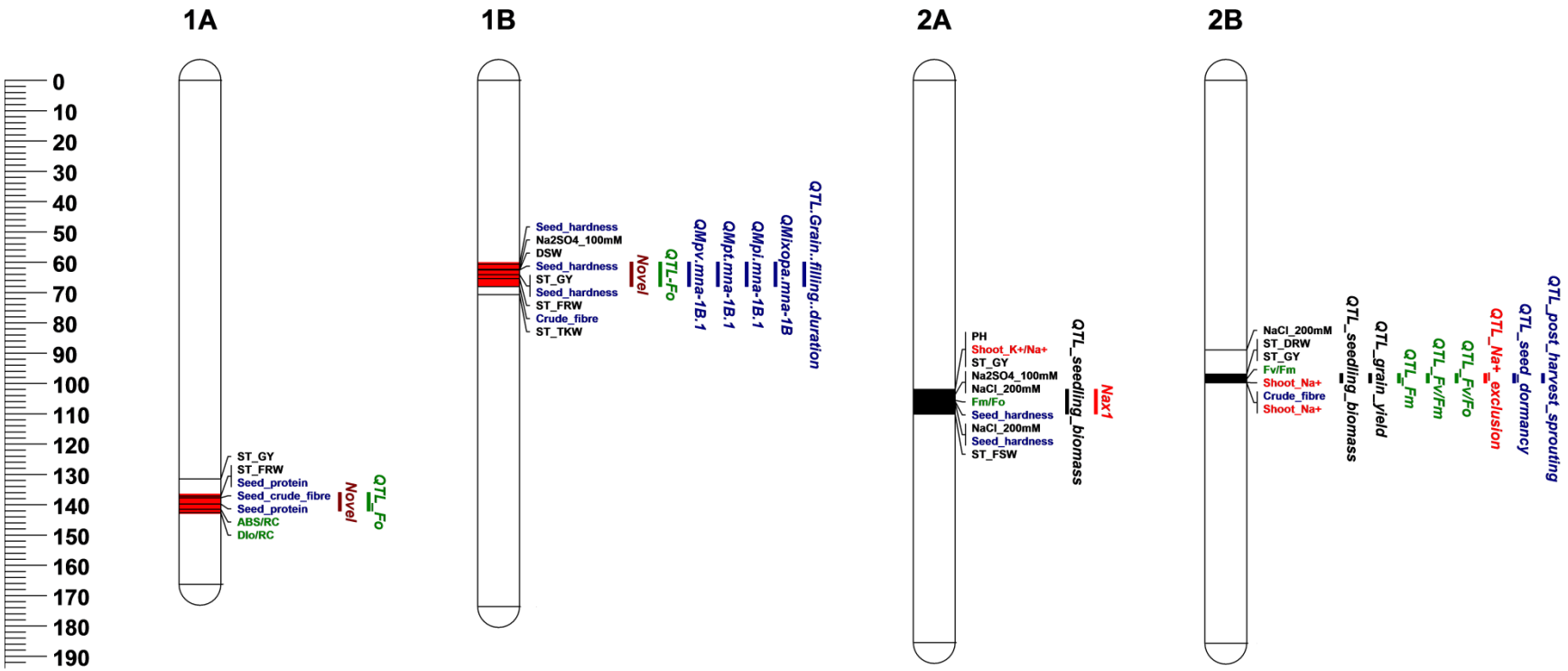
The QTL region on 2AL (101.97 to 110.13 cM) was strongly associated with ST traits at germination (under both 100 mM Na_2SO_4 and 200 mM NaCl), seedling (ST_FSW), field adult plant (PH and ST_GY) stages as well as leaf fluorescence (Fm/Fo) and ion homeostasis-related traits - shoot K^+ /Na^+. This region on 2AL coincided with the ST QTL for seedling biomass (Ma *et al*., 2007) and the Nax1 locus (Lindsay *et al*., 2004; James et al., 2006; Huang *et al*., 2006). It also showed sequence homology of ferredoxin-*dependent glutamate synthase enzyme* that has been implicated in ROS scavenging and ion homeostasis activities in plant. As has been reported in Chapter 3**,** *ferredoxin-dependent glutamate synthase* exhibited early response to salt stress and was highly expressed in the tolerant genotype compared to the sensitive genotype, suggesting the relevance of this gene in regulation of salt tolerance regulation in wheat. Report by Jang *et al*. (2012) indicated that ferredoxin-dependent glutamate synthase confers oxidative stress-tolerant into rice.

