The genetics of barley (*Hordeum vulgare*) salinity tolerance during germination and the instantaneous seedling endurance

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Doctor of Philosophy

By

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DECLARATION

I certify to the best of my knowledge that this thesis is my own work and its content has not been previously presented for any diploma/degree qualification at another institution of higher education or used for other purposes. I confirm that the intellectual content of this thesis is the product of my original work and that all the help rendered in its preparation and sources have been acknowledged and it does not contain any material that infringes copyright. All co-authors, where stated and certified by my principal supervisor, have agreed that the works presented in this thesis represent substantial contributions from myself.

Edward Kulecho Mwando

Date: **7.4.2021**

ABSTRACT

Salinity limits crop production through osmotic and ionic stress in combination with oxidative strain and nutrient imbalance. Osmotic tolerance, ionic exclusion, and tissue tolerance are some of the adaptive mechanisms in plants when exposed to salinity stress. These physiological adapative mechanisms are quantitative in nature and are manifiested genetically by affecting ~ 8% of genes expression. Barley, the fourth most important cereal crop in the world, is relatively salinity tolerant. However, salinity causes a significant reduction in its growth and grain yield. Adaptation to salinity in barley is varied with growth stage where germination and early growth stages are the most sensitive. This is because excessive salt accumulation in the rhizosphere affects the germinating seed and the subsequent developmental processes including revitalization of plants development after exposure to salinity stress during the sprouting stage. Studies are yet to close the lack of information between the germination and/or seedling stage salinity tolerance, and the genotypic differences in developing young plants regeneration after exposure to salinity stress. The current study explored the genetics of salinity tolerance during the germination stage and the seedling survival in barley after germination under salinity stress (NaCl). To detect the genetic loci and candidate genes responsible for salinity tolerance in barley during germination and early growth stages, four barley populations comprising of a diversity panel of 350 accessions from across the globe, two doubled haploid (DH) populations (CM72/Gairdner and Skiff/CM72), and a back-cross population of CM72/Gairdner/*Spartacus CL were used for phenotyping and mapping. These germplasm sets were exposed to different levels of salinity stress (75, 90, 120 AND 150 mM NaCl) along with a control treatment (deionized water) and various phenotypic traits recorded at germination and early seedling stages. Genome-Wide Association (GWAS) analysis was conducted on a diversity panel of 350 accessions using ~24,000 genetic markers, where 19 Quantitative Traits Nucleotides (QTNs) were detected across all 7 barley chromosomes and 4 genes predicted for salinity tolerance at germination. A study with CM72/Gairdner DH population mapped six Quantitative Traits Loci (QTLs) on chromosomes 1H, 3H and 4H for traits associated with seedling survival under salinity stress. Three QTLs on 1H (1) and 3H (2) with closely linked significant markers that were detected in more than one salinity survival traits were proposed as the regions with highest probability of having candidate genes. To narrow down the location of genetic regions asssiociated with salinity tolerance at germination on chromosome 2H, a major QTL was fine–mapped using CM72/Gairdner and Skiff/CM72 DH populations, F_2 and F_3 generations of CM72/Gairdner/*Spartacus CL to a region of ~ 0.341 Mb and designed 2 diagnostic markers. Further, this study reported two Receptor-like protein kinase 4 as the candidate genes for

enhanced germination under salinity stress. The diversity of seven reported genes in barley was explored further in 40 different species where three of them; dehydration-responsive element-binding (DREB) protein, somatic embryogenesis receptor-like kinase and aquaporin genes, were found to be the most varied. While all three gene families show great diversity in most plant species, the *DREB* gene family was more diverse in barley than in wheat and rice. Sixty-five barley homolog genes were identified from salinity tolerance genes characterized in *Arabidopsis*, maize, rice, soybean, and wheat. Besides, the homologs have been reported to express themselves in first three barley's developmental stages. The results of this study provide new genetic resources and information for further functional characterization of the identified candidate genes and to improve salinity tolerance at germination and early seedling stage *via* genomic and marker-assisted selection (MAS) in barley. The findings in this thesis together with other existing information will facilitate breeding and release of new high yielding barley varieties that can grow in extreme environment including saline soils of the wolrd.

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LIST OF ABBREVIATIONS

ABI 1 – ABA-Insensitive 1

ABI 2 – ABA-Insensitive 2

ADC – Arginine decarboxylase

APX – Cytosolic ascorbate peroxidase

BADH – Betaine aldehyde dehydrogenase

mt1D – Mannitol-1-phosphate dehydrogenase

CBL – Calcineurin N B-like protein

DBF – DRE binding factor

DH – Double haploid

DHAR – Dehydroascorbate reductase

DRE – Drought responsive element

DREB – Drought responsive element binding protein

GAMYB – GA-inducedMyb (myeloblastosis)-like protein

GWAS – Genome wide association analysis

GutD – Glucitol-6-phosphate dehydrogenase

HKT – High efficiency potassium transport

HvABA8′OH-1 – Barley ABA 8′ hydroxylase

HvGA2ox – Barley Gibberellin 2-oxidase1

HvKAO1 – Kaurenoic acid oxidase1

HvNCED – Barley Nine-cis-epoxycarotenoid dioxygenase

HvPTR – Barley scutellar peptide transporter

MAS – Marker assisted selection

MG – Methylglyoxal

MIPS L – Myo-Inositol-1-phosphate synthase

MYB – Myeloblastoma

NAC – No apical meristem, ATAF 1,2 and cup-shaped cotyledon

NDPK2 – Nucleoside diphosphate kinase 2

NHX-1 – Vacuolar Na+/H+ antiporter

ODC – Ornithine decarboxylase

OH – Hydroxyl radical

P5CR – P5C reductase

pH – The potential of hydrogen' or 'power of hydrogen' (acidity or basicity of an aqueous solution).

QTL – quantitative traits loci

ROS – Reactive oxygen species

SAMDC – S-Adenosyl methionine decaroboxylase

SAPK4 – Sucrose nonfermenting 1-related protein kinase2 (SnRK2)

SOD – Superoxide dismutase

SOS1 – Salt overly sensitive

SPDS – Spermidine synthase

TPS – Trehalose-6-phosphate synthase

p5cs D1 – Pyroline -5-carboxylate synthase codA Choline oxidase

TsVP H^+ - pyrophosphatase

UV – ultraviolet part of the radiation emitted in the solar spectrum

UVA – Ultraviolet A of 315-400nm wavelength, and is associated with skin aging

UVB – Ultraviolet B of 280-315nm wavelength, and is associated with skin burning

UVC – Ultraviolet C of wavelength 200-280nm

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Many thanks and God bless you all.

DEDICATION

This thesis is dedicated to my mother Margaret Meta Mwando who is my role model in persistence and positivity, my late father Charles Philip Mwando a guardian angel, brothers, sister and most importantly my friend Dr. Charles Obiero whose motivation has continuously remained my strength.

PUBLICATIONS

Journal Papers:

1. **Mwando, K. E.,** Han, Y., Angessa, T. T., Zhou, G., Hill, C. B., Zhang, X-Q., and Li, C. (2020). Genome-wide association study of salinity tolerance during germination in barley (*Hordeum vulgare* L.). ***Frontiers in Plant Science,*** 11:118. https://doi: https://10.3389/fpls.2020.00118.
2. **Mwando, K. E.,** Angessa, T. T., Han, Y., Zhou, G., and Li, C. (2021). Quantitative trait loci mapping for vigour and survival traits of barley seedlings after germinating under salinity stress. ***Agronomy,*** 11(1): 103. https://doi.org/10.3390/agronomy11010103.
3. **Mwando, K. E.,** Angessa, T. T., Han, Y., and Li, C. (2020). Salinity tolerance in barley during germination – homologs and potential genes. ***Journal of Zhejiang University-Science B,*** 21: 93–121. https://doi.org/10.1631/jzus.B1900400.

Manuscripts submitted or Ready for submission

1. **Mwando, K. E.,** Han, Y., Angessa, T. T., Zhang, X-Q., and Li, C. (Under review). Fine-mapping and characterisation of genes on barley chromosome 2H for salinity stress tolerance during germination.

CONFERENCE AND SEMINARS

1. **Mwando, K. E.,** Han, Y., Angessa, T. T., Zhang, X-Q., McFawn, L-A., and Li, C. (2019). Towards the identification of barley salinity tolerance genes during germination. ***111th American Society of Agronomy, 64th Crop Science Society of America and 83rd Soil Science Society of America Annual Meetings, 10th to 13th November 2019,*** The Henry B. Gonzalez Convention Center, San Antonio, Texas, USA.

2. **Mwando, K. E.,** Han, Y., Angessa, T. T., Zhou, G., and Li, C. (2019). Quantitative trait loci and markers associated with genes for barley salinity tolerance at germination. ***19th Australian Barley Technical Symposium, 10th to 23rd September 2019,*** Westin Hotel, Perth, Australia.

APPLICATION OF SKILLS

Developed near isogenic lines (NILs) of Spartacus CL background with salinity tolerance during germination on chromosome 2H, waterlogging and boron toxicity tolerance on 4H locus by back-crossing and MAS.

STATEMENT OF CO-AUTHORSHIP

During my enrolment as a PhD student in the College of Science, Health, Engineering and Education, School of Veterinary and Life Sciences at Murdoch University this thesis was completed. It is comprised of experimental outcomes that have not been previously presented for the award of a degree in this institution or anywhere else. This thesis contains eight chapters that includes introduction, expanded literature review, general discussion and conclusion. The contents of four research chapters in this dissertation, have been or prepared for publication in different journals with contribution from the following individuals and institutions.

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Journal Paper 1: Mwando, K. E., Han, Y., Angessa, T. T., Zhou, G., Hill, C. B., Zhang, X-Q., and Li, C. (2020). Genome-wide association study of salinity tolerance during germination in barley (*Hordeum vulgare* L.). ***Frontiers in Plant Science,*** 11:118. https://doi: https://10.3389/fpls.2020.00118.

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Candidate performed phenotyping experiments, conducted 80% of data analysis, interpretation and drafting of the paper.

Authors 4 and 6 conducted association analysis 20%. Authors 4, 5 and 6 generated the genotypic data. Authors 1, 2 and 3 conceived the project. All the author and candidate revised the paper and approved the final version for publication.

Journal Paper 2: Mwando, K. E., Angessa, T. T., Han, Y., Zhou, G., and Li, C. (2021). Quantitative trait loci mapping for vigour and survival traits of barley seedlings after germinating under salinity stress. ***Agronomy,*** 11(1): 103. https://doi.org/10.3390/agronomy11010103.

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Located in Chapter 6

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Authors 1 and 2 gave guidance on relevant literature search, information and data interpretation. Author 3 conceived the projects idea. All the authors and candidate revised the paper and approved the final version for publication.

Manuscript Paper 1: Mwando, K. E., Han, Y., Angessa, T. T., Zhang, X-Q., and Li, C. (Under review). Fine-mapping and characterisation of genes on barley chromosome 2H for salinity stress tolerance during germination.

Located in Chapter 5

Candidate performed validation, fine-mapping, gene expression and characterization experiments, data analysis, interpretation and drafting of the manuscript. Author 2 constructed the initial molecular linkage map, facilitated bulking of planting material and original QTL mapping.

Authors 2 and 5 guided in laboratory experiment. Author 3 conceived the project. All the authors and candidate revised the paper and approved the final version to be published.

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CHAPTER ONE
GENERAL INTRODUCTION

1.1 Background information

Climate change is a worldwide phenomenon triggering abiotic stress that affects agricultural land and interferes with plant growth and development (FAO, 2009; Grayson, 2013; Meena *et al.,* 2017; Porter *et al.,* 2019). Abiotic stresses encompasses all the non-biological factors whose variation impose pressure on plants and reducing their productivity (Boyer, 1982; Mittler, 2006; Ashraf *et al*., 2008; Athar and Ashraf, 2009; Acquaah, 2012; Pawel *et al.,* 2014; Sulmon *et al.,* 2015; Pereira, 2016; Kollist *et al.,* 2019; Priyadarshan, 2019). Consequently, stresses like salinity, drought, nutrient deficiency, waterlogging, cold, and heat are the primary abiotic constraints that reduce crop productivity (Shewry *et al.,* 2008; Kumar, 2013; Xue *et al.,* 2017; Cui *et al.,* 2018). Of these, drought, salinity, and temperature stress are the most destructive as they inhibit metabolic processes in plants (Akula and Gokare, 2011; Fujita *et al.,* 2011; Vishwakarma *et al.,* 2017; Kumari *et al.,* 2019; Rastogi *et al.,* 2019).

Among the most destructive, salinity stress is an important constraint that causes massive yield losses in crops predominantly in the arid, semi-arid and coastal regions of the world at the same time, it occurs in both humid and sub-humid landscapes (Munns, 2002; Munns and Tester, 2008; Fageria *et al*., 2011; Deinlein *et al.,* 2014; Bernstein, 2019; Cai and Gao, 2020). Soils are said to be saline when they have elevated levels of soluble salts (NaCl and Na_2SO_4), an electrical conductivity (EC) of not less than 4 ds/m or 40 mM NaCl, exchangeable sodium percentage (ESP) of less than 15% and a pH not exceeding 8 (USDA, 2008; Srivastava *et al.,* 2019). About 20% of cultivated area and 33 percent of the irrigated land around the world is saline (Shahid *et al.,* 2018; Ghonaim *et al.,* 2021). This is further aggravated by climate change that forces the use of poor-quality water for irrigation. Salinity stress in Australia is a serious threat in the semi-arid zones that receiving less than 450 mm of rainfall annually (Rengasamy, 2002). It affects more than 2.5 million hectares of land with projections indicating a likely increase to 15 million hectares in the coming decades. Dryland salinity puts 5.1 million hectares of the Australia farming land at risk and its predicted to increase to 17.1 million hectares by the year 2050 (NLWRA, 2000). Western Australia is the worst affected with a total salinity area of about 1.8 million hectares.

The increasing salinity due to human activities and climate change, and its seasonal fluctuations is worrying. This is because most of the cultivated crops are susceptible to salinity stress (Hasanuzzaman *et al.,* 2013; Sanower, 2019; Russe *et al.,* 2019; Zelm *et al.,* 2020). Thus, it

will be more challenging to meet the food demand of the growing world population because of the unavailability of agriculturally productive land due to environmental factors like salinity. Therefore, more considerable efforts will be needed to increase crop production in a stressful agricultural environment and to bring marginal lands such as those affected by salinisation under cultivation (Jamil *et al.,* 2011). According to Agarwal *et al.* (2013), salinity reduces crop yield in two ways: (i) It impedes plant access to soil water through increased osmotic potential of the soil thus curbing water and nutrient absorption (osmotic or water-deficit effect) and (ii) It causes ionic disproportion and toxicity in plants (salt-specific or ion-excess effect). However, the detrimental impact of salinity on crops varies with crop developmental stages. Germination and early seedling developmental stages being more susceptible than the other growth stages (Angesa *et al.,* 2017). Therefore, the yield reduction and death are just the manifestations of the damaging impacts of salinity stress on plants occurring at germination and early seedling developmental stages (Asish and Anath, 2004).

Plants are broadly categorized as halophytes or glycophytes depending on their response to salinity stress (Soundararajan *et al.,* 2019). Halophytes show the capacity to thrive under saline conditions (Volkov, and Flowers, 2019), while glycophytes cannot tolerate more than 40 mM NaCl without significant impact of growth and yield (Safdar *et al.,* 2019). Most of the cultivated crops in the world are glycophytes (Bose *et al*., 2013; Téllez *et al.,* 2020). They respond to salinity condition (>40 mM NaCl) in two phases: (i) A rapid response to an increase in external salt known as (osmotic phase), and (ii) Slower response with an accumulation of Na+ ions in vacuoles refer to as (ionic period). As the crop shows the above two reactions, there is a reduction in growth and yield (Munns and Tester, 2008; Behdad *et al.,* 2020). Some glycophytes, however, have a reasonable level of adaptation with the capacity to complete their life cycle under moderate salinity stress (40 -150 mM NaCl) with a satisfactory development (60-90%) and harvest (50-80%). For the plants to achieve this, they undergo complex phenomenon involving a variety of mechanisms (Flowers and Yeo, 1986; Colmer and Flowers, 2008; Devi and Arumugam, 2019).

Studies have shown a wide range of mechanisms in response to salinity stress in several plant parts that suggest adaptations at the whole plant level (Wyn and Gorham, 1983; Munns, 1993; Kamran *et al.,* 2020). Glycophytic plants have evolved to develop mechanisms that make them more tolerant to increasing salinity. According to Roy and Chakraborty (2014) and Acosta-Motos *et al.,* (2017), these mechanisms are grouped into three categories. (i) Osmotic stress tolerance controlled by long-distance signals that reduce shoot growth and are triggered by

shoot Na+ accumulation. (ii) Na+ or Cl^- exclusion that tends to prevent Na^+ and Cl− uptake and transport processes in roots to reduce these ions reaching toxic concentrations within leaves. (iii) Tolerance of tissue to accumulated Na^+ or Cl−, where, the Na^+ or Cl^- that succeeded in getting into the plants are compartmentalized into the leaf cells vacuole to prevent injury to the sensitive thylakoid membrane of the chloroplasts (Tester and Davenport, 2003; Kumari *et al.,* 2013). The above adaptation mechanisms entail multifaceted physiological traits, metabolic pathways, gene systems and its growth stage to enable the plant to survive salinity stress (Haq *et al.,* 2010; Bhaskar and Bingru, 2014; Manzoor *et al.,* 2020).

Some of the salinity management options available include; soil leaching, use of clean water for leaching and irrigation, good choice of irrigation method, applying amendments, planting deep rooted trees, improving field drainage among others (Hart *et al.,* 2020). Most of the above management options are expensive and need a lot of investment. At present, there are no practical physical techniques that are economically viable for managing salinity in agricultural lands (Hamdia and Shaddad, 2010). Growing adapted cultivars is currently the most economical and efficient way to increase food production in saline environments (Ashraf and Wu, 1994; Kamran *et al.,* 2020). Planting of adapted plants together with one or more of the above management practices would ensure that we optimise on our crops production potentials in saline soils (Hayat *et al.,* 2020). The process of developing adapted crops requires amongst others; physiological mechanisms and genetic knowledge of the contributing characters at different plant growing stages (Hamdia and Shaddad, 2010). Flowers and Yeo, (1995), proposed five possible genetic approaches to improving crop productivity under saline conditions: (i) Deployment of halophytes as alternative crops. (ii) Exploitation of genetic variation for salt tolerance already present in existing plant cultivars. (iii) Use of interspecific hybridization to increase salt tolerance of current commercial cultivars. (iv) Generate variation within existing crops via genetic mutation. (v) Breed for higher yield rather than salt tolerance. These approaches have different potentials depending on the targeted agricultural environments and plant species of interest. But they are only applicable if we can understand the genetic functioning of plants adaptation to salinity. Several genes controlling salinity tolerance traits in plants, fall into three main groups: (i) Those that mediate salt uptake and transport, (ii) Those that have an osmotic or protective function, and (iii) Those that promote plant growth in saline soil (Munns, 2005). The candidate genes express themselves differently in growth stages of plants and tissues influenced by environmental factors (Roy *et al.,* 2011). Most of the genes contributing to salinity adaptation are yet to be discovered even in model

crops like Arabidopsis, and rice (Colmer *et al.,* 2005; Grene *et al.,* 2019). Further, salinity tolerance is a multigenic trait (Khan *et al.,* 2016). Therefore, substantial improvement based on a modification of a single gene is not likely to occur (Mujeeb-Kazi *et al.,* 2019). Plants respond to salinity stress by exhibiting complex, quantitative traits, which involve the activity of many genes and physiological processes whose expression is influenced by many environmental factors (Frova *et al.,* 1999).

Since salinity tolerance genes are expressed differently at each developmental stage in crops (Foolad, 2004; Roy *et al*., 2011), therefore, it is only practical to screen varieties for adaptation at the correct phase. The first point that a plant gets in contact with the saline condition is at germination stage (Bewley *et al.,* 2013; Fatemeh *et al.,* 2016; Zörb *et al.,* 2019). Selection of salinity tolerance during germination under field conditions is not probable because different environmental factors affect the accuracy of the selections (Richards, 1996; Mujeeb-Kazi *et al.,* 2019). But, with the dawn of modern molecular breeding techniques, it is becoming possible to understand plant response to salt stress better and to improve plant salt tolerance through genetic approaches. Identification of new traits donating to adaptation to salinity can be done through traditional selection in stressful environments and based on mapping studies of quantitative trait loci (QTL) (Holland, 2007). Once the molecular basis of the characters donating to salinity tolerance is recognized, marker-assisted selection can be used to efficiently exploit the new traits at the gene level (Munns *et al.,* 2012; Hanin *et al.,* 2016). However, knowledge of candidate salinity tolerance genes is a prerequisite for efficient utilization of modern techniques for development of plants with improved salt tolerance (Jamil *et al.,* 2011).

Barley (*Hordeum vulgare* L.) is the fourth most important cereal crop after maize, rice, and wheat (Schulte *et al.,* 2009; Bhatta *et al.,* 2020; FAOSTAT, 2019). It grows in a wide range of environmental conditions some being tremendously unsuitable for plant growth, making it one of the hardiest cereal crops growing in extreme latitude and altitude where others are not adapted (Harlan, 1976; Harwood, 2019). Salinity is one of the adverse conditions that affects crops, but barley has shown the capacity to thrive under the environment because of its excellent ability to adapt to such soil conditions (Harlan, 1995). Because of this ability to endure salinity, barley is being used by breeders to improve other cereals as a source of favorable alleles through conventional and molecular approaches (Munns *et al.,* 2006; Harwood, 2019).

As some barley varieties express halophytic features, conversely, some varieties are equally affected by salinity, due to their reduced tolerance levels prompted by domestication when selecting for yield and quality under optimum environment (El-Esawi *et al.,* 2018). This has increased their sensitivity to salinity stress (Kiani-Pouya *et al.,* 2020). These varieties will show poor germination, slow growth, little survival, reduced development, low yield and mixed metabolic processes (Colmer *et al*., 2005; Xue *et al.,* 2009; Zhang *et al.,* 2010; Angessa *et al.,* 2017). Salinity tolerance in barley like other plants varies in different genotypes (Mano and Takeda, 1997; Flowers and Hajibagheri, 2001; Xue *et al.,* 2009). Screening of barley genotypes for salinity tolerance at various development stages is continuous, and a lot of progress has been made (Martinez-Cob *et al.,* 1987; Ahmed *et al.,* 2012; Askari *et al.,* 2017). Several QTLs associated with salt tolerance traits in barley have been mapped. They include yield and agronomic (Xue *et al.,* 2009; Eleuch *et al.,* 2008), germination and seedling (Mano and Takeda, 1997; Angessa *et al.,* 2017; Moghaddam *et al.,* 2020), plant survival (Zhou *et al.,* 2012), shoot sodium content or Na/K (NaK) ratio (Shavrukov *et al.,* 2010) and salt exclusion (Rivandi *et al.,* 2011).

Germination is a very sensitive stage when barley is exposed to salinity condition and the most significant phase in its life cycle because it gives vigour, determines seedling population and ultimately yield (El Madidil *et al.,* 2004; Tarawneh, 2019). Having varieties that can tolerate salinity at germination is essential in regions that experience hot and dry summer that increases salinity levels in the topsoil where germination occurs after sowing in autumn (Boyd *et al.,* 2003). Consequently, there are reports on phenotyping for various characters used in mapping quantitative traits loci (QTL) containing genes vital for salt tolerance at the germination stage (Mano and Takeda 1997; Hanen *et al.,* 2014). Uniform and timely germination and vigorous seedling growth under saline conditions are some of the key traits used when selecting for salinity tolerant genotypes. While several varieties have shown tolerance to salinity, little information on the candidate genes responsible at germination and early seedling stage survival has been documented. At the same time, there is scant knowledge on revival of seedling germinating under salinity stress to close the gap of limited information between germination and seedling survival. Also, limited work has been done to characterize the responsible genes and identify diagnostic markers. The current study generated important information to close the genetic gap by targeting germination and early seedling survival traits in barley after germination under salinity stress exploiting various seedling vigour indices.

1.2 Research objectives

1.2.1 Broad objective

The broad objective of this study was to understand the genetics of salinity tolerance during germination in barley, the nature of genes controlling it and their relationship with early seedling survival.

1.2.2 Specific objectives

1. To search for new loci of salinity tolerance during germination in barley by genome wide association analysis (GWAS).
2. To map QTLs for early seedling survival traits in barley after germinating under salinity stress.
3. To validate, fine map and design diagnostic markers for a major QTL on chromosome 2H of barley containing genes for salinity tolerance at germination stage.
4. To characterize the identified candidate gene(s) on chromosome 2H of barley.
5. To identify homologs and analyse barley genes for salinity tolerance during germination.

1.3 Justification

The projected annual production of barley in 2020 is 140 million tons from an area of 48 million hectares, which is a decline from the previous year production (143 million tons) and much below the recorded production of 156 million tons in 2008/2009 (FAOSTAT, 2019; USDA, 2020). But, world consumption of barley is projected to increase at 2% per year in the next five years due to the increased demand for animal feed and malting (FAOSTAT, 2019). However, climate change has caused variation in environmental factors to imposes stress on barley affecting its growth and yield. Salinity is an important abiotic stress in barley that causes an estimated 20% yield reduction around the world (Munns and Gilliha, 2015; Jamshidi and Javanmard, 2018). But there are reports of some barley varieties which can grow under adverse environmental conditions like salinity while others cannot (Belaid and Morris, 1991; Harlan, 1995), and their growth and yield are affected when exposed to high concentrations of salinity (Jamshidi and Javanmard, 2018). The degree of damage caused by salinity stress depends on the variety, the concentration of salinity and the development stage of the crop (Roy *et al.,* 2011). Germination and early seedling stages being the most critical phases in barley life cycle and the most sensitive to salinity (Bewley *et al.,* 2013; Fatemeh *et al.,* 2016). The detrimental

effect of salinity on germination and onward seedling growth can be mitigated by developing varieties with tolerance using various approaches.

To develop cultivars with high salinity tolerance there is a need to first understand the nature of gene action governing salinity tolerance in the germplasm (Inja *et al.,* 2017). Though the physiological and biochemical basis of salinity is well known, further studies on genetic factors at different growth phases especially at germination and at early seedling growth under salt stress and the traits controlling tolerance in barley are paramount. But there is limited information on the nature and type of genes for tolerance at these stages. Further, while several varieties have shown tolerance to salinity, little information is available on the candidate genes at germination and early seedling survival. Moreover, limited work has been done on characterization of the responsible genes and designing of the diagnostic markers for selecting the loci.

In this study, QTLs for salinity tolerance during germination and the immediate seedling survival were mapped using a panel of 350 diverse barley accessions and a double haploid population of CM72/Gairdner. This enabled the predicted of four candidate genes likely to enhance germination in barley under salinity stress. Additionally, a major QTL mapped on chromosome 2H for salinity tolerance during germination by Angesa *et al.,* (2017), was validated, fine mapped and two candidate genes reported. Relevant information about the candidate genes was generated and diagnostic markers for selecting the locus designed and validated across a diverse world barley panel of 265 accessions. Genes for salinity tolerance during germination from *Arabidopsis*, soybean, maize, wheat, and rice were blasted and mapped on the barley reference genome to identify homologs of candidate genes. Eventually, the diversity of functional salinity tolerance genes during germination in barley was explored in 40 different species. This study combined with other strategies will not only improve the understanding of the genetics of salinity tolerance during germination and early seedling survival but also it will facilitate engineering barley with improved genes.

1.4 References

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CHAPTER TWO
EXPANDED AND GENERAL LITERATURE REVIEW

2.1 Abiotic stresses affecting plants

Plants face a plethora of unfavourable conditions that cause abiotic stress (Boyer,1982; Mittler, 2006; Hasanuzzaman *et al.,* 2019); which is the negative impact of non-living factors on crops in an explicit environment (Priyadarshan, 2019; Gull *et al.,* 2019). Plant abiotic stress encompasses all elements from the atmosphere that can impose significant strain on a variety of species when they diverge from normal (Sulmon *et al.,* 2015). These factors include extreme levels of light (high and low), radiation (UV-B and UV-A), temperature (high and low), water (drought, flooding, and submergence) and chemical factors (heavy metals and pH). Others include salinity, deficiency or excess nutrients, gaseous pollutants (ozone and sulfur dioxide) and mechanical injury, (Versulues *et al.,* 2006; Suzuki *et al.,* 2014; Gull *et al.,* 2019).

It is estimated that abiotic factor causes over 70% of crop yield loss annually (Arun-Chinnappa *et al.,* 2017). They affect a plant as an individual factor or in combination at different growth stages, which may change the metabolic processes leading to reduced growth and development, and productivity (Tester and Bacic, 2005; Rupnarayan, 2017). A single abiotic factor can influence the impact of another by making it more severe or reducing the capability of a plant to tolerate other stresses (Tester and Bacic, 2005; Rafique *et al.,* 2020). Abiotic stresses interfere with the physiology, molecular processes and growth of plants leading to reduced crop performance and hence yields and sometimes ultimate death. The stresses make plants initiate an inimitable cellular response to minimize damage, ensure survival, and enhance plant growth and productivity (Bohnert *et al.,* 1995; Catalá *et al.,* 2020).

Shelford (1931) and Bechtold and Field, (2018) incorporated the element of how environmental factors vary through a range of intensity that organisms can tolerate, beyond which there is usually a change in metabolic processes that can cause stress (Figure 2.1). Extreme stress causes unbearable metabolic load on the cells leading to the death of a plant. Abiotic stress in general accelerates the creation of Reactive Oxygen Species (ROS) in plants that injure the membrane systems and other cellular processes (Dat *et al.,* 2000; Vranova *et al.,* 2002; Mittler *et al.,* 2004; Onyekachi *et al.,* 2019). Unlike other organisms that are mobile, crops are sessile, hence need to recognize changes in the environment and respond appropriately (Qi and Zhang, 2020). When a plant senses an environmental abiotic stress, it activates a signaling cascade that induces multiple biochemical processes to help the plants cope and induce expression of stress

tolerance genes (Shanker and Venkateswarlu, 2011). The expressed genes generate different molecules and metabolic pathways for stress acceptance (Munns and Tester, 2008; Iqbal *et al.*, 2011; Rupnarayan, 2017; Handayani *et al.*, 2019).

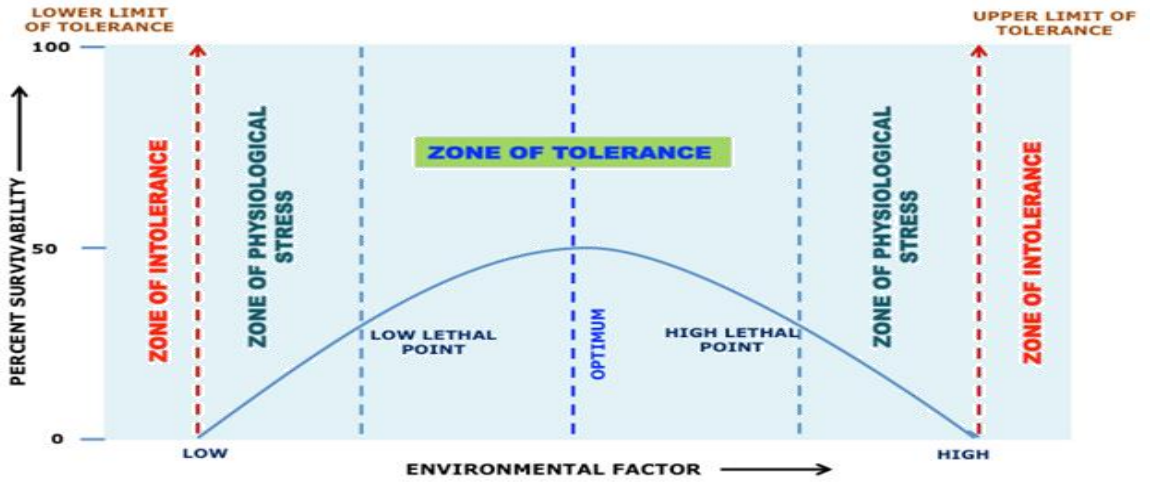


Figure 2.1 Organisms tolerance and resistance to environmental factors (Rupnarayan, 2017)

Plant's response to abiotic stress is hypothetically divided into four phases (Gerszberg and Hnatuszko-Konka 2017). These are: (i) Phase of alarm, (ii) Phase of resistance, (iii) Phase of exhaustion, and (iv) Regeneration phase that occurs only in cases of stress removal before the damage is too severe. The alarm phase is also known as the sensing phase and is experienced by the plant when one or more abiotic factors depart from the optimal. This phase is more complicated, and several processes are involved with no specific mechanism common to all the stresses (Urao *et al.*, 1999; Larcher, 2003; Rolland *et al.*, 2006). Sensing translates into signal and signal transduction that prepares the plant for a counter reaction (resistance phase), failure to which an acute damage or death occurs (Exhaustion phase). During this time functional activities like photosynthesis, germination, transport, accumulation of metabolites, nutrient uptake, and translocation slows down (Marques da Silva and Arrabac, 2004; Kosová *et al.*, 2018).

In response to abiotic stress, plants have developed intricate mechanisms at different levels of development to survive extreme conditions through the expression of a variety of genes (Tian *et al.*, 2017; Chang *et al.*, 2018; Qi and Zhang, 2020). The induced genes play an imperative role in plant response and tolerance mechanism to abiotic stresses. They protect the cell through the production of metabolic proteins and regulate genes for signal transduction (Kazuko and Shinozaki, 2006; Onaga and Wydra, 2016). The regulation of genes functions in three ways or

levels depending on the specific molecular element, molecular network, and cascades: (i) Transcriptional, (ii) Post-transcriptional, and (iii) Post-translational (Haak *et al.,* 2017). Transcriptional process is facilitated by three factors: (i) Chromatins, its modifications, and remodeling, (ii) Cis – regulating element that includes binding sites like enhancers, promoters located up and downstream of the coding region, and (iii) Trans-regulatory elements which are transcriptional factors (Luo *et al.*2012). Post-transcriptional regulation is the level of gene expression that has four groups of gene expression, that are; (i) Pre-messenger (mRNA) processing (capping, splicing, and polyadenylation), (ii) mRNA nucleocytoplasmic trafficking, and (iii) mRNA turn – over and stability and mRNA translation (Floris *et al.,* 2009; Trindade *et al.,* 2011). Three processes are involved in the modification of plant response to abiotic stress at post-translational level (i) Phosphorylation (ii) simulation, and (iii) Ubiquitination of proteins like ABA (Zhu, 2002; Yoshida *et al.,* 2006; Hashiguchi and Komatsu, 2016).

2.1.1 Plant nutrients imbalance and metal toxicity stress

On top of oxygen, carbon, and hydrogen (structural elements), plants require at least 14 other essential nutrient elements to successfully complete their life cycle (White *et al.,* 2012; El-Ramady *et al.,* 2018). Among the nutrients factors nitrogen, phosphorus, and potassium (NPK) are the primary macro-nutrient elements while calcium (Ca), magnesium (Mg), and Sulphur (S), are secondary macro-nutrient elements. Micro-nutrients are chlorine (Cl), boron (B), iron (Fe), manganese (Mn), copper (Cu), zinc (Zn), nickel (Ni), and molybdenum (Mo), (White and Brown, 2010; White *et al.,* 2012). Plants also require minute concentrations of the trace-nutrient elements including silicon (Si), aluminum (Al), sodium (Na), cobalt (Co), and nickel (Ni). Other elements like vanadium (V), lanthanum (La), cerium (Ce) and selenium (Se) are also essential in some plants (Tisdale *et al.,* 1993). Plants also accumulate heavy metals, but their function is not known and their cause toxicity. When they are available in limited or excess amount, plant processes are interrupted causing them to respond by displaying a range of symptoms that vary from morphological impairments, chlorosis, necrosis, and confused growth stages to premature death (White and Brown, 2010). Plants obtain nutrients from the soil solution through the roots or from the air through leaves in different forms. The process is continuous until it reaches a critical concentration that is enough in diagnostic tissues for the crop to attain 90% of its maximum yield (Table 2.1). However, in some cases, these levels can become low (deficiency) or excess (toxicity) to cause a yield reduction of more than 10% in crops (White *et al.,* 2012).

Table 2.1 Plant essential elements and their critical concentration in leaves of nontolerant plant

		Critical leaf concentrations (mg g^{-1} DM)	
Mineral element	**Form acquired**	**Enough concentrations**	**Toxic concentrations**
Nitrogen (N)	$NH4^{+}$, $NO3^{-}$	15 – 40	
Potassium (K)	K^{+}	5 – 40	>50
Phosphorus (P)	$H2PO4^{-}$	2 – 5	>10
Calcium (Ca)	Ca^{2+}	0.5 – 10	>100
Magnesium (Mg)	Mg^{2+}	1.5 – 3.5	>15
Sulphur (S)	$SO4^{2-}$	1.0 – 5.0	
Chlorine (Cl)	Cl^{-}	0.1 – 6.0	4.0 –7.0
Boron (B)	$B(OH)^3$	5 –100 × 10^{-3}	0.1 –1.0
Iron (Fe)	Fe^{2+} Fe^{3+}-chelates	50 – 150 × 10^{-3}	>0.5
Manganese (Mn)	Mn^{2+} Mn-chelates	10 – 20 × 10^{-3}	0.2 – 5.3
Copper (Cu)	Cu^{+}, Cu^{2+}, Cu-chelates	1 – 5 × 10^{-3}	15 – 30 × 10
Zinc (Zn)	Zn^{2+}, Zn-chelates	15 – 30 × 10^{-3}	100 – 300 × 10^{-3}
Nickel (Ni)	Ni^{2+}, Ni-chelates	0.1 × 10^{-3}	20 – 30 × 10^{-3}
Molybdenum (Mo)	$MoO4^{2-}$	0.1 – 1.0 × 10^{-3}	>1
Sodium (Na)	Na^{+}	—	2 – 5
Aluminum (Al)	Al^{3+}	—	40 – 200 × 10^{-3}
Cobalt (Co)	Co^{2+}	—	10 – 20 × 10^{-3}
Lead (Pb)	Pb^{2+}	—	10 – 20 × 10^{-3}
Cadmium (Cd)	Cd2+, Cd-chelates	—	5 – 10 × 10^{-3}
Mercury (Hg)	Hg^{2+}	—	2 – 5 × 10^{-3}
Arsenic (As)	$H2AsO^{-4}$, $H3AsO^3$	—	1 – 20 × 10^{-3}
Chromium (Cr)	Cr^{3+}, $CrO4^{2-}$, $Cr2O7^{2-}$	—	1 – 2 × 10^{-3}

Compiled from (White *et al.,* 2012; Mulder, 1950)

2.1.1.1 Plant nutrients deficiency

When nutrients are limited in the soil plants allocate more proportion of biomass to the affected plant part like roots and shoots, adjusting of the shoot to root ratio and modification of organs (Herman *et al.,* 2006; Kramer-Walter and Laughlin, 2017; Marschner, 1995; Kramer-Walter and Laughlin, 2017). Further, several metabolic processes are altered through the expression of several genes to facilitate the regulation and adjustments of metabolic processes (Herman *et al.,* 2006; Kumar *et al.,* 2020). For the purposes of this review we will use nitrogen, phosphorous, potassium, and magnesium as reference nutrients to sugar partitioning in different plant organs.

Nitrogen deficiency leads to increased sugars in the leaves causing a reduction in photosynthesis due to a feedback mechanism on the photosynthetic process (Tranbarger *et al.,* 2003; Koch, 2004). However, the effect of N deficiency in plants is related more to the ratio of carbon to nitrogen (C: N) in plant tissues and not to solely carbohydrates (Herman *et al.,* 2006; Luo *et al.,* 2019). On the other hand, phosphorous deficiency can increase sugar and starch concentration in leaves but not always in roots for some species (De Groot *et al.,* 2003; Ciereszko *et al.,* 2001; Sanchez – Calderon *et al.,* 2005; Cong *et al.,* 2019). Sugar can influence gene expression in P and N deficient plants and impact on development (Gibson, 2005). Increased supply of sugar affects plant morphology because it promotes cell differentiation, maturation, division and expansion (Herman *et al.,* 2006). Plants initiate two responses to N deficiency at a whole plant level depending on: (i) Ion concentration, and it involves local signaling, and (ii) The entire plant mineral status affecting long-distance signaling (Herman *et al.,* 2006). The responses signaling in plants with N deficiency accelerated root growth and augmented lateral root branching but when the roots contact nitrates, the lateral roots are further stimulated (Tranbarger *et al.,* 2003; Ning *et al.,* 2018; Herman *et al.,* 2006). Long distance signaling is essential for the shoot responses to low N. It lowers the cytokinin and increases auxins that are important for root growth (Tranbarger *et al.,* 2003). Reduced P leads to the development of highly branched roots near the surface of the soil because P inhibits primary roots elongation but activate lateral root elongation and proliferation of root hairs. The morphological changes due to P deficiency is related to hormones concentration in tissues. Auxin regulates branching but other aspects like primary root elongation and root hair are not enhanced (Huang *et al.,* 2019).

Potassium (K) deficiency causes accumulation of sugar in leaves but not starch. This reduces photosynthesis but does not accelerate root growth (Tighe-Neira *et al.,* 2018). Plants with K deficiency have reduced sugar concentration because sugar export to roots is low and attributed to the loading of K^+ into the phloem (Herman *et al.,* 2006; Pan *et al.,* 2017; Koch *et al.,* 2019). Signaling of K deficiency includes reactive oxygen species, ethylene, and jasmonic acid pathways (Shin and Schachtman, 2004). ABA signaling is also activated but this after a long time of K scarcity (Herman *et al.,* 2006; Hu *et al.,* 2017).

Magnesium (Mg) deficiency increases the sugar in the leaves due to down-regulation of genes involved in photosynthesis (Herman and Verbraggen, 2005; Kobayashi *et al.,* 2018). It inhibits both sugar metabolism and exports from the leaves and hence carbon allocation to young leaves (Herman and Verbraggen, 2005; Herman *et al.,* 2006). It has been hypothesized that reduced

sugar export due to Mg can be either be due to (i) Reduced metabolic activity at the sink organs or (ii) Impaired phloem loading as this process requires Mg (Herman *et al.,* 2006).

2.1.1.2 Plant nutrients and metal toxicity

Metals like zinc, iron and copper are essential for plant growth and development but at high concentration they cause toxicity. Others like arsenic, mercury, lead, totaling to 53 are non-essential but are potentially toxic (Krämer and Clemens, 2005; Asati *et al.,* 2016). Based on solubility under physiological conditions only 17 of the 53 metals are of biological significance (Weast, 1984; Lambers and Oliveira, 2019). At phytotoxic level, the metals inhibit bioprocesses (photosynthesis and transpiration) and induce secondary stresses (nutrient stress and oxidative stress) that affect plant growth and development (Zhao and Chengcai, 2011; Lambers and Oliveira, 2019). Equally the plants respond to the phytotoxic effect by developing a network of efficient homeostatic mechanisms that control the intake, buildup, movement, and detoxification channels of the metals (Clemens, 2001). The components of this system fall into three classes (Clemens, 2001; Zhao and Chengcai, 2011). The classes are: (i) Metal transporters in charge of nutrient uptake and vacuole transports, (ii) Chelator used for detoxification of metals through buffering cytosolic metal concentration, and (iii) Chaperons that assist in delivering and trafficking metal ions (Table 2.2). For plants to respond to metal stress signal, a coordination of complex physiological and biochemical processes, gene expression, protein modification and metabolic composition changes happens in a synchronized way that leads to tolerance (Urano *et al.,* 2010; Thapa *et al.,* 2012).

2.1.1.2.1 Boron

Boron (B) is a micro-nutrient plant element that plays an essential role in several metabolic processes. Its toxicity and deficiency in the soil are vital in several crops' growth and developmental (Pandey and Verma, 2017). Boron toxicity results in poor growth and change in plant metabolic processes that leads to leaf necrosis, deformed stems, buds and fruits. Below the ground, boron has been reported to affect the growth of roots in crops including, wheat, maize and barley (Landi *et al.,* 2019). Studies in wheat and barley suggest that B toxicity tolerance mechanism is a factor of an active efflux of B from the roots where gene encoding the efflux transporter has been identified. However, the utilization of the gene is futile because the soil always contains other stress like salinity (Reid, 2013). The toxicity effect is linked to the pressure of B to three metabolic processes: (i) Interference with cell division and development, (ii) Impairment of principal metabolism, and (iii) Interrupt protein synthesis (Landi *et al.,* 2019). Boron toxicity tolerance is the ability of a plant to: (i) Limit the absorption

of B from the soil and efflux it in high concentrations (Sutton *et al.,* 2007; Papadakis, 2016), (ii) Enhance the synthesis of B-chelating organic compounds (Papadakis *et al.,* 2018; Landi *et al.,* 2015; Lewis, 2019), (iii) Counter B-activated oxidative stress by enhancing antioxidant tackle (Landi *et al.,* 2012), and (iv) Boron compartmentalisation in organelles and spots (Wakuta *et al.,* 2016; Papadakis *et al.,* 2018) to reduce its impact on cellular processes.

2.1.1.2.2 Zinc

When Zinc (Zn) accumulated in developing grains it helps in reducing human health problems like; retarded growth, skeletal abnormalities, wound healing, abortion, and diarrhea when they are consumed (Shahzad et al., 2014). At the same time, Zn is an essential micronutrient in the plant at low concentration of 0.2mg g^{-1} above which it becomes toxic. Its toxicity to plants depends on plant species, age, environment, and its interaction with other metals. Though its toxicity is less common than deficiency, it is more often in mining, smelting, urban, and pre-urban soils with low PH (Zaman *et al.,* 2018). Zinc affects the ability of a plant to absorb and translocate water, reduce the effect of a short period of heat and salt stresses, and is involved in the synthesis of auxins, protein, and energy (Tsonko and Fernando, 2012). Plants respond to the toxicity of zinc in five ways that assist in the detoxification process and thus helping them tolerate the stress. These are: (i) Reduced intake of minerals, (ii) Repair of stress-damaged protein, (iii) Compartment of metals in vacuole (iv) Chelation of minerals in the cytosol, and (v) Reduction of metal uptake through extracellular exudate for binding to the cell wall and mycorrhiza (Hall, 2002; Torasa *et al.,* 2019).

2.1.1.2.3 Copper

Though an essential redox-active metal for many plant processes, copper (Cu) is potentially toxic. At very high concentration, it inhibits several cellular activities like photosynthesis and respiration making the plant chlorotic and have less mass accumulation (Yruela, 2005; Torasa *et al.,* 2019). Plants tolerate copper toxicity through several mechanisms: (i) Exclusion strategy where they avoid excessive Cu^{2+} uptake and minimize its movement to leaves. (ii) Accumulation strategy where in case the metal is transported to shoots, by compartmentation in vacuoles or complexation by organic ligands and then it is antioxidized (Qian *et al.,* 2005; Adrees *et al.,* 2015; Hossain *et al.,* 2019).

Table 2.2 Summary of metals toxic effect on plants and tolerance mechanisms

No.	Metal	Toxic effects on plants	Tolerance mechanisms
1	Arsenic	Hinders photosynthesis, impedes growth, inhibit Biomass accumulation, reduces crop yield, and eventually causing death (Sunitha *et al.,* 2013; Murugaiyan *et al.,* 2019).	Suppression of high affinity (AS) uptake system reducing its influx to a low level that the plant can detoxify. A single gene encoding achieves this for suppressed transporter (Yadav, 2010a).
2	Cadmium	It causes leaf chlorosis, hinders growth, lowers the rate of photosynthesis, and impedes water and nutrient uptake. Root tips start to show browning symptoms and death (Sunitha *et al.,* 2013).	Cell wall binding to avoid absorption and limit accumulation, chelation with Phyto -chelating that bind with the metal when it reaches toxic levels, the compartment of cadmium in vacuole and enrichment in leaf trichomes (Zhang and Shu, 2006; Sebastian and Prasad, 2014).
3	Chromium	Inhibits crop growth, causes chlorosis of young leaves, nutrient imbalance, wilting of leaf tips and root injury. It causes alteration of germination process, root, stem and leaf growth, causing reduced dry matter accumulation and yield. Affects plant activities like water relation and enzyme activities (Yadav, 2010a; Sunitha *et al.,* 2013; Sharma *et al.,* 2020).	Using mycorrhizal to protect the plant by accumulating chromium. Secretion of organic acids (glutathione and free amino acids) to antioxidize and chelating the metal (Shanker *et al.,* 2005).
4	Cobalt	Hurts Biomass accumulation affect translocation of P, S, Mn, Zn, and Cu from roots to shoot. It reduces the concentration of Fe, chlorophyll, protein, catalytic activities in the leaves and transpiration rate (Yadav, 2010a; Sunitha *et al.,* 2013).	Cell wall plays a crucial role in protecting the plant (Yadav, 2010a).
5	Mercury	Cause leaf stomata to close obstructing water flow, interferes with mitochondrial activities that induce oxidative stress which generates ROS that disturbs bio-membrane, lipids and cellular Metabolism in crops. It interrupts several bioprocesses (photosynthesis, respiration, transpiration, cell division, etc.) (Yadav, 2010a; Sunitha *et al.,* 2013; Shahid *et al.,* 2019).	Chelation seems to be the primary mechanism used by plants to tolerate mercury. However, cell repair, compartmentation, and biotransformation as also been reported (Shilpa *et al.,* 2015).
6	Nickel	It causes physiological alteration, chlorosis, necrosis, impaired plant growth, a disorder of cell membrane functions, water, and nutrient imbalance (Yadav, 2010a; Sunitha *et al.,* 2013; Hassan *et al.,* 2019).	Plants use physical barriers like cuticle, production of antioxidant and enzymes. Secretion of amino acids derivatives (as osmoprotectant) and organic acids in the root zone. Chelation and compartmentation Ni by phytochelatins (Sachan and Lal, 2017; Hassan *et al.,* 2019).

