Genetic Studies of Salinity Tolerance in Wheat

By

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B.Sc., M.Sc.

A thesis submitted for the degree of Doctor of Philosophy

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Discipline of Plant Breeding and Genetics

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THE UNIVERSITY of ADELAIDE

March 2013

Table of Contents

Abstract

Salinity is an important issue in arid and semi-arid regions of the world, both in irrigated and dryland agriculture. Increasing salinity tolerance of crops is a feasible approach to tackling salinity. Focusing on the physiological traits associated with salinity tolerance such as Na^+ exclusion and osmotic stress tolerance simplifies the strategies for improving tolerance.

The first aim of the study described in this thesis was the development of a high throughput technique for the measurement of osmotic stress tolerance in bread wheat. This technique was then applied to 162 recombinant inbred lines derived from crossing two Australian bread wheat cultivars (Gladius and Drysdale), to identify the loci associated with osmotic stress tolerance and 4th leaf Na^+ accumulation. This population was grown under two growth conditions – a pot-soil set-up with non-destructive imaging system (LemnaTec Scanalyzer 3D technology) for the estimation of osmotic stress tolerance using high through-put system (conveyor belt system) and a supported hydroponics set-up for 4th leaf Na^+ and 4th leaf K^+ accumulation measurements. In the soil based study, QTL analyses revealed two major QTL on the distal regions of the short arms of chromosomes 2B and 1B, where the salinity tolerance index (shoot biomass in saline conditions relative to shoot biomass in control conditions) and osmotic stress tolerance overlapped. Another significant QTL for osmotic stress tolerance was mapped onto the distal region of the long arm of chromosome 5D. In the hydroponics study, two QTL associated with 4th leaf Na^+ accumulation were mapped to the distal regions of the long arms of chromosomes 1D and 3B. Loci containing a vernalisation gene (*VRN-A1*), on the long arm of chromosome 5A, and a photoperiod gene (*Ppd-D1*), on the short arm of chromosome 2D, had an impact on tiller number, shoot biomass and shoot water content in salt and control conditions.

The second aim of the research program was to study the genetics of Na^+ exclusion in two Afghani durum wheat landraces, which accumulated half the amount of 3rd leaf Na^+ compared to Australian commercial durum wheat cultivars. These landraces were crossed with an Australian durum wheat (cv Jandaroi) and F_2 populations were developed. The parents and F_2 population were grown in a supported hydroponics system at 100 mM NaCl, and the Na^+ and K^+ concentrations in the third leaf was measured after ten days growth in salt. Selective genotyping analysis using DArT markers and bulked segregant analysis (BSA) using SNP markers were carried out to detect the putative genomic regions responsible for salinity tolerance. Both analyses revealed a locus on the distal region of the long arm of chromosome 4B associated with Na^+ and K^+ accumulation and the ratio of K^+/Na^+ in the third leaf; the favourable allele derived from the Afghani landraces. BSA identified another locus on the distal region of the long arm of chromosome 3B, associated only with 3rd leaf Na^+ accumulation and the favourable allele was inherited from Jandaroi. These loci on chromosomes 3B and 4B were validated in the entire F_2 population and marker regression analysis showed that both have a significant association with 3rd leaf Na^+ accumulation.

The putative genomic loci identified in this thesis can be validated further and these would lead to the identification of genes and the development of markers to facilitate the breeding of salt tolerant wheat cultivars.

Declaration

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Nawar Shamaya

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Acknowledgement

and support in measuring field capacity of soil and to Anzu Okada for the time to train and help me to carry out multiplex PCR for durum wheat project.

I would like to acknowledge Joanne Tilbrook, Ruth Harris and Christina Morris for their help, support and time in both editing my thesis and improving my English.

For providing words of wisdom, support and an enjoyable laboratory experience I would like to thank the salt research group at ACPFG and specially group leader Stuart Roy and Joanne Tilbrook, Melissa Pickering, Jan Nield. For laboratory and scientific help, guidance, friendship, generous assistance and unwavering support, I would like to acknowledge all ACPFG staff members. Many thanks to the administrative staff for their help and laughter, in particular to Ruth Harris, Monica Ogierman, Ann Pace, Andrea French and David Harris.

Also I want to say thanks to all the PhD students at ACPFG in particular I would like to thank Gordon Wellman and his girlfriend Sami for their great friendship and support.

Finally, but most importantly, I would like to thank my family and friends for their help and support over the four years I have been away from home. Thanks to my parents for their support over my many years of schooling and for always believing that I could get through it all! Thanks to my brothers, Bashar and Hazar for their help, support and gentle pressure. Also I want to give thanks to my friends specially Ismail for his words of wisdom.

List of figures

IX

List of tables

Chapter 1: General introduction

1.1 Introduction

Wheat is a major cereal crop which is grown throughout the world on both irrigated and rain-fed land. However, much of the arable land worldwide is affected by salinity, leading to significant limitations on wheat production. One approach to tackling this problem is to increase the salinity tolerance of wheat cultivars (Munns et al. 2006).

It has been shown that Na^+ exclusion and osmotic stress tolerance are associated with salt tolerance in plants, and these traits have been used successfully in the improvement of salt tolerance in maize (Schubert et al. 2009). An assay for osmotic stress tolerance in wheat has been developed and used in physiological studies (Rajendran et al. 2009). However, this assay has not been used to study the genetics underpinning osmotic stress tolerance in wheat due to the lack of a high through-put technology. The use of an osmotic stress tolerance assay and measurement of Na^+ exclusion in segregating populations in conjunction with the use of molecular markers will allow the genetic loci associated with these traits to be determined. This will enable us to identify the genes and to develop markers for use in selecting for salinity tolerance in wheat breeding programmes, leading to an improvement of wheat yield under saline conditions.

1.2 Literature Review

1.2.1 Salinity

Salinity is an important and serious problem for agricultural production in many regions. Salinity can increase rapidly in soil and adapting to this increase is challenging for plants (Munns 2002; Munns and Tester 2008). The main strategies used to combat salinity in cultivated land are land management and the development of salt tolerant crops (Flowers and Yeo 1995).

According to the UN Food and Agricultural Organisation (FAO 2008), over 800 million ha of land word-wide (which accounts for only 6% of the total land area of the world) are influenced by saline soil. Salinity affects approximately 32 million ha of the 1,500 million ha farmed in dryland agriculture. Of the 230 million ha of irrigated land, 45 million ha are salt affected (FAO 2008). Even though only 15% of the total cultivated area is irrigated, its production is twice that of dryland agriculture (Munns and Tester 2008). Consequently, the losses of yield due to the increase in soil salinisation in irrigated areas will have a disproportionately large effect.

It is predicted that salinity will continue to increase. For example, in Australia, salinity will affect 17 million ha of Australian agricultural land by 2050 (Rengasamy 2006). Clearly, salt affected land has an economic impact and significantly affects food production. It is estimated by the Australian Natural Resources Atlas (ANRA 2007) that the economic loss of agricultural productivity due to salinity ranges between $800 - $1090 for every ha of Australian land. It is estimated that salinity costs the Australian economy in the range of $130 M to $300 M per annum (Rengasamy 2002).

1.2.1.1 What is salinity?

Salinity is the increase of water-soluble salts in soil solution to a level at which plants start to suffer from the increase in external osmotic pressure outside roots. This causes a reduction in water availability to plant roots and a decrease in growth rate of plants. Another problem of salinity is a toxic effect which causes senescence of old leaves and the death of these leaves. Both salinity effects (external pressure outside roots and toxic effect) leads to a reduction in biomass and yield (Munns and Tester 2008). Plants start to show the effects of salt when the salinity increases above 4 dS/m in soil (United States Salinity Laboratory Staff 1954). A survey conducted in California revealed a decrease of 10% in plant yield as salinity rose by 1 dS/m (Rozema and Flowers 2008). Salinisation (the increase of salts such as sodium, chloride, calcium, magnesium and sulphate) in cultivated lands is caused by primary sources of salinity (which are natural processes, including water consumption by plants and evaporation) and secondary sources of salinity (processes caused by humans, land clearing and irrigation) (http://www.biosalinity.org). In some regions where the ground water may be saline, a rising water table can bring salt to the surface. Sea water flooding or seepage into the ground water can also increase salinity of the soil. These are examples of primary salinity (Rengasamy 2006). Another cause of rising salinisation is human intervention. The expansion of agricultural lands through clearing of native growth to remove deep-rooted vegetation and replacing this with shallow-rooted crops, followed by extensive irrigation, can cause the movement of ground water bringing salt to the surface. The application of certain chemical fertilisers and soil amendments can also lead to an increase in salinity. This kind of salinisation is referred to as secondary salinity (Rengasamy 2006).

Rengasamy (2006) identifies three types of salinity. The first are saline soils which are characterised by loose and sandy soils. This kind of soil contains high levels of water-soluble salts such as sodium, chloride, calcium, magnesium and sulphate. The electro-conductivity

(EC) of saline soils can exceed 4 dS /m (Rengasamy 2006), equivalent to 40 mM NaCl with an osmotic pressure of 0.2 MP (Munns and Tester 2008), and an exchangeable sodium percentage (ESP) of less than 15% with a pH below 8.5. The second are sodic soils which have low soluble salts content with an EC below 4 dS/m, ESP greater than 15% and a pH over 8.5. These soils can be defined as dense with a surface that appears crusty because they contain high concentrations of insoluble sodium carbonate and bicarbonate. The third are saline-sodic soils which account for a large proportion of soil in arid and semi-arid lands. These soils have an EC of over 4 dS/m, greater than 15% ESP, and a pH below 8.5 (Rengasamy 2006).

1.2.1.2 Phases of plant response and tolerance to salinity

1.2.1.2.1 First phase - osmotic effect

The phase of salt stress which can be observed immediately after plants are exposed to salinity is the osmotic effect. Munns and Tester (2008) suggested that osmotic stress affects plant growth by changing the water potential between the outside and inside of the plant cells. When salt level increases outside plant roots, water potential of soil will decline and the osmotic pressure will raise, this leads to a decrease of the amount of water entering the root and consequently causes a decrease in the transportation of water to the shoot. The decrease in water uptake causes slower growth. When soil becomes saline in early plant growth stages, the rate of leaf expansion will reduce and new leaves will emerge very slowly. Also, at this developmental stage, buds will grow slowly or stay quiescent leading to a decrease in the number of branches. All these plant growth responses result from the osmotic stress induced by the increase of salt outside the roots.

In cereals, the osmotic effect is evident in the reduction of the total leaf area and this leads to a decrease in the number of tillers. The salt stress also leads to death of old leaves and a decrease in the growth rate of young leaves. Although these effects are caused by osmotic

stress, the mechanism of this effect on shoot growth is unknown. At the flowering stage, the number of florets decreases and flowering will be early (Munns and Tester 2008).

An increase in stomatal closure is another plant response to salinity and this response is undoubtedly caused by the osmotic effect (Fricke et al. 2004; Munns and Tester 2008). Fricke et al. (2004) found a reduction in stomatal conductance in barley plants occurred immediately after salt application. They explained that when salt increased outside roots of barley plants, the water potential decline and this caused changed water relations and the production of ABA in the barley plants. Within 10 minutes of applying 100mM NaCl to barley, the ABA concentration rose in the photosynthetic tissues (Fricke et al. 2004; Fricke et al. 2006). Also, Davies et al. (2005) showed that when a plant grows in dry conditions, the plant roots will send information to the shoot in order to regulate the plant growth and development. This arises when ABA biosynthesis increases in root cells and transports from the root to the shoot via xylem vessel to regulate stomatal conductance. In saline conditions, the plant attempts to retain water by closing the stomata (Termaat et al. 1985).

1.2.1.2.2 Second phase - ion-specific effect

The ion-specific effect commences when salt accumulates in old leaves to a level that causes cells to die (Munns et al. 2006; Munns and Tester 2008). NaCl is the most common salt affecting plant growth. In this context, Na^+ will be the focus as it is the primary cause of ion-specific damage for most crops (Tester and Davenport 2003). Na^+ accumulates to higher level in leaves than in roots. The high concentration of leaf Na^+ causes a metabolic problem for plants and this is largely a result of metabolic toxicity of Na^+ which its ability to compete with K^+ at cellular level. K^+ is a co-factor which has a role in the activity of more than fifty enzymes in the cytoplasm of leaf cells, and when the concentration of K^+ declines significantly, most of these enzymes will be inhibited (Munns et al. 2006; Tester and Davenport 2003). Moreover, a high concentration of K^+ is necessary for protein synthesis (Blaha et al. 2000; Wyn Jones et al. 1979). In salt sensitive plants, or plants growing under

saline conditions, competition occurs between the Na^+ and K^+ ions at cellular level as mention above. Consequently, when Na^+ increases in the leaf, the activity of many enzymes in leaf cell is inhibited and that leading to dead leaves (Tester and Davenport 2003). In addition, when the number of old dead leaves is larger than the number of young leaves the growth rate of plants will be reduced because of the lack of photosynthetic ability (Munns and Tester 2008). Recent studies have focused on Na^+ exclusion (Ashraf and O'Leary 1996; McDonald et al. 2012; Munns et al. 2000; Poustini and Siosemardeh 2004; Rashid et al. 1999) and Na^+ tissue tolerance (Genc et al. 2007; Rajendran et al. 2009) since the toxic concentration of Na^+ in the shoot is more significant than Cl^-. However, Cl^- is more toxic in some crops, such as soybean, citrus and grapevine (Läuchli and Lüttge 2002; Munns and Tester 2008; Storey and Walker 1998).

1.2.1.3 Key conclusions

Salinity has a major impact on agricultural production which in turn affects food security. Salt affects plants in two phases; osmotic and ionic. Figure 1 illustrates the immediate effect of osmotic stress on the plant growth rate after salt application and this effect is greater than the ionic effect, especially in levels of moderate and low salinity (Munns and Tester 2008). Ion-specific stress shows its influence on growth later and is important at high levels of salt or when the plants are sensitive to salinity and cannot control Na^+ transport (Tester and Davenport 2003).
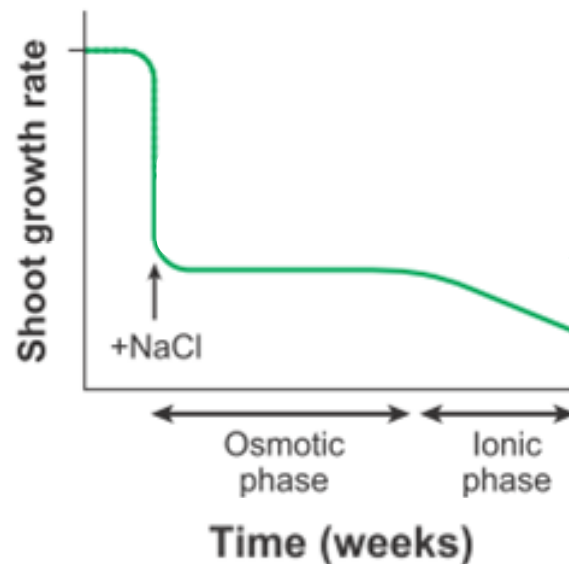
Figure 1. The change in the growth rate after application of NaCl. The two phases of the response to salinity of shoot growth: osmotic stress (rapid response to salinity) and ion-specific phase (slower response to salinity after accumulation of Na$^+$ in the shoot) (Munns and Tester 2008).

1.2.2 Physiological traits associated with salinity tolerance in plants

In order to improve the salinity tolerance in crops, the basis for salinity tolerance needs to be understood. The physiology and molecular biology of salinity tolerance have been studied and there are three physiological traits which enable the plant to adapt to this stress: osmotic stress tolerance, Na$^+$ exclusion, and Na$^+$ tissue tolerance.

1.2.2.1 Osmotic stress tolerance

Osmotic stress induced by salt, reduces the plant growth rate and stomatal conductance immediately after salt application. Plants which can tolerate osmotic stress are able to maintain stomatal conductance and maintain growth as if they were in a non-saline environment (James et al. 2008; Munns and Tester 2008; Rajendran et al. 2009). This is termed osmotic stress tolerance. Growth rate and stomatal conductance of plants have been used to measure the osmotic effect. The mechanisms associated with osmotic stress tolerance

are poorly understood but several studies have been carried out in an effort to explain the osmotic stress tolerance, as reviewed by Munns and Tester (2008).

1.2.2.1.1 Growth rate

The change in growth rate occurs before the build-up of salt (Na^+ and Cl^- which cause ion toxicity) in the leaf and shoot tissue (Fricke et al. 2004; Hu et al. 2007; Hu et al. 2005; Neves-Piestun and Bernstein 2005). Cramer and Bowman (1991) studied the effect of NaCl on the 3rd leaf elongation of maize (leaf elongation is function of the rate of cell production and expansion) and found that the reason for the change in 3rd leaf elongation was an alteration in cell wall properties of 3rd leaf which occurred when the plant is exposed to salinity. However, the change in wall properties is related to cellular and long-distance signalling, although this requires further study and confirmation (Munns and Tester 2008). The signalling which regulates the slow growth rate in plants under stress is controlled by growth regulators such as abscisic acid (ABA). ABA is important in drought responses and leads to reduced growth rate and stomatal conductance. Hartung et al. (1999) showed that when maize plants were exposed to drought conditions, ABA concentration increased in root cells. Davies et al. (2005); Zhu (2002) also showed that when the plants of most crops grown in drought conditions, ABA increase in roots and sends information to the shoot in order to regulate the plant growth and development. However, in barley and mutants of maize and tomato, ABA does not change significantly under saline conditions even though growth is reduced (Fricke et al. 2004; Mäkelä et al. 2003; Voisin et al. 2006). Gibberellins (GAs) are another growth regulator and are related to the leaf elongation. This regulator is inhibited by ABA and slow leaf growth may be due to the effects of ABA (Munns and Tester 2008). The DELLA proteins (which are members of a class of negative regulators of growth) mediate the effect of GAs and also integrate with the signalling induced in salt stress, so it may be that these proteins play a central role in plant adaption in abiotic environments (Achard et al. 2006).

After a plant was exposed to NaCl or other osmotica, it was observed that there was less effect on root elongation than on the growth rate of leaves (Munns 2002). Root elongation can

recover rapidly compared with the slower recovery of leaf growth. Reduced initiation of new seminal or lateral roots may occur but this requires further study (Munns and Tester 2008).

1.2.2.1.2 Stomatal conductance

The other immediate alteration which occurs when the salt increases outside the root is a decrease in stomatal aperture. This is related to the osmotic stress induced by salt (Munns and Tester 2008) and is due to the perturbed water status in the plant and the increase of ABA in the shoot. However, one study suggests that the change in water status does not cause the decrease in stomatal conductance. Termaat et al. (1985) found that plants treated with salt while the water status was kept high by applying a balance pressure to the rooting medium, still showed a decrease in stomatal conductance. Fricke et al. (2004); Fricke et al. (2006) found that 10 minutes after adding 100 mM NaCl to growth solution where barley plants were grown in a hydroponic system, the level of ABA in the photosynthetic 3rd leaf tissue suddenly increased. These studies suggest that local ABA may have an effect on stomatal conductance. However, Fricke et al. (2004); Fricke et al. (2006) demonstrated that although the concentration of ABA returned to control levels, the transpiration rate continued to decline. Munns and Tester (2008) assume that the change in stomatal conductance induced by salt is due to root signals, as Davies et al. (2005) showed that root signals control the stomata when the plant is grown under drought conditions.

1.2.2.1.3 Measurement of osmotic stress tolerance

Munns and Tester (2008) showed the relationship between the phases of salt stress and shoot growth (Figure 2). The shoot growth rate of two plants affected by osmotic stress reveals a difference in tolerance at the osmotic phase. Genetic variation in osmotic stress tolerance within a species was demonstrated in *Triticum monococcum* by Rajendran et al. (2009), who showed that the growth rate of accession AUS-90436 did not change immediately after exposure to salinity. Also, James et al. (2008) showed that there were genetic differences in the stomatal conductance among 50 lines of durum wheat in response to the osmotic effects induced by salinity. The variation in osmotic stress tolerance in plants can be used to explore

the genetics underlying this variation. Several methods of measuring the variation of osmotic

stress tolerance have been developed.
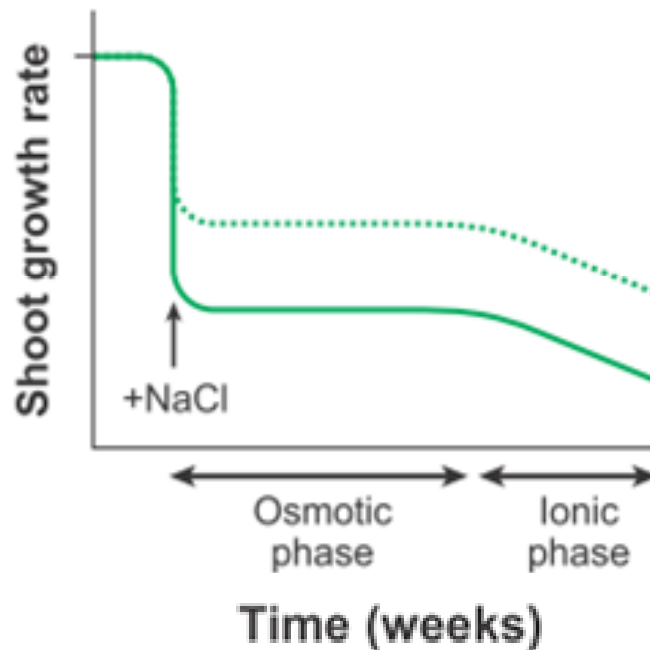
Figure 2. Increase in osmotic stress tolerance. The change in the growth rate after application of NaCl is denoted by the solid green line. The dashed green line shows the theoretical response of a plant with reduced sensitivity to the osmotic stress induced by salinity (Munns and Tester 2008).

James et al. (2008) used a steady-state porometer to measure the change in stomatal

conductance in leaves of 50 durum wheat genotypes immediately after salt application. They

found a positive relationship between stomatal conductance and relative growth rate, rate of

photosynthesis, and the CO_2 assimilation rate. It was also found that there is variation in

stomatal conductance between durum wheat genotypes in response to salinity. However, use

of the steady-state porometer has some limitations and Sirault et al. (2009) identified three

particular drawbacks. The differences in stomatal conductance between genotypes are small,

thus resulting in low confidence in the measurements. Also, in order to obtain reliable results,

many readings are needed per plant and genotype. Moreover, it was found that at low salt

concentrations the steady-state porometer has a low signal-to-noise ratio and significantly

interferes with the natural behaviour of the leaf. Another measurement technique related to

stomatal conductance and transpiration efficiency is Carbon Isotope Discrimination (CID) (Condon 2004). CID has been used successfully as an indicator of transpiration efficiency, and has been used in wheat breeding programmes to improving water-use efficiency. However, CID is expensive: US$30 per sample (Furbank and Tester 2011).

Leaf temperature differs with transpiration rate which is a function of stomatal conductance (Tanner 1963; Fuchs 1990). Infrared thermography measures leaf temperature or canopy temperature. Jones (1999) found a correlation between infrared thermography of stomatal conductance and estimates obtained using a diffusion porometer, areas of high temperature reflect stomatal closure and areas of low temperature reflect stomatal opening. Infrared thermography was used successfully to study the change in leaf temperature of barley subjected to three different salt treatments, and seventeen durum wheat genotypes at two different salt concentrations (Sirault et al. 2009). Leaf temperature increased with increasing salt concentration, and salt intolerant durum genotypes had a higher temperature than salt tolerant genotypes.

Observations of plant growth over time provide another method of measuring the osmotic stress induced by salinity. Evaluation of growth rates has involved harvesting plants at particular time points during growth and calculating the relative growth rate.

Non-destructive imaging over time using the LemnaTec Scanalyzer 3D was performed on the shoots of twelve *Triticum monococcum* accessions grown in 0 mM and 75 mM NaCl in a supported hydroponics system (Rajendran et al. 2009). Images were taken daily without the need for harvesting the plants and the growth rate of these accessions was calculated. This method has clear advantages over destructive measurements. Non-destructive imaging allows making multiple observation of the same plant over experimental period and the plant of interest can be taken through to seed. Also, this technology allows osmotic stress tolerance and Na^+ exclusion to be measured for the same plant. Rajendran et al. (2009) compared the

projected shoot area derived from three images at different plant angles with the shoot fresh biomass for the same plants harvested and measured with a physical balance. The linear correlation between the two methods was over 0.90, indicating that imaging the projected shoot area was a reliable way of measuring plant growth. Rajendran et al. (2009) measured the growth rate for four days after salt application and found the rate of growth in the salt treatment to be significantly lower than in control conditions. Also, the variation in growth rate between the accessions was significant and appeared to be heritable.

Rajendran et al. (2009) used a manually operated imaging system with plants grown in a supported hydroponics system. A high through-put imaging system is available through The Plant Accelerator, Waite Campus, University of Adelaide, South Australia, Australia. This uses a conveyor belt to move the plants automatically to the LemnaTec Scanalyzer 3D imaging station and watering is controlled and at defined intervals. Salt can be added to soil when the plant is at the three or four leaf stage to enable the measurement of plant growth rate immediately after salt application.

1.2.2.2 Na$^+$ exclusion

The leaf blade is the main site of Na$^+$ toxicity for most plants (Tester and Davenport 2003). Munns et al. (2006) suggested that around 50 times more water is transpired by plants than is retained in leaves, leaving Na$^+$ in the leaf tissue. Therefore exclusion of Na$^+$ from the leaf blades is vital. In order to exclude Na$^+$ from leaves, plants need to control the net uptake of Na$^+$ through the roots, and the net transport of Na$^+$ between the root and the shoot. Very small amounts of Na$^+$ can recirculate from the shoot to the root in the phloem, but the amount is too small to help plants exclude Na$^+$ from the shoot (Munns and Tester 2008).

According to Tester and Davenport (2003), there are four distinct components of Na$^+$ net delivery from the soil through the root to the xylem:

1. Influx of Na$^+$ from the soil into xylem parenchyma cells.

2. Efflux of Na^+ back to the soil solution via the root.

3. Efflux of Na^+ from xylem parenchyma cells to the xylem apoplast.

4. Influx of Na^+ from the xylem apoplast back into the xylem parenchyma cells.

The Na^+ influx into the outer half of the root (root cortex) can be mediated either by voltage-independent non-selective cation channels (Amtmann and Sanders 1999; Tester and Davenport 2003) or by Na^+ transporters encoded by some members of the high-affinity K^+ transporter (*HKT*) gene family (Haro et al. 2005; Laurie et al. 2002). The characteristics of genes that regulate the non-selective cation channels are unknown even though there are a number of candidates (Munns and Tester 2008).

A large amount of the Na^+ is pumped from the root cells across the plasma membrane via a Na^+/H^+ antiporter, back into the soil solution (Demidchik et al. 2002). Läuchli et al. (2008) suggested that the efflux of Na^+ out of the root cells could be mediated by a *SOS1* gene which encodes a protein similar to the plasma membrane Na^+/H^+ antiporter. A large family of *CHX* genes may also play an important role in Na^+ efflux out of the root cells (Pardo et al. 2006). Na^+ which remains in the root could be compartmentalised in vacuoles or delivered to the leaves. Na^+ can be sequestered into vacuoles by tonoplast Na^+/H^+ antiporters such as the Na/H exchanger (*NHX*) family in *Arabidopsis* (Pardo et al. 2006).

Using X-ray microanalysis of the root, Läuchli et al. (2008) found that the Na^+ concentration in the roots of two durum wheat varieties reduced across the root cortex from a high concentration in the epidermis to a low concentration in the endodermis. These results suggest that the epidermis and the sub-epidermis exclude a significant amount of the Na^+ from the transpiration stream as it flows through the root to the endodermis, and the movement of Na^+ is not controlled at the endodermis. The epidermis and outer cortical cells may be removing Na^+ from the cell wall and sequestering it into vacuoles (Läuchli et al. 2008).

1.2.2.2.1 Na⁺ exclusion in durum wheat

Low Na^+ accumulation (Na^+ exclusion) with a larger discrimination of K^+ to Na^+ in the shoot was identified as one of the major mechanisms associated with salt tolerance in *Triticeae* (Gorham et al. 1987). Durum wheat cultivars are more sensitive to salinity than bread wheat because they accumulate more Na^+ in the shoot (Francois et al. 1986; Maas and Grieve 1990). Figure 3 illustrates the differences in ionic stress tolerance between ionic intolerant and tolerant genotypes. An improvement in ionic tolerance would result in an improvement in the plant growth rate (Munns and Tester 2008). Several attempts have been made to improve Na^+ exclusion and the ratio of K^+/Na^+ in durum wheat.
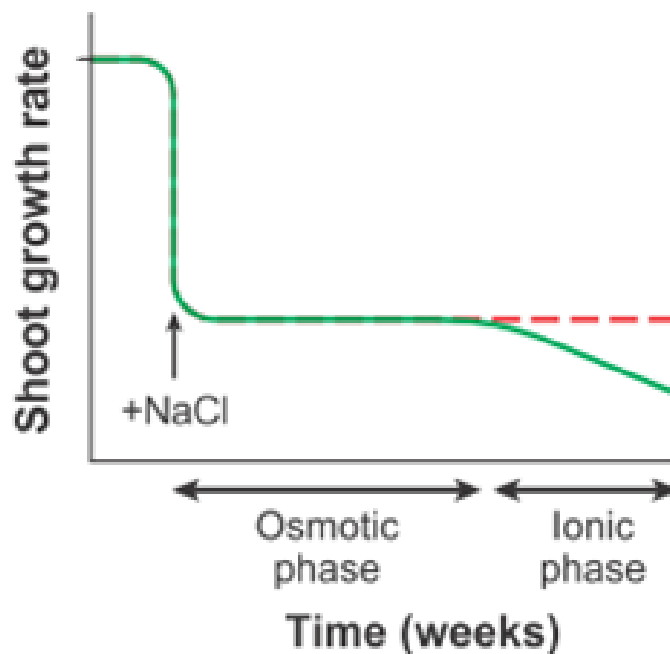
Figure 3. Increase in ionic tolerance. The change in the growth rate after application of NaCl is denoted by the solid green line. The dashed red line denotes the theoretical response of a plant with reduced sensitivity to the ion-specific phase induced by salinity (Munns and Tester 2008)

Dvořák et al. (1994) detected the *Kna1* locus on the long arm of chromosome 4D in bread wheat. This locus is strongly associated with ratio of K^+/Na^+ in the shoot. In order to introduce the *Kna1* locus to durum wheat, the pairing mutant *ph1c* which inhibits the normal

suppression of pairing between the homoeologous chromosomes, was used to induce a distil recombination of the chromosome 4D long arm with chromosome 4B (Dvořák et al. 1994). Although a novel durum line was generated with low Na^+ accumulation and higher ratio of K^+/Na^+ in the shoot, this line produced a lower biomass compared to other durum cultivars. It was later found that *Kna1* is linked with undesirable traits (Gorham et al. 1997). Subsequent work was carried out using the *phc1* mutant to reduce the size of the chromosome 4D fragment by another round of homoeologous recombination. However, the biomass of this novel durum wheat did not increase when grown in saline soil (Gorham et al. 1997).

Another attempt to enhance Na^+ exclusion in durum wheat involved investigating large numbers of accessions and landraces related to durum wheat to identify new sources of Na^+ exclusion. Wheat accessions which are the progenitors of current wheat have been used in breeding programmes for improving wheat yield under abiotic and biotic stress. Munns et al. (2000) found a source of Na^+ exclusion in a durum wheat breeding line (line 149) derived from a cross between the wild wheat accession C68-101 (*Triticum monococcum*) and a durum cv Marrocos. Two loci associated with low leaf Na^+ accumulation were detected on the long arm of chromosome 2A (*Nax1*) (Lindsay et al. 2004) and chromosome 5A (*Nax2*) (James et al. 2006). *Nax1* excludes Na^+ by unloading Na^+ in the leaf sheath to reduce leaf blade Na^+ and increase K^+, whereas *Nax2* excludes Na^+ ions from the xylem in the root before they can be transported to the shoot (James et al. 2006). The candidate gene for *Nax1* and *Nax2* loci are *HKT1;4* and *HKT1:5*, respectively. These genes are absent in modern tetraploid and hexaploid wheats and were derived from the wild species *T. monococcum*. *Nax2* has shown a 26% yield enhancement with near isogenic lines from Tamaroi background (Australian durum wheat cultivar) (Munns et al. 2012).

Landraces are an important source for enhancing yield potential of crops grown under biotic and abiotic stress. For example, the Indian bread wheat Kharchia 65 was shown to exclude Na^+ from leaves (Mujeeb-Kazi et al. 1993), and this landrace was used to breed and release

salt tolerant lines (Mahar et al. 2003). Shorawaki, a bread wheat landrace from Pakistan was used in a breeding programme for improving salt tolerance in Pakistani bread wheat. Breeding lines derived from crossing Shorawaki with other Pakistani bread wheat cultivars showed improved yield under high salt conditions (Jafari-Shabestari et al. 1995).

1.2.2.3 Na⁺ *tissue tolerance*

The third tolerance mechanism is the intracellular compartmentalisation of Na^+ which enables the leaves to tolerate high concentrations of Na^+ (Munns and Tester 2008). After Na^+ is transported to the leaves, the plant can tolerate ion-specific stress by compartmentalising Na^+ into vacuoles (Munns and Tester 2008).

In vitro experiments have indicated that leaf Na^+ concentration at 100 mM starts to inhibit the activity of enzymes extracted from leaf cells (Flowers and Dalmond 1992). Tester and Davenport (2003) demonstrated that most enzymes in leaf cells could function normally at a high concentration of both Na^+ and Cl^- (over 200 mM) and that because the toxicity of both Na^+ and Cl^- in the cell cytoplasm is reduced by compartmentalising these ions into the vacuole of the cells. Munns and Tester (2008) showed that sequestration of Na^+ is the main mechanism of salt tolerance in some halophytes because enzymes in these plants are intolerant of high concentrations of Na^+ even though these plants accumulate high concentration of Na^+ in leaves. Also, Staal et al. (1991) suggested that differences in Na^+ compartmentalisation in leaf cells are a good indicator of differences in salt tolerance between plant species . This is on the basis that the apoplast Na^+/H^+ antiporter is activated to a much greater degree in species (*Plantago maritima)* which are more salt tolerant than in salt susceptible species such as *Plantago media* (Staal et al. 1991).

Many studies have demonstrated that the *NHX* family of genes (Brini et al. 2007; He et al. 2005; Xue et al. 2004) and *AtAVP1* (Gaxiola et al. 2001) can control the compartmentalisation of Na^+ in leaf vacuoles in *Arabidopsis*.

When Na^+ is compartmentalised into the vacuole, the osmotic pressure outside and inside the vacuole needs to be balanced to maintain the volume of the other subcellular compartments, including the cytosol. This balancing can occur through either a rise in K^+ concentration to sub-toxic concentrations or by an increase in the concentration of compatible solutes such as sucrose, proline or glycine betaine outside the vacuole (Flowers et al. 1977; Wyn Jones et al. 1977). In several halophytes, proline or glycine betaine are synthesised in leaf cells to concentrations of over 40 mM to balance the osmotic pressure within the cell (Flowers et al. 1977; Hasegawa et al. 2000; Munns 2005). In cereals, glycine betaine is synthesised in maize and lines which carried *Bet1* (glycine betaine accumulation) gene yielded 10% - 20% higher biomass under saline conditions than control plants (Saneoka et al. 1995).

There are several mechanisms of Na^+ sequestration, including succulence and accumulation of Na^+ in salt glands or bladders which occur in dicotyledonous plants (Flowers et al. 1986). In monocotyledonous species, these mechanisms are uncommon, but James et al. (2006) found that some barley cultivars could maintain photosynthetic capacity at higher leaf Na^+ levels than durum wheat and this was attributed to higher levels of tissue tolerance.

1.2.2.4 Plant tolerance to two phases of salt stress (osmotic and ionic stress)

A hypothesis developed by Munns and Tester (2008) is that plants also go through two phases of salt tolerance, osmotic and ionic. If the plants are tolerant to both osmotic stress and ionic stress, they will have a higher growth rate than the plants with only one component (either osmotic or ionic stress tolerance) in a saline environment (Figure 4). Genc et al. (2007) found that variation in salinity tolerance in bread wheat is not only due to Na^+ exclusion but also other mechanisms. Rajendran et al. (2009) showed that three *T. monococcum* wheat accessions which employ both mechanisms have greater salt tolerance than the other accessions tested. Osmotic stress tolerance and Na^+ exclusion were incorporated in maize

breeding programme for improving salinity tolerance. The maize hybrids which tolerated osmotic and ionic stress showed significantly enhanced grain yield performance under saline soil (EC: 10 dS m^{-1}) (Schubert et al. 2009).
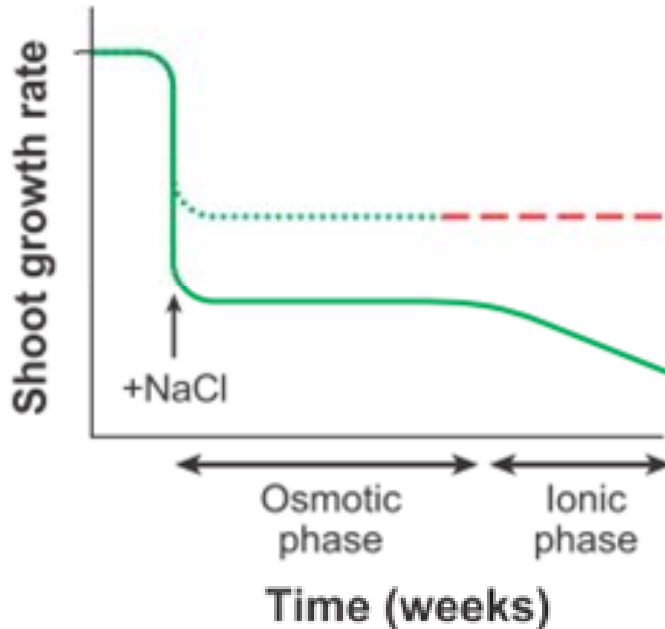
Figure 4. Increase in osmotic and ionic stress tolerance. The change in the growth rate after application of NaCl is denoted by the solid green line. The green-and-red line denotes the theoretical response of a plant with reduced sensitivity to both osmotic stress and ion–specific stress induced by salinity (Munns & Tester 2008).

1.2.3 Wheat

Wheat (*Triticum* ssp) is one of the oldest cultivated crops and for 8000 years has been the staple food in West Asia and North Africa. Wheat is grown on 240 million ha worldwide which is greater than any other cultivated crop (FAO 2009). Wheat provides a fifth of the total of humanity's food, and it is the second source of calories after rice and the first source of protein (Braun 2011). World wheat production for consumption, animal feed, seed and processed uses has remained around 550 million tonnes since 1990 (FAO 2009). According to a report issued by CIMMYT (Braun 2011), around 1.2 billion people are dependent on wheat as a food source and 2.5 billion people who consume wheat are poor, with an income of less than US$ 2 per day. Also, 30 million poor people are wheat producers. By 2050, it is

estimated that the number of people consuming wheat will increase by 60% as the global population continues to increase. However, the increase in temperature due to climate change is estimated to reduce wheat production by 20 to 30% (Braun 2011).

Wheat's phylogeny is: phylum *Angiospermatophyta*, class *Monocotyledonopsida*, order *Poales*, family *Poaceae*, subfamily *Pooideae*, tribe *Triticeae*, sub-tribe *Triticinae*, genus *Triticum* (Balint et al. 2000). Wheat is separated into three groups according to the ploidy classes (number of copies of chromosomes): diploid (2n=2x=14), such as *Triticum urartu*; tetraploid (2n=4x=28), such as *T. turgidum* ssp. *durum*, and hexaploid (2n=6x=42), such as *T. aestivum* (Goncharov 2011). *T. aestivum* (Bread wheat) and *T. turgidum* ssp. *durum* (durum wheat) are commercially important crops. Bread wheat is grown to produce grain for flour and baking which is used extensively for breads, cakes, biscuits and also fermented to make beer, vodka and biofuel (MacRitchie 1992; Uthayakumaran et al. 2002). Grain of durum wheat has high protein and gluten, which produces flour with qualities suitable for pasta and noodle production (Hatcher et al. 2009; Troccoli et al. 2000).

Bread wheat resulted from the combination of three diploid donors into one species through two distinct hybridisation events. Initially, the diploid *T. urartu* (AA) was naturally crossed with an unknown relative of *Aegilops speltoides* (BB) to produce tetraploid *T. turgidum,* ssp. *dicoccoides* around 0.2–0.5 million years ago (Huang et al. 2002). The tetraploid *T. turgidim* ssp. *dicoccoides* was naturally crossed with another diploid *Ae. tauschii* (DD), around 8500 years ago and the hexaploid *T. aestivum* was produced (Kihara 1944; McFadden and Sears 1946). The estimated size of the wheat genome is 17 Gbp (Paux et al. 2008). Between 75-90% of the wheat genome is made up of repeat sequences, largely retrotransposons (Wanjugi et al. 2009).

Increasing the potential yield of wheat using conventional breeding strategies requires phenotypic selection over many years (Kumar 1999). In Australia, it takes up to 14 years from

the first cross to the commercial release of a new wheat cultivar in most conventional breeding programmes (Lehmensiek et al. 2009). Understanding the mechanisms of abiotic stress, combined biotechnology tools will help breeders to increase wheat production and produce new varieties more rapidly.

1.2.4 Detection of the genetic loci associated with salinity tolerance

Biotechnology techniques can be used to accelerate breeding programmes and enhance trait introgression (Lehmensiek et al. 2009). Molecular marker technology is an important tool to understand and manipulate quantitative traits (Dubcovsky 2004). Molecular markers linked to qualitative genetics can be used to select for desirable alleles in segregating populations (Beecher et al. 2002). Many important traits for breeding show quantitative inheritance because they are controlled by multiple genes, and there are often interacting or epistatic loci. Quantitative trait loci (QTL) can be identified using a range of techniques (Chartrain et al. 2004; Chen et al. 2006) and molecular markers are used to track QTL in breeding programmes (Lehmensiek et al. 2009). Colmer et al. (2005); Munns and Tester (2008) explain that salinity tolerance traits such as osmotic stress tolerance and Na^+ exclusion are complex in plants and likely to show quantitative inheritance. Several genes associated with salinity tolerance traits have been identified and are summarised in Table 1.

Table 1. Plant processes and gene families associated with osmotic and ionic stress tolerance in the plant.

Process involved	Gene family	Salt tolerance mechanism	References
Accumulation of Na$^+$ in the shoots.	*HKT*	Na$^+$ exclusion	Schachtman and Schroeder (1994); Uozumi et al. (2000); Su et al. (2003); Horie et al. (2009); Møller et al. (2009); Xue et al. (2011); Munns et al. (2012)
Accumulation of Na$^+$ in the shoots. Sensing and signaling in roots.	*SOS*	Na$^+$ exclusion, Na$^+$ tissue tolerance and osmotic stress tolerance	Wu et al. (1996); Liu and Zhu (1998); Zhu et al. (1998); Shi et al. (2000); Liu et al. (2000); Shi et al. (2002); Shi et al. (2003)
Accumulation of Na$^+$ in vacuoles.	*NHX, AVP*	Na$^+$ tissue tolerance	Gaxiola et al. (1999); Apse et al. (1999); Gaxiola et al. (2001); Aharon et al. (2003); Bassil et al. (2011); Fukuda et al. (2011)
Accumulation of organic solute.	*P5CR, P5CS*	Osmotic stress tolerance	Yoshiba et al. (1995); Knight et al. (1997); Strizhov et al. (1997); Hare et al. (1999); Székely et al. (2008)

No genes associated with the reduction of shoot growth induced by salinity have been detected in wheat or in other species (Table 1) and the wheat homologues or orthologues of many of gene families have not been identified in wheat, except; *TmHKT1;4, TmHKT1;5* and *TaHKT1;5* which are member of *HKT* gene family and associated with Na$^+$ exclusion (Byrt et al. 2007; Dubcovsky et al. 1996; Huang et al. 2006). Although salinity tolerance traits are often genetically complex and controlled by many genes there has been considerable success (Table 1) in identifying some of the key genes controlling tolerance.

1.2.4.1 QTL analysis

QTL analysis combines traditional quantitative genetics and genomic mapping in order to search for genes (loci) which control complex or quantitative traits, on chromosomes. QTL associated with traits of interest can be detected by generating a population segregating for the trait in bi-parental or multi-parental populations, or through association mapping panels.