Chromosome 2BL

The QTL region found within a genetic distance that spanned 1.81 cM on 2BL showed pleiotropic effects on ST_DRW, ion homeostasis-related traits - shoot Na^+, Fv/Fm, grain yield and seed crude protein content. Moreover, several ST QTL for grain yield, seedling biomass (Quarrie *et al*., 2005; Ma *et al*., 2007; Genc *et al*., 2010b), Fm, Fv/Fm, Fv/Fo (Zhang *et al*., 2010; Li et al., 2012b), shoot Na^+ (Genc *et al*., 2010b) and seed dormancy and PHS loci (Chao *et al*., 2015) have also been reported in this region. This region also coded for NADPH-cytochrome P450 reductase, an oxidoreductase gene responsible for ROS scavenging and ion homoeostasis.

Chromosome 6AL

Two important QTL regions were identified on 6AL chromosome. We believed the first QTL region to be novel. It is located between 77.64 and 85.07 cM on 6AL and has strong effect on leaf fluorescence (Fj, Vj, Fj, ABS/RC, DIo/RC), seedling (ST_FRW, ST_DSW) and ion homeostasis-related traits (shoot Na^+) traits. Sequence analysis showed high sequence homology for a universal stress protein A-like protein in this region. The second QTL was detected at 90.04 cM on 6AL and was associated with ABS/RC, DIo/RC, and ion homeostasis-related traits (shoot Na^+) traits. It was found to possess high sequence identity of *myosin-J heavy chain*. The role of myosin-J heavy chain in salt tolerance in plant has also been discussed in Chapter 4. In addition, the MACE and RT-PCR analyses performed revealed that *myosin-J heavy chain* is highly and significantly expressed in the salt tolerant genotype when compared with the salt sensitive genotypes, an indication that it´s activity contributes to salt tolerance in wheat.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Seed_protein
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Dio/RC
Novel
QTL_Fo
Seed_hardness
Na2SO4_100mM
DSW
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ST_FRW
Crude_fibre
ST_TKW
Novel
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QMpt.mna-1B.1
QMpl.mna-1B.1
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PH
Shoot_K+/Na+
ST_GY
Na2SO4_100mM
NaCl_200mM
Fm/Fo
Seed_hardness
NaCl_200mM
Seed_hardness
ST_FSW
Nax1
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ST_GY
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Shoot_Na+
Crude_fibre
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QTL_seed_dormancy
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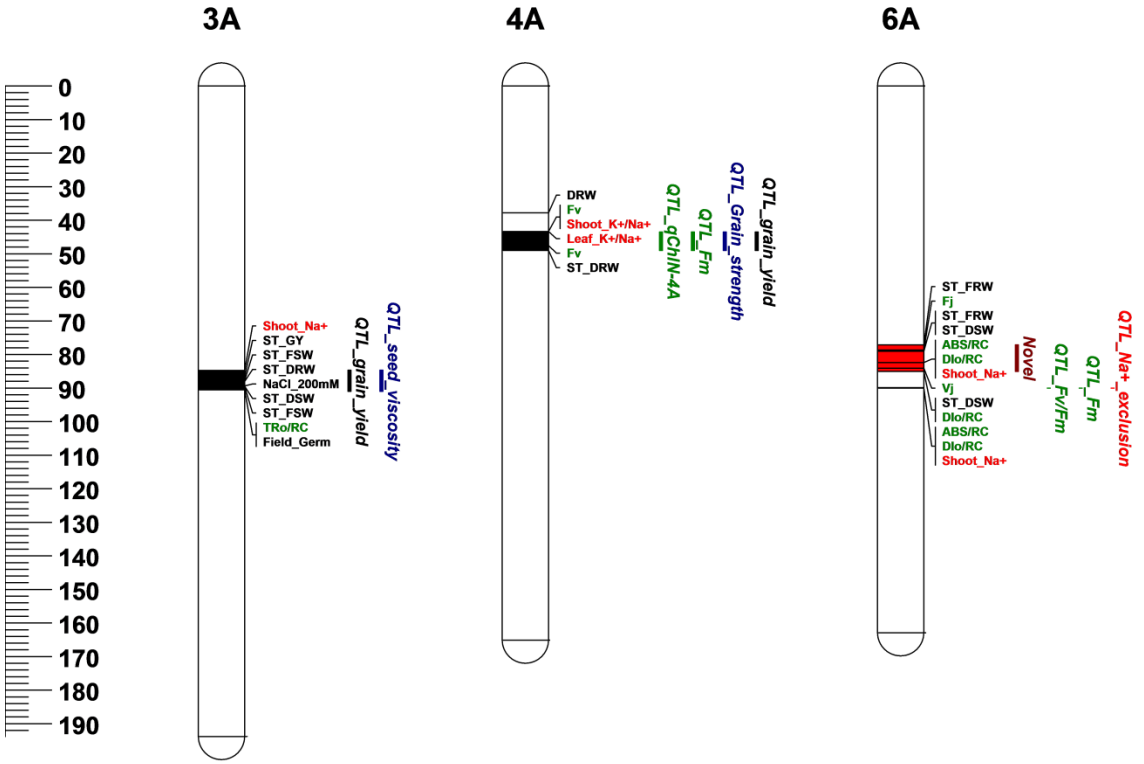