7	Silver	Damages the cell membrane, interrupts ATP production, DNA replication, and affects gene expression. It gives negative impact to root growth, reduces fresh biomass and root elongation of germinating seedlings. Interferes with a reproductive and photosynthetic system of plants, induces morphological modification on plant (Tripath *et al.,* 2017).	Production of plant antioxidant protein for neutralizing ROS (Tripath et al., 2017).
8	Selenium	Induces oxidative stress, destroy protein structure and function (Gupta and Gupta, 2017). Injure roots show stunting growth, chlorotic weathering drying leaves and death (Terry *et al.,* 2000).	Reduction of internal buildup by accumulating selenium in non-protein seleno-amino acids for detoxification, exclusion, and compartmentation into the vacuole (Terry *et al.,* 2000; Shahid *et al.,* 2018).
9	Lead	It affects plant morphology, growth, photosynthesis, inhibits enzymic activities, causes water imbalance disturbs permeability of the membrane, causes Oxidative stress and mineral imbalance (Sunitha *et al.,* 2013; Kumar and Prasad, 2018; Rizwan *et al.,* 2018; Mitra *et al.,* 2020).	Exclusion strategy to maintain low concentration levels until a critical point. Accumulation strategy to keep metal concentration within the plant tissue with high level specialized physiological processes. Avoidance, detoxification and biochemical tolerance (Sharma and Dubey, 2005; Kumar and Prasad, 2018).

2.1.2 Soil acidity

Acidic soil is toxic in Al, Mn, and Fe and it facilitates H^+ influx into the root tissue resulting in reduced plant growth (Iqbal, 2012; Bhuyan *et al.,* 2019). On the other hand, there is the formation of Al^- phosphate complex that result to low availability of P in the soil (Sanchez *et al.,* 1997; Kisinyo *et al.,* 2013). Plants tolerate this condition by several mechanisms. (i) Aluminium toxicity tolerance (exudation of the organic acid anion from the root, increasing rhizosphere PH, tissue tolerance to Al, and internal detoxification of Al). (ii) Manganese toxicity tolerance mechanism. (iii) Phosphorous deficiency tolerance (increased roots proliferation and relocation of sugars to roots) among others (Kochian *et al.,* 2004; Bhuyan *et al.,* 2019).

2.1.2.1 Aluminum

The most abundant element on earth is Aluminum (Al) but, it is absent as a nutrient or trace element in the biochemical pathway, and it is a plentiful factor in soil with low PH. (Zhou *et al.,* 2016). Miss-use of ammonium fertiliser and acidic rain lower the soil PH to go below 5.5 and causes Aluminum concentration to increase to toxic levels for plants (von Uexkull and Mutert, 1995). The toxic effect is manifested through lesion of the root tissue, nutrient deficiency due to interrupted intake and ceased root growth that eventually causes the whole plant to die (Ryan *et al.,* 1994; Kopittke *et al.,* 2015; Awasthi *et al.,* 2017). Crops respond to this ion in two ways (i) External tolerance where they increase the soil pH to prevent the solubility of toxic Al^{3+} (Taylor, 1991), or the chelation of the ion by organic acids in the soil produced by the plant (Watanabe *et al.,* 2008). (ii) The chelation of cation in the plant cell after intake (Kochian *et al.,* 2004; Sasaki *et al.,* 2004; Tovkach *et al.,* 2013; Zhou *et al.,* 2016). Aluminum affects seed germination by interfering with cell division in the root tips and increasing cell wall rigidity (Bennet *et al.,* 1991).

2.1.2.2 Manganese

Being an essential micronutrient Manganese (Mn) plays a pivot role in most metabolic, growth and development processes in plants (Yulong *et al.,* 2015). However, in low PH, organic matter and potential redox soils it can accumulate to toxic levels in crops (Hue and Mai, 2002), causing symptoms in aerial organs due to its mobility (Hue *et al.,* 1998). The most visible toxic symptoms in plants include dark vain, chlorosis, brown spots and crinkled older leaves and black specks on the stems (Yulong *et al.,* 2015). Plants have avoidance mechanisms to survive Mn toxicity, the most common one being sequestering it in the apoplast using organic acids.

Crops can also oxidize Mn in the cell wall of the roots, accumulate it in the cell to efflux it out and secrete it around trichomes (Millaleo *et al.,* 2010; Li *et al.,* 2019).

2.1.2.3 Iron

In well-aerated soils, iron (Fe) is present as ferric hydroxide (semi-soluble) hence available in low concentration to plants. However, in anaerobic fields, it is reduced to soluble forms prompting excess intake by the plants to catalyze the formation of hydroxyl radical (OH) and reactive oxygen species (ROS). The concentration of the two compounds causes damage to protein, membrane lipids, nucleic acids, and oxidation of chlorophyll that reduces photosynthesis (Wu *et al.,* 2014). According to Becker and Asch, (2005), we have three types of iron toxicity. (i) High concentration of Fe^{2+} in acidic sulfate soil and plants show symptoms throughout the season. (ii) In acidic clay soils where plants show signs of slow leaf browning late in the season and, (iii) Inland valley toxicity due to the inflow of water containing iron, and plants show symptoms only at early development stages, but later it is flushed out naturally. Crops respond to iron toxicity using three mechanisms. (i) Exclusion of iron at root level by oxidizing and precipitating it (Green and Etherington, 1977; Siqueira-Silva *et al.,* 2019). (ii) The inclusion but subsequent avoidance, i.e., Fe^{2+} is internally distributed but stored in the less reactive state. (iii) The inclusion and tolerance, the plant intake Fe^{2+} and forms antioxidants that can scavenge ROS formed (Gallie 2013; Wu *et al.,* 2014)

2.1.3 Temperature variations stress

Crop production everywhere in the world and on marginal lands is explicitly at the mercy of random environmental fluctuations due to the global climate change (Solomon *et al.,* 2007; Zinn *et al.,* 2010; Fahad *et al.,* 2017; Zandalinas *et al.,* 2018). High or low environmental temperatures can be detrimental to plants at all developmental stages (Zinn *et al.,* 2010; Zhang *et al.,* 2019). However, plants have developed mechanisms to survive as illustrated by changes in thousands of transcriptions in leaves, seedlings, roots, and reproduction to adapt (Frank *et al.,* 2009). Temperature stress in plants occurs in three categories namely: the high, chilling, and freezing temperature where extremes in each case affect the crop by lowering germination, reducing growth, interrupting photosynthesis and finally causing death (Kai and Iba, 2014).

High temperatures have damaging effects on plants growth and development due to a broad range of influence on physiological, biochemical and gene regulation (Bita and Gerats, 2013; Zandalinas *et al.,* 2018). When the temperature rises by 3 – 4°C crops experience yield reduction in the range of 15 to 35% (Ortiz *et al.,* 2008; Zhang *et al.,* 2019). However,

susceptibility of plants to high temperature is varied with developmental stages, genotypes and species and compounded by other abiotic factors like drought and salinity (Barnabas *et al.,* 2008). The effect of high temperature is more pronounced in crops during reproduction than in other developmental stages because it affects the yiled (Zinn *et al.*, 2010; Bita and Gerats, 2013; Siebers *et al.,* 2017; Lohani *et al.,* 2020). High temperature reduces dry matter accumulation, causes alterations in cellular structure, decreases protein synthesis and the production of protective molecules (Bita and Gerats, 2013). Heat stress shortens the life cycle of a plant by reducing the respiration and photosynthesis that lowers productivity (Zinn *et al.,* 2010; Akter and Islam, 2017; Behl *et al.,* 2019). Plants have developed mechanisms to survive under extremely high temperatures in different ways. These are: (i) Long-term phenological and morphological changes (leaf orientation and transpiration), (ii) Short-term stress avoidance and acclimatization mechanisms and, (iii) General mechanism (stress protein, osmoprotectant, and transcriptional control) (Hassan *et al.,* 2020). Further, there are two general plants adaptational mechanisms to high temperature: (i) Heat tolerance physiological aspect (amendment of essential processes) (Nagarajan *et al.,* 2010; Bita and Gerats, 2013) and (ii) Molecular aspects of heat tolerance (reprogramming their transcription, proteome, and metabolome) in the plant (Qi *et al.,* 2011; Bita and Gerats, 2013).

Low environmental temperatures limits crop growth and development that results in significant yield loss (Xin and Browse, 2000; Jan *et al.,* 2009; Liu *et al.,* 2019). Low temperatures of 0 – 15 ^{0}C associated with chilling while those below 0 ^{0}C is freezing (Gu *et al.,* 2008; Lobell *et al.,* 2015). Most crops in the temperate regions are tolerant to chilling condition than the tropical that are very susceptible (Jan *et al.,* 2009). Low-temperature stress causes reduced seed germination, stunted growth, chlorosis, wilting, and necrosis and interferes with reproduction. The significant damage is on the membrane due to an acute dehydration associated with freezing (Yadav, 2010b). Tolerance to the stress is exhibited in plants through the accumulation of chlorophyll that is inhibited by cold, reducing the sensitivity of photosynthesis and chloroplast that is the primary site for injury, improved seed germination, pollen fertility and seed set mainly the function of survival (Sanghera *et al.,* 2011; Hussain *et al.,* 2019).

2.1.4 Water stress

Plants experience water stress when there is limited (Drought) or excess (Flooding) supply of water in the root zone (Misra *et al.,* 2020). Drought stress in plants is when there is a limited supply of water to the roots caused by increased transpiration or high salinity (Seyed *et al.,*

2012; Kumar *et al.,* 2019). Drought is an abiotic stress that has the highest impact on crops as a single factor reducing its growth and productivity in a range of 3% and occasionally causing crop failures in extreme cases (Wang *et al.,* 2020). Drought has three categories of classes commonly called creeping phenomenon. The phenomenon starts with the meteorological dryness due to low season precipitation in the season. When meteorological dryness prolongs, it graduates into a hydrological drought which is the drying of rivers and decline of underground water. Agricultural drought is the most important in crop production since it occurs during the growing season and can cause wilting. It is independent of meteorological dryness and may persist even if there is no hydrological drought because of other factors that prevent the plant from absorbing the water (Aggarwal *et al.,* 2015). A plant experiencing drought will have reduced water content, low leaf water potential, loss of turgor, closure of stomata, reduced cell elongation, reduced growth, arrested physiological processes, interfered metabolism, and eventually death (Jaleel *et al.,* 2008; Misra *et al.,* 2020).

Plants response to drought depend on factors such as genotype, growth stage, other environmental stresses, duration and severity of the drought, physiological processes, gene expression, respiration and photosynthesis (Faruq *et al.,* 2013). It can induce several biochemical, physiological and molecular stresses to cause a response in plants in a way to tolerate the effect (Kalefetoğlu and Ekmekçi, 2005). Plants achieve tolerance to water stress in two ways: (i) Drought avoidance where plants have features and mechanisms to assist them in avoiding the drought (deep roots, economically use water, closing stomata, regulation of transpiration, and change in plants lifestyle) or (ii) Dehydration tolerance when there is limited water (Faruq *et al.,* 2013; Dodd and Ryan, 2016; Hadebe *et al.,* 2017). Heat stress can occur in combination with drought because of water loss from plant and soil surface under high temperature, both of which reduce nutrient uptake and photosynthesis capacity of plants (Li *et al.,* 2020; Ostmeyer *et al.,* 2020; Poudel *et al.,* 2020).

Flooding on the other hand decreases soil oxygen that limits respiration and reduction in oxidation potential (Armstrong, 1979; Wegner, 2010; Sasidharan *et al.,* 2018). Waterlogging occurs when only the roots of a plant are under anaerobic condition while flooding is when there is water above the ground either partially or complete submerging the crop (Stiker, 2012). Flooding reverses stomatal opening and closing (Mollard *et al.,* 2010) and reduces root water uptake (Jackson and Drew, 1984; Schulze *et al.,* 2019). This is the reason for wilting symptoms in flood sensitive plants (Else *et al.,* 1996) and finally death (Bradford and Hsiao, 1982; Sasidharan *et al.,* 2018). Flooding can reduce photosynthesis because of: (i) low leaf

chlorophyll, (ii) reduced activities of carboxylation enzyme, and (iii) oxidation damage on photosystem II by reactive oxygen species (Stiker, 2012; Yordanove *et al.*, 2004; Schulze *et al.*, 2019). Plants respond to flooding by producing aerenchyma in the tissues (Justin and Armstrong, 1987; Sharma, 2018), oxygen transport the shoot to roots, development of adventitious roots, and increment in plant height (Grimoldi *et al.*, 1999). One of the crops studied extensively on this aspect of flooding is rice, it can germinate under low oxygen levels induced by flooding because it can elongate coleoptile at the expense of radicle during this time as a tolerance mechanism (Magneschi and Perata, 2009; Zhou *et al.*, 2020).

2.1.5 Radiation stress

Plants primarily get its energy from the sun where ultra-violet (UV) is part of the radiation emitted in the solar spectrum. Inside the UV wavelength of the sun's electromagnetic spectrum, there are three sub-wavelengths that are important for the plant: (i) UV-A of 315-400 nm wavelength, (ii) UV-B of 280-315 nm wavelength, and (iii) UV-C of wavelength 200-280 nm. Radiation UV-A is entirely absorbed by the atmospheric gas. UV-B is partially absorbed by the ozone layer, while UV-C is not absorbed at all (Frohnmeyer and Staiger, 2003). Despite a small amount of penetration into the atmosphere and landing on the plant, UV-B has a high energy level and thus is very destructive (Simontacchi *et al.*, 2015; Kataria *et al.*, 2019). It interferes with plant morphological, physiological, biochemical, and molecular activities (Kataria *et al.*, 2014). The chloroplast is most sensitive to UV-B radiation because it triggers damage on photosystem II, prevents electron transport and causes oxidative damage to the reactive center (Aro *et al.*, 1993).

The protective mechanism employed by plants against the damage of UV-B radiation include: (i) Repair, negate and reduce the UV-B radiation damage, (ii) Reduce the amount of UV-B radiation reaching the plant (Stapleton 1992). Plants ensure repair by three central mechanisms: (i) Photoreactivation using a light-induced enzyme activity which cleavers pyrimidines dimers produced by UV-B, hence restoring correct base pairing. (ii) Excision and repair of UV products from DNA molecules using the undamaged template as a guide. (iii) Post-replication repair if DNA lesion is bypassed during replicating utilizing the information from the sister duplex to fill in the gap (Kataria *et al.*, 2019). Plants also screen sensitive tissues against the radiation using secondary structures and metabolites like flavonoid and cuticular wax (Stapleton 1992; Kataria *et al.*, 2019).

2.2 Osmotic, Ionic, Oxidative and Nutritional deficiency stresses under salinity

Extreme saline conditions in the soil severely affect plants physiochemical processes including growth and productivity by creating osmotic and ionic pressures that encourage oxidative stress and nutrients imbalances (Muchate *et al.,* 2019; Zhan *et al.,* 2019). High Na^+ in plant cells causes intracellular ionic imbalance hence inhibiting K^+ uptake, that is involved in mediating physiological and biochemical activities (Mohsin *et al.,* 2019; Yuan *et al.,* 2019). This increases the synthesis of reactive oxygen species (ROS) (Hasanuzzaman et al., 2018), and production of other metabolites like methylglyoxal (MG) that are highly toxic (Parvin *et al.,* 2020).

2.2.1 Osmotic stress

Low temperature, drought, and salinity increase the ground osmotic pressure which interferes with water absorption (Bartels and Nelson, 1994; Kosová *et al.,* 2014). According to Bartels and Nelson, (1994); Farhat and Debez, (2019), all the osmolytes cause a similar reaction because of dehydration, and a focus on one or two maybe a reflection of others. The salinity element of osmotic stress is denoted by high quantities of soluble ions in the root zones that decrease the water potential and limit its uptake by the plant. This osmotic stress factor is strong enough to reduce growth in plants like when non-ionic solutes is applied. (Köster *et al.,* 2019). Water is an essential environmental factor limiting all plant developmental stages (Singh *et al.,* 2019), including seed germination phase that is very susceptible. Some genotypes have shown some level of tolerance to osmotic stresses and have developed several adaptive strategies (Bohnert *et al.,* 1995; Farhat and Debez, 2019).

2.2.1.1 Methods used to screen osmotic stresses in plants

For a screening method to be evaluated, it is vital to creating an osmotic pressure in the growing environment of the crops. But, maintaining such conditions in the soil may not be feasible. Therefore, this type of conditions are simulated using several substances (Gharoobi *et al.,* 2012), or factors that can create stress to plants and they include; Sorbitol, polyethylene glycol, D – mannitol, Carbowax, Hydrogen peroxide, Ethanol, HCl, and Acetic acid among others.

2.2.1.1.1 Screening methods based on growth and yield

This are glasshouse-based screening methods that are recommended for moderate osmotic stress levels. They involve measurement of growth parameters like growth rate and yield (Greenway 1962), leaf and stem elongation (Cramer and Quarrie, 2002). These methods have little application in the field for large-scale screening, but long-term experiments can be used to generate data that is reproducible among genotype for genetic variation. Exposing plants to

extended salinity stress of at least two weeks to several months (Munns *et al.,* 1995), has necessitated detecting genetic differences among varieties. In rice, a relatively sensitive crop to salinity and fast-growing has been able to give reproducible results of tolerance variation among its genotypes (Zhu *et al.,* 2001). The setback of the long-term experiment is that they are labor and resource intensive, hard to replicate due to the length of time to maintain the conditions if the plants have different morphology or maturity time (Munns and James, 2003). When you want to get grain effect, it may be difficult to maintain many plants in the greenhouse (Munns *et al.,* 2006).

Germination is easy to measure (VonWell and Fossey, 1998), but it is unlikely to give the correct inference of the predictable performance of the plant at an advanced stage (Munns and James, 2003). In the field, seed germination may not get in touch with high salt concentration because it is likely to be leached from the topsoil by irrigation or planting after rains. On the filter paper, high salinity concentration may produce a plant that is too weak to break the soil crust or provide a viable crop (Shannon, 1978). Therefore, emergence rate and seed vigour can be more practical screening factors for soil that form a hard crust (Munns *et al.,* 2006). Survival at high concentrations may not reflect a healthy growth at the same level and better harvest. Field experiments must validate the yield because glass house may not provide the required space. But a setback to it is that stomatal conductance measurement by leaf temperature assessment (thermal imaging) or viscous flow porometry can only be used when ambient conditions are not varying which is the case for glasshouse environment (Munns *et al.,* 2006).

2.2.1.1.2 **Screening methods based on damage and tolerance**

They can be used in a very high concentration of salinity of over 200mM NaCl but may not show the genotype ability to tolerate lower levels (50-100mM NaCl). Again, there is hardly replication in genotype variation in germination or survival in the field (Munns and James, 2003). The method can handle many genotype and phenotypes including germination, plant survival, leaf injury by membrane damage and reduction of CO_2 assimilation (James *et al*., 2002). Chlorophyll loss and damage to photosynthetic parameters can also be recorded (Krishnaraj *et al*., 1993), among others. However, it may be difficult to tell the actual cause of damage. For example, the injury caused by NaCl can be due to water stress, Na^+ or Cl^- toxicity, and K^+ or Ca^{2+} deficiency (Greenway and Munns, 1980). Death of the leaf can be due to normal senescence or accelerated by osmotic stress caused by salinity (Munns and James, 2003).

2.2.1.1.3 Screening methods based on biochemical and physiological responses

Osmotic stress excites various physio-chemical responses that affects most plant processes and accumulation of compatible solutes whose measurement can be an indicator of tolerance (Hazman *et al.*, 2015; Sanoubar *et al.*, 2019), as explained in chapter 6 of this thesis.

2.2.2 Ionic stress

Because of the disproportionate entry of sodium (Na^+) and chloride (Cl^-) ions into the plant, they compete with their complementary cationic/anionic to enter the cell hence cumulating to cause ionic imbalances and/or toxicity (Khare *et al.*, 2020). Afterward, cellular ion-homeostasis is disturbed and plasmatic cellular partitions (cytosol, matrix and stroma) are filled with extreme amounts of (Na^+, Ca^{2+} or Mg^{2+}) cations and/or (Cl^-, SO_4^{2-} or PO_4^{3-}) anions; instead of their normal compartmentalisation in respective vacuoles. This inhibits the metabolic unevenness, energy production and redox homeostasis that can cause injury and death to the cell, cell components, organs and the whole plant (Zörb *et al.*, 2019).

2.2.3 Oxidative stress

Almost all abiotic stresses, including salinity cause oxidative stress in plants resulting to an upsurge in the levels of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), peroxy and alkoxy radicals ($RO^{\bullet}$, $RCO^{\bullet}$), hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot -}$) and hydroxyl radicals (OH.) that triggers programmed cell death (PCD) when the accumulate (Yin *et al.*, 2016; Kerchev *et al.*, 2019; Rohman *et al.*, 2019). Moreover, reactive nitrogen-oxygen species (RNS) including peroxynitrite (ONOO−), nitric oxide (NO•), etc. can be leaked out of the antioxidant protection system to induce metabolic dysfunction (Bhattacharjee, 2019).

2.2.4 Nutritional deficiency

Abundant of Na^+, Cl^-, or sulphate (SO_4^{2-}) in the soil due to salinity reduce the uptake and translocation or apportioning within the plant of crucial nutrients like phosphorus (P), potassium (K^+), nitrogen (N), and calcium (C^{2+}). At the same time, it decreases assimilation of nutrients, particularly K and Ca, subsequently causing ionic imbalances of K, Ca, and Mg (Parihar *et al.*, 2015; Kapoor *et al.*, 2019). Salinisation can lead to loss of soil fertility and crop productivity. To be precise, it disturbs soil microbial activities, biodiversity, and biological cycles including; organic residue decomposition, nitrification, denitrification and soil respiration among others. On top of that, salinity modify the physicochemical properties of soil, reducing its organic matter and sodification leading to clay particle dispersion and aggregate stability lose. This causes poor structured soil with destabilized hydraulic conductivity, low

water storage capacity, and poorly drained, this can lead to surface runoff and erosion (Calone *et al.,* 2020).

2.3 Salinity interaction with other environmental factors and their effects on plant

Growing in their natural habitats, plants are frequently confronted instantaneously by various stress factors including, abiotic and biotic (Kissoudis *et al.,* 2014). The highly synchronised, dynamic nature of plants growth and development requires them to distinguish and respond to various environmental signals in a collaborative manner (Kohli *et al.,* 2013). As the collective stresses undesirably affect plant growth, alternatively they occasionally enhance their survival approach and guard them. Therefore, shared stress conditions ought to be considered as novel and be studied in detail to understand their interaction in plants. If the combined stresses cause less damage than their individual ones in isolation then they are considered to have "positive effect" otherwise they have "negative effect" (Pandey *et al.,* 2019). Multiple environmental factors always have interactive effects on plants directly and indirectly, therefore salinity being one of them, it intermingles with both abiotic and biotic stresses (Syvertsen, and Yoseph, 2005).

Salinity stress hardly happens when other abiotic environmental factors are ideal, but it is common when they are sub-optimal like in poorly drained soil, places that experience high temperatures, arid and semi-arid areas among others. All osmotic stresses cause primary cell water loss (reduce cell osmotic potential) and they include cold, drought and salinity among others. However, there may be some variations of the actual water loss at cell level between the stresses for example, salinity cause the reduction of external water potential, cold due to physiological drought and drought is due to decreased cell water content as a factor of soil or/and atmosphere water shortage (Boudsocq and Laurière, 2005). Drought and salt stress are most destructive abiotic stress to plants when they occur at the same time in the environment, because they reduce growth, development, and productivity of crops by inducing osmotic, oxidative and ion toxicity stresses (Rao *et al.,* 2019; Szekely-Varga *et al.,* 2020).

Microbes coexist with plants in the ecosystem and they curve their own niche in association with plant roots to arbitrate vital physiological processes that enable them to tolerate numerous abiotic stresses. Microorganisms can endure diverse environmental variation that gives them massive capabilities to alleviate stresses (Egamberdieva *et al.,* 2017; Rao *et al.,* 2019). Salinity tolerant bacteria grow in the root rhizospheres, as they colonise the plant roots surface while exposing themselves to salt stress, they shield the plants (Rao *et al.,* 2019). Salt stress has a direct effect on root pathogens and on the other hand, pathogens like nematodes and

mycorrhizae makes the plant susceptible to increased chloride (Cl^-) uptake. Moderate salinity stress can decrease plant physiological activities and growth in citrus seedlings allowing them to survive cold stress and boosting flowering after the stress is withdrawn (Syvertsen, and Yoseph, 2005).

2.4 Barley

2.4.1 Introduction

Being one of the earliest domesticated crops (Shewry, 1992; El-Hashash and El-Absy, 2019) belonging to the family Poaceae, cultivated barley (*Hordeum vulgare* L.) is the fourth most crucial annual cereal crop after wheat, maize, and rice (Gujral and Gaur, 2005; Bhatta *et al.*, 2020). Its domestication can be traced back to about 10,000 years ago in the Middle East from the wild progenitor *H. vulgare* spp. *Spontaneum,* in the western part of the Fertile Crescent (Bard *et al*., 2000; Riehl, 2019). Its primary uses are animal feed (60%), malting (30%), and human food (3%) (Gujral and Gaur, 2005; Bhatta *et al.*, 2020). Additionally, 7% of the grain is for planting seeds while its straw gives forage for grazing, hay, and silage (Newman and Newman, 2008). Its world production is approximately 148.78 million tons annually from about 48 million hectares (USDA, 2017). In Australia, barley production is currently at 8.2 million tons per annum from 4 million hectares. Despite producing 5% of the world barley per volume, Australia exports represent 30% of malt and 20% of feed barley trade (ABARES, 2016).

2.4.2 Taxonomy and description

Barley forms part of the genus *Hordeum* in tribe *Triticeae* of the *Poaceae* family which is the largest in monocotyledonous plants. The genus *Hordeum* comprises of 32 species and 45 taxa including annual to perennial grasses. Majority of the species in *Hordeum* are diploid. However, there exist tetraploid, hexaploid as well as autoploid plants (Von Bothmer *et al*., 1995; El-Hashash and El-Absy, 2019). The genus, *Hordeum*, has a worldwide distribution with three species constituting the barley under cultivation. *Hordeum vulgare* L. is a 6-rowed barley species with a tough rachis or spike stem where all florets are fertile, while, *Hordeum distichon* L. is a 2-rowed barley species with a tough rachis but, only the central spikelets contain a fruitful flower. *Hordeum irregulare* E. Aberg and Wiebe., is an irregular barley species with a tough rachis, with lateral flowers that can reduce in some instances to a stem piece and others fertile, sterile or sexless the central spikelets contain fruitful flowers and set seeds (Badr *et al*., 2000; Kant *et al.*, 2016).

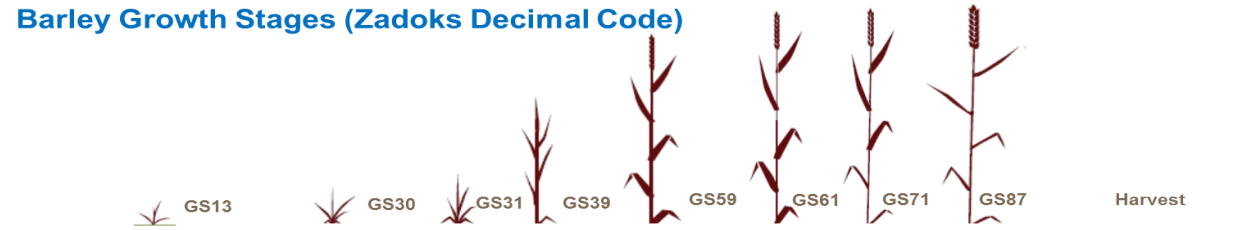
Hordeum vulgare ssp. *Vulgare* is the only species that has undergone domestication, and it is an annual self-pollinating diploid with a chromosome number of 2n=14 (Von Bothmer *et al*., 1995). It is a crop with a short season and can grow in nearly all the cultivated areas of the world (Newton *et al.*, 2011). According to Baik and Ullrich, (2008), barley has four broad groups depending on specific features. (i) Based on vernalization requirement (spring varieties or winter varieties). (ii) Based on fertile flowers and spike morphology (two rows or six rows). (iii) Based on end-user type and importance (malting or feed), and (iv) Based on presence or absence of hull tightly adhering to the grain (awned/hulled or hulless).

Barley has fibrous root system emanating in two ways, (i) Seminal roots (5 to 7) from coleorhiza tissues in the embryo, and (ii) Several adventitious roots initiated from the lower basal nodes of the crowns as tillers. The stem is a hollow cylindrical culm with a tubular node dividing to several internodes, to a height of about 60 to 120 cm. A whole barley plant is made up of several tillers arising from the axis of the basal leaves with each leaf consisting of a flattened blade and tubular sheath completely wrapping around the stem, each on the opposite side of the node alternating on opposite side of the progressive internode. The spike has a solid, flat, zigzag rachis carrying a triplet of spikelets alternating on opposite sides of each node named six-rows when all spikelets are fertile and two-rows when only the central spikelet is fertile. The grain is made of two parts (the endosperm and embryo) inside the seed coat (Briggs 1978, 1998; Horsley *et al*. 2009; Kant *et al.*, 2016).

2.4.3 Growth and development

Like other cereal crops, barley has ten growth stages based on ten principals 0 to 9. The stages form the respective Lifecyle phases that includes include 0 – Germination stage, 1 – Seeding growth, 2 – Tillering period, 3 – Stem elongation, 4 – Booting stage, 5 - Awn emergence, 6 - Flowering (anthesis), 7 - Milk development, 8 - Dough development, and 9 – Ripening. Each stage is further divides into ten subsequent steps (Figure 2.2), to extend the scale more from 00 to 99 (Zadoks *et al.*, 1974; Djanaguiraman *et al.*, 2020). In the first phase of germination (0), the seed undergoes several steps. Starting with a dry grain represented as 00, followed by 01 (start of water absorption), 03 (seed fully swollen), 05 (the first root emerged from seed), 07 (coleoptile appeared from seed) and finally 09 (first green leaf just at the tip of coleoptile to mark the beginning of a seedling). In the last stage of seed ripening phase (9) of barley lifecycle the seed undergoes several steps to reach inactive state. The last phase starts with step 91 (the hardening step challenging to divide) followed by 92 (not dented by thumbnail), 93 (grain

loosening in the daytime), 94 (over-ripe straw dead and collapsing), 95 (seed dormant), then 96 (viable seed giving 50% germination). The seed is not dormant at step 97, but Secondary dormancy is induced at 98, and Secondary dormancy is lost to mark the end of the lifecycle at 99 (Zadoks *et al.*, 1974).



Growth Stage	Description of stage	Growth Stage	Description of stage	Growth Stage	Description of stage	Growth Stage	Description of stage
Seedling Growth		**Stem Elongation**		**Ear Emergence**		**Dough Development**	
GS10	First leaf through coleoptile	GS30	Ear at 1cm (pseudostem erect)	GS51	First spikelet of ear just visible above flag leaf ligule	GS83	Early dough
GS11	First leaf unfolded (ligule visible)	GS31	First node detectable	GS55	Half of ear emerged above flag leaf ligule	GS85	Soft dough
GS13	3 leaves unfolded	GS32	Second node detectable	GS59	Ear completely emerged above flag leaf ligule	GS87	Hard dough (thumbnail impression held)
GS15	5 leaves unfolded	GS33	Third node detectable	**Flowering**		**Ripening**	
GS19	9 or more leaves unfolded	GS37	Flag leaf just visible	GS61	Start of flowering	GS91	Grain hard (difficult to divide)
Tillering		GS39	Flag leaf blade all visible	GS65	Flowering half-way	GS92	Grain hard (not dented by thumbnail)
GS20	Main shoot only	**Booting**		GS69	Flowering complete		
GS21	Main shoot and 1 tiller	GS41	Flag leaf sheath extending	**Milk development**			
GS23	Main shoot and 3 tillers	GS43	Flag leaf sheath just visibly swollen	GS71	Grain watery ripe		
GS25	Main shoot and 5 tillers	GS45	Flag leaf sheath swollen	GS73	Early milk		
GS29	Main shoot and 9 or more tillers	GS49	First awns visible	GS75	Medium milk		
				GS77	Late milk		

Figure 2.2 Defined growth stages of barley (Zadoks *et al.*, 1974)

2.4.4 Barley seed characteristics and germination

Barley seed is a product of double fertilization with a diploid embryo and triploid endosperm (Briggs, 1978). The seed has more cells than wheat or rice and this is the reason why it has more cell wall materials like B-glucans. Its varieties vary in morphology. They can be owned or own-less, husked or husk-less, different shapes and sizes, developing through several stages from watering ripe, milk, soft, hard dough, grain hardening to physiological maturity. The grain is a reproductive unit, and the end use product that has three necessary components; husk (7-13%), endosperm (70-80%), and embryo (2-5%) (Briggs,1978). The constituents of the components in barley grain is mainly made of 70% carbohydrates of which 97% is starch and protein (8-15%) that is important for brewing quality (Kirby and Appleyard 1984). Freshly harvested barley is usually considered dormant due to intrinsic factors (primary dormancy), but, in some instances, external factors can make the seed experience secondary dormancy

(Briggs, 1978). Germination process in barley like other monocots has three phases (Passioura, 2005).

2.4.4.1 Phase one: Water absorption (imbibition) where the seed needs to reach a moisture content of 35-45% of its dry weight for germination to start. In high humidity conditions (97%), grain can also gain this level of moisture to allow germination.

2.4.4.2 Phase two: Activation which starts by the production of hormones that stimulates enzymic activity to break down starch and protein into seed sugar and amino acids respectively to provide energy to the developing embryo. At this stage, moisture stress to the seed will cause it to dry but remains viable and dormant. However, activation phase is not complete until when the seed coat is ruptured.

2.4.4.3 Phase three: Visible germination where the embryo is visible, first the radicle, other primary roots, and coleoptile. On sensing light, coleoptile stops to grow to allow the first true leaves to push through the pore at the tip using the resource of energy within the seed. Emergence occurs when the coleoptile is visible above the soil surface. Temperature is an import factor for the extension of coleoptile (12-25^{0}C). Other factors include oxygen (20%), seed dormancy, quality, and storage conditions.

2.4.5 Barley genome organization, gene distribution, and Recombination

Barley (*Hordeum vulgare* L.), has two sub-species, (*vulgare* and *spontaneum*) which are diploid with seven pairs of chromosomes 2n=2x=14 representing the primary genome (Bothmer, 1992; Schreiber *et al.,* 2020). The chromosomes labeling is on the following features, (i) Size (chromosomes 1 – 5), and (ii) Other characteristics (presence of satellites, chromosomes 6, 7) (Shahbaz and Ashraf, 2013). The genome is relatively large estimated to contain close to 5.5 picograms of DNA (5.3x10^9 bp) per haploid nucleus (Bennett and Smith, 1976; Mascher *et al.,* 2017). The genome is the largest after hexaploidy bread wheat (*Triticum aestivum* L.), where it is 12 times that of rice and almost twice human genome (Bennett and Leitch, 1995). It is made up of a mixture of unique and repeated nucleotide sequence (Flavell, 1980), with a tandemly10-20% arranged in repeat sequences having 50-60% spread among one another or within the unique nucleotides' sequences (Rimpau *et al.,* 1980). The order gives approximately 80% of the repeat DNA on the barley genome (Flavell *et al.,* 1974). Almost 7% of barley genome comprises of interspersed *Copia*-like retrotransposon *BARE* (Manninen and Schulman, 1993), such as long terminal repeat (LTR) retrotransposons and DNA transposons

(Flavell *et al.*, 1974). The average distance between genes is 240kb and a gene density of one gene distributed equidistantly on 123-212kb (Panstruga *et al.*, 1998). Due to the variation in gene density, recombination rate differs between the chromosomes where most take place in gene-rich areas of the chromosome (Kunzel *et al.*, 2000).

2.4.5 Abiotic stress adaptation genes in barley

Barley often experience multiple stresses each year and has been coping with abiotic factors happening singularly or as a combination of stresses using several genes (Figure 2.3). The genes control many processes and synthesis of chemicals that play a vital role in protecting the plant against environmental stresses (Gürel *et al.*, 2016). They include transporter factors important for salinity tolerance in barley varieties (Mian *et al.*, 2011; Adem *et al.*, 2014). Genes responding to flowering time triggers vernalisation, photoperiod, and circadian clock to ensure natural tolerance of barley to abiotic factors (Turner *et al.*, 2005). While barley response by expression of antioxidant enzymes, osmolytes accumulation and heat- shock proteins synthesis induced ROS is regulated by detoxifying genes (Guo *et al.*, 2009). Induction of transcription factors elucidates expression of many genes controlling tolerance to abiotic stress in barley including vernalisation, frost and a group of genes that encode proteins binding to membrane phospholipids, ions, and water to protect the cells (Gürel *et al.*, 2016).

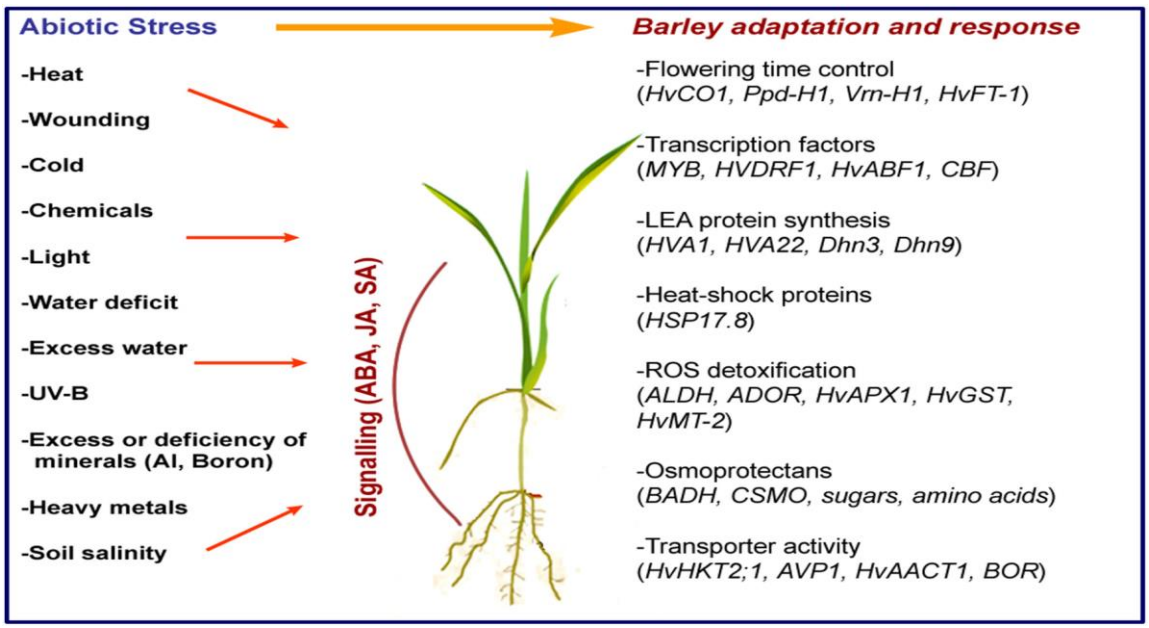


Figure 2. 3 Main genes involved in adaptation response to abiotic stress in barley (Gürel *et al.*, 2016).

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CHAPTER THREE

GENOME-WIDE ASSOCIATION STUDY OF SALINITY TOLERANCE DURING GERMINATION IN BARLEY (*Hordeum vulgare* L.)

The contents of this chapter is published as a research article in the *New Insights into Salinity Sensing, Signaling and Adaptation in Plants in the journal of* ***Frontiers plant science***. The full citation is: **Mwando, K. E.,** Han, Y., Angessa, T. T., Zhou, G., Hill, C. B., Zhang, X-Q., and Li, C. (2020). Genome-wide association study of salinity tolerance during germination in barley (*Hordeum vulgare* L.). *Frontiers plant science, New Insights into Salinity Sensing, Signaling and Adaptation in Plants, 11: 118.* https://doi: https://10.3389/fpls.2020.00118.

The contribution of each author is as follows: **EM** performed phenotyping experiments. **EM, GZ**, and **CH** conducted data analysis and interpretation. **GZ, X-QZ,** and **CH** generated the genotypic data. **TA** and **CL** conducted field experiments. **CL, YH,** and **TA** conceived the project. **EM** drafted paper with inputs from **YH, TA,** and **CH. CL** revised the paper and approved the final version for publication.

3.1 Abstract

Barley seeds need to be able to germinate and establish seedlings in saline soils in Mediterranean-type climates. Despite being a major cereal crop, barley has few reported quantitative trait loci (QTL) and candidate genes underlying salt tolerance at the germination stage. Breeding programs targeting salinity tolerance at germination require an understanding of genetic loci and alleles in the current germplasm. In this study, we investigated seed-germination-related traits under control and salt stress conditions in 350 diverse barley accessions. A genome-wide association study, using ~24,000 genetic markers, was undertaken to detect marker-trait associations (MTA) and the underlying candidate genes for salinity tolerance during germination. We detected 19 loci containing 52 significant salt-tolerance-associated markers across all chromosomes, and 4 genes belonging to 4 family functions underlying the predicted MTAs. Our results provide new genetic resources and information to improve salt tolerance at germination in future barley varieties *via* genomic and marker-assisted selection and to open up avenues for further functional characterization of the identified candidate genes.

3.2 Introduction

Soil salinity is a major global environmental factor limiting plant growth and productivity (Allakhverdiev et al., 2000; Ashraf et al., 2015). It causes two types of stress in plants, namely

osmotic pressure associated with non-ionic factors, and ionic stress induced by Na^+ and Cl^- ions (Bernstein, 1975; reviewed by Munns and Tester, 2008). High salt in the soil increases the osmotic pressure and creates a condition similar to drought (Leon, 1963; Bliss et al., 1986; Sayar et al., 2010) that impairs the ability of seeds to absorb water from the soil, hence prolonging or even inhibiting seed imbibition for subsequent germination. In addition, the absorption of excess Na^+ and Cl^- ions causes toxicity that impedes normal cellular processes (Hampson and Simpson, 1990), contributing to a decrease in seed germination rate (Dodd and Donovan, 1999; Zhihui et al., 2014). Ionic and osmotic stress interaction effects ultimately decrease the number of sprouted seeds and the germination rate (Kazemi and Eskandari, 2011).

Barley is one of the most saline-tolerant crops (Munns, 2005) and is often used as a model to understand salinity adaptation mechanisms in plants (Chen et al., 2007; Wu et al., 2011). Adaptation to salinity varies among barley genotypes and growth stages (Mano et al., 1996; Mano and Takeda, 1997; Xue et al., 2009). The germination process begins when a quiescent dry seed imbibes water and terminates on the emergence of the radicle (Gupta et al., 2019); barley is a model plant for studying the germination stage in monocots (Gorzolka et al., 2016). Depending on their ability to germinate and survive under salinity stress, barley genotypes are either tolerant or sensitive depending on their genetic diversity (Shelden et al., 2013; Shelden et al., 2016; Gupta et al., 2019). At this stage, several different loci control salinity tolerance (Mano and Takeda, 1997). Angessa et al. (2017) reported transgressive phenotypic segregation for germination rate and biomass at the seedling stage using a doubled haploid (DH) barley population derived from a CM72 Gairdner cross, with both traits controlled by different QTLs on chromosomes 2H and 3H, respectively. At the germination stage, Mano and Takeda (1997) reported 17 QTLs controlling abscisic acid (ABA) response on chromosomes 2H, 3H, 1H, and 5H in Steptoe Morex DH lines, and 9 QTL on 2H and 5H in a Harrington TR306 DH population. Loci located on chromosome 5H in both populations were closely linked to salinity tolerance. QTL mapping using a DOM REC Oregon Wolf Barley population identified several chromosomal regions on 2H, 5H, and 7H that were associated with salt stress response at the germination stage (Witzel et al., 2009). A single QTL on chromosome 5H, detected at three salt concentrations, was responsible for 42% of the phenotypic variation (Cattivelli et al., 2002).

There is little information linking the QTLs reported for salinity tolerance at the germination stage to specific genes and genetic mechanisms (Mano and Takeda, 1997; Hanen et al.,

2014; Angessa et al., 2017). Genome-wide association (GWAS) studies are increasingly used to discover and explain the genetic basis of agronomic traits that are often controlled by many genes of small magnitude, such as germination (Shi et al., 2017; Hazzouri et al., 2018; Naveed et al., 2018; Yu et al., 2018). GWAS relies on linkage disequilibrium (LD) to detect associations between a large number of genetic variants and traits across a large number of genotypes from natural populations. GWAS typically achieves higher mapping resolution due to higher recombination levels between the linked genetic loci and traits at the population level than conventional QTL mapping (Hu et al., 2011). With the current advances in genome-wide genotyping technology, hundreds of accessions encompassing thousands of gene loci can be genotyped using high-throughput markers to improve the efficiency of current breeding approaches (Russell et al., 2011; Kilian and Graner, 2012; Tondelli et al., 2013). GWAS can precisely locate polymorphisms and the underlying genetic loci that are accountable for phenotypic variations to allow gene-targeted searches (Naveed et al., 2018; Xu X. et al., 2018; Yu et al., 2018).

This study used GWAS analysis to identify salinity tolerance at the seed germination stage in 350 barley accessions from 32 countries. The germination rate of these accessions was assessed in 150 mM NaCl, and a tolerance index was calculated (the fraction of germination under salt and deionized water as a percentage), using seeds harvested from two trial locations in Western Australia. The GWAS analysis of two traits associated with salinity tolerance at germination was conducted using 24,138 diversity arrays technology (DArTseq) and single-nucleotide polymorphism (SNP) markers. This research aimed to identify quantitative trait nucleotides (QTNs) and predict genes that are highly associated with salt-tolerant traits at the germination stage to select markers for future breeding.

3.3 Materials and Methods

3.3.1 Barley Germplasm

A total of 350 barley genotypes selected from a larger set of 594 accessions in a worldwide collection were evaluated for salinity response at the germination stage to map the locations of genes associated with tolerance (Supplementary Tables 3.1 and 3.2). The genotypes originated from 32 countries in various geographic regions, including Europe, Asia, North and South America, Africa, and Australia (Supplementary Figure 3.1), and comprised landraces, domesticated cultivars, and breeding lines conserved at the Western Barley Genetics Alliance at Murdoch University Perth, Australia. The domesticated barleys were selected from various

breeding programs representative of all cultivated varieties, including two-row (92%) and six-row (8%) head types, and winter (7%), spring (92%), and facultative (1%) growth habits (Supplementary Table 3.2). All barley plants were grown at two Western Australian locations, Merredin (31.4756°S, 118.2789°E, 315 m asl, 324 mm annual rainfall) and Katanning (33.6856°S, 117.6064°E, 320 m asl, 470 mm annual rainfall) in the 2016 and 2017 cropping seasons, respectively, and harvested at maturity. Both sites experience Mediterranean-type climates with hot, dry summers, and winter-dominant rainfall (Supplementary Figure 3.2) and are affected by salinity. The hot, dry summer increases salinity levels through ion accumulation in the topsoil, just before the autumn sowing, that affects seed germination. After harvest, the seeds were stored for at least 2 months at room temperature and then incubated at 37°C for 48 h to break seed dormancy.

3.3.2 Evaluation of Salinity Tolerance at Germination

This study used a modified method based on those of Bliss et al. (1986) and Angessa et al. (2017) to determine salinity tolerance during germination. Barley seeds were surface sterilized for 5 min using 10% sodium hypochlorite, and then rinsed with sterile water. A set of 100 seeds of each genotype was placed in a 90 mm Petri dish on two layers of Whatman no. 1 filter paper to germinate. The treatments, with three replicates per treatment, contained either 4 ml deionized (DI) water (control) or 150 mM NaCl (salt treatment). The Petri dishes were sealed with parafilm and placed in a dark oven at 20°C. Germinated seeds were counted after 72 h of incubation; most domesticated barley malt varieties (mostly two-row)—selected for dormancy are expected to germinate (95–100%) within 2–4 days of imbibition (Briggs, 1978; Bothmer et al., 1995). However, the wild form (ssp. *Spontaneum*), those developed for feed, and most six-row varieties have not undergone such selection; hence, seed germination is irregular (Oberthur et al., 1995). To account for this variation, the tolerance index (TI) was adopted to reflect the stress effect on the same genotype over the period; any reduction in germination was considered to be caused by salinity stress (Askari et al., 2016). The germination percentage (G%) was calculated following the equation of Adjel et al. (2013), namely, G% = GS/TS×100%, where GS is the total number of germinated seeds, and TS is the total number of incubated seeds. The tolerance index was subsequently calculated as follows (Angessa et al., 2017), TI% = G_t%/G_c%×100%, where G_t% is the percentage of seeds germinated in the salt treatment, and G_c% is the percentage of seeds germinated in deionized water.

3.3.3 Statistical Analysis

The germination percentage of individual accessions from three replications and two locations were analysed by SAS software (version 9.4, SAS Institute Inc, 2013). Analysis of variance (ANOVA) was performed to test the interaction between germplasm, treatments, and locations. Correlation analysis between germination in the salt treatment and the tolerance index was calculated and visualized using IBM SPSS Statistics (Version 25.0, IBM Corp, 2017).

3.3.4 Genome-Wide Marker Profiling

We used a combination of three sequencing methods to capture variation in and around the gene-containing regions of 350 barley genotypes, namely targeted resequencing, low-coverage whole-genome resequencing, and DArTseq. We used SNP markers, based on a custom target-enrichment sequencing assay, that included loci implicated in the flowering pathway in barley and related plant species, as previously published by Hill et al. (2019a, 2019b). The remainder of the pre-capture DNA libraries were subjected to low-coverage whole-genome sequencing at BGI (Hongkong) on an Illumina HiSeq4000 instrument. DArTseq genotyping by sequencing (GBS) was performed using the DArTseq platform (DArT PL, Canberra, NSW, Australia) as described on the company website (https://www.diversityarrays.com/). The genetic position of each marker was determined based on the Morex physical reference assembly. All sequence files were post-run filtered and aligned to barley reference genome assembly (IBSC v2; Mascher et al., 2017). All genotype data were combined, filtered for duplicates, minor allele frequency (MAF), and imputed using BEAGLE v4.1 adopting MAF > 0.05, SNP call rate > 0.95, and missing values < 0.05 (Browning and Browning, 2007).

3.3.5 Population Structure and Linkage Disequilibrium Analysis

Population structure was analyzed by Structure software version 2.3 (Hubisz et al., 2009). The genotypic data were imported into the software; the burn-in period was set to 5,000, producing 5,000 MCMC (Markov Chain Monte Carlo) repetitions. Simulations were conducted to estimate the number of populations (K) using admixture models by running K from 2 to 10, as described by Evanno et al. (2005). The LD between every two linear markers and the correlation between a pair of markers, which is squared allele frequency correlations (r^2 value), was estimated using TASSEL software version 5.0 (Bradbury et al., 2007). Correlations between a pair of markers (r^2 value) and the genetic distance was selected to calculate LD using a fitted equation in the whole genome.