1.2.4.1.1 Mapping population

Bi-parental mapping populations are most commonly used for QTL mapping. Choosing the initial parents for the generation of a population is critical but several options can be used to

develop the mapping population (Figure 5). Ideally the chosen parents should show high levels of genetic polymorphism with minimal, common ancestral genome loci and they should also differ for the phenotypic or physiological traits of interest (Lehmensiek et al. 2009). After choosing the parents, segregating populations can be generated by crossing the parents. There are several options for population development (Table 2) and each type has advantages and disadvantages (Gupta et al. 2013). Multi-parental populations have the advantage of greater diversity and multiple alleles but when these are used for analysing of many traits the cost of genotyping and phenotyping multi-parental populations is higher than bi-parental populations (Table 2) (Morrell et al. 2012). RIL populations (either bi- or multi-parent) have greater genetic resolution due to the higher recombination rate (Gupta et al. 2013). F_2 and BC populations have low recombination rate, but these can be used for a trait where variation is due to major genes. Association mapping panels generally have the highest genetic resolution as a result of the long evolutionary history of the lines (Nordborg and Tavare 2002). However, association mapping panels needs high marker density and is strongly affected by population structure (Table 2) and may not detect QTL associated with rare alleles (ineffective sampling rare alleles) (Morrell et al. 2012). Integrating association mapping panels with linkage maps of bi-parental population is an approach used to overcome the limitations of both populations types (Gupta et al. 2013).
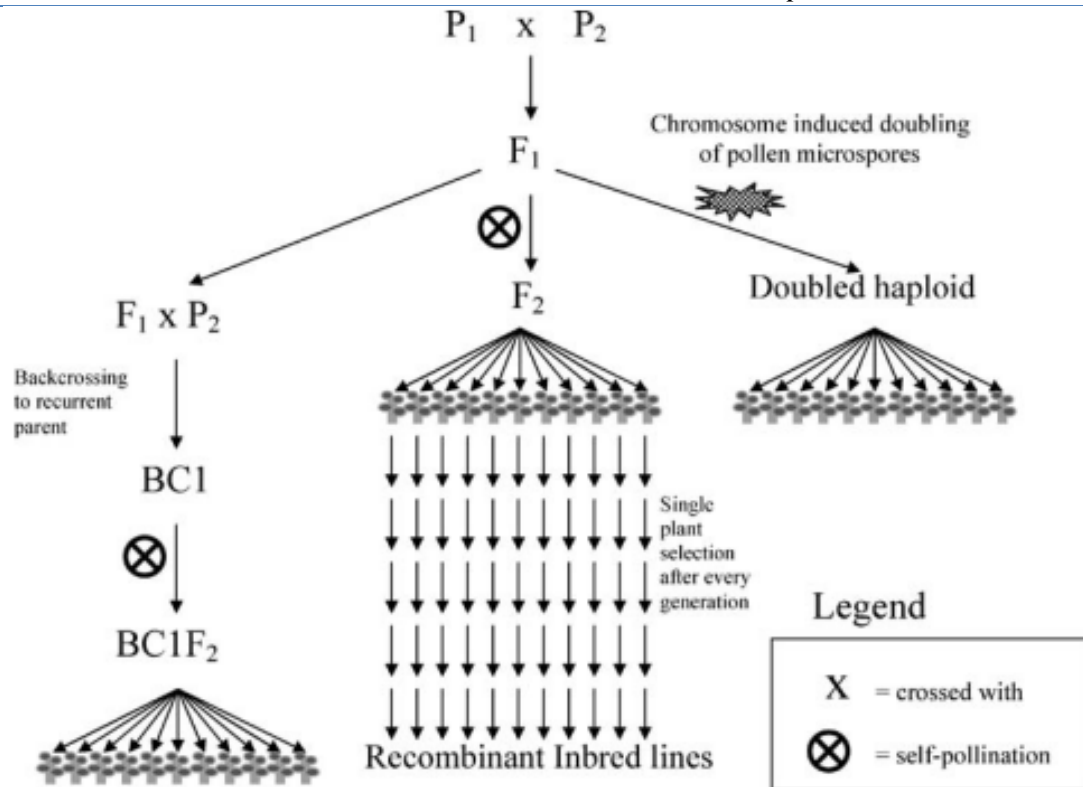
Figure 5. A scheme for generating bi-parental populations. Parent 1 (P$_1$) is crossed with parent 2 (P$_2$) to generate the first generation (F$_1$). Self-crossing of F$_1$ produces second generation (F$_2$) which can be used directly for mapping. It is also possible to develop genetically stable or immortal populations by going through single seed descent to produce a series of recombinant inbred lines, or through the generation of doubled haploids directly from the F$_1$. Backcross populations (one cycle of backcrossing) are produced by backcrossing F$_1$ to P$_2$. (Collard et al. 2005).

Table 2. Types of mapping populations used for QTL detection in crops and their advantages and disadvantages.

Type of population		Population production	Advantages	Disadvantages
Bi-parental populations	**Second generation (F$_2$) population**	The F$_2$ is produced by crossing two parents (inbreed lines) to produce the first generation (F$_1$). F$_1$ progeny will be self-pollinated to generate the F$_2$	• The production of F$_2$ population is rapid. • It can be used to study dominant genes or traits showing a clear phenotype	• The effective population size is reduced since half the lines are heterozygotes • QTL mapping cannot be replicated over different locations and years
	Backcrossing (BC$_1$) population	BC$_1$ is generated by backcrossing F$_1$ progeny with the recurrent parent	• As for F$_2$ populations	• Similar disadvantages as for F$_2$ populations
	Recombinant Inbred Line (RIL) population	RIL produced by six to eight cycles of selfing with single descent seed selection	• Generates high resolution linkage maps due to multiple cycles of recombination. • QTL analysis can be repeated over different locations and years	• Needs time to go through the six to eight generations
	Doubled Haploid (DH) population	DH populations can be generated from haploid cells (microspores or egg cells) and the chromosomes doubled through chemical treatment	• The same advantage as RIL populations • Can be generated directly from F$_1$ plants	• Not suitable for all plant species • Can be expensive and technically demanding • Plants have only been through a single meiosis so recombination levels are low
	Near isogenic lines (NILs)	NILs are generated by recurrent crossing and selection of lines with or without the target trait	• Detects a QTL and introgresses the putative QTL into elite cultivars at the same time • Epistatic interactions can be eliminated. • Provides high genetic resolution	• Time and labour intensive • Allows analysis of single or several loci

Table 2. Continued

Type of population		Population production	Advantages	Disadvantages
Multi-parental populations	**Multi-parent Advanced Generation Intercross (MAGIC)**	MAGIC is generated by crossing four to twenty inbred lines	• Allows for analysis of multiple alleles • Provides high levels of genetic diversity	• Can be expensive and time consuming to generate • The high level of diversity can mask major adaptive traits
	Nested Association Mapping (NAM)	NAM is generated by crossing one common parent (inbred line) with many donors to produce the F_1 generation. A series of related RIL populations is produced.	• Association mapping and linkage analysis can occur within the population. Historic and recent recombination events can be recorded • Large number of recombination events compared with bi-parental population • Large number of small QTL and epistatic effects can be detected	Similar disadvantages as a MAGIC population
	Multiline Cross Inbred Line	Represents standard population structures used in many breeding programs	Exploit populations and phenotypic data generated with breeding programs	• As the genotypes within the breeding programs are constantly changing between different trials, locations and years (breeding cycles), QTL analysis cannot be replicated • Limited to germplasm already within breeding programmes
Association mapping panels		Based around collections of elite lines, wild species or landraces	• High rate of recombination • It is not demanding in time, effort or cost because this population does not need to be generated as outlined Population production field.	• High density genotyping technology is needed due to the complex structure of the population • Rare alleles may not be detected.

1.2.4.1.2 Molecular markers

A variety of molecular marker systems are available for use in wheat mapping and genetic analysis. Marker development in cereals has progressed from restriction fragment length polymorphism (RFLP) through random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellite (simple sequence repeat, SSR), Diversity array technology (DArT), and single nucleotide polymorphism (SNP) markers (Lehmensiek et al. 2009).

The microsatellite markers are derived from library of genomic DNA sequences that were developed through a variety of enrichment procedures (named genomic SSR) (Edwards et al. 1996; Ostrander et al. 1992). Another type of microsatellite markers are generated from the expressed regions of the genome (named EST-SSR) (Holton et al. 2002; Thiel et al. 2003). The microsatellite markers were used as polymorphic markers in bread wheat which has a complex genome and a low level of polymorphism (Ganal and Röder 2007).

DArT markers were first developed in rice (*Oryza sativa*), which has a small genome of 430 Mbp (Jaccoud et al. 2001) and have since been extensively used in wheat mapping projects (http://www.diversityarrays.com/; Paux and Sourdille 2009). It has been found that DArT markers can distinguish between closely related cultivars of wheat or barley which allows for the generation of high density linkage maps (Paux and Sourdille 2009). However, the level of redundancy in DArT markers, at 27%, is larger than the redundancy in microsatellite markers (8.9%) and AFLP markers (1.8%) (Hearnden et al. 2007; Semagn et al. 2006). Nevertheless, DArT markers have become popular due to their low cost and the short time-frame required to genotype a population compared with microsatellite and AFLP markers (Lehmensiek et al 2009). Approximately 5000 to 7000 DArT markers have been developed for wheat (Akbari et al. 2006; Mantovani et al. 2008; Peleg et al. 2008), and have been used for QTL analysis for different traits in bread and durum wheat.

In wheat, SNPs can be classified as homoeologous or varietal. The varietal SNPs are the most suitable for molecular markers, whereas homoeologous SNPs, show differences between related wheat genomes (AA, BB and DD) which can help in differentiating varietal SNPs (Berkman et al. 2012). SNP markers can be developed from sequencing resources such as expressed sequence tags (EST sequence), amplicon sequencing, sequenced genomes and next generation sequencing (NGS) (Ganal et al. 2009). NGS has been used to generate several thousand SNP markers in durum (Trebbi et al. 2011) and bread wheat (Lai et al. 2012).

1.2.4.1.3 Linkage map

Molecular marker based genetic linkage maps can be constructed using the individual progeny scores. The distance between the markers is estimated on the basis of recombination events or cross-over between the markers (Collard et al. 2005). Several computer programmes are available for constructing the linkage maps, such as MapMaker/EXP (Lander et al. 1987), JoinMap (Stam 1993), MapManager QTX (Manly et al. 2001), Multipoint (Mester et al. 2003) and RECORD (Van Os et al. 2005).

1.2.4.1.4 QTL analysis methods

Three methods are widely used for detection of QTL. The first is single point or single-marker analysis which evaluates the segregation of a phenotype with respect to a marker genotype. Markers associated with the quantitative trait of interest can be identified. The single-marker analysis typically tests the null hypothesis whether the mean of the trait value is unrelated to the genotype at a particular marker (Doerge 2002). ANOVA, linear regression, likelihood analysis and the *t*-test (Lehmensiek et al. 2009) are the statistical methods used for single-marker analysis. Even though the advantage of this method is that a linkage map is not needed, it does not provide an accurate estimation of the QTL location (Figure 6), or the recombination frequency between the marker and the QTL. Despite these disadvantages, single-marker analyses are frequently used to identify individual markers which co-segregate with a trait. For large populations, this method is rapid and efficient in detecting associations

for a specific trait, such as disease resistance or Na^+ exclusion. In addition, these analyses can potentially detect numerous significant markers/trait associations with most standard statistical analysis packages.

The limitations of single-marker analysis can be overcome by simple interval mapping (SIM). The location of a QTL can be determined using the framework of a genetic linkage map. SIM detects a QTL incrementally within intervals (ordered pairs of markers) on a chromosome. The test of whether the probable QTL is located within the interval is calculated using standard statistical methods (Lander and Botstein 1989). Lander and Botstein (1989) compared the estimated likelihood function under a null hypothesis (no QTL) with the alternative hypothesis (QTL present at the testing position). This test is expressed as logarithm of the odds (LOD) scores. However, SIM often incorrectly detects multiple QTL (many peaks) within an interval (Figure 6). SIM can be improved by using covariance or multiple quantitative trait loci analysis to remove incorrect QTL (Zeng 1993) and this is called composite interval mapping (CIM).

The differentiation of linked and/or interacting QTL can be identified using CIM. This leads to an elimination of interacting QTL not associated with the target quantitative trait (Figure 6). Locating all single QTL by SIM, then building a statistical model with these QTL and their interactions, and finally identifying significant interactions in one dimension is an heuristic approach to conduct composite interval mapping (Basten et al. 2002).

Doerge (2002) stated that searching all potential QTL models can be difficult owing to the computational intensity of a multidimensional search. However, typical regression model building methods such as forward, stepwise or backward regression can be employed for multi-marker analysis (Boopathi 2013). After QTL and the associated markers are identified from SIM, forward regression is used to select adjacent significant markers (with highest significant LOD score (LOD $\geq$ 3)) which are then added to the model. The analysis will re-

estimate the significance of these two markers. If these markers remain significant, a third

significant marker will be added and tested for significance and so on. Markers which fail to

show a significant association with the trait at any step of re-evaluation, will be dropped from

the model, and markers that remain significant will be retained.
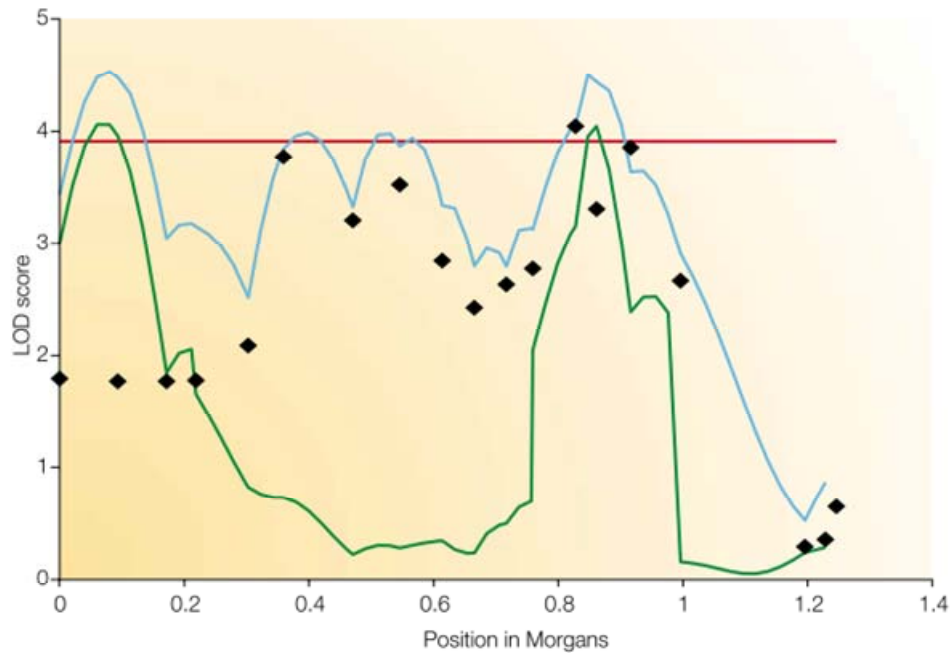
Figure 6. Different QTL analysis methods using QTL-Cartographer: single marker analysis using a *t*-test (black diamonds), simple interval mapping (SIM) (blue line) and composite interval mapping (CIM) (green line). The significant level (logarithm of odds (LOD) score) on the basis of 1000 permutations of the phenotypic data is denoted by the red line. Single marker analysis identified one significant marker, SIM detected four significant QTL and CIM located two maximum QTL regions on the LOD profile. The difference between single marker analysis and interval mapping (SIM and CIM) is due to the use of information obtained from an estimated genetic map. The difference between CIM and SIM is that two QTL identified from SIM have disappeared in CIM, because CIM allows detection of the interaction between these QTL and QTL identified in other regions (outside the region shown in the figure). This figure is taken from Doerge (2002) and is based on a study of experimental allergic encephalomyelitis (EAE) (Butterfield et al. 1999). The study mapped QTL for a quantitative trait on mouse chromosome 11. 633 F_2 mice were genotyped using microsatellite markers.

1.2.4.2 Selective genotyping analysis (SGA)

SGA, also known as distribution extreme analysis or trait-based analysis, is another method

for the detection of QTL associated with traits of interest. Two groups of individual plants are

selected from the two extremes of a phenotypic distribution (Collard et al. 2005). For

example, if a mapping population is phenotyped for a particular trait (e.g. disease resistance)

and distributes normally for the trait (Figure 7), SGA would be conducted by selecting

individuals representing the phenotypic extremes for marker genotyping. Subsequently,

linking markers could be then detected. The advantage of using SGA is that the reduced cost

compared to genotyping the entire population, but the disadvantage is that only one trait can

be tested at a time (Tanksley 1993). SGA was successfully used to detect markers associated

with salinity tolerance for germination in tomatoes (Foolad and Jones 1993).
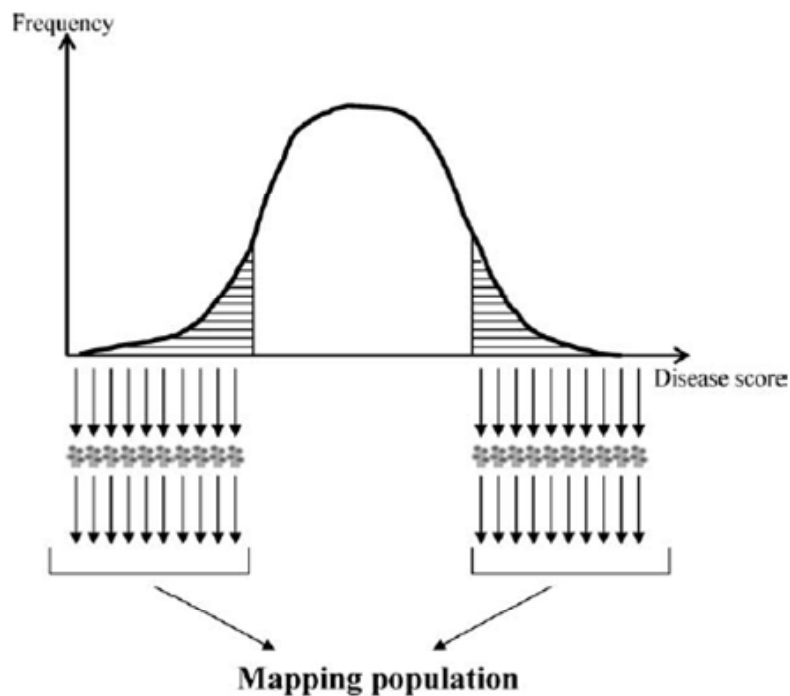
Figure 7. Selective genotyping analysis method involves selecting individual plants at end tails of a phenotypic distribution. The QTL can be detected by genotyping DNA of these selected individuals and constructing a linkage map. Axis x denotes the phenotypic values (e.g. disease score) and axis y denotes the number of plants of a population (Collard et al. 2005).

1.2.4.3 Bulked segregant analysis (BSA)

BSA is a rapid technique used to identify markers associated with traits of interest (Michelmore et al. 1991). The method involves combining the DNA of 10 to 12 selected individuals from each extreme of the segregated population into two pools or bulks. The bulks are genotyped with sufficient markers to give coverage of the full genome. The two DNA bulks differ in the phenotypic trait of interest (resistant vs. sensitive or tolerant vs. intolerant) and should be enriched in genomic regions associated with that trait; other genomic regions are assumed to be the same between the two bulks. Polymorphic markers can then be used to genotype the entire population to confirm the association (Ford et al. 1999). The *Nax1* locus, which is associated with Na^+ exclusion in durum wheat, was identified initially in a BSA analysis using AFLP markers (Lindsay et al. 2004). Recently, DArT markers (Wenzl et al. 2007) and SNP markers (Hayden et al. 2011) have been used to conduct BSA in wheat and barley.

1.2.4.4 QTL for salinity tolerance traits

QTL for different salinity tolerance traits have been mapped on different chromosomes in rice, barley and wheat (Table 3). Previous studies have found QTL associated with salinity tolerance traits on all wheat chromosome groups. The mapped traits include yield components, shoot biomass and tiller number, or physiological traits, but are predominantly associated with the accumulation of shoot Na^+, shoot K^+ and the ratio of K^+/Na^+ (Table 3). Some similarities have been found between barley and wheat. *Nax2,* associated with Na^+ exclusion, has been mapped on chromosome 5A of durum wheat and it was found that *Nax2* corresponded to QTL mapped on chromosome group 4 in wheat (De León et al. 2011; Dubcovsky et al. 1996) and 4H in barley (Long et al. 2013). *Nax1*, associated with Na^+ exclusion, was mapped to the short arm of the chromosome 2A in durum wheat (Lindsay et al. 2004) and this corresponded to QTL mapped on the short arm of the chromosome 2A in bread wheat (Genc et al. 2010). However, subsequently, few genes have been identified from

these QTL (Table 3) such as *HKT1;5*-like gene which is candidate for *Nax2* and *Kna1* loci and *HKT1;4*-like gene which is candidate for *Nax1* locus.

An example of utilising QTL analysis to improve salinity tolerance is found in the work of Munns et al. (2012). A locus, *Nax2,* which was associated with low Na^+ accumulation in the durum wheat shoot, was mapped to the distal region of the long arm of chromosome 5A (Byrt et al. 2007). F_1 plants were produced by crossing Tamaroi (which accumulates high Na^+ in the shoot), with breeding line 149, (which accumulates low Na^+ in the shoot) (James et al. 2006). Near isogenic lines (BC_4) derived from back-crossing F_1 plants with the recurrent parent (Tamaroi) were developed using two dominant microsatellite markers flanking *Nax2* at seedling stage. Two BC_4 lines yielded 26% more than the recurrent parent when grown in saline soils (Munns et al 2012).

No other genes or markers have been identified or developed from other QTL and used for improving yield in barley or wheat grown under saline conditions (Table 3). Also, even though osmotic stress tolerance is at least as important as Na^+ exclusion in bread wheat, no QTL associated with this trait have been detected. Na^+ exclusion is an important salinity trait in durum wheat and one gene has been used to improve salinity tolerance (Munns et al. 2012). Further studies are required to detect QTL associated with osmotic stress tolerance and Na^+ exclusion traits leading to the identification of genes and the development of markers which associated with these traits. This would assist breeding programmes of yield enhancement of wheat grown under saline soils.

Table 3. QTL and genes mapped in populations of rice, barley and wheat selected for salinity tolerance studies. QTL, gene location and chromosome, and wheat homoeologous groups are listed, along with likely osmotic and ionic stress tolerance.

Process involved	Candidate genes or loci	Trait measured	Salt tolerance mechanism	Species	Chromosome	Wheat homeologous group	Reference
Shoot growth		Seedling and palnt survival	Osmotic stress and ionic tolerance	Rice	1, 6, 7	3, 7, 2	Lin et al. (2004)
				Barley	2H, 5H, 7H	2, 5, 7	Zhou et al. (2012)
				Bread wheat	5D	5	Koebner et al. (1996)
				Bread wheat	5A	5	Semikhodskii et al. (1997)
		Tiller number	Osmotic stress and ionic tolerance	Rice	6	7	Gong et al. (2001)
				Barley	7H	7	Ellis et al. (2002)
				Barley	4H	4	Xue et al. (2009)
				Barley	6H	6	Long et al. (2013)
				Bread wheat	5A	5	Genc et al. (2010)

Table 3. Continued.

Process involved	Candidate genes or loci	Trait measured	Salt tolerance mechanism	Species	Chromosome	Wheat homeologous group	Reference
		Dry matter production	Osmotic stress and ionic tolerance	Rice	5, 6, 10	1, 7, 1	Prasad et al. (2000)
				Rice	6	7	Koyama et al. (2001)
				Barley	5H	5	Pakniyat et al. (1997)
				Barley	2H, 4H, 5H	2, 4, 5	Ellis et al. (2002)
				Barley	7H	7	Ellis et al. (1997)
Shoot growth				Barley	1H, 2H, 5H, 6H	1, 2, 5, 6	Mano and Takeda (1997)
				Barley	2H	2	Xue et al. (2009)
				Barley	3H	3	Nguyen et al. (2013)
				Barley	6H	6	Long et al. (2013)
				Bread wheat	1A, 3B	1, 3	Ma et al. (2007)
				Bread wheat	2A, 4B, 5A, 5B, 6A, 6D, 7A	2, 4, 5, 5,6, 6,7	Genc et al. (2010)

Table 3. Continued.

Process involved	Candidate genes or loci	Trait measured	Salt tolerance mechanism	Species	Chromosome	Wheat homeologous group	Reference
Photosynthesis	*ERA1, PP2C, AAPK, PKS3*	Chlorophyll content	ionic and osmotic stress tolerance	Rice	2, 3, 4	4, 2	Thomson et al. (2010)
				Barley	6H	6	Long et al. (2013)
				Bread wheat	3D,7A	3, 7	Ma et al. (2007)
				Bread wheat	5B	5	Genc et al. (2010)
Shoot Na$^+$ accumualtion	*HKT (Nax1, SKC1), SOS*	Shoot Na$^+$ accumulaion	Ionic tolerance	Rice	4, 6	2, 7	Koyama et al. (2001)
				Rice	7	2	Lin et al. (2004)
				Rice	1	3	Thomson et al. (2010)
				Rice	1, 4, 12	3, 2	Ul Haq et al. (2010)
				Barley	2H, 4H	2, 4	Xue et al. (2009)
				Barley	7H	7	Shavrukov et al. (2010)
				Barley	2H	2	Nguyen et al. (2013)
				Barley	4H	4	Long et al. (2013)
				Durum wheat	2A	2	Lindsay et al. (2004)
				Bread whaet	7A	7	Shavrukov et al. (2011)
				Bread whaet	2A, 2B, 7A	2, 2, 7	Genc et al. (2010)
				Bread whaet	2A, 6A	2, 6	Genc et al. (2013)
Shoot K$^+$ accumulation	*HKT (Nax2, SKC1)*	Shoot K$^+$ accumulation	Ionic tolerance	Rice	1, 4	3, 2	Koyama et al. (2001)
				Rice	1	3	Lin et al. (2004)
				Rice	1	3	Thomson et al. (2010)
				Barley	2H	2	Nguyen et al. (2013)
				Barley	4H	4	Long et al. (2013)

Table 3. Continued.

Process involved	Candidate genes or loci	Trait measured	Salt tolerance mechanism	Species	Chromosome	Wheat homeologous group	Reference
Ion transport	*HKT (Nax2, SKC1, Kna1)*	Na$^+$ / K$^+$ ratio	Ionic tolerance	Rice	1, 4	3, 2	Koyama et al. (2001)
				Rice	1	3	Bonilla et al. (2002)
				Rice	1, 9	3	Thomson et al. (2010)
				Rice	1, 4, 12	3, 2	Ul Haq et al. (2010)
				Barley	6H	6	Xue et al. (2009)
				Barley	2H	2	Nguyen et al. (2013)
				Barley	4H	4	Long et al. (2013)
				Durum wheat	5A	5, 4	Byrt et al. (2007)
				Bread wheat	4D	4	Dubcovsky et al. (1996)
Grain filling		Grain yield		Rice	7	2	Gong et al. (2001)
				Barley	6H	6	Xue et al. (2009)
				Bread wheat	1B, 2B, 3D, 4A, 4B	1, 2, 3, 4, 4	Quarrie et al. (2005)
				Bread whaet	2D, 4A	2, 4	De Leo´n et al. (2011)

1.3 Aim

Salinity is a major limitation to wheat production worldwide and this adds to the challenge of increasing in global food production to feed the rapidly growing human population. Increasing salt tolerance in elite lines provides an approach to maintaining yield for wheat grown under saline conditions.

Previous work has focussed on sodium exclusion but several recent studies have indicated that osmotic or tissue tolerance is at least as important. These traits have been difficult to screen particularly at the high throughput needed for genetic studies. The first task in this project was to improve the screening techniques for salinity in bread wheat.

The second task was to apply this technique to a bread wheat population segregating for salinity tolerance. A population based on a cross between two elite wheat cultivars, Gladius and Drysdale was used for this purpose. The aim was to identify novel loci associated with salt tolerance traits mainly osmotic stress tolerance and Na^+ exclusion.

Durum wheat is particularly susceptible to salt stress and although good progress has been made recently there appears to be little useful variation within the elite durum breeding pool. Therefore, emphasis was placed on two Afghani landraces recently shown to have a high level of tolerance. The third task was to identify novel genetic loci associated with Na^+ exclusion in durum wheat landraces.

The successful outcome of this project would be used in the detection of genes responsible for increasing salinity tolerance in wheat, and the development of molecular markers which could accelerate wheat breeding programmes to improve the salt tolerance of commercial cultivars.

Chapter 2 Improvement of screening techniques for salinity tolerance

2.1 Introduction

Salinity tolerance is a complex trait associated with many physiological processes and showing primarily quantitative inheritance (Colmer et al. 2005; Munns and Tester 2008). Three mechanisms are associated with salinity tolerance in plants (Munns and Tester 2008): Na^+ exclusion, Na^+ tissue tolerance and osmotic stress tolerance. The measurement of Na^+ exclusion is straightforward. However, Genc et al. (2007) established that there are other tolerance mechanisms which are important in bread wheat. Na^+ tissue tolerance was suggested as one mechanism associated with salinity tolerance but an appropriate high throughput assay for tissue tolerance in bread wheat has not been established (Genc et al. 2010; Rajendran 2012). An assay for osmotic stress tolerance has been developed using a non-destructive imaging system (LemnaTec Scanalyzer 3D technology) (Rajendran et al. 2009), but this has not been exploited to study the genetics underpinning osmotic stress tolerance in wheat.

The genetics of osmotic stress tolerance and associated quantitative trait loci (QTL) can be studied using bi-parental populations segregating for performance under salt stress. A cross between bread wheat cultivars, Gladius and Drysdale, was proposed as suitable for exploring salinity tolerance, since both cultivars are salt tolerant (Genc et al. 2007) but a reliable method for measuring osmotic stress tolerance in a large scale experiment was needed.

In order to measure the change in plant growth rate immediately after salt application, NaCl must be delivered to the root zone (Munns and Tester 2008). Plant growth should be measured shortly after application. In the conveyor system (The Plant Accelerator, Waite Campus, University of Adelaide, SA, Australia), the use of drained pots allows the salt to be added to the

soil while the plant is still a seedling. This enables measurement of plant growth rate immediately after salt application, and measurement of the effect of osmotic stress induced by NaCl on osmotic stress tolerance of individual lines of the population.

The aim of the experiment was to develop a screening method for osmotic stress tolerance by comparing Gladius and Drysdale grown at different salt levels (saline and non-saline soils), in different soils (coco peat mix and mixed Roseworthy) and using different pot types (closed or drained).

2.1.1 Gladius and Drysdale

Gladius is an Australian bread wheat cultivar widely adapted to drought and heat stressed environments, especially Southern Australia (Fleury et al. 2010). This cultivar had the highest yield compared with other Australian cultivars in two field trials conducted in 2001/2002 and 2006/2007 in drought and low rainfall environments (Wheeler 2007). Also, it was found that Gladius has a high level of resistance to rust and tolerates high concentrations of boron and acid soil (Wheeler 2007). Fleury et al. (2010) suggested that Gladius demonstrates high tolerance to heat, whereas Talukder et al. (2010) showed that Gladius had moderate level of heat tolerance ranked after Excalibur and Krichauff. The latter experiment was conducted by exposing the four Australian cultivars to heat in a single day at the flowering time. Gladius is considered to be an efficient Na^+ excluder and has a high salinity tolerance index (Relative shoot dry matter %) (Genc et al. 2007).

Drysdale is a high yielding cultivar in the low-medium rainfall area of southern New South Wales and has good grain size (AWB 2005). Drysdale tolerates acid soil, and has a moderate level of resistance to stem, leaf and stripe rusts (AWB 2005). In the 2006 trial, Drysdale was lower yielding than Gladius, and was sensitive to cereal nematodes (Wheeler 2007). It was, however, ranked among the drought tolerant cultivars and was the best cultivar in terms of rust

resistance (Wheeler 2007). In salinity experiments, Drysdale shows lower level of Na^+ exclusion compared to other Australian bread wheat cultivars (Fleury et al. 2010; Genc et al. 2007; Shavrukov et al. 2006). Genc et al. (2007) showed that Drysdale was the most tolerant cultivar compared to other bread wheats and it may have tissue tolerance traits, whereas El-Hendawy et al. (2005) found that Drysdale falls within the moderately tolerant group when compared with Egyptian and Indian bread wheats.

As Drysdale and Gladius both show drought tolerance (Fleury et al. 2010), they were selected by Australian Grain Technology (AGT) and ACPFG to produce a Recombinant Inbred Line (RIL) population to study the genetics of drought tolerance in bread wheat. They were also selected to investigate the carbon isotope discrimination trait which is associated with drought tolerance (water use efficiency) (Fleury et al. 2010).

Maphosa (2013) measured sensitivity to differences in photoperiod or response to vernalisation in (Zadoks score and days to heading) Gladius, Drysdale and their RILs. Both cultivars did not significantly differ in Zadoks score and days to heading trait at different locations across South Australia and New South Wales, Australia over three years 2009, 2010 and 2011except in the location Urrbrea, South Australia, Australia, in year 2010. However, these traits were segregated in RILs (Maphosa 2013). QTL for Zadoks score was mapped on region span on genes *Ppd-B1* and *Ppd-D1* on chromosomes 2A and 2D respectively, and day to heading trait was mapped on region spanning on genes *Ppd-B*, *Ppd-D*, *Vrn-A1* (on chromosome 5A) and *Vrn-D1* (on chromosome 5D) (Mophaso 2013).

While Gladius and Drysdale differ in shoot Na^+ accumulation (Genc et al. 2007; Fleury et al. 2010), both are considered to be salt tolerant (Genc et al. 2007).

2.2 Materials and methods

2.2.1 Plant Materials:

Gladius and Drysdale were used in the experiment. Gladius was derived from a complex cross including RAC875 (a line developed by AGT but not commercially released) and Kukri, (generated from a cross between Excalibur and Krichauff), and released in 2007 by AGT and the South Australian Research and Development Institute (SARDI).

Drysdale (Hartogx3/Quarrion) was released in 2002 by the Commonwealth Scientific and Industrial Research Organisation in conjunction with the New South Wales Department of Agriculture.

2.2.2 Growth conditions

Gladius and Drysdale seeds were placed on wet filter paper in petri dishes (10 seeds per petri dish) to germinate. The petri dishes were put in the cold room at $4^{\circ}C$ for 24h and then were placed at room temperature $(25^{\circ}C)$ for four days. Individual uniform size seedlings were transplanted into wet soil in the pots. Each pot had three seedlings. After five days, two seedlings were thinned out and one plant was left in each pot. Plants were grown for 50 d in the greenhouse and then harvested.

The temperature in the greenhouse was adjusted to between 22 and $24^{\circ}C$ during the day and $14{-}18^{\circ}C$ during the night. The humidity remained constant throughout the experiment. The light regime was a cycle of 14 h daylight/10 h dark. Lights (400 W/lamp) were used on cloudy days.

White plastic pots with a diameter of 14 cm and height of 19 cm were used. Closed and drained pots were used for the different watering systems. Closed pots were watered from the top,

whereas drained pots were placed in a saucer and watered into the saucer so water uptake into the soil was through the drain holes in the base of the pot. Blue beads were placed on the soil surface to reduce evaporation.

Two types of soil: Mixed Roseworthy and coco peat mix were used. Coco peat mix is an artificial soil which consists of organic and fibre materials. It is rich in macro and micro nutrients which are essential for plant growth (Abad et al. 2002; Awang et al. 2009). The mixed Roseworthy consisted of a field soil which is clay loam collected from Roseworthy Agricultural Campus farms, University of Adelaide, sand and coco peat mix in a ratio of 2:2:1. The Roseworthy soil was collected and steam sterilised. All three soils were mixed after they had been air dried.

In the closed pots, salt solutions of 0 mM and of 100 mM NaCl were applied onto soil at the top of the pot whereas these were applied into the saucers of the drained pots during watering. The 100 mM NaCl solution was applied incrementally. A volume (based on field capacity of the soil) of 25 mM NaCl solution was added daily from the time of third leaf emergence over a period of four days until the level reached 100 mM NaCl, along with a total of 3.3 mM $CaCl_2$.

2.2.2.1 Field capacity

The field capacity of mixed Roseworthy and coco peat mix was measured according to the gravimetric procedure (Smith and Mullins 2000). The aim of measuring field capacity is to estimate the volume of water required for plant growth during the experiment. A 5g sample of each soil type was dried in the oven (Contherm Scientific Ltd, Hutt City, New Zealand) at 105°C for five days before the sample was weighed. The oven dried soils were saturated with water and placed on a ceramic pressure plate. A pressure of -10 kPa was applied to these wet soils over 24 h. The wet soils were then weighed and field capacity (Θ_{fc}) at -10 kPa was calculated according to the formula (Smith and Mullins 2000):

$$\theta_{-10KPa}\ (g/g) = \frac{weight\ of\ wet\ soil - weight\ of\ dry\ soil}{weight\ of\ dry\ soil}$$

The results obtained from the 5g sample were used to estimate the total mass of water (Ψ_g) required for the volume of soil in the pots at -10kPa (Smith and Mullins 2000):

$$\Psi(g) =\ Mass\ of\ oven\ dried\ soil\ in\ pot\ (g) \times\ \theta_{-10KPa}\ (g/g)$$

These formulae were applied to the soil which had been air dried in the greenhouse by calculating the total mass of water (Ψ_g) from the difference in weight between the air dried and oven dried soils. All plants were watered to field capacity every second day. The weight of each pot and watering to the calculated field capacity was conducted manually.

Other soil characteristics were measured: electrical conductivity (EC), pH (Table 4) and nutrient content (Appendix III). Values of EC and pH were derived from a 1:5 soil: water suspension and the pH was measured in the suspension with and without the addition of 0.01 mM $CaCl_2$ (Smith and Mullins 2000).

To estimate the nutrient content, a saturated soil paste method was used. Distilled water was added to the dry soil until reflected light from the water was visible on the soil surface and the soil has consistency of sticky paste. The soil paste was left at room temperature for 24 h before the water was extracted and nutrient content of the extract measured. Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) Method-3VR was used to measure the nutrient content in soils and this was conducted by Waite Analytical Services, School of Agriculture, Food and Wine, Waite Campus, the University of Adelaide.

Table 4. Electrical conductivity (EC dS/m) and pH (with and without CaCl₂) of mixed Roseworthy and coco peat mix. The mean values of three replicates for EC and pH are given along with ± standard error (n=3).

Soil	$EC_{1:5}$ at 18° C (dS/m)	$pH_{1:5}$ in H_2O	$pH_{1:5}$ in $CaCl_2$
Mixed Roseworthy	0.56 ± 0.06	6.94 ± 0.09	6.8 ± 0.05
Coco peat mix	1.97 ± 0.1	5.78 ± 0.12	5.14 ± 0.1

2.2.3 Phenotypic characterisation

The fourth leaves were grown for 10 d in the salt treatment. The concentration of Na^+ and K^+ in fourth leaf was measured: after the leaf was harvested, the fresh weight was measured. The leaf was then dried at 65 °C in an oven (Contherm Scientific Ltd, Hutt City, New Zealand) for 48 h and dry weight of the leaf was assessed. After dried leaves were digested in 20 mL of 1% HNO_3 in a heat block (Adelab Scientific, Thebarton, Australia) for 4 h at 95 °C, the concentration of Na^+ and K^+ was measured using a flame photometer (Model 420, Sherwood Scientific, Cambridge, U.K) calibrated with of NaCl and KCl standard solutions. The Na^+ and K^+ readings were converted to concentrations using the following formula:

$$Coefficient = \frac{Standard(\mu M)}{unit\ in\ flame\ analysis} * Sample\ dilution\ factor * Volume\ for\ digestion\ (L)$$

$$Na^+, K^+ accumulation\ (mM) = \frac{Flame\ data * Coefficient}{Fresh\ leaf\ weight - Dry\ leaf\ weight}$$

After harvesting fully expanded fourth leaves for measuring Na^+ and K^+ accumulation, plants were grown for 15 days which then these were imaged and harvested for measuring Shoot biomass and plant height. Shoot plant biomass for all plants was measured non-distractively using plant image capture and analysis system (LemnaTec) and distractively using a physical

balance. The LemnaTec (LemnaTec Co, Würselen, Germany, http://www.lemnatec.com) is designed with two cameras and a rotating platform to allow plant images from different angles: from the top and two images from the side at a 90° horizontal rotation. Images were transferred on a computer as a RAW image file for further analysis.

In order to estimate basic leaf area from images, plant shoots need to be distinguished from the background and the pot. This was achieved by LemnaTec image analysis software (Version 2). The shoot area (Projected Shoot Area, PSA) of individual plants was calculated using the image based shoot sum (IBLS) model. The IBLS or PSA is defined as the sum of all areas viewed from each side of the shoot (IBSL = side 1area + side 2area + top area) (Rajendran et al. 2009). LemnaTec enables an assessment of the effect of salt stress on plants without requiring their destruction. Before plants were harvested (after 50 d of germination), these were imaged and the PSA of each plant for the control condition and salt treatment was estimated and used to calculate the salt tolerance index (STI_{PSA}) (Rajendran et al. 2009) as:

$$STI_{PSA} = \frac{PSA\ of\ salt\ treataed\ plants}{PSA\ of\ control\ plants}$$

After the imaging, plant height was measured from the soil surface to the tip of the stem. Plant shoots were harvested and shoot fresh weight (SFW) was collected before the plants were dried in an oven at 65 °C for 48 h for measuring the shoot dry weight (SDW). The salt tolerance index based on shoot fresh weight (STI_{SFW}) and shoot dry weight (STI_{SDW}) was then calculated:

$$STI_{SFW} = \frac{Shoot\ fresh\ weight\ of\ salt\ treated\ plants}{Shoot\ fresh\ weight\ of\ control\ plnats}$$

$$STI_{SDW} = \frac{Shoot\ dry\ weight\ of\ salt\ treated\ plants}{Shoot\ dry\ weight\ of\ control\ plants}$$

PSA derived from the captured images was compared with the conventional destructive measures. Also STI_{PSA} was also compared with STI_{SFW} and STI_{SDW}

2.2.4 Experimental Design

Uncompleted Random Block Design (URBD) was used to analyse data generated by growing Gladius and Drysdale under varying conditions in the Plant Accelerator, University of Adelaide. The URBD had four factors: two bread wheat cultivars (Gladius and Drysdale); two watering systems or pot types (drained pot and closed pot); two soil types (mixed Roseworthy and coco peat mix); and two salt treatments (0 and 100 mM NaCl). Each factor had four replicates except the cultivars * mixed Roseworthy * closed pot * 0 mM NaCl treatment which was replicated three times, where the separator symbol "*" between any factors represents the interaction term between them. The experiment was designed in four blocks, and randomised using GenStat 14 package (VSN International Ltd, Hemel Hempstead, UK) (Figure 8)

Figure 8. Uncompleted Random Block Design (URBD), with four factors: two bread wheat cultivars - Gladius (G) and Drysdale (D); two soil types - mixed Roseworthy (M) and coco peat (C); two salt concentrations - 0mM (0) and 100 mM (100) NaCl; and two pot types - closed (C) and drained (D) pots. All factors were repeated four times, each replicate (R) has 16 experimental units and four factors: for example, the first experimental unit in R1 is DM0D which means: D_rysdale * M_ixed Roseworthy soil * 0_mM NaCl * D_rained pot. Each experimental unit has a unique colour across replicates.

2.2.5 Statistical analysis

Linear mixed model and variance components estimation using the method of residual maximum likelihood (REML) (GenStat 14 package), were used to analyse the data. Two models were developed for both the fixed and the random terms. Four factors: cultivar, salt treatment, pot type, soil type, and the interactions between two, three and all four factors were treated in the fixed model as imposed treatment factors. Block (replicate) was treated in the random model as a random factor. The least significant difference (LSD) test was used in REML to identify significantly different means at a probability level of $P < 0.05$ or <0.01. The linear regression analysis package in Microsoft Excel was also used to study the relationship between the data generated from captured images and these derived from conventional destructive measures.

2.3 Results

Salinity tolerance traits (Shoot dry weight, salt tolerance index, 4th leaf Na^+ and 4th leaf K^+ accumulation and plant height) for Gladius and Drysdale grown at two levels of salinity were evaluated in order to test two different pot types and soil types.

2.3.1 Shoot dry weight (SDW)

A REML variance component analysis failed to reveal significant differences in SDW between cultivars, pot types and soil types, whereas the difference between control and salt treatment was highly significant for SDW ($p < 0.01$). The interaction between pot type and soil type shows similar high significance ($p < 0.01$). The analysis also shows that the interaction between cultivars and treatment was significant ($p < 0.05$), but the SDW differences between cultivars across all environments were not significant. The interaction between all four factors was also significant ($p < 0.05$).

Figure 9 a, b, c and d, SDW of plants in the control condition were consistently larger than those under salt treatment. The differences between cultivars were significant ($p < 0.05$) with respect to pot type and soil type. Both cultivars had almost the same SDW as in the control condition, whereas in the salt treatment, Gladius had larger SDW than Drysdale (Figure 9 a, b and d). In combinations of closed pots and two soil types (Figure 9a and b), Gladius shows better growth than Drysdale but this was not significant. In combination of coco peat mix and closed pot, the differences between Gladius and Drysdale were not significant (Figure 9c), but in the combination of mixed Roseworthy and drained pot the differences were significant ($p < 0.05$) (Figure 9d).
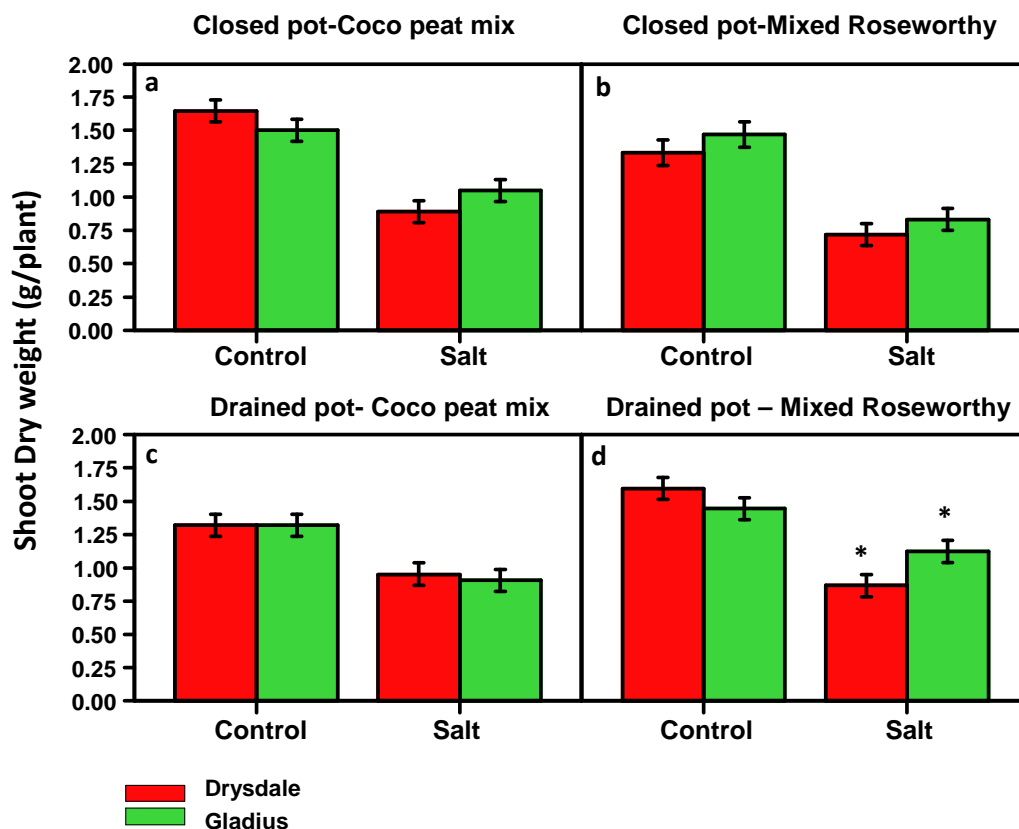
Figure 9. Shoot dry weight of Gladius and Drysdale grown under control and salt treatment conditions (X axis) in combinations of closed pot and coco peat mix (a), closed pot and mixed Roseworthy (b), drained pot and coco peat mix (c), and drained pot and mixed Roseworthy (d). Values are predicted mean ± s.e.d (n = 3-4) with asterisks (*) indicating a significant difference (LSD, p<0.05). LSD tests the significant differences of shoot dry weight between Gladius and Drysdale at each combination of pot and soil types.