Figure 1 Summary of major QTLs regions (in LD) detected in both chapter 3 and 4 association studies and their characteristics (associated traits and predicted genes) that be used for salt tolerance improvement.

Relationships among some of the studied traits

Our study identified a positive correlation between some of the ChlF traits [Fv, Fv/Fo, Fv/Fm, *TRo/RC*, Eto/RC and PI(ABS)] and shoot K^+ under saline conditions, which suggests that stomatal movement, energy transfer and photosynthetic activities in plant under saline conditions would depend on the K^+ uptake (Marschner *et al*., 2012). The seed starch content was also found to be positively correlated with Fo, *ABS/RC*, *TRo/RC* and *DIo/RC* but negatively correlated with *Fm/Fo*, *Fv/Fm*, *ETo/RC* and PI(ABS). The correlated traits are of interest because of the following three reasons: (i) to connect the genetic causes of correlation through the pleiotropic action of genes, (ii) to know how selection for one character will cause a simultaneous change in other characters, and (iii) to determine the relationship between traits and fitness (Sandhu *et al*., 2013). Establishing the coincidence of QTL for (morpho-) physiologically related traits provide powerful evidence for causal relationships amongst traits (Prioul *et al*., 1997). The high correlations observed for ChlF traits with the ion content and seed quality trait was also reflected in the co-localization of QTL with traits due to the effect of pleiotropy or very close linkage of genes. Therefore, our studies have shown that the ChlF techniques can be used for early detection of stress symptoms induced by salt stress among wheat genotypes; and thus, could be utilized as reliable screening criteria for evaluation of salt tolerance when screening for salt tolerance in large wheat population, and has been proposed by Li *et al*. (2014) as an indicator of stress responses in plant.