3.3.6 Genome-Wide Association Analysis

Marker-trait association analysis for the salinity tolerance index at germination was performed by TASSEL software version 5.0 (Bradbury et al., 2007), using the mixed linear model basing on: trait of interest = population structure + marker effect + individual + residual. Heritability was estimated with the formula proposed by Kruijer et al. (2015), using genetic variance simulated from tolerance index and marker data obtained from the TASSEL package. The effectiveness and appropriateness of the model were assessed by constructing quantile–quantile (Q–Q) plots. Manhattan plots were constructed to visualize the GWAS output, with chromosome position as the X-axis and –log (*P*-value) of all markers using the R "qqman" (Wickham, 2009; R Core Team, 2014; Bates et al., 2015). Markers with $P < 0.05$ were considered significant and corrected for multiple tests by calculating *q*-value (FDR adjusted *P*-value). False discovery rate (FDR) correction was performed using Benjamini-Hochberg multiple test correction to determine significant marker-trait associations (MTA) (Benjamini and Hochberg, 1995) and markers with *q*-values < 0.05 were selected.

The formula described by Li et al. (2016) was used to identify favorable alleles for markers significantly associated with salinity tolerance during germination. The tolerance allele effect (ai) was calculated as; $a_i = \sum x_{ij}/n_i - \sum N_k/n_k$, where a_i is the tolerance effect of the i_{th} allele, x_{ij} is the tolerance index value over the j_{th} material with the i_{th} allele, n_i is the number of germplasm with the i_{th} allele, N_k is the salinity tolerance value across all genotypes, and n_k is the number of germplasm (Mei et al., 2013). In this study, *ai* denotes the association of the average salinity tolerance value of germplasm with a specific allele with that of all genotypes; hypothetically, values > 0 have a positive effect on the trait, and < 0 have a negative effect (Zhang et al., 2013).

3.3.7 Database Search to Predict Putative Candidate Genes and Favorable Alleles

The barley reference genome assembly (IBSC v2; Mascher et al., 2017) was used to identify possible candidate genes by searching the region flanking the QTN range of significantly associated salinity tolerance markers, with a –log10 (P) (logarithm of the odds –LOD) value set at ≥ 3.

3.4 Results

3.4.1 Phenotypic Variation and Correlations Among Traits

Three hundred and fifty barley genotypes were evaluated for salinity tolerance under control (germination in DI water) and salt conditions (150 mM NaCl) using seeds obtained from barley grown in Merredin and Katanning (Western Australia). The ANOVA results showed that

genotype, treatment, and location differed significantly at $P < 0.01$. There were significant interactions between genotype and treatment and genotype by treatment by location (Table 3.1). In the salt treatment, the tolerance index and germination percentage had a positive correlation, such that a high percentage for the two traits indicated tolerance to salinity stress. There was a positive correlation coefficient between germination at 150 mM NaCl and tolerance index (R^2 = 0.85 for Merredin and R^2 = 0.90 for Katanning; see *Supplementary Information*), indicating that either of the two can be used to identify salinity tolerance during germination (Supplementary Figure 3.3).

Table 3.1 Phenotype analysis of variance (ANOVA) for barley germplasms

Source	DF	SS	Mean square	F value
Germplasm	349	156424.66	448.2081948	17.99*
Treatment	1	298428.89	298428.89	18018.50*
Location	1	1563.57	1563.57	94.41*
Germplasm × Treatment	349	76629.44	219.568596	8.81*
Germplasm × Location	349	50714.36	145.3133524	5.83*
Germplasm × Treatment × Location	350	17711.39	50.60397143	2.03*
Error	1048	26126.64	24.93	
Total	1412	627598.95		

** Significant at 1% probability level*

In the control (DI water), the average germination percentage at the two locations was 94.5% (Supplementary Figure 3.5). In the salt treatment (150 mM NaCl), the average germination percentage at Merredin was 76.8% (range 50–99%) and Katanning was 75.7% (range 49–98%) (Figure 3.1). The high average germination percentage in DI water indicates that the seeds were not dormant; therefore, the reduction in germination in the salt treatment can be attributed to salinity stress. The mean tolerance index for the two locations was 79.5%, with an average of 96.99% for the most tolerant germplasm WABAR2347 (Table 3.2) and 52.73% for the susceptible Torrens (Supplementary Table 3.4). At both sites (Merredin and Katanning), the tolerance index and germination rate in 150 mM NaCl had positive correlations of R^2 = 0.52 and 0.40, respectively (Figure 3.2). The top 10 genotypes at each location in terms of tolerance index are presented in Table 3.2 and Supplementary Figure 3.4.

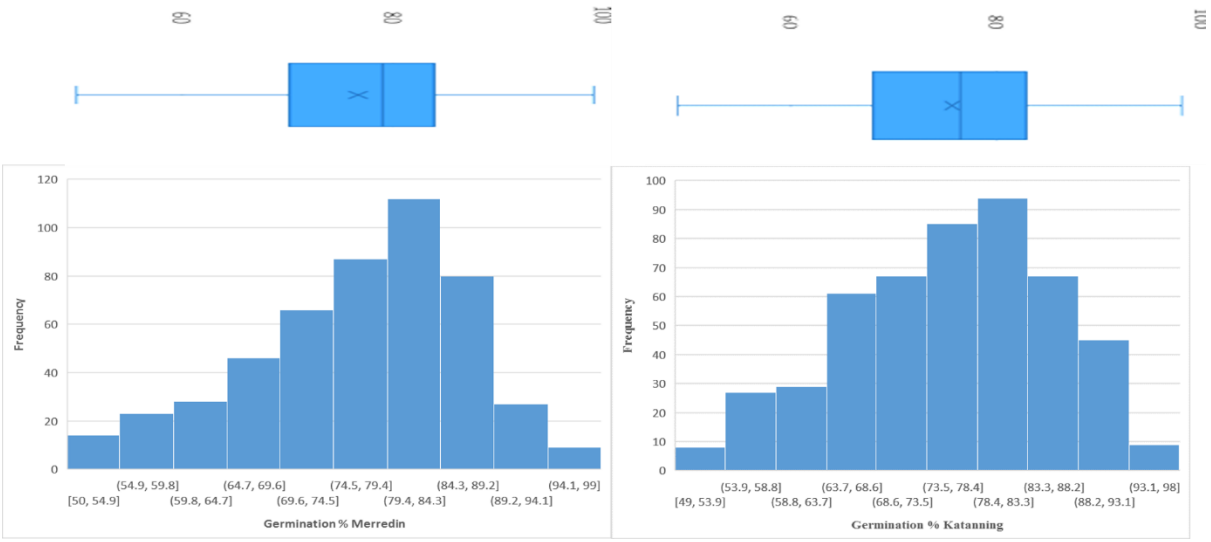

Figure 3.1 Combined histogram and plot block for germination percentage of 350 barley genotypes under 150 mM NaCl for seeds sourced from Merredin and Katanning, WA.

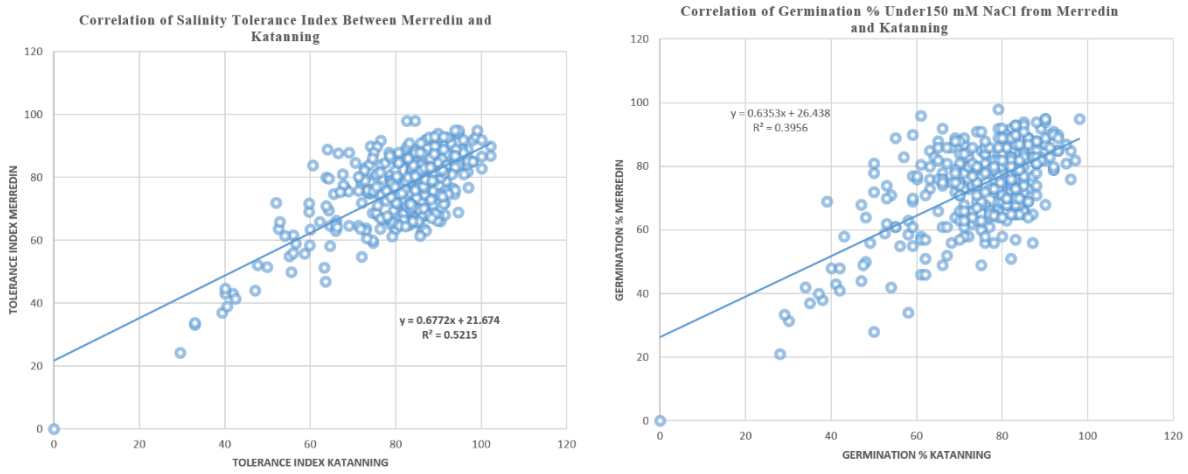

Figure 3.2 Correlation coefficient for seeds sourced from Merredin and Katanning for tolerance index and germination in 150 mM NaCl.

Table 3.2 List of most tolerant barley accessions (top 30) for seeds sourced from Merredin and Katanning (Western Australia)

		Merredin		Katanning		Average	
S/No.	**Accession**	**Germination %**	**Tolerance index**	**Germination %**	**Tolerance index**	**Germination %**	**Tolerance index**
1	WABAR2347	98	98.98	95	95	96.5	96.99
2	Har.Nan-35-	93	100	90	90	91.5	96.09
3	BM9647D-66	94.2	98.67	93	93	89	95.84
4	90SM193-34-	92	97.92	91	91.92	91.5	94.92
5	WVA22	90	94.77	95	95	92.5	94.88
6	WABAR2234	95	102.17	87	87	91	94.59
7	CDCGuardian	90	94.77	94	94	92	94.38
8	Yambla	90	93.75	95	95	92.5	94.38
9	90S205-45-4	89	94.44	94	94	89.5	94.22
10	H92036005Z	90	95.79	87	91.89	88.5	93.84
11	WABAR2425	91	100	92	97.42	91.51	93.32
12	ICB104039	90	96.79	89	89	89.5	92.89
13	ACMETCALFE	89	92.68	92	92	90.5	92.34
14	Patty	92	92	90	92.68	91	92.34
15	DH29287	86	95.56	89	89	87.5	92.28
16	HB08306	90	95.74	87	88.75	88.5	92.25
17	Landlord	82	95.45	89	89	85.5	92.23
18	WI4704	83	91.21	93	93	88	92.1
19	Tallon	88	89.75	94	94	91	91.88
20	CM67	92	92.9	79	90.8	85.5	91.85
21	VB0904	88	95.7	88	88	88	91.85
22	B697	85	96.61	86	86.76	85.5	91.69
23	WI4574	93	93.98	89	89	91	91.49
24	VTAdmiral	93	97.96	85	85	89	91.48
25	85SW:576	96	97.96	85	85	90.5	91.48
26	Mackay	88	90.14	90	92.73	86.5	91.44
27	BM9204-17	91	92.83	90	90	90.5	91.42
28	TR07393	97	100	82	82.78	89.5	91.39
29	CORGI	90	96.76	82	85.37	86	91.06
30	Tore*	86	89	93	93	88	91
	Mean	**90.37**	**95.48**	**89.5**	**90.67**	**89.58**	**92.93**

3.4.2 Marker Coverage, Population Structure, and Linkage Disequilibrium Analysis

Only DArTseq markers with a call rate of > 95% were selected, being 9,637 from a total of 22,241. An additional 28,502 SNP markers were identified by aligning the low-coverage sequences and targeted re-sequencing (Hill et al., 2019a; Hill et al., 2019b) of 350 barley accessions to the "Morex" reference genome and removing those with less than 5% allele frequency. In total, 24,138 DArTseq and SNP markers, anchored to the barley reference genome, were selected for population structure, linkage disequilibrium, and GWAS analysis.

Population structure analysis combined previously selected DArTseq and SNP markers to determine the genetic background of germplasm belonging to a group in a given number of populations (K). The number of genetic clusters (K values) for population structure was analysed in 350 barley genotypes with STRUCTURE software where parameter (ΔK) was used to determine the number of clusters suitable for association mapping analysis, with the cluster parameter K set from 2 to 10. The appropriate number of clusters was defined as 3, according to the method by Evanno et al. (2005)—when k was 3, ΔK would reach a top value of 21. The outputs were cross-confirmed to determine the optimal K-value, which was authenticated to be 3, according to the valley of the error rates of cross-validation (Supplementary Figure 3.4 and Supplementary Table 3.6).

Linkage disequilibrium (LD) decay (r^2) of individual chromosomes was analyzed and then summed to obtain an average value for the whole genome. The mean LD decay value for the 350 barley accessions was 3.5 Mb ($r^2 = 0.2$), with 24,138 were evenly spread and adequate for GWAS.

3.4.3 Genome-Wide Association Analysis of Salinity Tolerance at Germination

The GWAS was performed on 350 genotypes using both genotypic and phenotypic data. Given that the accuracy of association mapping analysis might be affected by population stratification, quantile–quantile (QQ) plots were generated to test the suitability of the model (Figure 3.3). The plots showed that the observed values (ordinate) initially matched the equivalent expected values (abscissa), but eventually, they were delineated and deviated to indicate a reasonable positive. Therefore, the GWAS results from all locations were reliable and not likely to give false negatives due to population stratification. Manhattan figure plots were created to visualize the significance of markers associated with the tolerance index (Figure 3.4). Heritability values of 0.18, 0.11, and 0.19 were obtained from the tolerance indices of Merredin, Katanning, and the average of the two locations, respectively.

3.4.4 Marker-Trait Associations for Salinity Tolerance Index During Germination

The threshold level was determined at a significance level of $P = 0.05$. Centered on the same, the genome-wide significance threshold for this study was $P = 1.39\times10^{-4}$ or –log10 (P) = 3.86 (rounded to 4.0). The selected markers were corrected for multiple testing, and those with *q*-values (FDR adjusted *P*-value) < 0.05 were considered accurately significant. Fifty-one markers (18 from Merredin, 13 from Katanning, and 21 from the average of the two sites), associated with the salinity tolerance index at germination were detected across all chromosomes, when –log10 (P) > 4.0. (Figure 3.4). Of these, L1H018492689 explained R^2 value of 11.03% and D7H016569501 for 6.33% as the highest and lowest, respectively (Supplementary Table 3.3). Five markers were detected at both locations (L1H018492798, L1H018492689, D1H528333687, L7H212035410, and D7H085710245), with eight at Katanning (L2H525371651, L5H070630348, L6H002587116, L6H004005746, D6H074421386, L7H004015622, C7H653619080, and D7H655103370), and 14 at Merredin (L1H018495748, C1H556900705, C1H556900787, D2H001502476, D3H598501321, L4H635824216, L5H044127079, L6H286731484, D6H471369639, L6H495910722, D7H016569501, L7H614807240, and D7H638672485). A hybrid of 21 markers at both locations was detected when the average was used (Supplementary Table 3.3). Some markers were detected only in one location, while others were present in both—an indication that some QTNs showed gene by environment (G×E) interactions.

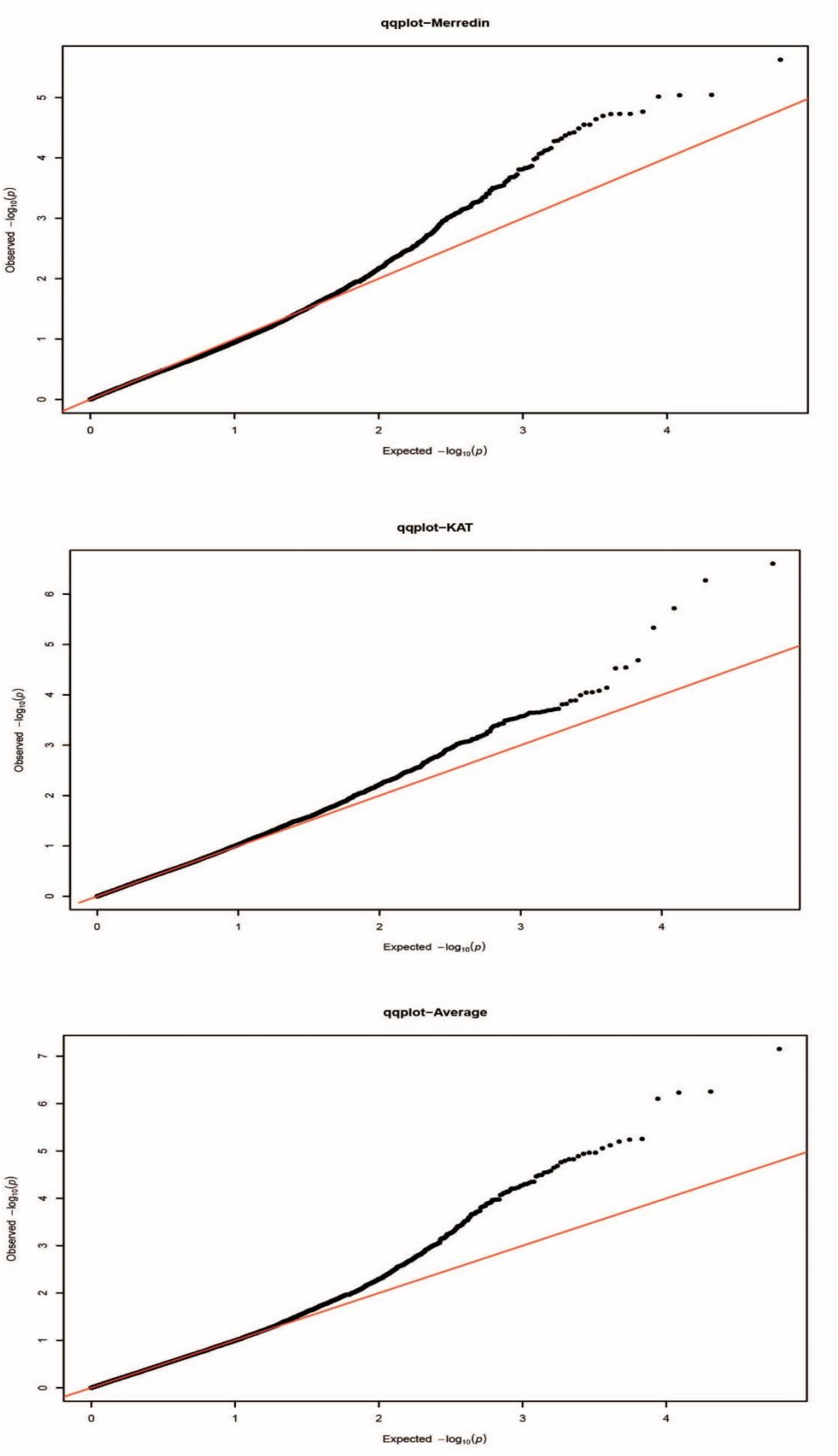

Figure 3.3 Quantile–quantile (Q-Q) plots for genome-wide association study (GWAS) of 350 barley accessions grown in Merredin, Katanning, and average for salinity tolerance index during germination under 150 mM NaCl. The Y-axis is observed –log (P) values, and X-axis the expected.

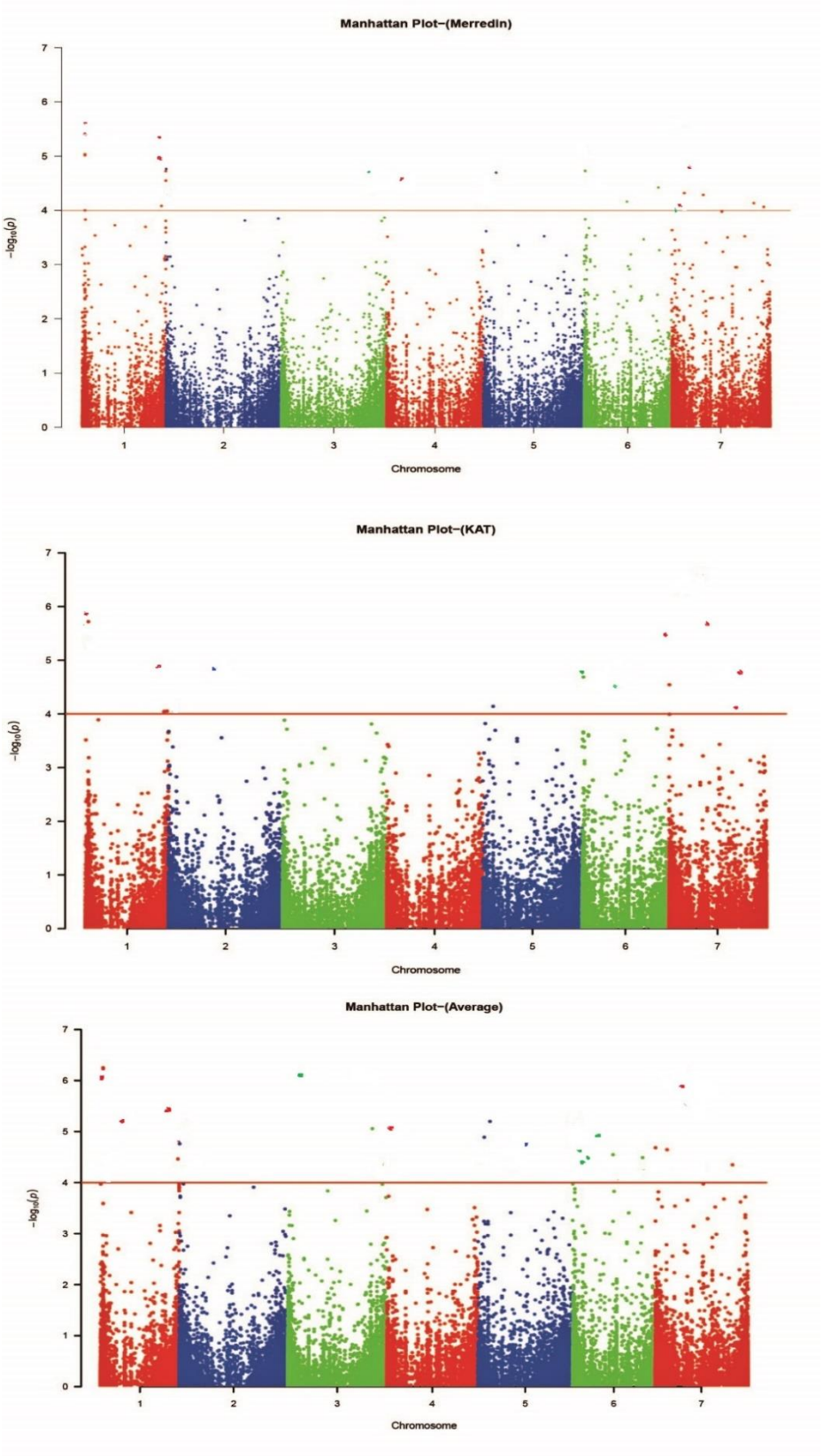

Figure 3.4 Manhattan plots for genome-wide association study (GWAS) of 350 barley accessions grown in Merredin, Katanning, and average for salinity tolerance index during germination under 150 mM NaCl. Each color indicates a different chromosome, the Y-axis is –log (P) values, and the dots above the red line are significant markers at –log10 (P) ≥ 4.0 (Krzywinski et al., 2009).

3.4.5 Mining of Favorable Marker Alleles Associated with Salinity Tolerance at Germination

The GWAS results are presented in Figure 3.4 and Supplementary Table 3.3 for significant markers at –log10 (P) > 4.0 and adjusted *P*-value (FDR) < 0.05. All significant markers from the two locations (Merredin and Katanning) were considered, and those detected in a range of 3.5 Mb were pooled to select the marker with the highest –log10 (P). Twelve representative significant markers were selected grounding on –log10 (P) > 4.0, overlapping both locations, and presence in one location and average (Table 3.4). Marker alleles with positive effects for tolerance index were considered favorable alleles, whereas marker alleles with negative effects were deemed unfavorable. Among the favorable marker alleles, L6H495910722, L6H286731484, and L7H614807240 had positive phenotypic effects on salinity tolerance at germination, being 5.3, 1.5, and 1.8%, respectively (Table 3.3). Salinity tolerance at germination in genotypes with favorable marker alleles was greater than those in genotypes with unfavorable marker alleles. Genotypes BM9204-17 and BM9647D-66 had favorable alleles for marker L6H495910722 and were present in the top 10 and 30 list (Supplementary Table 3.4 and Table 3.2) of varieties with the highest tolerance index from both locations. Accessions BM9647D-66 and TR06390 had favorable alleles for markers L6H495910722 and L7H614807240, while genotype SM02544 had favorable alleles for markers L6H495910722 and L6H286731484.

3.4.6 Quantitative Trait Nucleotides Controlling Salinity Tolerance During Germination in Barley

Significant markers flanking a range of 3.5 Mb were considered under one QTN, with only the highest –log10 (P) selected. Nineteen QTNs for salinity tolerance index during germination were identified from the two locations. Two QTLs were mapped on chromosomes 1H, 2H, and 4H, four each on 5H, 6H, and 7H, and one on 3H (Table 3.4). In Katanning, we detected ten QTNs—two on chromosome 1H and 6H, four on 7H, and one on 5H and 2H. Twelve QTNs were detected at the Merredin site, four on 7H, one each on 2H, 3H, 4H, and 5H, and two on 2H and 6H. When the average tolerance index value from the two locations was used for the analysis, a hybrid of the QTNs detected from Katanning and Merredin were realized (Table 3.4). For further analyses, we only considered QTNs that were present at both locations and their average. Four QTNs were present at Merredin, Katanning, and when the average was used, with two each on chromosome 1H and 7H (Table 3.5).

The estimated boundaries of the four QTNs were determined using –log10 (P) (logarithm of the odds –LOD) of the markers, setting the threshold at LOD ≥ 3, i.e., the borders for the intervals were the markers immediately below LOD 3. The most significant marker within the borders was selected as the representative QTN in the region. Two QTNs on chromosome 1H were flanked within an average range of 1.18 Mb for marker L1H018492689 and 1.39 Mb for C1H556900757, while on 7H they oscillated at an average of 1.49 Mb for L7H212035410 and 3.39 Mb for D7H085710245.

Table 3.3 Favourable alleles, their phenotypic effects (ai), and the number of accessions

Marker	Chromosome	Ref-Allele	Alt-Allele	Favourable allele	ai * Value	% Accessions
L1H018492689	1H	C	T	T	0.377	227
D1H528333687	1H	G	C	G	0.262	299
D2H001502476	2H	G	C	G	0.429	295
L2H525371651	2H	G	A	G	0.291	155
D3H598501321	3H	A	G	G	0.201	299
L5H044127079	5H	G	T	G	0.311	314
L6H286731484	6H	G	A	G	1.521	300
L6H495910722	6H	A	C	C	5.321	21
L7H004015622	6H	C	T	C	0.381	108
L7H212035410	7H	T	C	T	0.475	327
D7H085710245	7H	T	G	T	0.451	331
L7H614807240	7H	C	T	T	1.183	305

Table 3.4 Association mapping QTNs for salinity tolerance at germination in barley

Location	Marker	Chr	Position (bp)	MarkerR2	–log10 (P)	q-FDR
Merredin	L1H018492689	H1	18492689	0.087	5.514	0.028
	C1H556900705	H1	556900705	0.082	5.402	0.028
	D2H001502476	H2	1502476	0.087	4.864	0.048
	D3H598501321	H3	598501321	0.079	4.869	0.048
	L4H635824216	H4	635824216	0.075	4.676	0.050
	L5H044127079	H5	44127079	0.082	4.941	0.047
	L6H286731484	H6	286731484	0.074	4.261	0.071
	L6H495910722	H6	495910722	0.078	4.722	0.049
	D7H016569501	H7	16569501	0.063	4.049	0.088
	D7H085710245	H7	85710245	0.079	4.420	0.054
	L7H212035410	H7	212035410	0.079	4.730	0.049
	L7H614807240	H7	614807240	0.072	4.425	0.053
Katanning	L1H018492689	H1	18492689	0.102	5.934	0.018
	D1H528333687	H1	528333687	0.088	4.915	0.036
	L5H070630348	H5	70630348	0.082	4.841	0.037
	L6H004005746	H6	4005746	0.087	4.878	0.037
	D6H074421386	H6	74421386	0.073	4.482	0.051
	L7H004015622	H7	4015622	0.099	5.443	0.020
	D7H085710245	H7	85710245	0.071	4.403	0.059
	L7H212035410	H7	212035410	0.114	5.816	0.019
	D7H655103370	H7	655103370	0.085	5.312	0.029
Average	L1H018492689	H1	18492689	0.110	6.352	0.006
	D1H528333687	H1	528333687	0.097	5.440	0.013
	D2H001502476	H2	1502476	0.090	4.961	0.030
	D3H598501321	H3	598501321	0.100	6.357	0.006
	L4H007417825	H4	7417825	0.082	5.215	0.016
	L5H017667933	H5	17667933	0.090	4.893	0.030
	L5H044127079	H5	44127079	0.086	5.289	0.028
	L5H232131131	H5	232131131	0.087	4.626	0.035
	L6H015979347	H6	15979347	0.075	4.553	0.043
	L6H042597693	H6	42597693	0.073	4.396	0.050
	L6H286731484	H6	286731484	0.080	4.947	0.023
	L6H495910722	H6	495910722	0.079	4.589	0.043
	L7H004015622	H7	4015622	0.083	4.984	0.030
	D7H085710245	H7	85710245	0.082	4.845	0.030
	L7H212035410	H7	212035410	0.100	5.802	0.009
	L7H614807240	H7	614807240	0.075	4.496	0.050

Table 3. 5 Quantitative trait nucleotides present at both locations, estimated flanking region, and gene numbers

QTN	Flanking markers	Length of region (bp)	Number of genes
L1H018492689	L1H017315659 - L1H018494015	1178356	21
C1H556900757	L1H556830379 - L1H556830379	1392219	30
L7H212035410	L7H212035367- L7H226963761	1492840	62
D7H085710245	L7H082317438 - D7H085710245	3392807	30

3.4.7 Candidate Gene Prediction

A search for possible salt-tolerant candidate genes within the regions flanking each marker, based on the estimated QTNs boundaries above (Table 3.5), was conducted on the recently published barley reference genome assembly, with 143 genes found (Supplementary Table 3.5). Of these, four were very close to the most significant markers, or the markers were inside them; hence, they were given a high confidence as possible candidates (Table 3.6). Genes associated with the following four markers, *Piriformospora indica*-insensitive protein 2 (L1H018492689), lipase 1 (L7H212035410), protein kinase superfamily protein (C1H556900757), and heat shock protein 21 (D7H085710245), most likely play role in enhancing salinity tolerance during germination, as indicated by their –log10 (P) and % R^2 values (Supplementary Table 3.3). The frequency of B3 domain-containing protein, glutamate-1-semialdehyde-2;1-aminomutase, heat shock protein 21, leucine-rich repeat protein kinase family protein, MADS-box transcription factor family protein, protein kinase superfamily protein, RING/U-box superfamily protein, ubiquitin-like superfamily protein, and zinc finger protein family more than once at different chromosome locations indicated their involvement in enhancing salinity tolerance during germination (Supplementary Table 3.5).

Table 3.6 Genes close to or embedding significant markers associated with salinity tolerance at germination

Marker	Chromosome	Genes associated ID	Start	End	Function description
L1H018492689	1H	HORVU1Hr1G008420	18484404	18485253	Piriformospora indica-insensitive protein 2
C1H556900757	1H	HORVU1Hr1G094990	556905147	556910542	Protein kinase superfamily protein
L7H212035410	7H	HORVU7Hr1G053930	212741878	212744393	Lipase 1
D7H085710245	7H	HORVU7Hr1G036570.2	85583651	85584754	heat shock protein 21

3.5 Discussion

3.5.1 Salt Stress Significantly Inhibited Seed Germination

Seed germination is the first and most crucial stage in crop growth and development (Almansouri et al., 2001). It starts with the imbibition of water, which is repressed in the presence of salinity stress, hence disturbing the progression of germination (Othman et al., 2006). Earlier reports have shown that salinity delays the initiation processes, thus reducing

germination percentage and vigor (Dodd and Donovan, 1999; Zhihui et al., 2014). The biochemical and physical processes involved are incredibly complex and attributed to osmotic stress and ionic toxicity (Yu et al., 2018). Barley is a Mediterranean field crop that is directly sown in soil in autumn after hot summer, and salt tolerance during seed germination is essential. In this study, salinity reduced the average germination percentage across locations in the barley germplasm by 18.25%, confirming the compound effect of the stress (El Madidi et al., 2004; Abdi et al., 2016). At both locations, germination under salt stress and the tolerance index had a positive correlation (R^2 = 0.85–0.90), indicating that adapted barley germplasm has the capacity to withstand salt stress (Munns, 2005; Tajbakhsh et al., 2006; Zhang et al., 2010; Negrão et al., 2017).

Different methods of screening for salinity tolerance have been proposed, including non-stress conditions (Betran et al., 2003), stress conditions, and midway (non-stress and stress) (Ashraf et al., 2015). Selection criteria for salinity stress tolerance include the capacity of germplasm to produce high yields under stress (Rosielle and Hamblin, 1981), the stress susceptibility index being the degree of damage caused (Fischer and Maurer, 1978), and the stress tolerance index, being the percentage of yield under stress and non-stress conditions in the same germplasm (Angessa et al., 2017).The stress tolerance index is not suitable for genotypes that produce low yields under non-stress conditions (Kumar et al., 2014); it can be used to identify genotypes that produce high yields under both stress and non-stress conditions (Askari et al., 2016). Allel et al. (2019) evaluated various indices for salinity tolerance screening and confirmed that the salt-tolerance index is a better selection tool for highly salt-tolerant and productive barley genotypes under salinity, as reported by others (Ali et al., 2007; Shahzad et al., 2012; Senguttuvel et al., 2016). Traits with high rates of variation are among the most indicative and responsive under stress and can be used for the selection using tolerance indices, such as the tolerance index (TOL), salinity susceptibility index (SSI), geometric mean productivity (GMP), mean productivity (MP), and stress tolerance index (STI). Traits with low rates of variation are not suitable for selecting tolerant barley genotypes using tolerance indices under stress (Jamshidi and Javanmard, 2018). Nayyeripasand et al. (2019) reported a positive correlation among stress tolerance indices, including STI, SSI, and TOL, but not in the subgroups. Yu et al. (2018) reported that salt-tolerance levels in rice (*O. sativa*) were not strongly correlated with rice subgroups, which was confirmed in a maize population the following year (Luo et al., 2019). Tolerance indices do not accurately distinguish cultivars under severe stress (Mardeh et al., 2006; Mohammadi, 2019), but can be used as indicators for

high-yielding, salt-tolerant lines in stress, and non-stress environments or for traits like germination (Nayyeripasand et al., 2019; Sedri et al., 2019).

3.5.2 Barley Reference Genome and High-Density Markers Facilitate the Prediction of Candidate Genes Through Genome-Wide Association

To boost barley production in salt-prone areas, unique genes and alleles linked to salt tolerance at germination must be identified in a wider range of barley accessions. GWAS is an alternative and complementary approach that takes advantage of historical recombination's in a high-resolution genome scan to identify regions that are responsive to the traits (Zhao et al., 2011). Several QTLs for salt-tolerant traits at the germination stage have been reported (Mano and Takeda, 1997; Hanen et al., 2014; Angessa et al., 2017). Fan et al. (2015) reported two QTLs for salinity tolerance and N^+ content on chromosomes 7H and 2H in a DH population of TX9425 × Franklin that were closely linked to markers D7H085710245 and D2H001502476, respectively. A QTL for salinity tolerance mapped on 1H in a YYXT × Franklin DH population was closely linked to marker C1H556900757 (Zhou et al., 2012), as reported in this study. Using 206 barley accessions collected worldwide, 408 Diversity Arrays Technology (DArT) markers, and GWAS, Fan et al. (2016) reported a QTL on 2H that is closely linked to marker D2H001502476. Direct comparisons of our GWAS findings with other studies is tricky, as the marker-trait linkages and chromosomal locations we identified were based on a worldwide barley panel not previously investigated for salinity traits.

Our GWAS for the salinity tolerance index during germination was undertaken on 350 barley accessions using 24,138 DArTseq and SNP markers. Our findings will be a source of new understanding into the genetic basis of salt tolerance at germination and the identification of alleles underlying variation in the trait and candidate genes. Markers with significant effects identified at both locations were selected. We detected 19 QTNs for the tolerance index during germination across the barley genome, showing the complex genetic architecture of salinity tolerance in barley during germination, which is genetically and physiologically controlled by multiple small-effect genes (Flowers, 2004). The significant markers associated with the QTNs will form a basis for marker-assisted selection in barley breeding programs. Conferring with the released genome sequence of barley (Mascher et al., 2017) and gene annotation information, four candidate genes for the tolerance index during germination, belonging to four families, were predicted around the reliable QTNs in the QTN clusters (Table 3.6). In a GWAS study on rice, Naveed et al. (2018) reported 20 QTNs within 22 genes associated with salinity stress

at the germination and seedling stages, including kinase family protein, as found in our study. Yu et al. (2018) and Cui et al. (2018) identified 17 and 66 genes, respectively, contributing to salinity tolerance during germination in rice.

Nine markers were identified in Katanning and 12 in Merredin, with four overlapping at both locations (Supplementary Table 3.3). This indicates that salinity tolerance encompasses a complex of mechanisms at both the molecular and plant level that is controlled by many genes affected by the environment and genotype-by-environment (G × E) interactions (Arzani and Ashraf, 2016). The heritability values observed in this study indicated that the variation in tolerance index (germination in salt divided by germination in DI water) was mainly a factor of salinity concentration with a small genetic component. However, salinity tolerance is an important trait in barley that is inherited quantitatively and strongly influenced by environmental conditions (Jabbari et al., 2018), as indicated by the significant interactions among genotypes, salinity tolerance, and location in this study (Table 3.1). Estimated heritability defines how a trait is affected by genotype; however, it is not a total quantifier of how genes and the environment govern a phenotype, but specific to the population and environment under study. It does not account for the effect of missing or the lack of variable factors in the population (Yu et al., 2016).

3.5.3 Candidate Genes Reveal the Possible Molecular Basis of Salinity Tolerance at Germination

Of the 4 genes associated with salinity stress tolerance traits identified in this study, *P. indica*-insensitive protein 2 is reportedly involved in salinity tolerance through its interaction with phytohormones (auxins, cytokinin, gibberellins, abscisic acid, ethylene, salicylic acid, jasmonates, and brassinosteroids) in *Arabidopsis* (Xu L. et al., 2018). When barley and rice roots were colonized by endophytic basidiomycete fungi (*P. indica*), the host plants enhanced performance under salinity stress (Baltruschat et al., 2008; Vahabi et al., 2016; Jogawat et al., 2016). The protein kinase superfamily is another important gene family that has been characterized in several plants; e.g., for drought tolerance in barley (Cieśla et al., 2016; Yang et al., 2017) and salinity stress tolerance in wheatgrass (Shen et al., 2001). Protein kinase gene family, regulated by transcription factors (TFs) and microRNAs (miRNAs), plays key roles in salt stress tolerance in cotton (Shehzad et al., 2019). Overexpressed transgenic plants of soybean with protein kinase showed significantly increased tolerance to salt stress, suggesting that it plays a pivotal role in salinity tolerance (QIU et al., 2019). *Arabidopsis thaliana*, abscisic

acid (ABA)-non-activated protein kinases regulates reactive oxygen species (ROS) homeostasis and triggers genes expression under salinity stress (Szymańska et al., 2019).

In *Arabidopsis*, lipase expression is prompted by NaCl; its overexpression enhances salinity tolerance in transgenic plants, relative to non-transformed control plants, which facilitates seed germination, vegetative growth, flowering, and seed set (Naranjo et al., 2006). Studies have suggested that heat shock protein are likely to be involved in tolerance to other abiotic stresses such as salinity apart from thermal stresses (Song and Ahn, 2011; Mu et al., 2013). Transgenic tobacco plants with heat shock protein of alfalfa exhibited enhanced tolerance to salinity in comparison to wild type plants, in terms of germination rates (Lee et al., 2012). Overexpression of maize heat shock transcription factor enhanced thermo, increased the sensitivity to abscisic acid and salinity stress tolerance in transgenic *Arabidopsis* (Jiang et al., 2018). High expression of heat shock protein genes in barley have been reported in tissue — specific manner salinity stress (Chaudhary et al., 2019). The gene families mentioned above have been associated with stress tolerance, including salinity, in barley, related relatives, and other organisms. This finding will form the basis of more detailed studies to discover and validate the mechanism by which candidate genes play roles in salinity tolerance during germination in barley.

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3.7 Supplementary Material

3.7.1 Supplementary Tables

Supplementary Table 3.1 Regional representation of barley accessions used in this study

Place of origin	No. total varieties	Percentage	No. varieties used	Percentage
Argentina	1	0.17	1	0.29
Australia	211	35.52	122	34.86
Austria	2	0.34	1	0.29
Brazil	2	0.34	1	0.29
Canada	72	12.12	42	12.00
Chile	1	0.17	1	0.29
China	7	1.18	4	1.14
Czech	17	2.86	10	2.86
Denmark	1	0.17	1	0.29
Ethiopia	2	0.34	1	0.29
Europe	12	2.02	7	2.00
Finland	3	0.51	2	0.57
France	1	0.17	1	0.29
Germany	13	2.19	8	2.29
ICARDA	68	11.45	39	11.14
India	1	0.17	1	0.29
Japan	12	2.02	7	2.00
Mexico/CIMMYT	15	2.53	9	2.57
Morocco	1	0.17	1	0.29
Netherlands	2	0.34	1	0.29
New Zealand	1	0.17	1	0.29
Portugal	1	0.17	1	0.29
Russia	2	0.34	1	0.29
Scotland	2	0.34	1	0.29
Slovakia	1	0.17	1	0.29
South Africa	16	2.69	9	2.57
Spain	1	0.17	1	0.29
Sweden	3	0.51	2	0.57
UK	11	1.85	7	2.00
Unknown	18	3.03	11	3.14
Uruguay	18	3.03	11	3.14
USA	74	12.46	43	12.29
Uzbekistan	2	0.34	1	0.29
Total	**594**	**100.00**	350	100.

Supplementary Table 3.2 List of genotypes used in this study, their origin and growth habits

No.	Genotype ID	Accession name	Origin	Region	Head type	Growth habit
1	P0004	02S169-51-45	AUS	Australia	two-row	spring
2	P0005	04053-034	AUS	Australia	two-row	spring
3	P0007	04055-119	AUS	Australia	two-row	spring
4	P0010	04S213D-B-11	AUS	Australia	two-row	spring
5	P0014	04S213D-B-20	AUS	Australia	two-row	spring
6	P0029	07T741	AUS	Australia	two-row	spring
7	P0035	83SL:517	AUS	Australia	two-row	n.d.
8	P0036	83SM:522	AUS	Australia	two-row	n.d.
9	P0038	84SL:551	AUS	Australia	two-row	n.d.
10	P0039	85SW:576	AUS	Australia	two-row	spring
11	P0043	90S205-129-19	AUS	Australia	two-row	spring
12	P0044	90S205-45-46	AUS	Australia	two-row	spring
13	P0045	90S228-151-2	AUS	Australia	two-row	spring
14	P0051	90SM193-34-32	AUS	Australia	two-row	spring
15	P0053	91HBSN24	MEX	North America	two-row	spring
16	P0054	91IBON100	MEX	North America	two-row	spring
17	P0057	91IBON45	MEX	North America	six-row	spring
18	P0058	91IBON5	MEX	North America	two-row	spring
19	P0062	94S909G-20-19	AUS	Australia	two-row	spring
20	P0063	94S920W-18-6	AUS	Australia	two-row	spring
21	P0066	95S008-108-27	AUS	Australia	two-row	spring
22	P0067	95S009-81-33	AUS	Australia	two-row	spring
23	P0075	96B543	CAN	North America	two-row	spring
24	P0084	AB 47-6	AUS	Australia	two-row	spring
25	P0086	AC METCALFE	CAN	North America	two-row	spring
26	P0087	AC Oxbow	CAN	North America	two-row	spring
27	P0101	Andre	USA	North America	two-row	spring
28	P0118	Atem	GBR	Europe	two-row	spring
29	P0121	Atribut	CZE	Europe	two-row	spring
30	P0127	B521	CHN	Asia	two-row	n.d.
31	P0129	B559	CHN	Asia	two-row	n.d.
32	P0132	B645	CHN	Asia	two-row	n.d.
33	P0133	B697	CHN	Asia	two-row	n.d.
34	P0139	Barke	DEU	Europe	two-row	spring
35	P0145	BEARPAW	USA	North America	two-row	spring
36	P0146	Beatrice	FRA	Europe	two-row	spring
37	P0148	BEKA	FRA	Europe	two-row	spring
38	P0149	Bellini	FRA	Europe	two-row	spring
39	P0150	Bentley	CAN	North America	two-row	spring
40	P0152	Binalong	AUS	Australia	two-row	spring
41	P0154	BM9204-17	CAN	North America	two-row	spring
42	P0155	BM9311-35	CAN	North America	two-row	spring
43	P0156	BM9507-109	CAN	North America	two-row	spring
44	P0158	BM9645-96	CAN	North America	two-row	spring
45	P0159	BM9647D-43	CAN	North America	two-row	spring
46	P0160	BM9647D-66	CAN	North America	two-row	spring

47	P0161	BM9752D-125	CAN	North America	two-row	spring
48	P0162	BM9857-263-1	CAN	North America	two-row	spring
49	P0167	BoaFe	PRT	Europe	two-row	spring
50	P0169	BOLRON	USA	North America	six-row	facultative
51	P0171	BOWMAN	USA	North America	two-row	spring
52	P0175	Braemar	GBR	Europe	two-row	spring
53	P0177	Bridge	CAN	North America	two-row	spring
54	P0178	Brindabella	AUS	Australia	two-row	spring
55	P0180	BT558	CAN	North America	six-row	winter
56	P0181	BT634-AC Lacombe	CAN	North America	six-row	spring
57	P0183	Buloke	AUS	Australia	two-row	spring
58	P0184	Burton Malt	GBR	Europe	two-row	spring
59	P0187	BVDV-026	MEX	North America	two-row	n.d.
60	P0190	C01P-37	URY	South America	two-row	n.d.
61	P0191	C01P-53	URY	South America	two-row	n.d.
62	P0192	C01P-66	URY	South America	two-row	n.d.
63	P0194	C04A-34	URY	South America	two-row	n.d.
64	P0196	C2-05-10/263	URY	South America	two-row	n.d.
65	P0197	C2-05-10/437	URY	South America	two-row	n.d.
66	P0199	C2-05-337-2	AUS	Australia	two-row	spring
67	P0201	C2-05-63/710	URY	South America	two-row	n.d.
68	P0203	C2-05-89/827	URY	South America	two-row	n.d.
69	P0205	C2-05-89/878	URY	South America	two-row	n.d.
70	P0210	C98Prel-29	URY	South America	two-row	n.d.
71	P0212	C98Prel-60	URY	South America	two-row	n.d.
72	P0226	CBSS98M00022T-0TOPY-0M-1Y-2M-0Y	MEX	North America	six-row	spring
73	P0230	CDC Dolly	CAN	North America	two-row	spring
74	P0233	CDC Guardian	CAN	North America	two-row	spring
75	P0237	CDC Meredith	CAN	North America	two-row	spring
76	P0245	CDC Thompson	CAN	North America	two-row	spring
77	P0242	CDC TISDALE	CAN	North America	six-row	spring
78	P0243	CDC UNITY	CAN	North America	two-row	spring
79	P0244	CDC Yorkton	CAN	North America	six-row	spring
80	P0250	Chapais	CAN	North America	six-row	spring
81	P0251	Charger	AUS	Australia	two-row	spring
82	P0253	Charlottetown	CAN	North America	two-row	spring
83	P0255	CHERI	DEU	Europe	two-row	spring
84	P0261	CI5791	ETH	Africa	two-row	spring
85	P0262	CI9819	ETH	Africa	two-row	spring
86	P0266	Clark	USA	North America	two-row	spring
87	P0275	CLE235	URY	South America	two-row	spring
88	P0277	CLE268	URY	South America	two-row	spring
89	P0278	CLE270	URY	South America	two-row	spring
90	P0282	CM67	USA	North America	six-row	spring
91	P0284	Commander	AUS	Australia	two-row	spring
92	P0285	Compass	AUS	Australia	two-row	spring
93	P0291	CORGI	GBR	Europe	two-row	spring

94	P0292	Cowabbie	AUS	Australia	two-row	spring
95	P0303	Defra	DEU	Europe	two-row	spring
96	P0307	Derkado	GBR	Europe	two-row	spring
97	P0309	DH29287	AUS	Australia	two-row	spring
98	P0313	DH29400	AUS	Australia	two-row	spring
99	P0316	DIABAS	CZE	Europe	two-row	spring
100	P0324	DVORAN	SVK	Europe	two-row	spring
101	P0325	E Dong 85-1	CHN	Asia	two-row	spring
102	P0327	EB1111	AUS	Australia	two-row	spring
103	P0330	EMIRCOMPLEX	SWE	Europe	two-row	spring
104	P0332	ESPERANCE ORGE 289	n.d.	n.d.	two-row	spring
105	P0334	EUROPA	NLD	Europe	two-row	spring
106	P0337	Fairview	NZL	Australia	two-row	spring
107	P0342	FILIPPA	SWE	Europe	two-row	spring
108	P0343	Finniss	AUS	Australia	two-row	spring
109	P0346	Flagon	GBR	Europe	two-row	winter
110	P0347	Flagship	AUS	Australia	two-row	spring
111	P0348	Fleet	AUS	Australia	two-row	spring
112	P0349	Flinders	AUS	Australia	two-row	spring
113	P0350	FORMULA	GBR	Europe	two-row	spring
114	P0352	Foster	USA	North America	six-row	spring
115	P0353	FR/DAYTON	USA	North America	six-row	winter
116	P0355	France 30161	FRA	Europe	two-row	winter
117	P0361	GALAN	CZE	Europe	two-row	spring
118	P0371	GrangeR	AUS	Australia	two-row	spring
119	P0372	Granifen	CZE	Europe	two-row	spring
120	P0373	Grimmett	AUS	Australia	two-row	spring
121	P0374	Grout	AUS	Australia	two-row	spring
122	P0376	GSHO 2483 (in XV2334-6R from Indian Dwarf, slender dwarf 5 mutant)	USA	North America	six-row	spring
123	P0379	H92014002X	CAN	North America	two-row	winter
124	P0380	H92036005Z	CAN	North America	two-row	winter
125	P0383	H96009006	CAN	North America	six-row	winter
126	P0386	Hamelin	AUS	Australia	two-row	spring
127	P0389	Hannan	AUS	Australia	two-row	spring
128	P0390	HANNCHEN	SWE	Europe	two-row	spring
129	P0391	Har.Nan-35-24	AUS	Australia	two-row	spring
130	P0392	Har.Nan-35-28	AUS	Australia	two-row	spring
131	P0394	HARRINGTON	CAN	North America	two-row	spring
132	P0398	Haruna Nijo	JPN	Asia	two-row	spring
133	P0399	Hassan	NLD	Europe	two-row	spring
134	P0400	Havanna	CZE	Europe	two-row	spring
135	P0401	HB08306	CAN	North America	two-row	spring
136	P0402	HB09309	CAN	North America	two-row	spring
137	P0404	HB344-SB93666	CAN	North America	two-row	spring
138	P0405	HB352	CAN	North America	two-row	spring
139	P0407	HB380	CAN	North America	two-row	spring