2.3.2 Salt tolerance index (STI$_{SDW}$)

The STI$_{SDW}$ was derived by dividing the shoot dry weight of salt treated plants by the shoot dry weight of plants under control conditions (Section 2.2.3). REML analysis showed no significant difference in STI$_{SDW}$ between cultivars (Gladius and Drysdale), soil and pot types. Also, the differences of the interaction between cultivars and pot type, the interaction between cultivars and soil type, and the interaction between pot and soil type were not significant. However, the analysis revealed a significant difference (p<0.05) in STI$_{SDW}$ between the interaction of cultivars, pot and soil types. There was a significant difference between Gladius

and Drysdale in particular pot and soil types. The differences of STI_{SDW} between Gladius and Drysdale on combination of drained pots and mixed Roseworthy were highly significant (p<0.01) (Figures 10 b). The significant differences between Gladius and Drysdale for a single combination: drained pot-mixed Roseworthy may support the findings from the analysis of SDW (Section 2.3.1) and also suggest that drained pot–mixed Roseworthy is the better environment for screening of wheat plants in both salt treatment and control conditions.
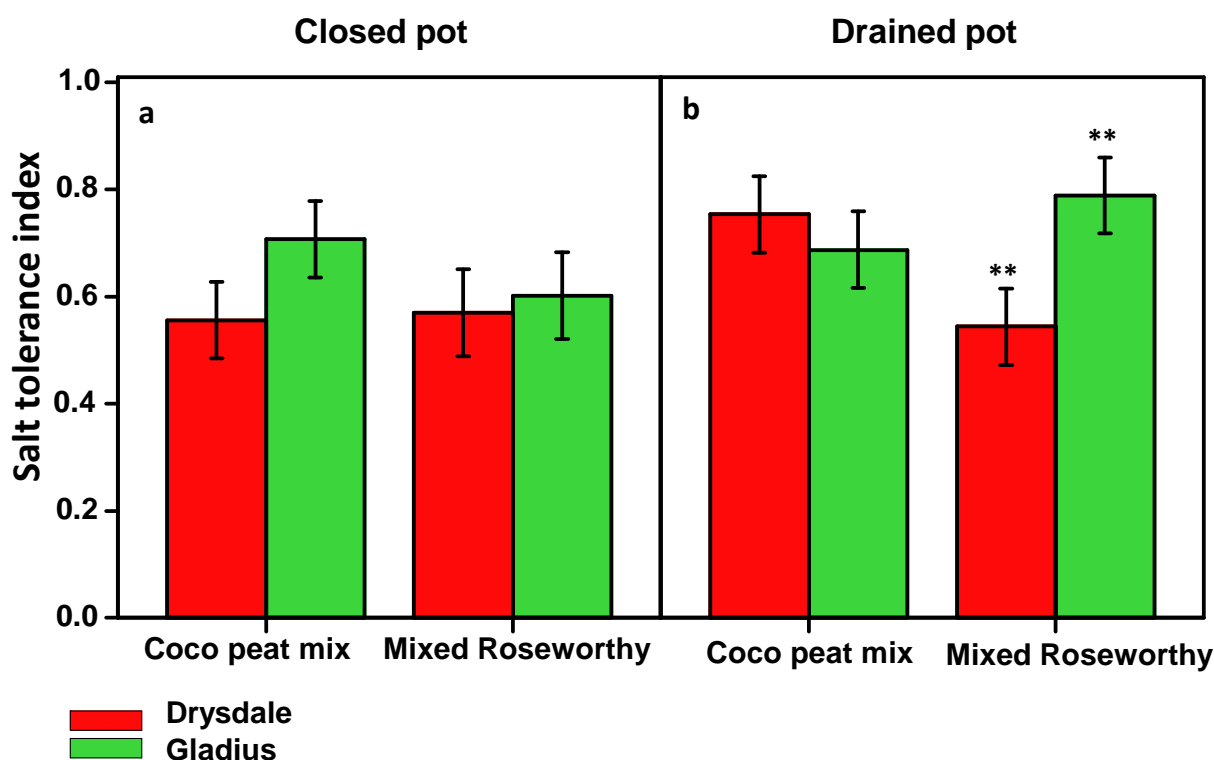
Figure 10. Salt tolerance index (relative shoot dry weight) of Gladius and Drysdale in combinations of closed pot and two types of soil (a) and combinations of drained pot and two types of soil (b). Values are predicted mean ± s.e.d (n = 3-4) with asterisks (**) indicating a significant difference (LSD, p<0.01). LSD tests the significant differences of salt tolerance index between Gladius and Drysdale at each combination of pot and soil types.

2.3.3   4th leaf Na+ accumulation

Na$^+$ accumulation was measured in the fully expanded fourth leaf after 20 d of 100 mM NaCl treatment (Section 2.2.3). The REML variance component analysis shows that there are highly significant differences ($p<0.01$) in accumulation of 4th leaf Na$^+$ between cultivars, between pot types and between soil types. The differences between most interactions were not significant except for the interaction between cultivars and soil types. Gladius accumulates lower Na$^+$ in the fourth leaf than Drysdale across pot and soil types (Figure 11a and b). The differences between cultivars in both pot types and in mixed Roseworthy were highly significant ($p<0.01$) (Figure 11a and b). 4th leaf Na$^+$ accumulation and the variation between cultivars in closed pots was larger than in drained pots ($p<0.01$, Figure is not illustrated in this section). Also, the variation in Na$^+$ accumulation between Gladius and Drysdale was significantly larger in mixed Roseworthy ($p<0.01$) in contrast to the coco peat mix, which was not significant (Figure 11a and b).This implies that the mixed Roseworthy is a better environment than coco peat mix for salt experiments.
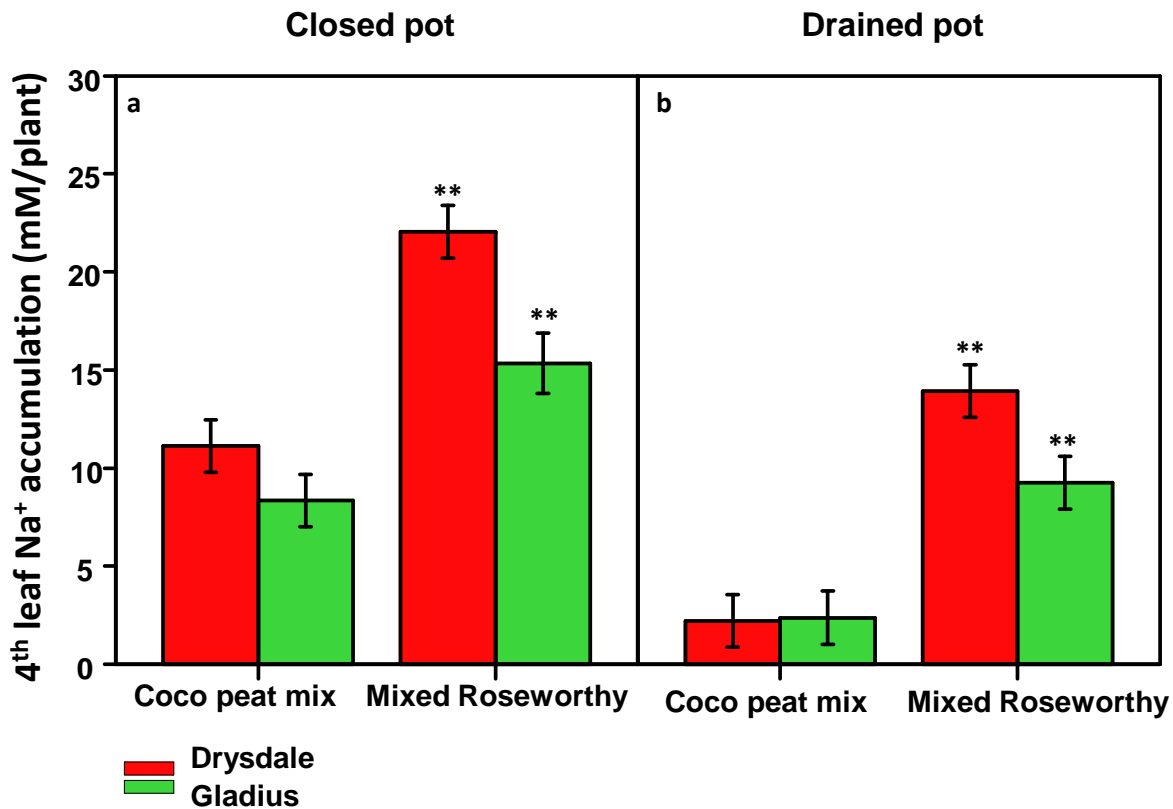
Figure 11. 4th leaf Na+ accumulation (tissue water based) of Gladius and Drysdale in combinations of closed pot and two types of soil (a) and combinations of drained pot and two types of soils (b). Values are predicted mean ± s.e.d (n = 3-4) with asterisks (**) indicating a significant difference (LSD, p<0.01). LSD tests the significant differences of 4th leaf Na+ accumulation between Gladius and Drysdale at each combination of pot and soil types.

2.3.4   4th leaf K+ accumulation

K^+ accumulation in the fully expanded fourth leaf was evaluated (Section 2.2.3). It was found

that there were highly significant differences in 4th leaf K^+ accumulation between cultivars and

between soil types (p<0.01), whereas differences between pot types were not significant. The

differences in 4th leaf K^+ accumulation between most interactions were not significant except

the interaction between cultivars and soil. Figures 12 a and b show that Gladius had higher 4th

leaf K^+ accumulation than Drysdale. This was opposite to the finding for 4th leaf Na^+

accumulation, as Gladius accumulated less 4th leaf Na^+ than Drysdale. The 4th leaf K^+

accumulation of Gladius was significantly larger than Drysdale in combination of closed pots and mixed Roseworthy (p<0.01) (Figure 12a) and in combination of drained pots with mixed Roseworthy (p<0.5) (Figure 12b). In combination of drained pots with coco peat mix, Gladius accumulate lesser 4th leaf K^+ than Drysdale but this was not significant (Figure 12b). This implies that the closed pot or drained pot and mixed Roseworthy soil are the better condition for the study of the salinity tolerance in wheat plants on the basis of 4th leaf K^+ accumulation. This result is also in agreement with the outcome from 4th leaf Na^+ accumulation.
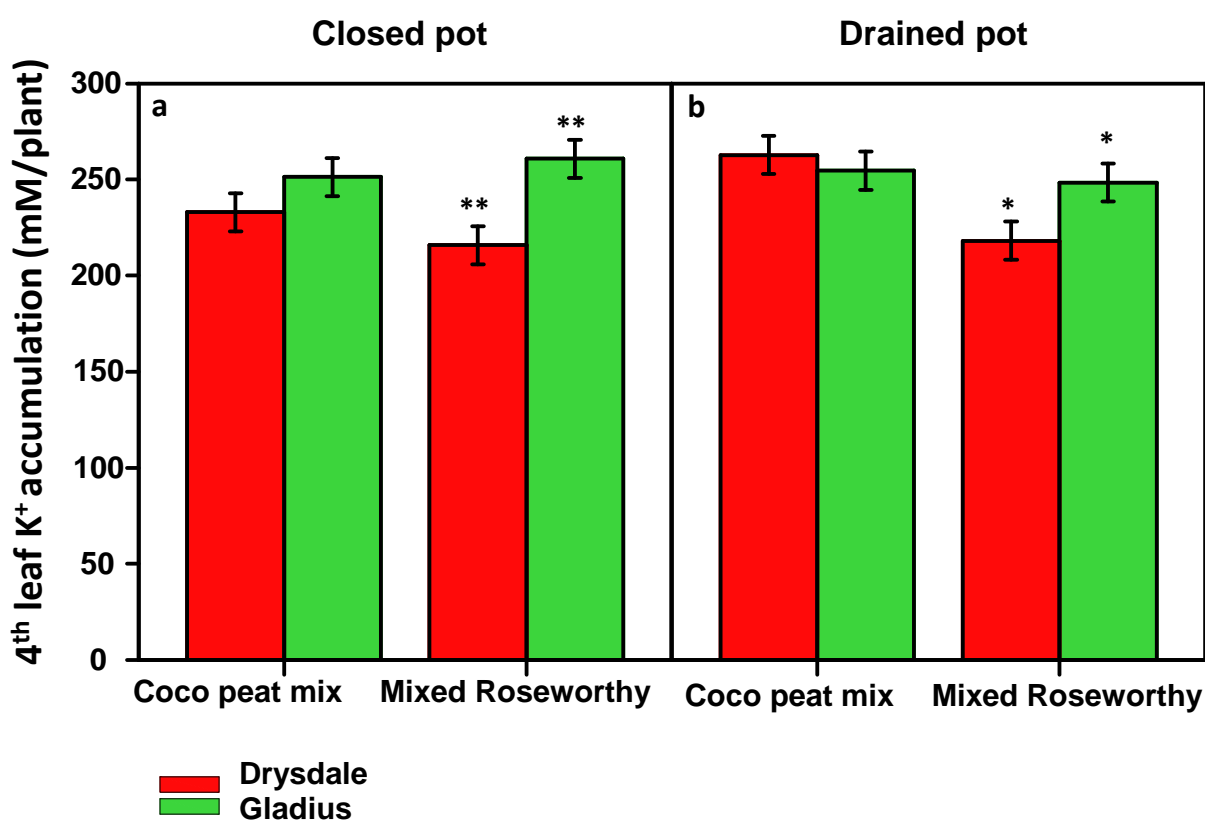
Figure 12. 4th leaf K^+ accumulation (tissue water based) of Gladius and Drysdale in combinations of closed pot and two types of soil (a) and combinations of drained pot and two types of soils (b). Values are predicted mean ± s.e.d (n = 3-4) with asterisks (*, **) indicating a significant difference (LSD, p<0.05 or p<0.01). LSD tests the significant differences of 4th leaf K^+ accumulation between Gladius and Drysdale at each combination of pot and soil types.

2.3.5 Plant height

The REML analysis revealed significant differences in plant height between cultivars ($p<0.05$) and between soil types ($p<0.05$), whereas there are no significant differences between pot types, treatments and the interactions between the factors. Drysdale was significantly taller than Gladius ($p<0.01$) across all environments (Figure 13). Plant height was found not to be influenced by salt (Figures 13a, b, c and d) and these results imply that plant height is not a promising salt tolerance trait in these materials.
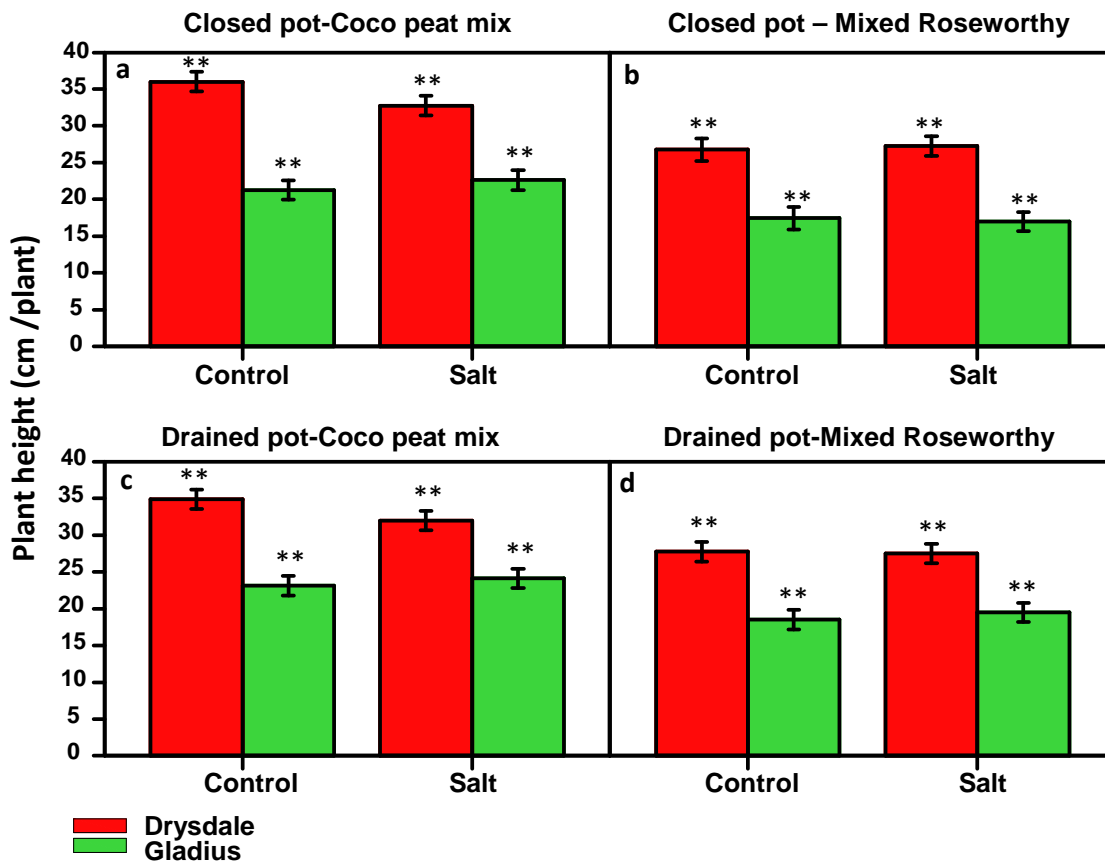
Figure 13. Plant height of Gladius and Drysdale grown under control and salt treatment conditions (X axis) in combinations of closed pot and coco peat mix (a), closed pot and mixed Roseworthy (b), drained pot and coco peat mix (c), and drained pot and mixed Roseworthy (d). Values are predicted mean ± s.e.d (n = 3-4) with asterisks (**) indicating a significant difference (LSD, p<0.01). LSD tests the significant differences of shoot dry weight between Gladius and Drysdale at each combination of pot and soil types.

2.3.6 A comparison of projected shoot area (PSA) with shoot fresh weight (SFW) and with shoot dry weight (SDW)

Plant imaging technology provides an advantage for plant shoot biomass measurements since it does not require destructive harvesting of the plants. However, it is necessary to compare the conventional destructive measures (SFW, SDW) with PSA derived from the captured images. Rajendran et al. (2009) showed a strong linear relationship between PSA and shoot fresh weight in *Triticum monococcum*. To illustrate that PSA is an adequate measure of SFW and of SDW in bread wheat (*T. aestivum*), the PSA was compared with SFW and SDW on the last day of the experiment. Plants under control and saline conditions across cultivars, pot and soil types were harvested and fresh and dry weights were measured. The relationship of PSA with SFW and PSA with SDW was linear, with r^2 values of 0.97 and 0.93, respectively (Figure 14 a and b). Also, the estimated salt tolerance index (STI_{FSW}) based on relative fresh shoot biomass was compared with a similar STI_{PSA} based on relative PSA. The relationship between the values was also linear with an r^2 of 0.95 (Figure 15a). When the STI_{SDW} calculated from the relative dry shoot weight was compared to the STI_{PSA} estimated from PSA, the relationship was still linear with an r^2 of 0.89 (Figure 15b). These results imply that the imaging technology is accurate enough for estimation of SFW and SDW and to evaluate the growth rate of the plants which was used to calculate osmotic stress tolerance.
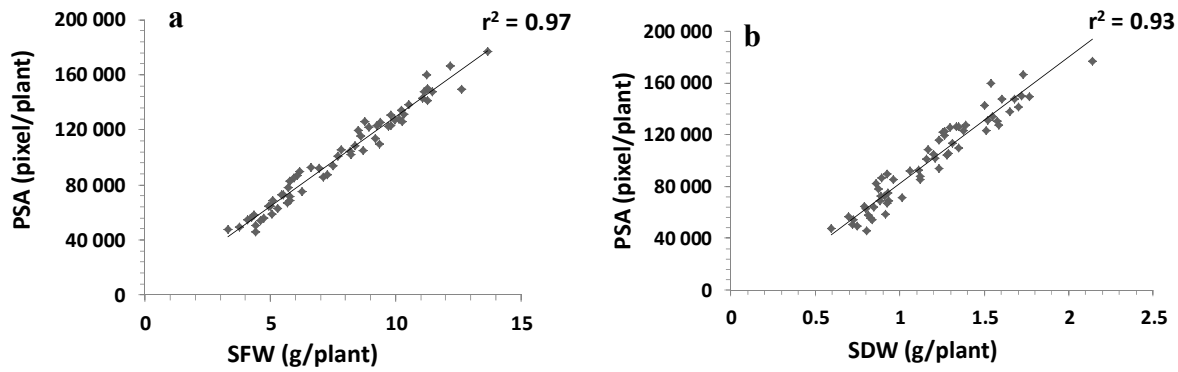
Figure 14. Relationships between projected shoot area (PSA) and (a) shoot fresh weight (SFW) and (b) shoot dry weight (SDW) of Gladius and Drysdale, grown in both 0 mM and 100 mM NaCl, in two soil and two pot types. Fitted linear regression equations: (a) *Y=130x – 881.5* and (b) *Y=97543x – 14941*.
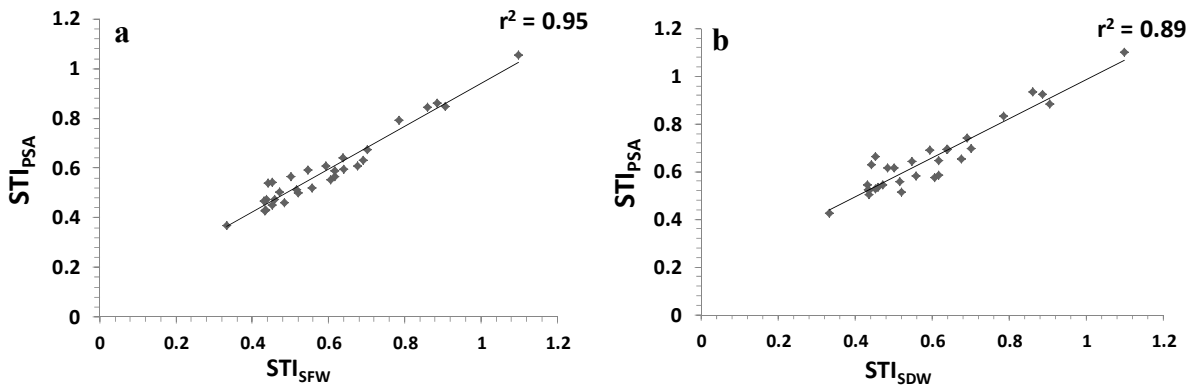
Figure 15. Relationships between salt tolerance index calculated from relative projected shoot area (STI_PSA) and (a) STI_SFW calculated from relative shoot fresh weight, and (b) STI_SDW calculated from relative shoot dry weight of Gladius and Drysdale, grown in both 0 mM and 100 mM NaCl in two soil and two pot types. Fitted linear regression is described by following equation: (a) *Y=0.86x + 0.079* and (b) *Y=0.82x+0.16*.

2.4 Discussion

An osmotic stress tolerance assay has been developed for small scale experiments (Rajendran et al. 2009), but a technique for screening large germplasm collections or populations has not been designed or assessed. The aim of the experiments was to establish an appropriate method for high-throughput screening for osmotic stress tolerance. Two types of pot and soil were evaluated by comparing two cultivars (Gladius and Drysdale) grown under control and salt conditions.

The REML analysis revealed significant differences ($P<0.01$) in 4th leaf Na^+ accumulation trait between the factors of mixed Roseworthy and coco peat mix and this difference is probably due to differences in the nutrient content of these soils. Coco peat mix had approximately three times greater K^+ content than mixed Roseworthy, whereas the Na^+ content of both soils was similar (Appendix III). Also, coco peat mix contains 10-fold more Fe^{2+} than mixed Roseworthy (Appendix III). The high content of K^+ and Fe^{2+} may moderate Na^+ accumulation in the shoot in both cultivars. Neid and Biesboer (2005) found that applying a low level of KNO3 could reduce the effect of NaCl on seed germination in certain grass species. Zheng et al. (2008) found that different levels of KNO3 can alleviate the negative impact of 100 mM NaCl in salt tolerant and salt sensitive wheat cultivars by increasing shoot and root biomass, reducing Na^+ accumulation and increasing K^+ accumulation in the shoot. These researchers concluded that increasing the concentration of K^+ in the soil will reduce the effects of ionic toxicity and improve osmotic stress tolerance in spring bread wheat. This suggests that coco peat mix is not a suitable growth medium for experiments where salt is applied.

All seedlings in pots were watered from the top at the beginning of the experiment to allow seedling root establishment. Salt solution was then added either into the saucer underneath the pot for drained pots or the top of each pot for closed pots after the surface of the soil had been allowed to dry. Results of 4th leaf Na^+ accumulation showed a high significant difference ($p > 0.01$) between closed pot (plants were watered from the top) and drained pots (plants watered from underneath) whereas the difference in SDW, STI_{SDW} and 4th leaf K^+ accumulation was not significant between the pot types. These results do not clearly show which the watering regime is favourable for screening salinity tolerance in wheat plants. However, the results of SDW, STI_{SDW}, and 4th leaf K^+ accumulation showed that the differences between Gladius and Drysdale were affected by the interaction between pot type and soil type. In SDW and ST_{SDW}, the differences between Gladius and Drysdale were significant ($p<0.05$, Figure 9d) and highly significant (LSD, $p<0.01$, Figure 10b) in combination of drained pot and mixed Roseworthy. Also, the differences between Gladius and Drysdale in 4th leaf Na^+ and 4th leaf K^+ accumulation were highly significant $p<0.01$, (Figure 11b) and significant ($p<0.05$, Figure 12b) in the combination of drained pot and mixed Roseworthy, and highly significant ($p<0.01$, Figure 11 a and Figure 12a) in the combination of closed pot and mixed Roseworthy. These results of four traits suggest that drained pot–mixed Roseworthy is the better environment for screening of the RIL population in both control and saline conditions.

SDW and STI_{SDW} of plants grown in closed pots was lower than in drained pots, and 4th leaf Na^+ accumulation for plants grown in closed pots was greater than for these grown in drained pots. This suggested that the reduction of SDW and STI_{SDW} of plants grown in closed pots was due to the elevated Na^+ concentration in the shoots. Munns (1993); Munns et al. (2002) showed that sodium-specific damage is correlated with high concentration of Na^+ in leaf tissues and this causes necrosis of older leaves where Na^+ accumulates to toxic levels . This shortened the life

of individual leaves which led to a reduction in net plant productivity and crop yield. However, Gladius and Drysdale showed symptoms of necrosis only in the old leaves for the combination of closed pot-mixed Roseworthy with a salt treatment. However, this damage accounted for about 0.019% of the SDW in affected plants which was statistically insignificant. This raised a question: was the STI_{SDW} in these cultivars due to shoot Na^+ accumulation, osmotic stress tolerance, Na^+ tissue tolerance or a combination of these physiological traits? This question was addressed in subsequent experiments conducted in pot soil (Chapter 3).

Under salt conditions Gladius was superior to Drysdale for all traits except the plant height which was not affected by salinity (Figures 9, 10, 11 and 12). It was observed that Gladius accumulated less 4th leaf Na^+ and more 4th leaf K^+ when compared with Drysdale. Also, SDW and STI_{SDW} in Gladius were greater than for Drysdale under salt treatment. However, these differences were affected by the interaction between pot and soil types. These results imply that 4th leaf Na^+ accumulation contributes to salinity tolerance in Gladius and Drysdale but this requires further study. Fleury et al. (2010) and Genc et al. (2007) showed that Gladius was classified as a low leaf Na^+ accumulator relative to Drysdale, and Shavrukov et al. (2006) demonstrated that Gladius accumulates less leaf Na^+ than other bread wheat cultivars. In the experiments described here it was not possible to determine which physiological traits made a major contribution to salinity tolerance. Therefore, experiments were designed and to study the contribution of 4th leaf Na^+ accumulation and osmotic stress tolerance to the salinity tolerance of bread wheat (Chapter 3).

In conclusion, the results revealed that coco peat mix may prevent the increase of 4th Na^+ accumulation in Gladius and Drysdale leaf tissue, and may reduce the salt effect and help plants to grow normally. Also, the interaction between pot and soil types had a significant impact on the studied traits. The main aim of the experiment was to select the most suitable environment

for screening osmotic stress tolerance in the Gladius * Drysdale RIL population. On the basis

results achieved in this experiment the drained pot - mixed Roseworthy was selected as the

most suitable medium for screening the RIL population.

Chapter 3: Enhancing salinity tolerance in elite bread wheat cultivars

3.1 Introduction

The genetic analysis of salinity tolerance has been based around the salinity exclusion trait and this has led to significant improvement in the tolerance of bread and durum wheat cultivars. However, exclusion accounts for only part of the overall salinity tolerance but has been the easiest and most reliable salinity tolerance trait to measure. In the previous chapter, a series of salinity tolerance traits were assessed for the parental lines Gladius and Drysdale. Gladius was more salt tolerance than Drysdale according to most traits under salt treatment over most soil and pot types, and recombinant inbred line population derived from the cross between these two lines provides an opportunity to dissect the genetic basis for the differences in response of these two cultivars to salt stress.

An osmotic stress tolerance assay has been developed for the A genome progenitor of wheat, *Triticum monococcum,* using a non-destructive imaging system (LemnaTec Scanalyzer 3D technology) (Rajendran et al. 2009). Rajendran (2012) used this technique on a bread wheat mapping population to detect QTL for osmotic stress tolerance, but the results of QTL analysis were less robust because they measured growth rate manually and could only assess a limited number of lines, reducing the accuracy of the measurement. In addition the population was grown in solution culture and, as shown in Chapter 2, the growth medium significantly affects the salinity response with the best results obtained using an actual soil based mix (Mixed Roseworthy-drained pots).

The aim of the study in this chapter was to determine the genetic control of osmotic stress tolerance in a segregating population derived from a cross between two Australian bread wheat cultivars (Gladius and Drysdale). 162 RILs and a linkage map enabled us to study

osmotic stress tolerance and associated QTL. Two experiments were conducted; using soil based conditions and a supported hydroponics system.

3.2 Materials and methods

3.2.1 Plant materials

The study described in Chapter 2 identified differences in salinity tolerance traits between two cultivars, Gladius being more salt tolerant than Drysdale. RILs derived from the cross of these cultivars were therefore selected to study the genetic of osmotic stress tolerance. Initially, 200 RILs were selected from the initial set of 250 RILs. The 50 RILs were excluded from the population due to the absence or poor quality of many of their genotyping scores (DArT and microsatellite markers). Drysdale and Gladius both were selected by AGT and ACPFG to produce a RIL population to study the genetic control of drought tolerance (Fleury et al. 2010). RILs were developed by selecting lines randomly from the fourth, fifth and sixth generations using a single seed descent method. The initial population size was 5000 RILs. A subset of 250 RILs was selected randomly and genotyped with DArT and microsatellite markers.

The soil based and supported hydroponics experiments were designed and carried out for the selected 200 RILs. Subsequently, when the linkage map was reconstructed (Section 3.3.1), some RILs were merged, leading to a reduction in population size from 200 to 162 RILs. The merger was deemed necessary since some RILs proved to be clones. Finally, the statistical and QTL analysis were carried out on these 162 RILs.

In addition, two additional bread wheat cultivars (Excalibur and Kukri) were used as controls in the soil based experiment.

3.2.2 Growth conditions

Two different growth conditions were used to screen plants at different salt concentrations: soil based conditions and a supported hydroponics system.

3.2.2.1 Soil based experiment

The soil based experiment was conducted in a glasshouse and SmartHouse in the Plant Accelerator, Waite Campus, University of Adelaide, South Australia. GL potting mix was used instead of the mixed Roseworthy soil as Roseworthy soil was not available. GL potting mix consisted of 35% UC potting soil (sandy soil from University of California protocol), 35% coco peat mix and 15% clay loam soil from Angle Vale, South Australia, Australia. Seeds were germinated in wet soil without using petri dishes. The seedlings were grown for 24 d in the greenhouse and then moved to the SmartHouse for imaging every second day over 15 d. At fourth leaf emergence, salt solution was applied as described in Section 2.2.2 with the exception that the concentration of 75 mM NaCl with supplementary 2.3 mM $CaCl_2$ was used and the salt was added to seedlings in one step and not incrementally. Control plants were grown in pots with no salt application. The temperature and humidity in the glasshouse and the SmartHouse were adjusted as described in Section 2.2.2. The experiment was conducted during autumn (March).

3.2.2.2 Hydroponics experiment

The hydroponics experiment was carried out in the greenhouse at SARDI, Waite Campus, South Australia, Australia. Seeds were germinated as described in Section 2.2.2. The uniform size seedlings were then transplanted into a supported hydroponics system (Figure 16) which is based on the method of Munns and James (2003). The seedlings were grown in polycarbonate cylinders (4 cm diameter x 28 cm depth) containing cylindrical black poly carbonate pellets (approximately 2–4 mm long and 1–2 mm in diameter) with one seedling per tube. These tubes were placed in a tub with 42 tubes per tub. Each trolley had two tubs. Eighty litres of modified Hoagland's solution was used, and consisted of: NH_4NO_3 (0.2 mM);

KNO$_3$ (5.0 mM); Ca(NO$_3$)$_2$•4H$_2$O (2.0 mM); MgSO$_4$•7H$_2$O (2.0 mM); KH$_2$PO$_4$ (0.1 mM); Na$_2$SiO$_3$ (0.5 mM); NaFe(III)–EDTA (0.05 mM); H$_3$BO$_3$ (0.05 mM); MnCl$_2$•4H$_2$O (0.005 mM); ZnSO$_4$•7H$_2$O (0.01 mM); CuSO$_4$•7H$_2$O (0.0005 mM) and Na$_2$MoO$_4$ •2H$_2$O (0.0001 mM). The growth solution was pumped from the reservoir tank to the tubs for 20 min, and then allowed to drain back to the tank for 20 min. During the experimental period, the growth solution was pumped continually back and forth. Every ten days, the growth solution was changed. The pH and EC of the solution were monitored to maintain the solutions with a pH between 6-7, and the EC between 1-2 dS/m in the control conditions and 11-12 dS/m in the salt treatment.
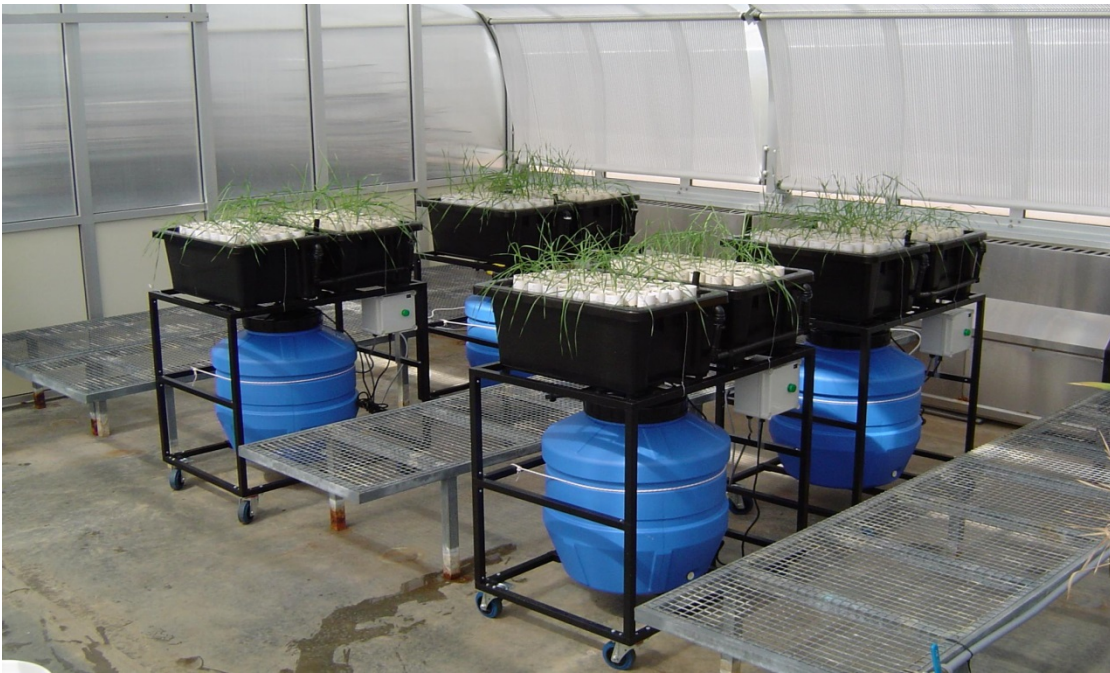
Figure 16. The supported hydroponics system used to grow RILs drived from crossing Gladius and Drysdale in control (0 mM NaCl) and salt (100 mM NaCl) treatments.

Ten days after transplanting and at the emergence of the third leaf, NaCl was applied to trolleys. 25 mM NaCl with supplemental CaCl$_2$.2H$_2$O was added daily to the solution until

100 mM NaCl was achieved. Supplemental calcium was added to give a Na^+: Ca^{2+} ratio of 30:1. Plants in the control trolley were grown without any salt addition.

The RIL population was grown for 45 d in the supported hydroponics system. The temperature in the greenhouse was adjusted as described in Section 2.2.2 The hydroponics experiment was conducted in spring (September).

3.2.3 Experimental design

3.2.3.1 Soil based experiment

Two experiments were designed: the first design was used in the greenhouse and the other in the SmartHouse. Plants were grown initially in a greenhouse at the Plant Accelerator and then moved to a SmartHouse 24 d after sowing. The population of 200 RILs was partially replicated: with 20% of the population duplicated. The parents (Gladius and Drysdale) were replicated six times and two control cultivars (Excalibur and Kukri) were replicated five times. Two experimental designs were undertaken with using DiGGer software (Coombes 2002). The greenhouse experiment was split into two areas (sides). Each area or side had five rows which were divided into four blocks. Blocks 1, 2 and 3 in each side had sixteen columns, and block 4 in each side had five columns. Each block had 80 plants (pots) except for block 4 which had only 22 plants (Figure 17). In the SmartHouse, all plants (524 plants) were grown in three groups. Each group had eleven rows and sixteen columns (Figure 18).

In the greenhouse and SmartHouse, each genotype was grown in two soil pots: one being used as a control and another pot being treated with 75 mM NaCl. Both pots for each genotype were located in one particular area and each pair was referred to as a 'whole'.

Once in the SmartHouse, the seedlings were imaged using a LemnaTec platform and data for projected shoot area were collected. These data were used to assess the impact of the greenhouse spatial factors (rows and columns). Then, the plants were imaged every second

day. Before the plants were harvested 39 d after sowing, these were imaged and this information was used to calculate the impact of position in the SmartHouse.
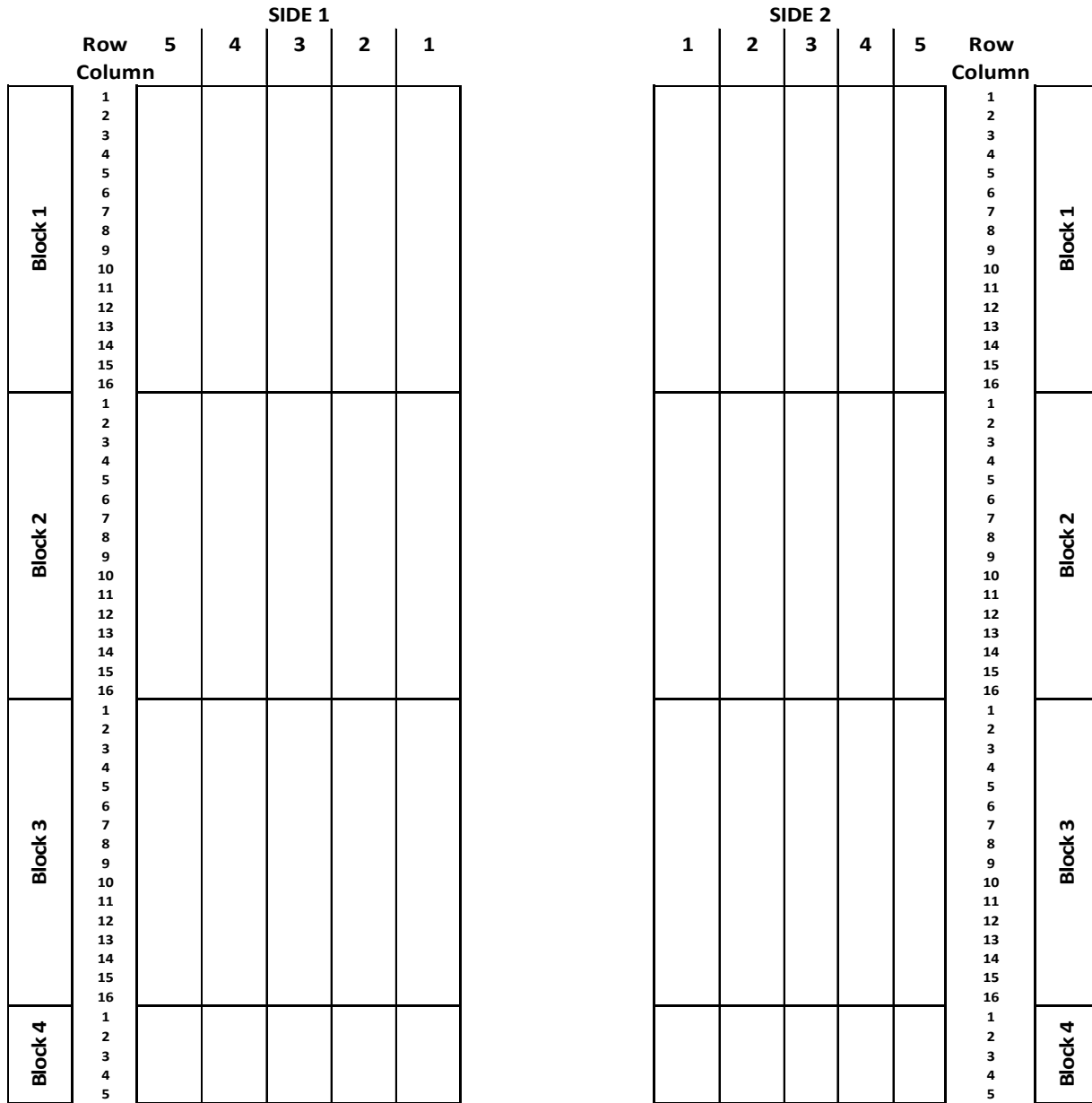
Figure 17. Layout of the soil based experiment in the greenhouse. The greenhouse was split into two sides. Each side had five rows which were divided into four blocks. Blocks 1, 2 and 3 in each side had sixteen columns, and block 4 in each side had five columns. Each block had 80 plants (pots) except for block 4 which had only 22 plants. The RILs, parents and standard Australian bread wheat cultivars were sown in the greenhouse and grown for 24 d before the plants were moved to the SmartHouse.

Figure 18. Layout of the soil based experiment in the SmartHouse. Gladius is shown in red, Drysdale in green, Excalibur in pink, Kukri in bright blue, RILs which were replicated twice in tan, RILs which were not replicated in sky blue, and empty pots in yellow. The plants were divided into three groups. The plants were moved to the SmartHouse before the emergence of leaf 4. Plants were grown in the SmartHouse for 15 d in total; 4 d before and 11 d after salt application.

3.2.3.2 Hydroponics experiment

The experimental design was carried out with using DiGGer software (Coombes 2002). Of the 200 members of the RIL population, 20% were duplicated and the parents were replicated six times in each treatment. The RILs and parents were allocated across three trolleys per treatment: three trolleys for control condition and three trolleys for salt treatment. Each trolley had two tubs which were divided into rows and columns (Figure 19).