Expression analysis

Chapter 3 and 4 also describe some salt-responsive and specific gene expression of some of the candidate genes predicted to be controlling the observed genetic variation for salt tolerance in the studied panel. By using the PCoA, we have shown that the identified polymorphisms were able to discriminate against most of the identified salt tolerant genotypes from the salt sensitive ones, an indicative of the regulatory involvement of the SNPs in the salt tolerance mechanisms in wheat. Thus, having insight into the regulatory networks of the genes that are co-segregating with the identified polymorphisms would help us to know which genetic pathways and mechanisms each of the polymorphism is involved in and thus, may elaborate further the functional relevance of such locus in offering salt tolerance in wheat. In general, the genes that are involved in the stress/defense related (24%) activity along with those regulating the antiport and transmembrane (18%), transcription and translation (14%) and redox homoeostasis and detoxification (11%), repair/protection/cell wall modification (7%), carbohydrate/sucrose metabolism (7%), plant

hormone/osmo-protectant (6%), photosynthesis (4%) activities, etc at stressed condition were detected (**Figure 2**). The genes that are identified here may be quantitatively regulating salt stress and, from the PCoA analysis, it is evident that the tolerant genotypes may have higher number of these useful alleles that enabled them to cope and sustain growth under the applied salt treatment conditions, as has been observed in our previous report (Oyiga *et al*., 2016). Our findings would help to explain the potential fundamental mechanisms of salinity tolerance active in the diverse natural genetic background of wheat. The identified candidate genes can also be considered partly as the product of the genetic variation among all the genetic blocks existing within the studied panel, since we have analyzed all the genotypes simultaneously. Therefore, we took this into account by using only the salt tolerant and salt sensitive wheat genotypes identified in the population to identify the differentially expressed transcripts/genes due to salt treatment, which is described in the next section.