140	P0408	HB382	CAN	North America	two-row	spring
141	P0409	HB385	CAN	North America	two-row	spring
142	P0410	HB395	CAN	North America	two-row	spring
143	P0412	HB805-BZ594-26	CAN	North America	two-row	spring
144	P0413	Heart	GBR	Europe	two-row	spring
145	P0417	Heriot	GBR	Europe	two-row	spring
146	P0418	Heris	CZE	Europe	two-row	spring
147	P0419	Hindmarsh	AUS	Australia	two-row	spring
148	P0424	I01-106-2-2	USA	North America	two-row	n.d.
149	P0425	I01-173-1	USA	North America	two-row	n.d.
150	P0428	I01-179-4	USA	North America	two-row	n.d.
151	P0429	I01-302-1	USA	North America	two-row	n.d.
152	P0435	I90-137-1	USA	North America	two-row	spring
153	P0436	I91-454	USA	North America	two-row	spring
154	P0437	I91-495	USA	North America	two-row	spring
155	P0440	I91-696	USA	North America	two-row	spring
156	P0445	I93-608	USA	North America	two-row	spring
157	P0451	I97-415	USA	North America	two-row	spring
158	P0453	ICB 104039	AFG	Middle East	six-row	facultative
159	P0454	ICB78-0058-7AP-2AP-1AP-4AP-0AP	SYR	Middle East	two-row	spring
160	P0456	IG 16957	UZB	Asia	six-row	spring
161	P0460	IGB1120	AUS	Australia	two-row	spring
162	P0462	IGB1133	AUS	Australia	two-row	spring
163	P0463	IGB1138	AUS	Australia	two-row	spring
164	P0469	IGB1234	AUS	Australia	two-row	spring
165	P0471	IGB1243	AUS	Australia	two-row	spring
166	P0472	IGB1244	AUS	Australia	two-row	spring
167	P0475	IGV3-313 (ATLAS46)	USA	North America	six-row	spring
168	P0476	Ilka	DEU	Europe	two-row	spring
169	P0477	Inari	FIN	Europe	two-row	spring
170	P0479	Ishuku Shirazu	JPN	Asia	two-row	winter
171	P0480	Jantar	CZE	Europe	two-row	spring
172	P0482	Jubilant	SVK	Europe	two-row	spring
173	P0483	Jyoti-PI 428399	IND	Asia	six-row	spring
174	P0484	Kalkreuther Fruhe	DEU	Europe	six-row	winter
175	P0485	Kaputar	AUS	Australia	two-row	spring
176	P0490	Keel	AUS	Australia	two-row	spring
177	P0495	Kinukei 18	JPN	Asia	two-row	spring
178	P0496	Kinukei 19	JPN	Asia	two-row	spring
179	P0499	KLAXON	GBR	Europe	two-row	spring
180	P0505	KRYSTAL (Mla13)	YUG	Europe	two-row	winter
181	P0507	Kustaa	SWE	Europe	two-row	spring
182	P0513	Landlord	GBR	Europe	two-row	spring
183	P0517	Larker	USA	North America	six-row	spring
184	P0518	Legacy	USA	North America	six-row	spring
185	P0521	Lindwall	AUS	Australia	two-row	spring
186	P0525	LOCEB-30	MEX	North America	two-row	spring

187	P0526	Lockyer	AUS	Australia	two-row	spring
188	P0531	Luxor	CZE	Europe	six-row	winter
189	P0537	Mackay	AUS	Australia	two-row	spring
190	P0538	Macquarie	AUS	Australia	two-row	spring
191	P0541	Maltine	NLD	Europe	two-row	spring
192	P0544	Maresi	DEU	Europe	two-row	spring
193	P0546	Maritime	AUS	Australia	two-row	spring
194	P0550	MC9924-012	CAN	North America	two-row	winter
195	P0552	MC9924-031	CAN	North America	two-row	winter
196	P0553	MC9939-007	CAN	North America	two-row	winter
197	P0555	MC9939-016	CAN	North America	two-row	winter
198	P0557	MC9939-039	CAN	North America	two-row	winter
199	P0558	MC9939-048	CAN	North America	two-row	winter
200	P0563	MN599	BRA	South America	two-row	spring
201	P0564	MN607	BRA	South America	two-row	spring
202	P0570	Moondyne	AUS	Australia	two-row	spring
203	P0572	Morex	USA	North America	six-row	spring
204	P0574	Moroc9-75/ArabiAswad/4/Hml-02/ArabiAbiad/3/Api/CM67//Nacta	SYR	Middle East	two-row	n.d.
205	P0575	MoroccanLandrace	MAR	Africa	six-row	spring
206	P0573	Morovian	USA	North America	six-row	spring
207	P0576	Mosane	BEL	Europe	two-row	spring
208	P0581	Natasha	FRA	Europe	two-row	spring
209	P0583	NB1054/ALELI	MEX	North America	six-row	spring
210	P0584	NBX05019-08-099	AUS	Australia	two-row	spring
211	P0587	ND22170	USA	North America	two-row	n.d.
212	P0588	ND23265	USA	North America	two-row	n.d.
213	P0589	ND23275	USA	North America	two-row	n.d.
214	P0592	Newdale	CAN	North America	two-row	spring
215	P0594	Nirasaki Nijo 9	JPN	Asia	two-row	spring
216	P0602	NRB08308	AUS	Australia	two-row	spring
217	P0606	O'Connor	AUS	Australia	two-row	spring
218	P0613	Optic	GBR	Europe	two-row	spring
219	P0615	ORBIT	SVK	Europe	two-row	spring
220	P0616	OTIS	USA	North America	two-row	spring
221	P0619	Oxford	AUS	Australia	two-row	spring
222	P0621	Patty	FRA	Europe	two-row	spring
223	P0622	Pearl	GBR	Europe	two-row	winter
224	P0624	PERUN	CZE	Europe	two-row	spring
225	P0625	PEWTER	DNK	Europe	two-row	spring
226	P0626	PICCOLO	NLD	Europe	two-row	spring
227	P0635	Prosa	AUT	Europe	two-row	spring
228	P0638	Quasar	AUS	Australia	two-row	spring
229	P0641	Rawson	USA	North America	two-row	spring
230	P0645	Regina	DEU	Europe	two-row	winter
231	P0646	RETROARUPOBV-9225	MEX	North America	two-row	spring

232	P0648	Riviera	GBR	Europe	two-row	spring
233	P0650	Roe	AUS	Australia	two-row	spring
234	P0655	RUSSIA24	RUS	Europe	two-row	spring
235	P0657	Ruti	CZE	Europe	two-row	spring
236	P0661	SANALTA	CAN	North America	two-row	spring
237	P0662	Satsuki Nijo	JPN	Asia	two-row	spring
238	P0663	SB01513	GBR	Europe	two-row	spring
239	P0664	SB03180	GBR	Europe	two-row	spring
240	P0667	SB99252	GBR	Europe	two-row	spring
241	P0670	Schooner	AUS	Australia	two-row	spring
242	P0674	SE612.01	DEU	Europe	two-row	n.d.
243	P0676	SEEBE	MEX	North America	two-row	spring
244	P0679	SH040468	CAN	North America	two-row	winter
245	P0682	Shepherd	AUS	Australia	two-row	spring
246	P0684	Shinonome	JPN	Asia	six-row	spring
247	P0685	SHN094	USA	North America	two-row	n.d.
248	P0687	SHYRI	MEX	North America	two-row	spring
249	P0689	Sissy	DEU	Europe	two-row	spring
250	P0690	Skiff	AUS	Australia	two-row	spring
251	P0692	Sloop	AUS	Australia	two-row	spring
252	P0693	Sloop SA	AUS	Australia	two-row	spring
253	P0694	Sloop VIC	AUS	Australia	two-row	spring
254	P0697	SM02544	CAN	North America	two-row	winter
255	P0698	SM060103	CAN	North America	two-row	winter
256	P0703	SMBA11-1771	AUS	Australia	two-row	spring
257	P0709	SpanishLandrace-355	ESP	Europe	n.d.	n.d.
258	P0714	Steffi	DEU	Europe	two-row	spring
259	P0715	Stella	SWE	Europe	two-row	spring
260	P0716	Stellar-ND	USA	North America	six-row	spring
261	P0718	Stirling	AUS	Australia	two-row	spring
262	P0720	Sublette	USA	North America	two-row	spring
263	P0723	SVB21	ZAF	Africa	two-row	n.d.
264	P0724	SVB24 (lfrR)	ZAF	Africa	two-row	n.d.
265	P0726	SVC5	ZAF	Africa	two-row	n.d.
266	P0728	Syn6058-06	AUS	Australia	two-row	spring
267	P0730	Tallon	AUS	Australia	two-row	spring
268	P0731	TANKARD	CAN	North America	two-row	spring
269	P0732	Tantangara	AUS	Australia	two-row	spring
270	P0736	Tilga	AUS	Australia	two-row	spring
271	P0737	Tipper//WI2291/WI2269	SYR	Middle East	two-row	spring
272	P0739	Tocada	DEU	Europe	two-row	spring
273	P0740	Toddy	GBR	Europe	two-row	spring
274	P0743	Tore*	NOR	Europe	two-row	spring
275	P0744	Torrens	AUS	Australia	two-row	spring
276	P0749	TR06390	CAN	North America	two-row	spring
277	P0751	TR07393	CAN	North America	two-row	spring
278	P0756	TR117a	CAN	North America	two-row	spring
279	P0757	TR145	CAN	North America	two-row	spring

280	P0762	TR245	CAN	North America	two-row	spring
281	P0763	TR257	CAN	North America	two-row	spring
282	P0768	TR638	CAN	North America	two-row	spring
283	P0769	TR645	CAN	North America	two-row	spring
284	P0777	Tulla	AUS	Australia	two-row	spring
285	P0779	Unicorn	JPN	Asia	two-row	spring
286	P0781	Urambie	AUS	Australia	two-row	winter
287	P0782	Ursa	CZE	Europe	two-row	spring
288	P0786	UWA94TK18-18	AUS	Australia	two-row	spring
289	P0788	UWA96T45-07-26	AUS	Australia	two-row	spring
290	P0794	Valeta	NLD	Europe	two-row	spring
291	P0796	VB0330	AUS	Australia	two-row	spring
292	P0800	VB0904	AUS	Australia	two-row	spring
293	P0801	VB0916	AUS	Australia	two-row	spring
294	P0806	VIC--8717	AUS	Australia	two-row	spring
295	P0809	Vlamingh	AUS	Australia	two-row	spring
296	P0810	VODKA	FRA	Europe	two-row	spring
297	P0813	VT Admiral	AUS	Australia	two-row	spring
298	P0814	W2 2010-5-17	AUS	Australia	six-row	spring
299	P0817	WABAR2228	AUS	Australia	two-row	spring
300	P0818	WABAR2231	AUS	Australia	two-row	spring
301	P0819	WABAR2234	AUS	Australia	two-row	spring
302	P0825	WABAR2259	AUS	Australia	two-row	spring
303	P0830	WABAR2347	AUS	Australia	two-row	spring
304	P0832	WABAR2377	AUS	Australia	two-row	spring
305	P0833	WABAR2378	AUS	Australia	two-row	spring
306	P0834	WABAR2411	AUS	Australia	two-row	spring
307	P0835	WABAR2421	AUS	Australia	two-row	spring
308	P0837	WABAR2425	AUS	Australia	two-row	spring
309	P0841	WABAR2547	AUS	Australia	two-row	spring
310	P0843	WABAR2592	AUS	Australia	two-row	spring
311	P0844	WABAR2609	AUS	Australia	two-row	spring
312	P0854	WB146	AUS	Australia	two-row	spring
313	P0858	WI2553	AUS	Australia	two-row	spring
314	P0860	WI2816	AUS	Australia	two-row	spring
315	P0862	WI2868	AUS	Australia	two-row	spring
316	P0883	WI4574	AUS	Australia	two-row	spring
317	P0885	WI4584	AUS	Australia	two-row	spring
318	P0887	WI4597	AUS	Australia	two-row	spring
319	P0889	WI4619	AUS	Australia	two-row	spring
320	P0891	WI4638	AUS	Australia	two-row	spring
321	P0897	WI4666	AUS	Australia	two-row	spring
322	P0899	WI4683	AUS	Australia	two-row	spring
323	P0901	WI4704	AUS	Australia	two-row	spring
324	P0905	WI4715	AUS	Australia	two-row	spring
325	P0912	WI4741	AUS	Australia	two-row	spring
326	P0920	WI4768	AUS	Australia	two-row	spring
327	P0924	WI4801	AUS	Australia	two-row	spring

328	P0925	WI4843	AUS	Australia	two-row	spring
329	P0926	WI4847	AUS	Australia	two-row	spring
330	P0927	WI4849	AUS	Australia	two-row	spring
331	P0928	WI4854	AUS	Australia	two-row	spring
332	P0931	WI4870	AUS	Australia	two-row	spring
333	P0932	WI4874	AUS	Australia	two-row	spring
334	P0933	WI4876	AUS	Australia	two-row	spring
335	P0935	WI4879	AUS	Australia	two-row	spring
336	P0936	WI4882	AUS	Australia	two-row	spring
337	P0937	WI4885	AUS	Australia	two-row	spring
338	P0938	WI4886	AUS	Australia	two-row	spring
339	P0941	WI4890	AUS	Australia	two-row	spring
340	P0943	WI4893	AUS	Australia	two-row	spring
341	P0944	Wicket	GBR	Europe	two-row	spring
342	P0951	WVA22	ZAF	Africa	two-row	n.d.
343	P0952	WVB35	ZAF	Africa	two-row	n.d.
344	P0953	WVC3	ZAF	Africa	two-row	n.d.
345	P0959	XVE7	ZAF	Africa	two-row	n.d.
346	P0960	XVH11	ZAF	Africa	two-row	n.d.
347	P0962	Yambla	AUS	Australia	two-row	spring
348	P0967	Z019Q008R	USA	North America	two-row	n.d.
349	P0972	ZBC9322	MEX	North America	two-row	spring
350	P0974	ZBC934	MEX	North America	two-row	spring

Supplementary Table 3.3 Genome wide association analysis for salinity tolerance index at germination stage

Location	Marker	Chr	Position (bp)	MarkerR2	–log10 (P)	q-FDR
Merredin	L1H018492689	H1	18492689	0.087	5.514	0.028
	L1H018492798	H1	18492798	0.090	5.437	0.028
	L1H018495748	H1	18495748	0.087	5.300	0.039
	D1H528333687	H1	528333687	0.083	4.982	0.045
	C1H556900705	H1	556900705	0.082	5.402	0.028
	C1H556900787	H1	556900787	0.077	5.277	0.038
	D2H001502476	H2	1502476	0.087	4.864	0.048
	D3H598501321	H3	598501321	0.079	4.869	0.048
	L4H635824216	H4	635824216	0.075	4.676	0.050
	L5H044127079	H5	44127079	0.082	4.941	0.047
	L6H286731484	H6	286731484	0.074	4.261	0.071
	D6H471369639	H6	471369639	0.067	4.324	0.064
	L6H495910722	H6	495910722	0.078	4.722	0.049
	D7H016569501	H7	16569501	0.063	4.049	0.088
	D7H085710245	H7	85710245	0.079	4.420	0.054
	L7H212035410	H7	212035410	0.079	4.730	0.049
	L7H614807240	H7	614807240	0.072	4.425	0.053
	D7H638672485	H7	638672485	0.066	4.241	0.076
Katanning	L1H018492689	H1	18492689	0.102	5.934	0.018
	L1H018492798	H1	18492798	0.100	5.889	0.019
	D1H528333687	H1	528333687	0.088	4.915	0.036
	L2H525371651	H2	525371651	0.096	4.893	0.037
	L5H070630348	H5	70630348	0.082	4.841	0.037
	L6H002587116	H6	2587116	0.085	4.722	0.039
	L6H004005746	H6	4005746	0.087	4.878	0.037
	D6H074421386	H6	74421386	0.073	4.482	0.051
	L7H004015622	H7	4015622	0.099	5.443	0.020
	D7H085710245	H7	85710245	0.071	4.403	0.059
	L7H212035410	H7	212035410	0.114	5.816	0.019
	C7H653619080	H7	653619080	0.067	4.139	0.074
	D7H655103370	H7	655103370	0.085	5.312	0.029
Average	L1H018492689	H1	18492689	0.110	6.352	0.006
	L1H018492798	H1	18492798	0.110	6.172	0.006
	L1H021222161	H1	21222161	0.095	5.263	0.013
	D1H528333687	H1	528333687	0.097	5.440	0.013
	D2H001502476	H2	1502476	0.090	4.961	0.030
	D3H598501321	H3	598501321	0.100	6.357	0.006
	L3H687771598	H3	687771598	0.089	5.086	0.029
	L4H007417825	H4	7417825	0.082	5.215	0.016
	L5H017667933	H5	17667933	0.090	4.893	0.030
	L5H044127079	H5	44127079	0.086	5.289	0.028
	L5H232131131	H5	232131131	0.087	4.626	0.035
	L6H015979347	H6	15979347	0.075	4.553	0.043
	L6H042597682	H6	42597682	0.073	4.310	0.051
	L6H042597693	H6	42597693	0.073	4.396	0.050
	L6H286731484	H6	286731484	0.080	4.947	0.023
	L6H495910722	H6	495910722	0.079	4.589	0.043
	L6H502983510	H6	502983510	0.075	4.480	0.050
	L7H004015622	H7	4015622	0.083	4.984	0.030
	D7H085710245	H7	85710245	0.082	4.845	0.030
	L7H212035410	H7	212035410	0.100	5.802	0.009
	L7H614807240	H7	614807240	0.075	4.496	0.050

Supplementary Table 3.4 The top 10 best and worst salinity-tolerant accessions and Australian commercial varieties based on the salinity tolerance index at two sites (Merredin and Katanning) and their average.

		Salinity tolerance index		
		Merredin	**Katanning**	**Average**
Top 10 best accessions				
1	WABAR2347	98.98	95.00	96.99
2	Har.Nan-35-	100.00	90.00	96.09
3	BM9647D-66	98.67	93.00	95.84
4	90SM193-34-	97.92	91.92	94.92
5	WVA22	94.77	95.00	94.88
6	WABAR2234	100.00	87.00	94.59
7	CDCGuardian	94.77	94.00	94.38
8	Yambla	93.75	95.00	94.38
9	90S205-45-4	94.44	94.00	94.22
10	H92036005Z	95.79	91.89	93.84
	Mean	**96.91**	**92.68**	**95.01**
Top 10 worst accessions				
1	HB09309	54.01	61.55	57.78
2	CLE268	56.49	59.00	57.74
3	C2-05-63/71	63.25	51.43	57.34
4	ESPERANCEOR	58.57	55.75	57.16
5	EB1112	56.00	56.04	56.02
6	96B543	63.46	46.96	55.21
7	04S213D-B-1	55.04	54.97	55.01
8	Torrens	55.47	50.00	52.73
9	Shepherd	59.92	51.56	55.74
10	Inari	57.69	52.10	54.90
	Mean	**57.99**	**53.94**	**55.96**
Representative of Australian commercial varieties				
1	Flinders	80.44	78.43	79.43
2	GrangeR	73.62	75.00	74.31
3	Buloke	69.00	64.67	66.84
4	Commander	65.91	63.00	64.45
5	Hamelin	79.82	82.31	81.06
6	Vlamingh	76.32	81.63	78.98
7	Compass	84.63	65.63	75.13
8	Lockyer	86.27	68.18	77.23
	Mean	**77.00**	**72.36**	**74.68**

Supplementary Table 3.5 Genes in QTN flanking regions of significant markers associated with salinity tolerance during germination

Marker	Chr	Genes associated ID	Start	End	Function description
L1H018492689	1H	HORVU1Hr1G008110.1	17447097	17449114	B3 domain-containing protein
	1H	HORVU1Hr1G008120.1	17566211	17571908	dehydroascorbate reductase 2
	1H	HORVU1Hr1G008130.1	17580274	17583426	11S seed storage protein
	1H	HORVU1Hr1G008140.1	17584434	17585571	TSA: Wollemia nobilis Ref_Wollemi_Transcript_13814
	1H	HORVU1Hr1G008150.1	17623756	17625773	B3 domain-containing protein
	1H	HORVU1Hr1G008160.3	17670835	17672723	COP9 signalosome complex subunit 5b
	1H	HORVU1Hr1G008170.2	17712400	17712697	Ubiquitin-like superfamily protein
	1H	HORVU1Hr1G008230.4	17901407	17905134	dual specificity protein phosphatase 1
	1H	HORVU1Hr1G008250.12	17907383	17913396	beta-hexosaminidase 1
	1H	HORVU1Hr1G008270.1	17919392	17919843	Anthocyanin 5-aromatic acyltransferase
	1H	HORVU1Hr1G008290.1	18102516	18109705	MADS-box transcription factor 27
	1H	HORVU1Hr1G008300.3	18158350	18163296	MADS-box transcription factor 27
	1H	HORVU1Hr1G008330.5	18353095	18359860	evolutionarily conserved C-terminal region 5
	1H	HORVU1Hr1G008340.2	18398661	18399623	unknown function
	1H	HORVU1Hr1G008350.2	18408388	18414274	UDP-galactose transporter 5
	1H	HORVU1Hr1G008360.2	18458166	18463957	laccase 7
	1H	HORVU1Hr1G008380.1	18468091	18468734	Heparanase-like protein 2
	1H	HORVU1Hr1G008370.2	18472177	18474612	undescribed protein
	1H	HORVU1Hr1G008420.1	18484404	18485253	Piriformospora indica-insensitive protein 2
	1H	HORVU1Hr1G008430.1	18487438	18488152	undescribed protein
	1H	HORVU1Hr1G008440.2	18489346	18490548	unknown function
C1H556900757	1H	HORVU1Hr1G094980.1	556900386	556904840	Early flowering 3
	1H	HORVU1Hr1G094990.2	556905147	556910542	Protein kinase superfamily protein
	1H	HORVU1Hr1G095010.1	556924891	556929224	Tudor/PWWP/MBT superfamily protein
	1H	HORVU1Hr1G095020.1	556950985	556953259	casein kinase I-like 5
	1H	HORVU1Hr1G095060.2	557105950	557109459	U6 snRNA-associated Sm-like protein LSm8
	1H	HORVU1Hr1G095080.1	557108008	557115788	WD-40 repeat family protein / notchless protein; p
	1H	HORVU1Hr1G095090.1	557118354	557118541	U-box domain-containing protein 4
	1H	HORVU1Hr1G095100.1	557122078	557125772	ATP-dependent RNA helicase DeaD

	1H	HORVU1Hr1G095110.2	557387532	557391034	unknown function
	1H	HORVU1Hr1G095120.1	557544024	557544124	cDNA clone:001-043-A08; full insert sequence
	1H	HORVU1Hr1G095130.3	557707978	557709129	unknown function
	1H	HORVU1Hr1G095140.1	557764284	557767320	histone deacetylase 2B
	1H	HORVU1Hr1G095150.1	557774147	557779964	AT1 protein
	1H	HORVU1Hr1G095160.1	557826700	557826926	undescribed protein
	1H	HORVU1Hr1G095170.6	557837872	557840541	Rho GDP-dissociation inhibitor 1
	1H	HORVU1Hr1G095180.1	557898136	557899011	unknown function
	1H	HORVU1Hr1G095190.1	557908922	557909609	undescribed protein
	1H	HORVU1Hr1G095210.4	557940300	557942187	unknown function
	1H	HORVU1Hr1G095220.1	557944582	557946336	RING/U-box superfamily protein
	1H	HORVU1Hr1G095230.35	557948038	557952685	structural maintenance of chromosomes 5
	1H	HORVU1Hr1G095240.1	557960891	557964049	Two-component response regulator ORR42
	1H	HORVU1Hr1G095250.1	557981437	557982203	unknown function
	1H	HORVU1Hr1G095270.16	557990565	557997508	Zinc finger CCCH domain-containing protein 37
	1H	HORVU1Hr1G095300.15	558019323	558024901	Argonaute family protein
	1H	HORVU1Hr1G095310.7	558025868	558026702	Reticulon family protein
	1H	HORVU1Hr1G095330.1	558119435	558121492	Eukaryotic aspartyl protease family protein
	1H	HORVU1Hr1G095340.3	558135613	558140825	Leucine-rich receptor-like protein kinase family p
	1H	HORVU1Hr1G095370.1	558185054	558186084	undescribed protein
	1H	HORVU1Hr1G095390.1	558216056	558217013	Zinc finger protein CONSTANS-LIKE 4
	1H	HORVU1Hr1G095400.1	558218311	558221262	Pleckstrin homology (PH) domain-containing protein
L7H212035410	7H	HORVU7Hr1G053930.9	212741878	212744393	lipase 1
	7H	HORVU7Hr1G053940.1	213013811	213019550	uridine kinase-like 2
	7H	HORVU7Hr1G053950.1	213078230	213078879	Late embryogenesis abundant (LEA) hydroxyproline-r
	7H	HORVU7Hr1G053970.1	213255282	213255655	1-aminocyclopropane-1-carboxylate synthase 11
	7H	HORVU7Hr1G054000.4	213613194	213616315	Chromosome 3B; genomic scaffold; cultivar Chinese
	7H	HORVU7Hr1G054010.1	213651429	213654111	60S ribosomal protein L17-2
	7H	HORVU7Hr1G054020.2	213855291	213857668	exocyst subunit exo70 family protein F1
	7H	HORVU7Hr1G054040.1	213889904	213890312	Major facilitator superfamily protein
	7H	HORVU7Hr1G054060.3	214453499	214459125	Nitrate reductase [NADH]

7H	HORVU7Hr1G054070.1	214455835	214461060	Far1-related sequence 3 isoform 1
7H	HORVU7Hr1G054090.4	214811865	214815839	purple acid phosphatase 27
7H	HORVU7Hr1G054120.1	215037587	215037757	Chlorophyll synthase; chloroplastic
7H	HORVU7Hr1G054130.4	215040274	215045086	microtubule-associated protein 65-8
7H	HORVU7Hr1G054140.1	215040171	215062445	Transducin family protein / WD-40 repeat family pr
7H	HORVU7Hr1G054160.3	215063343	215070088	Serine/threonine-protein kinase Rio1
7H	HORVU7Hr1G054190.14	215393914	215397113	Threonylcarbamoyl-AMP synthase
7H	HORVU7Hr1G054220.1	216028773	216035915	MADS-box transcription factor 7
7H	HORVU7Hr1G054230.1	216113703	216120774	Protein kinase superfamily protein
7H	HORVU7Hr1G054320.1	216662723	216664112	MADS-box transcription factor family protein
7H	HORVU7Hr1G054360.1	216704870	216706683	glutamate-1-semialdehyde-2;1-aminomutase
7H	HORVU7Hr1G054380.1	216784253	216786999	glutamate-1-semialdehyde-2;1-aminomutase
7H	HORVU7Hr1G054390.1	216788559	216822414	MADS-box transcription factor family protein
7H	HORVU7Hr1G054440.1	217284728	217287615	nuclear transport factor 2A
7H	HORVU7Hr1G054460.2	217289596	217289820	NAM-like protein
7H	HORVU7Hr1G054500.2	217854577	217858795	Nodulin-like / Major Facilitator Superfamily prote
7H	HORVU7Hr1G054510.1	218220114	218223625	Peroxidase superfamily protein
7H	HORVU7Hr1G054520.2	218228667	218230555	zinc ion binding;transcription regulators
7H	HORVU7Hr1G054530.1	218520818	218524923	Lipid transfer protein-like
7H	HORVU7Hr1G054550.1	218865361	218869367	Transmembrane emp24 domain-containing protein
7H	HORVU7Hr1G054580.1	219675948	219676121	Tubby-like F-box protein 9
7H	HORVU7Hr1G054610.3	220370264	220373270	Bifunctional uridylyltransferase/uridylyl-removing
7H	HORVU7Hr1G054660.6	221197966	221199221	Chromosome 3B; genomic scaffold; cultivar Chinese
7H	HORVU7Hr1G054670.1	221347389	221350163	60S ribosomal protein L7a
7H	HORVU7Hr1G054690.4	221603430	221606339	ARM repeat superfamily protein
7H	HORVU7Hr1G054710.2	221745516	221747264	Sugar transporter SWEET
7H	HORVU7Hr1G054730.9	221809550	221814569	Membrane fusion protein Use1
7H	HORVU7Hr1G054760.2	221851240	221855098	Protein S-acyltransferase 8
7H	HORVU7Hr1G054850.2	222085959	222089913	Multiple organellar RNA editing factor 3; mitochon
7H	HORVU7Hr1G054880.1	222639811	222641222	basic helix-loop-helix (bHLH) DNA-binding superfam
7H	HORVU7Hr1G054890.2	222644027	222646598	B12D protein

	7H	HORVU7Hr1G054910.1	222704979	222706366	Leucine-rich repeat protein kinase family protein
	7H	HORVU7Hr1G054920.1	222709151	222711551	Mitochondrial import inner membrane translocase su
	7H	HORVU7Hr1G054980.28	222924348	222932515	Glycerophosphodiester phosphodiesterase GDE1
	7H	HORVU7Hr1G055010.1	222934962	222936149	RING/U-box superfamily protein
	7H	HORVU7Hr1G055080.2	223284188	223288321	NAC domain protein;
	7H	HORVU7Hr1G055090.3	223540999	223548166	Pyruvate dehydrogenase E1 component subunit beta
	7H	HORVU7Hr1G055100.6	223549926	223554903	unknown function
	7H	HORVU7Hr1G055160.1	225222379	225222765	unknown function
	7H	HORVU7Hr1G055180.1	225250259	225253008	Transcription factor bHLH137
	7H	HORVU7Hr1G055190.1	225320897	225321325	trehalose-6-phosphate synthase
	7H	HORVU7Hr1G055200.1	225322578	225323273	Elongated mesocotyl1
	7H	HORVU7Hr1G055220.4	225589997	225616772	ADP-ribosylation factor GTPase-activating protein
	7H	HORVU7Hr1G055270.2	225748834	225749980	Histone-lysine N-methyltransferase
	7H	HORVU7Hr1G055280.1	225947017	225950048	Ubiquitin thioesterase otubain-like
	7H	HORVU7Hr1G055290.6	225951783	225965924	O-fucosyltransferase family protein
	7H	HORVU7Hr1G055300.1	225960676	225960924	undescribed protein
	7H	HORVU7Hr1G055310.1	226129155	226130687	Cathepsin B-like cysteine proteinase 6
	7H	HORVU7Hr1G055320.1	226172292	226173072	Heat stress transcription factor C-2a
	7H	HORVU7Hr1G055330.1	226285016	226286367	rRNA N-glycosidase
	7H	HORVU7Hr1G055340.1	226514183	226519506	Glycine--tRNA ligase
	7H	HORVU7Hr1G055370.6	226851895	226866971	receptor-like protein kinase 1
	7H	HORVU7Hr1G055390.1	226892424	226893019	chitin elicitor receptor kinase 1
D7H085710245	7H	HORVU7Hr1G036090.2	82420627	82440422	kinesin 4
	7H	HORVU7Hr1G036100.6	82606397	82608783	laccase 14
	7H	HORVU7Hr1G036110.1	82650592	82654618	Mediator of RNA polymerase II transcription subuni
	7H	HORVU7Hr1G036120.1	82861778	82862187	Speckle-type POZ protein-like
	7H	HORVU7Hr1G036130.1	82946101	82953029	MADS-box transcription factor 55
	7H	HORVU7Hr1G036140.1	82948556	82952709	Elongation factor Ts
	7H	HORVU7Hr1G036160.1	83249502	83255889	Peptidyl-prolyl cis-trans isomerase D
	7H	HORVU7Hr1G036180.1	83278444	83279568	Copper ion-binding protein; putative
	7H	HORVU7Hr1G036170.8	83280307	83285878	Pentatricopeptide repeat-containing protein

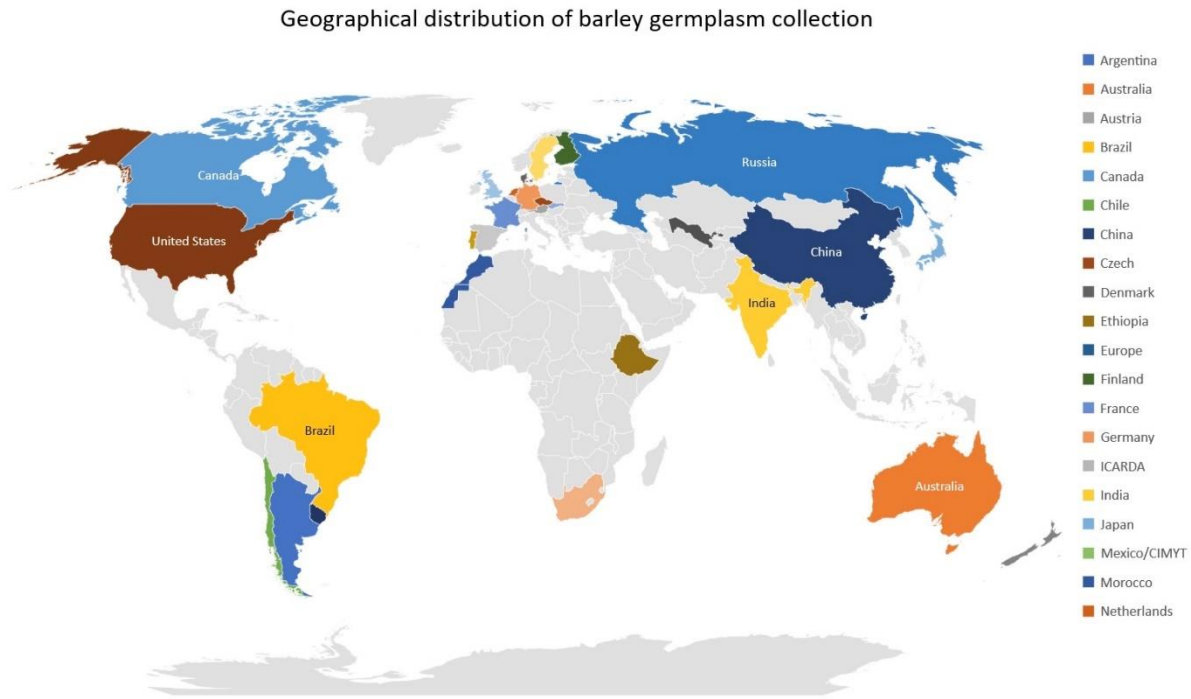
7H	HORVU7Hr1G036210.1	83526144	83527023	Cullin-associated NEDD8-dissociated protein 1
7H	HORVU7Hr1G036220.2	83526145	83528738	Leucine-rich repeat protein kinase family protein
7H	HORVU7Hr1G036280.1	83610089	83611469	Protein CURVATURE THYLAKOID 1D; chloroplastic
7H	HORVU7Hr1G036290.1	83636776	83636944	undescribed protein
7H	HORVU7Hr1G036310.3	83687930	83688669	alpha/beta-Hydrolases superfamily protein
7H	HORVU7Hr1G036330.1	84006342	84008301	Signal recognition particle 54 kDa protein 2
7H	HORVU7Hr1G036370.1	84228373	84229694	RING/U-box superfamily protein
7H	HORVU7Hr1G036380.2	84258258	84259714	RING/U-box superfamily protein
7H	HORVU7Hr1G036390.1	84513601	84514975	Blue copper protein
7H	HORVU7Hr1G036410.1	84565373	84565873	unknown function
7H	HORVU7Hr1G036420.10	84566492	84569524	LMBR1 domain-containing protein 2 homolog B
7H	HORVU7Hr1G036430.2	84582203	84583778	DNA helicase MCM9
7H	HORVU7Hr1G036440.1	84627463	84627646	DNA replication licensing factor MCM9
7H	HORVU7Hr1G036450.20	84642784	84646994	O-fucosyltransferase family protein
7H	HORVU7Hr1G036460.1	84700862	84702674	PATATIN-like protein 4
7H	HORVU7Hr1G036470.3	84846602	85031958	heat shock protein 21
7H	HORVU7Hr1G036500.7	84965457	84973258	heat shock protein 21
7H	HORVU7Hr1G036520.4	85198618	85200204	Pectin lyase-like superfamily protein
7H	HORVU7Hr1G036540.3	85341216	85462034	heat shock protein 21
7H	HORVU7Hr1G036560.1	85490557	85490959	Chitinase family protein
7H	HORVU7Hr1G036570.2	85583651	85584754	heat shock protein 21

Supplementary Table 3.6 Multiple comparison of subpopulations mean tolerance index

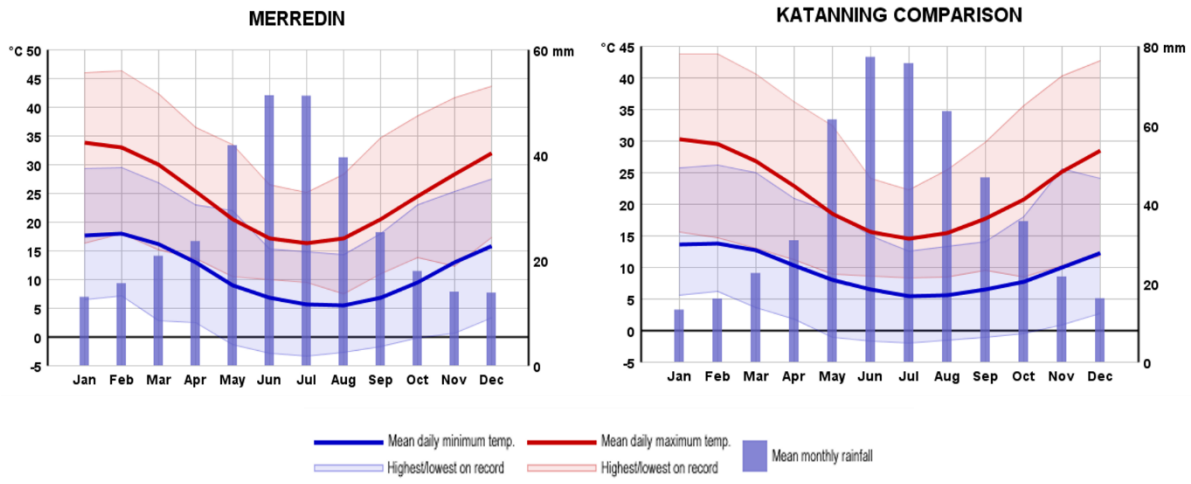
Subpopulation	T. Index	Comparison	Mean Difference	Std. Error	Sig.
1	79.43	-	-	-	-
		2	2.13	2.78	0.44
		3	0.06	2.62	0.98
		4	0.47	1.84	0.80
		5	2.15	2.12	0.31
		6	4.78	2.52	0.06
2	77.30	3	-2.07	2.94	0.48
		4	-1.66	2.28	0.47
		5	0.03	2.51	0.99
		6	2.66	2.86	0.35
3	79.37	4	0.41	2.08	0.84
		5	2.10	2.33	0.37
		6	4.73	2.70	0.08
4	78.96	5	1.69	1.40	0.23
		6	4.32*	1.96	0.03
5	77.27	6	2.63	2.23	0.24
6	74.64	-	-	-	-

* The mean difference is significant at the 0.05 level.

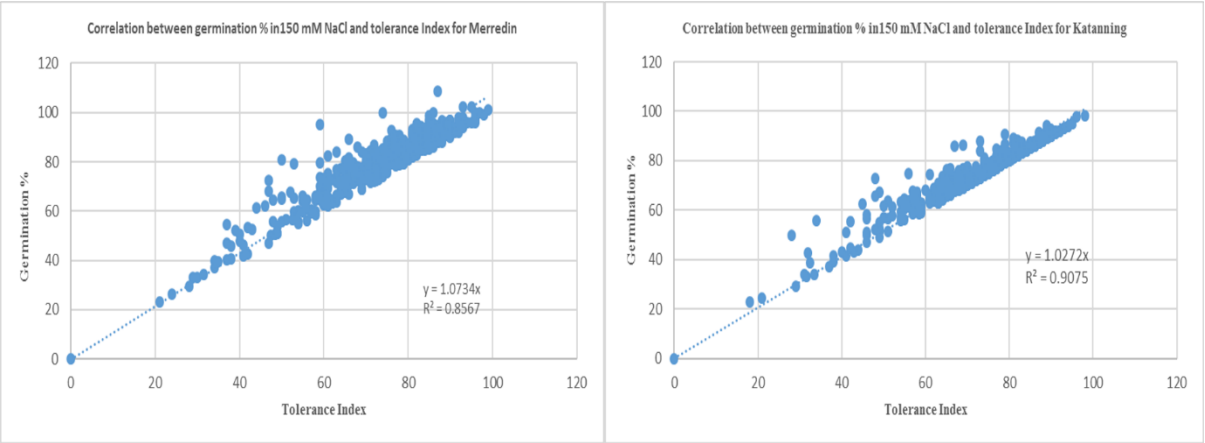
3.7.2 Supplementary Figures



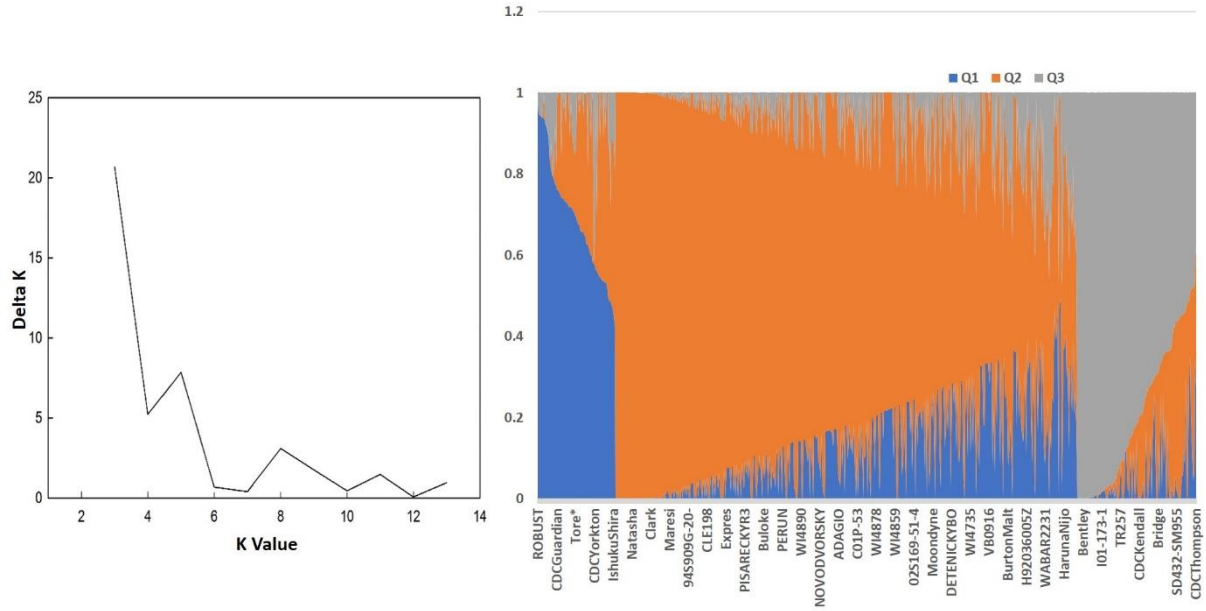
Supplementary Figure 3.1 A map showing geographical representation and origin of barley accessions used in this study.



Supplementary Figure 3.2 Annual average weather pattern for Merredin and Katanning as reported by the Australian Bureau of Meteorology (http://www.bom.gov.au/index.php).



Supplementary Figure 3.3 Correlation coefficient for germination in 150 mM NaCl against tolerance index for seeds from Merredin and Katanning.



Supplementary Figure 3.4 Estimate of the most probable number of clusters (k) and population structure.

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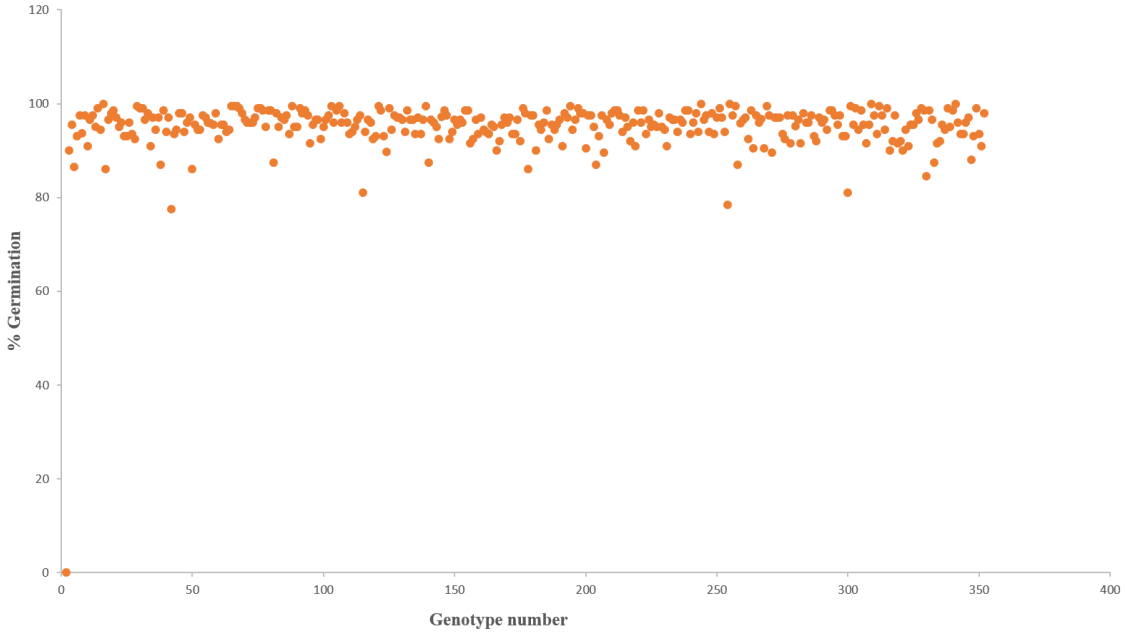

Supplementary Figure 3.5 Average germination percent distribution of 350 barley accession in deionized water.

CHAPTER FOUR

QUANTITATIVE TRAIT LOCI MAPPING FOR VIGOUR AND SURVIVAL TRAITS OF BARLEY SEEDLINGS AFTER GERMINATING UNDER SALINITY STRESS

The inside of this chapter institute a published research article in ***Agronomy journal.*** The full citation is: **Mwando, K. E.,** Angessa, T. T., Han, Y., Zhou, G., and Li, C. (2021). Quantitative trait loci mapping for vigour and survival traits of barley seedlings after germinating under salinity stress. *Agronomy*, 11(1): 103. https://doi.org/10.3390/agronomy11010103.

The authors contribution is as follows: **E.M.** performed the experiments, data analysis, interpretation and drafting of the manuscript; **G.Z.** constructed the molecular linkage map and QTL mapping; **Y.H.** and **T.A**. guided in experiment; **C.L.** conceived the project; **E.M., Y.H., T.A**. and **C.L.** revised the paper and approved the final version to be published.

4.1 Abstract

Seed germination and seedling establishment are the most critical stages in the barley (*Hordeum vulgare* ssp. *vulgare* L.) life cycle that contribute substantially to grain yield. These two phases are exposed to several forms of environmental stresses such as salinity due to high level of salt accumulation in the soil rhizosphere where seed germination takes place and seedlings emerge from. Previously, we have reported genotypic variability and independent QTLs associated with salinity tolerance at seedling and germination stages. However, genotypic studies on revival of a seedling germinating under salinity stress are yet to close the lack of information between germination and seedling stages. Here, we attempt to close the genetic gap by targeting early seedling survival traits in barley after germination under salinity (NaCl) stress and the various seedling vigour indices. Seedling vigour parameters formed the basis for Quantitative trait locus (QTL) linkage mapping in 103 Doubled Haploid (DH) lines of CM72/Gairdner population, and validated the phenotypic response using a selected diverse panel of 85 barley germplasm. The results indicate that 150 mM NaCl stress significantly reduced all the recorded phenotypic traits compared to 75, 90

and 120 mM NaCl. In both DH population and diversity panel barley germplasm, the highest percentage reduction was recorded in shoot length (65.6% and 50.3%) followed by seedling vigour index length (56.5% and 41.0%), while root length (28.6% and 15.8%) and root dry weight (29.3% and 28.0%) were least reduced when control was compared to150 mM NaCl stress treatment. Six QTLs containing 13 significant markers were detected in the DH population, 3 on chromosomes 1H, 8 on 3H and 2 on 4H with LOD values ranging from 3 to 8 associated with seedling survival traits under salinity stress. Three QTLs one on 1H and two on 3H with closely linked significant markers (Bmac0032, bPb-9418 and bPb-4741), (bPb-4576 and bPb-9624) and (bPb-3623, bPb-5666 and bPb-6383) for 1H and two on 3H respectively formed the regions with high possibility of candidate genes. A QTL on 3H flanked with markers bPb-4576 and bPb-9624 that were detected in more than one salinity survival trait and were closely linked to each other will form a basis for detailed studies leading to gene functional analysis, genetic transformation and marker assisted selection (MAS).

4.2 Introduction

Barley (*Hordeum vulgare* ssp. *vulgare* L.) is an important crop grown globally for its multipurpose uses for malting/ brewing purposes, human food and animal feed. While it has been reported to naturally acclimatize to varying environmental conditions, cultivated barley varieties have a narrower genetic diversity to most stresses occasioned by deliberate breeding programmes concentrating on fewer traits. (Angessa *et al.,* 2020; Hill et al., 2020; Mikołajczak *et al.,* 2020). Most programmes focus on minimising the gap between yield capacity and actual yield under stress by identifying the Quantitative trait loci (QTL) or responsible genes through phenotypic screening and then incorporating it into adapted backgrounds (Berger *et al.,* 2010). Seedling stage is one of the most important phases in barley growth and development and is determined by seed germination. It governs production aspects including uniform stand, good nutrients uptake, environmental stresses tolerance and yield (Wang *et al.,* 2017). Germination is a stage prompted by water uptake into the embryo

leading to root and shoot emergence. It is a foundation for the succeeding seedling stage, and thus plays an imperative role in plant growth and the ultimate yield (Xue *et al.,* 2019).