Columns

Rows

Tub 1 (Trolley 1, Control)

Row	1	2	3	4	5	6	7	8	9
1	0	59	2	73	162	Gladius	225	149	140
2	114	Drysdale	77	126	127	220	180	209	25
3	244	51	46	67	165	23	241	54	196
4	47	230	219	235	39	247	90	226	115
5	0	14	239	57	124	216	215	202	0

Tub 3 (Trolley 2, Salt)

Row	1	2	3	4	5	6	7	8	9
1	0	59	2	73	162	Gladius	225	149	140
2	114	Drysdale	77	126	127	220	180	209	25
3	244	51	46	67	165	23	241	54	196
4	47	230	219	235	39	247	90	226	115
5	0	14	239	57	124	216	215	202	0

Tub 2 (Trolley 1, Control)

Row	1	2	3	4	5	6	7	8	9
1	0	32	226	103	146	48	46	64	44
2	219	121	195	71	82	62	174	100	Gladius
3	55	203	216	182	68	186	175	65	246
4	116	188	Drysdale	214	231	84	165	40	236
5	0	50	52	23	98	57	17	153	0

Tub 4 (Trolley 2, Salt)

Row	1	2	3	4	5	6	7	8	9
1	0	32	226	103	146	48	46	64	44
2	219	121	195	71	82	62	174	100	Gladius
3	55	203	216	182	68	186	175	65	246
4	116	188	Drysdale	214	231	84	165	40	236
5	0	50	52	23	98	57	17	153	0

Tub 5 (Trolley 3, Salt)

Row	1	2	3	4	5	6	7	8	9
1	0	29	178	201	83	10	189	4	137
2	80	148	36	152	104	158	86	72	132
3	198	64	111	58	122	74	200	47	45
4	11	88	Gladius	215	163	173	207	91	250
5	0	42	20	133	194	169	Drysdale	78	0

Tub 7 (Trolley 4, Control)

Row	1	2	3	4	5	6	7	8	9
1	0	29	178	201	83	10	189	4	137
2	80	148	36	152	104	158	86	72	132
3	198	64	111	58	122	74	200	47	45
4	11	88	Gladius	215	163	173	207	91	250
5	0	42	20	133	194	169	Drysdale	78	0

Tub 6 (Trolley 3, Salt)

Row	1	2	3	4	5	6	7	8	9
1	0	243	151	163	135	67	20	34	142
2	242	45	118	70	Gladius	104	18	92	166
3	205	158	22	147	43	161	76	31	7
4	240	248	40	138	132	96	143	201	195
5	0	238	102	140	Drysdale	79	123	60	0

Tub 8 (Trolley 4, Control)

Row	1	2	3	4	5	6	7	8	9
1	0	243	151	163	135	67	20	34	142
2	242	45	118	70	Gladius	104	18	92	166
3	205	158	22	147	43	161	76	31	7
4	240	248	40	138	132	96	143	201	195
5	0	238	102	140	Drysdale	79	123	60	0

Tub 9 (Trolley 5, Control)

Row	1	2	3	4	5	6	7	8	9
1	0	134	97	212	129	Gladius	199	9	218
2	177	150	161	221	245	21	233	176	181
3	63	31	61	190	224	108	32	127	228
4	120	69	125	4	186	139	107	249	160
5	0	5	141	184	192	Drysdale	93	3	0

Tub 10 (Trolley 6, Salt)

Row	1	2	3	4	5	6	7	8	9
1	0	134	97	212	129	Gladius	199	9	218
2	177	150	161	221	245	21	233	176	181
3	63	31	61	190	224	108	32	127	228
4	120	69	125	4	186	139	107	249	160
5	0	5	141	184	192	Drysdale	93	3	0

Tub 11 (Trolley 5, Control)

Row	1	2	3	4	5	6	7	8	9
1	0	159	245	134	25	171	211	109	125
2	75	119	249	105	85	49	61	51	87
3	37	74	13	99	76	234	68	15	Drysdale
4	Gladius	136	1	229	130	198	150	94	128
5	0	206	26	139	191	185	144	223	0

Tub 12 (Trolley 6, Salt)

Row	1	2	3	4	5	6	7	8	9
1	0	159	245	134	25	171	211	109	125
2	75	119	249	105	85	49	61	51	87
3	37	74	13	99	76	234	68	15	Drysdale
4	Gladius	136	1	229	130	198	150	94	128
5	0	206	26	139	191	185	144	223	0

Legend:
- Trolley 1, Control
- Trolley 2, Salt
- Trolley 3, Salt
- Trolley 4, Control
- Trolley 5, Control
- Trolley 6, Salt

Figure 19. Layout of the experimental design for the hydroponics experiment. Three trollies were used for the control conditon (0 mM NaCl) and three for the salt treatment (100 mM NaCl). Each trolley has two tubs. Plants were grown in nine columns and in five rows in each tub. 200 RILs and parents (Gladius and Drysdale) were used. RILs were partially replicated, whereas the parents were replicated six times.

3.2.4 Linkage analysis

A subset of 250 RILs derived from the cross between Gladius and Drysdale was genotyped with 875 markers (672 DArT and 203 microsatellite markers) and four markers that are diagnostic for vernalisation (*Vrn-A1* and *Vrn-D1*) and photo period (*Ppd-B1* and *Ppd-D1*) genes. The initial linkage map of the RIL population was constructed with MapManager QTX version QTXb20 (Manly et al. 2001) using the Kosambi mapping function with a threshold value of P = 0.01. The initial linkage map had 30 linkage groups as some chromosomes contain two linkage groups and some chromosomes were over 200 cM. The initial linkage map was constructed by Dr. Ken Chalmers (The University of Adelaide, South Australia, Australia).

In this Chapter, the linkage map of Gladius and Drysdale RIL population was improved by being re-constructed with advice and assistance of the statisticians Mr. Chris Lisle (Charles Sturt University, New South Wales, Australia) and Mr. Paul Eckermann (University of Adelaide, South Australia, Australia). The `read.interval` function from the Wgaim package in R software (Version 2.12.1) was used for removing coincident markers and markers with the most number of missing values. The `ripple` function in the R/QTL package was also applied using the likelihood analysis method to re-order markers. In addition, the `gn.clones` function in the Wgaim package in R was used to calculate the genetic correlation between line pairs. The map was drawn using MapChart 2.1 (Voorrips 2002).

3.2.5 Phenotypic characterisation

In the soil based experiment, after 24 d of sowing, the plants were imaged every second day for 15 d. The total projected shoot area was determined as outlined in Section 2.2.3.

The projected shoot area was used with two assays to establish an osmotic stress tolerance value for the Gladius * Drysdale RIL population. OST1 involved measuring and calculating

the growth rates for the RILs in the two environments (control condition and salt treatment) over a period of 10 d after salt application in the SmartHouse. The OST2 was measured using the growth rate of the RILs over the four days before and after the application of salt.

The salt tolerance index was calculated based on relative projected shoot area (PSA) as described in Section 2.2.3. Tiller number in the soil based conditions experiment was recorded before the plants were harvested.

In the supported hydroponics experiment shoot fresh biomass, shoot dry biomass, tiller number, water content, salt tolerance index and water content index of RILs and the parents were evaluated in both control and salt treatments. After 45 d, the plant shoots were harvested and the shoot fresh biomass and tiller number were recorded. The shoot fresh and dry weights from the control condition and salt treatment and the salt tolerance index were evaluated as described in Section 2.2.3.

The water content of the control and salt treated plants and the water content index were then calculated using the following formula:

$$Water\ content = \frac{fresh\ shoot\ biomass - dry\ shoot\ biomass}{dry\ shoot\ biomass}$$

$$Water\ content\ index = \frac{water\ content\ of\ salt\ treated\ plants}{water\ content\ of\ control\ plants}$$

The 4th leaf Na^+ and 4th leaf K^+ accumulation was measured in the soil based and the hydroponics experiments as outlined in Section 2.2.3.

3.2.6 Statistical analysis

The growth rate per plant was estimated by generating fitted models using a cubic smooth spline which was incorporated in linear mixed model. The analysis was conducted in asreml-R (version 2.12.1). The growth rate values were used to estimate OST1 and OST2.

Heritability and the value for best linear unbiased estimates (BLUE) for each trait were estimated using linear mixed model and variance components in the method of residual maximum likelihood (REML) (GenStat 14th edition, VSN International Ltd, Hemel Hempstead, UK). In order to calculate heritability and to generate BLUE, models were developed for the fixed and random terms in which a polygenic effect and non-genetic sources of variation were included.

To estimate heritability in soil based experiment, genotypes (Type) were treated in the fixed model as imposed factors. The factor Type had five levels (Gladius, Drysdale, Kukri, Excalibur and RIL population). RILs, blocks, sides, row, columns and whole (greenhouse factors) in the greenhouse (Figure 17) and also rows, columns and whole (SmartHouse factors) in the SmartHouse (Figure 18) were treated in the random model as random factors.

Similarly in the hydroponics experiment, genotypes (Type) were treated the fixed model as imposed factors. The factor Type had three levels (Gladius, Drysdale and RIL population) RILs, rows, columns, tubs, trollies (trolley factors) (Figure 19) were treated in the random model as random factors.

In both experiments, each studied trait had different models in the random terms but, in the fixed model, Type was used as an imposed treatment factor for all the traits (Appendix IV, Sections 6.4.1 and 6.4.2).

To calculate the value for BLUE for each studied trait which was used in the QTL analysis, the individual RIL was used as an imposed treatment factor in the fixed model. Greenhouse

and SmartHouse factors in the soil based experiment and trolley factors in the hydroponics experiment and were used in the random model (Appendix IV, Sections 6.4.1 and 6.4.2).

In addition, the correlation analysis package in GeneStat 14th was used to estimate the correlation and its coefficient between the studied traits in both the soil based and the hydroponics experiments.

3.2.7 QTL analysis

QTL analysis was conducted using the mixed model QTL package in GenStat 14th edition (VSN International Ltd, Hemel Hempstead, UK). Phenotypic (BLUE), genotypic and map data were exported to GenStat and analysis was undertaken in three steps. Firstly, Simple Interval Mapping (SIM) was performed with a step size of 10 cM. SIM tests the association between phenotypic and genotypic data across the genomic grid using a genetic predictor. Several QTLs were detected and the significant locus for each found QTL was selected automatically. The second step was performed using Composite Interval Mapping (CIM). The function of CIM is the same as SIM, but it controls the background QTL segregating across the genome by including covariates representing the background QTL (markers and genetic predictors). CIM was conducted three or four times. The third step was the backward regression or final QTL model which uses the information from both SIM and CIM. The backward regression develops a model for candidate QTL and detects the largest significant loci across the genome. The percentage of the phenotype explained by QTL effects, the significant size interval for loci in cM, and the additive effect of candidate QTL were also calculated from backward regression. The resulting QTL were classified as ''significant'' or ''not significant'' according whether the associated LOD was greater or less than 3.

3.3 Results

3.3.1 Linkage analysis

The initial linkage map was constructed with 875 markers. The map had 30 linkage groups as some chromosomes contain two linkage groups and some chromosomes were over 200 cM, for example, chromosome 7B was 516.83 cM. This indicated significant quality problems in the map. The linkage map of the Gladius * Drysdale was therefore required to re-construct. The `read.interval` function was used for re-constructing the map. This function removed 136 coincident markers and markers with the most number of missing values. 739 markers were retained from the initial 875 markers on the map (Table 5). The `ripple` function was also applied in order to re-order markers. After several iterations, the length of each chromosome and the number of crossovers along the chromosome were reduced. As an example, the total length of chromosome 1B-3 was reduced from 277.82 cM to 209.8 cM (Table 5) and the number of crossovers was reduced by 511 to 378. After further investigation, when using marker information, it was found that the some lines were clones. The genetic correlation between line pairs was calculated using the `gn.clones` function in the Wgaim package in R. The correlation between clustered lines was high when only DArT markers were used and lower when only microsatellite markers were used (Appendix II). As an example, the correlation of DArT markers in Cluster 1 was 98% whereas the correlation of microsatellite markers was 89% for the same cluster (Appendix II). Therefore, the clustered lines were merged to one line which resulted in the population size being reduced from 200 to 162 RILs.

Table 5. Improved linkage maps of RILs derived from the cross between Gladius and Drysdale. The first column shows 30 linkage groups across the 21 chromosomes of bread wheat. The second column indicates the number of markers on each chromosome. The third column represents the improved map re-constructed using R software (version 2.12.1).

Linkage group	Number of markers	Length (cM)
1A	15	121.8
1A	29	12.4
1B	31	209.8
1B	4	2.1
1D	21	132.2
1D	19	9.9
2A	53	169.1
2B	45	260.2
2D	11	198.2
2D	18	35.8
3A	21	246.7
3A	14	63.5
3B	70	335.8
3D	17	39.9
4A	55	146
4B	15	200.1
4D	6	0
5A	13	260.4
5B	49	197.1
5D	4	11.4
5D	3	43.8
5D	2	0
6A	28	142.1
6A	8	109.2
6B	55	133
6B	15	19.3
7A	19	176.4
7A	20	37.4
7B	74	288.3
7D	5	41.3
Total	**739**	**3643.2**

3.3.2 Soil based experiment

3.3.2.1 Phenotyping

Osmotic stress tolerance (OST) and the contribution of OST to salt tolerance in the RIL population was assessed for six traits: salt tolerance index (STI), osmotic stress tolerance-assay1 (OST1), osmotic stress tolerance-assay2 (OST2), tiller number (TN), and 4th leaf Na^+ and 4th leaf K^+ accumulation.

The growth rate of RILs, parents and the control cultivars grown in saline and non-saline conditions was estimated and used in two assays (OST1 and OST2). These assays were used to establish an osmotic stress tolerance value for the Gladius * Drysdale RIL population. The growth rate for each plant was estimated from the fitted model with cubic splines using asreml-R (Version 2.12.1). Typically (the estimation of growth rate for OST1 is given as an example), the projected shoot area (PSA) of each plant (Figure 20 a) was transformed to a logarithm 10 scale (log_{10}) to fix the growth curve of each plant to a line as shown in Figure 20 b. Cubic splines were incorporated with linear mixed model in order to find the best fitted model. Within the mixed model framework, PSA per plant and at a single time point was modelled to be a sum of fixed and random terms as shown in models 1, 2 and 3 below. When, the final fitted model was identified, the fitted value for log_{10} of PSA per plant was estimated in order to fit parametric curves on the trend of the growth rate over time (Figure 20 c). Missing values of PSA was estimated (predicted values) (Figure 20 d). Fixed coefficients were then calculated from predicted fitted values. The growth rate of each plant over a time period was obtained from fixed coefficients.
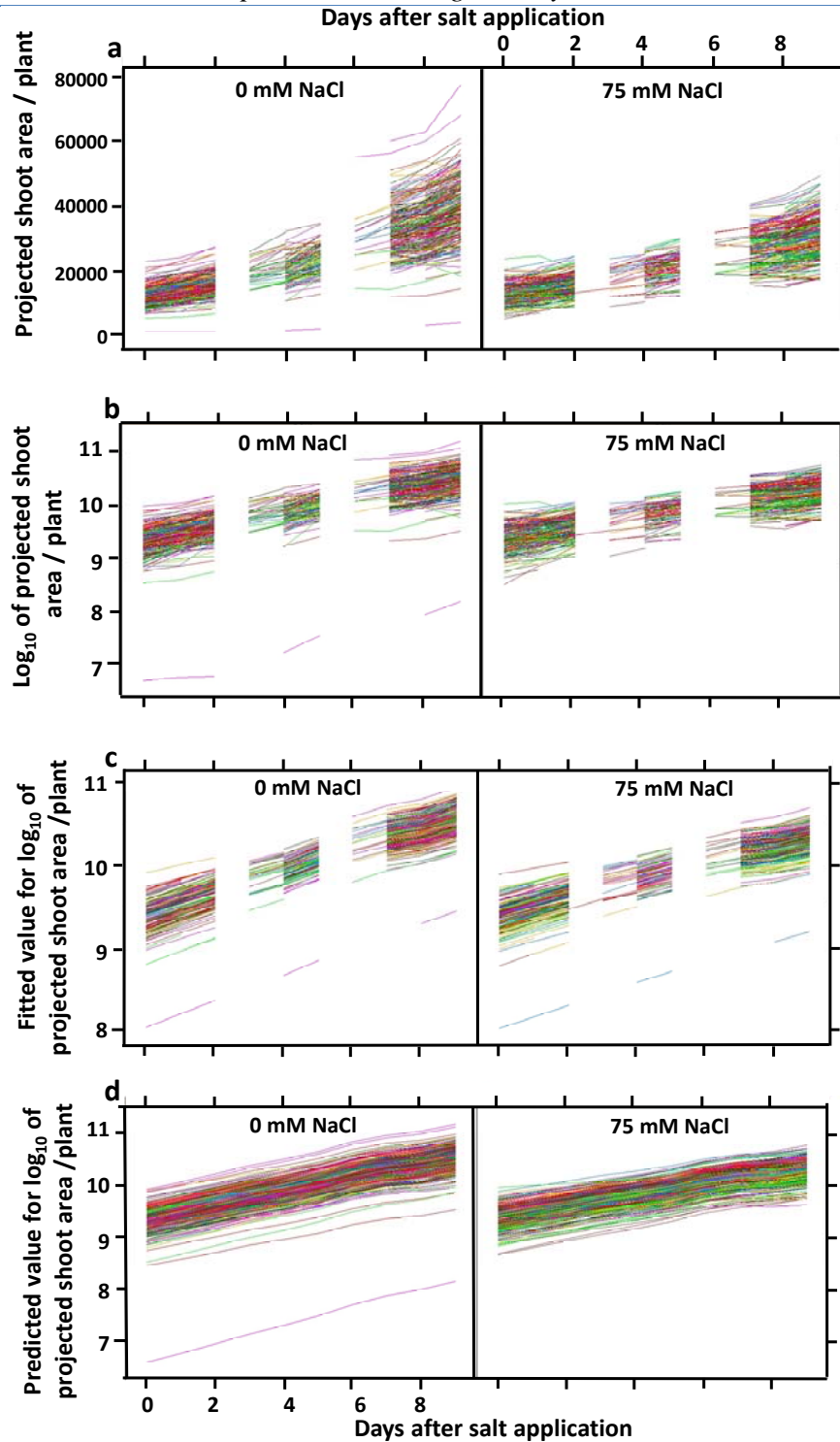
Figure 20. The evaluation of growth rate (which was used to estimate OST1) for RILs, parents and control cultivars grown over 10 days of salt application (0 mM NaCl and 75 mM NaCl). Projected shoot area (PSA) was evaluated by taking images from three angles of the plant and analysing the images using LemnaTec software (Version 2) (a). PSA per plant was then transformed using logarithm 10 ($\log_{10}$) (b). $\log_{10}$ of PSA per plant was used to estimate fitted value per plant using the final fitted model (c). Finally, missing values of PSA were estimated and fixed coefficient was calculated (d). The growth rate for each plant was evaluated from the fixed coefficient. X axis denotes day 0 – 9 shown in table 6.

The growth rate for OST1 was calculated using one fitted model for the period from day 0 to day 9 (Table 6) as:

Model 1: Fitted model for growth rate for the period after salt application (days 0 to 9)

*Days + PSA + Days * PSA | spline (Days) + spline (Days) * Treatment + spline (Days) * PSA*

Where *PSA* is the projected shoot area for each plant, *Days* is days of imaging as shown in Table 6 and *Treatment* is salt treatments (0 and 75 m M NaCl). The fixed terms are written to the left of the '|' and random terms to the right. The separator symbol "*" between any factors represents the interaction term between them.

OST1 was estimated as the ratio of the growth rate of salt treated plants to the growth rate of control treated plants as:

$$OST1 = \frac{Growth\ rate\ for\ salt\ treated\ plant}{Growth\ rate\ for\ control\ plant}$$

The growth rate for OST2 was estimated using two models fitted for the period before salt application (days -4 to -1) and for the period after salt application (days 0 to 3) (Table 6) as:

Model 2: Fitted model for growth rate before salt application (days -4 to -1)

*Days + PSA + Days * PSA | spline (Days)*

Model 3: Fitted model for growth rate after salt application (days 0 to 3)

*Days + PSA + Days * PSA| spline (Days) * PSA*

Where *PSA* is the projected shoot area for each plant and *Days* is days of imaging as shown in Table 6. The fixed terms are written to the left of the '|' and random terms to the right. The separator symbol "*" between any two factors represents the interaction term between them.

OST2 was calculated as the ratio of growth rate after salt application to the growth rate before salt application as:

$$OST2 = \frac{Growth\ rate\ for\ salt\ treated\ plant\ after\ salt\ application\ (day\ 0 - day\ 3)}{Growth\ rate\ for\ salt\ treated\ plant\ before\ salt\ application\ (day - 4 - day - 1)}$$

Table 6. Dates of the daily plant imaging before 4 d and 10 d after application of 75 mM NaCl. The plants were grown in the SmartHouse in The Plant Accelerator as mentioned in Sections 3.2.2

Date	10/03/2011	11/03/2011	12/03/2011	13/03/2011	14/03/2011	15/03/2011	16/03/2011	17/03/2011
Day	-4	-3	-2	-1	0	1	2	3
Date	18/03/2011	19/03/2011	20/03/2011	21/03/2011	22/03/2011	23/03/2011	24/03/2011	25/03/2011
Day	4	5	6	7	8	9	10	11

These two methods were evaluated to confirm that both assays are correlated with STI in the Gladius * Drysdale RIL population as outlined in Section 3.3.2.2.

The RIL population showed a normal distribution for most traits, with the except for 4th leaf Na$^+$ accumulation which is skewed towards a high Na$^+$ concentration, and TN which did not have normal distribution (Figure 21). The means for all traits for the parents (Gladius and Drysdale), the standard Australian bread wheat cultivars (Excalibur and Kukri) and 162 RILs and also the range for each trait in the RIL population are shown in Table 7. For all traits apart from TN, the variation between the parents is large, and the distribution of traits in the population shows a wider range of values than the parents. Most of the traits, therefore, showed transgressive segregation in both directions implying that several genes could be associated with these traits with desirable alleles at some loci for either parent. Heritability for all traits was small, ranging between 0.24 – 0.15 (table 7)
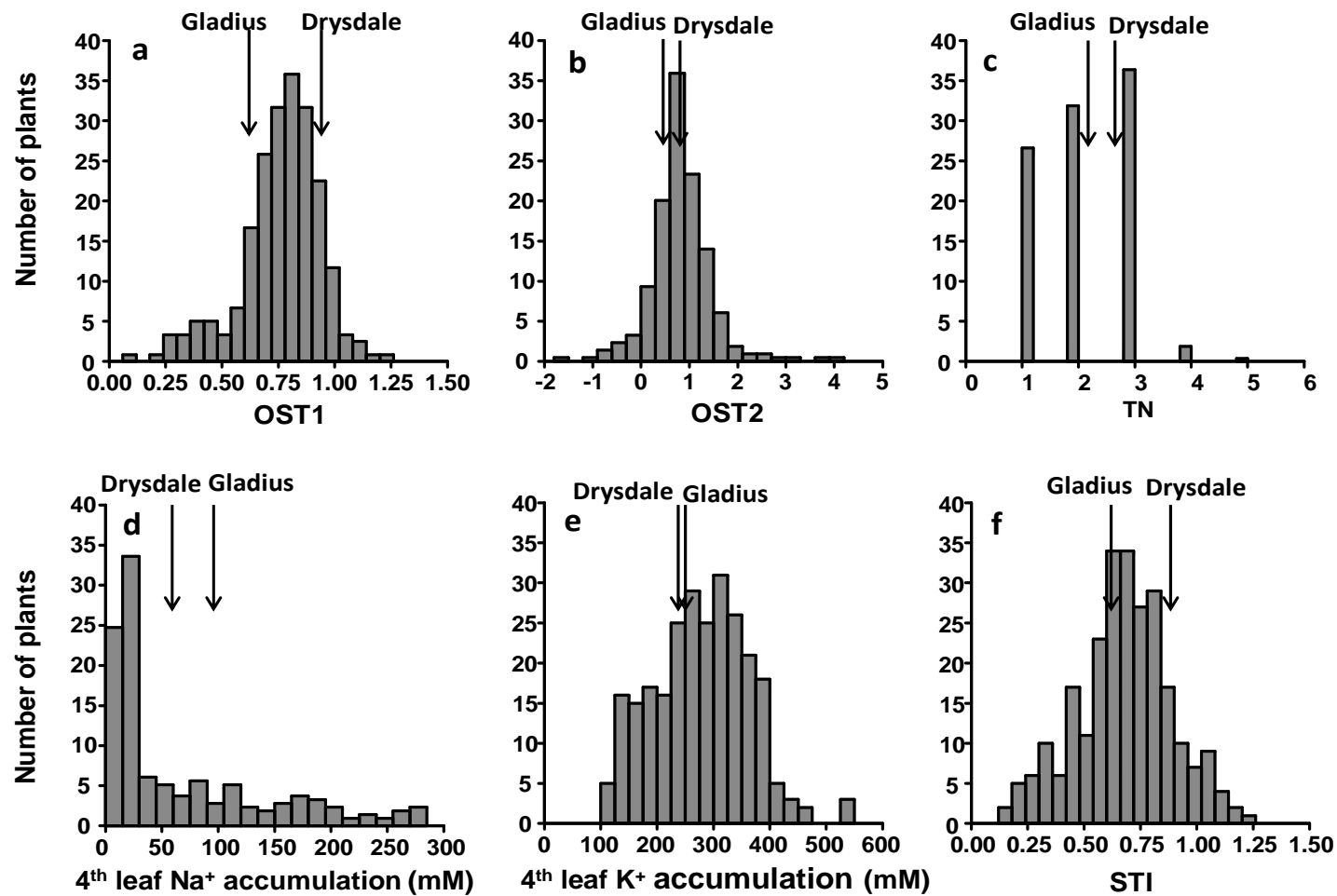
Figure 21. Frequency distribution of osmotic stress tolerance – assay 1(OST1) (a), assay 2 (OST2) (b), number of tiller on salt treated plants (TN) (c) , 4th leaf Na$^+$ accumulation (mM) (d), 4th leaf K$^+$ accumulation (mM) (e), salt tolerance index (STI) (f) in the Gladius * Drysdale RILs. Arrows indicate the trait mean for parents, Gladius and Drysdale.

OST was estimated as a ratio of the growth rate of a genotype growing under salt treatment relative to the same genotype growing in the control treatment. In general, the ratio must be between 0 (the genotype does not grow in salt treatment) and 1 (when the genotype maintains normal growth under salt treatment), but some lines were outside this scale due to tissue loss under the salt treatment (negative OST) or accelerated growth in response to salt (OST greater than 1). The range of values for OST1 across the RIL population was 0.11 to 1.22, whereas the range of OST2 was between -1.7 and 4.1. When stresses are imposed on plants, some plants will stop growing or grow very slowly, and others maintain their growth rate. The large range of OST2 scores show that some genotypes had negative values for OST and that means that the seedlings for these genotypes died under salt treatment. The method based on period of four days before and after salt application may not adequate to estimate the reduction of growth rate induced by salt stress. This suggest that OST2 may not be a reliable assay for studying osmotic stress tolerance and therefore this was not used for further genetic analysis in the Gladius * Drysdale RIL population.

The leaf 4th Na^+ accumulation of Gladius is larger than Drysdale (Table 7). These results do not agree with the results presented in Chapter 2 where Gladius accumulated less 4th leaf Na^+ the Drysdale. Also, 4th leaf Na^+ accumulation by both parents generated in the experiment described in Section 2.3.3 (Chapter 2) was much lower than 4th leaf Na^+ accumulation of Gladius and Drysdale generated in the soil based experiment. The 4th leaf Na^+ measured in soil based experiment required validation.

Table 7. Salt tolerance index (STI), osmotic stress tolerance–assay 1 (OST1) and assay 2 (OST2), number of tiller on salt treated plants (TN), 4th leaf Na$^+$ accumulation (Na) and 4th leaf K$^+$ accumulation (K) for Gladius, Drysdale, standard cultivars and RILs - mean, range (min–max) and heritability (h^2). The values are means ± s.e.m (n = 2, 5 or 6). The accumulation of Na$^+$ and K$^+$ were measured in the fully expanded fourth leaf after 10 d in salt.

| Trait | Parents | | Control cultivars | | RILs | | h^2 |
	Gladius	Drysdale	Excalibur	Kukri	Mean	Min - Max	
STI	0.64 ± 0.07	0.84 ± 0.10	0.54 ± 0.10	0.80 ± 0.10	0.67 ± 0.01	0.16 - 1.20	0.22
OST1	0.58 ± 0.08	0.88 ± 0.06	0.64 ± 0.10	0.70 ± 0.020	0.76 ± 0.01	0.10 - 1.20	0.24
OST2	0.50 ± 0.35	0.86 ± 0.13	1.15 ± 0.27	0.99 ± 0.10	0.85 ± 0.04	-1.7 – 4.1	-
TN	2.30 ± 0.40	2.83 ± 0.10	3.60 ± 0.40	2.40 ± 0.40	2.00 ± 0.05	1 - 4	0.21
Na (mM)	97.10 ± 48.30	60.20 ± 16.8	76.30 ± 52.30	65.30 ± 49.6	68.80 ± 5.00	5.40 - 280.70	0.15
K (mM)	240.60 ± 34.2	233.20 ± 21.3	263.60 ± 49.20	266 ± 55.50	280.40 ± 5.50	101.4 - 542.2	0.20

3.3.2.2 Trait correlation

In order to study the association between salinity tolerance and salt tolerance components (4th leaf Na$^+$ and 4th leaf K$^+$ accumulation, osmotic stress tolerance) in the Gladius * Drysdale RIL population, a correlation analysis was conducted of STI with other traits. The results show that the correlation of STI with 4th leaf K$^+$ accumulation and with OST2 was small and positive, and that between STI and 4th leaf Na$^+$ accumulation was negative and moderate (Figure 22, Table 8). However, a large positive correlation was observed between STI and OST1. A strong negative correlation between 4th leaf K$^+$ accumulation and 4th leaf Na$^+$ accumulation was observed. The correlation between OST1 and 4th leaf Na$^+$ accumulation was large and negative (Table 8) whereas, the trend between OST1 and 4th leaf Na$^+$ accumulation was slightly negative (Figure 22). There was no clear correlation between TN and any other trait (Figure 22) although the correlation coefficient was moderate between TN and two other traits (STI and OST1). OST2 does not have a strong correlation with STI (r=0.21), whereas the correlation coefficient between STI and OST1 is high (r=0.56). The

correlation analysis show that STI strongly correlated with OST1 and poorly with OST2. This suggests that the OST1 assay provides a reliable assay of osmotic stress tolerance in this population.

On the basis of these phenotypic (Section 3.3.2.1) and correlation analyses, OST2 was eliminated and OST1 was used for further QTL analysis.
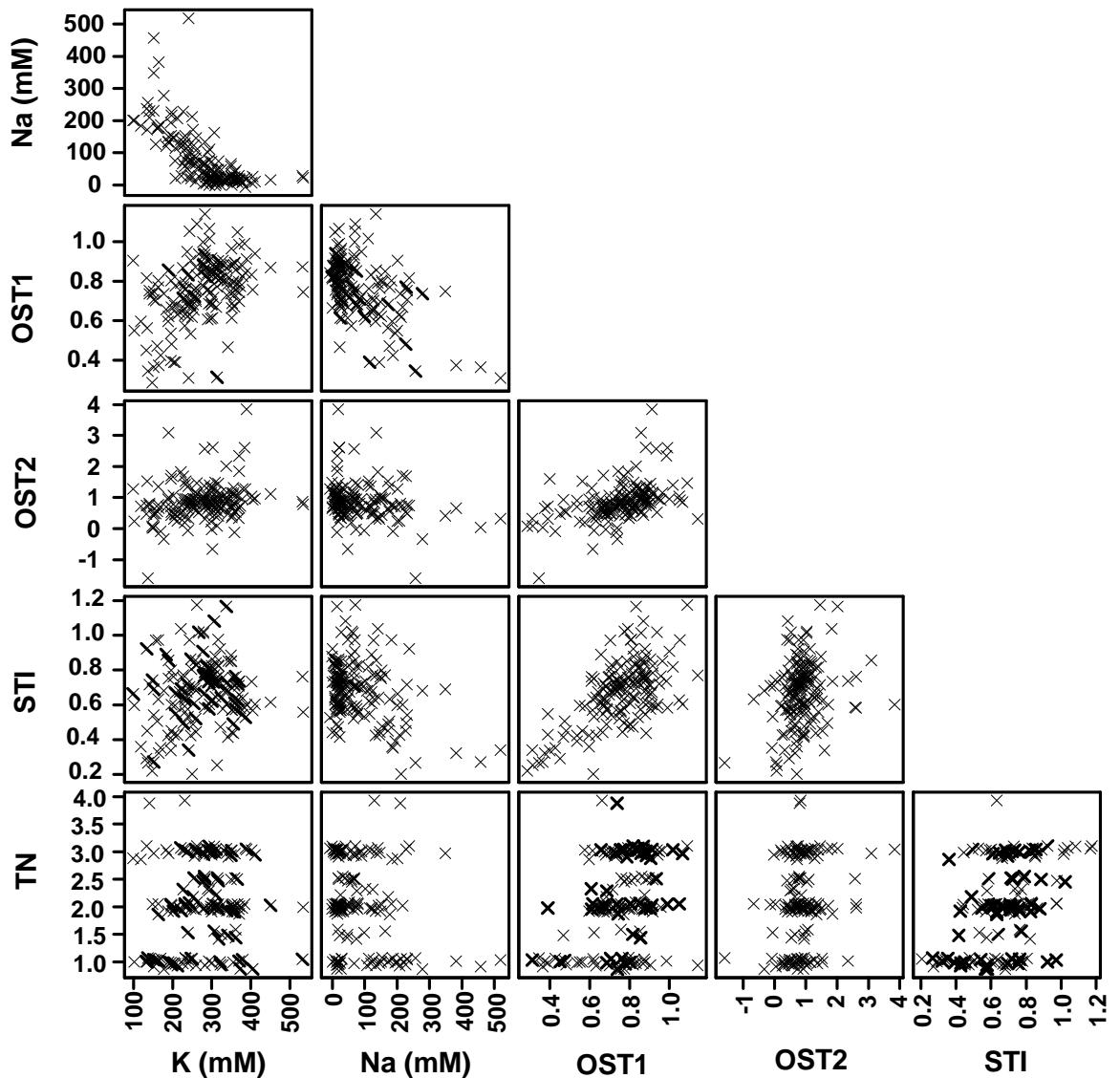
Figure 22. Phenotypic correlation between salt tolerance index (STI) and osmotic stress tolerance–assay 1 (OST1) and assay 2 (OST2), number of tiller (TN), 4th leaf Na+ accumulation (Na) and 4th leaf K+ accumulation (K) for the Gladius * Drysdale RIL population under salt treatment.

Table 8. Coefficient of phenotypic correlation (r) between salt tolerance index (STI) and osmotic stress tolerance–assay 1 (OST1) and assay2 (OST2), number of tiller (TN), 4th leaf Na$^+$ accumulation (Na) and 4th leaf K$^+$ accumulation (K) of the Gladius * Drysdale RIL population under salt treatment.

	K (mM)	Na (mM)	OST1	OST2	STI
Na (mM)	-0.70	-			
OST1	0.43	-0.55	-		
OST2	0.21	-0.25	0.46	-	
STI	0.13	-0.39	0.56	0.22	-
TN	0.14	-0.28	0.33	0.19	0.41

3.3.2.3 QTL analysis

QTL analysis was conducted to detect the genetic loci associated with salinity tolerance in the Gladius * Drysdale RIL population. This enabled identification of molecular markers for marker-assisted selection and genes associated with salinity tolerance traits. The calculated BLUE value (Section 3.2.6) was used for QTL analysis. Many QTL were identified across the genomes with LOD scores ranging between 2.15 and 4.90 (Table 9).The variation explained by QTL for all traits was between 3.70–11.60 and the additive effect varied across the traits (Table 9). Figure 25 shows results of the genetic map of the Gladius * Drysdale RIL population and QTL for all the traits.

Three significant QTL for STI were mapped onto the distal regions of chromosomes 1B, 2B and 3B with LOD scores of 3.25, 3.20 and 3.34, respectively, while one suggestive QTL was identified on chromosome 7B (LOD score of 2.90). These four QTL explained 6.50, 6.20, 6.85 and 7.04 % of the variation in STI respectively. Most QTL for STI had a negative additive effect derived from the Gladius parent. For the exceptions was a QTL on chromosome 2B, which had a positive effect originating from the Drysdale parent.

The QTL analysis also detected four QTL associated with OST1. Two QTL on chromosomes 2B and 5D were significant, with LOD scores of 4.15 and 3.10 which accounted for 11.60%

and 7.70% of the phenotypic variation, respectively. Another two suggestive QTL were identified on chromosomes 1B and 7B with LOD scores of 2.30 and 2.15, respectively. The first QTL on chromosome 1B explained 4.20% and the second QTL explained 3.70% of the phenotypic variation. Most positive effects for OST1 were derived from the Gladius parent, except for the QTL on chromosome 2B which had a positive effect from the Drysdale parent.

A significant QTL for TN was identified on chromosome 2B with a LOD score of 4.90, and this QTL explains 11.40% of the phenotypic variation. Another QTL was detected on chromosome 3A with a LOD score of 2.87. The additive effect of both QTL was positive and derived from the Drysdale parent.

Table 9. QTL for salt tolerance index (STI), osmotic stress tolerance-assay1 (OST1), number of tiller (TN), 4th leaf Na⁺ accumulation (Na) and 4th leaf K⁺ accumulation (K) under salt treatment for the Gladius * Drysdale RIL population grown in the soil based conditions. Locus, chromosome, phenotypic variation explained by the QTL (% Variation), LOD score, additive effect, and parental allele are shown.

Trait	Locus	Chromosome	LOD score	% Variation	Additive effect	Allele
STI	*XwPt-6229*	1B	3.25	6.50	-0.046	**Gladius**
	XwPt-7619	2B	3.20	6.20	0.045	**Drysdale**
	XwPt-1867	3B	3.34	6.85	-0.047	**Gladius**
	XwPt-1266 - XwPt-8615	7B	2.90	7.04	-0.048	**Gladius**
OST1	*XwPt-6229*	1B	2.30	4.20	-0.032	**Gladius**
	Xwmc0154 - XwPt-7619	2B	4.15	11.60	0.054	**Drysdale**
	Xcfd0007	5D	3.10	7.70	-0.043	**Gladius**
	XwPt-5069	7B	2.15	3.70	-0.03	**Gladius**
TN	*XwPt-7619*	2B	4.90	11.40	0.26	**Drysdale**
	Xcfa2076	3A	2.87	6.08	0.19	**Drysdale**
Na	*XwPt-5987*	7A	2.87	6.67	-0.11	**Gladius**
K	*XwPt-2141*	4B	2.35	4.56	16.80	**Gladius**

Identical QTL for STI and OST1 were found on the same region of chromosomes 1B and 2B, as might be expected given the strong correlation (r = 0.56) between these traits (Table 8).

However, the 7B QTL for STI mapped well away from the OST1 QTL with a genetic distance of 92.19 cM (Figure 25). The QTL analysis suggests that the genomic loci of interest for salinity tolerance in the Gladius * Drysdale RIL population are mostly located on the distal regions of the short arms of chromosomes 1B and 2B. These results also imply that the OST1 assay provides the best assay for salinity tolerance in this population. QTL for STI and TN on chromosome 2B are close to the photoperiod gene (*PpdB1*) at a genetic distance of 4.8 cM which suggests that the putative QTL for OST1 did not co-segregate with *PpdB1*.

A suggestive QTL for 4th leaf Na^+ accumulation which explained 6.67% of the phenotypic variation was identified on chromosome 7A close to *XwPt*-5987, with LOD score of 2.87. Another suggestive QTL was detected on chromosome 4B near *XwPt-2141*. This QTL was associated with 4th leaf K^+ accumulation, had a LOD score of 2.35 and accounted for 4.56 % of the phenotypic variation. The positive effect for both QTL originated from Gladius.

3.3.3 Hydroponics experiment

3.3.3.1 Phenotyping

Several traits were measured in RILs and their parents grown in a supported hydroponics system at two levels of NaCl (0 and 100 mM): 4th leaf Na^+ and 4th leaf K^+ accumulations, shoot fresh weight (SFW), shoot dry weight (SDW), number of tiller (TN), water content (WC), salt tolerance index (STI) , and water content index (WCI) .

The heritability for all traits was calculated as a ratio between the total genetic variance and phenotypic variance (Section 3.2.6). Heritability was moderate for most traits, except for WCI and 4th leaf K^+ accumulation (Table 10). The heritability of SFW, SDW and TN in the control treatment was smaller than the heritability of SFW, SDW and TN in the salt treatment (Table 10).

Table 10. 4th leaf Na$^+$ accumulation (Na), 4th leaf K$^+$ accumulation (K), shoot fresh weight (SFW), shoot dry weight (SDW), number of tiller (TN), water content (WC), salt tolerance index (STI) and water content index (WCI) for Gladius, Drysdale and RILs mean, range (min–max) and heritability (h^2) in control and salt treatment. Na and K were measured after 20 d in salt. Other traits were measured at day 45 after germination (at day 30 in salt).