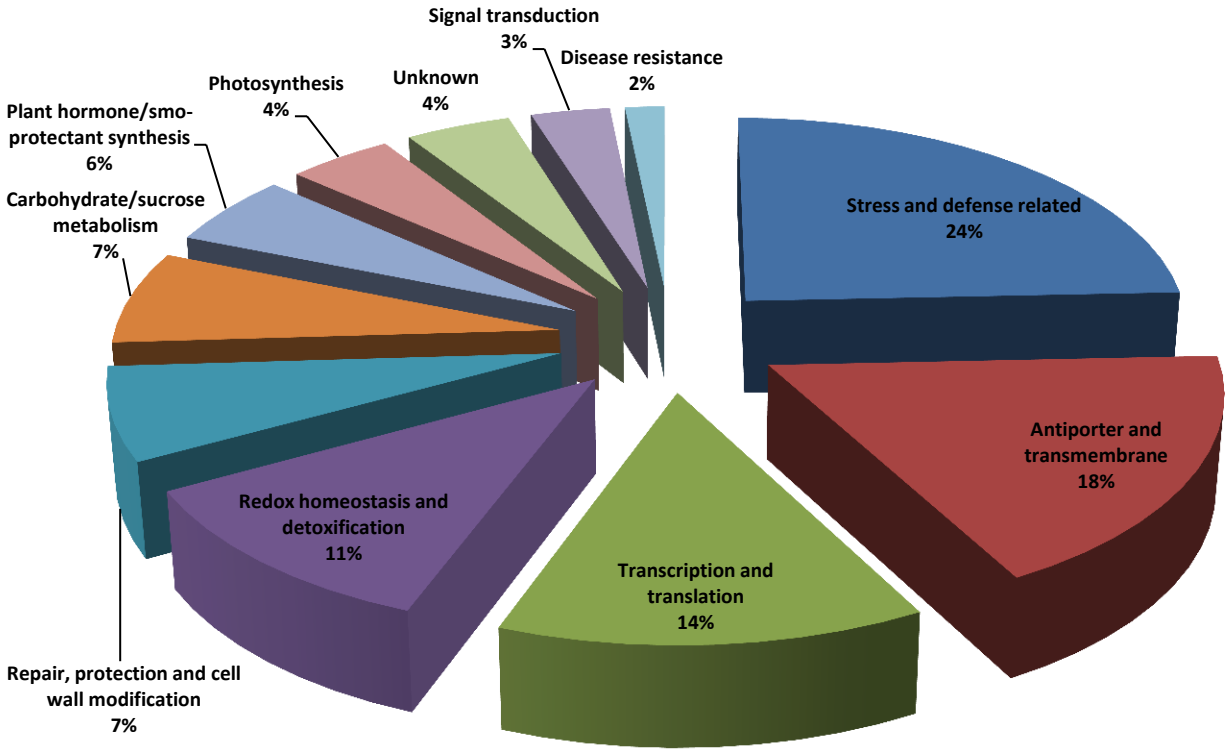

Figure 2 Functional analyses of the associated SNPs identified in the GWAS studies in Chapter 3 and 4

Differentially expressed transcripts

The salt tolerant wheat genotypes used for this investigation were chosen accordingly based on our previous salt screening study (Oyiga et al., 2016). The comparative gene expression study involving some of the identified genotypes was necessary in other to verify the tolerance status of some of the identified genotypes. The results revealed that all the analyzed genes were differentially expressed between the contrasting wheat genotypes with their expressions mostly favoring the tolerant wheat genotypes, suggesting that both genotypes are genetically different in terms of allele constitution for salt tolerance. 46 out of the 50 candidate genes analyzed were found to be highly expressed in the tolerant genotypes and were mostly down-regulated in the sensitive genotypes. Reports in wheat (Aprile *et al*. 2009; Liu *et al*. 2012) and barley (Ueda *et al*. 2006; Talamè *et al*. 2007) have shown that numerous genes are involved in abiotic stresses tolerance mechanisms. The functional roles of these differentially expressed candidate genes have also been linked to salinity tolerance mechanism, as has also been discussed in the previous Chapters. Thus, one may conclude that the tolerant genotypes are better prepared to overcome the salt stress vis-à-vis the number of stress responsive genes that were over-expressed compared with the sensitive genotypes. The expressions of 6 candidate genes identified in this study were also validated by RT-PCR.

Concluding Remarks

The association mapping technology highly increased the power of detection and mapping resolution. The genotypes and QTLs identified in this study suggested the involvement of salt stress tolerance genes to be of importance for salt tolerance breeding. Therefore, our specific conclusions are:

I. There is an extensive genetic variation for salt tolerance in the studied germplasm that can be exploited for wheat improvement. Among the most consistent extreme salt tolerant wheat genotypes identified across growth stages in this study, *Altay2000*, *14IWWYTIR-19* and *UZ-11CWA-8* (salt tolerant), and Bobur (salt sensitive) identified across the three growth stages. The tolerant genotypes - *Altay2000* and *UZ-11CWA-8*, exhibited higher capacity for Na^+ exclusion, higher K^+ inclusion, lower osmotic potential, higher membrane stability and optimal photochemical activities under high salinity than the sensitive genotypes - *Bobur* and *UZ-11CWA-24*. Further genetic and molecular analyses of these extreme genotypes indicate that the tolerant genotypes contain higher number of positive alleles that are quantitatively regulating salt stress tolerance in their favor than the salt sensitive genotypes. We therefore

recommend these genotypes for further genetic studies for wheat improvement and breeding program.

II. GWAS with 90K SNPs is able to unravel the genetic architecture for salt tolerance using some growth, physiological and seed quality salt-related traits in wheat. The gene ontology analysis of the associated polymorphisms revealed that the plausible candidate genes linked to the detected polymorphisms are involved in salt tolerance. The identified candidate genes were categorized according to the biological processes (BPs) and molecular functions (MFs) of which the genes that were associated with BPs (i.e., stress response, signaling and signaling process) and MFs (i.e., antiporter/transporter activity, transcription factor, transcription regulator activity and antioxidant activity) have been discussed in Chapter 3 and 4. The genes identified here provide a picture of the complex and quantitative nature of salinity response in wheat with new insights into the mechanisms that are active in the wide natural variation of wheat genotypes under salinity stress.