Seedling establishment, which is a vital phase in the plant life cycle that lays foundations for the succeeding growth stages, is exposed to numerous environmental stress factors (Batool *et al.,* 2018; Sedzik *et al.,* 2019). Salinity is among the main factors that affects germination and seedling establishment of most crops including barley (Angessa *et al.,* 2017; Williams *et al.,* 2019; Priyadharshini *et al.,* 2019; Thanh and Bharali, 2019; Aminifard and Bayat, 2020). Salinity interferes with seed germination and plants growth and developments through (i) osmotic pressure imbalance i.e. initiating water shortage, (ii) ion toxicity, (iii) essential nutrients uptake imbalance and (iv) production of reactive oxygen species (ROS) that act at cellular or at whole plant level to cause physiological and biochemical defects that result to reduced germination, suppressed seedling growth and poor harvest (Xue *et al.,* 2019; Rajabi Dehnavi *et al.,* 2020).

Uniform and timely germination and vigorous seedling growth under saline conditions are some of the key traits when selecting for salinity tolerant genotypes. The ability of a plant to grow under salinity stress determined by the proportion of dissolved salts depends on the potential of the seed to germinate and grow under declining soil osmotic potentials, and the plants varied internal cellular ionic compositions (Kanbar, 2014). Barley is grown on every continent because of its ability to adapt to various environmental conditions (Ko *et al.,* 2019). It is produced in a wider topographical area than most other cereals spreading across more than 100 countries around the world and is the fourth most important cereal crop (Giraldo *et al.,* 2019; Ko *et al.,* 2019). The top country by production in the world is Russian Federation at 19, 939 thousand tonnes which is 21.4% of the world's barley production of 93,392 thousand tonnes followed by Canada, Ukraine, Australia, and Turkey that account for 60.8% collectively (USDA, 2020). The world’s land affected by salinity is ~1125 million hectares which is approximately 6% of total global area including 20% of cultivated and 33% of the irrigated land (Hossain, 2019). At the same time, more land area on the planet is being lost

to high salinity at the rate of ~1.5 million hectares annually, an indication that ~50% of arable lands will be saline by 2050 (Hasanuzzaman *et al.,* 2014). Most of the world's barley producing countries are affected by the salination problem including but not limited to the following Russian Federation, Australia, Bangladesh, USA, China, Egypt, Turkey, India, Mexico, Iran, Syria, Iraq and Pakistan (Hossain, 2019). Several barley growing environments are prone to salinity stress due to high level of salt accumulation in the topsoil due to high evapotranspiration and capillary actions from dry and hot summers. Under such environments seed germination and seedling development phases are severely impacted by salinity stress until the excessive salt level is leached out of the rootzone by rain or irrigation later in the season by the time when the plants are likely to be rather hardened and vigorous (El Goumi *et al.,* 2014; Ho *et al.,* 2020).

Few QTLs for salinity stress tolerance have been reported at seedling level in barley including those governing response to ionic stress (Witzel *et al.,* 2010; Xue *et al.,* 2017), osmotic stress (Bálint *et al.,* 2008; Wójcik-Jagła *et al.,* 2013) and salinity tolerance (Angessa *et al.,* 2017). Genome Wide Association analysis of salinity tolerance in a collection of barley accessions from across the globe totaling to 350 identified genomic regions linked with germination stage salinity tolerance (Mwando *et al.,* 2020a). Angessa *et al. (*2017) mapped QTLs associated with germination stage and/ or seedling stage salinity tolerance in a DH population of 103 lines developed from CM72 (tolerant) and Gairdner (sensitive). Previous studies demonstrated that salinity tolerance associated QTL at seedling phase vary from those controlling similar response in other development stages (Angessa et al., 2017; Xue *et al.,* 2009). However, studies on salinity tolerance at the germination and seedling stages interphase, referred to as seedling survival in this paper, appears to be scant in barley. Seedling survival (The singular vigorous growth of the immediate post-germination phase without seedling loss) is an important trait in growing regions where dry seeding is practicing to fitting the crops growth period into the narrow growing season where a delayed seeding exposes flowering and grain filling stages to abiotic stress factors that cause massive yield and grain quality reduction. Lack of previous reports on seedling survival as a measure of

salinity tolerance and our previous findings of independent QTLs linked with salinity tolerance at germination and seedling stages lead us to; (i) to study seedling survival as a measure of salinity tolerance, (ii) to identify QTL associated with seedling survival, (iii) to identify phenotypic trait as a measure of seedling survival. Building on our previous study, our current study explored salinity tolerance in the stage between germination and seedling in two sets of barley germplasm.

4.3 Materials and methods

4.3.1 Germplasm

Two barley germplasm sets were used for this study. These are (i) 103 Doubled Haploid (DH) lines developed from a cross between CM72 (salinity tolerant parental genotype) and Gairdner (salinity susceptible parental genotype) (Angessa et al., 2017), and (ii) A diverse panel of selected 85 barley entries that included Australian barley varieties, breeding lines and landraces from across the globe representing six continents characterized by different head types and growth habits (Supplementary Table 4.1). The diversity panel of 85 barley entries were selected from our recent study conducted on salinity tolerance during germination (Mwando *et al.,* 2020a). Categorized based on the observed germination percentage (GP) under 150 mM NaCl stress, the diversity panel was randomly selected to represent four salinity tolerance categories namely, tolerant group (> 90% GP) (31 entries), moderately tolerant group (80-89% GP) (37 entries), susceptible group (65-79% GP) (10 entries) and sensitive group (< 65% GP) (seven entries) (Supplementary Table 4.2).

4.3.2 Germination Assays

Germination experiment of both germplasm sets was conducted using two treatments, namely 150 mM NaCl or deionised water (DI). Petri dishes with 9 cm diameter were fitted with two filter papers and 100 surface sterilized seeds placed in each. Surface sterilization of barley seeds was done using 10% sodium hypochlorite for 5 mins, followed by thorough rinsing with sterile water (Mwando *et al.,* 2020). All entries from both germplasm sets were subjected to two treatments in three replications and placed in a dark oven set at ~24^{0}C

temperature level. Sprouted seeds were counted after 72 hours from which Germination Percentage (GP) and germination tolerance index (GTI) were calculated as show in equation (i) and (ii) below. GP was the number of sprouted seeds expressed as a percentage of the total number of incubated seeds multiplied by 100 (Adjel *et al.,* 2013). GTI was the ratio of GP under NaCl stress treatment and GP of the same entry under DI water as detailed by Angessa *et al.,* (2017); Mwando *et al.,* (2020a).

$$\text{Germination Percentage (GP)} = \frac{\text{Number of germinated seeds}}{\text{Total number of seeds incubated}} * 100 \quad \text{(i)}$$

$$\text{Germination tolerance index (GTI)} = \frac{\text{Number of seeds germinated under NaCl stress}}{\text{Number of seeds germinated under deionised water}} * 100 \quad \text{(ii)}$$

4.3.3 Seedling survival and vigour evaluation.

Evaluation of entries from both sets of barley germplasm for seedling survival and seedling vigour was conducted on germinated seeds in a glasshouse with a controlled temperature level set to a maximum level of ~24 ºC using a hydroponic system (Wang *et al.,* 2017; Han *et al.,* 2018), (Supplementary Figure 4.1). Approximately 150 plump and uniform barley seeds pre genotype were selected, and surface sterilized in 10% sodium hypochlorite solution for ~5 minutes (Mwando eat al., 2020). The seeds were rinsed thoroughly with distilled water, and then germinated as explained in germination experiment using DI water and four different NaCl concentration levels namely 75, 90, 120 and 150 mM. After 4 days of incubation in control or salinity stress in an oven at ~24 ºC in dark condition, six uniform seedlings per treatment in three replicates were transplanted and placed in the holes of the lids of the plastic tanks assigned with control or salinity stress treatment as described below.

Six sets of 6L containers with a dimension of 25 cm length, 25 cm width × 12 cm depth were filled with improved Hoagland's nutrient solution (Han *et al.,* 2018). The modified Hoagland solution comprised of 19.9 µmol/L Fe (III)EDTA, 0.2 mmol/L KH_2PO_4, 2 mmol/L NH_4NO_3, 0.3 mmol/L K_2SO_4, 0.4 mmol/L $CaCl_2$, 46.9 µmol/L H_3BO_3, 0.4 mmol/L $MgSO_4$, 4.5 µmol/L $MnCl_2$, 0.19 µmol/L $CuSO_4$, 1 µmol/L Na_2MoO_4 and 0.38 µmol/L $ZnSO_4$ (Han *et al.,* 2018).

The containers were then covered with lids with holes that were in turn covered by Kimberly Clark Professional Kleenex Compact Towel tissues. The containers were assigned with control treatment which received pure modified Hoagland's nutrient solution, or four salinity stress levels. Salinity stress assigned containers received modified Hoagland's nutrient solution and four levels of salinity stress. All the containers were fitted with an electric pump that constantly aerated the solution. Completely randomized design with three replications was used and the experiments were repeated four times. The solution in each tank was replaced after seven days and the pH adjusted to 6.5 through addition of NaOH and/or HCl (Karunarathne *et al.,* 2020).

Fourteen days after transplanting, three plants per replication were harvested from each entry per treatment and replication. The roots (R) were separated from the shoots (S) at the base, to record the length (L) of each; root length (RL) and shoot length (SL). Harvested root and shoot were put in labelled bags and oven dried at 80 ^{0}C temperature for 72 hours after which dry weight (DW) of was recorded independently; root dry weight (RDW) and shoot dry weight (SDW). Using length and weights measurements recorded on shoots and roots for both treated (tr) and control (ck), Tolerance Indices (I) of roots, shoots were calculated as follow.

$$\text{Root length index (RLI)} = \frac{\text{salt treated root L}}{\text{control root L}} * 100 \quad \text{(iii)}$$

$$\text{Root dry weight index (RDWI)} = \frac{\text{salt treated root DW}}{\text{control root DW}} * 100 \quad \text{(iv)}$$

$$\text{Shoot length index (SLI)} = \frac{\text{salt treated shoot L}}{\text{control shoot L}} * 100 \quad \text{(v)}$$

$$\text{Shoot dry weight index (SDWI)} = \frac{\text{salt treated shoot DW}}{\text{control shoot DW}} * 100 \quad \text{(vi)}$$

$$\text{Root to shoot ratio by Length (R/SL)} = \frac{\text{root length}}{\text{shoot length}} \quad \text{(vii)}$$

Root to shoot ratio by DW (R/SDW) = $\frac{\text{root DW}}{\text{shoot DW}}$ (viii)

Root/shoot ratio by length Index (R/SLI) = $\frac{R/_{SL}\ Treated}{R/_{SL}\ Control}$ (ix)

Root/shoot ratio by DW Index (R/SDWI) = $\frac{R/_{SDW}\ Treated}{R/_{SDW}\ Control}$ (x)

Note: - Seedling length or dry weight is the sum of roots and shoots length or dry weight.

Assessment for seedling vigour (SV) was based on previous study that used germination capacity, which is the ability of germinating seeds to give normal seedlings, to estimate seedling vigour and represent the extent of salinity damage to the seedling (Zhao *et al.*, 2016). SV was calculated as the sum of average of the root and shoot vigour, seedling vigour index (SVI) estimated by modified formula suggested by Abdul-Baki and Anderson, (1973); Sagar *et al.*, (2019); Podder *et al.*, (2020) as the product of seedling vigour (root and shoot length/weight) by germination percentage, while the Relative seedling vigour indices (RSVI) were the percentage of trSVI divided by ckSVI.

Treated SV by length (trSVL) = $\sum(\frac{\text{(Root L + shoot L) Treated}}{n})$ (xi)

Control SV by length (ckSVL) = $\sum(\frac{\text{(Root L + shoot L) Control}}{n})$ (xii)

Treated SV by DW (trSVDW) = $\sum(\frac{\text{(Root DW + shoot DW) Treated}}{n})$ (xiii)

Control SV by DW (ckSVDW) = $\sum(\frac{\text{(Root DW + shoot DW) Control}}{n})$ (xiv)

Treated seedling vigour index (SVI) by length (trSVIL) = $\sum(\frac{\text{(Root L + shoot L) Treated}}{n}) * GP\ Treated$ (xv)

Control SVI by length (ckSVIL) = $\sum(\frac{\text{(Root L + shoot L) Control}}{n}) * GP\ Control$ (xvi)

Relative seedling vigour index by length (RSVIL) = $\frac{\text{trSVIL}}{\text{ckSVIL}} * 100$ (xvii)

Treated seedling vigour index (SVI) by DW (trSVIDW) = $\sum(\frac{(\text{Root DW + shoot DW) Treated}}{n}) * GP\ Treated$ (xviii)

Control SVI by DW (ckSVIDW) = $\sum(\frac{(\text{Root DW + shoot DW) Control}}{n}) * GP\ Control$ (xix)

Relative seedling vigour index by DW (RSVIDW) = $\frac{\text{trSVIDW}}{\text{ckSVIDW}} * 100$ (xx)

where n refers to number of individual counts.

4.3.4 Phenotypic data analysis.

The presented data unless where it is specified, are the means of three independent replications. Pearson correlation coefficients was used to estimate the association between phenotypic traits pairs and analysis of variance (ANOVA) was computed to determine if there were significant differences between treatments with the differences significance among mean values tested with Duncan's Multiple Range tests (DMRT) at $p < 0.05$. These analyses together with plotting of the graphs were performed using SPSS statistical computer software program (IBM Corporation, 2017).

4.3.5 DNA extraction and molecular marker selection

Fresh leaf tissues were collected from all DH lines and their parental genotypes (used to build up the linkage map) when their seedlings reached three leaves stage and their genomic DNA extracted using CTAB method as described by Stein *et al.,* (2001), and further decontaminated through RNase treatment. Polymorphisms of each markers was then determined by polymer chain reaction (PCR) reactions in a volume of 10 µl and electrophoresis; first using the two parents (CM72 and Gairdner), and then followed by the analysis of individual lines using the selected primers (Wang *et al.,* 2003). The Simple Sequence Repeat (SSR) markers were separated by either 2% agarose gel or 6% polyacrylamide gel electrophoresis (PAGE) and visualized under UV following ethidium

bromide staining. A total of 350 (Diversity Array Technology Pty Ltd, Australia) (DArT) markers (http://www.diversityarrays.com) and 84 single sequence repeat (SSR) markers were selected to survey DH population.

4.3.5. Genetic linkage map construction and QTL analysis

An integrated genetic linkage map was developed using MapQTL 5.0 software (Ooijen, 2004), with 350 Diversity Array Technology (DArT) and 84 Simple Sequence Repeat (SSR) markers. Interval mapping (IM) function was used to perceive significant QTLs passing a logarithm of odds (LOD) and a threshold score of 3.0 at $P < 0.05$ was used to declare a significant locus for phenotype data. This was predicted by execution genome-wide permutation analysis done in MapQTL 5.0 with 1000 permutations of the original data on each salinity seedling survival trait that resulted in a 95% LOD threshold of around 3.0. The intervals flanking each QTL on the right and left was determined, by taking two positions peak, with LOD values lower than the maximum after performing restricted multiple QTL model (MQM) mapping. R^2 which is the percentage variance elucidated by each QTL was attained by restricted MQM mapping (Ooijen, 2004). The graphical image of the linkage map and QTL was done in MapChart 2.2 (Voorrips, 2002). Broad sense heritability (H^2) estimates were done using the formula proposed by Sayed et al., (2017) using genotypic ($\sigma^2 g$) and phenotypic (σ^2 p) variances. The respective genotypic and phenotypic variances were calculated from the mean squares of the genotypes (MSg) and experimental error (MSe) as shown below.

$$\text{Genotypic variance } (\sigma^2 g) = \frac{MSg - Mse}{r} \quad \text{(xxi)}$$

$$\text{Error variance } (\sigma^2 e)\ \sigma^2 e = MSe \quad \text{(xxii)}$$

$$\text{Phenotypic variance } (\sigma^2 p) = \sigma^2 g + {}^{\sigma^2}/_{r} \quad \text{(xxiii)}$$

$$\text{The coefficient of broad sense heritability } (H^2) = \frac{\sigma^2 g}{\sigma^2 p} \quad \text{(xxiv)}$$

where r refers to number of replicates.

4.4 Results

4.4.1 Phenotypic response to salinity stress

Salinity stress reduced seedling survival traits recorded on young plants from seeds germinated under various salinity levels in the DH population (103 lines) and diverse barley germplasm panel (85 entries) without any mortality. Salt concentration of 150 mM NaCl had a higher effect on DH lines seedling survival than 75, 90 and 120 mM NaCl treatment levels (Figure 4.1A). In our second experiment of diverse barley germplasm panel, we only used two salinity levels of 75 and150 mM NaCl because we didn't record much variation among 75, 90 and 120 mM NaCl in the DH population trial (Figure 4.1A and 4.1B). The lowest salinity stress level of 75 mM NaCl increased root length (18.9cm) with almost no variation observed in the whole seedling length (4.7cm) and root weight (1.0 mg) compared to observations in the control treatment (Figure 4.1B). Overall, among the four levels of salinity concentration treatments used in this study, the highest level of 150 mM NaCl adversely and consistently affected shoot, root and seedling length or dry weight in both the DH population and diverse barley germplasm panel. Compared with observations made under control treatment, the 150 mM NaCl concentration treatment caused almost 50% reduction in most traits (Figure 4.1A and 4.1B).

Due to strong effect of 150 mM NaCl level on almost all the traits, our further investigation into genotypic differences in seedling survival in two barley germplasm sets in this study was based on observations made under the highest salinity level and the control treatments. Salinity stress with 150 mM NaCl reduced all the traits in both DH population and diversity panel barley panel (Figure 4.2). Salinity stress didn't only reduced germination, but it also impacted negatively on seedling growth to an extent of 50% (Table 4.1). High germination tolerance index was not a direct reflection of a high Relative seedling vigour index (RSVI) in selected barley accessions, for instance, WABAR2347 had the highest germination index (97.0%) but with (50.2% and 53.1%) RSVI by length and DW respectively (Figure 4.3). Starting with DH population then diversity panel barley set, the highest reduction was recorded in shoot length (65.6 & 50.4%) followed by seedling vigour index by length (56.5

& 41.0%), while root length (28.6 & 15.8%) and root dry weight (29.3 & 28.0%) were least reduced when control was compared to150 mM NaCl stress treatment respectively. Seedling vigour indices best explained the seedling survival of sprouted plants after germinating in salinity stress since it related the two traits (germination and early seedling growth). Barley entries seedling stage phenotypic traits variation assessed under 150 mM NaCl stress treatment exhibited considerable variations in both CM72/Gairdner DH population and barley germplasm diversity panel (Table 4.1). The average GTI was highest for both DH population and diversity panel at 81.3 and 83.7%, while R/SDWI was the lowest at 12.4 and 13.4% respectively. BmnL-75 had the highest RSVI for both length (84.5%) and weight (79.9%) but with a GTI of 88.9% among the diversity panel germplasm set (Figure 4.3). Interestingly, C01P-53 recorded the lowest level of indices in GIT (42.4%), RSVIL (25.5%) and 21.6% for RSVIDW (Figure 4.3 and Supplementary table 4.3).

In DH population, trSVIL ranged from 46.3 - 131.6 cm, RSVIL from 26.1 - 80.3%, trSVIDW from 0.5 – 1.9 g. and RSVIDW from15.1 - 76.7%. The range of values for seedling vigour indices in barley diversity panel were; 23.0 – 118.4cm for trSVIL, 25.4 – 88.3%for RSVIL, 0.8 – 3.3 g for trSVIDW and 22.0 – 84.8% for RSVIDW. The highest value Relative seedling vigour indices (RSVI) for both L and DW were recorded in BmnL-75 ((84.4 and 79.9%) while the lowest was (25.5 and 21.6%) recorded in C01P-53 (Table 4.1). Frequency distributions of RSVIL and RSVIDW are shown in Supplementary Figure 4.2, the traits showed a normal distribution and the DH population was transgressive.

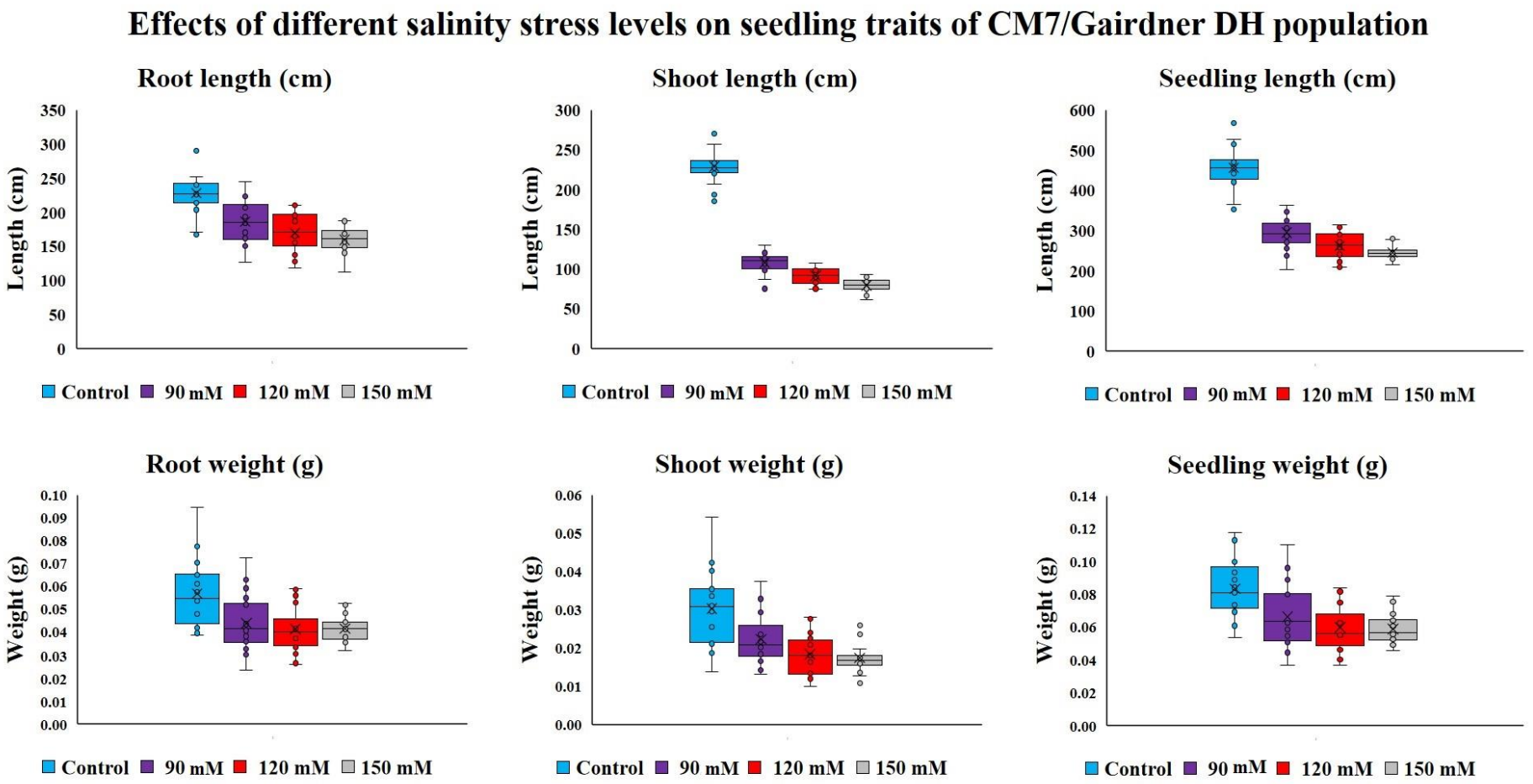

Figure 4.1A Block plots for seedling survival traits of CM72/Gairdner DH populations under different levels of NaCl concentrations (mM) grown in hydroponic solutions after germinating under the same conditions.

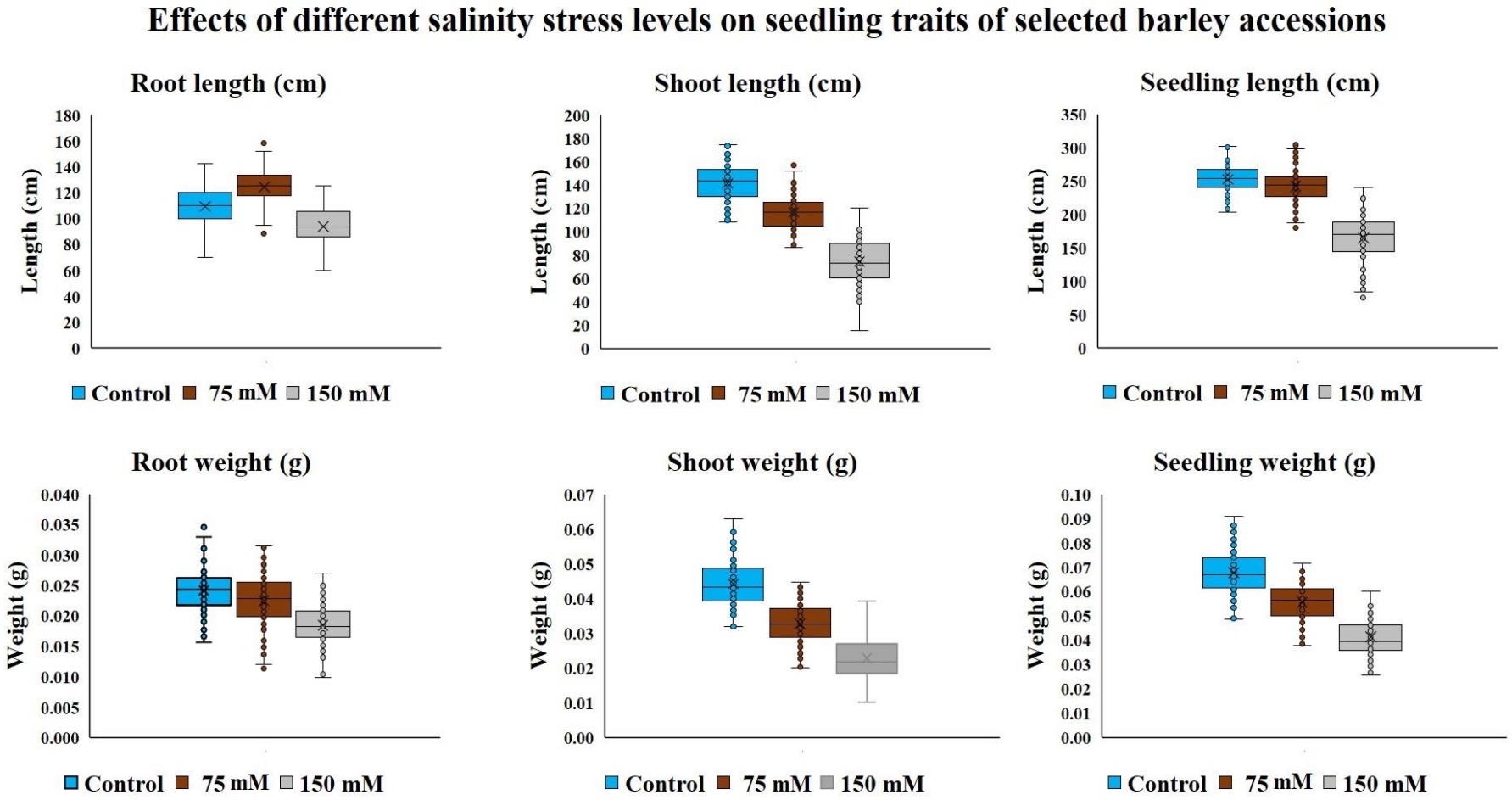

Figure 4.1B Block plots for seedling survival traits of selected barley accessions under different levels of NaCl concentrations (mM) grown in hydroponic solutions after germinating under the same stress.

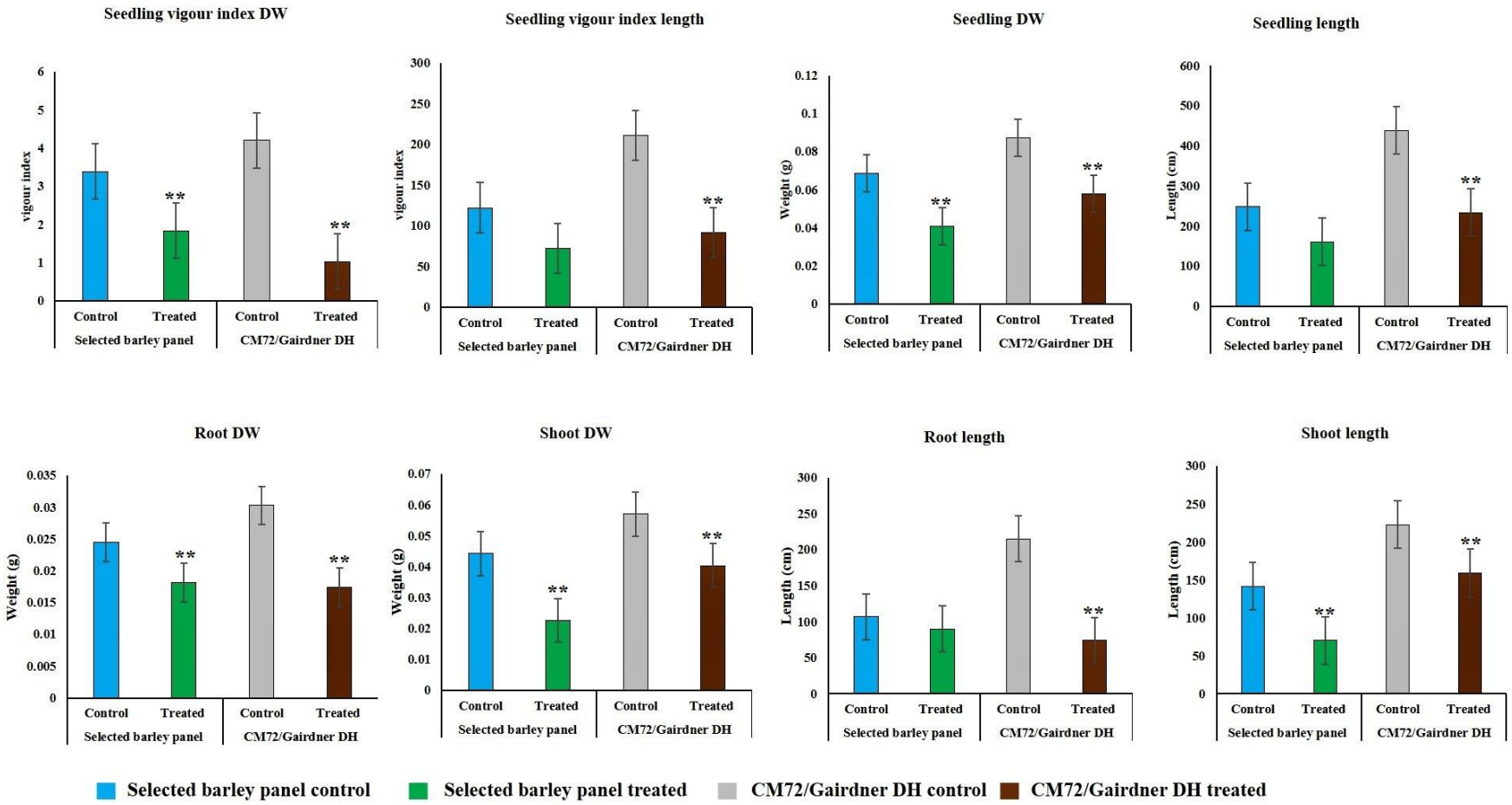

Figure 4.2 Effect of 150 mM NaCl on seedling survival traits and seedling vigour indices of CM72/Gairdner DH populations and selected barley diversity panel. Values are given as mean±SD of six experiments in each group. Error bars which are not overlapping differ significantly at P≤0.05 and ** indicate significant difference between control and in the salt treatment at P < 0.05.

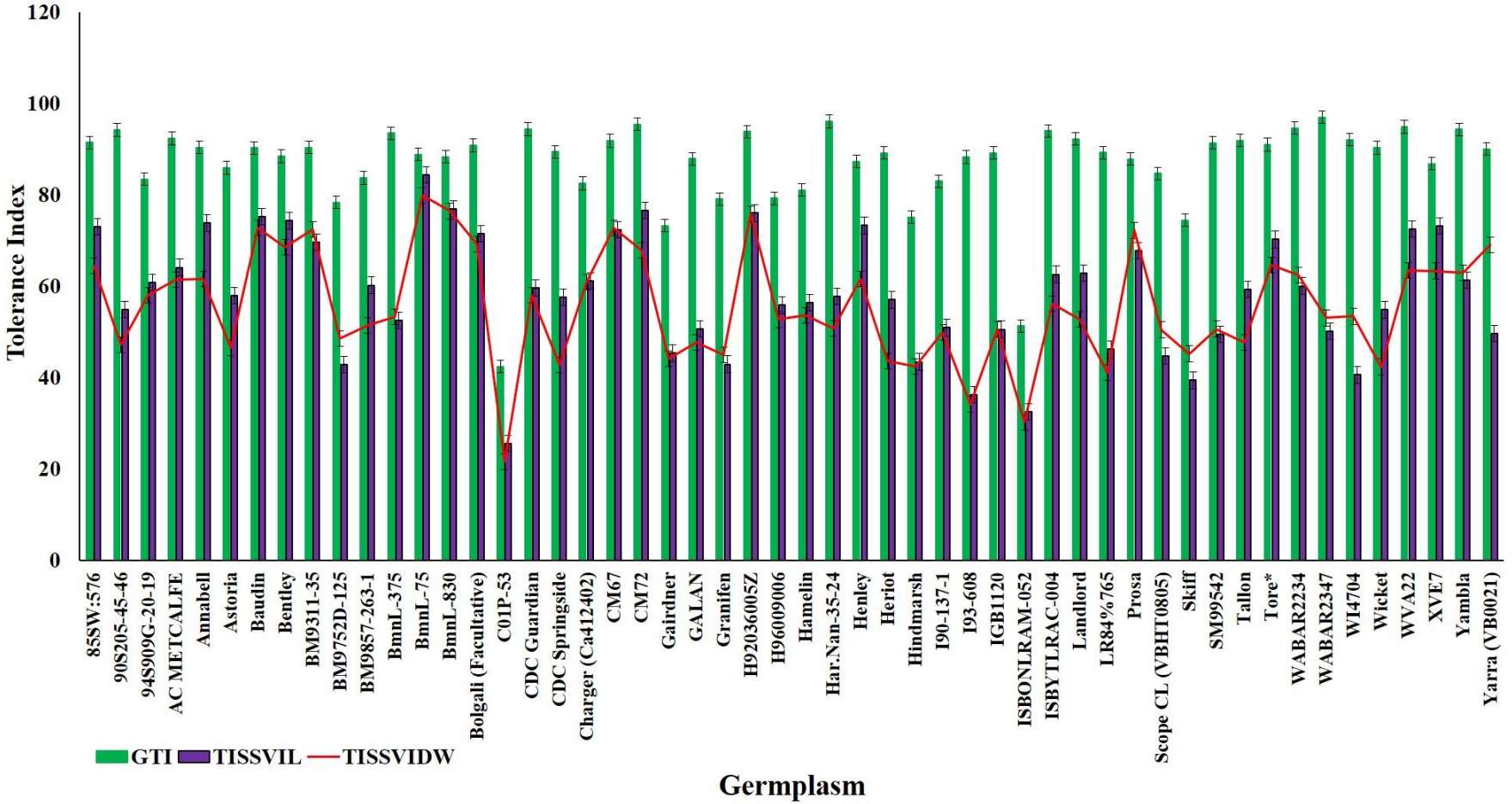

Figure 4.3 A Representation of the highly tolerant and extremely sensitive 15 genotypes for germination tolerance index (GTI) blue line, Relative seedling vigour index by length (RSVIL) red line and Relative seedling vigour index by DW (RSVIDW) green line.

Table 4.1 Seedling survival extent of variations in barley germplasms in response to 150 mM NaCl salinity stress.

Germplasm set	Variable	Mean	Max	Min	Standard Deviation	Coefficient of variations	Standard error	Between genotypes
DH population	GTI	81.3	97.0	47.4	16.3	0.2	4.1	**
	SLI	35.5	62.5	23.3	9.2	0.3	2.3	**
	RLI	72.7	91.8	59.8	10.0	0.1	2.5	**
	R/SLI	21.5	37.2	11.6	5.6	0.3	0.1	**
	SDWI	60.6	78.8	40.0	12.6	0.2	3.1	**
	RDWI	72.9	100.8	51.0	15.2	0.2	3.8	**
	R/SDWI	12.4	20.0	6.5	3.1	0.2	0.1	**
	trSL (cm)	74.4	93.3	40.0	15.0	0.2	3.7	**
	trRL (cm)	159.1	186.7	112.5	19.3	0.1	4.8	**
	trR/SL	2.2	3.3	1.7	0.4	0.2	0.1	**
	trSDW (g)	1.70	2.60	1.10	4.1	0.2	1.1	**
	trRDW (g)	4.02	5.33	2.04	8.2	0.2	2.4	**
	trR/SDW	2.3	3.0	1.9	0.3	0.1	0.1	**
	trSVIL	91.7	131.6	46.3	23.9	0.3	6.0	**
	RSVIL	44.0	80.3	26.1	11.2	0.2	2.8	**
	trSVIDW	1.0	1.9	0.5	0.3	0.3	0.1	**
	RSVIDW	25.6	76.7	15.1	9.7	0.4	2.4	**
Diversity panel	GTI	83.7	97.0	42.4	12.2	0.2	1.5	**
	SLI	42.8	90.0	11.4	13.7	0.5	2.4	**
	RLI	71.3	101.7	57.2	13.3	0.4	3.4	**
	R/SLI	14.9	62.1	9.5	7.2	0.6	0.1	**
	SDWI	43.8	96.6	13.4	13.1	0.5	2.3	**
	RDWI	64.8	100.0	44.2	12.1	0.5	3.2	**
	R/SDWI	13.4	47.7	8.6	5.7	0.6	0.1	**
	trSL (cm)	59.1	120.0	13.3	22.3	0.5	3.5	**
	trRL (cm)	78.0	125.0	30.0	19.1	0.5	3.9	**
	trR/SL	1.2	7.3	0.7	0.9	0.8	0.1	**
	trSDW (g)	1.69	2.74	0.79	3.8	0.5	1.4	**
	trRDW (g)	1.92	3.95	0.57	6.8	0.5	1.2	**
	trR/SDW	0.7	3.3	0.4	0.4	0.7	0.1	**
	trSVIL	60.2	118.4	23.0	18.4	0.5	3.4	**
	RSVIL	47.9	88.3	25.4	13.3	0.5	2.7	**
	trSVIDW	1.5	3.3	0.8	0.5	0.5	0.1	**
	RSVIDW	44.6	84.8	22.0	11.7	0.5	2.5	**

Note: **, Significant at the 0.05 probability levels between genotypes. Where: GTI – Germination tolerance index, SLI –Shoot length index, RLI –Root length index, R/SLI – Root to shoot length index, SDWI –Shoot dry weight index, RDWI –Root dry weight index, R/SDWI – Root to shoot ratio dry weight index, trSL – Shoot length treated, trRL – Root length treated, trR/SL – Root to shoot ratio length treated, trSDW – Shoot dry weight treated, trRDW – Root dry weight treated, trR/SDW – Root to shoot ratio dry weight treated, trSVIL – Treated seedling vigour index length, RSVIL – Relative seedling vigour index length, trSVIDW - Treated seedling vigour index dry weight and RSVIDW – Relative seedling vigour index dry weight.

4.4.2 Correlation among seedling traits

Pearson correlation between seedling survival traits under 150mM NaCl are presented separately for CM72/Gairdner DH population and barley diverse panel (Table 4.2). Associations among all the recorded seedling character ranged from positive to negatives, for both DH population and diverse panel. The highest correlation coefficients (r = 0.8**) was detected between trRDW and trSDW for the DH population and (r = 0.9**) observed between

trSVIL and RSVIL for diversity panel set. The results indicated that there were significant positive correlations among trSVIL, RSVIL, trSVIDW treated and RSVIDW at $p < 0.05$ in both sets of genotypes (Table 4.2). GTI showed positive correlations with trSVIL, RSVIL, trSVIDW and RSVIDW in both sets with varying strengths, but significant ($p < 0.05$).

4.4.3 Bi – parent QTL mapping for seedling survival traits

The CM72/Gairdner DH population showed a transgressive segregation in relation to their individual parents for seedling survival forming the basis for mapping of QTL for salinity stress tolerance (Supplementary Figure 4.2). We did not do QTL and marker – trait association analysis for GTI for DH population and diverse panel because we had already reported them in our previous studies. Three hundred and fifty DArT and 84 SSR markers in our Bi parent population were polymorphic amongst (CM72 and Gairdner), and were thereafter evaluated in the remaining 103 lines before the scores being used for genetic linkage map construction. We detected new QTLs for seedling survival traits under salinity stress totaling to 13 on chromosomes 1H, 3H and 4H (Table 4.3 and Supplementary Figure 4.3). Two markers bPb-9624 (173.172 cM) and bPb-1278 (78.006 cM) were mapped on chromosome 3H and 4H for trRL with LOD values of 7.7 and 3.6 explaining 29.0 and 7.6% of the phenotypic variations respectively. Marker bPb-9624 was contributed by CM72 with an additive effect of 3.5, while bPb-1278 had -1.8 from Gairdner.

Two other marker bPb-3623 (190.268 cM) and bPb-5666 (189.664 cM) on 3H were detected for trSVL at position 190.268 cM and 189.664 cM having LOD values of 5.1 and 5.0 respectively that explained between 19.0 – 21.0% of the phenotypic variation from CM72 parent (Table 4.3). Almost at the same position (190.981 cM) as trSVL, marker bPb-6383 (190.981 cM) was mapped on 3H for trSVDW, it recorded LOD value of 3.5 that contributed 10.5% of the variation from CM72. We further mapped 8 markers, 3 for trSVIL (bPb-4576 & bPb-9624 on 3H and bPb-9820 on 4H) and 5 for trSVIDW (Bmac0032, bPb-9418 & bPb-4741on 1H respectively and bPb-4616 & bPb-9624 on 3H). The position of trSVIL markers were 184.281 & 173.172 cM for bPb-4576 & bPb-9624 on 3H and bPb-9820 on 4H was at 244.943 cM, while trSVIDW markers were located at 28.004, 40.875 & 25.075 cM on 1H respectively and 142.225 & 173.172 cM on 3H. All the trSVIL and trSVIDW markers except bPb-9820 were contributed by CM72 and were explaining between 8.1 – 21.8% of the total phenotypic variation. Its worthy noting that marker bPb-9624 was detected for trRL, trSVIL and trSVIDW (Table 4.3).

Table 4.2 Pearson correlation among seedling survival traits of diverse barley panel (above) and CM72/Gairdner DH (below main diagonal) in 150 mM NaCl

	GTI	trSL	trRL	trR/SL	trSDW	trRDW	trR/SDW	trSVIL	RSVIL	trSVIDW	RSVIDW
GTI	1	0.002	0.529 **	0.189	0.002	0.281 **	0.14	0.471 **	0.461 **	0.390 **	0.462 **
trSL	0.043	1	0.401 **	−0.698 **	0.831 **	0.350 **	−0.678 **	0.747 **	0.694 **	0.639 **	0.549 **
trRL	0.588 **	0.689 **	1	0.07	0.291**	0.653 **	0.036	0.799 **	0.729 **	0.542 **	0.502 **
trR/SL	0.299 **	−0.859 **	−0.251 **	1	−0.584 **	−0.064	0.872 **	−0.319 **	−0.305 **	−0.334 **	−0.297 **
trSDW	0.390 **	0.470 **	0.614 **	−0.211**	1	0.392 **	−0.726 **	0.626 **	0.569 **	0.803 **	0.618 **
trRDW	0.177	0.676 **	0.770 **	−0.383 **	0.815 **	1	0.064	0.588 **	0.524 **	0.709 **	0.499 **
trR/SDW	0.365 **	0.278 **	0.162	−0.277 **	−0.392 **	0.202	1	−0.335 **	−0.316 **	−0.413 **	−0.374 **
trSVIL	0.809 **	0.188	0.486 **	−0.01	0.031	0.337 **	0.463 **	1	0.932 **	0.848 **	0.760 **
RSVIL	0.831 **	−0.172	0.042	0.216 **	−0.427 **	−0.155	0.439 **	0.744 **	1	0.779 **	0.837 **
trSVIDW	0.442 **	0.612 **	0.631 **	−0.403 **	0.483 **	0.684 **	0.234**	0.314 **	0.251 **	1	0.799 **
RSVIDW	0.339 **	0.443 **	0.306 **	−0.346 **	−0.119	0.144	0.414 **	0.203	0.496 **	0.719 **	1

1 0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 0 -0.1 -0.2 -0.3 -0.4 -0.5 -0.6 -0.7 -0.8 -0.9 -1

Pearson moment correlation coefficients (r) between the analysed traits (yellow – green) denotes high positive correlation ($r \rightarrow 1$), (yellow – red) high negative correlation ($r \rightarrow -1$) and blue the diagonal. Where: GTI – Germination tolerance index, trSL – Shoot length treated, trRL – Root length treated, trR/SL – Root to shoot ratio length treated, trSDW – Shoot dry weight treated, trRDW – Root dry weight treated, trR/SDW – Root to shoot ratio dry weight treated, trSVIL – Treated seedling vigour index length, RSVIL – Relative seedling vigour index length, trSVIDW - Treated seedling vigour index dry weight and RSVIDW – Relative seedling vigour index dry weight. ** Significant Pearson correlation coefficient at 0.05 probability levels.

4.4.4 Core QTLs regions for seedling survival traits under salinity stress mapped on 3H and 1H

A total of 8 markers were detected on chromosome 3H, with a region flanked by markers bPb-4576 and bPb-9624 in the DH population being detected in more than one trait (trRL, trSVIL and trSVIDW). Notably three more markers detected on 3H, bPb-3623, bPb-5666 and bPb-6383 were closely linked to the above flanking makers while, bPb-4616 was a distance from them (Table 4.3). On 4H we mapped 2 markers, bPb-1278 and bPb-9820 that were distance from each other whereas, on 1H we recorded 3 markers Bmac0032, bPb-9418 and bPb-4741 that were closely linked to each other. The region mapped by markers on 3H and 1H can therefore be considered for further validation and fine mapping.

Table 4. 3 Quantitative Traits Loci (QTL) linked with seedling survival traits mapped using CM72/Gairdner DH population

Trait	Marker	Chr	Position cM	LOD	Variance	% Explained	Additive effect	Tolerance source	H2
trRL	bPb-9624	3	173.172	7.67	27.117	29.00	3.507	CM72	0.71
	bPb-1278	4	78.006	3.55	24.200	7.60	-1.792	Gairdner	
trSVL	bPb-3623	3	190.268	5.14	86.774	20.50	4.972	CM72	0.82
	bPb-5666	3	189.664	4.95	87.511	19.90	4.899	CM72	
trSVDW	bPb-6383	3	190.981	3.45	0.051	10.50	0.086	CM72	0.77
trSVIL	bPb-4576	3	184.281	4.10	0.056	17.40	0.113	CM72	0.79
	bPb-9624	3	173.172	4.06	0.056	17.20	0.110	CM72	
	bPb-9820	4	244.943	3.01	0.050	10.10	-0.081	Gairdner	
trSVIDW	bPb-4616	3	142.225	3.40	0.089	15.80	0.140	CM72	0.68
	Bmac0032	1	28.004	3.21	0.077	8.60	0.100	CM72	
	bPb-9418	1	40.875	3.17	0.077	8.40	0.100	CM72	
	bPb-4741	1	25.075	3.09	0.077	8.10	0.098	CM72	
	bPb-9624	3	173.172	5.36	0.031	21.80	0.105	CM72	

4.5 Discussions

4.5.1 The dynamic reaction of barley seedlings to salinity induced stress

In a plant life cycle, seeds have maximum capacity to tolerate environmental pressures, while germination and seedlings growth stages are extremely delicate. Therefore, to establish a good crop population stand requires well adapted genotypes both at germination and seedling growth stages (Qu *et al.,* 2008; Debez *et al.,* 2019). Seed germination is impaired by salinity stress in barley and its tolerance depends on the genotype (Angessa *et al.,* 2017; Mwando *et al.,* 2020a). Salinity stress delays/ inhibits the imbibition process of a seed during germination by reducing the surrounding osmotic potential making the emerging seedling less vigorous. In our context, NaCl reduced seed germination and seedling vigour of barley genotypes as indicated by Figure 1 and 2. The findings indicated the difference in responses for seed germination and seedling developmental traits of DH population and selected barley diversity panel under salinity stress. As expected, there was higher negative impact of the salt on both germination and seedling traits when the concentration of NaCl was increased. This results were in line with those reported by Kilic and Kahraman, (2016), using 0.0, 0.25, 0.275, 0.30 M salt concentrations in barley (*Hordeum vulgare* cv. Bülbül 89), 0.0, 1.0, 1.5, 2.0 and 2.5% in Sorghum (*Sorghum bicolor* L. Moench) and 160 mM NaCl in maize (*Zea mays* L.) by (Wang *et al.,* 2014; Cui *et al.,* 2015; Rajabi Dehnavi *et al.,* 2020).

At 150mM NaCl, the highest concentration level used in our study, it was recorded that the germination tolerance index and seedling vigour indices was varied among the barley genotypes. After seed germination (root emerging from seed), the growth of shoot starts through a process of intense cell divisions that is very responsive to water scarcity (Nonogaki *et al.,* 2010), and more sensitive to hostile ionic ratio because of undeveloped vacuole (Alam *et al.,* 2002). Consequently, the suppressed growth of barley seedlings can be linked to inhibition effect of salinity stress during germination (Kilic and Kahraman, 2016). This is an indication that the negative effect of salinity on seedling survival in this study was initially due to osmotic stress. The findings reported by Kilic and Kahraman, (2016), suggest similar mechanisms when hydrogen peroxide (H_2 O_2) was applied as an osmo-protectant in barley to alleviate salinity stress effect during germination and early seedling growth. The findings also go in line with what was reported in Grass Pea (*Lathyrus sativus* L.) (Tokarz *et al.,* 2020), rice (*Oryza sativa*), soybean (*Glycine max*), wheat (*Triticum aestivum*) and *Arabidopsis* plants (Kataria and Verma, 2018).