Trait	Treatment	Parents		RILs		h^2
		Gladius	Drysdale	Mean	Min - Max	
Na (mM)	Salt	27.3 ± 4.3	48.2 ± 10.5	39.9 ± 1.3	10.4 - 147.3	0.31
K (mM)	Salt	246.1 ± 7.2	218.2 ± 13.6	231.5 ± 1.9	139.5 - 355.6	0.06
SFW (g)	Control	10.7 ± 1.1	10.6 ± 1.7	10.8 ± 0.2	101.4 - 542.2	0.29
	Salt	3.8 ± 0.2	3.2 ± 0.1	4.7 ± 0.08	1.4 - 7.8	0.48
SDW (g)	Control	1.2 ± 0.2	1.3 ± 0.2	1.2 ± 0.03	0.23 - 2.85	0.20
	Salt	0.6 ± 0.04	0.55 ± 0.01	0.7 ± 0.01	0.24 - 1.26	0.46
TN	Control	4.3 ±0.3	3.7 ± 0.5	3.9 ± 0.09	1 - 10	0.28
	Salt	2.2 ± 0.4	1.16 ± 0.16	2.5 ± 0.07	1 - 5	0.56
WC (g)	Control	0.89 ± 0.003	0.87 ± 0.0	0.89 ± 0.0	0.85 - 0.92	0.57
	Salt	0.83 ± 0.003	0.82 ± 0.0	0.84 ± 0.0	0.79 - 0.87	0.48
STI	-	0.50 ± 0.04	0.48 ± 0.07	0.63 ± 0.01	0.16 - 1.07	0.31
WCI	-	0.94 ± 0.0	0.94 ± 0.0	0.94 ± 0.0	0.91 - 0.97	0.2

The RILs had a normal distribution for most traits (Figure 23 a, b, c, d, e, g, j, i and l), except for 4th leaf Na$^+$ and 4th leaf K$^+$ accumulation (Figure 23 h and k), which was skewed towards lower 4th leaf Na$^+$ concentrations and larger 4th leaf K$^+$ concentration. Variation between parents was low for all traits but all traits showed a wide range across RILs (Figures 23). Therefore, most traits showed transgressive segregation in both directions which implies that many genes are involved and that both parents contribute favourable alleles. However, the TN distribution in salt treatment seems to be bimodal (Figure 23 f). This implies that major genes may be associated with TN in the saline environment.
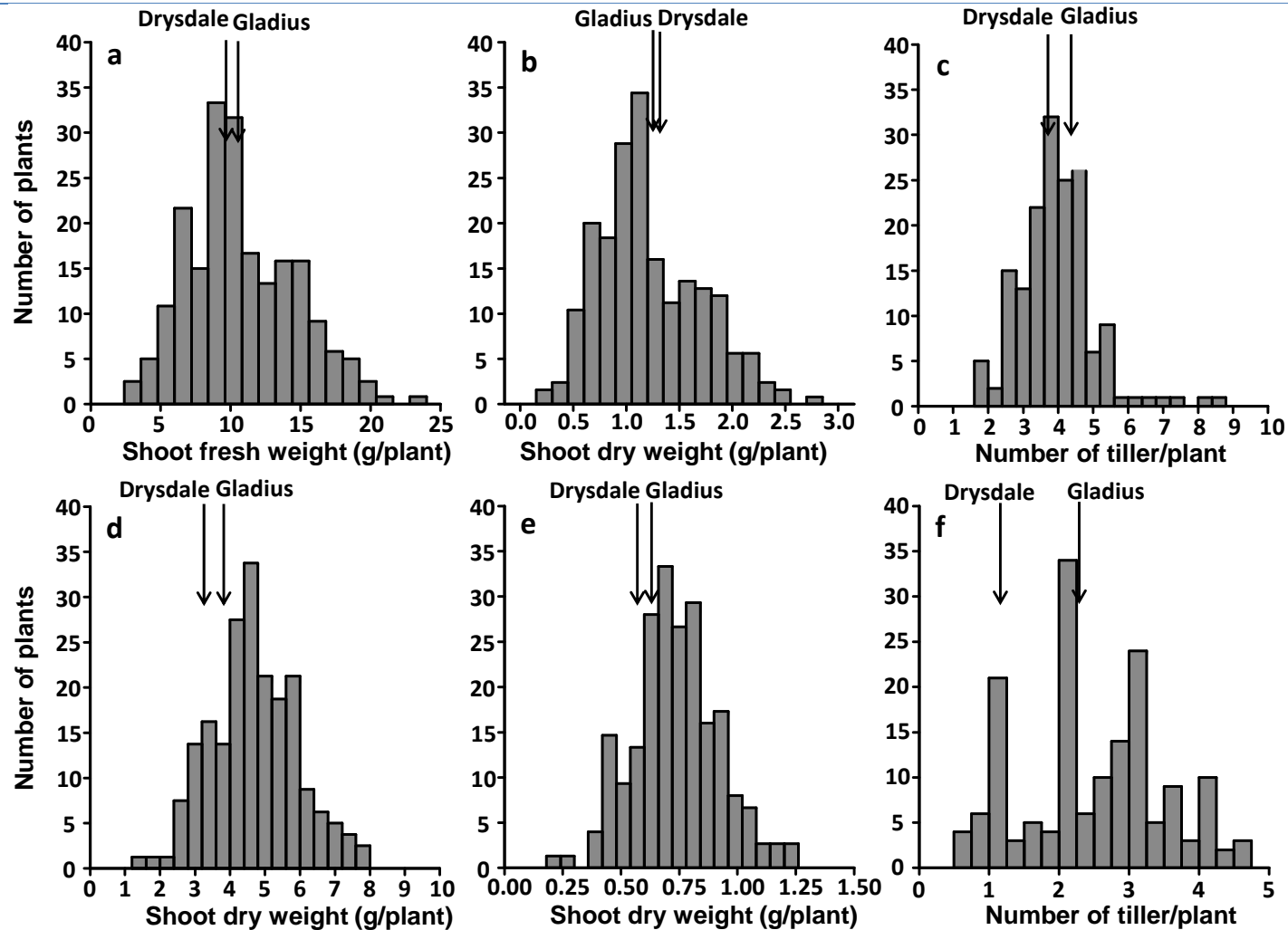
Figure 23. Frequency distribution of shoot fresh weight (a), shoot dry weight (b), number of tiller (c), water content (g) in the Gladius * Drysdale RILs in control condition. Also, frequency distribution of shoot fresh weight (d), shoot dry weight (e), number of tiller (f), water content (j), 4th leaf Na+ accumulation (h), 4th leaf K+ accumulation (k) in the Gladius * Drysdale RILs in salt treatment. Figures i and l denote the frequency distribution of salt tolerance and water content indexes. Arrows indicate the trait means (n = 6) for both parents, Gladius and Drysdale. Values of X-axis for salt treatment are different from values of X-axis for control condition.
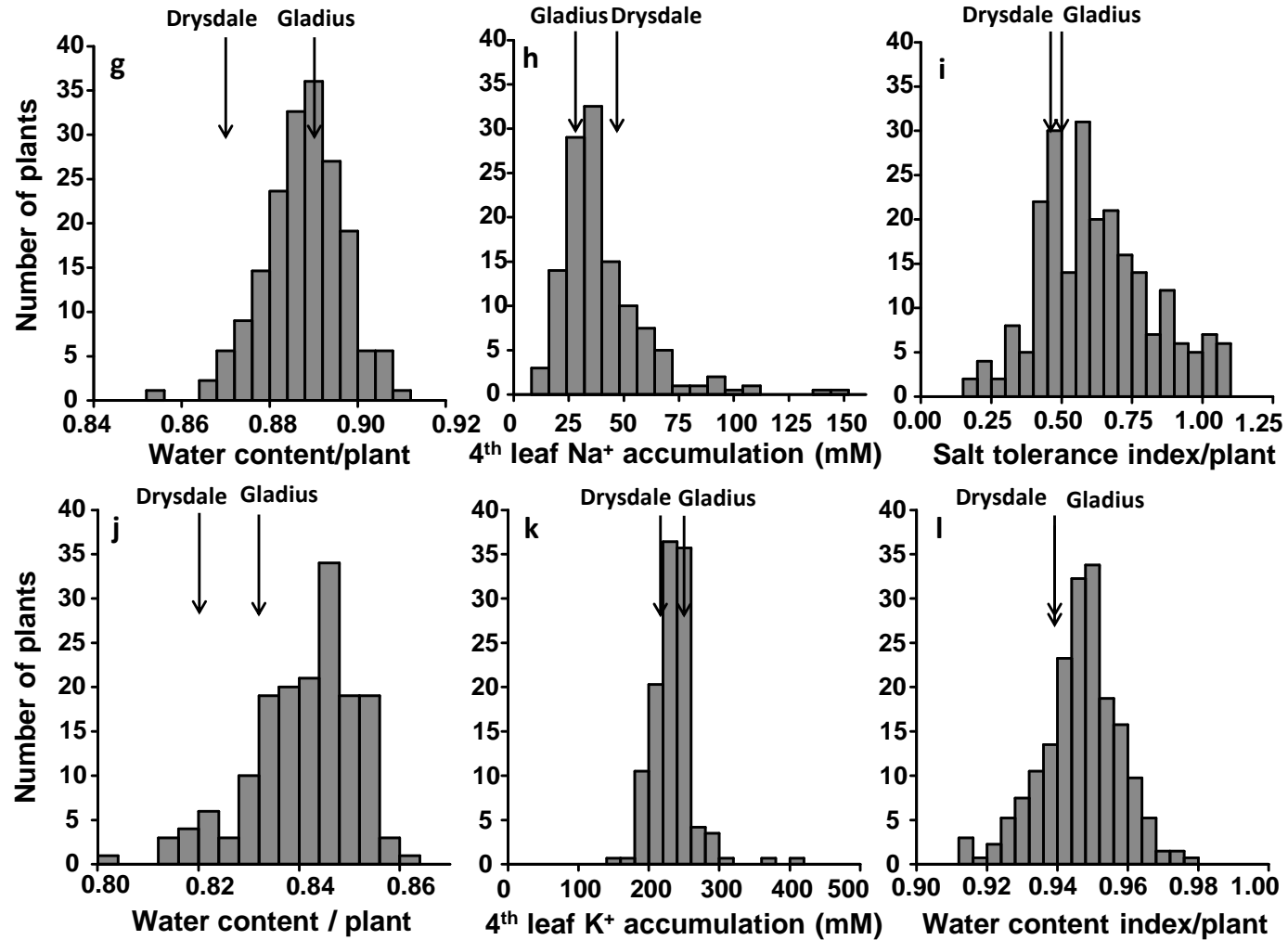
Figure 23. Continued

3.3.3.2 Trait correlation

The correlation analysis revealed a moderate correlation between STI and the other three traits (SDW, SFW and TN) in both treatments (Figure 24, Table 11). A large positive correlation was observed between three traits (SFW, SDW and TN) in the salt treatment. The correlations among these three traits were also seen in the control conditions (Figure 24 and Table 11). WCI (relative water content) has a high correlation with WC in both treatments and also with TN in the salt treatment (Figure 24 and Table 11). This may be due to a large correlation between WC and TN in the salt treatment. The correlation between STI and Na^+ accumulation was slightly negative, while the correlation between STI and K^+ accumulation was slightly positive (Figure 24 and Table 11). This implies that low Na^+ accumulation and high concentration of K^+ in the leaf may not be key factors in determining STI. This suggests that other mechanisms such as osmotic stress tolerance may represent the main component of STI in this population.
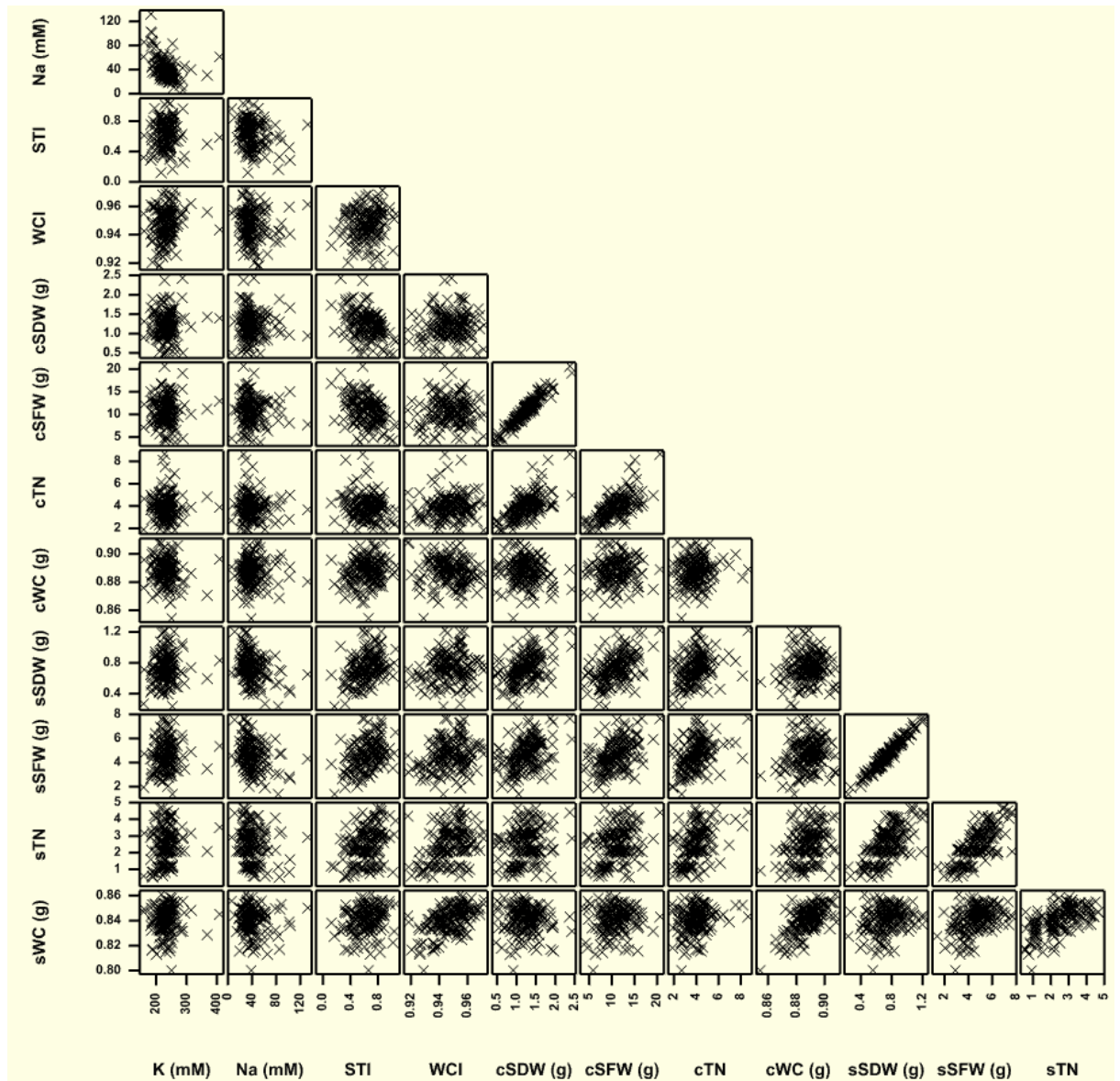
Figure 24. Phenotypic correlation between salt tolerance index (STI) with 4th leaf Na$^+$ (Na) and 4th leaf K$^+$ (K) accumulations, shoot fresh weight in control condition (cSFW) and on salt treatment (sSFW), shoot dry weight in control condition (cSDW) and on salt treatment (sSDW), number of tiller in control condition (cTN) and on salt treatment (sTN), water content in control condition (cWC) and on salt treatment (sWC) and water content index (WCI) of the Gladius * Drysdale RIL population.

Table 11. Coefficient of phenotypic correlation (r) between salt tolerance index (STI) with 4th leaf Na⁺ (Na) and 4th leaf K⁺ (K) accumulation, shoot fresh weight in control condition (cSFW) and on salt treatment (sSFW), shoot dry weight in control condition (cSDW) and on salt treatment (sSDW), number of tiller in control condition (cTN) and on salt treatment (sTN), water content in control condition (cWC) and on salt treatment (sWC) and water content index (WCI) of the Gladius * Drysdale RIL population.

	K (mM)	Na (mM)	STI	WCI	cSDW(g)	cSFW(g)	cTN(g)	cWC(g)	sSDW(g)	sSFW(g)	sTN
Na (mM)	-0.55										
STI	0.06	-0.21									
WCI	0.22	-0.08	0.12								
cSDW(g)	0.04	-0.03	-0.40	0.07							
cSFW(g)	0.00	-0.04	-0.35	0.04	0.96						
cTN	0.03	-0.07	-0.13	0.11	0.63	0.66					
cWC(g)	-0.16	0.00	0.19	-0.12	-0.12	0.14	0.15				
sSDW(g)	0.13	-0.32	0.39	0.15	0.47	0.50	0.37	0.08			
sSFW(g)	0.12	-0.31	0.41	0.25	0.44	0.51	0.40	0.22	0.97		
sTN	0.13	-0.15	0.35	0.37	0.24	0.33	0.46	0.33	0.65	0.74	
sWC(g)	0.02	-0.07	0.21	0.53	-0.02	0.15	0.25	0.66	0.22	0.42	0.59

3.3.3.3 QTL analysis

In order to detect markers flanking the genomic loci of interest and to identify genes associated with salt tolerance traits, QTL analysis was carried out (Section 3.2.7).

The QTL package in GenStat was used for simple and composite interval mapping. LOD scores of QTL for all traits ranged from 2.08 to 10.07 and the phenotypic variation explanation for all traits ranged from 4.04 to 24.87% (Table 12). Figure 25 shows results of the genetic map of the Gladius * Drysdale RIL population and QTL for all the traits.

Two suggestive QTL for 4th leaf Na^+ accumulation were detected on chromosomes 1D and 3B. The LOD score of the QTL on chromosome 1D is 2.60 at *Xwmc0216* and this explained 5.30 % of the phenotypic variation. The other QTL was detected between the loci *XwPt-0021* and *Xgwm299* on chromosome 3B with a LOD score of 2.60 and this QTL accounted for 6.71% of the phenotypic variation. The additive effect for both QTL was high and negative inherited from the Gladius parent. Also, two suggestive QTL associated with 4th leaf K^+ accumulation were detected on chromosome 2D at *wPt-3692* and on chromosome 3A at *wPt-2938* with LOD scores of 2.50 and 2.08 and these explained 5.02% and 4.04% of 4th leaf K^+ accumulation variation, respectively.

In the control conditions, three significant QTL were detected for SFW. These QTL were mapped to the distal regions of chromosomes 1B, 2B and 5A with LOD scores of 3.06, 3.60 and 3.67 and these QTL explained 5.97, 7.50 and 7.80%, respectively of the SFW variation. The QTL on chromosome 1B is located at the *XwPt*-1070 locus and the QTL on chromosome 2B is located at *Xgwm*0388, whereas the QTL on chromosome 5A is close to the *VRN-A1* gene, which is associated with vernalisation and maturity. In the salt treatment, two QTL for SFW were detected on chromosomes 1B and 5A. The QTL on chromosome 1B was suggestive with a LOD score of 2.67, and this QTL accounted for 5.40% of the SFW variation. Another QTL was significant at the interval between *XwPt-4262* and *VRN-A1* with a LOD score of 3.50 and this explained 19.62% of the phenotypic variation for SFW and the positive effect for this QTL

was from Drysdale. This QTL was close to a vernalisation gene (*VRN-A1)* (Figure 25). This suggests that *VRN-A1* on chromosome 5A may have a significant effect on QTL for SFW.

No significant QTL was found for SDW in either control condition or salt treatment even though the heritability of SDW was moderate in the salt treatment ($h^2 = 0.46$). There were many minor suggestive QTLs implying that many genes may control SDW but none of sufficient strength for detection in this population (Appendix I, 6.1.2).

Table 12. QTL for 4th leaf Na$^+$ accumulation (Na), 4th leaf K$^+$ accumulation (K), shoot fresh weight (SFW), shoot dry weight (SDW), number of tiller (TN), water content (WC), salt tolerance index (STI) and water content index (WCI) for the Gladius * Drysdale RIL population grown in a hydroponics system. Locus, chromosome, phenotypic variation explained by QTL (% Variation), LOD score, additive effect and parental allele are shown.

Trait	Treatment	Locus	Chromosome	LOD score	% Variation	Additive effect	Allele
Na	Salt	*Xwmc0216*	1D	2.60	5.30	-4	Gladius
		XwPt-0021 - Xgwm299	3B	2.60	6.71	-4.5	Gladius
K	Salt	*XwPt-3692*	2D	2.50	5.02	6.69	Gladius
		XwPt-2938	3A	2.08	4.04	-6	Drysdale
SFW	Control	*XwPt-1070*	1B	3.06	5.97	0.72	Gladius
		Xgwm0388	2B	3.60	7.50	0.80	Drysdale
		VRN-A1	5A	3.67	7.80	0.82	Drysdale
	Salt	*XwPt-8240*	1B	2.67	5.40	0.28	Gladius
		XwPt-4262 - VRN-A1	5A	3.50	19.62	0.54	Drysdale
SDW	Control	-	-	-	-	-	-
	Salt	-	-	-	-	-	-
TN	Control	*VRN-A1*	5A	3.00	12.08	0.37	Drysdale
	Salt	*XwPt-2600*	2B	3.87	8.20	0.28	Drysdale
		PpdD1	2D	3.22	6.84	-0.26	Drysdale
		VRN-A1	5A	4.50	10.52	0.33	Drysdale
WC	Control	*VRN-A1*	5A	5.06	11.93	0.003	Drysdale
		Xgdm0150 - Xbarc0235	6A	2.54	6.05	0.002	Gladius
	Salt	*PpdD1*	2D	3.16	5.99	-0.003	Drysdale
		VRN-A1	5A	10.07	24.87	0.005	Gladius
STI		-	-	-	-	-	-
WCI		*XwPt-4607*	4B	3.03	6.40	-0.003	Drysdale
		XwPt-7201 - Xwpt-4262	5A	2.26	7.66	0.003	Gladius

A QTL at *VRN-A1* on chromosome 5A was detected in the control condition for TN with LOD score of 3.00 and this QTL accounted for 12.08 % of TN variation (Table 12). The positive effect for this QTL was from Drysdale. In the salt treatment, three QTL for TN were identified at three loci on chromosomes 2B, 2D and 5A with LOD scores of 3.87, 3.22 and 4.50, respectively. The QTL on chromosome 2B was located at *XwPt-2600* which is close to *Ppd-B1* gene and this QTL explained 8.20% of the phenotyping variation. Interestingly, the QTL on chromosome 2D was at the position of the *Ppd-D1* gene, with 6.84% of phenotyping variation being explained by this QTL. The third QTL is located at *VRN-A1* on chromosome 5A and this QTL accounted for 10.52% of TN variation (Table 12). The three QTL in salt treatment had a positive and negative effect from Drysdale. Photoperiod and vernalisation genes have a strong impact on TN in the Gladius * Drysdale RIL population implying a significant effect of maturity on tiller number.

Two QTL were detected on chromosomes 5A and 6A for WC in the control treatment. The former QTL was significant with an LOD score of 5.06 and it accounted for 11.93% of the phenotyping variation, whereas the other QTL was suggestive with a LOD score of 2.54 and explaining 6.05% of the WC variation. The highly significant QTL may explain why WC has a large heritability (0.57) in the control treatment. The locus for this QTL is close to *VRN-A1* (Figure 25). In the salt treatment, another two QTL associated with WC were detected on chromosome 2D (*PpdD1* gene) and on chromosome 5A (*VRN-A1*gene) with LOD scores of 3.16 and 10.07, respectively. These QTL accounted for 5.99% (on chromosome 2D) and 24.87% (on chromosome 5A) of the phenotyping variation. The QTL on chromosome 2D had a negative effect derived from Drysdale, whereas the QTL on chromosome 5A had a positive effect from Gladius. These results show that the *VRN-A1* gene has a strong association with WC in both treatments.

For WCI, a significant QTL was mapped onto the distal region of chromosome 4B at *XwPt-4607* with a LOD score of 3.03 and it accounted for 6.40% of the phenotyping variation. This

QTL shows a positive effect of the Gladius allele. Another suggestive QTL was detected on chromosome 5A at the interval between *XwPt-7201* and *XwPt-4262* with an LOD of 2.26. This QTL explained 7.66 % of phenotypic variation for WCI and the negative effect of this QTL was from Drysdale.

No significant QTL for STI were identified in the Gladius * Drysdale RIL population. The possible explanation for the failure to detect QTL for STI that the STI distribution (Figure 23 I) is broad and the QTL analysis (Appendix I, Section 6.1.2) detected many small but non-significant QTL across the genomes. This implies that STI may be controlled by a large number of genes.
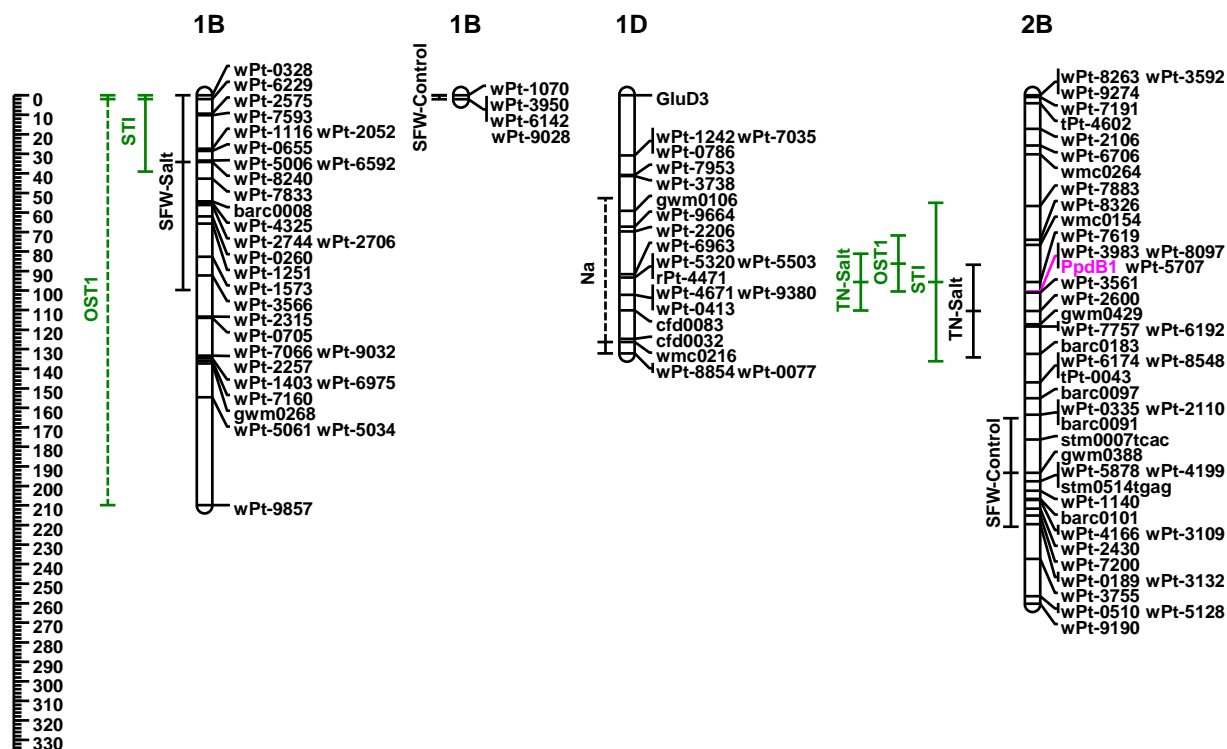
Figure 25. Genetic map of chromosomes 1B, 1D, 2B, 2D, 3A, 3B, 4B, 5A, 5D, 6A, 7A and 7B of the Gladius * Drysdale RIL population and quantitative trait loci (QTL) for 4th leaf Na+ accumulation (Na), 4th leaf K+ accumulation (K), shoot fresh weight (SFW), water content (WC), tiller number (TN), salt tolerance index (STI), water content index (WCI) and osmotic stress tolerance-assay1 (OST1) for the hydroponics experiment (QTL shown in black bars) and for the soil based conditions experiment (QTL shown in green bars). SFW-control, WC-control, TN-control indicate these traits from the control treatment, whereas SFW-Salt, TN-Salt and WC-Salt indicate these traits from the salt treatment in the hydroponics experiment. The sold bars denote QTL that are significant (LOD ≥ 3) whereas the dashed bars denote QTL that are not significant (LOD < 3). A dash on the QTL bar represents the peak or maximum LOD score associated with the most significant markers. Molecular markers (on the right of the chromosome) and genetic distance cM (the ruler at the left of the page) are indicated. The photoperiod genes on chromosomes 2B and 2D are shown in pink, and the vernalisation genes on chromosomes 5A and 5D are shown in brown.
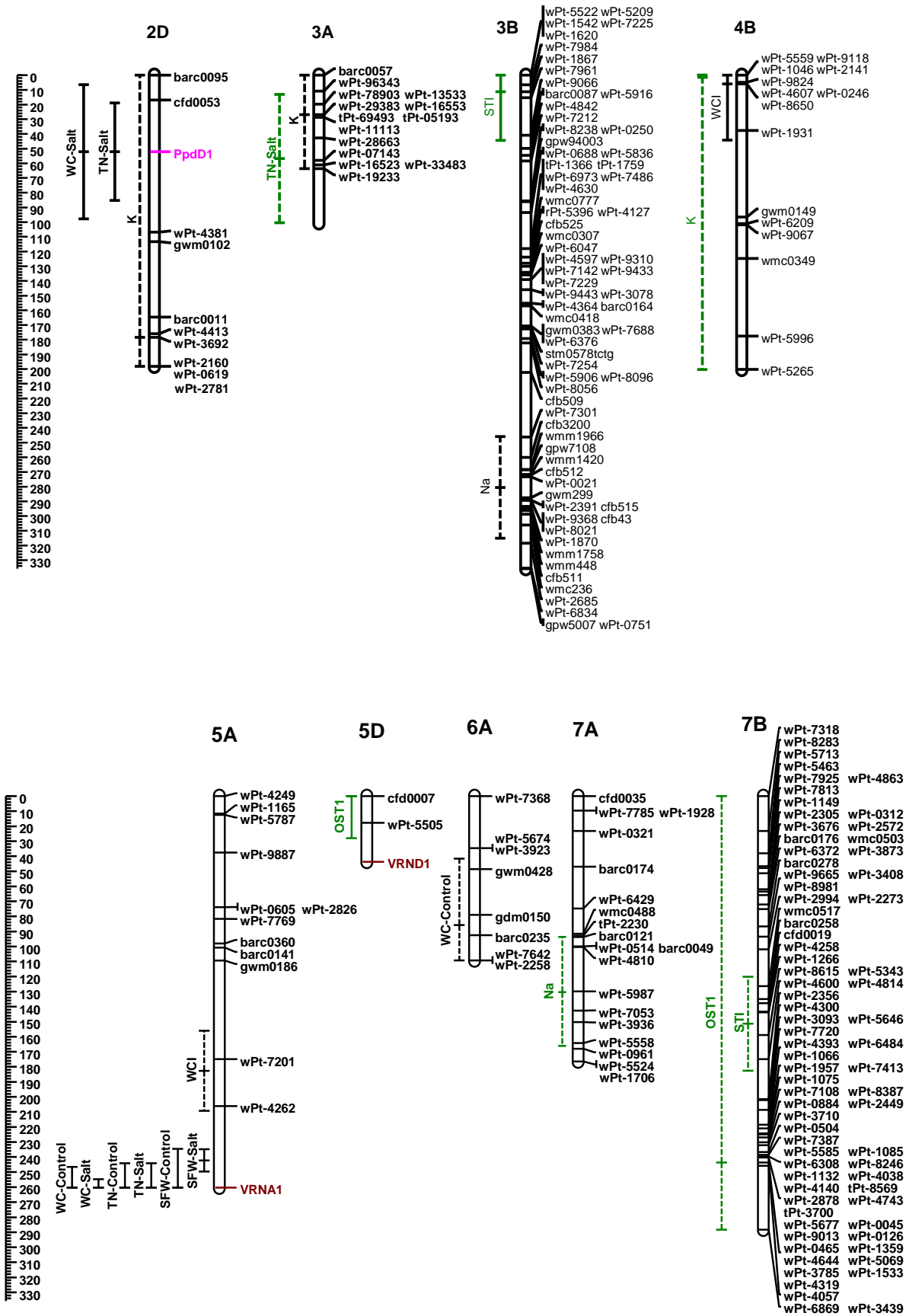
Figure 25. Continued.

3.3.4 The response of RILs to saline conditions varies between soil based and hydroponics experiments

To investigate the plant response to salt under the different growth conditions (pots with soil or hydroponics), the correlation analysis package in GeneStat 14 was used to compare two sets of data: shoot fresh and dry weights as measured in the hydroponics experiment for control (hSFWc and hSDWc, respectively) and salt treatments (hSFWs and hSDWs, respectively) were compared with the projected shoot area as measured in the soil based experiment for control (pPSAc) and saline conditions (pPSAs). Data collected from the hydroponics experiment on the accumulation of 4th leaf Na^+ and 4th leaf K^+ (hNa and hK) were compared with data collected from the soil based experiment (pNa and pK). BLUE values of phenotypic traits (biomass and accumulation of ions) of the RILs were used. The correlation analysis showed no relationship between biomass measured in the hydroponics system (hSFWc and hSDWc) and biomass measured in the soil based experiment (pPSAc) for the control condition. Similarly, no correlation was found between biomass data for the salt treatment in the two experiments (hSFWs and hSDWs compared with pPSAs) (Figure 26; Table 13). The correlation between 4th leaf ion accumulation as measured in the hydroponics system and in the pots with soil was also very low (Figure 27; Table 14). This suggests that the RILs responded differently in different experiments (soil based and hydroponics experiments) under control conditions and salt treatments.
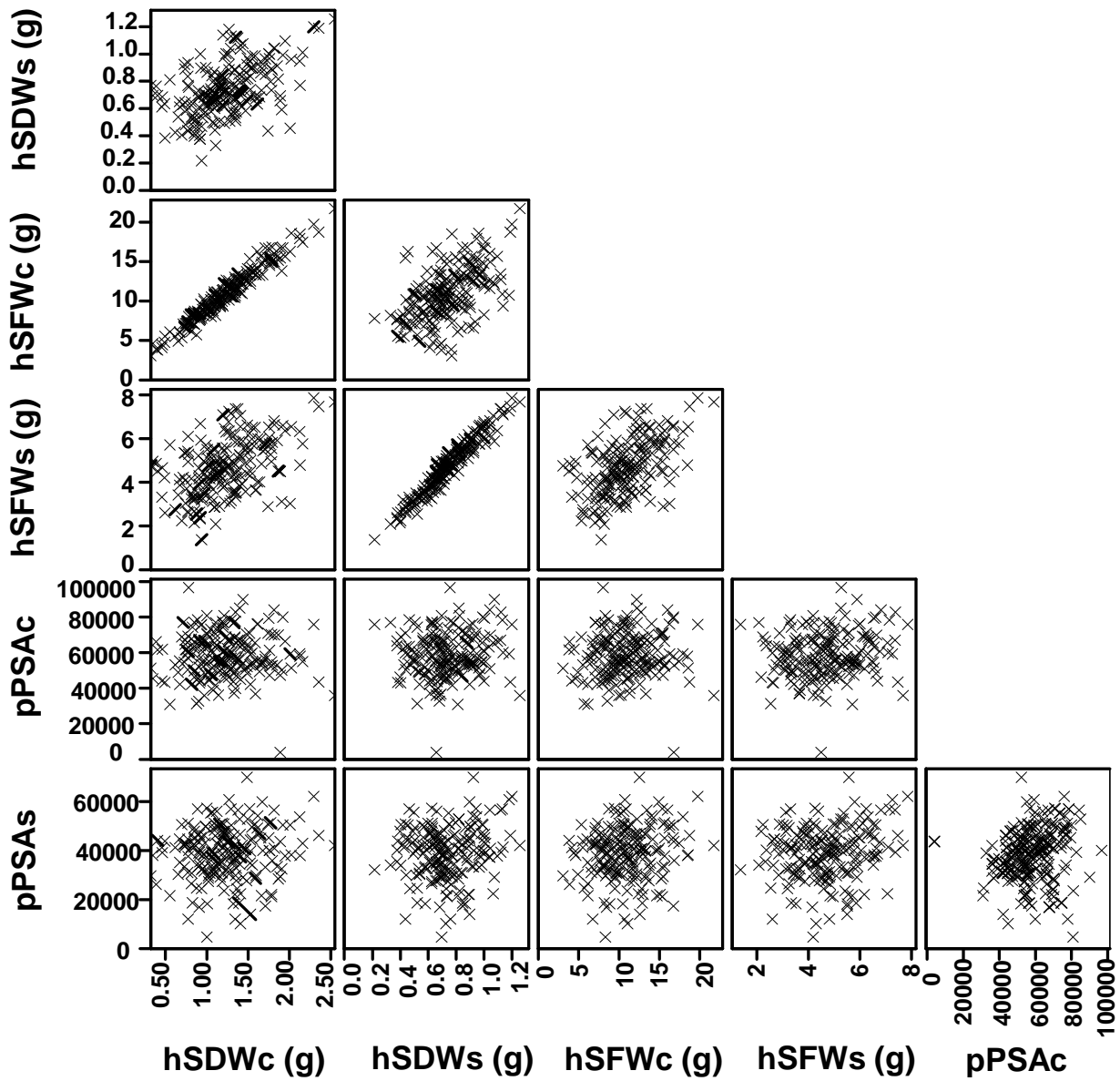
Figure 26. Phenotypic correlations between biomass [shoot fresh weight (hSFWc) and shoot dry weight (hSDWc) in control condition. Shoot fresh weight (hSFWs) and shoot dry weight (hSDWs) in salt treatment] collected from the hydroponics experiment and biomass [projected shoot area (pPSAc) in control condition and (pPSAs) in salt treatment] collected from the soil based experiment. BLUE data of the Gladius * Drysdale RIL population was used to conduct the correlation analysis.

Table 13. Coefficient of phenotypic correlation (r) between biomass [shoot fresh weight (hSFWc) and shoot dry weight (hSDWc) in control condition, shoot fresh weight (hSFWs) and shoot dry weight (hSDWs) in salt treatment] collected from the hydroponics experiment and biomass [projected shoot area (pPSAc) in control condition and (pPSAs) in salt treatment] collected from the soil based experiment. BLUE data of the Gladius * Drysdale RIL population was used to conduct the correlation analysis.

	hSDWc (g)	hSDWs (g)	hSFWc (g)	hSFWs (g)	pPSAc	pPSAs
hSDWc (g)	-					
hSDWs (g)	0.50	-				
hSFWc (g)	0.97	0.52	-			
hSFWs (g)	0.46	0.97	0.52	-		
pPSAc	0.00	0.11	0.05	0.14	-	
pPSAs	0.11	0.13	0.15	0.16	0.19	-
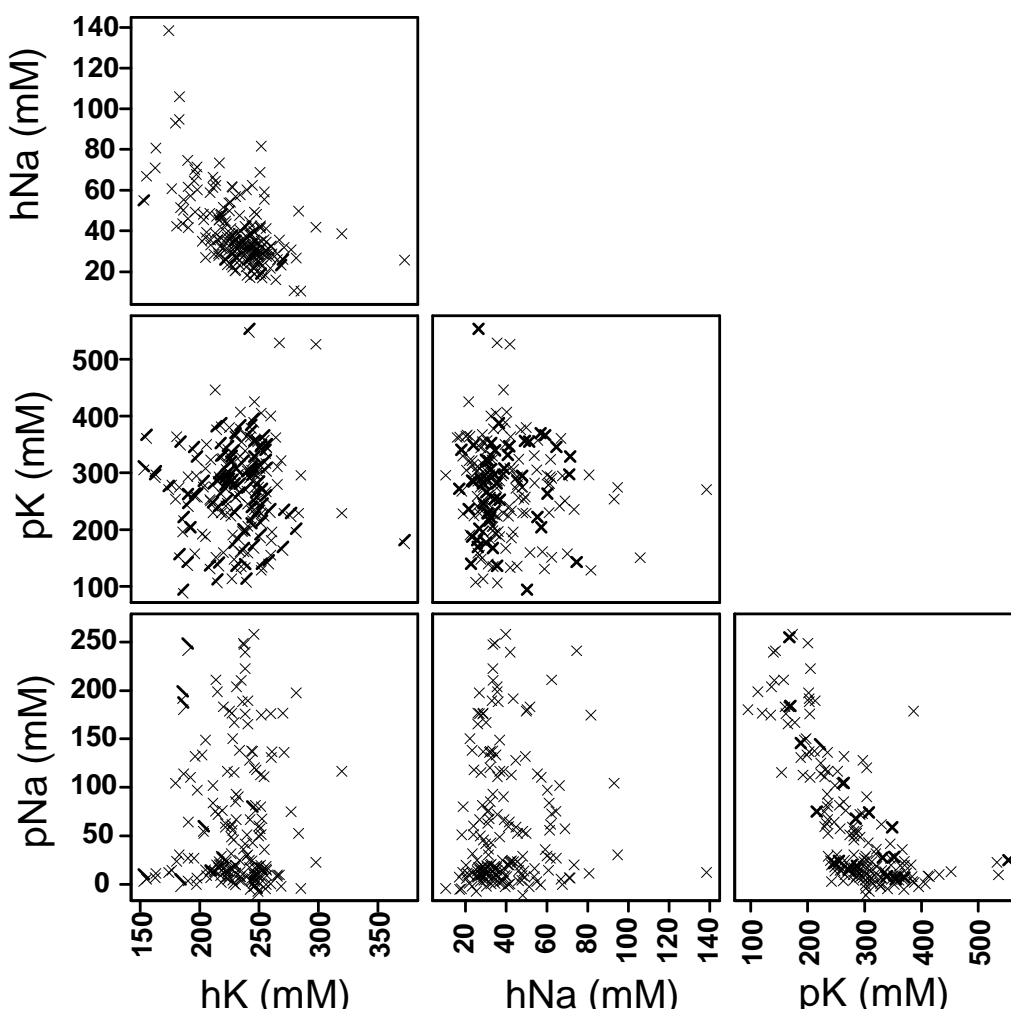
Figure 27. Phenotypic correlation between accumulation of 4th leaf Na^{+} and 4th leaf K^{+} (hNa and hK) collected from the hydroponics experiment and accumulation of 4th leaf Na^{+} and 4th leaf K^{+} (pNa and pK) collected from the soil based experiment. BLUE data of the Gladius * Drysdale RIL population was used to conduct the correlation analysis.

Table 14. Coefficient of phenotypic correlation (r) between accumulation of 4th leaf Na$^+$ and 4th leaf K$^+$ (hNa and hK) collected from the hydroponics experiment and accumulation of 4th leaf Na$^+$ and 4th leaf K$^+$ (pNa and pK) collected from the soil based experiment. BLUE data of the Gladius * Drysdale RIL population was used to conduct the correlation analysis.

	hK (mM)	hNa (mM)	pK (mM)	pNa (mM)
hK (mM)	-			
hNa (mM)	-0.55	-		
pK (mM)	0.07	-0.05	-	
pNa (mM)	0.03	0.03	-0.70	-

3.4 Discussion

3.4.1 Soil based experiment

The aim of the experiments was to determine the genetic control of osmotic stress tolerance in wheat using a mapping population derived from a cross between Gladius and Drysdale. The basis for assessing tolerance was the reduction in plant shoot growth rate induced by salinity stress before ionic toxicity builds in the shoot (Munns and Tester, 2008). The growth rate of salt treated and non-salt treated plants was measured every second day non-destructively using the LemnaTec Scanalyzer 3D.

Two clustered QTL were found in the soil based experiment: one on the distal region of the short arm of chromosome 2B for OST1, ST and TN which confirmed that OST1 was strongly associated with STI. In previous studies (De León et al. 2011; Genc et al. 2010; Quarrie et al. 2005), no important QTL associated with salinity tolerance traits have been found on the short arm of chromosome 2B. Ma et al. (2007) and Ogbonnaya et al. (2008) found QTL for fresh shoot biomass and Na$^+$ accumulation on 2B but they did not indicate their significance, importance, or their specific location. However, Long et al. (2013) detected QTL related to salinity tolerance, fresh shoot weight and ionic accumulation (Na$^+$, K$^+$ and Cl$^-$) on the short arm

of chromosome 2H in a doubled haploid population of barley. The authors showed a positive correlation between Na^+ accumulation and salinity tolerance which suggested that salinity tolerance was related to osmotic adjustment but not to Na^+ exclusion. Gorham et al. (1985); Horie et al. (2012); Munns and Tester (2008); Teakle and Tyerman (2010) suggested that plants are able to lower osmotic potential by increasing net solute accumulation in response to water stress. These solutes consist of organic compounds (such as glycine betaine and proline) and ions (K^+ in the cytosol, and Na^+ and Cl^- in the vacuole). It has been suggested that the QTL mapped on the short arm of the chromosome 2H (Long et al. 2013) was related to osmotic tolerance and it may correspond to the QTL identified on 2B here. A comparative genome analysis would be needed to align the 2B and 2H loci.

The QTL on the short arm of chromosome 2B was located near the *Ppd-B1* photoperiod gene. This is a well-known gene associated with flowering time and maturity in wheat. However, the genetic distance between the OST1 QTL and *Ppd-B1* was 15 cM. Drought experiments conducted in the field in south eastern Australia, using the same RIL population as used in the soil based experiment (Gladius * Drysdale), led to the detection of a number of QTL in the same region of chromosome 2B near the *Ppd-B1* gene. These QTL were associated with yield and yield components (Maphosa 2013). It is likely that *Ppd-B1* is important for both drought and salt tolerance, and is involved in the reaction of plants to a range of abiotic stresses. The maturity time for the Gladius * Drysdale RIL population was estimated in the Roseworthy field in 2009 by Australian Grain Technology based on Zadoks growth scale (Zadoks et al. 1974). The maturity trait was mapped onto the short arm of homeologous chromosomes 2B and 2D with high LOD scores of 17.90 and 29.57. In the experiment described here the impact of the *Ppd-B1* gene on the OST1 trait was tested. A correlation analysis was carried out between OST1 and maturity in the population showed no relationship (r = 0.01) between these traits (Figure 28). This indicates that the QTL for OST1 on the distal region of the short arm of

chromosome 2B is unlikely to be related to the *Ppd-B1* gene but genes encoding both traits are closely linked.

A significant QTL for STI (LOD>3) and A suggestive QTL for OST1 (LOD=2.3) was mapped onto the short arm of chromosome 1B (Figure 25). The identified region of the short arm of chromosome 1B has not been previously reported as associated with any traits of salinity tolerance (Quarrie et al. 2005; Ma et al. 2007; Ogbonnaya et al. 2008; Genc et al. 2010; De León et al. 2011). However, in drought experiments, Edwards (2012) detected a QTL on the short arm of chromosome 1B which was associated with osmotic adjustment induced by drought stress in an Excalibur*Kukri doubled haploid population of bread wheat. The QTL identified here may correspond to the QTL identified by Edwards (2012) as osmotic stress is important for both drought and salt. It will be interesting to compare both loci.

On the distal region of chromosome 5D, a significant QTL for OST1 (LOD>3) was detected. The genetic distance between the OST1 QTL and the vernalisation gene (*VRN-D1*) on chromosome 5D is approximately 40 cM meaning that these are two distinct loci.

A suggestive QTL for 4th leaf Na^+ accumulation was mapped onto the distal region of the long arm of chromosome 7A. Similar but significant QTL for shoot Na^+ accumulation was mapped to the same genetic region in another bread wheat population derived from a cross between Krichauff and Berkut (Genc et al. 2010). The coincidence of both QTL suggests that this QTL is important for Na^+ accumulation. Also, Ma et al. (2007) identified QTL for chlorophyll content on the long arm of the chromosome 7A in a RIL population of bread wheat ten days after application of a 250 mM NaCl stress. However, no QTL for STI was detected on the long arm of 7A in this study, and this implies that Na^+ accumulation may be a minor contributor to salinity tolerance in the wheat population.

3.4.2 Hydroponics experiment

The supported hydroponics experiment was carried out largely to investigate the accumulation of Na^+ and K^+ in the RIL population as these ions were unexpectedly high in the shoots of plants grown in the soil based experiment.

In this section the correlation between the physiological traits (4th leaf Na^+ and 4th leaf K^+ accumulation) and salt tolerance index (STI) and QTLs for these traits will be discussed. Other traits, tiller number (TN), shoot fresh weight (SFW), shoot dry weight (SDW), water content (WC) were also studied in control and salt treatments.

The correlation analysis (Figure 24; Table 11) showed that 4th leaf K^+ accumulation had a very low positive correlation with STI (r=0.03), and also 4th leaf Na^+ accumulation has low negative correlation with STI (r=0.21). Therefore, 4th leaf Na^+ accumulation made a small contribution to the salinity tolerance in this population relative to 4th leaf K^+ accumulation. No correlation between 4th leaf Na^+ and 4th leaf K^+ ions accumulation and STI has been reported previously in bread wheat (Genc et al. 2007). In our experiments, 4th leaf Na^+ accumulation showed a moderate correlation with SFW and SDW in plants under salt treatment, which means plants accumulating higher concentrations of 4th leaf Na^+ in the shoot had lower biomass in general. Similar negative correlation between 4th leaf Na^+ accumulation in the shoot and SDW was also found in the Krichauff * Berkut DH population (Genc et al. 2010). SFW, SDW, TN and WC of plants under salt treatment showed a moderate positive correlation with STI. The heritability of SFW, SDW and TN was higher in salt treated than in control plants (Table 10). The difference in heritability between the salt and control conditions implied these traits were responsive to salt and may be controlled by salt-inducible genes. Our results also suggest that the shoot biomass, number of tiller and maintaining water content in the shoot had important contributions to salt tolerance in this population.

The QTL analysis revealed two suggestive QTL associated with 4th leaf Na^+ accumulation, one of which was mapped to the distal region on the long arm of chromosome 1D and the other to

the long arm of 3B. Neither QTL has been previously reported (De León et al. 2011; Genc et al. 2010; Quarrie et al. 2005) indicating potentially novel genes for 4th leaf Na^+ accumulation. A preliminary screen in a supported hydroponics using the Gladius * Drysdale RIL population identified a suggestive QTL for 4th leaf Na^+ accumulation in the same region of 1D. Other experiments using the same population under drought stress have been conducted in the field at different locations in south eastern Australia (Maphosa 2013). The analysis found QTL for yield and thousand kernel weight mapped on the long arm of chromosome 3B (Maphosa 2013), the same region reported here for the 4th leaf Na^+ accumulation QTL. Both loci on chromosomes 1D and 3B are likely to be important for Na^+ accumulation. However, the QTL analysis showed that these QTL were not highly significant and explained on average only 6% of the variance in Na^+ accumulation. This result may be due to the limited population size of only 162 lines as a factor affecting the power of identifying QTL is population size (Lehmensiek et al. 2009). The chance of recombination between two markers is low in a small population and this leads to an increase in statistical uncertainty associated with ordering markers which are close to each other (Wu et al. 2003). Therefore further evaluating of Na^+ accumulation in leaf tissue using additional RIL lines would be desirable.