III. In general, out of the 50 candidate gene transcripts analyzed upon exposure to salt stress using MACE microarray, 46 of them were uniquely up-regulated in tolerant genotypes and/or are commonly up-regulated in both tolerant and susceptible genotypes (but at a higher rate in tolerant genotypes). For example, among the uniquely up-regulated genes in tolerant genotypes, 12 genes are involved in antiport/transporter activity, 8 genes are involved in response to stress and 10 genes are involved in antioxidant/ROS savaging activities and thus providing the strongest candidates for salinity tolerance in wheat.

    a. Among the genes involved in antiport/transporter activity, the most highly induced gene is Q5Z653 (increased by713.98% in *Atlay2000* and decreased by 22.19% in *Bobur*) encoding a '*Zinc transporter* 7' on the novel QTL region of 1BS, which was also validated with the RT-PCR and were found in QTL region conferring all growth stage salt tolerance in the studied panel. Another important transporter gene whose expression was validated by RT-qPCR is in this study is *Myosin-J heavy chain* (increased by 198.03% in *Atlay2000* and increased by 32.94% in *Bobur*) and was associated with shoot Na^+, Dio/RC, and ABC/RC at 90.04 cM on 6AL.
    b. Among the genes involved in antioxidant/ROS savaging activities is the gene encoding for *Glutathione S-transferase* (increased by ~250% in *Atlay2000* and increased by ~80% in *Bobur*) located at 101.92 cM on 2AL and was strongly

associated with agronomic, leaf fluorescence and shoot K^+ /Na^+ (ion homeostasis-related) traits. The antioxidant NADH dehydrogenase complex (plastoquinone) assembly associated with metal transporter showed 321.52% increase in *Atlay2000* and -65.78% decrease in *Bobur*.

IV. The candidate genes were also assessed in terms of whether they are located within previously reported salt stress related QTL. Among the uniquely up-regulated genes in the tolerant genotypes, three were found in QTL region previously described for salt tolerance genes.

   a. The *glutathione-regulated potassium-efflux system protein* (kefC) (increased by ~ 60% in *Atlay2000* and decreased by ~ 30 in *Bobur*) associated with dry shoot ST related trait in this study was found on 2AL region that has been described to carry Na^+ exclusion gene controlling biomass (Lindsay *et al*., 2004; Huang *et al*., 2006; Genc et al., 2010).
   b. An *ABC transporter F family member 3* found on 6AL region (detected with the shoot Na^+ trait) that has reported also been reported for three wheat plasma membrane transporters (Pearce *et al*., 2014).
   c. Lastly, Morgan (1991) and Morgan and Tan (1996) reported osmoregulation gene regions on 7AS. We detected a gene fructan *6-fructosyltransferase* (*6-SFT)* that functions as osmo-protectants synthesis, anti-oxidation and membrane stability (Valluru and Van den Ende, 2008; He *et al*., 2015) in the same region of 7AS. The expressions of kefC and 6-SFT were also validated using RT-qPCR.

V. A novel QTL associated with leaf K^+ content and whose sequence was highly homologous to O13726 (increased by ~ 90% in *Atlay2000* and decreased by ~ 10% in *Bobur*) encoding an uncharacterized *Na^+/H^+ antiporter protein* was also located on 1DL.

VI. Finally, the amino acid sequence analyses of the putative candidate genes identified non-synonymous substitutions between the contrasting salt tolerant wheat genotypes at the associated gene coding regions, suggesting that the detected SNPs are linked to salt-responsive genes that can be utilized for future genetic studies and salt tolerance improvement in Wheat.

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LIST OF PUBLICATION

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MANUSCRIPT UNDER PREPARATION

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