Early seedling vigour (ESV), determines even emergence and speedy development of plants in stressing condition in which they grow and stabilise the ultimate yield (Wen *et al.,* 2018). In most cases, it is expressed in terms of seedling height and weight with little regards to the germination capacity (Lu *et al.,* 2007; Capo-chich *et al.,* 2019). However, seedling emergence under field condition is an indication of a rapid germination and early seedling vigour which can be best expressed by Seedling vigour index (SVI). SVI is influenced by physiological, genetic, storage and the germinating condition of a seed (Capo-chich *et al.,* 2019). In the current study, storage and germinating conditions was held constant therefore, the physiological and genetic aspects were evaluated though genotype-phenotype mapping by QTL analysis (Zhu *et al.,* 2008).

4.5.2 Major QTLs locations and the comparison of the two analysis methods

The QTLs reported for salinity tolerance at germination stage in our previous studies (Angesa *et al.,* 2017; Mwando *et al.,* 2020), were different from what is reported here for seedling survival traits. Using the same DH population included in this study, Xue *et al.,* (2009) and Angessa *et al.* (2017) mapped QTLs for early seedling growth stage, late phenotypic agronomic and physiological traits on all chromosomes. Comparable to their findings, our seedling survival QTLs were located on chromosomes 1H, 3H and 4H (Table 5). The QTLs located on 3H and 4H and were closely linked to those reported by Angessa *et al.,* (2017). Two QTLs on 3H contributed by CM72 and recognized by markers bPb-4576 (184.281 cM) and bPb-9624 (173.172 cM) were closely linked to bPb-6504 (176.5 cM) associated with 50% seedling emergence in 150 mM NaCl dry weight, fresh weight and dry weight of 50% full 1st leaf expansion under 150 mM NaCl reported by Angessa *et al.,* (2017). The same markers (bPb-4576 and bPb-9624) were distantly linked to bPb-1961 (161.1 cM) and bPb-1579 (159.8 cM) reported previously together with bPb-3634 (192.8 cM) for fresh weight of 50% full 1st leaf expansion under 150 mM NaCl (Angessa *et al.,* 2017) and dry weight at late growth stage under control condition (Xue *et al.,* 2009).

Using 172 DH lines generated from YYXT (salinity-tolerant) and Franklin (salinity-sensitive) Zhou *et al.,* (2012) identified 5 QTLs for salinity tolerance on 1H, 2H, 5H, 6H and 7H, accounting for not less than 50% of the fore-leaf chlorosis and plant survival difference. The experiment also included the two parents CM72 and Gairdner used in this study whose results confirmed their difference in response to salinity stress as reported here. Mano and Takeda, (1997), used two DH populations derived from the crosses, Steptoe/Morex and

Harrington/TR306 by interval mapping analysis to detect 5 QTLs governing salinity stress tolerance at germination and the seedling stage. The study findings indicated that QTLs for salinity tolerance at germination is different from those at seedling stage. Later, twenty-three QTLs were reported by Siahsar and Narouei, (2010) for salt tolerance using Steptoe×Morex DH population at seedling stage on all chromosomes except 4H. Further in hydroponic experiment Xu *et al.,* (2012), reported one major QTL on 2H controlling 50% of phenotypic variation to salinity tolerance in Chinese landrace of barley using 188 DH lines generated from TX9425 (salinity tolerant) and Naso Nijo (salinity sensitive) by leaf chlorosis and plant survival. Using the same population as Xu *et al.,* (2012), Aminfar *et al.,* (2011) reported four QTLs on 1H, 2H, 5H and 7H for physiological traits linked with salt tolerance at seedling stage in barley. Like our study, Fan *et al.,* (2016) used 206 barley genotypes from across the globe in a potting experiment inside a glasshouse to identify 24 markers associated with salinity stress on all chromosomes except 1H by combining scores for plant survival and leaf chlorosis. Marker bPb-6504 reported in that study on 3H was closely linked to two markers bPb-4576 and bPb-9624 reported in the current study illumination a possibility of them being the same QTL. Using 2,671 barley lines a USDA mini-core collection Hazzouri *et al.,* (2018), identified a SNPs on 4H associated with salt tolerance in a region with *HKT1;5* ion transporter (HORVU4Hr1G087960, location; 638634849 – 638636785bp) that is responsible for withdrawing Na^+ from the xylem. The location of the SNPs is very close to the marker (bPb-9820) on 4H (638546732bp) reported in our current results. Also, Fan *et al.*, (2016), predicted the possibility of a salinity tolerance genes on 4H related to ion homeostasis linked to marker bPb-9668. Using Association Mapping in a structured barley population of 103 Mohamed *et al.,* (2015) reported 9 markers for salt tolerance, 12 for Potassium ion (K^+) and Sodium ion (Na^+) across the whole barley genome.

We predicted a region on chromosome 3 that is likely to harbour putative genes for seedling salinity survival after salinity stress germination. A search in barley genome (Mascher *et al.*, 2017) gave us 36 genes (Supplementary table 4) from the flanking region. Late embryogenesis abundant protein Lea, receptor-like kinase and Dehydrin families were noticeable, all of which have been associated with drought and salinity stress (Kosová *et al.,* 2014; Passricha *et al.,* 2020; Mwando *et al.,* 2020b). The finding of this research can be further validated and fine mapped by either GWAS or other DH populations. Using GWAS approach, a diverse panel of barley accessions with a wide geographic origin having 632 genotypes and 30,543 SNP markers by Jia et al., (2020) is perfect. Alternatively, using a DH population developed form

parents with contrasting salinity tolerance like TX9425 salinity tolerant and Naso Nijo salinity sensitive by Xu *et al.*, (2012) and YYXT (salinity tolerant) and Franklin (salinity-sensitive) by Zhou *et al.*, (2012) in relation to the current DH population can be used.

4.6 Conclusions and recommendations

In the current study, we recorded a large phenotypic difference among early seedling growth traits in response to salinity stress. Two major QTLs for seedling vigour indices were mapped on chromosome 3H using CM72/Gairdner DH population in addition to 2 more on 1H and 4H. Further literature search indicated that some genes in the hotspot on 3H have been reported to enhance salinity and other abiotic stresses in plants. The supposed candidate genes and identified markers will be a source of important information for studies on gene finding and functional analysis and marker-assistant selection (MAS) breeding. Before that, we recommend validation studies to evaluate the allele effects precisely using a larger number of accessions by Genome-Wide Association Analysis (GWAS) and other DH populations. Extra fine mapping of the region on 3H and 1H will enable narrowing down to the putative gene and development of stable barley varieties with vigorous seedlings under salinity through genetic transformation and associated studies. The identified QTLs will form an important basis for development of new barley lines that can survive salinity stress during germination and early seedling stage. The markers will be used for pyramiding the seedling salinity survival QTLs by MAS and allow for release of barley varieties that will have a vigorous seedling growth in saline prone region of the world.

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4.8 Supplementary Material

4.8.1 Supplementary Tables

Supplementary Table 4.1 Accessions used for association analysis

No.	Accession name	Origin	Region	Head type	Growth habit
1	04S213D-B-125	AUS	Australia	two-row	n.d.
2	85SW:576	AUS	Australia	two-row	spring
3	90S205-45-46	AUS	Australia	two-row	spring
4	91IBON45	MEX	North America	six-row	spring
5	94S909G-20-19	AUS	Australia	two-row	spring
6	95S009-81-33	AUS	Australia	two-row	spring
7	AC METCALFE	CAN	North America	two-row	spring
8	Annabell	DEU	Europe	two-row	spring
9	Astoria	CZE	Europe	two-row	spring
10	Barke	DEU	Europe	two-row	spring
11	Baudin	AUS	Australia	two-row	spring
12	Bentley	CAN	North America	two-row	spring
13	BM9204-17	CAN	North America	two-row	spring
14	BM9311-35	CAN	North America	two-row	spring
15	BM9752D-125	CAN	North America	two-row	spring
16	BM9857-263-1	CAN	North America	two-row	spring
17	BmnL-375	USA	North America	two-row	n.d.
18	BmnL-75	USA	North America	two-row	n.d.
19	BmnL-830	USA	North America	two-row	n.d.
20	Bolgali (Facultative)	UZB	Asia	two-row	winter
21	Braemar	GBR	Europe	two-row	spring
22	C01P-53	URY	South America	two-row	n.d.
23	C2-05-10/263	URY	South America	two-row	n.d.
24	C2-05-337-2	AUS	Australia	two-row	spring
25	CDC Guardian	CAN	North America	two-row	spring
26	CDC Springside	CAN	North America	six-row	spring
27	Charger (Ca412402)	AUS	Australia	two-row	spring
28	CLE196	URY	South America	two-row	spring
29	CM67	USA	North America	six-row	spring
30	CM72	USA	North America	six-row	spring
31	Coor's	USA	North America	two-row	spring
32	EB1110	AUS	Australia	two-row	spring
33	Fairview	NZL	Australia	two-row	spring
34	France30161	FRC	Europe	two-row	n.d.
35	Gairdner	AUS	Australia	two-row	spring
36	GALAN	CZE	Europe	two-row	spring
37	Granifen	CZE	Europe	two-row	spring
38	H92036005Z	CAN	North America	two-row	winter
39	H96009006	CAN	North America	six-row	winter
40	Hamelin	AUS	Australia	two-row	spring
41	Har.Nan-35-24	AUS	Australia	two-row	spring
42	Heart	GBR	Europe	two-row	spring
43	Henley	AUS	Australia	two-row	spring
44	Heriot	GBR	Europe	two-row	spring
45	Hindmarsh	AUS	Australia	two-row	spring
46	I90-137-1	USA	North America	two-row	spring
47	I93-608	USA	North America	two-row	spring
48	ICARDA016	ICARDA	ICARDA	two-row	n.d.
49	IGB1120	AUS	Australia	two-row	spring
50	IGB1138	AUS	Australia	two-row	spring
51	IGB1244	AUS	Australia	two-row	spring
52	ISBONLRAM-052	ICARDA	ICARDA	two-row	n.d.
53	ISBYTLRAC-004	ICARDA	ICARDA	two-row	n.d.
54	ISBYTLRAC-015	ICARDA	ICARDA	two-row	n.d.
55	Kinukei7	JPN	Asia	two-row	spring
56	Landlord	GBR	Europe	two-row	spring
57	LR84%3065	AUS	Australia	two-row	spring

58	LR84%765	AUS	Australia	two-row	spring
59	Lumar	CZE	Europe	two-row	spring
60	Moroc9-75/AA/4/Hml-02/AA/3/Api/CM67//Nacta	SYR	Middle East	two-row	n.d.
61	NAIRN	GBR	Europe	two-row	spring
62	Prosa	AUT	Europe	two-row	spring
63	Schooner	AUS	Australia	two-row	spring
64	Scope CL (VBHT0805)	AUS	Australia	two-row	spring
65	Skiff	AUS	Australia	two-row	spring
66	Skipper (WI4446)	AUS	Australia	two-row	spring
67	Sloop VIC	AUS	Australia	two-row	spring
68	SM02544	CAN	North America	two-row	winter
69	SM99542	CAN	North America	two-row	winter
70	SMBA10-2324	AUS	Australia	two-row	spring
71	SMBA12-2297	AUS	Australia	two-row	spring
72	Tallon	AUS	Australia	two-row	spring
73	Tantangara	AUS	Australia	two-row	spring
74	Tore*	NOR	Europe	two-row	spring
75	TR350	CAN	North America	two-row	spring
76	UWA96T45-07-26	AUS	Australia	two-row	spring
77	WABAR2234	AUS	Australia	two-row	spring
78	WABAR2347	AUS	Australia	two-row	spring
79	WABAR2609	AUS	Australia	two-row	spring
80	WI4704	AUS	Australia	two-row	spring
81	Wicket	GBR	Europe	two-row	spring
82	WVA22	ZAF	Africa	two-row	n.d.
83	XVE7	ZAF	Africa	two-row	n.d.
84	Yambla	AUS	Australia	two-row	spring
85	Yarra (VB0021)	AUS	Australia	two-row	spring

Supplementary Table 4.2 Germination % of barley varieties to 150mM NaCl

Number	Variety	Germination response	Category
1	WABAR2347	96.99	T
2	Har.Nan-35-24	96.09	T
3	CM72	95.48	T
4	WVA22	94.88	T
5	WABAR2234	94.59	T
6	CDC Guardian	94.38	T
7	Yambla	94.38	T
8	90S205-45-46	94.22	T
9	ISBYTLRAC-004	93.98	T
10	H92036005Z	93.84	T
11	BmnL-375	93.50	T
12	AC METCALFE	92.34	T
13	Landlord	92.23	T
14	WI4704	92.10	T
15	Tallon	91.88	T
16	CM67	91.85	T
17	85SW:576	91.48	T
18	BM9204-17	91.42	T
19	NAIRN	91.40	T
20	SM99542	91.37	T
21	SM02544	91.23	T
22	Tore*	91.00	T
23	Bolgali (Facultative)	90.84	T
24	IGB1244	90.84	T
25	CLE196	90.76	T
26	Lumar	90.54	T
27	BM9311-35	90.39	T
28	Annabell	90.37	T
29	Wicket	90.33	T
30	Baudin	90.31	T
31	Yarra (VB0021)	90.01	T
32	Barke	89.66	MT
33	CDC Springside	89.45	MT
34	LR84%765	89.23	MT
35	IGB1120	89.22	MT
36	Heriot	89.19	MT
37	BmnL-75	88.86	MT
38	Bentley	88.45	MT
39	UWA96T45-07-26	88.43	MT
40	BmnL-830	88.32	MT
41	I93-608	88.28	MT
42	95S009-81-33	88.23	MT
43	GALAN	87.93	MT
44	Prosa	87.87	MT
45	Coor's	87.87	MT
46	Fairview	87.35	MT
47	Henley	87.33	MT
48	Moroc9-75/AA/4/Hml-02/AA/3/Api/CM67//Nacta	87.23	MT
49	Heart	87.14	MT
50	Tantangara	86.97	MT
51	IGB1138	86.80	MT
52	XVE7	86.80	MT

53	TR350	85.95	MT
54	Astoria	85.92	MT
55	SMBA10-2324	85.89	MT
56	Kinukei7	85.17	MT
57	Braemar	84.71	MT
58	Scope CL (VBHT0805)	84.67	MT
59	WABAR2609	83.96	MT
60	C2-05-337-2	83.90	MT
61	BM9857-263-1	83.70	MT
62	94S909G-20-19	83.41	MT
63	Schooner	83.25	MT
64	I90-137-1	82.96	MT
65	Charger (Ca412402)	82.52	MT
66	Hamelin	81.06	MT
67	Skipper (WI4446)	80.71	MT
68	SMBA12-2297	80.16	MT
69	H96009006	79.29	SC
70	Granifen	79.08	SC
71	France30161	78.86	SC
72	Sloop VIC	78.39	SC
73	BM9752D-125	78.37	SC
74	Hindmarsh	75.12	SC
75	EB1110	74.73	SC
76	91IBON45	74.50	SC
77	Skiff	74.44	SC
78	Gairdner	73.25	SC
79	04S213D-B-125	55.01	SE
80	LR84%3065	54.19	SE
81	ISBONLRAM-052	51.32	SE
82	C01P-53	49.40	SE
83	ISBYTLRAC-015	49.21	SE
84	C2-05-10/263	49.07	SE
85	ICARDA016	46.91	SE

T - Tolerance, MT - Moderately Tolerance, SC - Susceptible and SE – Sensitive

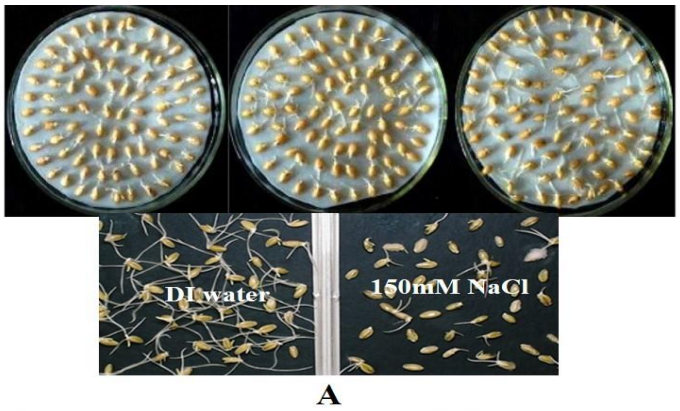
Supplementary Table 4.3 List of top and bottom 15 genotypes for germination tolerance index, Tolerance index salinity seedling vigour index length and weight

Variety	GTI	SVI TI length	SVITI DW
85SW:576	91.479	73.002	64.497
90S205-45-46	94.222	54.855	47.171
94S909G-20-19	83.411	60.824	58.049
AC METCALFE	92.342	64.113	61.367
Annabell	90.374	73.803	61.551
Astoria	85.918	57.936	46.546
Baudin	90.307	75.235	72.831
Bentley	88.449	74.341	68.554
BM9311-35	90.394	69.654	72.397
BM9752D-125	78.372	42.853	48.601
BM9857-263-1	83.702	60.221	51.488
BmnL-375	93.500	52.523	53.265
BmnL-75	88.864	84.408	79.936
BmnL-830	88.316	76.985	76.435
Bolgali (Facultative)	90.844	71.480	69.227
C01P-53	42.396	25.531	21.577
CDC Guardian	94.385	59.647	57.982
CDC Springside	89.452	57.573	42.759
Charger (Ca412402)	82.521	61.176	60.892
CM67	91.851	72.321	72.752
CM72	95.475	76.561	67.895
Gairdner	73.250	45.342	44.139
GALAN	87.932	50.716	47.733
Granifen	79.079	42.949	45.030
H92036005Z	93.838	76.031	75.766
H96009006	79.287	55.864	52.713
Hamelin	81.061	56.408	53.567
Har.Nan-35-24	96.087	57.811	50.765
Henley	87.335	73.320	61.609
Heriot	89.187	57.032	43.595
Hindmarsh	75.122	43.450	42.409
I90-137-1	82.964	50.925	49.945
I93-608	88.284	36.267	34.223
IGB1120	89.221	50.550	50.642
ISBONLRAM-052	51.319	32.520	30.191
ISBYTLRAC-004	93.979	62.607	56.181
Landlord	92.227	62.864	52.744
LR84%765	89.230	46.204	41.036
Prosa	87.867	67.781	72.202
Scope CL (VBHT0805)	84.668	44.697	50.483
Skiff	74.441	39.398	45.226
SM99542	91.369	49.430	50.662
Tallon	91.875	59.321	47.657
Tore*	91.000	70.245	64.610
WABAR2234	94.587	60.041	62.430
WABAR2347	96.990	50.173	53.051
WI4704	92.104	40.593	53.390
Wicket	90.333	54.837	42.351
WVA22	94.885	72.574	63.510
XVE7	86.796	73.173	63.360
Yambla	94.375	61.349	62.940
Yarra (VB0021)	90.006	49.615	68.992

Supplementary Table 4.4 Genes from barley genome in the two important regions on 3H

Region	Transcript ID	Chr	Start	End	Function Description
629154913bp - 628265323bp	HORVU3Hr1G089290.3	chr3H	628015148	628016247	Late embryogenesis abundant protein Lea5-A
	HORVU3Hr1G089300.1	chr3H	628030631	628032075	Dehydrin DHN3
	HORVU3Hr1G089330.38	chr3H	628115699	628124992	RNA-binding protein 10
	HORVU3Hr1G089350.1	chr3H	628125540	628126774	undescribed protein
	HORVU3Hr1G089360.2	chr3H	628164744	628170097	CMP-sialic acid transporter 3
	HORVU3Hr1G089370.3	chr3H	628213099	628215684	Protein of unknown function (DUF1685)
	HORVU3Hr1G089380.4	chr3H	628222876	628224487	Protein of unknown function (DUF1685)
	HORVU3Hr1G089410.1	chr3H	628276869	628277428	NAC domain protein;
	HORVU3Hr1G089420.1	chr3H	628355427	628356316	Agglutinin isolectin 3
	HORVU3Hr1G089430.1	chr3H	628465079	628465480	undescribed protein
	HORVU3Hr1G089440.1	chr3H	628523331	628523600	Ribosomal protein-like
	HORVU3Hr1G089450.13	chr3H	628540971	628547483	ARM repeat superfamily protein
	HORVU3Hr1G089480.17	chr3H	628711641	628721787	sister chromatid cohesion 1 protein 4
	HORVU3Hr1G089490.10	chr3H	628756973	628762509	receptor-like kinase 1
	HORVU3Hr1G089500.1	chr3H	628889265	628890381	DNA topoisomerase 2
	HORVU3Hr1G089510.1	chr3H	628923468	628927818	formin homology 1
	HORVU3Hr1G089520.5	chr3H	628930353	628937043	beta glucosidase 42
	HORVU3Hr1G089540.1	chr3H	629103777	629106091	pentatricopeptide repeat 336
	HORVU3Hr1G089550.10	chr3H	629145058	629147085	ribonuclease 2
	HORVU3Hr1G089570.1	chr3H	629148937	629149135	vacuolar ATP synthase catalytic subunit-related /
	HORVU3Hr1G089580.5	chr3H	629154100	629157076	ribonuclease 2
	HORVU3Hr1G089590.2	chr3H	629164844	629167239	unknown protein; Has 1807 Blast hits to 1807 prote
	HORVU3Hr1G089600.1	chr3H	629185150	629185355	vacuolar ATP synthase catalytic subunit-related /
	HORVU3Hr1G089620.1	chr3H	629249798	629250352	unknown protein; BEST Arabidopsis thaliana protein
	HORVU3Hr1G089630.1	chr3H	629257831	629261447	nuclear transport factor 2B
	HORVU3Hr1G089640.2	chr3H	629276593	629278988	unknown protein; Has 1807 Blast hits to 1807 prote
	HORVU3Hr1G089650.2	chr3H	629321677	629326506	Rhodanese/Cell cycle control phosphatase superfami
	HORVU3Hr1G089670.1	chr3H	629334432	629339341	Lectin-domain containing receptor kinase A4.1
	HORVU3Hr1G089680.1	chr3H	629338385	629338564	Mixed-linked glucan synthase 2
	HORVU3Hr1G089690.4	chr3H	629445289	629449466	IQ-domain 13
	HORVU3Hr1G089700.1	chr3H	629468653	629469496	AT2G18410-like protein
	HORVU3Hr1G089710.1	chr3H	629573890	629574698	Ribonucleoside-diphosphate reductase large subunit
	HORVU3Hr1G089720.1	chr3H	629625302	629625686	DNA mismatch repair protein MLH1
	HORVU3Hr1G089750.1	chr3H	629720265	629721322	unknown function
	HORVU3Hr1G089760.1	chr3H	629934519	629939622	unknown function
	HORVU3Hr1G089770.3	chr3H	629955127	629960035	unknown function
	HORVU3Hr1G089780.1	chr3H	629961114	629961513	undescribed protein

4.8.2 Supplementary figures



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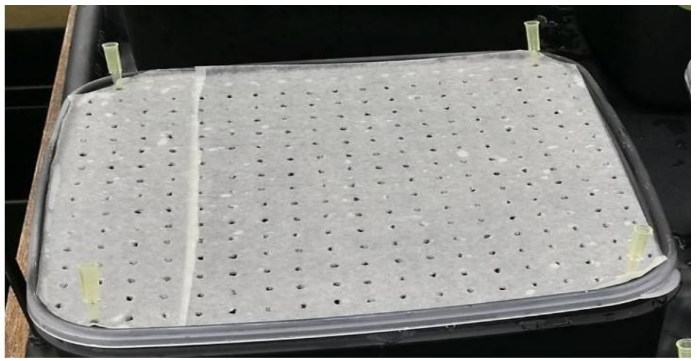
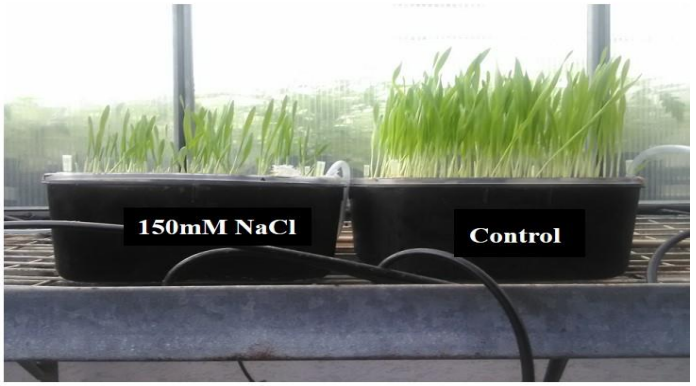


B

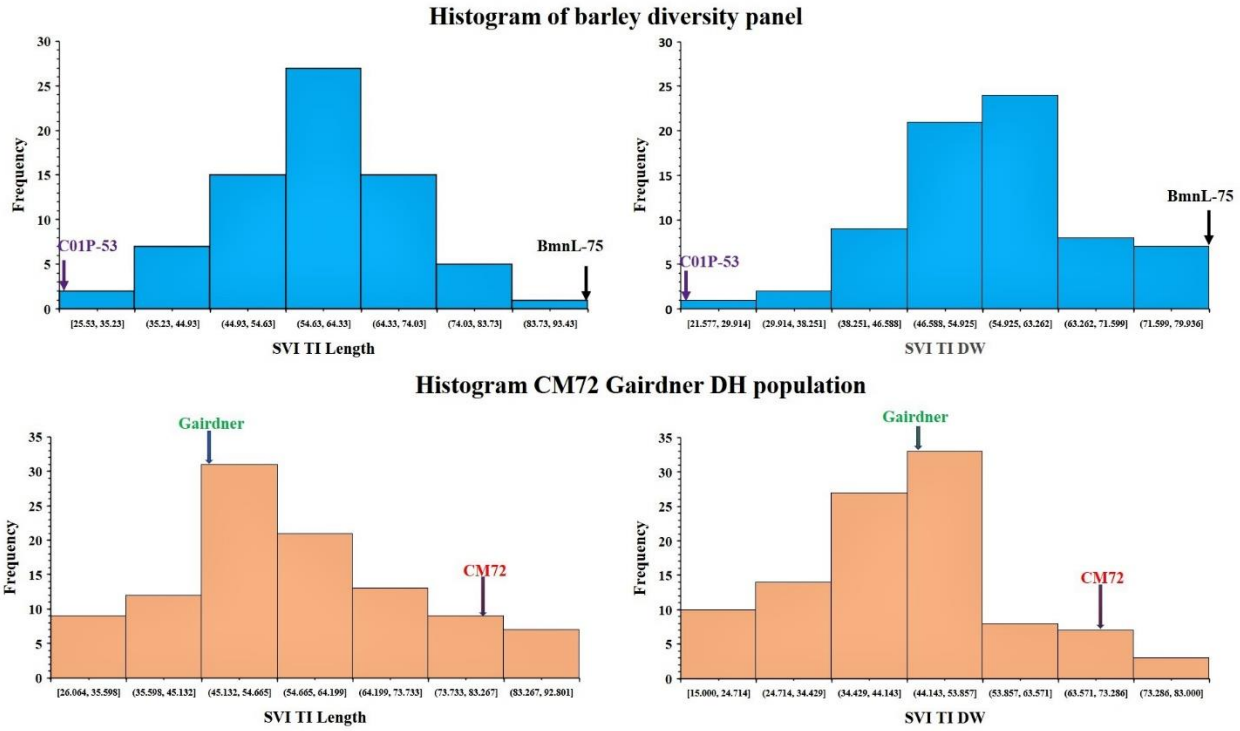
C



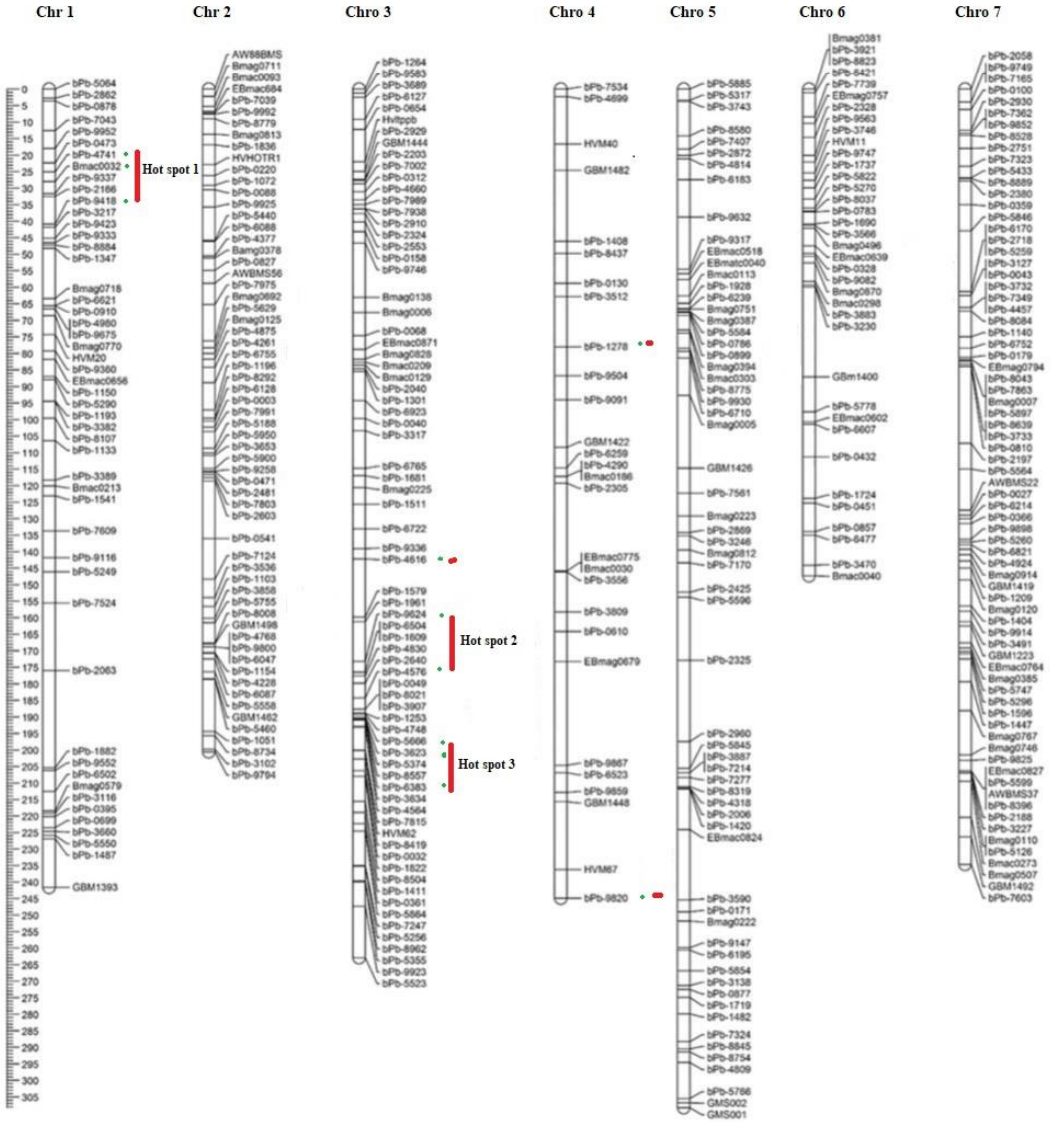
D

Supplementary Figure 4.1 Production of barley seedling under hydroponic solution phenotyping.

A Germination of seedling in 9cm diameter Petri dishes with filter paper in deionised water and 150mM NaCl. **B** Plastic tanks with Hoagland's nutrient solution ready for seedling transplanting. **C** Transplanted seedling into the holes of plastic tanks covered with Kimberly Clark Professional Kleenex Compact Towel tissues and fitted with the solution aerating hydroponic pump system. **D** Seedlings growing in 150 mM NaCl and control treatments.



Supplementary Figure 4.2 Histograms of seedling vigour index tolerance index (SVI TI) Length (L) and weight (DW) for selected barley genotypes and CM72/Gairdner DH population under 150 mM NaCl.



Supplementary Figure 4.3 Locations of the QTLs for seedling survival traits in barley after germinating under salinity stress in CM72/Gairdner DH population.

QTLs are shown by red color and the green indicates all significant marker with LOD > 3.0. On the left of the figure is the map distances in Kosambi centimorgan while on the right of every chromosome are markers.

CHAPTER FIVE

FINE-MAPPING AND CHARACTERISATION OF GENES ON BARLEY CHROMOSOME 2H FOR SALINITY STRESS TOLERANCE DURING GERMINATION

The content of this chapter is composed of a manuscript under review: **Mwando, K. E.,** Han, Y., Angessa, T. T., Zhang, X-Q., and Li, C. (under review). Fine-mapping and characterisation of genes on barley chromosome 2H for salinity stress tolerance during germination.

The authors contribution is as follows: **EM** performed fine-mapping experiments, data analysis, interpretation and drafting the manuscript. **TA** constructed the molecular linkage map, facilitated bulking of planting material and initial QTL mapping. **X-QZ** and **YH** guided laboratory experiment. **CL** conceived the project. All authors revised the paper and approved the final version for publication.

5.1 Abstract

Salinity causes a detrimental impact on plant growth, particularly when the stress occurs during germination and early development stages. Barley is one of the most salt-tolerant crops; previously we mapped two quantitative trait loci (QTLs) for salinity tolerance during germination on the short arm of chromosome 2H using a CM72/Gairdner doubled haploid (DH) population. Here, we narrowed down the major QTL to a region of 0.341 Mb containing nine candidate genes belonging to six functional gene families, using two DH populations of CM72/Gairdner and Skiff/CM72, F_2 and F_3 generations of CM72/Gairdner/*Spartacus CL. Two Receptors'-like protein kinase 4 (RLPK4) could be the candidates for enhanced germination under salinity stress because of their upregulated expression in salt-tolerant variety CM72. Besides, several insertion/deletion polymorphisms were identified within the 3rd exon of the genes between CM72 and Gairdner. The sequence variations resulted in shifted functional protein domains, which may be associated with different in salinity tolerance. Two molecular markers were designed for selecting the locus, and one was in the first receptor-like protein kinase 4 gene families. The diagnostic markers will allow for pyramiding of 2H locus in barley varieties and facilitate genetic improvement for saline soils. Further, validation of the genes to elucidate the mechanisms involved in enhancing salinity tolerance at germination and designing RLPK4 specific markers is proposed.

5.2 Introduction

Salinity is a global problem affecting more than 6% of the world's area, being more than 20% of the total arable land and 50% of cropland (Akram et al. 2009; Barhoumi 2018; FAO 2010; Gupta and Huang 2014; Mickelbart et al. 2015; Meena et al. 2017; Dagar et al. 2019). Saline land is increasing at a disturbing level due to human activities, climate change and seasonal variations (Long et al. 2013; Shen et al. 2020). Salinity is likely to exacerbate the challenge of meeting the world's food demands as most cultivated crops are susceptible to salinity stress (Flowers and Flowers 2005; Hasanuzzaman et al. 2013; Lin et al. 2006; Mustafa et al. 2019). In Australia, salinity is a serious abiotic stress that is threatening crop production, especially in semi-arid zones that receive less than 450 mm of annual rainfall (Rengasamy 2002). According to earlier studies by Agarwal et al. (2013); Tabatabaei and Ehsanzadeh (2016), salinity reduces crop yields by (i) impeding plant access to soil water through increased soil osmotic potential that curbs water and nutrient absorption (osmotic or water-deficit effect), and (ii) causing ionic disproportion and toxicity in plants (salt-specific or ion-excess effect). Crops are more susceptible to salinity during germination and early developmental stages than other growth phases (Agarwal et al. 2013; Mwando et al. 2020).

At present, there is no practical solution for managing salinity on agricultural land (Dagar et al. 2019; Hamdia and Shaddad 2010). The development of varieties that can withstand salinity stress is a long-term option that requires knowledge of the physiological mechanisms and genetic elements contributing to the characters at different plant growth stages (Hamdia and Shaddad 2010; Mwando et al. 2020). Studies have identified various adaptive mechanisms in different plant parts in response to saline conditions that can be summarized into three: osmotic stress tolerance, Na^+ or Cl^- exclusion, and tissue tolerance to accumulated Na^+ or Cl^- (Bernstein, 2019; Kumar et al. 2013; Tester and Davenport, 2003; Munns 1993; Wyn and Gorham 1983; Roy et al. 2014). These mechanisms involve multifaceted physiological traits, metabolic alleyways, hormonal pathways, transcriptional responses, gene systems and growth stages to enable plants to survive salinity stress (Bhaskar and Bingru 2014; Haq et al. 2010; Nimbolkar et al. 2020).

Barley (*Hordeum vulgare* L.) is the fourth most important cereal crop in production and consumption worldwide (Alan 2018; Meng et al. 2016; Noaman 2017; Schulte et al. 2009; Zeng et al. 2018). It grows in a wide range of environmental conditions, including extreme latitude and altitude, and is one of the hardiest cereal crops (Barhoumi 2018; Forsberg et al.

2019; Harlan 1976). The salt-tolerant barley varieties, Numar and ZUG293, exhibited halophytic features to exclude Na^+ from root uptake, relative to the salt-sensitive varieties, Gairdner and ZUG403 (Chen et al. 2007). Barley is a crop of interest because it can survive saline conditions by accumulating high Na^+ in the leaves if the ions find their way into the plant (Munns et al. 1988; Munns and Tester 2008; Zhu et al. 2020). Its capacity to tolerate salinity is attributed to the sequestration of Na^+ in its intracellular vacuoles and synthesis of compatible solutes in the cytoplasm that balance the osmotic potential of vacuolar Na^+ (Houston et al. 2020; Mian et al. 2011; Shabala et al. 2010; Widodo et al. 2009). As a result, barley is used by breeders as a source of favourable alleles to improve other cereal crops using conventional and molecular approaches (Colmer et al. 2006; Huang et al. 2020; Monteagudo et al. 2019; Munns et al. 2006).

Like other plant species, salinity tolerance in barley varies between genotypes (Flowers and Hajibagheri 2001; Mano and Takeda 1997; Xue et al. 2009) and is controlled by multiple genes that express themselves at various growth phases (Ahmed et al. 2012; Fu et al. 2019; Qiu et al. 2011; Shen et al. 2020). Saline-susceptible barley varieties will have poor germination, slow growth and development, low survival, and reduced yield and grain quality (Angessa et al. 2017; Colmer et al. 2005; Mwando et al. 2020; Xue et al. 2009; Zhang et al. 2010). Screening barley genotypes for salinity tolerance at various development stages is ongoing (Ahmed et al. 2012; Askari et al. 2017; Martinez-Cob et al. 1987). Several quantitative trait loci (QTLs) associated with salt tolerance traits in barley have been mapped, including those related to yield and agronomic factors (Eleuch et al. 2008; Xue et al. 2009), germination and seedling (Angessa et al. 2017; Mano and Takeda 1997; Mwando et al. 2020), plant survival (Zhou et al. 2012), shoot Na^+ content or Na/K ratio (Shavrukov et al. 2010) and salt exclusion (Rivandi et al. 2011; Xue et al. 2009).

The degree of damage depends on the variety, salinity concentration and developmental stage of the crop (Rogers et al. 1995; Roy et al. 2011). Germination is the most critical phase in the crop life cycle but the most sensitive to salinity (Bewley et al. 2013; Fatemeh et al. 2016; Huang and Redmann 1995; Mahdi et al. 2012) because it determines plant vigour and population which ultimately affect yield (Zhang et al. 2010). Having varieties that can acclimatize to salinity at this stage is essential in salinity-prone cropping regions such as the drier areas of Western Australia that experience hot and dry summers and increased salinity levels just before sowing in autumn (Boyd et al. 2003). Also, germination and initial seedling growth occur in the topsoil where there is high salt accretion due to evapotranspiration (El Goumi et al. 2014).

The development of improved cultivars requires an understanding of the gene actions governing salinity tolerance (Inja et al. 2015). While the physiological and biochemical basis of salinity in crops (barley) are well known, further studies on genetic factors at different growth phases, especially germination, are paramount. QTLs for salt tolerance at the germination stage have been screened and mapped (Hanen et al. 2014; Mano and Takeda 1997), most recently by Angessa et al. (2017), and Mwando et al. (2020), but there is limited information on the nature and function of the tolerance genes at this stage.

Our previous study mapped two stable quantitative loci on chromosome 2H for salinity tolerance at germination using a doubled haploid (DH) population of CM72/Gairdner (Angessa et al. 2017). Here, we validate the QTLs and fine-mapped to a region of 0.341 Mb, predicted two receptor-like protein kinase 4 genes as candidates through expression and sequence analysis and designed two molecular markers for selecting the locus.

5.3 Materials and Methods

5.3.1 Plant materials

The plant materials used in this study included two barley DH populations (CM72/Gairdner and Skiff/CM72), their parents (CM72, Gairdner, and Skiff), a salt-sensitive cultivar Spartacus CL, and F_2 and F_3 generations of CM72/Gairdner/*Spartacus CL and 265 global barley accessions. CM72/Gairdner comprised 102 DH lines, that had been previously used to map salinity tolerance QTLs on chromosome 2H at germination (Angessa et al. 2017), and Skiff/CM72 comprised 88 lines. Gairdner with Tas83-587/Onslow pedigree, Skiff with Abed Deba/WI-2235//CD28/WI-2231 pedigree and Spartacus CL with Scope/4*Hindmarsh//HMVB0325-106 pedigree are salinity sensitive Australian varieties. While, CM72 is salinity tolerant genotype derived from California Mariout*4/CI1179 (Algerian)//2*California Mariout/Club Mariout/3/CM67.

To develop an F_2 population of CM72/Gairdner/*Spartacus CL segregating for salinity tolerance at germination, the CM72/Gairdner salt-tolerant DH lines, previously selected for CM72 genotype on chromosome 2H (Angessa et al. 2017), were crossed with Spartacus CL and the F_1 plants allowed to self-pollinate. The process involved crossing 2 DH lines (WADH13531 and WADH13543) and screening them for salinity tolerance using marker Bmac134. Lines that had the CM72 genotype on the 2H locus were selected for crossing with Spartacus, which yielded 32 F_1 seeds. The progeny was confirmed to be heterozygous before crossing them back to Spartacus CL and allowing them to self-pollinate. A total of 2020 F_2

seeds were harvested and cut in half while observing polarity (the embryo and the endosperm sides). One half was used for genotyping with InDel markers from the 2H fine-mapped region and the other half containing the embryo was grown to produce the F_3 population for phenotyping salinity tolerance at germination.

5.3.2 Assessment of Salinity Tolerance during Germination

Three replicates of 100 surface-sterilized seeds from the two DH populations were spread on a double layer of Whatman No.1 filter paper placed in a 90 mm Petri dish. Each Petri dish received 4 mm of either (DI) water (control) or 150 mM NaCl (salt treatment) and was then covered with a lid and labelled. Five Petri dishes were bundled together using cling wrap and placed in a dark oven at 20 ^{0}C. After 72 hrs incubation, the germinated seeds were counted. The tolerance index was calculated as the percentage of seeds germinated in the salt treatment divided by those in deionized water multiplied by 100. While germination reduction was the percentage of seed germinated in DI water less those in 150 mM NaCl.

5.3.3 Phenotypic data analysis

The germination percentage of individual lines from the three replicates was used for analysis of variance with SAS version 9.4 (SAS Institute 2013). Spearman's rank correlation coefficient analysis was undertaken with IBM SPSS Statistics for Windows, Version 25.0 (IBM Corporation 2017).

5.3.4 DNA extraction

Genomic DNA was extracted from individual barley seeds of each DH line including the parents using the modified cetyltrimethylammonium bromide (CTAB) method as described by Stein et al. (2001). The CTAB method isolates protein from the tissue, separates it from DNA with a salt solution, and purifies the DNA using ethanol. DNA was dissolved in 150 µL TE buffer, and its concentration measured using a NanoDrop spectrophotometer (Thermo Scientific™ NanoDrop™ One).

5.3.5 Marker development and genotyping

Insertion-deletion (InDels) markers were designed from an in-house barley genomic database (http://146.118.64.11/BarleyVar/), between and slightly outside the two flanking markers bPb-3858 and bPb-1103 of the two mapped QTLs on chromosome 2H (Angessa et al. 2017; Mascher et al. 2017). Since none of the barley parents of the DH populations was among the database varieties, InDels with polymorphism in more than half of the barley genotypes, with

insertion or deletion >10 nucleobases and an amplifying sequence of 100–200 bp amplicon size flanking equally were selected. The primers were blasted on the IPK website http://webblast.ipk-gatersleben.de/barley_ibsc/ to ensure that they amplified a single region on the barley genome then tested for polymorphism among the DH parents (CM72, Gairdner and Skiff). The polymorphic InDels were selected and run in the two DH populations (Supplementary table 5.1). A polymer chain reaction (PCR) of 10 µl was used to amplify the template DNA using a Thermal Cycler (Applied Biosystems) machine with the settings adjusted to an initialization at 95°C for 3 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension/elongation at 72°C for 30 s, final elongation at 72°C for 5 min, and the final hold at 14°C. The amplification products were visualized on either 2% agarose gel or 6% polyacrylamide gel electrophoresis (PAGE).

5.3.6 QTL validation and fine mapping

The previously mapped diversity array technology (DArT) flanking markers bPb-3858 and bPb-1103 (Angessa et al. 2017) were the benchmarks for placing markers on chromosome 2H. The new InDels were combined with 315 DArT and 84 simple sequence repeat (SSR) markers to construct a new map using the CM72/Gairdner DH population. JoinMap 3.0 (Van and Voorrips 2001) was used for linkage analysis and determination of the likely order of the markers in the DH population. Recombinant lines from CM72/Gairdner and Skiff/CM72 DH populations were selected between new flanking markers and re-evaluated for response to 150 and 225 mM NaCl at germination stage. The tolerance index for each recombinant line was related to the polymorphic InDel marker genotype of their respective DH population. All the markers within the new flanking QTL region were used to screen a larger F_2 population with 2020 individuals derived from M72/Gairdner DH/* Spartacus CL. The resulting recombinant lines were bulked into individual F_3 populations and tested for germination with 150 and 225 mM NaCl levels.

5.3.7 RNA extraction and genes expression profile

Seeds were germinated using DI water and embryos collected at 24 hrs and 48 hrs from AC Metcalfe, Morex, Harrington, Stirling and Bass varieties. Germination stage salinity tolerance was previously determined for these five varieties (Supplementary table 5.2). The embryos were snap frozen immediately using liquid nitrogen and stored at −80°C before RNA extraction and gene expression analysis was conducted following Zhang et al. (2016a, b).

5.3.8 Molecular marker identification for salinity tolerance loci on chromosome 2H during germination

All the markers within the new flanking region were considered to have potential for use in marker-assisted selection (MAS). To validate them, individual recombinant lines were tested using each marker and their genotype associated with their respective phenotype. Selected markers were tested in a wider collection of 265 barley germplasm from around the world including Australian commercial varieties. The salinity tolerance of the diverse barley panel was previously determined as part of the germplasms used in Mwando et al. (2020). Hierarchical clustering of phenotype to genotype was done with IBM SPSS Statistics for Windows, Version 25.0 (IBM Corporation 2017), after data transformation.

5.3.9 Estimation of gene expressions under salt stress by real-time quantitative PCR

Seeds of CM72 and Gairdner were used for this experiment. First, the seeds were cut into half to reduce starch content and then germinated in 150mM NaCl and DI water separately after sterilization. Embryos were collected by separating them from the endosperm at 4 time points (TP) whereTP1was 16hrs, TP2 40hrs, TP3 64hrs and TP4 88hrs after germination. The embryos were snap-frozen using liquid nitrogen and stored at -80 ^{0}C. Tissue samples were crushed to a smooth powder in frozen liquid nitrogen by a mortar and pestle. Total RNAs was isolated using TRIsure^{TM} reagent (Bioline Australia). RNA was extracted with chloroform, precipitated by isopropyl alcohol before the pellet was washed in 75% ethanol, air-dried and dissolved in DEPC-treated water. Eventually total RNA was purified using RNeasy Plant Mini kit (Qiagen) by being treated with DNase I to digest DNA and chromatins. DNA free RNA was recovered by precipitating using sodium acetate in 100% ethanol washed with 75% ethanol and dissolved in DEPC-treated water. The integrity of RNAs was determined using formaldehyde-agarose gel electrophoresis and the concentration measured using UV spectrometry (NanoDrop 1000, Thermo Scientific).

The first-strand cDNA was synthesized through reverse transcription using SensiFAST^{TM} cDNA Synthesis Kit following manufactures procedure (Bioline Australia). Quantitative polymerase chain reaction (qPCR) was performed using gene-specific primers (Supplemental table 5.3), in the reaction system of SensiFAST^{TM} SYBR® L0-ROX Kit on a Roche 480 real-time PCR machine. *HvGAPDH* was employed as our inside reference control (Han et al. 2018). Real-time quantitative polymerase chain reaction (RT – qPCR) was done in three biological and technical repetitions. The comparative quantification $2^{-\Delta\Delta CT}$ method was used to estimate

the relative expression levels and evaluate quantitative variation of genes following the equations (i – v) below as modified from Livak and Schmittgen, (2001), where embryos harvested at specific time point was related to the respective time point from the same variety between target gene and housekeeping *HvGAPDH* for salinity treated and control separately (fold change) as exemplified in equations i and ii. The fold change in treated correlated to control for individuals and individuals to averages (comparative fold change) equations iii and iv and gene expression (relative fold change) equation v. The expression levels and quantitative variation of genes data were calculated from independent replications and subjected to the analysis of variance (ANOVA) done to determine the significant differences between the replicates before testing the means by Duncan's Multiple Range tests (DMRT) at $p < 0.05$. SPSS statistical computer software program (IBM Corporation, 2017) was used to plot the graphs.