QTL for TN, SFW and WC in the control and salt treatments were found in the distal region on the long arm of chromosome 5A, where the vernalisation gene (*VRN-A1)* is located. These QTL were significant (LOD > 3) in the salt and control conditions. This cluster was strongly associated with the *VRN-A1* gene. Koebner et al. (1996) studied the responses of Chinese Spring and a number of its aneuploids involving the chromosomes of homoeologous group 5 to salt stress in a hydroponics system under 200 mM NaCl. It was reported that lines lacking the long arm on 5D showed improved survival under salt stress and it was concluded that the salt stress response is associated with the *Vrn3* gene. Genc et al. (2010) detected QTL for most traits studied at the *VRN-A1* gene on the long arm of chromosome 5A for a doubled haploid population of bread wheat grown under control and saline conditions. De León et al. (2011)

also found three QTL for tiller number, plant height and mean ear length that mapped onto the distal region of the long arm of chromosome 5A (where the *VRN-A1* gene is located) in both control conditions and saline soils. Other experiments mapped QTL for yield and yield components in non-saline environments and found QTL for number of tiller, spikelet and yield that had strong associations with the *VRN-A1* gene on the long arm of chromosome 5A (Kato et al. 2000; Kumar et al. 2007). In these studies, it was concluded that the yield advantage was associated with the late flowering allele of *VRN-A1* in non-saline soil, and high potential yield was related to the response to a longer period of growth. However, in saline soil, De León et al. (2011) found that earlier flowering RILs were more salt tolerant than late flowering RILs. In the hydroponics experiment, the maturity date (number of days to booting or to head emergence) was not collected and therefore no estimation was made of whether early maturing lines were more salt tolerant or not in this population. It is clear that flowering time and salt tolerance traits require further study in future experiments.

The other QTL for TN and WC were found on the distal region of the short arm of chromosome 2D in the salt treated plants. This cluster of QTL was located in the region of the photoperiod gene (*Ppd-D1*). These QTL were significant (LOD>3) and accounted for 6.4% of the phenotypic variance for each trait. De León et al. (2011) detected a cluster of QTL for maturity and yield on the short arm of chromosome 2D when growing 114 RILs derived from crossing Opata 85 * W7984 for two seasons in control and saline fields. This cluster of QTL was mapped onto the *Ppd-D1* gene. As mentioned earlier, this gene has also been shown to have an important impact on yield and yield component for the Gladius * Drysdale RIL population grown under drought conditions. It has been found that QTL for yield and yield components were mapped on a distal region of chromosome short arm of 2D and this region contains *Ppd-D1* gene (Maphosa 2013). These results imply that the *Ppd-D1* gene has a strong effect on a number of traits when plants are grown under abiotic stress.

Previous studies found that the *VRN-A1* and *Ppd-D1* genes play a major role in salt tolerance in bread wheat and they are likely to have also been important in this study.

3.4.3 The response of RILs to saline conditions varies between soil based and hydroponics experiments

The data for plant biomass and 4th leaf ionic accumulation measured in soil based and hydroponic experiments were used to validate the ionic accumulation and to compare the response of RILs to salinity when grown in soil in pots or solution culture. Only a weak relationship was found between the soil based and hydroponics experiments (Section 3.3.4). There are three possible reasons for the lack relationships.

Firstly, the traits measured in each experiment were on plants at different growth stages. The RIL plants in the hydroponics experiment were grown for 45 d after germination, whereas in the soil based experiment plants were grown for 30 d. Rajendran et al. (2009) found that some accessions of *T. monococcum* were able to recover their growth rates after long term salt exposure even though these accessions showed an overall reduction in growth rate for 7 d after salt application. This indicated that the plant responses to salt stress may vary with developmental stage. Flowers and Flowers (2005) stated that the detection of QTL associated with salinity tolerance traits was dependent on growth conditions and the developmental stage of plants. It has been reported that QTL analysis differs with the developmental stage of various crops (tomato, rice, barley and citrus) grown in saline conditions (Flowers and Flowers 2005).

A second possible reason for the inconsistent experimental results is different growth conditions (soil based and solution culture). Tavakkoli et al. (2010) compared the responses of two barley genotypes (Clipper and Sahara) grown in supported hydroponics to those grown in soil in pots with different salt concentrations. They found that the growth rate and ion accumulation (Na^+, K^+ and Cl^-) of both genotypes varied depending on the growth conditions. However, some wheat studies have found a positive relationship between two data sets on

shoot Na^+ accumulation measured while plants grew in solution culture or soil (Edwards 2012; Ramsey 2007). A significant correlation was found between shoot Na^+ accumulation from the hydroponics system and from the saline field in two doubled haploid populations of bread wheat (Edwards 2012; Ramsey 2007). These two studies suggest that the data from plants grown in hydroponics system can give an indication of salinity tolerance based on Na^+ exclusion for plants grown in saline field for bread wheat.

In place of saline fields, the measurement of 3rd leaf Na^+ accumulation for plants grown in hydroponics system has been used for breeding salinity tolerance cultivars in durum wheat (Munns et al. 2012). A locus for 3rd leaf Na^+ accumulation (*Nax2)* was mapped onto the long arm of chromosome 5A of durum wheat (Byrt et al. 2007). Two near isogenic lines (NILs) produced from back-crossing Tamaroi (Australian durum wheat, which accumulates high 3rd leaf Na^+) with Line 149 (a breeding line derived from crossing durum wheat cultivar Marrocos and *T. monococcum* accession C68-101, which accumulates low 3rd leaf Na^+) (James et al. 2006). The NILs were evaluated in a hydroponics system using molecular markers flanking *Nax2* and the phenotypic trait (3rd leaf Na^+ accumulation) for selection (Munns et al. 2012). An increase of 26% in yield was found in the NILs compared to the recurrent parent Tamaroi growing in a saline field (Munns et al. 2012).

The results from these studies in wheat (Edwards 2012; Ramsey 2007) do not agree with the findings from a comparable study in barley (Tavakkoli et al. 2010). Barley accumulates much more shoot Na^+ and is more salt tolerant than bread wheat (Munns and Tester 2008). However, the basis for the difference for salinity tolerance between barley and wheat is not understood.

A third possible explanation for the unusual 4th leaf Na^+ accumulation observed in the soil based experiment was the difference in methods of salt application. In the hydroponics experiment NaCl was applied incrementally (25mM NaCl per day until it reached to 100 mM) whereas 75 mM NaCl was added to the plants in one step in the soil based experiment (Section

3.2.2.1). The method of salt application in the soil based experiment may have caused osmotic shock to the plants (Munns 2002).

Other pot based experiments conducted in the Plant Accelerator showed that applying a large volume of salt solution would avoid delivering a shock to the plants (Tilbrook et al. unpublished data). It is possible that the accumulation of high 4th leaf Na^+ in plants grown in the soil based experiment may be due to the small volume of salt solution that was applied, which led to a localised, high concentration in the pot.

3.5 Conclusion

The results suggest that osmotic stress tolerance has a higher contribution to salinity tolerance than 4th leaf Na^+ accumulation in the Gladius * Drysdale RIL population.

For future work, the QTL for osmotic stress tolerance that mapped to the distal regions of the short arms of chromosomes 1B, 2B and the long arm of chromosome 5D should be validated. A lager member of Gladius * Drysdale RILs and conditions shown in Section 3.2.2.1 with in exception in salt application are required for the validation of three OST QTL. This experiment would enable confirmation of the position of these QTL and lead to the identification of closely linked markers and possible candidate genes.

Since it has been established that osmotic stress tolerance and Na^+ exclusion are components of salinity tolerance in plants (Munns and Tester 2008), field experiments should be conducted to examine whether the RILs which accumulate low Na^+ and have osmotic stress tolerance, have a high or maintain yield potential in saline fields.

The validation of QTL for osmotic stress tolerance and leaf Na^+ accumulation, the correlation of these QTL with QTL for salt tolerance index, and the identification of the impact of vernalisation and photoperiod genes on biomass and yield in saline soil are likely to reveal the most important QTL for salt tolerance in the Gladius * Drysdale population. These QTL could

be used for further fine mapping, leading to the identification of candidate genes and the development of molecular markers for these salinity tolerance loci. The genes and markers will be valuable tools in breeding programmes aimed to improve salinity tolerance in bread wheat. Also, the identification of the putative alleles for Na^+ exclusion and osmotic stress tolerance would allow the additive and epistasis effects of these traits to be studied.

Chapter 4 Genetic behaviour of Na⁺ exclusion in Afghani landraces of durum wheat

4.1 Introduction

Cereals vary in their tolerance to salinity, with barley showing higher tolerance than bread wheat and durum wheat being particularly sensitive to salinity (Munns and Tester 2008). Durum wheat is a commercial cereal crop grown throughout temperate regions of the world, in both irrigated and rain-fed systems (Moragues et al. 2006). Much of the world's arable land, however, is affected by salinity, and this is a major limitation to durum wheat production (Francois et al. 1986; Maas and Grieve 1990). Low Na$^+$ accumulation (Na$^+$ exclusion) with a high ratio of K$^+$ to Na$^+$ in the shoot was identified as one of the major mechanisms associated with salt tolerance in *Triticeae* (Gorham et al. 1987).

Several genes associated with Na$^+$ exclusion (depending on the number of genes for tolerance segregating in a population) can show either quantitative or simple (one or two gene) inheritance. Two genes, (*Nax1* and *Nax2*), which control Na$^+$ exclusion, have been identified in durum wheat, however, separately or in combination these do not account for the large differences in shoot or 3rd leaf Na$^+$ exclusion seen in durum when compared to bread wheat (James et al. 2006). Therefore, additional loci or genes controlling Na$^+$ exclusion may be present in diploid and tetraploid wild wheats or durum wheat landraces.

Recently, two Afghani landraces (*T. turgidum* ssp. *Durum*, lines 740 and 752) had been found to accumulate low concentrations of Na$^+$ in 3rd leaf tissue when compared with other durum wheat cultivars and landraces screened in hydroponics experiments (Figure 29). A large difference in Na$^+$ exclusion was shown between Jandaroi and two Afghani landraces (Shavrukov et al. 2011). These lines were found to not contain the *Nax1* gene, but were not

assayed for the presence or absence of the *Nax2* locus (Shavrukov et al. 2011). Therefore, it was possible that these lines could be sources of novel loci or genes. The aim of this study was to determine the genetic control of Na$^+$ exclusion in two lines of Afghani durum landrace wheat.
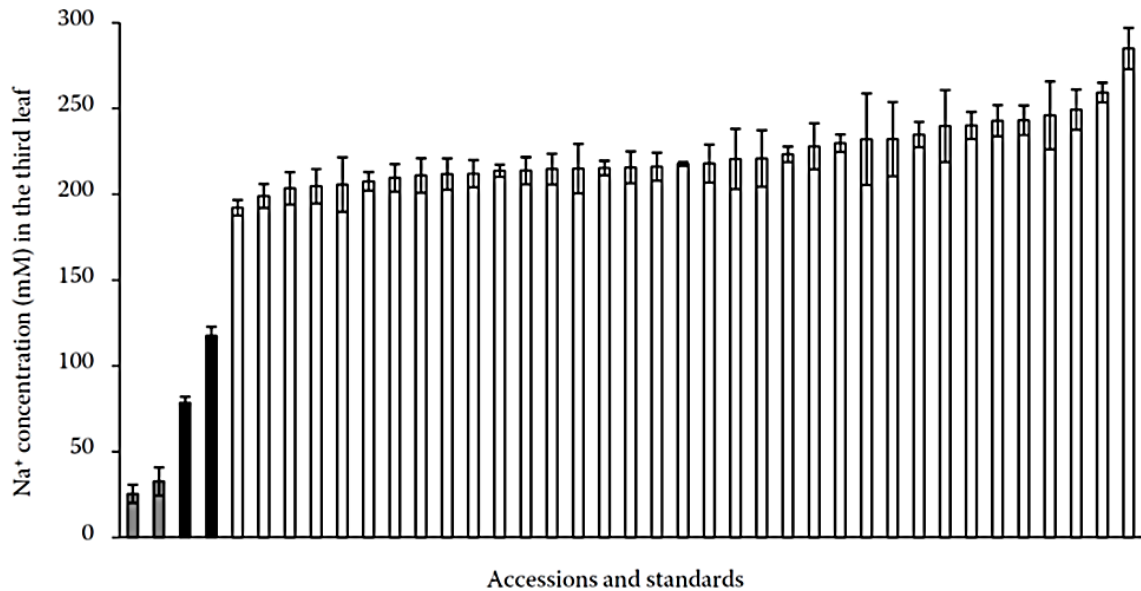
Figure 28. Na$^+$ concentration in fully expanded third leaves of wheat genotypes; two bread wheat cultivars (Krichauff and Kharchia-65) are controls and represented by the shaded bars; two durum wheat landraces (line 740 and line 752) are represented by the black bars and 35 durum wheat cultivars and accessions are depicted as clear bars. Plants were grown in a supported hydroponics system at 100 mM NaCl for 10 d. Each value is the mean of four replicates ± standard error (Shavrukov et al. 2011)

4.2 Materials and methods

4.2.1 Plant materials

Lines 740 and 752 were obtained from the Australian Winter Cereal Collection, Tamworth, New South Wales. In contrast, Jandaroi is an Australian durum cultivar released in 2007 by the New South Wales Department of Primary Industries and is adapted to central and southern Queensland and some parts of New South Wales, Western Australia and South Australia. The study in this Chapter generated two F_2 populations derived from crossing between Jandaroi selected as the elite parent, and lines 740 and 752. These F_2 populations were used to study the genetic behaviour of 3rd leaf Na$^+$ accumulation in lines 740 and 752. 130 F_2 plants derived from the cross between Jandaroi * line 740 and 200 F_2 plants from the cross Jandaroi * line 752. These populations were grown in a supported hydroponics system. After the measurement of 3rd leaf Na$^+$ concentration, lowest and highest 3rd leaf Na$^+$ accumulation F_2 plants of both populations were selected for selective genotyping analysis (Section 4.2.6.2) and transplanted into non-saline soil to grow those to the seed stage and to produce $F_{2:3}$ progeny. These progeny were used to study the relationship between 3rd leaf Na$^+$ accumulation of selected F_2 plants and 3rd leaf Na$^+$ accumulation of $F_{2:3}$ progeny.

4.2.2 Crossing

Jandaroi (maternal) and the two Afghani landraces (paternal) were crossed to produce F_2 populations. After spikelet formation in the maternal plants, the central spikelets of the immature ear were removed using forceps, leaving two side spikelets. The emasculation was conducted by removing immature anthers and the emasculated ear was then isolated in a paper bag. When the spikelets were mature, they were pollinated by mature anthers taken from the paternal plants and put directly onto the stigmas of the female plants. The pollinated ear was re-bagged and pollination details were recorded. All tools (scissors and forceps) and hands were disinfected with ethanol between the emasculation of each genotype.

4.2.3 Growth conditions

The two F_2 populations were grown in a supported hydroponics system in a controlled environment to test the accumulation of Na$^+$ and K$^+$ in 3rd leaf tissue. Each population was grown in one separate trolley. Each trolley had two tubs which were divided into ten rows and 16 columns. Plants from both F_2 populations were grown in tubs filled with black pellets (Figure 30) in growth solution and under conditions described in Section 2.2.2. A single salt concentration of 100 mM NaCl was applied as outlined in Section 3.2.2.2 (The hydroponic experiment). These conditions and the salt application were also used in growing of selected $F_{2:3}$ progeny.

Figure 29. A supported hydroponics system providing growth conditions used to evaluate two F_2 populations derived from crossing Jandaroi and two Afghani landraces under 100 mM NaCl

4.2.4 Experimental design

A spatial design was used to conduct the experiment of measuring 3rd leaf Na$^+$ and 3rd leaf K$^+$ accumulations of each F$_2$ population by locating F$_2$ plants in rows and columns per a tub. As both segregation populations were F$_2$ which are not replicable (Section 1.2.4.1.1, Table 2), each parent was replicated ten times per tub and were randomised using lattice square design (GenStat, version 14th edition package, VSN International Ltd, Hemel Hempstead, UK). The replicates of the parents were used to estimate the experimental error and environmental effects, using a linear mixed model. This enabled us to estimate the heritability of studied traits.

Similarly, the 3rd leaf Na$^+$ accumulation in selected F$_{2:3}$ progeny experiment was designed according the experimental design of the F$_2$ populations.

4.2.5 Measurement of 3rd leaf Na$^+$ and 3rd leaf K$^+$ accumulation

Plants of the two F$_2$ populations and the parents were grown for 10 days in the salt solution prior to harvest and to the stage when 3rd leaf was fully expanded. The 3rd leaf of each plant was then harvested and the Na$^+$ and K$^+$ concentration was measured in these leaves according to Section 2.2.3. The ratio of K$^+$ /Na$^+$ was calculated by dividing the value of 3rd leaf K$^+$ concentration to 3rd leaf Na$^+$ concentration. This measurement was also used to assess 3rd leaf Na$^+$ accumulation in the selected F$_{2:3}$ progeny.

4.2.6 Genotyping characterising

After 3rd leaf Na$^+$ accumulation was measured, DNA of two F$_2$ populations' plants and the parents was extracted and analysed through selective genotyping analysis and balked segregant analysis. DArT markers were used in selective genotyping analysis and these DArTs which showed a strong association with 3rd leaf Na$^+$ accumulation were validated using microsatellite markers. The Jandaroi * line 740 F$_2$ population was only used for further genetic analysis as an association was detected in this population. The association between microsatellite markers and

3rd leaf Na$^+$ accumulation in the entire Jandaroi * line 740 F_2 population was analysed using marker regression analysis.

SNP markers were used in bulked segregant analysis. The putative SNP markers identified from bulk segregant analysis were designed and used in genotyping of the entire Jandaroi * line 740 F_2 population. The association between putative SNP markers and 3rd leaf Na$^+$ accumulation in the entire Jandaroi * line 740 F_2 population was analysed using marker regression analysis.

4.2.6.1 DNA extraction

Two methods were used for extracting DNA from plants. Firstly, a freeze dry method was used. In this method, DNA extraction was conducted in racks containing 96 x 1.1 mL Micro Tubes (wells). A section of the fourth leaf (about 30 mm long) was cut from each plant and each piece of leaf was put in a well. DNA extraction was performed using a method described in Shavrukov et al. (2010). This method was used to extract DNA from the entire F_2 population for both crosses.

 Secondly, the DNA extraction was performed using a DNA midi-prep method. Two g of green leaf of F_2 plants were harvested at botting stage and collected into a yellow capped tube (10 mL, Sarstedt, Australia). The DNA extraction was carried out as described in Rogowsky et al. (1991). This was used to extract DNA from F_2 plants which selected for selective genotyping analysis in both F_2 populations.

4.2.6.2 Selective Genotyping Analysis (SGA)

In each F_2 population, two groups of F_2 plants were selected: 20 F_2 plants from each tail of the distribution of 3rd leaf Na$^+$ accumulation: (20 plants accumulated the lowest 3rd leaf Na$^+$ concentration and 20 plants accumulated the highest 3rd leaf Na$^+$ concentration). The DNA of 40 individual F_2 plants from each population was extracted from leaf material and the concentration of DNA was estimated in 1% agarose gel in the presence of a 1 kb ladder. On the basis of DNA concentration and quality, 19 of the lowest and 16 of the highest 3rd leaf Na$^+$

accumulating F$_2$ plants were selected. The DNA concentrations were adjusted to 50 ng/μL and then these were sent to Triticarte Pty Ltd. (http://www.tritcarte.com.au) for DArT platform genotyping. To detect the loci associated with 3rd leaf Na$^+$ accumulation, SGA was conducted by measuring the co-segregation of alleles of each DArT marker in the selected F$_2$ plants with lowest and highest Na$^+$ accumulation. After identifying DArT loci of interest, microsatellite markers adjacent to these DArT loci were selected and identified from the published genetic maps of the crosses: Ajana * WAWHT2074, Cadoux * Reeves, EGA Blanco * Millewa (Francki et al. 2009), Carnamah * WHWAT2046 (Ryan 2005), Cranbrook * Halberd (Kammholz et al. 2001), Synthetic * Opata (Somers et al. 2004). The linkage maps of these populations were taken from Triticarte (http://www.triticarte.com.au/content/further_development.html) and GrainGenes (http://wheat.pw.usda.gov/) web sites. The parents and F$_1$ plants were genotyped with the selected microsatellite markers in order to test the polymorphism of these markers. The markers showed polymorphism between the parents, were then used in SGA to genotype selected F$_2$ plants with the lowest and highest 3rd leaf Na$^+$ accumulation.

4.2.6.2.1 DArT markers

Diversity Array Technology (DArT) is a high-throughput microarray hybridisation technique enabling the detection of hundreds of polymorphic markers across a genome (Jaccoud et al. 2001; Wenzl et al. 2004). Approximately 20 μL of 50 ng/μL DNA is needed to genotype 5000–7000 genomic loci in parallel in a single-reaction assay. Gupta et al. (2008) reported that DArT markers are reproducible and cost-effective, and have been successfully used in different plants such as rice (Jaccoud et al. 2001), barley (Wenzl et al. 2004), cassava (Xia et al. 2005) and wheat (Akbari et al. 2006; Semagn et al. 2006).

5000 of DArT markers was used to genotype 20 μL of 50 ng/μL DNA of the selected F$_2$ plants that exhibited lowest and highest 3rd leaf Na$^+$ accumulation from two populations. DArT

markers are dominant markers and their allele frequency were used in SGA by comparing the dominant and recessive alleles with the 3rd leaf Na$^+$ accumulation of selected F$_2$ plants in order to detect the association between DArT markers and 3rd leaf Na$^+$ accumulation in two F$_2$ populations.

4.2.6.2.2 Microsatellite markers

Microsatellite markers or simple sequence repeats (SSR) are PCR-based markers which amplify a stretch of DNA that is comprised of repeats of sets of nucleotides (Paux and Sourdille 2009). These nucleotides can be repetitive and distributed throughout the wheat genome. The variation in microsatellite length is due to the differences in the repeat numbers of the microsatellites. These variations are consistently inherited and, therefore, can be used as genetic markers (Ganal and Röder 2007). The advantages of using microsatellite markers, described by Röder et al. (1998), are that they are co-dominant and multiallelic; have a high level of polymorphism at each locus; have a genomic specificity which allows the analysis of three homoeologous chromosomes in bread wheat or two homoeologous chromosomes in durum; and many have been mapped and published for bread and durum wheat populations. Microsatellites were used together with DArT markers in this study. 140 microsatellite markers located near the significant DArT loci were selected from published genetic maps and used to genotype the parents (Jandaroi, line 740 and line 752). Polymorphic markers were then used for SGA. Multiplex-ready marker technology was applied as described by Hayden et al. (2007) to genotype the parents and the F$_2$ populations that were generated. A detailed protocol is provided at http://www.genica.net.au. The markers showing a strong association with 3rd leaf Na$^+$ accumulation in the SGA were used to genotype plants of the entire F$_2$ populations.

In addition, microsatellite markers were used to test whether Na$^+$ exclusion in lines 740 and 752 was due to alleles at the *Nax1* and/or *Nax2* loci, microsatellite markers, *Xgwm291* and *Xgwm410,* which co-segregate with *Nax2* (Byrt 2008) and the marker *Xgwm312* which co-

segregates with *Nax1* locus (Lindsay et al. 2004) were used to genotype the entire two F$_2$ populations.

4.2.6.3 Bulked segregant analysis (BSA)

BSA was conducted in the Jandaroi * line 740 F$_2$ population. Two bulks were generated: DNA samples from 19 F$_2$ plants which exhibited the lowest 3rd leaf Na$^+$ accumulation were pooled in equal quantities to construct a low Na$^+$ accumulation bulk (Bulk 1). Similarly, DNA samples from 16 F$_2$ plants which exhibited the highest 3rd leaf Na$^+$ were pooled to create a high Na$^+$ accumulation bulk (Bulk 2). The DNA of parents, Bulk 1 and Bulk 2 were genotyped with 9000 SNP markers using Infinium SNP genotyping assays which were performed on the Illumina BeadStation and iScan instruments according to the manufacturer's protocols (Illumina) as described by Cavanagh et al. (2013). SNP allele clustering and genotype calling was performed using GenomeStudio v2011.1 software (Illumina) (Cavanagh et al. 2013). The TaGENmap_v1.7 describes SNP behaviour observed from genotyping about 8000 wheat lines. All Gen- Call data were manually checked, and positive hits for BSA were recorded when a SNP was polymorphic between Jandaroi and line 740. Bulk 1 (low Na$^+$ accumulation bulk) clustered tightly with line 740 and bulk 2 (high Na$^+$ accumulation bulk) clustered tightly with Jandaroi in the GenCall output. The markers with strong and medium association with 3rd leaf accumulation were considered to be putative SNP markers. The genotyping analysis was carried out at Victorian AgriBiosciences Centre, Bundoora, Australia.

4.2.6.3.1 SNP markers

The DNA samples were analysed using a 9000 SNP marker platform (Cavanagh et al. 2013; Hayden et al. 2011) in BSA. These SNPs were generated based on sequencing whole wheat genome using Roche 454 and Illumina (GAIIx and HiSeq2000) next-generation sequencing (Cavanagh et al. 2013). A single nucleotide polymorphism (SNP) is defined as the difference between genotypes of one or more nucleotides at a given DNA sequence (Ganal and Röder 2007). SNP markers have been used in many species, and are especially useful for crops with

complex genomes (e.g. allopolyploid species such as wheat) (Ganal et al. 2009). SNP markers provide a high level of genetic polymorphism between wheat genotypes (Edwards et al. 2012). SNP markers were used in this project as this markers are associated with cost-efficient and high-throughput multiplexing analysis of genotyping (Ganal and Röder 2007).

After the putative SNP markers were identified from BSA, the sequence of each marker obtained from the Infinium wheat SNP platform (http://wheat.pw.usda.gov/ggpages/9K_assay_available.html) was then utilised to generate the forward and the reverse primers, using Primer 3 software (http://frodo.wi.mit.edu/). All primers were designed in the range between 100–300 bp. The annealing temperature for all primers was 60°C. The GC content (CG%) of the designed primers ranged between 40-60%. The possibility of dimerisation and hairpins on the designed primers were tested using NetPrimer software (http://www.premierbiosoft.com), and those with a ΔG of more than -5 kcal/mol were re-designed. After the sequences of primers were designed, these were generated by GeneWorks (http://www.genework.com.au/). Initially primers were tested on the parents, the F$_1$ hybrids and 12 F$_2$ lines to validate them. The fragments were amplified with designed primers and genotyped using High Resolution Melting Point technology.

4.2.6.4 Marker regression analysis (MRA)

To analyse the association between markers (microsatellites and SNPs) and phenotype (accumulation of 3rd leaf Na$^+$ and 3rd leaf K$^+$, and the ratio of K$^+$/Na$^+$) in the entire F$_2$ population, an MRA was conducted using QTX software (MapManager) (Manly et al. 2001). The analysis was carried out by inserting the phenotypic and the genotypic score data into the MapManager program. Each marker was analysed individually without the need for constructing the linkage map. The report from the analysis lists the chromosome name, the locus (marker) name, the likelihood ratio statistic (LRS), the phenotypic variation explained by

the locus (percentage), and the parental allele. The LRS value was converted to a LOD score using the formula **LOD score = LRS / 4.61** (Liu 1998)**.**

If the presence of a paternal allele tends to increase the trait, then the parental allele has a positive effect and if the presence of parental allele tends to decrease the trait, then the parental allele was estimated as negative.

4.2.7 Statistical analysis

A linear mixed model and variance components estimation, using the method of residual maximum likelihood (GenStat 14 edition package, VSN International Ltd, Hemel Hempstead, UK), were used to estimate the broad-sense heritability and best linear unbiased estimates (BLUEs) of phenotypic traits: 3rd leaf Na$^+$ and 3rd leaf K$^+$ accumulation and the ratio of K$^+$/Na$^+$ in both populations (Appendix IV, Section 6.4.3). Each population was treated and analysed individually. The broad-sense heritability is defined as the genetic proportion of phenotypic variance. Usually, heritability is estimated as the ratio of genotypic variation to phenotypic variation (the total of genetic variation and variation due to environment and experimental errors) (Lynch and Walsh 1998). To estimate the heritability for each trait, two models were developed for both fixed and random terms. Genotypes (Type) were treated in the fixed model as imposed factors. The factor Type has three levels (Jandaroi, line 470 or line 752, and plants of the F$_2$ population). In random model, the F$_2$ plants, rows, columns, and tubs were used as random factors.

BLUE values are an estimated value for phenotypic traits which is due to genetic variation of F$_2$ plants across the effects of rows, columns and tubs. To estimate the BLUE values which were then used in the MRA (Section 4.2.6.4), two models were developed. The individual F$_2$ plants were used as an imposed treatment factor in the fixed model, whereas rows, columns, and tubs were used in the random model.

Linear regression analysis package in Microsoft Excel was used to study the relationship between 3rd leaf Na$^+$ accumulation of selected F$_2$ plants and 3rd leaf Na$^+$ accumulation of F$_{2:3}$ progeny.

4.3 Results

4.3.1 Phenotyping

Approximately 130 F$_2$ seeds from crossing Jandaroi with line 740, and approximately 200 F$_2$ seeds were obtained from crossing Jandaroi with line 752. The distribution of 3rd leaf Na$^+$ accumulation for both populations (Figure 31, a and d) appears to be bimodal and segregated closely to the ratio 3:1. Also the 3rd leaf K$^+$ accumulation (Figure 31 b and e) and the K$^+$:Na$^+$ ratio (Figure 31 c and f) distributions appeared to be bimodal. This implies that a major gene is associated with 3rd Na$^+$ accumulation in the durum wheat landraces. The variation in the Jandaroi * line 740 F$_2$ population was larger than the variation in the Jandaroi * line 752 F$_2$ population, even though the population size of the former was smaller (94 plants) than the latter (176 plants). This suggests that the genetic effect in the Jandaroi * line 740 F$_2$ might be larger than in the Jandaroi * line 752 F$_2$ population.
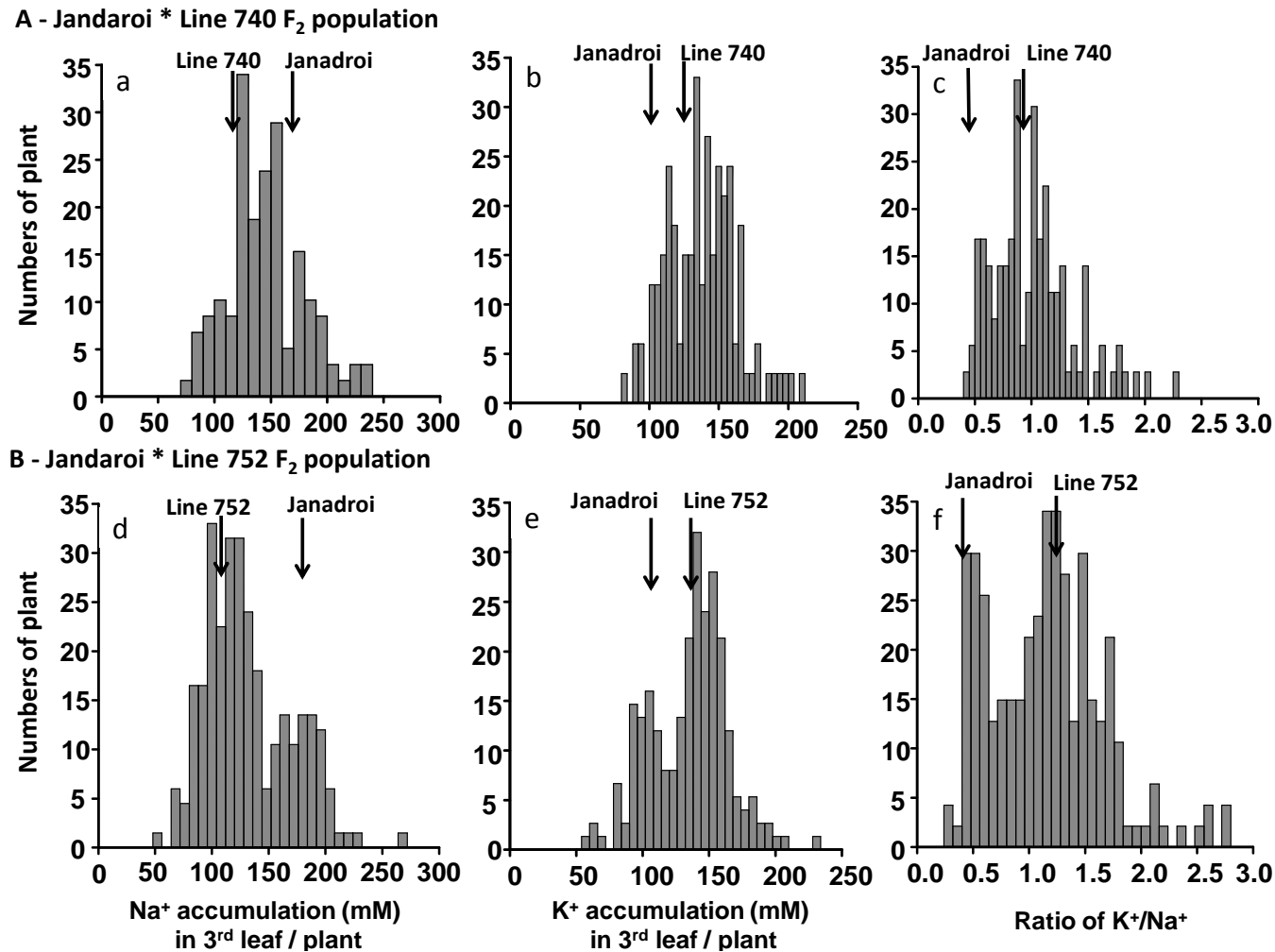
Figure 30. Distributions of Na$^+$ accumulation (a and d), K$^+$ accumulation (b and e) and K$^+$/Na$^+$ ratio (c and f) of two F$_2$ populations derived from crossing Jandaroi and line 740 (A) and line 752 (B). Arrows indicate parental means (n=10). 100 mM NaCl was applied at the time of the third leaf emergence. Na$^+$ and K$^+$ concentrations were measured in the fully expanded third leaf 10 d after salt application.

Table 15. Na$^+$ accumulation (Na), K$^+$ accumulation (K) and K$^+$/Na$^+$ ratio values for Jandaroi, line 740, line 752 and F$_2$ population mean, range (min–max) and heritability (h^2) in 100 mM NaCl. Na and K were measured in the fully expanded third leaf 10 d after salt application.

Population	Trait	Parents		F$_2$		h^2
		Jandaroi	line 740	Mean	Min - Max	
Jandaroi * line 740	Na (mM)	168.5 ± 7.2	137.7 ± 8.86	149.7 ± 3.9	79.8 - 284.3	0.89
	K (mM)	119.1 ± 6.52	147.4 ± 5.9	141.2 ± 2.5	83.8 - 209.2	0.41
	K/Na	0.7 ± 0.04	1.10 ± 0.066	1.01 ± 0.035	0.45 - 2.25	0.79
		Jandaroi	line 752			
Jandaroi * line 752	Na (mM)	185.7 ± 5.9	123.2 ± 14.8	129.8 ± 2.7	49.27 - 266.1	0.78
	K (mM)	116.9 ± 10.63	140.2 ± 9.0	138.0 ± 2.35	60.36 - 315.7	0.11
	K/Na	0.64 ± 0.07	1.26 ± 0.14	1.20 ± 0.04	0.26- 2.92	0.66

To estimate the genetic contribution to the phenotypic variation of the traits, the broad-sense heritability of 3rd leaf Na$^+$ and 3rd leaf K$^+$ accumulation and ratio of K$^+$/Na$^+$ in both populations were calculated. The heritability of 3rd leaf Na$^+$ accumulation for the F$_2$ populations was 0.89 and 0.78 for Jandaroi * line 740 and Jandaroi * line 752, respectively. The heritability of the K$^+$/Na$^+$ ratio was also large in both populations, whereas the heritability of 3rd leaf K$^+$ accumulation was moderate in the Jandaroi * line 740 F$_2$ population (0.41) and low in the Jandaroi * line 752 F$_2$ population (Table15). Based on these results, the objective of this project was directed toward detecting the genetic loci associated with 3rd leaf Na$^+$ accumulation in lines 740 and 752 using molecular markers.

4.3.2   3rd leaf Na$^+$ accumulation in lines 740 and 752 and *Nax1, Nax2* loci

Both *Nax1* and *Nax2* genes are associated with low Na$^+$ accumulation in the shoot (James et al. 2006). Marker *Xgwm312,* which is linked to *Nax1* (Lindsay et al. 2004), and markers *Xgwm291* and *Xgpw2181*, which flank *Nax2* (Byrt et al. 2007), were used to validate whether 3rd leaf Na$^+$ accumulation in lines 740 and 752 was associated with variation at the *Nax1* and/or *Nax2* loci. Firstly, the parents were genotyped and results showed that markers flanking *Nax2* (*Xgwm291* and *Xgpw2181*) were monomorphic, whereas *Xgwm312* linked to *Nax1* was polymorphic between Jandaroi and line 740. In contrast, markers linked to both

Nax1 and *Nax2* were polymorphic between Jandaroi and line 752. The F$_2$ plants of the two populations were then genotyped and analysed using MRA in Map Manager. The analysis showed very low association between *Xgwm312* and 3rd leaf Na$^+$ accumulation, with a LOD score of only 0.8, and this marker explained only 4 % of the phenotypic variation in the Jandaroi * line 740 F$_2$ population (Table 16). Similarly, the markers *Xgwm312*, *Xgwm291* and *Xgpw2181* had a low association with 3rd leaf Na$^+$ accumulation in the Jandaroi * line752 F$_2$ population, with LOD scores of 0.3, 1.3, and 2.0 respectively. These results imply that reduced 3rd leaf Na$^+$ accumulation in the two Afghan landraces is not due to variation at either the *Nax1* or *Nax2* loci. A further genetic analysis was carried out to identify novel genetic loci associated with 3rd leaf Na$^+$ accumulation derived from lines 740 and 752.

Table 16. The association analysis between 3rd leaf Na$^+$ accumulation in two F$_2$ populations derived from crossing Jandaroi and two lines (740 and 752), and microsatellite markers flanking loci *Nax1* and *Nax2*. This analysis was conducted using MRA in QTX MapManager software. The table shows the LOD scores of each association and the percentage of 3rd leaf Na$^+$ accumulation variation explained by each microsatellite marker (% Variation).

Population	Locus	Flanked marker	Chromosome	LOD score	% Variation
	Nax1	*Xgwm312*	2AL	0.8	4
Jandaroi * line740 F$_2$	*Nax2*	*Xgwm291*	5AL	Monomorphic	
	Nax2	*Xgpw2181*	5AL	Monomorphic	
	Nax1	*Xgwm312*	2AL	0.3	1
Jandaroi * line 752 F$_2$	*Nax2*	*Xgwm291*	5AL	1.3	4
	Nax2	*Xgpw2181*	5AL	2.0	6

4.3.3 Selective genotyping analysis (SGA)

SGA was conducted to detect loci associated with 3rd leaf Na$^+$ accumulation. DNA extracted from lines at the tails of the bimodal distribution (very low and very high 3rd leaf Na$^+$ accumulation) of both populations was analysed using 5000 DArT markers. Between Jandaroi and line 740 there were 616, and between Jandaroi and line 752 there were 707 polymorphic markers. In the F$_2$ Jandaroi * line 740 plants with lowest and highest in 3rd leaf Na$^+$ accumulation, SGA showed that a region on the long arm of the chromosome 4B was strongly

associated with that difference in 3rd leaf Na$^+$ accumulation (Table 17A1); 18 plants with lowest 3rd leaf Na$^+$ accumulation carried dominant alleles (BB or AB) derived from line 740, whereas 12 of 16 plants with highest 3rd leaf Na$^+$ accumulation showed the null alleles (AA) derived from Jandaroi. It should be noted that DArT markers are scored as present (dominant) or nulls. Therefore, in an F_2 population, the assay can only determine a difference between homozygous nulls, because heterozygous and homozygous positives are indistinguishable. Three loci which were detected on chromosomes 1B, 4A and 7B were partially associated with 3rd leaf Na$^+$ accumulation. In the second population, five loci on chromosomes 2B, 3B, 4A and 6A were partially associated with 3rd leaf Na$^+$ accumulation (Table 17B).

Table 17. Allele frequency of DArT markers in selected F_2 plants with lowest and highest 3rd leaf Na$^+$ accumulation. The alleles were categorised into AA (homozygous for the allele from Jandaroi parent), BB (homozygous for the allele from line 752 and 740 parents) and AB is heterozygous. DArT are dominant markers, where homozygotes and heterozygotes cannot be distinguished. Therefore, the dominant alleles from DArT analysis were labelled as AA/AB or BB/AB. Selected F_2 plants derived from the cross Jandaroi * line 740 (A1 and A2) show that DArT markers located on chromosome 4B have a strong association with the trait, whereas other markers show a partial association with 3rd leaf Na$^+$ accumulation. Selected F_2 plants derived from the cross Jandaroi * line 752 (B) show a partial association with 3rd leaf Na$^+$ accumulation in both lowest and highest 3rd leaf Na$^+$ accumulation with all DArT markers.

A– Jandaroi * line 740 F_2 population

A1

DArT marker	Chromosome	Line 740	Allele								Jandaroi
			Lowest 3rd leaf Na$^+$ plants				Highest 3rd leaf Na$^+$ plants				
			BB/AB	AA	failed	total	BB/AB	AA	failed	total	
wPt-2988	1B	BB	12	7		19	11	3	2	16	AA
wPt-3477	1B	BB	12	7		19	12	3	1	16	AA
wPt-2485	1B	BB	16	2	1	19	12	4		16	AA
wPt-7094	1B	BB	12	7		19	13	3		16	AA
wPt-8267	1B	BB	12	7		19	13	3		16	AA
wPt-2395	1B	BB	12	7		19	13	3		16	AA
wPt-6370	1B	BB	12	7		19	13	3		16	AA
wPt-7138	1B	BB	12	7		19	13	3		16	AA
wPt-3411	1B	BB	12	7		19	13	3		16	AA
wPt-7259	1B	BB	12	7		19	13	3		16	AA
wPt-1568	1B	BB	11	7	1	19	13	2	1	16	AA
wPt-3804	4B	BB	18	1		19	4	12		16	AA
wPt-5996	4B	BB	18	1		19	4	12		16	AA
wPt-3917	4B	BB	18	1		19	4	8	4	16	AA

Table 17. Continued

A2

DArT marker	Chromosome	Line 740	Lowest 3rd leaf Na$^+$ plants				Highest 3rd leaf Na$^+$ plants				Jandaroi
			AA/AB	BB	failed	total	AA/AB	BB	failed	total	
wPt-5212	4A	BB	11	8		19	12	4		16	AA
wPt-8167	4A	BB	11	8		19	13	3		16	AA
wPt-8271	4A	BB	11	8		19	13	3		16	AA
wPt-3108	4A	BB	11	8		19	13	3		16	AA
wPt-2985	4A	BB	11	8		19	13	3		16	AA
wPt-6390	4A	BB	11	8		19	5	11		16	AA
wPt-5578	4A	BB	12	7		19	10	2	4	16	AA
wPt-6900	4A	BB	12	7		19	13	3		16	AA
wPt-4272	4A	BB	16	3		19	14	2		16	AA
wPt-5172	4A|2R|3R	BB	13	6		19	14	2		16	AA
rPt-0238	4A|2R|3R	BB	13	6		19	14	2		16	AA
wPt-9183	4A|4R	BB	11	8		19	13	3		16	AA
wPt-3796	4A|4R	-	11	8		19	12	3	1	16	-
wPt-2933	7B	BB	16	2	1	19	11	5		16	AA
tPt-8569	7B	BB	16	3		19	10	6		16	AA
wPt-4140	7B	BB	17	2		19	10	5	1	16	AA
wPt-0884	7B	BB	18	1		19	10	6		16	AA
wPt-9746	7B	BB	17	2		19	11	5		16	AA
wPt-9813	7B	BB	17	2		19	11	5		16	AA
wPt-5069	7B	BB	17	2		19	11	5		16	AA
wPt-6320	7B	BB	17	2		19	11	5		16	AA
wPt-0126	7B	BB	17	2		19	11	5		16	AA
wPt-9515	7B	BB	17	2		19	11	5		16	AA
tPt-8504	7B	BB	16	2	1	19	11	5		16	AA

Table 17. Continued

B – Jandaroi * line 752 F$_2$ population

DArT marker	Chromosome	Line 752	Allele								Jandaroi
			Lowest 3rd leaf Na$^+$ plants				Highest 3rd leaf Na$^+$ plants				
			AA /AB	BB	failed	total	AA/AB	BB	failed	total	
wPt-9527	2B	BB	16	1	1	18	10	6		16	AA
wPt-6477	2B	BB	17	1		18	10	6		16	AA
wPt-1140	2B	BB	17	1		18	10	6		16	AA
wPt-6174	2B	BB	17	1		18	10	6		16	AA
wPt-8548	2B	BB	17	1		18	10	6		16	AA
wPt-2117	2B	BB	17	1		18	10	6		16	AA
wPt-5825	3B	BB	16	2		18	10	6		16	AA
wPt-0280	3B	BB	16	2		18	10	6		16	AA
wPt-0995	3B	BB	16	2		18	10	6		16	AA
wPt-4576	3B	BB	16	2		18	10	6		16	AA
wPt-8983	3B	BB	16	2		18	10	6		16	AA
wPt-8352	3B	BB	15	2	1	18	10	6		16	AA
wPt-4370	3B	BB	16	2		18	10	6		16	AA
wPt-0365	3B	BB	16	2		18	10	6		16	AA
wPt-1822	3B	BB	16	2		18	10	6		16	AA
wPt-8396	3B	BB	16	2		18	10	6		16	AA
wPt-8141	3B	BB	16	2		18	10	6		16	AA
wPt-7266	3B	BB	16	2		18	10	6		16	AA
wPt-0405	3B	BB	16	2		18	10	6		16	AA
wPt-0438	3B	BB	16	2		18	10	6		16	AA
wPt-10407	3B	BB	16	2		18	10	6		16	AA
wPt-8167	4A	BB	11	7		18	14	2		16	AA
wPt-8271	4A	BB	11	7		18	14	2		16	AA
wPt-3108	4A	BB	11	7		18	14	2		16	AA
wPt-3766	4A	BB	13	5		18	14	1		16	AA
wPt-7327	4A	BB	11	7	2	18	15	1		16	AA
wPt-6214	4A	BB	13	5		18	15	1		16	AA
wPt-9833	4A	BB	13	5		18	14	1		16	AA
wPt-669203	4A	BB	12	6	1	18	15	1		16	AA
wPt-5749	4A	BB	13	5		18	15	1		16	AA
wPt-0105	4A	BB	13	5		18	15	1		16	AA
wPt-9872	4A	BB	13	5		18	15	1		16	AA
wPt-8078	4A	BB	13	5		18	15	1		16	AA
wPt-6520	6A	BB	11	6	1	18	12	3	1	16	AA
wPt-4016	6A	BB	12	6		18	12	3	1	16	AA
wPt-3468	6A	BB	12	6		18	13	3		16	AA
wPt-8006	6A	BB	12	6		18	13	3		16	AA
wPt-2636	6A	BB	9	7	2	18	13	3		16	AA
wPt-4017	6A	BB	12	6		18	12	3	1	16	AA
wPt-7330	6A	BB	11	6	1	18	11	4	1	16	AA
wPt-9280	6A	BB	12	6		18	12	3	1	16	AA
wPt-7754	6A	BB	12	6		18	13	3		16	AA
wPt-7623	6A	BB	12	6		18	13	3		16	AA
wPt-3965	6A	BB	12	6		18	13	3		16	AA
wPt-9832	6A	BB	12	6		18	13	3		16	AA
wPt-4270	6A	BB	12	6		18	13	3		16	AA
wPt-4589	6A	BB	10	6	2	18	12	3	1	16	AA
wPt-665782	6A	BB	12	6		18	13	3		16	AA
wPt-666416	6A	BB	12	5	1	18	13	3		16	AA
wPt-6904	6A	BB	12	6		18	13	3		16	AA

To validate the SGA results, 140 microsatellite markers flanking and spanning the regions of putative DArT loci were used. The parents Jandaroi, lines 740 and 752 were genotyped using microsatellite markers to identify polymorphic markers. Multiplex PCR was used to genotype the parents. Nine markers showed polymorphisms between Jandaroi and line 740 (two markers on each of chromosomes 4B, 1B and 7B, and three markers on chromosome 4A), whereas six markers showed polymorphisms between Jandaroi and line 752 (two markers on each chromosome, 4A, 2B and 6A). Fifty six markers were monomorphic and 65 markers failed to generate useful amplification products. The polymorphic markers were used to genotype the individual lowest and highest 3rd leaf Na$^+$ accumulation plants in both F$_2$ populations. The marker *Xgwm160* on chromosome 4A and two markers (*Xbarc60* and *Xgwm375*) on chromosome 4B showed a high association with 3rd leaf Na$^+$ accumulation in the lowest 3rd leaf Na$^+$ plants in the Jandaroi * line 740 F$_2$ population, but they showed low association in the highest 3rd leaf Na$^+$ plants. Other microsatellite markers showed either no associations or failed (Table 18 A). The lowest and highest 3rd leaf Na$^+$ F$_2$ plants from the Jandaroi * line 752 cross showed no association with 3rd leaf Na$^+$ accumulation (Table 18 B).