(i) $\Delta CT_{Control} = CT_{Target\ gene} - CT_{house\text{-}keeping\ gene}$

(ii) $\Delta CT_{Treated} = CT_{Target\ gene} - CT_{house\text{-}keeping\ gene}$

(iii) $\Delta CT_{(Treated - Control)} = \Delta CT_{Treated} - \Delta CT_{Control}$

(iv) $\Delta\Delta CT = \Delta CT_{Individual} - \Delta CT_{Average}$

(v) Gene expression $= 2^{-\Delta\Delta CT}$

5.3.10 Amplification and full-length sequencing of genes from genomic DNA of barley

Genomic DNA extraction that followed the method by Stein et al. (2001) was used to extract DNA from both CM72 and Gairdner seedlings and subjected to polymerase chain reaction (PCR) as described earlier but using genes specific markers (Supplemental table 5.4) in a volume of 25 µl using 1X TAE buffer. The primers were designed from genes sequences obtained from the NCBI databases that amplified sections of each gene into several fragments. Purified fragments for sequencing reaction were obtained from gel fragment by Clean Up, where excised gel was centrifuged for 30 – 60 s at 14,000 rpm. The sequencing reaction contained 4 µL dGTP: BDV3.1 in a ratio of 1:3, 3uL template and 3uL of forward primer in a 10uL reaction volume. The sequencing reaction was processed under the following conditions: 96 ^{0}C for 2 mins, 35 cycles of (96 ^{0}C for 10 sec, 50 ^{0}C for 5 sec, 60 ^{0}C for 4 mins) and 14 ^{0}C hold. This was followed by ethanal precipitation where 1 µL of 125mM EDTA (disodium salt), 1 µL of 3 M sodium acetate and 25 µl of 100% ethanol was added into each tube succeeding the order after spinning down. This was mixed by pipetting and incubated at room temperature for 20 mins, then centrifuged using maximum speed of 14,000 rpm at 4 ^{0}C for 30 mins. The supernatants were removed by inverting the tubes on filter paper and centrifuging at low speed

of 200 rpm for 1 min. The DNA pellets were rinsed by adding 125 µL of 75% ethanol into each tube and centrifuging at 14,000 rpm for 5 min. The cleaning solution was removed for the pellet to dry at room temperature. Finally, the samples were sequenced by sequencing apparatus in Australian State Agricultural Biotechnology Centre (SABC), Murdoch University. Computer software MEGA-X (Kumar et al. 2018) and Geneious 6.0 (Kearse et al. 2012) were used to align and analyse the sequences, while the online tool Multalin version 5.4.1 used to generate the images (Corpet, 1988).

5.3.11 Candidate genes analysis

Protein sequences of the two RLPK4's were extracted from barlex (https://apex.ipk-gatersleben.de/apex/f?p=284:10::::::) (Colmsee et al. 2015) and blasted on NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins). Then a single representative sequence from each of the different species including wheat, rice, maize and sorghum having a percentage resemblance of more than 70% selected and extracted. Additionally, the protein sequence of all hits above 70% were alignment at (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins). The actual positions of the conserved domains in the middle of both genes was positioned using the respective protein sequences at https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi.

5.4 Results

5.4.1 Germplasm response to salinity stress at germination

Germination of the three parental lines (tolerant CM72, and sensitive Skiff and Gairdner) in 150 mM NaCl was 96.7, 78.0, and 68.7%, respectively (Figure 5.1). The two DH populations differed significantly for germplasm, treatment and their interaction (Table 5.1). Both DH populations had an average germination of the parental lines in DI water of 97.58%, while in 150 mM NaCl it was 82.72% for CM72/Gairdner and 82.02% for Skiff/CM72 population, respectively. Germination ranged from 46.7–99.3% in the CM72/Gairdner population and 42.7–96.0% in the Skiff/CM72 population exposed to salinity stress.

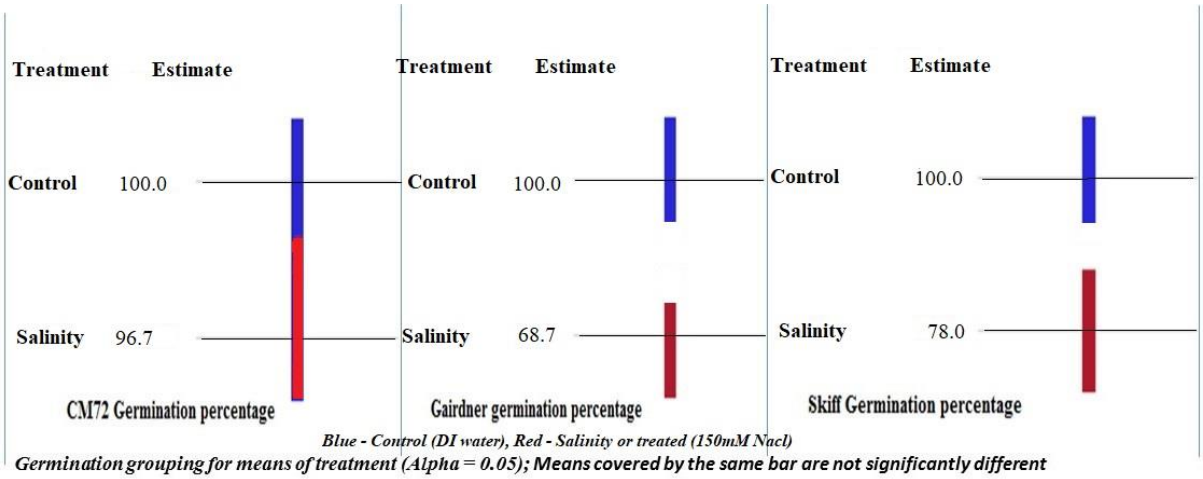


Figure 5.1 Germination variation among the three parents in DI water and 150 mM NaCl

The tolerance index of CM72 (96.7%) indicated that it was least affected by 150 mM NaCl at germination followed by Skiff (78.0%). However, Gairdner was the most affected variety at 68.67%. Across the two DH populations, a transgressive tolerance index was exhibited by individual lines where some lines were below and above the parents (Supplementary figure 5.1). There was an inverse relationship between tolerance index and germination reduction due to salinity stress with a correlation coefficient of $R^2 = –0.97$ (Figure 5.2A) for CM72/Gairdner and –0.95 for Skiff/CM72 (Figure 5.2B) indicating that either tolerance index or reduction in germination can explains the tolerance capacity of each DH line.

Table 5.1 Analysis of variance for germination percentage of doubled haploid populations of CM72/Gairdner and Skiff/CM72

DH population	Source	DF	SS	Mean square	F value
CM72/Gairdner	Treatment	1	32273.7	32273.7	3929.3*
	Germplasm	102	32857.3	322.1	39.2*
	Treatment × Germplasm	102	17199.3	168.6	20.5*
	Total	**205**	**82330.3**		
Skiff/CM72	Treatment	1	34048.1	34048.1	6549.6*
	Germplasm	88	21391.7	243.1	46.7*
	Treatment × Germplasm	88	10183.5	115.7	22.2*
	Total	**177**	**65623.3**		

** Significant at the 1% probability level*

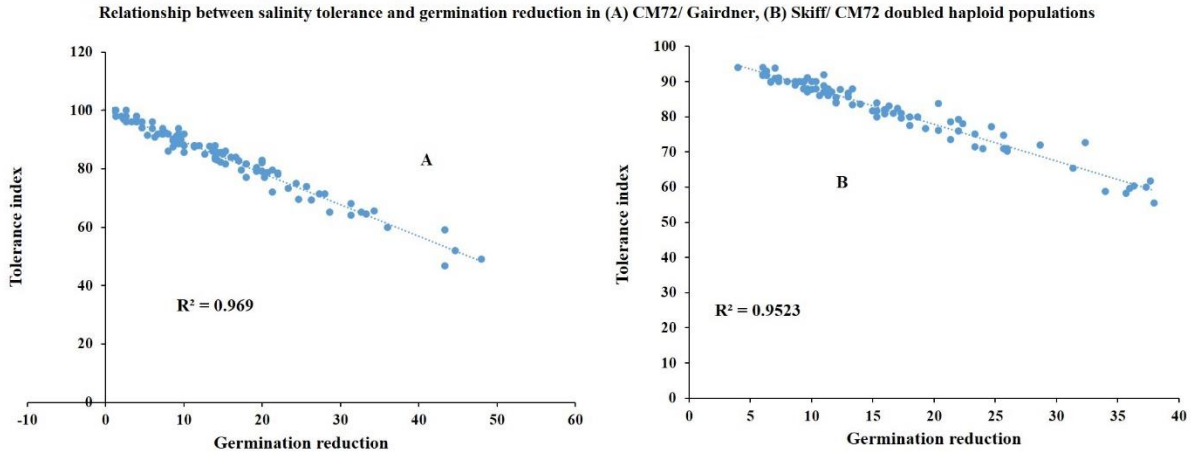


Figure 5.2 Correlation coefficient of tolerance index and germination reduction due to 150 mM NaCl in the two DH populations

5.4.2 Validation and refining genomic interval of chromosome 2H QTL

Among the 200 tested InDel markers in the two DH populations, 41 in CM72/Gairdner and 12 in Skiff/CM72 were polymorphic (supplementary table 5.1). A total of 53 InDel markers that were polymorphic in either of the two DH populations were evaluated in their respective DH populations and a new genetic map constructed using the CM72/Gairdner population. A total of 82 markers were scanned on chromosome 2H that included 42 new InDel, 33 DArT, and 7 SSR markers. The new linkage analysis confirmed the two QTLs previously mapped on 2H for germination percentage at 150 mM and 300 mM NaCl associated with markers bPb-3858, bPb-1103 (Angessa et al. 2017) and InDel 74-48 (Table 5.2). Thirteen recombinant lines from CM72/Gairdner and 16 from Skiff/CM72 were selected for their clear-cut phenotypic responses and marker information.

Table 5.2 Chromosome 2H QTLs linked to salinity tolerance during germination

Trait	Chromosome	Position	Marker	LOD	Variance	% explained	Additive effect
Germination % in 150 mM NaCl	2	40.004	bPb-3858	3.6	130.402	19.0	5.7
Tolerance index at 150 mM NaCl	2	41.776	InDel74-48	2.7	123.91	14.7	4.8
Germination % in 300 mM NaCl	2	45.278	bPb-1103	3.5	379.094	14.8	8.5
Tolerance index at 300 mM NaCl	2	45.278	bPb-1103	3.2	405.228	13.6	8.3

5.4.3 Physical mapping and comparative analysis on 2H QTL interval

Flanking markers InDel 74-47 and InDel 20-44 were used as the starting point to narrow the region likely to contain the tolerant genes using the new recombinant lines. The two markers were chosen for their polymorphism in both DH populations. The region had 21 polymorphic markers in both DH populations with 16 in CM72/Gairdner, two in Skiff/CM72 and three

overlapping. The genotype data indicated that six lines from CM72/Gairdner [WADH13529, WADH13534, WADH13538 (sensitive) and WADH13536, WADH13537, and WADH13561 (tolerant)] and three lines from Skiff/CM72 [WADH13772 and WADH13820 (sensitive) and WADH13806 (tolerant)] were recombinant within the region (Figure 5.3).

Among all the markers, InDel 15-008 was considered the left boundary for the gene region and InDel 74-63 was best suited for the right boundary because the same lines above showed similar genotype-phenotype combinations (Figure 5.3). Three lines WADH13529, WADH13534, and WADH13538 of CM72/Gairdner DH population had Gairdner genotype for InDel 15-008 and were salt-sensitive, while three other lines WADH13536, WADH13537, and WADH13561 had CM72 genotype and were salinity tolerant. The genotype of these six recombinant lines in between the left and right boundary markers region matched with their phenotype response to salinity stress. Therefore, the candidate gene(s) were likely located close to and/ or in between InDel 13-010 and InDel 15-013 on chromosome 2H. While there were fewer polymorphic markers (5) and lines (3), the Skiff/CM72 DH population indicated a likelihood of candidate gene(s) around the region. Lines WADH13772 and WADH13820 had Skiff genotype for InDel 74-56, which were sensitive, while line WADH13806 was CM72 and tolerant (Figure 5.3).

Four recombinant lines with similar allele were identified after screening 2020 F_2 lines of CM72/Gairdner/*Spartacus CL using markers InDel 15-008, 15-009, 74-61, and 74-63. The genotype of the lines was CM72 type for InDel 15-008 and 15-009 markers, and Spartacus CL for InDel 74-61 and 74-63 markers. Germination stage tolerance index of the F3 progeny lines drived from these four lines was similar to that of Spartacus CL (sensitive) in 150 and 225 mM NaCl treatment. The genotype of the lines for markers InDel 15-008, 15-009 was the opposite of phenotypic expression (Figure 5.3) indicating that the gene responsible for increased germination under salinity stress was likely to be located between markers InDel15-009 (724,170,810) and InDel15-013 (724,511,661).

Genotype	Markers																					Phenotype		
Identification	InDel 74-47 (723218508)	InDel 74-48 (723220505)	InDel 74-49 (723247735)	InDel 15-001 (723294358)	InDel 13-001 (723294359)	InDel 74-56 (723599320)	InDel 13-010 (723835777)	InDel 15-008 (723886744)	InDel 15-009 (724170810)	InDel 74-61 (724202721)	InDel 74-63 (724367188)	InDel 15-013 (724511661)	InDel 74-67 (724511886)	InDel 74-68 (724518959)	InDel 15-29 (725602923)	InDel 13-44 (725773819)	InDel 15-44 (725784256)	InDel 16-44 (725813140)	InDel 17-44 (725814356)	InDel 21-29 (725850287)	InDel 20-44 (726080570)	Tolerance index at 150mM NaCl	Tolerance index at 225mM NaCl	Reaction Classification
CM72/Gairdner DH																								
CM72	CC	CC	CC	CC	CC	-	CC	CC	CC	CC	CC	CC	CC	CC	-	CC	CC	CC	CC	CC	CC	97.33	87.33	TT
Gairdner	GG	GG	GG	GG	GG	-	GG	GG	GG	GG	GG	GG	GG	GG	-	GG	GG	GG	GG	GG	GG	76.00	52.00	SS
WADH13529	CC	CC	CC	CC	CC	-	GG	GG	GG	GG	GG	GG	GG	GG	-	GG	GG	GG	GG	GG	GG	74.07	45.45	SS
WADH13534	GG	GG	GG	GG	GG		GG	GG	GG	GG	GG	GG	GG	GG		GG	CC	CC	CC	CC	CC	79.33	54.67	SS
WADH13536	GG	GG	GG	GG	GG	-	GG	CC	CC	CC	CC	GG	GG	GG	-	GG	GG	GG	GG	GG	GG	92.52	71.63	TT
WADH13537	GG	GG	GG	CC	CC	-	CC	CC	CC	CC	CC	CC	CC	CC	-	CC	CC	CC	CC	CC	CC	91.33	70.00	TT
WADH13538	GG	GG	GG	GG	GG	-	GG	GG	GG	GG	GG	GG	GG	GG	-	CC	CC	CC	CC	CC	CC	68.67	43.33	SS
WADH13561	GG	GG	GG	CC	CC	-	CC	CC	CC	CC	CC	CC	CC	CC	-	CC	CC	CC	CC	CC	CC	95.33	74.00	TT
Skiff/CM72 DH																								
Skiff	SK	SK	-	-	-	SK	-	-	-	-	-	-	-	-	SK	-	-	-	-	-	SK	78.00	56.57	SS
WADH13772	SK	SK	-	-	-	SK	-	-	-	-	-	-	-	-	CC	-	-	-	-	-	CC	72.92	50.67	SS
WADH13806	SK	SK	-	-	-	CC	-	-	-	-	-	-	-	-	CC	-	-	-	-	-	CC	93.33	86.20	TT
WADH13820	SK	SK	-	-	-	SK	-	-	-	-	-	-	-	-	CC	-	-	-	-	-	CC	75.00	77.55	SS
CM72/Gairdner DH/*Spartacus F2-F3																								
CM72	-	-	-	-	-	-	-	CC	CC	CC	CC	-	-	-	-	-	-	-	-	-	-	95	85	TT
spartacus	-	-	-	-	-	-	-	SP	SP	SP	SP	-	-	-	-	-	-	-	-	-	-	79.74	57.14	SS
F2/B2P3	-	-	-	-	-	-	-	CC	CC	SP	SP	-	-	-	-	-	-	-	-	-	-	79.23	57.44	SS
F2/B2P15	-	-	-	-	-	-	-	CC	CC	SP	SP	-	-	-	-	-	-	-	-	-	-	76.17	45.65	SS
F2/B4P10	-	-	-	-	-	-	-	CC	CC	SP	SP	-	-	-	-	-	-	-	-	-	-	76.16	45.23	SS
F2/B4P26	-	-	-	-	-	-	-	CC	CC	SP	SP	-	-	-	-	-	-	-	-	-	-	64.91	44.23	SS

Figure 5.3 The genotype of recombinant lines from CM7/Gairdner and Skiff/CM72 DH populations and BCF2 lines from CM72/Gairdner DH/*Spartacus CL using InDel markers between the flanking region of 2H germination salinity tolerance QTL and phenotypic response under 150 and 225 mM NaCl. Where, CC = CM72; GG = Gairdner; SK = Skiff; SP = Spartacus CL for the Markers category, and TT = Tolerant; SS = Sensitive for the phenotypic reaction classification.

5.4.4 Gene annotation and identification

Overall nine candidate genes were identified between markers InDel 15-009 and InDel 15-013 (Table 5.3), the function of which has been found to belong to six different families and one undescribed protein (Mascher et al. 2017). Of the nine genes in the region, three were belonged to the receptor-like protein kinase 4 family in a repeat sequence (Figure 5.4). The salinity tolerance gene co-segregated with the two InDel markers 74-61 and 74-63 (Figure 5.4), because CM72 allele of the markers was associated with tolerance while Gairdner with sensitivity. InDel 74-61 is within receptor-like kinase 4 (HORVU2Hr1G111760.1), indicating the likelihood of the gene to be enhancing salinity tolerance during germination.

Table 5.3 Gene annotation for region within chromosome 2H QTL for salinity tolerance at germination

Gene transcript identification	Chromosome	Physical location	Function description
HORVU2Hr1G111740.1	2H	724172453	Cyclin family protein (CFP)
HORVU2Hr1G111750.5	2H	724174334	Disease resistance protein (DRP)
HORVU2Hr1G111760.1	2H	724183111	Receptor-like protein kinase 4 (RLPK4 – 1)
HORVU2Hr1G111780.3	2H	724201765	Receptor-like protein kinase 4 (RLPK4 – 2)
HORVU2Hr1G111790.7	2H	724208872	Receptor-like protein kinase 4 (RLPK4 – 3)
HORVU2Hr1G111840.5	2H	724373641	Glutathione-S-transferase (GST) family protein
HORVU2Hr1G111880.1	2H	724410823	Endoglucanase 3 (EDG3)
HORVU2Hr1G111920.1	2H	724425792	Undescribed protein (UP)
HORVU2Hr1G111930.1	2H	724473502	Transmembrane emp24 domain-containing protein 3 (TEDCP3)

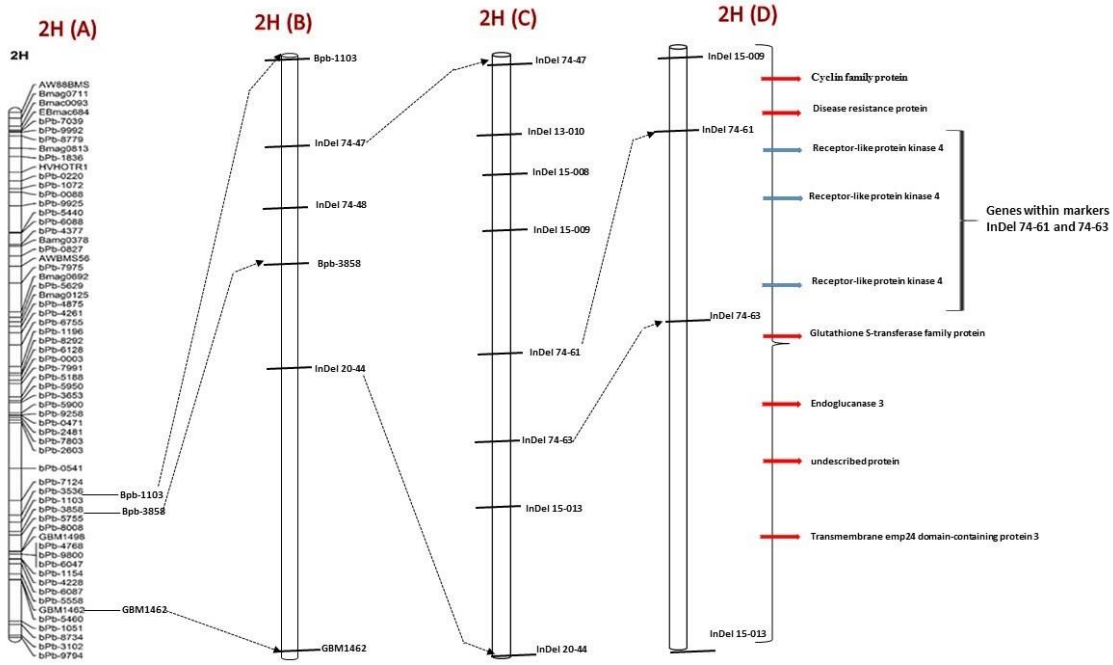


Figure 5.4 Fine-mapping results and gene marker alignment.

(A) 2H QTL of DArT and SSR markers initially detected as flanking between bPb-3858 and bPb-1103, (B) genetic map created using InDel, DArT and SSR markers within the QTL region, (C) fine-mapped 2H QTL region flanking between markers InDel 15-008 and 74-63, and (D) genes close to (red) and between (blue) InDel markers 74-61 and 74-63.

5.4.5 Candidate gene profiles and potential expression prediction

To find out if the genes are possible candidates, their level of expression was explored in tolerant (AC Metcalfe and Bass) and sensitive (Morex, Harrington and Stirling) varieties (Supplementary table 5.5) during the early stages of germination at two time points (24 and

48hrs). The expression levels of HORVU2Hr1G111840.5 (Glutathione-S-transferase (GST) family protein) was generally high across all the varieties with Bass recording the highest expression (1046 RPKM) at 48hrs followed by HORVU2Hr1G111780.3 Receptor-like protein kinase 4 – 2 (RLPK4 – 2) (73 RPKM) at 48hrs in Stirling. HORVU2Hr1G111760.1, a Receptor-like protein kinase 4 – 1 (RLPK4 – 1) was consistently expressed in all varieties at 24 and 48hrs, but at moderately low levels than GST family protein)and RLPK4 – 2with its best expression of 36 RPKM in AC Metcalfe at 48hrs. Expression of three genes, namely HORVU2Hr1G111750 Disease resistance protein (DRP), HORVU2Hr1G111790.7 Receptor-like protein kinase 4 (RLPK4), and HORVU2Hr1G111880.1 Endoglucanase 3 (EDG3) recorded very low or no expression level at all, in some of its time points (Supplementary figure 5.2, Supplementary table 5.5).

A search for each of the gene was undertaken in barley genome explorer (https://apex.ipk-gatersleben.de/apex/f?p=284:10::::::) (Colmsee et al. 2015) to identify the expression profile at different growth stages in fragments per kilobase million (FPKM) (supplementary table 5.6). Three genes (HORVU2Hr1G111760.1, HORVU2Hr1G111780.3, and HORVU2Hr1G111840.5) expressed themselves at early growth stages and are most likely to be candidate genes for salinity tolerance at germination (Supplementary figure 5.3). This finding paved the way for identifying candidate genes for salinity tolerance in barley on the chromosome 2H locus mapped by Angesa et al. (2017).

5.4.6 Identification of potential molecular markers for marker-assisted selection

In total 21 InDel markers within the newly defined flanking region of markers InDel 74-47 and InDel 20-44 inclusive on chromosome 2H locus was considered to have potential for marker-assisted selection (MAS) for salinity tolerance at germination (Figure 5.3). The 19 markers identified within the salinity tolerance region of 2H in the CM72/Gairdner DH population were categorized into seven groups that explained tolerance index variation of 9.06 - 11.12% (Supplementary table 5.7). The InDel markers in salinity tolerance region of 2H in the six recombinant lines of CM72/Gairdner DH population selected for their association between markers and phenotypic expression in response to salinity stress had similar grouping compared with the whole lines but explaining wider range of phenotypic variations of 1.84 - 19.61% (Supplementary table 5.7).

In Skiff/CM72 DH population, the five markers associated with salinity tolerance in the region were divided into three groups explaining phenotypic variation of 10.63 to 14.52%. The three

recombinant lines selected from this population within the region, belonging to three different groups of markers explaining phenotypic variation of 6.65 to 20.03% (Supplementary table 5.7). The Skiff/CM72 DH population recombinant lines had a genotype-phenotype similarity of 66.7% for 4 markers and 100% for one (Figure 5.3). Only markers that were showing 100% genotype-phenotype consistencies' in their respective recombinant populations and explaining the highest phenotypic variations were selected for further analysis. Therefore, markers InDels 15-008, 15-009, 74-61, and74-63 in the CM72/Gairdner DH population explaining 19.62% and InDel74-56 explaining 20.03% of the phenotypic variations in the recombinant lines and 9.89% in the whole population were considered to be possible diagnostic markers (Table 5.4 and supplementary table 5.7).

Table 5.4 Details of the InDel markers considered as candidate diagnostic markers

Marker ID	Chromosome	Physical location	Forward sequence	Reverse sequence	Size (bp)
InDel 15-008	2H	723886744	TGAGTGGCAGAGTGTTGGTC	TCAAACCGGGTAAGATCATGCA	164
InDel 15-009	2H	724170810	GCTCAAAGTTGGTTCCGTCG	TAAGCGAGGGAGTCTTCCGA	195
InDel 74-56	2H	723599320	TACGTCTCCCGAAAGCAACC	ACCGGTTTTGAAAGGTCCGT	121
InDel 74-61	2H	724202721	GGTCATAACCATGGCCGTGA	CGGCTTCTCGTTGAGGATGT	187
InDel 74-63	2H	724367188	TCGGTCCGAGTCCAAAAAGG	GAAAGTTGAGCCGGACTGGT	105

The fine-mapping results from the CM72/Gairdner DH population located InDel 74-56 outside the boundary region likely to have tolerant genes. Therefore, only InDel 15-008, 15-009, 74-61 and 74-63 were considered for further evaluation. These four InDel markers were genotyped in a panel of 265 barley germplasm from across the globe including Australian commercial varieties. Markers InDel 74-61 and 74-63 were consistent with each other for both the A (CM72) and B (Gairdner) allele while, InDel 15-009 was consistent but uniformly opposite and InDel 15-008 was neither stable nor consistent hence not considered for further analysis. Genotyping of InDel 15-008, 15-009, 74-61 and 74-63 on the F_2 generation of CM72/Gairdner DH/*Spartacus CL resulted in four recombinant lines whose phenotypic expression indicated that the two markers (InDel 74-61 and 74-63) were the closest to the gene(s) of salinity tolerance at germination on the 2H locus. Therefore, two markers namely, InDel 74-61 and 74-63 explaining phenotypic variation of 3.04% and 2.97% respectively, in the 265 diverse barley germplasm were considered candidate Diagnosti**c** markers on chromosome 2H containing gene(s) for salinity tolerance at germination. Cluster analysis of these two InDel markers and the tolerance index of the 265 diverse barley germplasm formed two major clusters, CM7 (C – type) and Gairdner (G – type) groups with average tolerance indices of 81.38 and 78.02%

respectively. The G – type had higher number of genotypes (210) than the C – type (55). Furthermore, the G – type was distinguished into two minor groups of 201 and 9 entries, with respective average salinity tolerance indices of 77.35 and 78.69% each. Tolerance index of the whole population of 265 accessions was 79.01% which was above the G – group but below C – group (Supplementary figure 5.4), and a standard deviation of ±1.23 was calculated. Therefore, the average tolerance index of C – type was significantly different from G – type and the whole population.

5.4.7 Expressions of 4 genes in CM72 and Gairdner under salinity stress by real-time qPCR

All the seven genes from the fine mapped region had a noticeable expression during germination under deionised (DI) water (control) in the two varieties CM72 and Gairdner. Under DI water generally, the expression levels of the genes were higher in Gairdner than in CM72. Disease resistance protein (DRP) (HORVU2Hr1G111750.5) recorded the highest fold changes while Cyclin family protein (CFP) (HORVU2Hr1G111740.1) had the lowest in Gairdner. (Supplementary figure 5.5 and Supplementary Table 5.7). In the 150mM NaCl treatment, the relative expression level of the genes made a shift in the two varieties. Apart from CFP that had lower values for CM72, the fold changes were generally higher in CM72 in the rest of the genes. The expression levels of Receptor-like protein kinase 4 – 1 (RLPK4 – 1) (HORVU2Hr1G111760.1), RLPK4 – 2 (HORVU2Hr1G111780.3) and RLPK4 – 3 (HORVU2Hr1G111790.7) were relatively higher in CM72, but not significant at all time points. GST family protein (HORVU2Hr1G111840.5) and EDG3 (HORVU2Hr1G111880.1) recorded higher and significant fold change under stress at 40hrs 64hrs and 88hrs in CM72. CFP expression was inhibited in both CM72 and Gairdner at 16 and 40hrs of salt treatment, but Gairdner showed increased expression at 64hrs. A possible reason may be that the salt-sensitive variety has reached a critical point for growth and development under stress, so the gene is triggered to increase the rate of cell division. This pattern maybe in response to salinity stress rather than a tolerance mechanism. DRP expression was induced in Gairdner at the late stage of salinity treatment, however, it's generally inhibited by salt stress in both varieties (Supplementary figure 5.5).

Comparative analysis of fold changes under salinity and control treatments of the seven genes indicated that RLPK4 – 1, RLPK4 – 2, RLPK4 – 3, GST family protein and EDG3 were upregulated in CM72 and suppressed in Gairdner under salinity stress. It was significantly

induced at 40hrs, 64hrs and 88hrs in RLPK4 – 1, RLPK4 – 2, RLPK4 – 3 and EDG3 and at 16hrs, 64hrs and 88hrs in GST family protein and EDG3. Interestingly, salt stress upregulated the comparative expressions of RLPK4 – 1, RLPK4 – 2 and RLPK4 – 3 in Gairdner at 16hrs. The upregulation at 16hrs was just momentous in RLPK4 – 2 as it was quickly suppressed at 40hrs and started to increase in the subsequent times of 64 and 88hrs even though in all cases Gairdner's levels were less than that of CM72. RLPK4 – 3 expression was higher in Gairdner at 16hrs and dropped below CM72 at 40, 64, and 88hrs, but noticeably at 40 and 88hrs the comparative expression level of Gairdner was the same. Relative expression of RLPK4 – 1 in Gairdner remained unchanged in all the hours and relatively below CM72 at 40, 64 and 88hrs (Figure 5.5).

RLPK4 – 1, RLPK4 – 2, RLPK4 – 3, GST family protein and EDG3 were highly induced in Gairdner but not CM72 under control (consistent with Supplementary figure 5.2 & 5.3, Supplementary table 5.5 & 5.6). They were gradually upregulated in CM72 under treatment but not substantial in Gairdner, since the expression level was much higher than CM72 under control condition. Based on the relative and comparative expression patterns, we selected genes focusing on: - (i) genes that were expressed in both CM72 and Gardner under control treatment in all time points. (ii) genes that displayed a continuous up-regulation pattern in both varieties with little variation among them or Gairdner being higher initially and eventually CM72 progresses to outshine in the subsequent time points under salinity treatment (iii) genes whose comparative expression (salinity treatment less control treatment), were up-regulated at time point 1 more than or equivalent to that in Gairdner than CM72 but were down-regulated or remained unchanged in the subsequent time points. (iv) genes with initial low proportional expression (treated less control) at time point 1 in CM72 but eventually increased at time points 2 onwards to more than that of Gairdner.

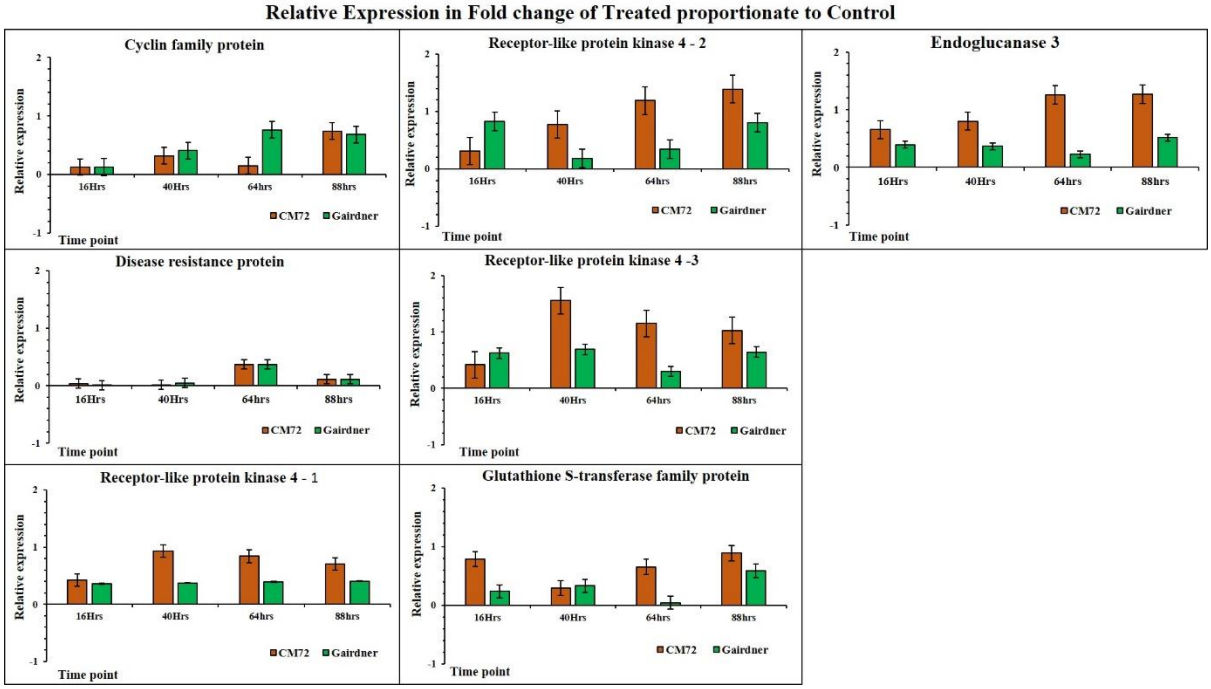


Figure 5.5 Quantitative variation of genes in proportional expression levels of seven genes in the embryo during germination of CM72 and Gairdner under 150mM NaCl (treated) relative to deionized (DI) water (control).

Cyclin family protein (HORVU2Hr1G111740.1), Disease resistance protein (HORVU2Hr1G111750.5), Receptor-like protein kinase 4 – 1 (HORVU2Hr1G111760.1), Receptor-like protein kinase 4 – 2 (HORVU2Hr1G111780.3), Receptor-like protein kinase 4 – 3 (HORVU2Hr1G111790.7), Glutathione-S-transferase family protein (HORVU2Hr1G111840.5), Endoglucanase 3 (HORVU2Hr1G111880.1). Expression of 7 genes were analysed from RT-qPCR results using comparative quantification $2^{-\Delta\Delta CT}$ method with HvGAPDH as internal control under 150mM NaCl stress and DI water at 16, 40, 64 and 88 hrs. Data was shown as means ± S.D. Error bars which are not overlapping differ significantly at $P \leq 0.05$.

The three Receptor-like protein kinase 4 were fitting most of the four groups outlined earlier. While RLPK4 – 3 displayed a continuous up-regulation pattern in both varieties, there was no variation among them at 16 and 40hrs, but eventually CM72 progresses to outshine Gairdner at 64hrs and the two varieties were equal again at 88hrs, therefore it was not likely to be the candidate gene. RLPK4 – 1 was fitting the description, but notable, was the up regulation of Gairdner to levels higher than CM72 at time point 2 under treated conditions making it prospective to salinity stress at germination (Supplementary figure 5.5). RLPK4 – 2 was fulfilling all the descriptions above almost impeccably and hence forthcoming as a possible

gene. Therefore, RLPK4 – 1 and 2 (HORVU2Hr1G111760.1 and HORVU2Hr1G111780.3) are proposed to be the most likely genes contributing to salinity stress tolerance at germination by enhancing germination in barley.

5.4.8 Candidate genes structure analysis

We amplified the three Receptor-like protein kinase 4 including, GST family protein and compared the sequences between the two varieties (CM72 and Gairdner). Though it was not fulfilling the threshold outlined in 4 points earlier, GST family protein was included because of its high expression level during germination. Based on barley genome explorer (https://apex.ipk-gatersleben.de/apex/f?p=284:10::::::) (Colmsee et al. 2015) and genomic database (http://146.118.64.11/BarleyVar/) (Mascher et al. 2017) three Receptor-like protein kinase 4 were initially selected as possible candidate genes and analysed (Table 5.5). RLPK4 – 2 with 4633bp full length was the shortest compared with RLPK4 – 1 with 6614bp and RLPK4 –3 with 6754bp. RLPK4 – 2 and RLPK4 – 1 had 3 exons each with almost similar coding sequences of 1551 and 1548bp translating into 516 and 515 amino acids, respectively. GST family protein was the largest gene with 7999bp full length, 2 exons and 804bp coding sequence translatable to 267 amino acids.

The sequences comparison between genomic DNA of the two varieties (CM72 and Gairdner) in the four genes revealed similarities between the parents. There were not many variations in the promoter regions and the exons of RLPK4 – 3 and GST family protein (between CM72 and Gairdner that may be associated with dissimilar expression levels. However, in exon 3 of RLPK4 – 1, 724182861 – 724185561 on 2H of barley genome we observed deletions at 6 different sites of Gairdner sequence totalling to 12bp, 1bp in CM72 and 1bp insertion in both varieties separately in comparison with Morex. Further, the alteration caused a probable loss of 6 amino acids (3 Ser, 1 Arg, 1 Pro and 1 Ala) and some substitutions in Gairdner (Ala to arg, 2 leu to 2 Ala, Ser to Glu, Ala to Ser and Thr to Ser) (Figure 5.6). An insertion of 18bp back to back in exon 3, in CM72 and 1bp in Gairdner of RLPK4 – 2, 724201515 – 724203996 on 2H was observed. More deletions totalling to 22bp at different locations within CM72 sequence was detected in RLPK4 – 2. The inserted bases in CM72 were translating in to 6 extra projected amino acids added (1 Ala, 2 Pro,1 Glu and 2 Thr) and the deletions causing a likely loss of 12 amino acids (2 Arg, 1 Tyr, 1 Glu, 1Pro, 3 Ala, 1 Asp, 1 Phe, 1 Asp and 1 Thr) (Figure 5.7). The insertions and deletions in the exons of the two genes may be important for the difference in salinity tolerance levels between the two varieties during germination.

Table 5.5 Analysis of the four genes in the fine mapped region based on barley genome explorer and genomic database

Gene ID	Name	InDels	SNPs	Gene length (bp)	Exons No. and length (bp)	Coding sequence (bp)	Intron No. and length (bp)	Upstream sequence (bp)	Downstream sequence (bp)	Amino acids No.
HORVU2Hr1G111760.1	Receptor-like protein kinase 4	22	138	6614	3 (110, 71 & 1370)	1551	2 (510 & 134)	2371	2047	516
HORVU2Hr1G111780.3	Receptor-like protein kinase 4	14	157	4633	3 (155, 69 & 1324)	1548	2 (707 & 119)	629	130	515
HORVU2Hr1G111790.7	Receptor-like protein kinase 4	5	105	6754	2 (19 & 1238)	1257	1 (94)	2000	3403	418
HORVU2Hr1G111840.5	Glutathione-S-transferase family protein	21	88	7999	2 (417 & 387)	804	1 (102)	2002	5091	267

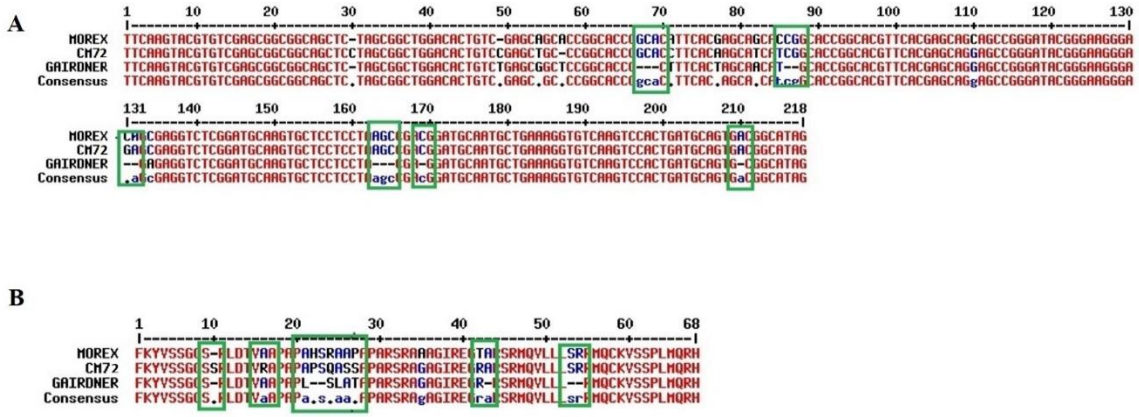


Figure 5. 6 Representation of A – sequences at the tail end of exon 3 location 4349 to 4566 of Receptor-like protein kinase 4 – 1 (HORVU2Hr1G111760.1) and B – the predictable amino acids.

The highlights in green A – indicates the deletions and insertions of the bases while B – is the changes in predictable amino acids due to the alterations in bases.

Figure 5.7 Receptor-like protein kinase 4 – 2 (HORVU2Hr1G111780.3) exon 3 extract from 3179 to 3990 of the gene showing A – the bases sequences and B – the likely amino acids with the green colour boxes indicating the discrepancies in the respective orders.

5.4.9 Relationship between the two RLPK4 of barley and homolog genes from other species

The two RLPK4 (HORVU2Hr1G111760.1 and HORVU2Hr1G111780.3) had similar and identical 10 hits that were aligned using MEGA-X (Kumar et al. 2018) as shown in Supplementary figure 5.6. G-type lectin S-receptor-like serine/threonine-protein kinase SD2-5 of *Triticum urartu* had the highest similarity of 86.83% while G-type lectin S-receptor-like serine/threonine-protein kinase SD2-5 of *Setaria viridis* was the least at 71.71%. Phylogenetic tree was constructed by means of maximum likelihood (ML) process executed in MEGA-X and it indicated that barley was related to wheat with a slight distance from it. However, it was more distinct from *Setaria* (Supplementary figure 5.7).

5.4.10 Comparison of the conserved domains in the two RLPK4

All the hits above 70% (Supplementary figure 5.8) of the two genes indicated that they were more diverse at the beginning and at the end, but more conserved in the middle. The preserved area for RLPK4 – 1 was between amino acids 150 – 435 (285 amino acids) and RLPK4 – 2 was 165 – 450 (285 amino acids) both of which are within the third exon (Figure 5.8). The preserved domains for RLPK4 – 1 contains Protein Kinases, catalytic domain (PKc) _ like superfamily) that is composed of catalytic domains of serine/threonine and tyrosine-specific protein kinases, RIO kinases, (typical serine protein kinases), aminoglycoside phosphotransferases, and choline kinases. The ATP binding site on conserved domain of RLPK4 – 1 have been mapped in Figure 5.8A as follows: i. STE**LGSG**GF**G**V**V**YKGELPNGLPV**A**V**K**VL ii. HL**V**RLYGFCFDPDTKALV**YEYL**ENG and iii. VHY**D**IKP**PN**I**L**LTADFTPKV**AD**FG the (amino acid sequences underlined and bold are conserved domains for ATP binding site). Conserved domain of RLPK4 – 2 has Serine/Threonine kinases (STKc), Interleukin-1 Receptor Associated Kinases (IRAK), related STKs and PKc _ like superfamily (Figure 5.8B). The ATP binding, active, polypeptide substrate binding and activation loop (A-loop) sites on conserved domain have been mapped in Figure 5.8B as follows: i. STE**LGSGG**FGVVYKGELPNRLSV**A**V**K**LL ii. HVHL**V**RLYGFCFDPDTKAL**VYEYL**ENG**S**L**E**KY and iii. HY**D**I**K**P**AN**I**L**LTADFTPKV**ADFGLARL**GERENTHMS**SLT**GGG**RGTPG**YA (the amino acid sequences underlined and bold are conserved domains for the sites). The occurrence of conserved area in exon 3 of the genes (that are hypothetically starting on amino acid 61 and 76 to the end for RLPK4 – 1 and RLPK4 – 2 respectively) is important because any variation in

this regions as reported here is likely to cause changes in amino acids that can affect the gene function.

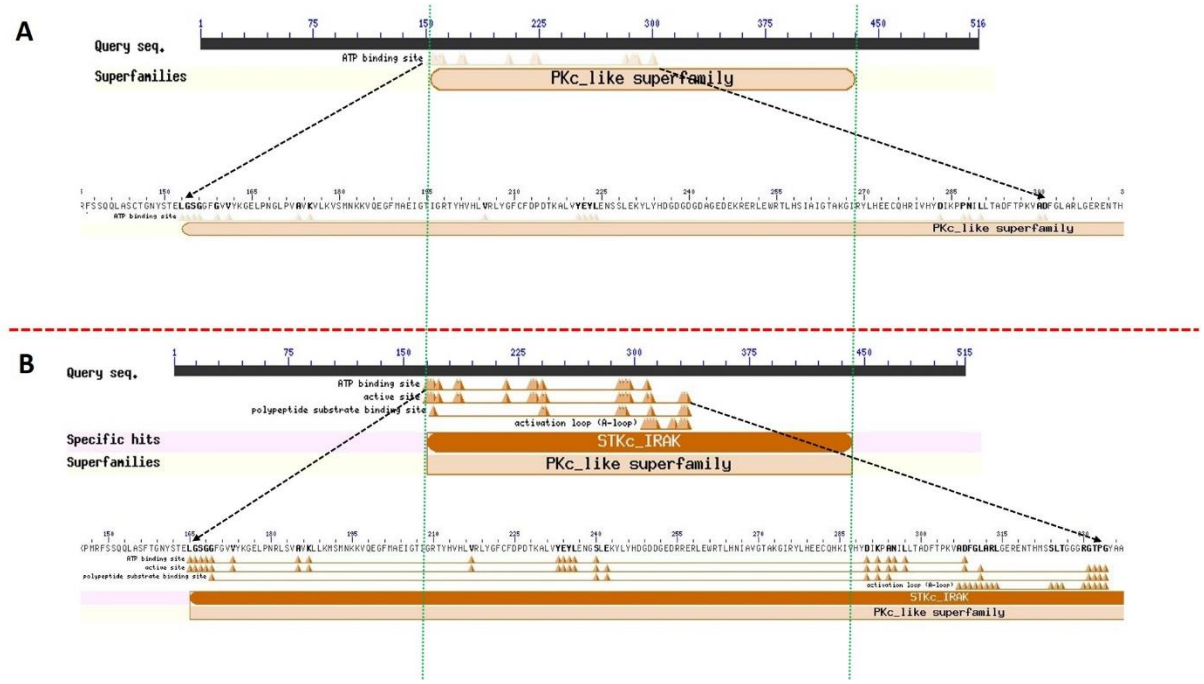


Figure 5.8 Conserved domains for Receptor-like protein kinase 4 – 1 (HORVU2Hr1G111760.1) A Receptor-like protein kinase 4 – 2 (HORVU2Hr1G111780.3) B. PKc is Protein Kinases, catalytic domain, STKc is Serine/Threonine kinases and IRAK is Interleukin-1 Receptor Associated Kinases.

The red dotted line is the boundary between the two genes while the 2 blue are for the conserved domain. The amino acids sequences bolded are reserved domains for the ATP binding, active, polypeptide substrate binding and activation loop (A-loop) sites.

5.5 Discussion

5.5.1 Fine-mapping and gene annotation

The double haploid (DH) lines from the CM72/Gairdner and Skiff/CM72 populations showed continuous distribution but varied germination percentage. This demonstrates the quantitative nature of salinity stress tolerance in barley. Some lines showed transgressive tolerance index in both directions, indicating the likelihood of favourable and unfavourable allelic combinations in parents. Two studies conducted on barley reported different QTLs for salinity tolerance at germination stage and confirmed that they differed from those at the seedling stage (Angesa et al. 2017; Mano and Takeda 1997).

Saturating markers in a specific chromosomal region is the first step in map-based gene isolation and to improve the accuracy of MAS (Jia et al. 2016; Lüpken et al. 2013; Silvar et al.

2012). In our previous study, the QTLs conferring salinity tolerance at germination were mapped to a big interval on the short arm of chromosome 2H (Angesa et al. 2017). In the present study, we increased the marker density on the QTL flanking region before identifying gene(s) and developing molecular markers. Screening the parents with InDel markers identified 42 of polymorphic markers in CM72/Gairdner compared with 12 Skiff/CM72 DH Populations. This confirms the fact that CM72/Gairdner DH population is derived from varieties that vary more between susceptible (Gairdner) and resistant (CM72) parents compared to Skiff/CM72 DH population from Skiff (susceptible) and CM72.

Using InDel markers is advantageous because they give an accurate nature of allelic variation of haplotypes and provide high density near the locus (Batley et al. 2003; Bian et al. 2013). The inclusion of new markers within the QTL region increased their density enabling the construction of a new genetic map and validation of the previous flanking markers. The screening of recombinant lines and phenotypic matching allowed us to narrow the interval to a region of ~0.341 MB. Within this region, we identified 9 genes belonging to five families including; cyclin family protein, disease resistance protein, glutathione S-transferase family protein, endoglucanase 3, Undescribed protein, and transmembrane emp24 domain-containing protein 3. The receptor-like protein kinase 4 was repeated, three in a series following each other. The receptor-like protein kinase 4 subfamily enhances the reverse inhibitory effect of salinity during germination in rice (Anuradha et al. 2001) and has been characterized for salinity tolerance in maize (Baudino et al. 2001), wheat (Singla et al. 2008), soybean (Yang et al. 2011), rice (Singla et al. 2009), cotton (Pandey and Chaudhary 2014) and model *Arabidopsis* (Hecht et al. 2001). Overexpression of receptor-like kinases improves salinity, oxidative stress and ABA tolerance in their seeds during germination and enhances early root growth in *Arabidopsis* (Wang et al. 2017), soybean (Qiu et al. 2019), boosts shoot Na^+ elimination and improves biomass in both *Arabidopsis* and barley (Amarasinghe et al. 2020).