Table 18. Allele frequency of microsatellite markers in selected F$_2$ plants. The alleles were categorised into AA (homozygous for the allele from Jandaroi parent), BB (homozygous for the allele from lines 752 or 740 parents) and AB (heterozygous). Selected F$_2$ plants derived from crossing Jandaroi with line 740 (A) show that *Xgwm160*, *Xbarc60* and *Xgwm375* have an association with lowest 3rd leaf Na$^+$ accumulation plants, whereas other markers do not show an association. Selected F$_2$ plants derived from crossing Jandaroi with line 752 (B) do not show any association with the trait.

A - Jandaroi * line 740 F$_2$ population

Microsatellite marker	Chromosome	Line 740	Lowest 3rd leaf Na$^+$ plants					Highest 3rd leaf Na$^+$ plants					Jandaroi
			BB	AB	AA	Failed	Total	BB	AB	AA	Failed	Total	
Xgwm779	1B	BB	3	3	5	6	17	3	2	8	2	15	AA
Xwmc216	1B	BB	2	10	3	3	17	4	5	5	1	15	AA
Xgwm160	4A	BB	7	5	0	5	17	3	4	4	4	15	AA
Xwmc219	4A	BB	7	0	0	10	17	2	1	2	10	15	AA
Xwmc283	4A	BB	5	2	4	6	17	2	6	3	4	15	AA
Xbarc60	4B	BB	9	5	2	1	17	5	5	4	1	15	AA
Xgwm375	4B	BB	12	4	1	-	17	2	8	5	-	15	AA
Xcfa223	7B	BB	3	9	4	1	17	3	6	3	3	15	AA

B - Jandaroi * line 752 F$_2$ population

Microsatellite marker	Chromosome	Line 752	Lowest 3rd leaf Na$^+$ plants					Highest 3rd leaf Na$^+$ plants					Jandaroi
			BB	AB	AA	Failed	Total	BB	AB	AA	Failed	Total	
Xbarc55	2B	BB	1	10	3	2	16	3	7	1	1	12	AA
Xbarc101	2B	BB	1	10	3	2	16	3	7	1	1	12	AA
Xwmc283	4A	BB	4	8	4	0	16	1	7	5	0	12	AA
Xwmc160	4A	BB	4	0	3	9	16	4	0	4	4	12	AA
Xwmc398	6A	BB	2	9	3	2	16	3	7	1	2	13	AA
Xwmc256	6A	BB	5	8	3	0	16	0	9	4	0	13	AA

The entire Jandaroi * line 740 F$_2$ population were genotyped using markers *Xbarc60* and *Xgwm375*. The association between 3rd leaf Na$^+$ accumulation of the entire population and genotyping data of these microsatellite markers was analysed using MRA in QTX software. The results of the MRA showed a low association between 3rd leaf Na$^+$ accumulation and locus *Xgwm375* with a LOD score of 1.5, and this locus explained only 7% of the phenotypic variation. The other locus did not show any association (Table 19). This implies that the DArT loci associated with 3rd leaf Na$^+$ accumulation which identified from SGA are distant from the loci tested with the microsatellite markers.

SGA and MRA using microsatellite markers revealed no association between two markers located on the long arm of the chromosome 4B and 3rd Na$^+$ accumulation. These unexpected results needed the validation of 3rd leaf Na$^+$ accumulation in the Jandaroi * line 740 F$_2$

population by measuring 3rd leaf Na$^+$ accumulation in their progeny (F$_3$ population) of F$_2$

generation. Also, high-throughput genotyping (9K SNP markers) was required in order to

substantiate SGA.

Table 19. Association analysis between the 3rd leaf Na$^+$ accumulation for the entire Jandaroi * line 740 F$_2$ population and microsatellite markers was conducted using MRA in QTX MapManager software. The LOD score of the association, percentage of 3rd leaf Na$^+$ accumulation variation explained by each marker (% Variation.), and the parental allele responsible for the trait, are shown.

Locus	Chromosome	LOD score	% Variation	Parental allele
Xbarc60	4B	0.0	0.0	0.0
Xgwm375	4B	1.5	7	line 740

4.3.4 3rd leaf Na$^+$ accumulation in selected F$_{2:3}$ progeny

To validate the lowest and highest 3rd leaf Na$^+$ F$_2$ plants of the Jandaroi * line 740 F$_2$

population, progeny derived from those F$_2$ plants (F$_{2:3}$) were grown in a supported

hydroponics system. The 3rd leaf Na$^+$ accumulation of the F$_{2:3}$ progeny was measured and

compared with the 3rd leaf Na$^+$ accumulation of the F$_2$ parents.

The average of 3rd leaf Na$^+$ accumulation of the parents in this experiment was 240 mM

(Jandaroi) and 176 mM (line 740) (Table 20). Accordingly, the selected F$_{2:3}$ plants which

accumulated less than 200 mM Na$^+$ were scored as low Na$^+$ accumulators, and those which

accumulated over 200 mM Na$^+$ were scored as high Na$^+$ accumulators. The results showed

that most selected F$_{2:3}$ progeny derived from the F$_2$ families with lowest Na$^+$ accumulation in

3rd leaf tissue had less than 200 mM Na$^+$, except F$_2$ families 19, 55, 59, and 131. These

families had some F$_{2:3}$ progeny that accumulated more than 200 mM Na$^+$ (Table 20). This is

expected as several of the F$_2$ lines are likely to be heterozygous for Na$^+$ exclusion.

The DArT analysis showed that the allele of a putative locus on the long arm of chromosome

4B in the low Na$^+$ F$_2$ plants is dominant, and these F$_2$ plants can be either heterozygous or

homozygous (Table17, A1). In the group at the highest 3rd leaf Na$^+$, most F$_{2:3}$ progeny accumulated high Na$^+$ (more than 200 mM) with the exception of F$_2$ family 116, where some of the F$_{2:3}$ plants accumulated less and others more (Table 20).

The 3rd leaf Na$^+$ accumulation means of selected F$_{2:3}$ progeny were calculated to estimate the relationship of this trait with the 3rd leaf Na$^+$ accumulation in the F$_2$ families. In the lowest 3rd leaf Na$^+$, the F$_2$ families for which the F$_{2:3}$ progeny accumulated less than 200 mM of Na$^+$ did not segregate for 3rd leaf Na$^+$ accumulation. The F$_2$ families in which the F$_{2:3}$ progeny accumulated less or more than 200 mM Na$^+$ did segregate for 3rd leaf Na$^+$ accumulation. This scoring of F$_2$ families was also applied for the highest 3rd leaf Na$^+$ side. Figure 32 shows a linear correlation for 3rd leaf Na$^+$ accumulation of the lowest and highest F$_2$ family (which do not segregate for 3rd leaf Na$^+$ accumulation) with 3rd leaf Na$^+$ accumulation means of their selected F$_{2:3}$ progeny, with a correlation coefficient (r^2) of 0.80. The relationship was strong between the selected F$_2$ plants and the F$_{2:3}$ progeny, which implies that the 3rd leaf Na$^+$ accumulation in the lowest and highest 3rd leaf Na$^+$ F$_2$ plants was estimated accurately. However, it is possible that some errors were generated in the glasshouse environment as the residual maximum likelihood methods (GenStat 14 package) (Appendix V) revealed that environmental factors (tubs, rows and columns) and residual values (variations which are not due to genotype or environmental factors) have an impact on 3rd leaf Na$^+$ accumulation.

In the highest 3rd leaf Na$^+$ plants, the Na$^+$ accumulation means of selected F$_{2:3}$ progeny, associated with allele of the putative DArT loci of F$_2$ families, whereas the 3rd Na$^+$ accumulation of selected F$_{2:3}$ plants does not associate with alleles of two microsatellite markers (*Xbarc60, Xgwm375*) (Table 20). These results suggest that the microsatellite markers assessed are not sufficiently close to the 3rd leaf Na$^+$ accumulation QTL and to the putative DArT markers on the long arm of chromosome 4B to yield strong associations.

Table 20. Na$^+$ accumulation in the fully expanded third leaf and allele frequency of DArT and microsatellite markers of parents, selected F$_2$ plants and F$_{2:3}$ progeny grown at 100 mM NaCl.

Parents	3rd leaf Na$^+$ accumulation in F$_2$ experiment	3rd leaf Na$^+$ accumulation in selected F$_{2:3}$ experiment		DArT marker alleles in F$_2$ families	Microsatellite marker alleles in F$_2$ families
Jandaroi	162.22	240.09		AA	AA
Line 740	132.60	176.69		BB	BB
F$_2$ families	**3rd leaf Na$^+$ accumulation in F$_2$ Families**	**Means 3rd leaf Na$^+$ accumulaion in familis homogenous in selected F$_{2:3}$ progeny**	**Means 3rd leaf Na$^+$ accumulaion in familis segregating in selected F$_{2:3}$ progeny**		
19	107.1		207.85	AB/BB	BB
55	123.00		198.03	AB/BB	AB
57	93.08	171.63		AB/BB	BB
59	117.57		205.07	AB/BB	BB
61	120.79	163.89		AB/BB	-
65	107.00	171.25		AB/BB	BB
86	96.74	155.45		AB/BB	BB
90	83.47	165.09		AB/BB	BB
99	110.32	159.62		AB/BB	AA
107	90.63	134.21		AB/BB	-
131	121.95		203.99	AB/BB	AB
10	190.90	272.45		AA	AA
52	177.18	206.39		AA	AA
76	183.96	237.52		AA	AB
79	186.59	229.27		AA	-
102	180.02	277.69		AA	AB
103	222.93	260.46		AA	AA
116	195.35		199.42	AB/BB	BB
123	227.12	237.74		AA	BB
129	232.24	258.70		AA	AB
130	204.21	227.17		AA	AA

Lowest 3rd leaf Na$^+$ (families 19–131)

Highest 3rd leaf Na$^+$ (families 10–130)
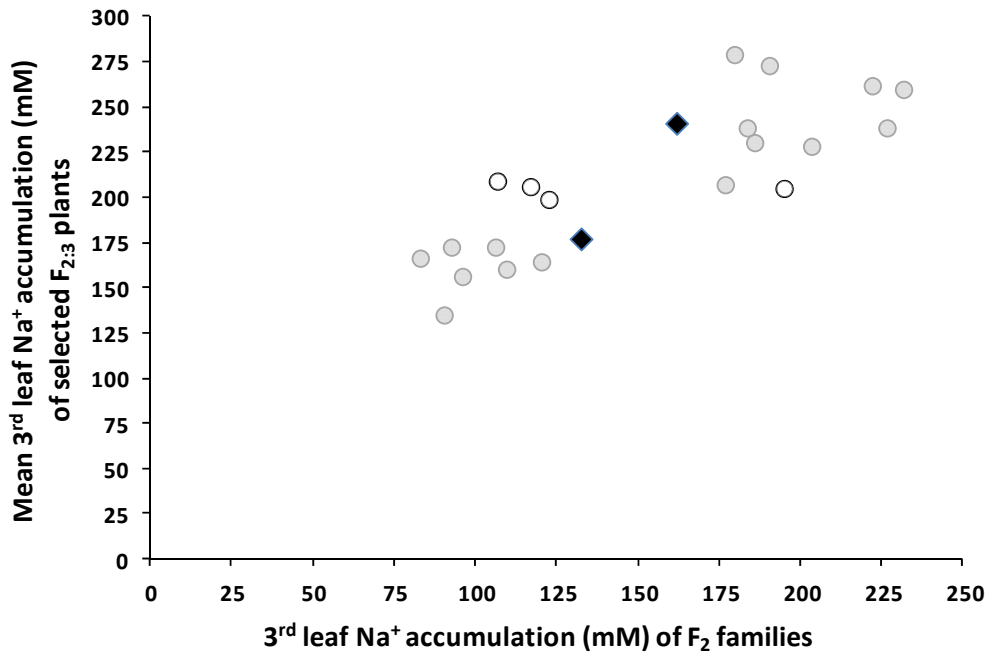
Figure 31. Relationship between low- and high-selected F_2 families and their $F_{2:3}$ progeny means for 3rd leaf Na⁺ accumulation at 100 mM NaCl for Jandaroi * line 740 cross. Families homogenous in the selected $F_{2:3}$ generation (●), families segregating in the selected $F_{2:3}$ generation (○), means for high (Jandaroi) and low (line 740) Na⁺ parents (♦) are shown. The least-squares relationship for 3rd leaf Na⁺ accumulation of F_2 families and the mean of 3rd leaf Na⁺ accumulation of $F_{2:3}$ progeny was *y=0.7819x + 85.517* (r^2=0.80).

4.3.5 Bulked segregant analysis (BSA)

Two bulks of the DNA of F_2 plants (low Na⁺ accumulation bulk and high Na⁺ accumulation bulk) was developed and analysed using the wheat 9K Infinium SNP genotyping assay (Section 4.2.6.3). Seventeen markers showed an association with 3rd leaf Na⁺ accumulation in the plants. The BSA analysis revealed three significant QTL on the long arm of chromosomes 3B, 4B and 7A. Four SNP markers on chromosome 3B showed a strong association, with the favoured allele derived from Jandaroi, whereas three markers on chromosome 4B and two markers on chromosome 7A had a strong association with 3rd leaf Na⁺ accumulation with the favoured allele inherited from line 740. The association on chromosome 7A was apparent only in the low Na⁺ accumulation bulk and not the high Na⁺ accumulation bulk. Other QTL on chromosomes 1A, 4A and 6B showed an association with 3rd leaf Na⁺ accumulation.

Primers for the putative SNP markers were designed using Primer3 software and their quality checked using NetPrimer software. These primers amplified the SNP markers for genotyping the entire Jandaroi * line 740 F$_2$ population. First, the parents, F$_1$ plants and 15 F$_2$ plants were genotyped using the 17 SNP markers. Seven SNP markers amplified successfully in High Resolution Melting Point technology and their alleles had a clear melting curve. These seven markers were used to genotype the entire F$_2$ population. The MRA (Table 21a) showed that *Xm5511* on the long arm of chromosome 3B and *Xm564* on the long arm of chromosome 4B have a strong association with 3rd leaf Na$^+$ accumulation, with LOD scores of 3.50 and 3.40 respectively. Each marker explains 18% of the variation in 3rd leaf Na$^+$ accumulation. The other markers did not show any association with 3rd leaf Na$^+$ accumulation. *Xm564* also showed a strong association with 3rd leaf K$^+$ accumulation and the ratio of K$^+$/Na$^+$, with LOD scores of 3.90 and 5.58 respectively. This locus accounted for 20% of the variation in 3rd leaf K$^+$ accumulation and 27% of the K$^+$/Na$^+$ ratio, but *Xm5511* showed no association with either trait (Table 21 b and c). No association was detected between other markers and the phenotypic traits (Table 21 a, b and c). *Xm5511* on the long arm of chromosome 3B had an association with 3rd leaf Na$^+$ accumulation whereas *Xm7512,* also on chromosome 3B, did not show any association. In the published genetic map the genetic distance between these markers is approximately 40 cM. Also, the same genetic distance was found between *Xm564* and *Xm6828* but *Xm6828* did not have any association with the phenotypic traits. This implies that *Xm7512* and *Xm6828* are not linked to the QTL of interest on the long arm of chromosomes 3B and 4B. The MRA suggests that the loci or QTL of interest are close to *Xm5511* on chromosome 3B and close to *Xm564* on chromosome 4B. Future work will focus on these two loci by mapping the F$_2$ population with more markers adjacent to *Xm5511* and *Xm564* to validate the identified QTL. This will lead to the identification of candidate genes and the development of markers associated with 3rd leaf Na$^+$ accumulation in line 740.

Table 21. Association analysis between SNP markers of interest and phenotypic traits (3rd leaf Na$^+$ accumulation, 3rd leaf K$^+$ accumulation and K$^+$/Na$^+$ ratio) in the F$_2$ population derived from the cross Jandaroi * line 740. This analysis was conducted using MRA in QTX MapManager. The table shows the LOD score of the association, the percentage of phenotypic trait variation explained by each SNP markers (% Variation) and parental allele which has a positive effect on the trait.

a - 3rd leaf Na$^+$ accumulation

Chromosome	Locus	LOD score	% Variation	Allele
1A	*Xm2584*	0.6	3	Jandaroi
1A	*Xm1608*	0.0	0.0	-
3B	*Xm7512*	0.0	0.0	-
3B	*Xm5511*	3.4	18	Jandaroi
4B	*Xm6828*	0.3	2	Line 740
4B	*Xm564*	3.4	18	Line 740
7A	*Xm3054*	0.1	0.0	Line 740

b - 3rd leaf K$^+$ accumulation

Chromosome	Locus	LOD score	% Variation	Allele
1A	*Xm2584*	0.02	0.0	Jandaroi
1A	*Xm1608*	0.0	0.0	-
3B	*Xm7512*	0.0	0.0	-
3B	*Xm5511*	0.3	2	Line 740
4B	*Xm6828*	0.6	4	Line 740
4B	*Xm564*	3.9	20	Line 740
7A	*Xm3054*	0.78	4	Line 740

c - K$^+$/Na$^+$ ratio

Chromosome	Locus	LOD score	% Variation	Allele
1A	*Xm2584*	0.28	1.3	Line 740
1A	*Xm1608*	0.0	0.0	-
3B	*Xm7512*	0.0	0.0	-
3B	*Xm5511*	1.06	6	Jandaroi
4B	*Xm6828*	0.93	6	Line 740
4B	*Xm564*	5.58	27	Line 740
7A	*Xm3054*	1.5	7	Line 740

4.4 Discussion

Previous work identified two Afghani landraces (Lines 740 and 752) which accumulated half the concentration of Na$^+$ in leaf tissue when compared with Australian durum wheats and other durum wheat landraces (Figure 29) (Shavrukov et al. 2011). The aim of this study was to detect the genetic loci for 3rd leaf Na$^+$ accumulation in these two durum landraces.

The heritability for the studied traits in both populations is large except for 3rd leaf K$^+$ accumulation which was medium in the Jandaroi * line 740 F$_2$ population and very low in the Jandaroi * line 752 F$_2$ population. The large heritability for 3rd leaf Na$^+$ accumulation in both populations implies that genetic variance had a greater impact than environment. Heritability is an important factor in detecting QTL (Collard et al. 2005) and is defined as the genetic proportion of phenotypic variance.Tanksley (1993) stated that traits with a large genetic effect are likely to be associated with major QTL. Munns et al. (2003) estimated a moderate to high heritability for shoot Na$^+$ accumulation in two F$_2$ populations of durum wheat. Two major QTL (*Nax1* and *Nax2)* were identified (Byrt et al. 2007; Lindsay et al. 2004) and markers flanking these putative QTL were used to develop salt tolerant durum wheat cultivars (Munns et al. 2012).

The phenotyping results showed that the distributions of all traits in both populations are bimodal and this suggests that major genes are associated with ionic accumulation in the lines of durum wheats studied. High heritability of 3rd leaf Na$^+$ accumulation in both populations also supports the claim that this trait is associated with major gene(s). However, salinity tolerance traits such as Na$^+$ exclusion can be regarded as quantitatively inherited traits controlled by many genes. Lindsay et al. (2004) and Byrt et al. (2007) identified two QTLs associated with Na$^+$ exclusion in breeding line 149 of durum wheat, but these did not explain the total variation of the trait. Therefore, additional minor genes also influence Na$^+$ exclusion. Na$^+$ exclusion in lines 470 and 752 may be similarly controlled by several major and many

minor genes. This assumption was tested by selective genotyping analysis (SGA) using DArT markers to detect QTL for 3rd leaf Na$^+$ accumulation in the two F$_2$ populations derived from crosses with Jandaroi.

SGA showed a strong association between three DArT markers on the long arm of chromosome 4B and 3rd leaf Na$^+$ accumulation in the Jandaroi * line 740 F$_2$ population (Table 17 A1). Dubcovsky et al. (1996) detected a QTL associated with ratio of K$^+$/Na$^+$ on the long arm of the chromosome 4D in bread wheat. Also, Long et al. (2013) identified QTL for the shoot Na$^+$ accumulation on the long arm of the chromosome 4H in an association mapping study of barley which may be homoeoloci for the QTL mapped on the long arm of the chromosome 4B in this study. The candidate gene in this region is an *HKT*-like gene (Huang et al. 2008).

However, the putative QTL on the long arm of the chromosome 4B was not validated in this study using microsatellite markers. Subsequently, bulked segregant analysis (BSA) detected two QTL on the long arm of chromosomes 4B and 3B for 3rd leaf Na$^+$ accumulation (Table 21a). This confirmed the strong association found on 4B detected in SGA using DArT markers.

The results showed differences between the analysis of DArTs and SNPs. Five thousand DArTs were used in the SGA whereas 9000 SNPs were analysed in BSA. Also, the SNP markers are derived from coding sequences and provide a better distribution than DArT markers, which tend to be highly clustered (Paux and Sourdille 2009). Further, there were 1,216 polymorphic SNP markers between Jandaroi and line 740 compared to only 616 DArT markers. The DArT markers which represent a medium-density marker platform did not detect an association on the long arm of chromosome 3B and 7A in the Jandaroi * line 740 F$_2$ population in SGA , whereas this association was found in BSA using SNP markers which are a high throughput platform (Akhunov et al. 2009).

Both SGA and BSA showed three DArT markers and three SNP markers associated with 3rd leaf Na$^+$ accumulation on the long arm of chromosome 4B. The genetic distance or co-segregation of both markers in the Jandaroi * line 740 F$_2$ population was not tested. The other three SNP markers which showed an association with 3rd leaf Na$^+$ accumulation, with the favourable allele derived from Jandaroi, were mapped to the distal region of the long arm of chromosome 3B. However, SGA did not show any associations on chromosome 3B in the Jandaroi * line 740 F$_2$ population (Table 17 A). A partial association between the DArT loci and 3rd leaf Na$^+$ accumulation on the long arm of chromosome 3B, with a favourable allele derived from Jandaroi, was shown in the Jandaroi * line 752 F$_2$ population (Table 17B). This raises a question: are the putative SNP and DArT markers close to the 3rd leaf Na$^+$ accumulation QTL on the long arms of chromosomes 3B and 4B? This question can be answered by mapping the distal regions of the long arms of these chromosomes. Both DArT and SNP markers can be designed to develop PCR-based assays for genotyping the entire F$_2$ population. A fine linkage map of these regions now needs to be constructed.

In the Jandaroi * line 740 F$_2$ population, the locus *Xm456* was identified in the distal region of the long arm of chromosome 4B and has a strong association with the accumulation of 3rd leaf Na$^+$, 3rd leaf K$^+$ and the ratio of K$^+$/Na$^+$ (Table 21). The *Kna1* locus was mapped on the distal region of chromosome 4DL in bread wheat (Dubcovsky et al. 1996). This locus is associated with the selective accumulation of K$^+$ over Na$^+$ in the shoot and was found to correspond to *Nax2* when physically mapped onto the distal part of chromosome 5AL in durum wheat (Byrt et al. 2007). In rice (*Oryza sativa*), a QTL for shoot K$^+$ content was mapped onto chromosome 1 in an F$_2$ population derived from cross between a salt tolerant *indica* (Nona Bokra) and an intolerant elite *Japonica* variety (Koshihikari). This QTL was named *SKC1* (Ren et al. 2005). Low Na$^+$ concentration in the leaves, improved selection of K$^+$ over Na$^+$ in transport from roots to shoots, and a high ratio of K$^+$ / Na$^+$ in the leaves are common phenotypic characteristics between *Nax2*, *Kna1* and *SKC1* (Byrt et al. 2007). These characteristics were

seen in the F_2 population derived from Jandaroi with line 740. The candidate gene of the *SKC1* locus is *OsHKT1;5*, which reduces the amount of Na$^+$ transported from the root to the shoot (Ren et al. 2005). An *HKT1*-like gene was cloned in *Kna1* and *Nax2* and *HKT1;5* is a candidate for both loci; named as *TaHKT1;5-D* and *TmHKT1;5-A*, respectively (Byrt et al. 2007). *Kna1* is found in bread wheat (*T. aestivum* L.) whereas *Nax2* was derived from *T. monococcum*. It is possible that the *Xm456* locus identified in this study corresponds to the *Kna1* and *Nax2* loci and the candidate gene for *Xm456* on the long arm of chromosome 4B is an *HKT1*-like gene. Huang et al. (2008) found four bands of *HKT1;5*-like genes in bread wheat using a *HKT1;5* probe in Southern blots. Three hybridisation bands were located on the long arm of chromosome 4B and one band on the long arm of chromosome 4D. The *HKT1;5* gene has only found in bread wheat and *T. monococcum*, and has not been identified in durum wheat (Munns et al. 2012) . The results of this Chapter suggest that Na$^+$ exclusion in line 740 is likely to be due to the gene *HKT1;5*. This hypothesis needs to be tested by genotyping *HKT1*-like genes in the Jandaroi * line 740 F_2 population.

4.5 Conclusion

As BSA identified several polymorphic markers between Jandaroi and line 740, the putative loci on the long arms of chromosomes 4B and 3B should be further mapped using SNP markers. This would facilitate construction of a fine linkage map of the region.

F$_2$ populations derived from crossing Australian durum wheats and the Afghani landraces were generated in addition to the Jandaroi F$_2$ populations in this study. These can be used to validate the regions on the long arms of chromosomes 3B and 4B.

The *HKT1;5*-like gene on the long arm of chromosome 4B identified in this work should be examined in the Jandaroi * line 740 F$_2$ population using primers designed and used for bread and durum wheats (Byrt 2008). This would determine whether the *HKT1;5* like gene is a candidate for the 4B locus.

Chapter 5 General Discussion

Salinity is a major limitation to wheat production worldwide (Mujeeb-Kazi and Diaz de Leon 2002) and adds to the challenge of increasing global food supply to feed the rapidly growing human population (FAO 2009; Tester and Langridge 2010). Enhancing the salt tolerance of elite wheat cultivars while maintaining yield when grown under saline and non-saline conditions is desirable (Munns et al. 2006). Conventional plant breeding has achieved significant improvements in salinity tolerance in wheat in India (Hollington 2000), Pakistan (Ashraf 2002) and Egypt (Munns et al. 2006), but has occurred over a long period of time (Chinnusamy et al. 2005). Molecular marker-assisted selection offers the opportunity to accelerate breeding for desired traits (Lehmensiek et al. 2009). However, salinity tolerance is genetically and physiologically complex (Colmer et al. 2005; Munns and Tester 2008) and it is difficult to use biotechnology as a tool for improvement. Munns and Tester and (2008) suggested that salinity tolerance can be dissected into separate traits that can be measured in controlled environments at specific developmental stages, therefore reducing the complexity. Osmotic and ionic stress tolerances are two components of salinity tolerance (Munns and Tester 2008). Bread wheat shows moderate tolerance due to the ability to limit shoot Na^+ accumulation (Maas and Hoffman 1977; Munns and Tester 2008; McDonald et al. 2012). To further increase the tolerance in bread wheat, osmotic stress tolerance can be targeted even though it has been largely ignored in previous studies in wheat. This component is based on measuring the growth rate of plants grown in saline and non-saline conditions using platforms such as plant images generated by the LemnaTec platform (Rajendran et al. 2009). To measure osmotic stress tolerance and study the genetics of osmotic stress tolerance in bread wheat, the LemnaTec platform imaging system was applied to a mapping population of recombinant inbred lines (RIL) from a cross between bread wheat cultivars (Gladius and Drysdale) (Chapter 3). The results showed that osmotic stress tolerance is a major contributor

to salinity tolerance (Chapter 3). Broad sense heritability for this trait was moderate (0.25) and three QTL were identified, which explained 24% of the trait variation and virtually all of the genetic variation. These results suggest that the osmotic stress tolerance trait measured on this platform is largely affected by environmental variables and further experiments will be required to improve the heritability values.

In contrast to the moderate level of salinity tolerance in bread wheat, durum wheat is generally salt intolerant because most accessions lack an effective Na^+ exclusion mechanism (Gorham et al. 1987; Munns et al. 2000). The measurement of ionic tolerance (Na^+ exclusion) trait is straightforward and new sources of variation have been identified through screening of a wide range of germplasm. A source of Na^+ exclusion was identified from a breeding line of durum wheat derived from the wild wheat accession *T. monococcum* and a durum wheat cultivar Marrocos (Munns et al. 2000). Na^+ exclusion in this breeding line is due to two loci (*Nax1* and *Nax2*) (James et al. 2008). *Nax2* has recently been used to improve yield of elite Australian durum wheat grown under saline conditions (Munns et al. 2012). Shavrukov et al (2011) identified new sources of Na^+ exclusion in two Afghani durum landrace wheats. The genetic basis for Na^+ exclusion in these landraces was studied in experiments reported in Chapter 4 and two new loci associated with Na^+ exclusion were identified.

A further two QTL for 4th leaf Na^+ accumulation in seedlings growing under saline conditions were identified in the RIL population (Chapter 3). The QTL mapped on the long arm of the chromosome 3 B in bread wheat and were coincident with QTL in durum wheat. However, fine mapping and more detailed analysis will be needed to determine if these QTL are indeed the same.

It is unclear whether the different tolerance mechanisms are additive and can be combined to give high levels of salinity tolerance. Will Na^+ exclusion increase salinity tolerance in wheat plants which also have good osmotic stress tolerance? Munns and Tester (2008) hypothesised

that plants with both osmotic stress tolerance and ionic tolerance (Na^+ exclusion and Na^+ tissue tolerance) will show a higher growth rate than plants with only one of these mechanisms. This strategy was successful in maize. In a cross between a Na^+ excluding line and an inbred line with good osmotic stress tolerance, the resulting hybrid produced higher yield than either parent under saline conditions (Schubert et al. 2009). In diploid wheat, Rajendran et al (2009) showed that the two most salt tolerant *T. monococcum* accessions were both osmotically tolerant, but one showed high Na^+ tissue tolerance and the other excluded Na^+ from the leaf. They concluded that both accessions were likely to use two mechanisms to achieve whole-plant salt tolerance.

The study in maize indicates that osmotic stress tolerance and Na^+ exclusion are likely to have an additive effect, although Rajendran et al. 2009 were unable to demonstrate an additive effect. The Gladius * Drysdale RIL population provided an opportunity to investigate additive effects of Na^+ exclusion and osmotic stress tolerance because Gladius excludes Na^+ while Drysdale shows a higher level of osmotic tolerance. The results presented here indicate that these mechanisms can be combined to generate lines with levels of tolerance well above that of either parent. However, additional studies will be needed to indicate if there is any disadvantage associated with high tolerance in non-saline conditions.

An additive effect of salinity tolerance components will allow engineering of salt tolerant bread and durum wheat. The QTL for osmotic stress tolerance and Na^+ exclusion in bread and durum wheat identified in this work could be pyramided into agronomically superior genotypes. Marker-assisted selection provides an opportunity to select for desirable alleles at multiple loci and pyramid tolerance. Importantly, marker assisted selection does not require identification of the causal gene underlying the QTL. However, marker-assisted selection may not be straightforward if the total variation of a trait is based on many genes with a small effect. Another limitation of marker-assisted selection is linkage drag. Linkage drag occurs

where desirable genes are closely linked to deleterious alleles at neighbouring loci (Tanksley and Nelson 1996). Back-crossing can reduce the size of the introgressed segment, but this can be slow and represents a considerable amount of work. The use of molecular linkage maps can accelerate progress of back-crossing by selecting individuals containing recombinant chromosomes (Tanksley and Nelson 1996). However, small fragments of a chromosome carrying undesirable alleles may remain if high marker densities around the target QTL are not available (James et al. 2012). High density genotyping platforms, such as genotyping by sequencing (Berkman et al. 2012) can improve the detection of fine recombination events (Tester and Langridge 2010).

Results presented here form a base for further studies; in particular the validation of putative genomic regions associated with different components of salinity stress tolerance. Further work can lead to the identification of the genes underlying the identified QTL and the development of diagnostic markers to facilitate the breeding of salt tolerant wheat cultivars. Releasing tolerant cultivars will contribute to the maintenance of a sustainable food supply for the world's growing population.

Appendix

6.1 Appendix I

6.1.1 Soil based Experiment

QTL detected with Composite Interval Mapping (CIM) for phenotypic traits in the RIL population derived from the cross between Gladius and Drysdale. CIM was performed using QTL package in GenStat 14. The title of each graph shows the phenotypic trait. Horizontal axes represent 30 linkage groups across the 21 chromosomes of bread wheat. Some chromosomes have two linkage groups as shown in Section 3.3.1 and Table 5. The vertical axis show the $-\log_{10}$ (P) values of QTLs according to the Wald test. The horizontal red line indicates the $-\log_{10}$ (P) threshold (3.6). The QTL effect which represents favourable allele inherited from the parents (Gladius in blue colour and Drysdale in red colour) is shown on the horizontal axis below the graphs.

Test profile: salt tolerance index
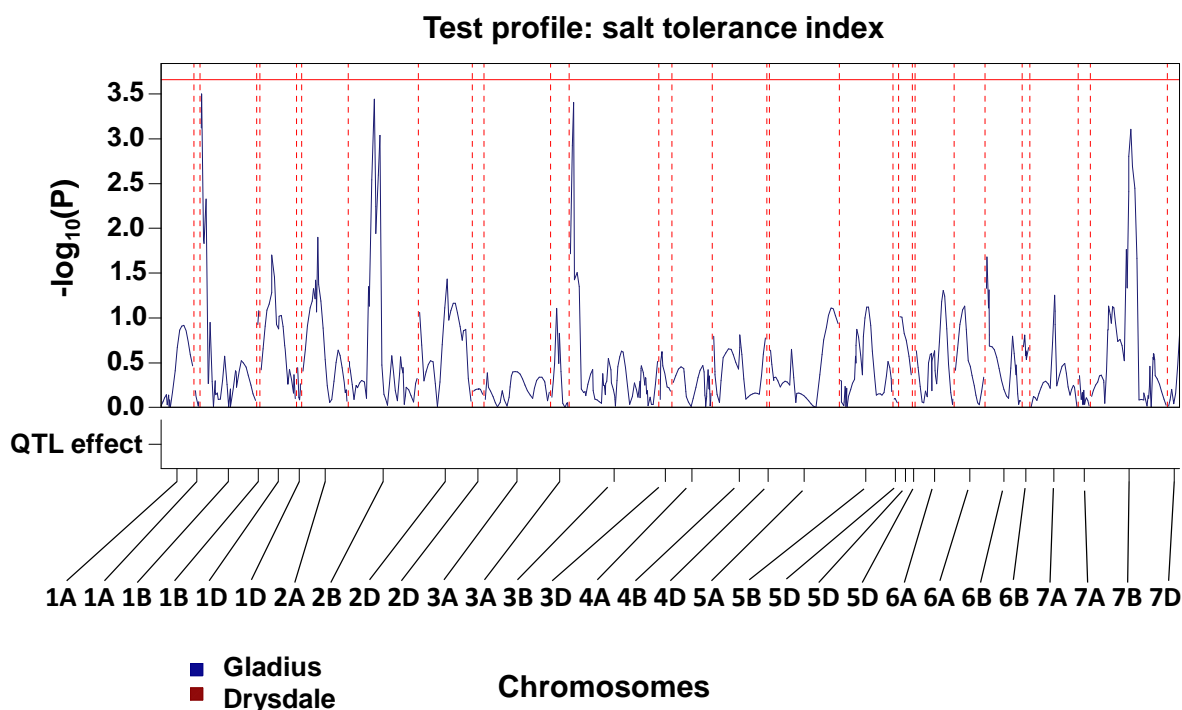
Test profile: osmotic stress tolerance-assay 1

QTL effect

1A 1A 1B 1B 1D 1D 2A 2B 2D 2D 3A 3A 3B 3D 4A 4B 4D 5A 5B 5D 5D 5D 6A 6A 6B 6B 7A 7A 7B 7D

Chromosomes
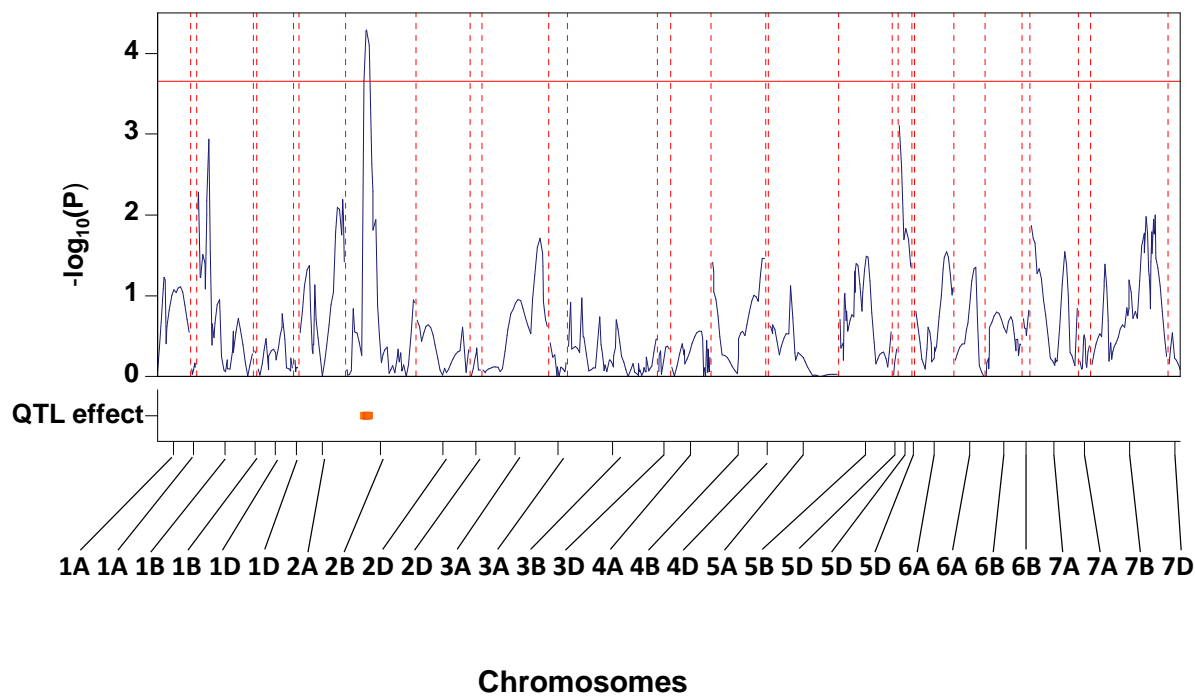
Test profile: number of tiller on the salt treatment
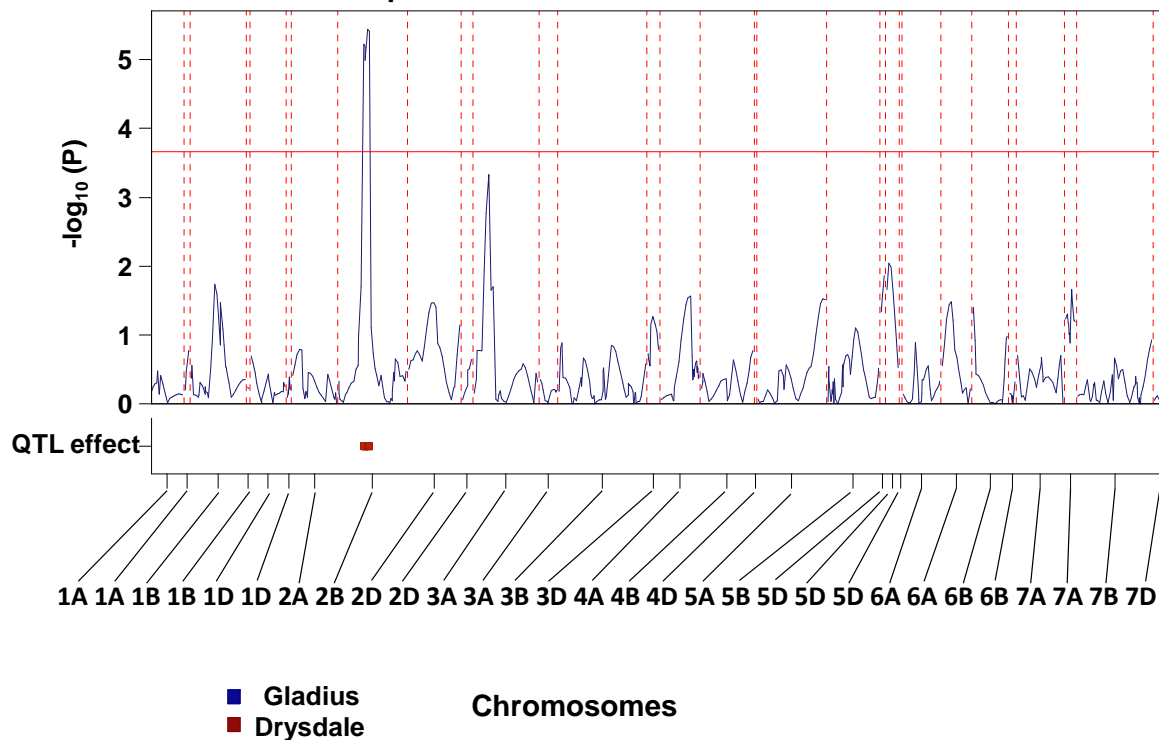
QTL effect

1A 1A 1B 1B 1D 1D 2A 2B 2D 2D 3A 3A 3B 3D 4A 4B 4D 5A 5B 5D 5D 5D 6A 6A 6B 6B 7A 7A 7B 7D

■ Gladius
■ Drysdale

Chromosomes

Test profile: 4th leaf Na+ accumulation

Test profile: 4th leaf K+ accumulation

■ Gladius
■ Drysdale

Chromosomes
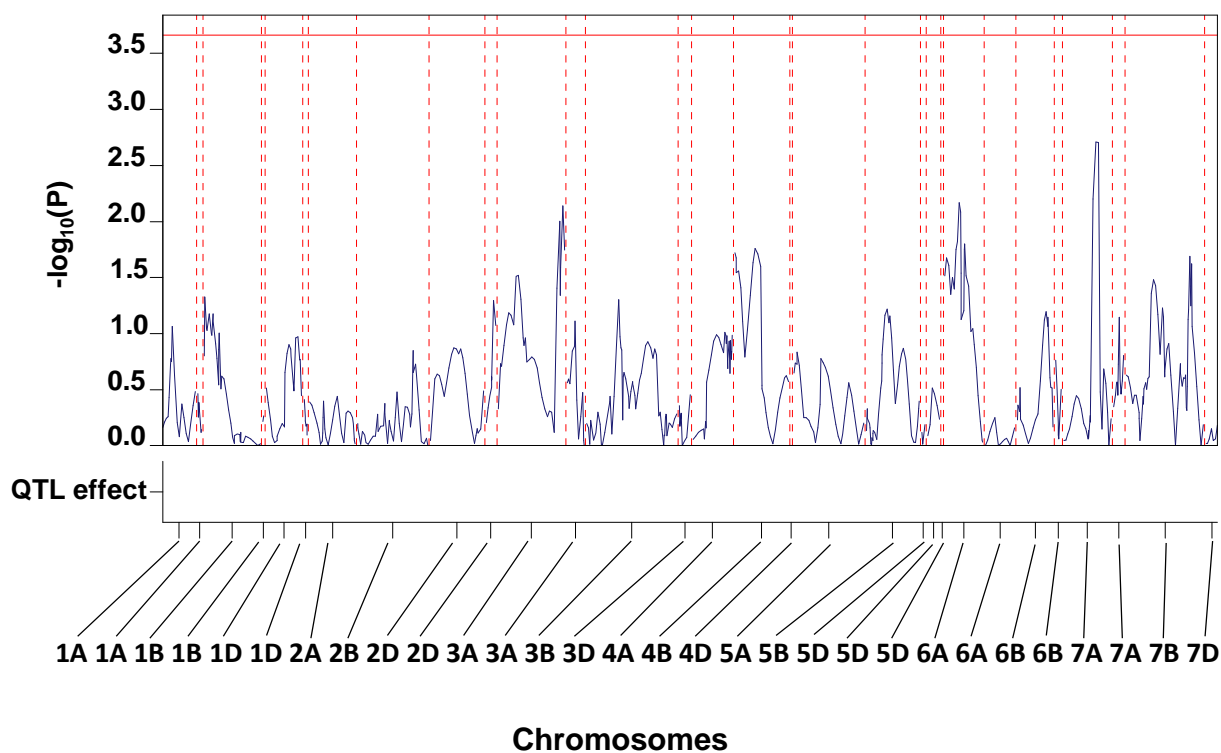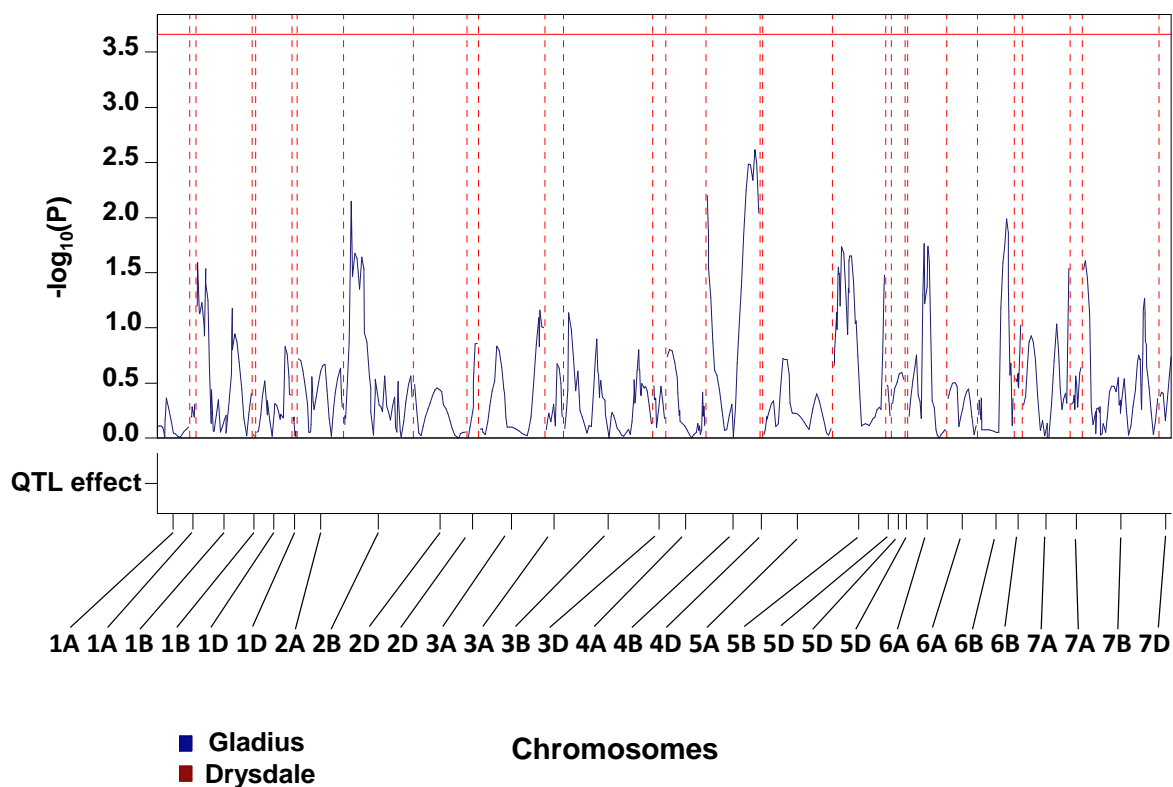
6.1.2 Hydroponics Experiment

QTL detected with Composite Interval Mapping (CIM) for phenotypic traits in control and salt treatments in the RIL population derived from the cross between Gladius and Drysdale. CIM was performed using QTL package in GenStat 14. The title of each graph shows the phenotypic traits and the salt treatments used. The horizontal axes represent 30 linkage groups across the 21 chromosomes of bread wheat. Some chromosomes have two linkage groups as shown in Section 3.3.1 and Table 5. The vertical axis show the -log 10 (P) values of QTLs according to the Wald test. The horizontal red line indicates the -log 10 (P) threshold (3.6). The QTL effect which represents the favourable allele inherited from the parents (Gladius in blue colour and Drysdale in red colour) is shown on the horizontal axis below the graphs.

Test profile: shoot fresh weight – control treatment

Test profile: shoot dry weight – control treatment

Test profile: number of tiller– control treatment

- Gladius
- Drysdale

Test profile: water content – control treatment

Test profile: soot fresh weight – salt treatment
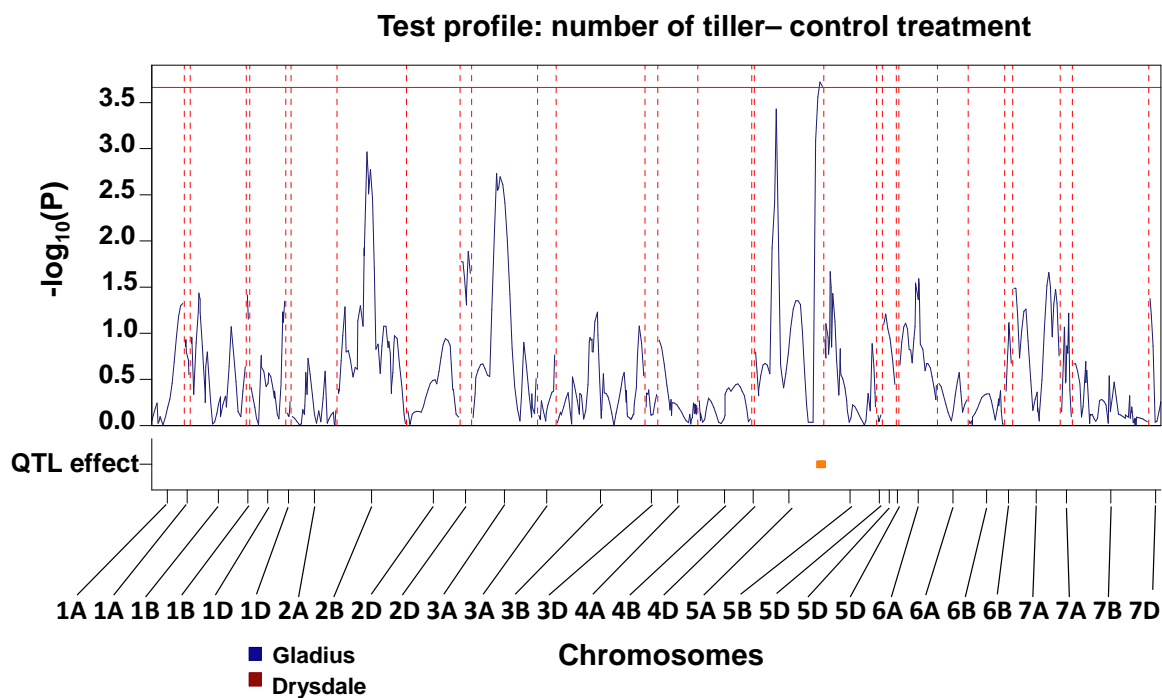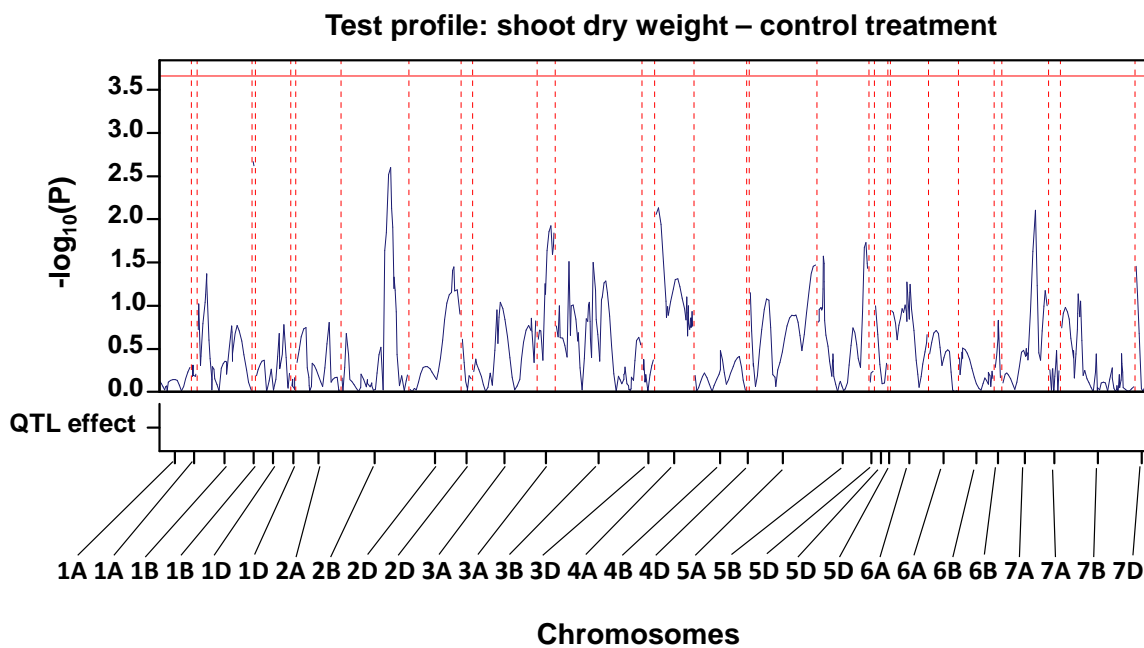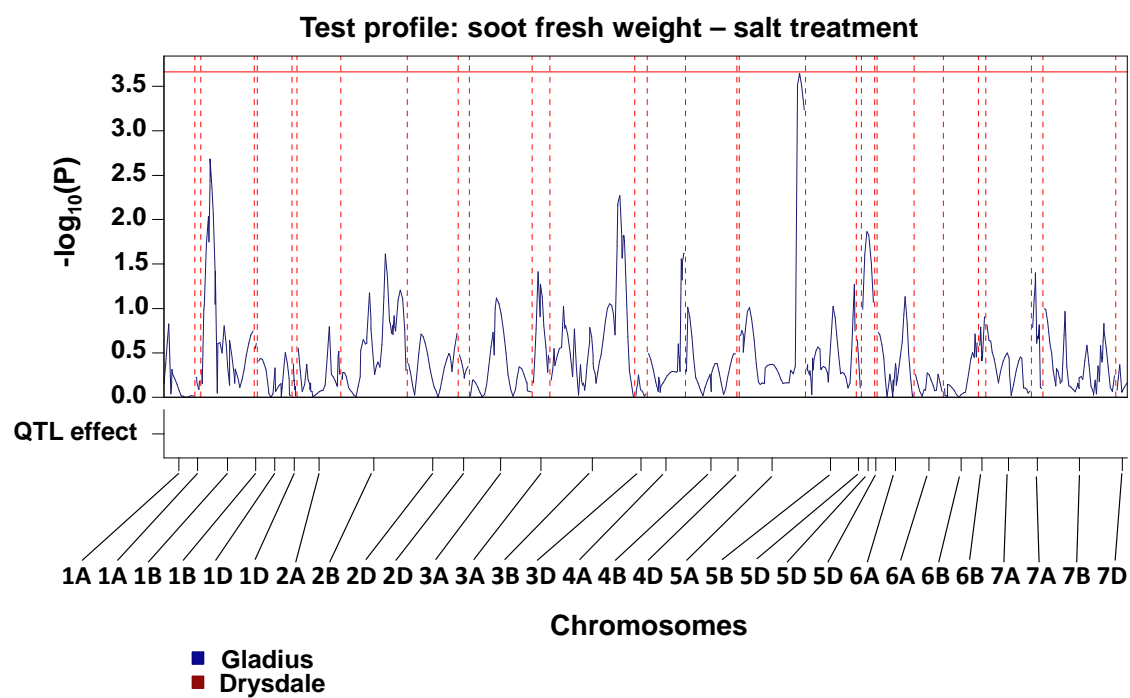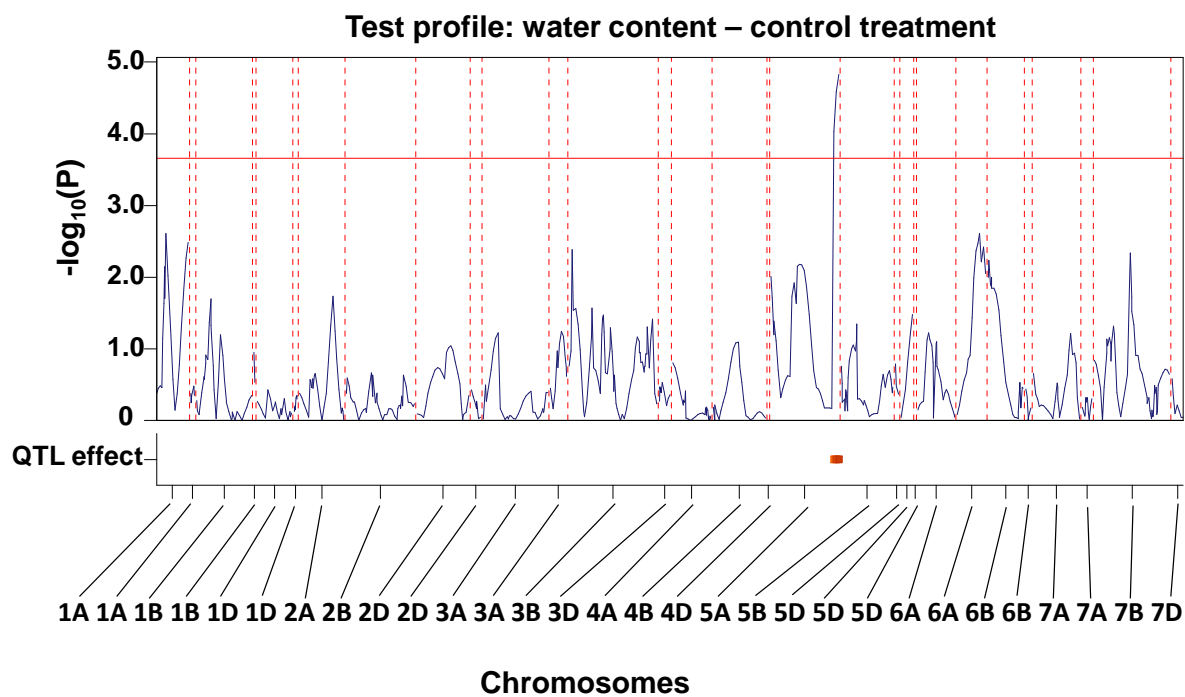
■ Gladius
■ Drysdale

Test profile: shoot dry weight – salt treatment
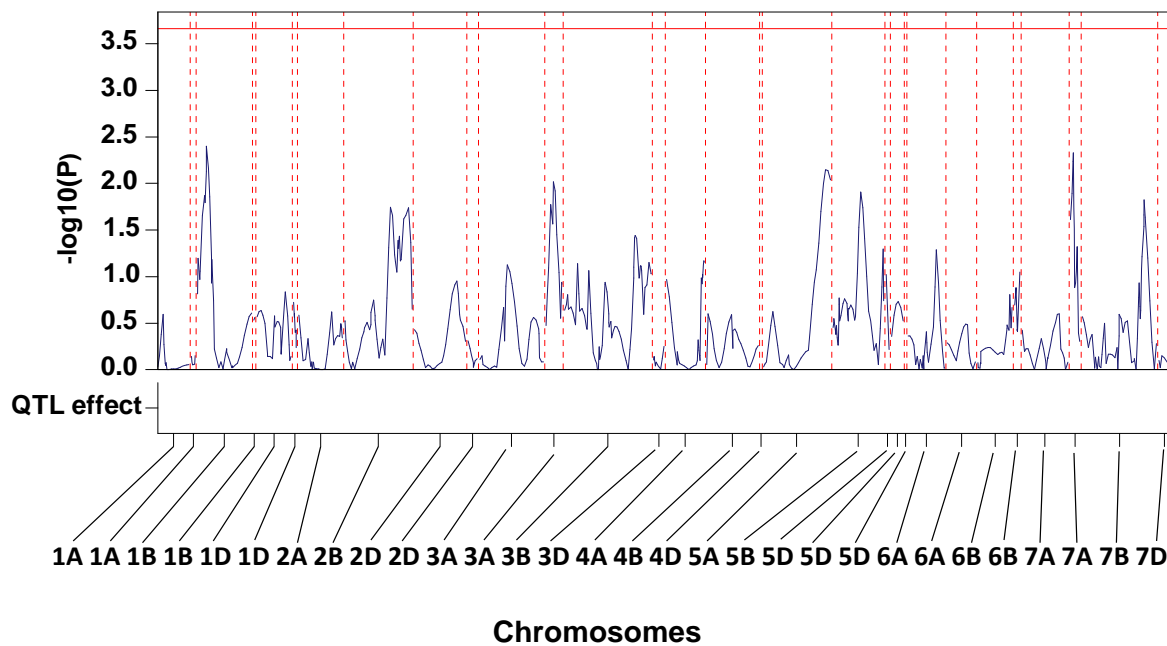
Chromosomes

Test profile: number of tiller – salt treatment
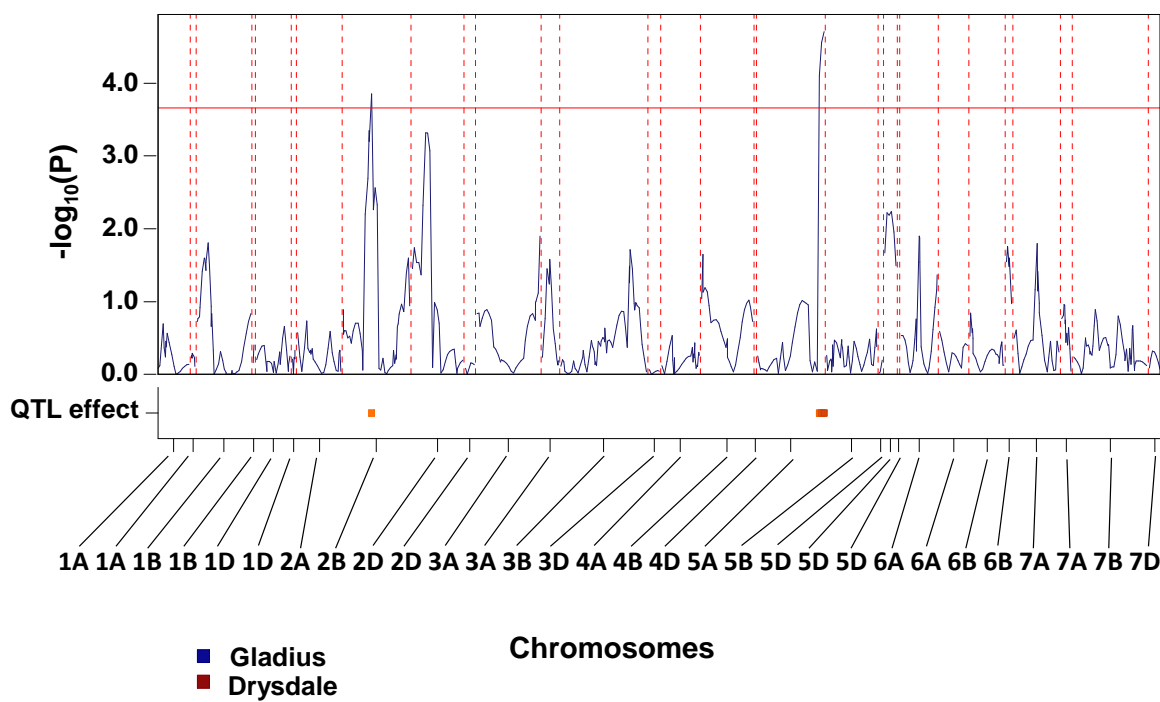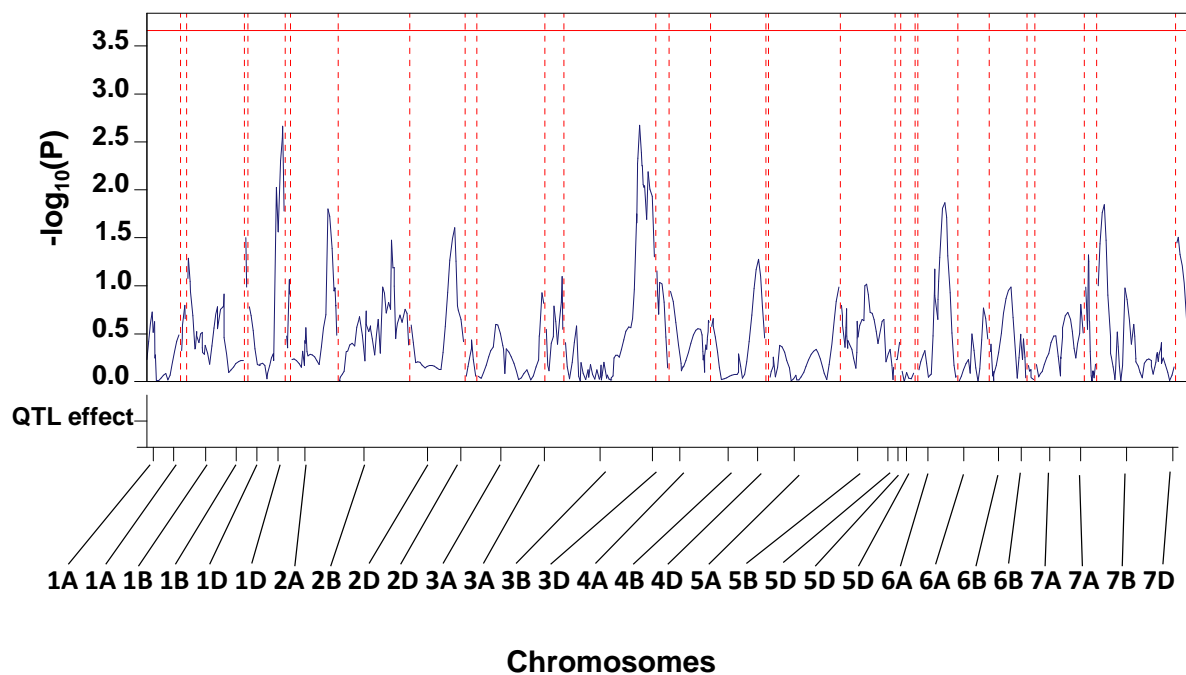
Chromosomes

■ **Gladius**
■ **Drysdale**

Test profile: 4th leaf Na+ accumulation

Test profile: 4th leaf K+ accumulation
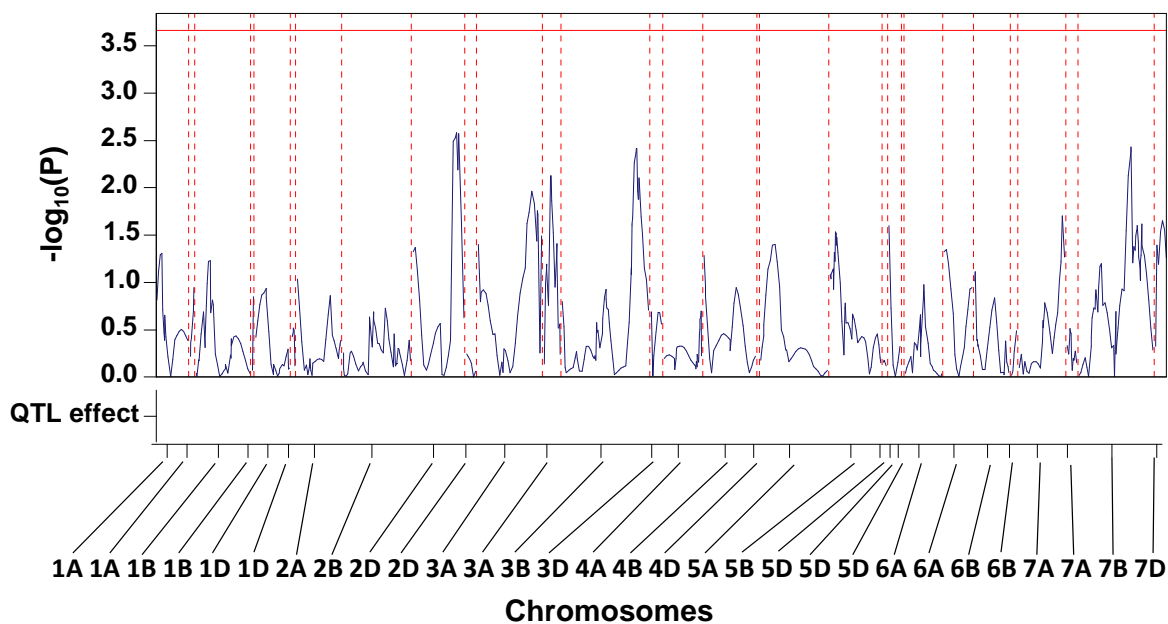
■ Gladius
■ Drysdale

Test profile: salt tolerance index
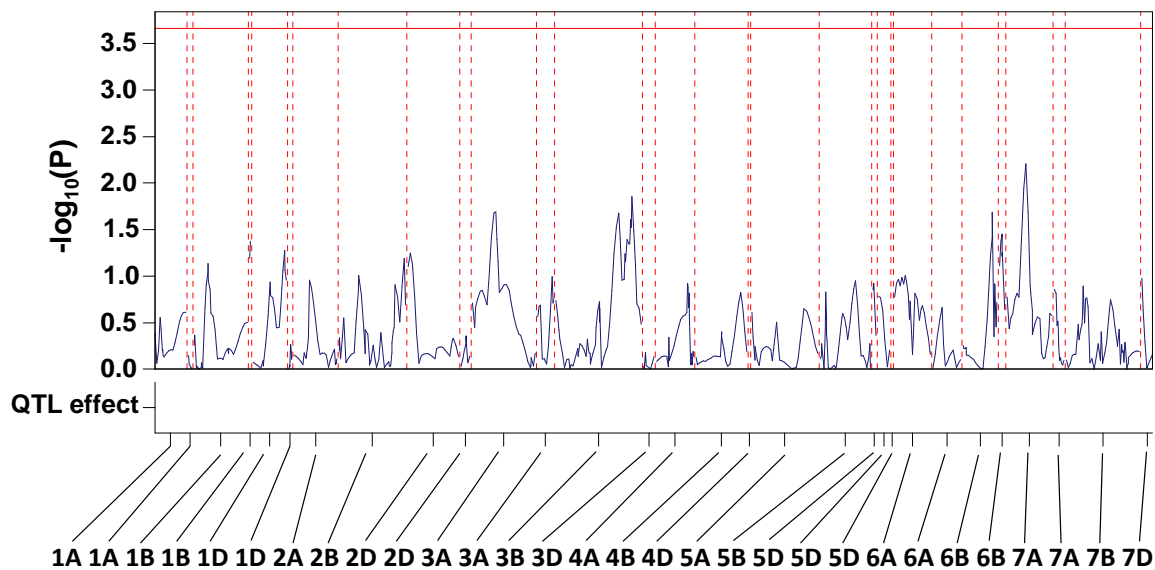
Test profile: water content index
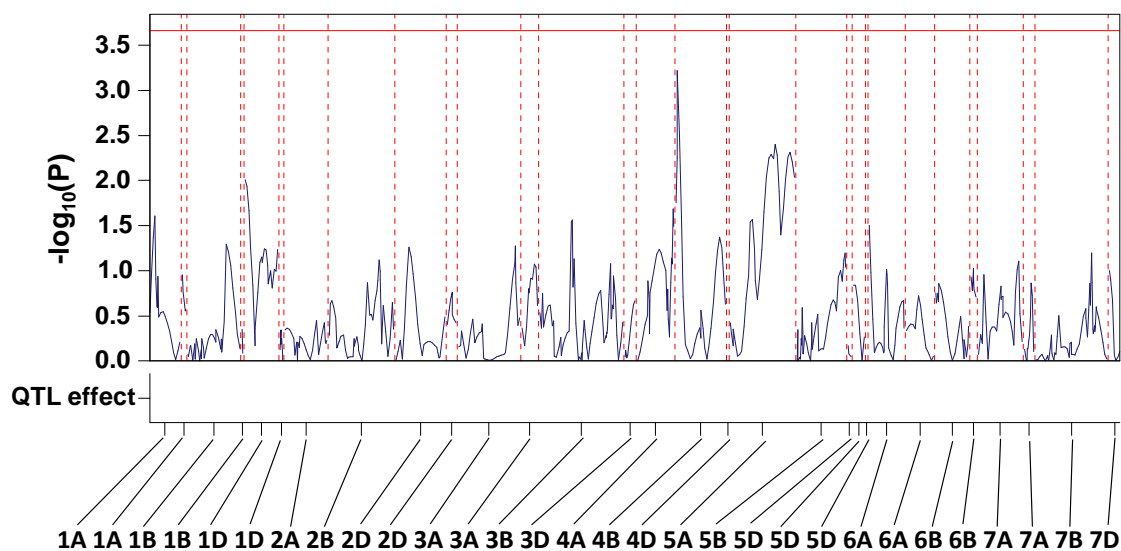
■ Gladius
■ Drysdale

6.2 Appendix II

The genetic correlation between RILs derived from crossing Gladius and Drysdale (Sections 3.2.4 and 3.3.1). Column 1 represents name of RILs from the Gladius * Drysdale population. Column 2 represents cluster number. Two or more RILs with a correlation coefficient close to 1 were considered as one cluster. Column 3 represents the correlation coefficient between RILs using DArT genotyping data. Column 4 represents the correlation coefficient between RILs using microsatellite genotyping data. Column 5 represents the correlation coefficient between RILs using DArT and microsatellite data. (-) corresponds to those RILs which were not genotyped with DArTs or microsatellites.

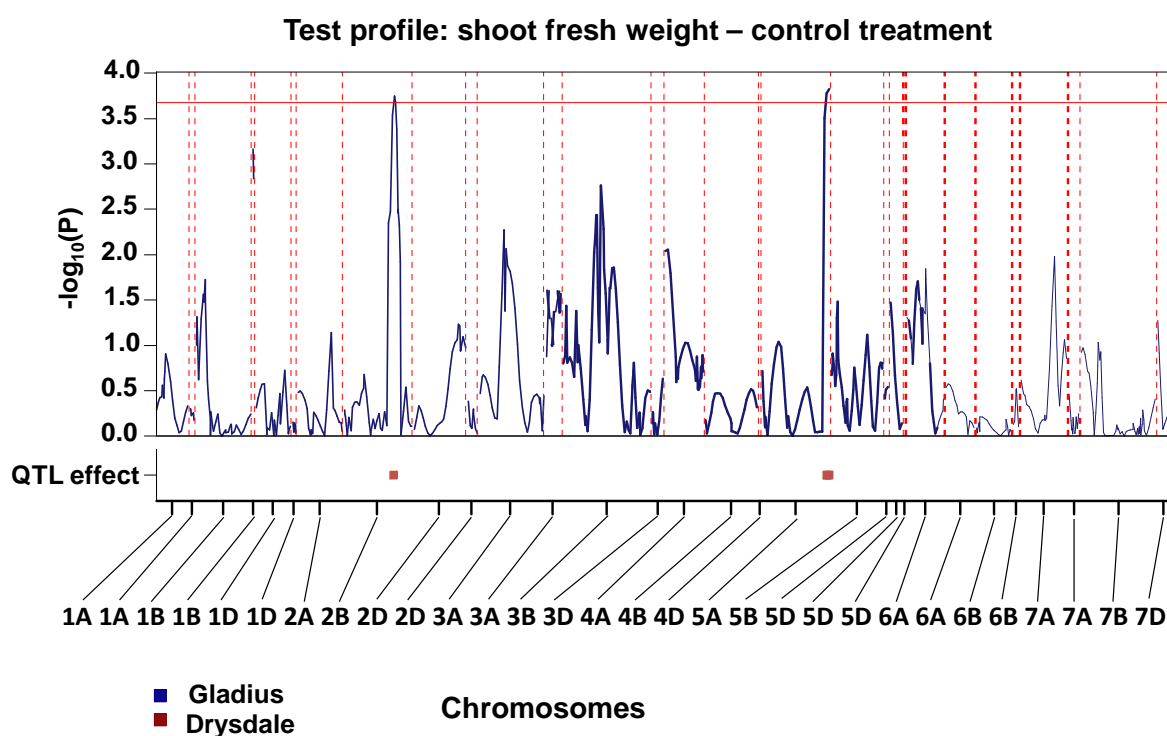| Column 1	Column 2	Column 3	Column 4	Column 5	Column 1	Column 2	Column 3	Column 4	Column 5
RILs	Cluster	DArT	microsatellite	All	RILs	Cluster	DArT	microsatellite	All
GD001	1	0.98	0.89	0.95	GD121	14	1	0.81	0.94
GD040	1	0.98	0.89	0.95	GD126	14	1	0.81	0.94
GD091	1	0.98	0.89	0.95	GD156	14	1	0.81	0.94
GD022	1	0.98	0.89	0.95	GD137	15	0.99	0.84	0.95
GD002	2	1	0.88	0.96	GD149	15	0.99	0.84	0.95
GD023	2	1	0.88	0.96	GD119	16	1	0.83	0.96
GD205	3	0.95	0.95	0.95	GD248	16	1	0.83	0.96
GD236	3	0.95	0.95	0.95	GD210	16	-	-	0.87
GD090	4	0.97	0.94	0.96	GD174	17	0.99	0.69	0.90
GD206	4	0.97	0.94	0.96	GD184	17	0.99	1.69	0.90
GD129	5	0.97	0.83	0.93	GD042	18	1	0.86	0.96
GD191	5	0.97	0.83	0.93	GD099	18	1	0.86	0.96
GD034	6	1	1	1	GD123	19	0.99	0.84	0.90
GD083	6	1	1	1	GD194	19	0.99	0.84	0.90
GD031	7	0.95	0.69	0.89	GD169	19	0.99	0.84	0.90
GD036	7	0.95	0.69	0.89	GD197	19	-	0.84	0.90
GD144	7	0.95	0.69	0.89	GD240	20	0.99	0.76	0.92
GD153	7	0.95	0.69	0.89	GD249	20	0.99	0.76	0.92
GD057	8	0.98	0.87	0.95	GD173	21	0.98	0.87	0.95
GD062	8	0.98	0.87	0.95	GD218	21	0.98	0.87	0.95
GD061	8	0.98	0.87	0.95	GD183	21	0.98	0.87	0.95
GD059	8	0.98	0.87	0.95	GD135	22	0.87	0.87	0.86
GD058	8	0.98	0.87	0.95	GD148	22	0.87	0.87	0.86
GD060	8	0.98	0.87	0.95	GD203	23	-	0.94	0.94
GD189	9	1	0.97	0.99	GD227	23	-	0.94	0.94
GD207	9	1	0.97	0.99	GD142	24	-	0.86	0.85
GD128	10	0.99	0.80	0.94	GD170	24	-	0.86	0.85
GD181	10	0.99	0.80	1.94	GD095	25	-	0.95	0.94
GD014	11	1	0.84	0.95	GD192	25	-	0.95	0.94
GD063	11	1	0.84	0.95	GD204	26	-	0.93	0.95
GD075	11	1	0.84	0.95	GD208	26	-	0.93	0.95
GD118	11	1	0.84	0.95	GD157	27	-	0.82	0.83
GD108	12	0.99	0.86	0.95	GD166	27	-	0.82	0.83
GD241	12	0.99	0.86	0.95	GD164	28	-	0.90	0.90
GD243	12	0.99	0.86	0.95	GD195	28	-	0.90	0.90
GD009	13	1	0.97	1	GD176	29	-	0.88	0.88
GD070	13	1	0.97	1	GD179	29	-	0.88	0.88

6.3 Appendix III

The nutrient (mg/L) analysis of two types of soil (Mixed Roseworthy and coco peat mix) used in the experiment described in Chapter 2 was measured using inductively coupled plasma atomic emission spectrometry (ICP-AES) with CIROS axial and radial as outlined in Materials and Methods, Section 2.2.2.1

Soil type	Fe	Mn	B	Cu	Mo	Co	Ni	Zn	Ca
Mixed Roseworthy	0.37	5.84	0.73	0.27	0.01	0.44	0.30	0.60	880.00
Coco peat mix	3.69	6.90	0.88	0.21	< 0.01	< 0.01	0.17	0.46	613.33

Soil type	Na	K	P	S	Al	Ti	Cr	Cd	Pb
Mixed Roseworthy	240.00	303.33	4.35	503.33	<0.002	<0.002	<0.008	0.04	0.06
Coco peat mix	159.37	1023.33	63.45	576.67	0.08	< 0.002	< 0.008	< 0.003	< 0.03

6.4 Appendix IV

6.4.1 Soil based experiment

Two models were developed. Model 1 was used to calculate the proportion of the relative contribution of genetic effects to the overall phenotype for each trait (Heritability),where Types (parents, standard cultivars and RIL population) were considered as fixed terms (written to the left of the '|') and RILs, greenhouse and SmartHouse factors were considered as random terms (written to the right of the '|'). Model 2 was used to estimate Best Linear Unbiased Estimates (BLUE) values for each trait, where RILs were considered as fixed terms (written to the left of the '|') and greenhouse and SmartHous factors were considered as random terms (written to the right of the '|'). The BLUE value was used for QTL analysis. Block, whole, row, column are the greenhouse and SmartHouse factors (Section 3.2.3.1). Traits used in this experiment were osmotic stress tolerance – assay 1(OST1), tiller number on salt treated plants (TN), 4th leaf Na^+ accumulation (mM) (Na), 4th leaf K^+ accumulation (mM) (K), salt tolerance index (STI). The separator symbol "." between any two factors represents the interaction term between them. "G" represents the greenhouse factors and "S" the SmartHouse factors.

	Model 1	Model 2
STI	Types | RILs + Gblocks.Gwhole + Sblocks.Swhole + Sblocks.Srows.Swhole	RILs | Gblocks.Gwhole+ Sblocks.Swhole + Sblocks.Srows.Swhole
Na	Types | RILs + Gblocks.Gwhole + Sblocks + Sblocks.Srows + Sblocks.Swhole + Sblocks.Srows.Swhole	RILs | Gblocks.Gwhole + Sblocks + Sblocks.Srows + Sblocks.Swhole + Sblocks.Srows.Swhole
K	Types | RILs + Grows + Gblocks.Grows + Sblocks+ Sblocks.Swhole + Sblocks.Srows.Swhole	RILs | Grows + Gblocks.Grows + Sblocks + Sblocks.Swhole + Sblocks.Srows.Swhole
OST1	Type | RILs + Sblocks + Sblocks.Srows.Swhole	RILs | Gblocks.Gwhole+ Sblocks.Srows.Swhole
TN	Types | RILs Gblocks + Gblocks.Gwhole + Sblocks.Srows + Swhole + Sblocks.Srows.Swhole	RILs | Gblocks + Sblocks.Srows + Swhole + Sblocks.Srows.Swhole

6.4.2 Hydroponics experiment

Two models were developed. Model 1 was used to calculate the proportion of genetic to the phenotypic effect for each trait (Heritability), where Types (parents and RIL population) were considered as fixed terms (written to the left of the '|') and RILs and trolley factors were considered as random terms (written to the right of the '|'). Model 2 was used to estimate Best Linear Unbiased Estimates (BLUE) values for each trait, where RILs were considered as a fixed terms (written to the left of the '|') and trolley factors were considered as a random terms (written to the right of the '|'). The BLUE value was used for QTL analysis. Trolley, Tub, Row, Column and Edge are the trolley factors (Section 3.2.3.2). Traits used in this experiment were 4th leaf Na^+ accumulation (Na), 4th leaf K^+ accumulation (K), salt tolerance index (STI), water content index (WCI), shoot fresh weight (SFW), shoot dry weight (SDW), tiller number (TN) and water content (WC). The separator symbol "." between any two factors represents the interaction term. "c" represents the control treated trollies and "s" the salt (100 NaCl mM) treated trollies.

Control plants

	Model 1	Model 2
SFW	Types | RILs + cTrolley + cTrolley.cRow + cTub.cEdge	RILs | cTrolley + cTrolley.cRow + cTub.cEdge
DSW	Types | RILs + cTrolley + cTrolley.cRow + cTub.cEdge	RILs | cTrolley + cTrolley.cRow + cTub.cEdge
TN	Types | RILs + cTrolley + cTrolley.cRow + cTub.cEdge	RILs | cTrolley + cTrolley.cRow + cTub.cEdge
WC	Types | RILs + cTrolley + cTrolley.cRow + cTub.cColumn + cTub.cEdge	RILs | cTrolley + cTrolley.cRow + cTub.cColumn + cTub.cEdge

Salt treated plants

	Model 1	Model 2
SFW	Types | RILs + sTrolley + sTub.sColumn + sTub.sEdge	RILs |sTrolley + sTub.sColumn + sTub.sEdge
DSW	Types | RILs + sTrolley + sTub.sColumn + sTub.sEdge	RILs |sTrolley + sTub.sColumn + sTub.sEdge
Na	Types | RILs + sTrolley + sTrolley.sRow	RILs |sTrolley + sTrolley.sRow
K	Types | RILs + sTrolley +sTrolley.sRow + sTub.sEdge	RILs |sTrolley + sTub.sColumn + sTub.sEdge
TN	Types | RILs + sTrolley + sTrolley.sRow + sTub.sEdge	RILs |sTrolley + sTrolley.sRow + sTub.sEdge
WC	Types | RILs +sTrolley + sTub + sTrolley.sRow + sTub.sColumn + sTub.sEdge	RILs |sTrolley + sTub + sTrolley.sRow + sTub.sColumn + sTub.sEdge
STI	Types | RILs + cTrolley + cTub + cTrolley.cRow + cTub.cColumn + cTub.cEdge + sTrolley + sTub +sTrolley.sRow + sTub.sColumn + sTub.sEdge	RILs |cTrolley + cTub + cTrolley.cRow + cTub.cColumn + cTub.cEdge + sTrolley + sTub + sTrolley.sRow + sTub.sColumn + sTub.sEdge
WCI	Types | RILs + cTrolley + cTub + cTrolley.cRow + cTub.cColumn + cTub.cEdge + sTrolley + sTub + sTrolley.sRow + sTub.sColumn + sTub.sEdge	RILs |cTrolley + cTub + cTrolley.cRow + cTub.cColumn + cTub.cEdge + sTrolley + sTub + sTrolley.sRow + sTub.sColumn + sTub.sEdge

6.4.3 Durum wheat

Two models were developed to calculate the proportion of genetic effect to the phenotypic (Heritability) and Best Linear Unbiased Estimates (BLUE) values for each trait evaluated in two F_2 populations derived from crossing Jandaroi and two Afghani landraces (lines 752 and 740) (Section 4.2.4). Traits used in this experiment were 3rd leaf Na$^+$ accumulation (Na), 3th leaf K$^+$ accumulation (K) and the ratio of K$^+$/Na$^+$ (K/Na). Model 1 was used to calculate heritability where Type (parents and F_2) is considered as a fixed terms (written to the left of the '|') and F_2 plants and trolley factors were considered as random terms (written to the right of the '|'). Model 2 was used to estimate BLUE values for each trait, where F_2 plants were considered as fixed term (written to the left of the '|') and trolley factors were considered as random terms (written to the right of the '|'). The BLUE values were used for marker regression analysis.

	Jandaroi * line 740	
	Model 1	**Model 2**
Na	Types | F_2 + column + row	F_2 | column + row
K	Types | F_2 + column	F_2 | column
K/Na	Types | F_2 + column + row + tub	F_2 | column + row + tub

	Jandaroi * line 752	
	Model 1	**Model 2**
Na	Types | F_2 + column + tub	F_2 | column + tub
K	Types | column	F_2 | column
K/Na	Types | F_2 + column + row + tub	F_2 | column + row + tub

6.5 Appendix V

Estimated variance components (random model) and residual variance model with their standard error (S.E.) of two F_2 populations derived from crossing Jandaroi and two Afghani landraces. Each population was grown in one trolley. Each trolley had two tubs which were divided into ten rows and 16 columns. The F_2 plants, rows, columns, and tub (trolley factors) were used in the random model as random terms (Appendix IV, Section 6.4.3). Estimated variance components for both populations showed that the F_2 random term component of 3rd leaf Na^+ (Na) accumulation and the ratio of K^+/Na^+ (K/Na) was significantly larger than the other components, and also larger than the residual variance.

Random term	Jandaroi * line 740 F_2 population					
	Na		K		K/Na	
	Component	S.E.	Component	S.E.	Component	S.E.
F_2 plants	1617.9	251.1	272.8	176.2	0.111	0.021
column	255.0	114.6	12.7	45.5	0.013	0.009
row	0.0	bound	0.0	bound	0.000	bound
tub	112.6	57.8	0.0	bound	0.005	0.008
Residual term	0.0	1.1	366.9	156.4	0.015	0.011

Random term	Jandaroi * line 752 F_2 population					
	Na		K		K/Na	
	Component	S.E.	Component	S.E.	Component	S.E.
F_2 plants	1052.1	178	0	bound	0.203	0.047
column	60.5	57.4	4	45	0.009	0.013
row	0	bound	0	bound	0.004	0.012
tub	7.5	29.7	0	bound	0.003	0.009
Residual term	238.2	105.8	1004	110	0.092	0.037

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