5.5.2 Identification of potential molecular markers

Selecting for a trait based on phenotyping is slow, laborious, environment-dependent and needs lots of space. However, cultivar release could be accelerated by identifying parental tolerant germplasm and choosing progeny through MAS using dependable molecular markers, such as PCR-based, that are easy to run (Yang et al. 2018). With dependable molecular markers already developed, MAS would be able to quickly identify salt-tolerant germplasm at the germination stage for crossing and selection in barley breeding programs (Collard and Mackill

2008). In this study, we designed two molecular markers from the fine-mapped region with a 100% phenotype-genotype match in the recombinant lines of CM7/Gairdner DH likely to be associated with the salt-tolerant gene(s) located in the chromosome 2H locus. Furthermore, our results indicate that the gene(s) responsible for the QTL effect is/are likely to be between or close to markers InDel 74-61and 74-63. InDel 74-61 is inside one of our target genes (HORVU2Hr1G111780.3) that belongs to the receptor-like protein kinase 4 family. In the longer term, other markers (SNP-based) within InDel 74-61and 74-63 will need to be designed to allow for a more informative marker haplotype that will remove issues of marker-gene recombination. It is likely that the above markers could be converted for this purpose or do SNP screening as per the methods in this study using F_2/F_4 populations.

A collection of 265 barley accessions from across the globe offered a retrospective analysis for validation of the identified markers and tested their usability as a selection tool in a commercial barley breeding program. The cluster analysis divided germplasm into subpopulations that exhibit a mean tolerance index that corresponds with that of the DH or F_2 populations. However, there was some variation in the means of the subpopulations in the worldwide barley germplasm, most likely due to factors such as the linkage between markers broken by recombination, other genes playing a role since the trait is polygenic, and genetic variation at other loci. Four parents, constituting the two DH and F_2 backcross populations, were included in the genotyping analysis of barley germplasm; notably, Gairdner and Spartacus CL fell into the same group, distinct from CM72, while Skiff was in a distinct smaller subgroup but the same major group as CM72, as predicted from our fine-mapping results. The above results suggest that the markers could be confidently used to practice negative selection to remove genotypes without markers because it is rare for the most tolerant or sensitive germplasm to be erroneously predicted by the marker genotype.

It is clear from this study and other reports that no single gene can offer complete salinity tolerance at germination or any other growth stage, which confirms the polygenic nature of this trait (Colmer et al. 2005; Liu et al. 2017; Roy et al. 2014; Saade et al. 2016). Using MAS to combine multiple tolerant loci with divergent functions could widen the tolerance range of varieties and result in additive effects on tolerance levels. However, combining different tolerant loci does not necessarily produce an additive effect (Melese 2018). Marker-assisted selection is the best way to identify salinity tolerance gene/loci pyramiding from different sources and the most effective loci combinations (Ashraf et al. 2012). The markers developed

in this study will enable the 2H locus to be combined with others to breed barley varieties with a high level of mixed salinity tolerance, used for germplasm selection targeting QTLs, and provide a base for the development of gene-specific markers.

5.5.3 Expression studies of tolerant and sensitive verities revealed the involvement of Receptor-like protein kinase 4 in salinity stress tolerance during germination

Without overemphasizing salinity affects most plant growth aspects including germination through osmotic stress, ionic imbalance, nutrition imbalance and oxidation (Deinlein et al. 2014), as well as, changing gene expression levels that affect plant response indirectly (Byrt et al. 2020). Germination involves a process where a quiescent, dry seed imbibes water to facilitate embryonic axis elongation that is enhanced by several internal (like gene expression) and external factors including those with potential resistance like salinity stress (Yang et al. 2020). The processes by which barley seeds can maintain high germination under salt stress is not well investigated and little is known about the genes involved. To circumvent the adverse impact of environmental stress, plants either evade or sidestep the harmful effects by varying the expression of genes associated with stress management signalling (Passricha et al. 2020). Key categories of genes displaying upregulation under stress conditions are linked to cellular activities like metabolite synthesis for osmoregulation, transportation of ions, hormone secretion, signal recognition and signal transmission (Passricha et al. 2020). The essential high expression levels of genes under stress are hypothetically associated with tolerance capacity of a variety.

In this study, we compared a salt tolerance variety (CM72) and a salt-sensitive variety (Gairdner) in 150mM NaCl to investigate the variation in genetic responses, which is predictable to be associated to salinity tolerance. Based on the real time-qPCR analysis, we observed that 2 RLPK4 (HORVU2Hr1G111780.3 and HORVU2Hr1G111760.1) were significantly upregulated in salt-tolerant variety (CM72) under salt stress than sensitive Gairdner. In consistence with our results, Real-time PCR results showed that the expression levels of Receptor-like protein kinases (RLKs) in soybean were principally induced by salt stresses and their overexpression in *Arabidopsis* promoted seed germination, as well as primary root and rosette leaf growth during the early stages of salinity stress (Sun et al. 2013; Qiu et al. 2019).

Nanda et al. (2019) reported a combination of two receptor-like kinases working together synergistically to regulate the timing of germination and were responsive to salt and osmotic

stress in *Arabidopsis* seeds. Salinity susceptible mutants seeds also showed a hypersensitive reaction to ABA at germination, at the same time, exhibiting heightened upregulation of germination repressors and dormancy inducers (ABA-insensitive-3, ABA-insensitive-5, DELLA encoding RGL2 and Delay-Of-Germination-1) under salinity. Receptor-Like Kinases (RLKs) are plasma membrane receptors/proteins molecules involved in detection of external and endogenous cell signals or stimuli both biotic and abiotic (Passricha et al. 2019). Embryogenesis Receptor-like Kinases (*SERK*) genes, a subgroup of RLKs play a role in several signalling that are vital for plant, development and immune system (Cueva-Agila et al. 2020). In specific, they are vastly expressed in the early stages of somatic embryogenesis (Salaj et al. 2008), and hence, the study of their involvement in salinity tolerance during germination is of supreme standing to advance the commercial production of barley. In the coding sequence, of exon three in the two Receptor-like protein kinase's 4 (HORVU2Hr1G111780.3 and HORVU2Hr1G111760.1) we reported variation in bases sequences that could be associated with the variation in the expression levels of the genes in CM72 and Gairdner varieties. Any variation in the coding sequences of genes is likely to change the amino acids that will be spliced and eventually modify its expression. Similar to our study, Do et al. (2020) reported a variation of 4 bases in coding region of OsHKT1;1 gene of rice leading to substitution of 4 amino acids that had no potential effects to resulting protein structure, but caused variations in post-translational modifications.

Homolog G-type lectin S-receptor-like serine/threonine-protein kinase SD2-5 from *Triticum urartu* and *Setaria viridis* had the highest and lowest percentage (87 and 72%) similarity for both Receptor-like protein kinase's 4 (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins). The genes may be closely related in structure and function however, there was none with a 90 – 100% resemblance in amino acids meaning that RLP4 is somewhat unique. Receptor-like protein kinase's superfamily have been reported to duplicate themselves in organisms with variation in the copy numbers and size due to variance in expansion in species genome size differences and have redundant function within a cluster (Liu et al. 2018). G-type lectin S-receptor-like serine/threonine protein kinase (GsSRK) have been reported to positively regulate salinity tolerance in plants, especially in wild soybean. Real-time PCR has shown GsSRK is upregulated by ABA, salt, and drought stresses. Its overexpression enhanced seed germination, main root and growth of rosette leaf during the initial stages of salt stress and eventually higher chlorophyll content, taller plants, better yields, lower ion leakage and more siliques at the adult growing phase in *Arabidopsis* (Sun et al. 2013).

In alfalfa, overexpression of GsSRK resulted to more twigs but petite shoots, healthier growth, reduced ion leakage (low Na^+ and high K^+) and MDA content, increased SOD activity, proline content (plays a role in ROS scavenging, ion homeostasis, and osmotic regulation) and salt stress tolerance (Sun et al. 2018).

The highly conserved sites with binding abilities in plants suggest that any alteration in their structure units can eliminate protein function, and consequently evolutionary less favoured (Sharma and Pandey 2016). Transcription factor domain capability to confer protein binding and arbitrating structural communications is drawn from many biological activities like signal transduction, biological molecule modification and cellular biosynthesis (Boulard et al. 2018). Protein kinases function in big numbers of diverse signalling pathways, with their catalytic activity being very critical in regulating growth and protection of the organisms. Different active kinases assume patently comparable structure of catalytic domains, but inactive ones are flexible to allow for adoption of different conformations to a precise controlling protein in response to communications (Huse and Kuriyan 2020).

The 2 protein kinases are composed of catalytic domains of serine/threonine and tyrosine-specific protein kinases (STKs), RIO kinases, (typical serine protein kinases), aminoglycoside phosphotransferases, and choline kinases that are involved in catalysing the transfer of gamma-phosphoryl from ATP to hydroxyl groups in substrates of proteins (Hanks and Hunter 1995). The conserved domains of ATP binding site $GXGXXGX_{14}K$ (X being any amino acid) (Hanks et al. 1988), active site, polypeptide substrate binding site and activation loop in the two Receptor-like protein kinase's 4 (Figure 5.8) were highly conserved. IRAKs plays a role in Toll-like receptor (TLR) and interleukin-1 (IL-1) signalling pathways, thus critical in regulating innate immune responses and inflammation (De Nardo et al. 2018; Flannery and Bowie 2010; Ringwood and Li 2008). Different types of IRAKs have been reported (IRAK-1, -2, -3 (or -M), and -4) that exhibit different functions and levels of expression, subcellular distribution and dissimilarly arbitrate TLR signalling (Chu et al. 2019). Generally, -1, -2, and -4 are universally expressed as active kinases, while IRAK-M is only induced in monocytes (barley) and macrophages and usually is an inactive kinase (Liu et al. 2019). IRAK-M contains a central kinase domain (a pseudokinase domain) on top of an N-terminal Death domain (DD), a proST region (rich in serines, prolines, and threonines), and a C-terminal domain like other IRAKs (Wesche et al. 1999). They are hormonal regulated pathways for resisting attack and binds to signalling peptides to limit stem cell proliferation, maintenance of shoot and root apical

meristem in embryos (Couto and Zipfel, 2016; Zhou et al. 2018). The presence of inactive kinase IRAK – 3 or M (Figure 5.8B) in the conserved domain of RLPK4 – 2, is an indication that it's likely to show more plasticity to external stimulus like salinity stress.

5.6 References

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5.7 Supplementary Material

5.7.1 Supplementary Tables

Supplementary Table 5.1 List of all Markers used and their polymorphism in the respective DH populations

InDel_ID	Code	Position	Forward Primer	Reverse Primer	Size (bp)	CM72/Gairdner	Skiff/CM72
IND|chr2H|720098942	74-1	720098692-720099242	CCGATACGACATACCCCACG	GCTAGCCAGGTTCCAGTCAG	144	Polymorphic	Not Polymorphic
IND|chr2H|720348280	74-2	720348030-720348580	GAAGTTTCAGATGCACGGGC	TCAGTAGACACCGCATGCTG	135	Not Polymorphic	Not Polymorphic
IND|chr2H|720584153	74-3	720583903-720584453	AGGAGGTCTTCAGTCGAGCT	TTCGACCTGCACAGCCTAAG	168	Polymorphic	Not Polymorphic
IND|chr2H|720618379	74-4	720618129-720618679	CGCTGGACATGCATCCCTTA	ATGATGCCTTGGGTGTGTGT	116	Not Polymorphic	Not Polymorphic
IND|chr2H|720620802	74-5	720620552-720621102	GGGGTTTTTACACGGGGACA	AGTATGTTTTCCTGGCGGCA	136	Not Polymorphic	Not Polymorphic
IND|chr2H|720661583	74-6	720661333-720661883	AGAGGTCTTAGCACTTCACCT	GATTACAGCCTCGCCTCACA	100	Polymorphic	Not Polymorphic
IND|chr2H|720741307	74-7	720741057-720741607	TGGTCAGGAAACCTTGACCAT	TTGCATTTCCTTGCTCGTGA	158	Not Polymorphic	Not Polymorphic
IND|chr2H|720992664	74-8	720992414-720992964	TTTCCGTAGCAGTTGGGCTC	GCGTCGGGCCCGTAAATATA	137	Not Polymorphic	Not Polymorphic
IND|chr2H|720993636	74-9	720993386-720993936	ATAAGGGCGCACATTGTCCA	GACGGCATGGATCGATGGAT	194	Polymorphic	Not Polymorphic
IND|chr2H|721113030	74-10	721112780-721113330	TGTGTTGTAACCTGGCCACC	TAGGTCGGAGTGTGAGAGCA	111	Polymorphic	Not Polymorphic
IND|chr2H|721201859	74-11	721201609-721202159	GGCTTCCTACCCATCGGATC	CTGGCCTCTCCAACAGACAG	150	Polymorphic	Not Polymorphic
IND|chr2H|721203925	74-12	721203675-721204225	CTTGTAGCTCGCATCGCAAC	CGAAGTTGTGAAGCACGTCG	138	Not Polymorphic	Not Polymorphic
IND|chr2H|721207068	74-13	721206818-721207368	TTGCTAGCTTCGTCGTCCTC	GGCTCCACCTGGTTACCATT	117	Not Polymorphic	Not Polymorphic
IND|chr2H|721210499	74-14	721210249-721210799	TCGTGAGTTGCAGACCACAG	GTCTCTCAAAGGTGCCCGTA	175	Not Polymorphic	Not Polymorphic
IND|chr2H|721644485	74-15	721644235-721644785	ACCTAGAATGCCCAAACTTGAGT	GCATGGGCTACATCAACCAT	151	Polymorphic	Not Polymorphic
IND|chr2H|721677674	74-16	721677424-721677974	AAGCAGTGCAAGGCGTCTAT	AATGAGCCACCAACCTGGAG	195	Polymorphic	Not Polymorphic
IND|chr2H|721923841	74-17	721923591-721924141	CCATCACCCACGCATTGTTG	TTTCATGCGGTGTCGGGTAA	135	Not Polymorphic	Not Polymorphic
IND|chr2H|721942794	74-18	721942544-721943094	GGTGAGCACGGGAAAGAAGA	GCCCAACGAGCAACACAATT	173	Polymorphic	Not Polymorphic
IND|chr2H|721945132	74-19	721944882-721945432	TCTGCTGCCAACTCTGGATC	TTCAGGACAGCGAGGTTGAC	130	Polymorphic	Not Polymorphic
IND|chr2H|721985284	74-20	721985034-721985584	GCCGGCTTCTTTCAACACTC	AGCACGACAACAGATGTGGA	110	Not Polymorphic	Not Polymorphic
IND|chr2H|722118491	74-21	722118241-722118791	CTGCAGCTAGACTCACTCCG	CAGCTGCCGAATTACCAAGC	189	Not Polymorphic	Not Polymorphic
IND|chr2H|722124583	74-22	722124333-722124883	GGCGACTAGGATGAGTCTGC	CGTTGCACCGTGTGGATTTT	110	Not Polymorphic	Not Polymorphic
IND|chr2H|722129984	74-23	722129734-722130284	ATCTCTCTCCACCCTCGACC	GCGGTTAGGGTTTGAGCTCT	135	Not Polymorphic	Not Polymorphic
IND|chr2H|722132111	74-24	722131861-722132411	GTCTTCGGACACATGCATGC	TCTGTGTGAACTGAAACATGCA	102	Not Polymorphic	Not Polymorphic
IND|chr2H|722150458	74-25	722150208-722150758	TTGTTTCCCATGCGACGTTC	GGTGAAGAACCCGGAATGGA	146	Not Polymorphic	Not Polymorphic
IND|chr2H|722153870	74-26	722153620-722154170	TGCTGCGATTTGGTGGTAGT	ATGGGTCATGTACGCTTGGG	135	Polymorphic	Not Polymorphic

IND|chr2H|722284991	74-27	722284741-722285291	AAAGGAGGGCAGCGACAATT	TTGTGAACGAGCCAAGCTGA	189	Not Polymorphic	Not Polymorphic
IND|chr2H|722298434	74-28	722298184-722298734	CTCATCCTAGAGCCGCTTGG	GCCAATGTTCGTCGTGTGAC	158	Not Polymorphic	Not Polymorphic
IND|chr2H|722310460	74-29	722310210-722310760	AACATATTGCAAGCGTCGGC	CCCCGCGTCAAAAACAGTTT	110	Not Polymorphic	Not Polymorphic
IND|chr2H|722319078	74-30	722318828-722319378	TCTGTGTCCGCATCTTGTCC	CAGATCCGTAAGTACCGGCC	122	Not Polymorphic	Not Polymorphic
IND|chr2H|722344032	74-31	722343782-722344332	AGGCGTTAGTAAGCGATGCA	CGGAGACCAAAATTTCTCGCC	200	Not Polymorphic	Not Polymorphic
IND|chr2H|722345853	74-32	722345603-722346153	GGCCGTGGGGAAGTATCTTC	TCTTTTCCCTTTCGGCAGCT	110	Not Polymorphic	Not Polymorphic
IND|chr2H|722389078	74-33	722388828-722389378	TCTGCTGTGCACGGTTAAGT	TCCTGGTGAACTCGAGCAAC	188	Not Polymorphic	Not Polymorphic
IND|chr2H|722456686	74-34	722456436-722456986	CTACCGCTCTACTGCCAGTG	TGCCTTTGCCCATTTTGAGC	171	Not Polymorphic	Not Polymorphic
IND|chr2H|722466365	74-35	722466115-722466665	GCAGCAAAGCAAAGCAAAGC	ATTTCCAGCGCGATCTCACA	174	Not Polymorphic	Not Polymorphic
IND|chr2H|722483114	74-36	722482864-722483414	CTTTTCCTGATCGCGTGCAC	GCCACACAAAAATGACGCGA	119	Not Polymorphic	Not Polymorphic
IND|chr2H|722626555	74-37	722626305-722626855	GCACAGTCCCCCTACACTTG	ACGTTGCCGGTTGTAGTACA	111	Not Polymorphic	Not Polymorphic
IND|chr2H|722704586	74-38	722704336-722704886	TCGTTGAGAAATGCAGGGGG	GCAACGGTCGCTCAGGAATA	129	Not Polymorphic	Not Polymorphic
IND|chr2H|722705372	74-39	722705122-722705672	CCACATTGCTCCACCATCCA	GCTGCAGCCACATTCAACAA	199	Not Polymorphic	Not Polymorphic
IND|chr2H|722720224	74-40	722719974-722720524	CCTAAACACTGATGCGGGGT	ACACCGAAGAAGCGTGCATA	155	Not Polymorphic	Not Polymorphic
IND|chr2H|722729203	74-41	722728953-722729503	CCACTGACGGGAGAGGTTTC	CGACGGGGAATTGGGGATAG	143	Not Polymorphic	Not Polymorphic
IND|chr2H|722764576	74-42	722764326-722764876	CAAGAAACCATTGGTGCCGG	CCTCTGCTTCCCCGTCAAC	119	Not Polymorphic	Not Polymorphic
IND|chr2H|722782141	74-43	722781891-722782441	TGGAGTTCAGGTGTGTCGTG	TCCCGACGCCGAACAATAAT	139	Not Polymorphic	Not Polymorphic
IND|chr2H|723092858	74-44	723092608-723093158	TACGGGTTGTCGCCCTAGTA	GCCTCGGCCATCTAAGTTGT	102	Not Polymorphic	Not Polymorphic
IND|chr2H|723108985	74-45	723108735-723109285	GCGCTAGTACCGGTACTCAC	TACCGTGTACGCAGCACTTT	109	Not Polymorphic	Not Polymorphic
IND|chr2H|723130816	74-46	723130566-723131116	TGTTTCTTTAGGCCGACGGG	CCGGACATGACTTGCTCTGT	144	Not Polymorphic	Not Polymorphic
IND|chr2H|723218508	74-47	723218258-723218808	ATCACCACACGCGGTTGTAT	GGGACGAGAAGGACAAGTGG	136	Polymorphic	Polymorphic
IND|chr2H|723220505	74-48	723220255-723220805	CTGCAGTCGCGACTCGTT	GGATGAGATTGTATTGCCGGC	154	Polymorphic	Polymorphic
IND|chr2H|723247735	74-49	723247485-723248035	CCTCCTGGGCTTTGGCATTA	CCCTTAACCAAACGAGGCCT	139	Polymorphic	Not Polymorphic
IND|chr2H|723294519	74-50	723294269-723294819	GACCGATAAGGGGACGACAC	ACCTCAGTACGGTCAAAGCC	103	Not Polymorphic	Not Polymorphic
IND|chr2H|723321668	74-51	723321418-723321968	GCACTTCTGCCACTCAAATCA	CCCCCATGTCCAGAAACAGA	157	Not Polymorphic	Not Polymorphic
IND|chr2H|723376260	74-52	723376010-723376560	GGCATGGGTATGGACGGTAA	TAAAGCGGCCGGATTTCTGT	137	Not Polymorphic	Not Polymorphic
IND|chr2H|723388142	74-53	723387892-723388442	GAGAGAACTAGTGACCGGCG	TTCACAACACTACGCGCATG	133	Not Polymorphic	Not Polymorphic
IND|chr2H|723405764	74-54	723405514-723406064	CCTCCAACAAGCGCTCTTTG	AGGCTAGGATGTGGGGATGT	194	Not Polymorphic	Not Polymorphic
IND|chr2H|723496904	74-55	723496654-723497204	CCAAAAACCATGGCGTTGCT	ACGCCACTGACAATAGCGTT	161	Not Polymorphic	Not Polymorphic
IND|chr2H|723599320	74-56	723599070-723599620	TACGTCTCCCGAAAGCAACC	ACCGGTTTTGAAAGGTCCGT	121	Not Polymorphic	Polymorphic
IND|chr2H|723606230	74-57	723605980-723606530	TCAAAACCACCCCCTCCATG	TGGATTTTGGCCGGATTGGA	198	Not Polymorphic	Not Polymorphic

IND|chr2H|723648084	74-58	723647834-723648384	CTGTTTGCGGCTTATGGTTCA	GCACGGGTCCTTTTGCTAGT	123	Not Polymorphic	Not Polymorphic
IND|chr2H|723650212	74-59	723649962-723650512	TGCTTTGGAATCGCAAGCAG	GGGCGATGTGTACGTGAGAA	129	Not Polymorphic	Not Polymorphic
IND|chr2H|724148616	74-60	724148366-724148916	TCCGTGTCCATGGTGTGAAG	CTTCCGACCCTTGTCTCACC	105	Not Polymorphic	Not Polymorphic
IND|chr2H|724202721	74-61	724202471-724203021	GGTCATAACCATGGCCGTGA	CGGCTTCTCGTTGAGGATGT	187	Polymorphic	Not Polymorphic
IND|chr2H|724299263	74-62	724299013-724299563	CAACGCGCCATCCCTACATA	TCCATCTTCTCTGCCCTGGA	122	Not Polymorphic	Not Polymorphic
IND|chr2H|724367188	74-63	724366938-724367488	TCGGTCCGAGTCCAAAAAGG	GAAAGTTGAGCCGGACTGGT	105	Polymorphic	Not Polymorphic
IND|chr2H|724372841	74-64	724372591-724373141	CAATGGTGGACTCCGATGCT	AAACGAAGCGGTAGACACGT	156	Not Polymorphic	Not Polymorphic
IND|chr2H|724500304	74-65	724500054-724500604	TAACGGTCACGCACTGCATA	ATATCAAGGGTGTGGCAGGC	102	Not Polymorphic	Not Polymorphic
IND|chr2H|724502620	74-66	724502370-724502920	GCCATACTCTGCCACCAACT	AGCGTTGTTCGAGTGTGTCA	200	Not Polymorphic	Not Polymorphic
IND|chr2H|724511886	74-67	724511636-724512186	TCGTGCCGCAATTCTGATTG	GCGGAAAGGTCAGCTGGTAG	127	Polymorphic	Not Polymorphic
IND|chr2H|724518959	74-68	724518709-724519259	AGGGCCATCAAGTTCTCAGC	ACCGGACGTCTTGGTAGAGA	123	Polymorphic	Not Polymorphic
IND|chr2H|724566092	74-69	724565842-724566392	TTGCTGTCGGCTCACTCTTC	CTCACGTCTTCTCCCGAACC	134	Not Polymorphic	Not Polymorphic
IND|chr2H|724576218	74-70	724575968-724576518	TCGTACCGGCGTAGAACAAC	AGCACCATGGACCGTTTGAT	121	Not Polymorphic	Not Polymorphic
IND|chr2H|724608904	74-71	724608654-724609204	CTTGCTCTGGTGGTACGTGT	ATCGTCCAGCCACTTGTCTG	129	Not Polymorphic	Not Polymorphic
IND|chr2H|724670693	74-72	724670443-724670993	AGGTTGAATCCCGAACCCAA	CCCAACAGAGTGCTTTTGCC	182	Not Polymorphic	Not Polymorphic
IND|chr2H|724711155	74-73	724710905-724711455	GGCAGGCAAAACCCTAGCTA	CGGCCACGTCTCCACATATT	102	Not Polymorphic	Not Polymorphic
IND|chr2H|724727505	74-74	724727255-724727805	GCCGGGTAATCGATCTAGCC	TGCAGGGCCAAGTTGGTTAT	133	Not Polymorphic	Not Polymorphic
IND|chr2H|723294359F	13-001	723294109-723294659	CGATGGGAATTAGCGCATGC	ACGGACACTGGCACATGTTA	190	Polymorphic	Not Polymorphic
IND|chr2H|723297482F	13-002	723297232-723297782	GATGTCACGAGGAGCTACGG	ACCCATCCAAACTCTTCCGG	160	Not Polymorphic	Not Polymorphic
IND|chr2H|723313398F	13-003	723313148-723313698	ACCTCCCTGAATGGTCCCTT	TCCCGAAGTTCACACCCTTG	154	Not Polymorphic	Not Polymorphic
IND|chr2H|723370972F	13-004	723370722-723371272	CCACTCCTATCGCGTCACTC	TATGAGCTGACATCACCGCC	166	Not Polymorphic	Not Polymorphic
IND|chr2H|723405779F	13-005	723405529-723406079	TACCACAACCGAAATGGGGG	AGGTGTGGCATGAAGTCGTC	158	Not Polymorphic	Not Polymorphic
IND|chr2H|723496900F	13-006	723496650-723497200	AGTCTACCTTGCGTTGCAGT	GCAATGGTGACCCTGGTTGA	174	Not Polymorphic	Not Polymorphic
IND|chr2H|723645633F	13-007	723645383-723645933	ATAGCGGGCATTTTCCCCAT	GGCTCGGCCCAACTACATTT	181	Not Polymorphic	Not Polymorphic
IND|chr2H|723659439F	13-008	723659189-723659739	TTCCGTTCCACACCGTTTCT	CGCCAGGCAACAAAATCTCC	178	Not Polymorphic	Not Polymorphic
IND|chr2H|723709459F	13-009	723709209-723709759	GGTTTCAACCCAAGTCTAAAATTTTCT	TGGCTGAAAGCGAGTAGACA	194	Not Polymorphic	Not Polymorphic
IND|chr2H|723835727F	13-010	723835477-723836027	ACTAACCACATGGACCGCTG	TGCGGATCCGTAAGAGCATC	153	Polymorphic	Not Polymorphic
IND|chr2H|724372831F	13-011	724372581-724373131	TAGATGGACGGTTGTGACGC	TCTGCTGATCGTGGGTTGTT	183	Not Polymorphic	Not Polymorphic
IND|chr2H|724396640F	13-012	724396390-724396940	GGACGATCTTGTCAGCCCAA	GAAAGGTAGGTCAGCGGCAA	168	Not Polymorphic	Not Polymorphic
IND|chr2H|724499306F	13-013	724499056-724499606	TATGGTCGTCCGGTCCCTT	GATCGTGTTTGCTCGTTGCT	155	Not Polymorphic	Not Polymorphic
InDel2103	2103	713775885-713776385	GCTGAATGACTGAGTCAGGTTG	TCCTTCCAAGACTTGATCTGCA	111	Not Polymorphic	Not Polymorphic

InDel2109	2109	719499585-719500085	TATGAGGATACGCGGACTGT	CAATCTCTCACACAACCGCG	124	Not Polymorphic	Not Polymorphic
InDel2111	2111	720285252-720285752	AAAACTTTCCCATCGGCAGC	AATTCCCTTCCTGCCACCTG	113	Not Polymorphic	Not Polymorphic
InDel2113	2113	722125013-722125513	TGGTGATGCCATCGGTTGAT	CGTATTCAGACCACACCGCA	126	Not Polymorphic	Not Polymorphic
InDel2114	2114	724946232-724946732	GGCGATACACCTTGAGACGA	CGGCGGTGGAAATGAAAACA	135	Not Polymorphic	Not Polymorphic
InDel2118	2118	728238441-728238941	ATGTGCCACATAACCGTCGT	GCCTCATGAGATGTCAGCGA	126	Not Polymorphic	Not Polymorphic
InDel2119	2119	730356461- 730356961	AACGGTTACGGGACAACACA	TCCTATGAATCAAAGGACCAACGT	130	Not Polymorphic	Not Polymorphic
InDel2122	2122	732453401-732453901	TGCTGCTCAACGTCTCAACT	GTACTTTTCTTTGCGGCCGG	100	Not Polymorphic	Not Polymorphic
InDel2123	2123	728009749-728010249	GCCACCTGTCGTCTATCCAG	GGAGACGGCTTTTCCTGCT	93	Polymorphic	Not Polymorphic
InDel2126	2126	734870786-734871286	ACGTACGCAAACGACATGTG	CAGCTGCAAACTGGATCTT	100	Not Polymorphic	Not Polymorphic
InDel2130	2130	735669035-735669535	TGTTCTTTGGATGATGAATGTTT	TGATCCTCGTAGCACTCATCC	146	Not Polymorphic	Not Polymorphic
InDel2133	2133	739148162-739148662	CCCCTCGTTGCACATGGT	GTTGTGGTTGTGTGCTGCAA	131	Polymorphic	Polymorphic
INDEL 2 H 721400673	01	721400429-721400929	TCCCCGAGATATGCCATGGT	GCTTCAGAGCTCTTGGTCGT	142	Not Polymorphic	Not Polymorphic
INDEL 2H 721526682	02	721526473-721526973	TGTAAGAGTGCGAAAACCACA	TTGCCATGCAACTCCCACT	156	Not Polymorphic	Not Polymorphic
INDEL 2H 721657162	03	721656935-721657035	TGTGTTTTCATCAAGTTTTATTCACCC	ATGCAGACAGGACAAGGCTG	164	Not Polymorphic	Not Polymorphic
INDEL 2H 721818931	04	721818721-721819221	TGGTGCCTCTCGTTGTTTCA	TCGTAGGCTGACGCAGAATT	170	Not Polymorphic	Not Polymorphic
INDEL 2H 722209816	05	722209587-722210087	AAATGAGTCCGTCGTCACCG	TGCACATGTTAGCGCAAGGA	141	Not Polymorphic	Not Polymorphic
INDEL 2H 722298169	06	722297938-722298438	TGACTAGTCTTTAGCTGACGCA	TCCTCTTCGATGGTAAATTCGGA	136	Not Polymorphic	Not Polymorphic
INDEL 2H 722348706	07	722348490-722348990	GCCAGGAGTACCCCCTCTC	AGATCAGAGCAGGACATGGC	191	Not Polymorphic	Not Polymorphic
INDEL 2H 743654366	08	743654119-743654619	GCCAAGTTGCAGGGAAGTTG	TCCAACCTGAGGGCTACAGA	163	Not Polymorphic	Not Polymorphic
INDEL 2H 743719799	09	743719583 -743720083	GTCGGACAGACCCACTTTGA	CCGAGTCGATAGTTAGTAGCGG	177	Not Polymorphic	Not Polymorphic
IND|chr2H|723294358F	15-001	723294108-723294658	ACCAGGAAGTTGGAGCGATG	GTGGGCCTTTGGGGTAGATC	151	Polymorphic	Not Polymorphic
IND|chr2H|723371941F	15-002	723371691-723372241	TCTGCGCTGCATCACAAGTA	CCAACTCCACCGCCTTCTTC	189	Not Polymorphic	Not Polymorphic
IND|chr2H|724266837F	15-003	724266587-724267137	GACCCCTCGACATCCGACTA	CGTGTTAGGGGGCAGCTG	163	Not Polymorphic	Not Polymorphic
IND|chr2H|723599326F	15-004	723599076-723599626	ACGGACCTTTCAAAACCGGT	TCCATAAAATTTGGAAACGAAAAAGGA	150	Not Polymorphic	Not Polymorphic
IND|chr2H|723603561F	15-005	723603311-723603861	GTTTGGCGAGGTACAAAGCG	TCAAACCGGGTAAGATCATGCA	197	Not Polymorphic	Not Polymorphic
IND|chr2H|723606365F	15-006	723606115-723606665	TCAAAACCACCCCCTCCATG	TGGATTTTGGCCGGATTGGA	198	Not Polymorphic	Not Polymorphic
IND|chr2H|723884447F	15-007	723884197-723884747	TGCTGGCATCGGACTGAATT	CTGCAGGGGATCGAGGAATC	162	Not Polymorphic	Not Polymorphic
IND|chr2H|723886744F	15-008	723886494-723887044	TGAGTGGCAGAGTGTTGGTC	TCAAACCGGGTAAGATCATGCA	164	Polymorphic	Not Polymorphic
IND|chr2H|724170810F	15-009	724170560-724171110	GCTCAAAGTTGGTTCCGTCG	TAAGCGAGGGAGTCTTCCGA	195	Polymorphic	Not Polymorphic
IND|chr2H|724181598F	15-010	724181348-724181898	TTTTGGTGGGGTGAAACGTG	GAAATCAGAGCACGCGTGAC	165	Not Polymorphic	Not Polymorphic
IND|chr2H|724367279F	15-011	724367029-724367579	ACCAGTCCGGCTCAACTTTC	GCCCAAAGGGTAAAGTCCGA	199	Not Polymorphic	Not Polymorphic

IND|chr2H|724411424F	15-012	724411174-724411724	GCACTGTCGGTCCCAAGATT	AACTAGCCGAACGTGCATGA	169	Not Polymorphic	Not Polymorphic
IND|chr2H|724511661F	15-013	724511411-724511961	GTCGTTGAGCTACTTGCCCT	GCCGAACCACAAACGAGGAA	193	Polymorphic	Not Polymorphic
IND|chr2H|724531409F	15-014	724531159-724531709	AGATTGGACGGTTGCTCCAA	AATATGCAACTTGCCGTGCG	170	Not Polymorphic	Not Polymorphic
IND|chr2H|724572508F	15-015	724572258-724572808	CTGCTTATTTGGTGCGTCCG	CTGTGGGAAGGGGTCAAAGG	163	Not Polymorphic	Not Polymorphic
IND|chr2H|725504819	1-29	725504569-725505119	TGCACTTTCACCTTCCACCA	TGACGGCACAAGTCATGAGA	182	Not Polymorphic	Not Polymorphic
IND|chr2H|725520027	2-29	725519777-725520327	AAAGCATCTCTCACCGCCTC	CTTCTCGAGAAAGAGGCGGG	175	Not Polymorphic	Not Polymorphic
IND|chr2H|725523382	3-29	725523132-725523682	GCATCTTCAGCAGCACGATG	GAAAGGCTGCCACATTTGGG	183	Not Polymorphic	Not Polymorphic
IND|chr2H|725524760	4-29	725524510-725525060	TGATCACATCCACGCATCGT	ATCATGCCTCTCGCGAAAAA	248	Not Polymorphic	Not Polymorphic
IND|chr2H|725525222	5-29	725524972-725525522	CCATGTGAGACAGGGTTTTTCT	CAATTGGGTTTCTCGCGTCG	185	Not Polymorphic	Not Polymorphic
IND|chr2H|725526667	6-29	725526417-725526967	GAGGGTGGTAGAGGGTGTCT	GTGTTGGATGTTCGGCTTCG	249	Not Polymorphic	Not Polymorphic
IND|chr2H|725529852	7-29	725529602-725530152	AGTAGTGACAAGCCCAATGGT	CAGGGCAGTCCTTGACAGAT	174	Not Polymorphic	Not Polymorphic
IND|chr2H|725532232	8-29	725531982-725532532	TCGAGCTTCAGGACCTTGTG	CACCTGTGAACATGTGTTTCAT	191	Not Polymorphic	Not Polymorphic
IND|chr2H|725538871	9-29	725538621-725539171	CGAGGAATCTGGGATGGCTG	CGTCCCACGGCTGAAGAATA	160	Not Polymorphic	Not Polymorphic
IND|chr2H|725539471	10-29	725539221-725539771	TGCTTGCGTATGTACCGTGT	AAGTTCAAAGGCCACCACCA	222	Not Polymorphic	Not Polymorphic
IND|chr2H|725539584	11-29	725539334-725539884	ATGCAGTAGTGGGAGGAGCT	AAGTTCAAAGGCCACCACCA	166	Not Polymorphic	Not Polymorphic
IND|chr2H|725540590	12-29	725540340-725540890	TTTTAAGCCGACGGTGTGGT	TGAGGGAAAAGGAATGGCGG	186	Not Polymorphic	Not Polymorphic
IND|chr2H|725557441	13-29	725557191-725557741	AACACCAACCAGGCCCACC	TCATGAGTTCATCAGCCAGAA	193	Not Polymorphic	Not Polymorphic
IND|chr2H|725559134	14-29	725558884-725559434	TACTTCCGCAGCCTTTTCGT	CGGCACTTCTTAACTGGGCA	153	Not Polymorphic	Not Polymorphic
IND|chr2H|725602973	15-29	725602723-725603273	AAGGGAGCTTGAACGGAGTG	ACTCCAAGCTAACCGGCATC	191	Not Polymorphic	Polymorphic
IND|chr2H|725705941	16-29	725705691-725706241	CTGACACGTACGCTCACGAT	ACATTTTGTACCCGTCTGAAAAA	186	Not Polymorphic	Not Polymorphic
IND|chr2H|725764298	17-29	725764048-725764598	AGGCTCCTGGTCGAGAGATC	ATTTTGGGTGCGTGGGGTAT	193	Not Polymorphic	Not Polymorphic
IND|chr2H|725778165	18-29	725777915-725778465	TGAGACGGGCCGCTTTTTAT	TGTAACTTCCAGTGGGGCTG	186	Not Polymorphic	Not Polymorphic
IND|chr2H|725779684	19-29	725779434-725779984	TGATTAGTCCGGACGCGATG	GGAGCTCGTCGGCATTCTTA	180	Not Polymorphic	Not Polymorphic
IND|chr2H|725820007	20-29	725819757-725820307	TCTGGAGGGTCAAGAACGGA	GCACTCCCCCTCTCGTAGAT	216	Not Polymorphic	Not Polymorphic
IND|chr2H|725860187	21-29	725859937-725860487	CTCGACTAAGTGCGTGTTGC	AAATCAGGGCGGTGCATCAT	159	Polymorphic	Not Polymorphic
IND|chr2H|726084054	22-29	726083804-726084354	GCCGTCCACACAGCAAATTC	CCCAGTGGATCGCTGTGATT	186	Not Polymorphic	Not Polymorphic
IND|chr2H|726262622	23-29	726262372-726262922	TCCCACTATGGCCAGCCTTA	GGCTGAGACCATCATCAGGG	188	Not Polymorphic	Polymorphic
IND|chr2H|726566561	24-29	726566311-726566861	AGTCTAGCCTTTTGCCGTCC	CAGTGGGGCACGACATGG	222	Not Polymorphic	Not Polymorphic
IND|chr2H|726957567	25-29	726957317-726957867	GATTCGTTCACCCACCGTCT	CAAAGAGCTTGCACCCAACG	196	Not Polymorphic	Not Polymorphic
IND|chr2H|726962555	26-29	726962305-726962855	CTCCAATCCGCCTTGGGATT	ATTTCCTGCAGTGTACCGGG	153	Not Polymorphic	Not Polymorphic
IND|chr2H|726972282	27-29	726972032-726972582	GTCACTGACACGAAACCGGA	TGCAAGCACCATACTAGCCA	171	Not Polymorphic	Not Polymorphic

IND|chr2H|727254438	28-29	727254188-727254738	GCGGGGGTAAGGGAGTTTTT	GTGCCTCACGATGCCATACT	201	Not Polymorphic	Not Polymorphic
IND|chr2H|727362043	29-29	727361793-727362343	ACGGAGCGTCACAAATGAGT	TATTTGGGCCCTGGCTGAAG	244	Not Polymorphic	Not Polymorphic
IND|chr2H|725440703	1-44	725440453-725441003	TGTAGCAAGGGACAAGCCTG	CGTAGTTGCGGATTCTTGCG	236	Not Polymorphic	Not Polymorphic
IND|chr2H|725471439	2-44	725471189-725471739	CTACGAAAATTGCACCGGCC	AGACTTTGCGTTAGGTGGTGT	236	Not Polymorphic	Not Polymorphic
IND|chr2H|725473777	3-44	725473527-725474077	CGCCTGCGTTGTTTTGTTCT	GAGGAGTTCCGAAGTGCACA	163	Not Polymorphic	Not Polymorphic
IND|chr2H|725476581	4-44	725476331-725476881	ATGTCGTGTCGTACACTCGT	AGCATTGTTCATGACGTGCC	220	Not Polymorphic	Not Polymorphic
IND|chr2H|725519356	5-44	725519106-725519656	TTTGACCCGTTGACCGTGAT	CAGGCAGAAAAGCGCCATAC	199	Not Polymorphic	Not Polymorphic
IND|chr2H|725523405	6-44	725523155-725523705	GCATCTTCAGCAGCACGATG	GAAAGGCTGCCACATTTGGG	183	Not Polymorphic	Not Polymorphic
IND|chr2H|725525884	7-44	725525634-725526184	TTCACCCATCGCCGTCTTC	GTAAGTACTGGGCGTCCGAG	155	Not Polymorphic	Not Polymorphic
IND|chr2H|725530021	8-44	725529771-725530321	AGGCTGTAACACACTGTTCCC	TAGCTTTCGTCTTCGTGGCC	215	Not Polymorphic	Not Polymorphic
IND|chr2H|725533387	9-44	725533137-725533687	ACTCCCCTGTCTTTCCTACCA	TCTCCGTGTCAAAGTTCAGGA	249	Not Polymorphic	Not Polymorphic
IND|chr2H|725538256	10-44	725538006-725538556	TGCAGGAGAGCAGGGATGAT	TCTTGGGCATCTGTTAGCCG	156	Not Polymorphic	Not Polymorphic
IND|chr2H|725598373	11-44	725598123-725598673	CAACCAACGTCCCGTCTCTT	AGGCATGTCACGAAAGAGGG	204	Not Polymorphic	Not Polymorphic
IND|chr2H|725704142	12-44	725703892-725704442	GCTGCACTTTTTAGCTACCGT	GCATTTGTCGCGAGAAATCCA	154	Not Polymorphic	Not Polymorphic
IND|chr2H|725773819	13-44	725773569-725774119	CGGTGCTGTCAGATTCAGGT	GTAACTGTGCTCCGAGGAGG	157	Polymorphic	Not Polymorphic
IND|chr2H|725777903	14-44	725777653-725778203	CGAAGCAGCATCCTGACTGA	TACAGCAGATTTAGGGCGGG	192	Not Polymorphic	Not Polymorphic
IND|chr2H|725784256	15-44	725784006-725784556	TGAGGGTATTTCGTGGCAAATC	CGGAGTGGTTGATACGGCAA	250	Polymorphic	Not Polymorphic
IND|chr2H|725813140	16-44	725812890-725813440	CGACTCGTGGTCCTTGAGAA	GGCCCGACGTACATAACCAT	175	Polymorphic	Not Polymorphic
IND|chr2H|725814356	17-44	725814106-725814656	CCAGCGATAATGCCGTCAAC	GTTGTTCGGACTTGGCTTGC	237	Polymorphic	Not Polymorphic
IND|chr2H|725867871	18-44	725867621-725868171	GCGTACGCGAGCCCTAATAT	CATTTACAAACGGCGCGTCA	187	Not Polymorphic	Polymorphic
IND|chr2H|726011965	19-44	726011715-726012265	ACGTGGTGTTAATCCAGGCT	TCAATGGATTTGGGGCTGAGT	197	Not Polymorphic	Not Polymorphic
IND|chr2H|726080570	20-44	726080320-726080870	TCCCTGGACACATGTGCATT	TACCCCCATATGCAAGACGC	200	Polymorphic	Polymorphic
IND|chr2H|726084052	21-44	726083802-726084352	GCCGTCCACACAGCAAATTC	CCCAGTGGATCGCTGTGATT	186	Not Polymorphic	Not Polymorphic
IND|chr2H|726195033	22-44	726194783-726195333	AGCGCTTCTCAATGCAACAT	GCACACGAGATTAGGCTAGTCA	152	Polymorphic	Polymorphic
IND|chr2H|726240854	23-44	726240604-726241154	ACGTTTTGGCAATTGGTCGG	TGGAAGATATAGCCGGGGGA	157	Not Polymorphic	Not Polymorphic
IND|chr2H|726682083	24-44	726681833-726682383	CCAGAGTCGTTTCGAGCGAT	ATTGAGTTCGGTCGTGCCAT	176	Not Polymorphic	Not Polymorphic
IND|chr2H|726868461	25-44	726868211-726868761	CAATTATGCCGGCGTGGTTC	CATCGTCGGGAGCTCTGAAA	196	Polymorphic	Not Polymorphic
IND|chr2H|726899972	26-44	726899722-726900272	CGCACATGCTTTGCAAAAGG	TCTGTTGTGATGGAGGACGG	197	Polymorphic	Not Polymorphic
IND|chr2H|727176784	27-44	727176534-727177084	GCCTCCCCGCATCCAATAAT	ACCGTTGTTTCAGAGTTGCG	166	Not Polymorphic	Not Polymorphic
IND|chr2H|727195951	28-44	727195701-727196251	ACGATGATGATGCGATGCCT	CGAGTGACCCACATCCGTTT	178	Not Polymorphic	Not Polymorphic
IND|chr2H|727203266	29-44	727203016-727203566	GCACGCTCGTCTAGACATGA	AGAGAGTTAGAGGAGGCGCT	214	Not Polymorphic	Not Polymorphic

IND|chr2H|727347506	30-44	727347256-727347806	CACGGTCTCCCATTCTAGGC	GAGCAGGCATCCACGAAGAT	241	Polymorphic	Not Polymorphic
IND|chr2H|727448005	31-44	727447755-727448305	GCAACCGAAATCGACACACA	ACAGTGGAGAGCTCTGACCT	221	Polymorphic	Polymorphic
IND|chr2H|727515074	32-44	727514824-727515374	TGTTCAACGGGCTAAAGGGG	CATCCACCTGAACACGGCTA	219	Not Polymorphic	Not Polymorphic
IND|chr2H|727541239	33-44	727540989-727541539	TGTCCTCACGCATCCATCAA	GAGGCTCAAATCTCCTCCGG	195	Not Polymorphic	Not Polymorphic
IND|chr2H|727542763	34-44	727542513-727543063	TGTAACCCACTGTGCCTTGT	TCTGCTTGCTAGTCAACCCTT	197	Not Polymorphic	Not Polymorphic
IND|chr2H|727576221	35-44	727575971-727576521	GCCACTATCACATCCCGGTT	AGGAAACCGTGTGAAGTAACA	200	Not Polymorphic	Polymorphic
IND|chr2H|727576801	36-44	727576551-727577101	ACATCACAGGCCAATCTAACGA	TGTGTAACATGGCAGTTGCT	200	Not Polymorphic	Not Polymorphic
IND|chr2H|727615057	37-44	727614807-727615357	ACATCAAGGATACACACAGCCA	GGTAACGAGTCTAACGCCCA	182	Not Polymorphic	Not Polymorphic
IND|chr2H|727640717	38-44	727640467-727641017	GAAGTGCCCATGTGTTTCGG	GGCGTCTGCGTGATTGATTG	151	Not Polymorphic	Not Polymorphic
IND|chr2H|727782420	39-44	727782170-727782720	CTTCGGTGGTGTCTACGGC	CGTCCCTCCCTCAATTCCTC	195	Not Polymorphic	Not Polymorphic
IND|chr2H|727797884	40-44	727797634-727798184	GTGCCTCGTGGATCATCTCC	GAATCCCTCCGACTGCTTCC	191	Not Polymorphic	Not Polymorphic
IND|chr2H|727808343	41-44	727808093-727808643	CGATACGCGCTTCCGAAATC	ATTTGAACCAGACGGAGGCC	150	Not Polymorphic	Not Polymorphic
IND|chr2H|727925718	42-44	727925468-727926018	AACGTACGACACCACGATCC	CTGAGCCATCACGTCTCCTC	241	Not Polymorphic	Not Polymorphic
IND|chr2H|725159120	43-44	725158870-725159420	GGGTCACATCACAACGGTCT	CAGAACCTCACTCTTCGCGA	174	Not Polymorphic	Not Polymorphic
IND|chr2H|725396173	44-44	725395923-725396473	TAGGGGAAGGGGAAGTGAGG	TTGGGTCGTCGGTTCTATGC	156	Not Polymorphic	Not Polymorphic
InDel2121	2121	732337677-732338177	GCCCTCGCACATACCATCAT	CACGTGTGTCACAACTCACA	140	Polymorphic	Not Polymorphic
InDel2131	2131	735740736-735741236	TTGCCAGTCTCCCGATTCAA	GCTGGCTCTGACGCACTAAA	114	Polymorphic	Not Polymorphic
InDel2137	2137	740254344-740254844	CGCAACGTGTAGTCAAACGG	GGTTATCCGGAGTCGACTGAC	139	Polymorphic	Not Polymorphic
GBM 1462	1462	743651104 -743651604	CTGTGGCTAAAGAAGGCACC	AAGATTGCTGCAGGATAGGC	154	Polymorphic	Polymorphic

Supplementary Table 5.2 Tolerance Index of five varieties used for gene expression analysis for seed from Merredin and Katanning

Variety	Merredin	Katanning	Average
AC_Metcalfe	92.68	92.00	92.34
Morex (WA4655)	76.25	78.76	77.51
Harrington	73.80	79.00	76.40
Stirling	77.49	72.28	74.89
Bass	83.28	78.00	80.64

Supplementary Table 5.3 The oligonucleotide sequences used for RT-qPCR for different genes

Primer Name	Name	Chromosome	Sequence Name
HORVU2Hr1G111740.1F	Cyclin family protein	2H	ATGTGTTCCTTGTGCGCTTT
HORVU2Hr1G111740.1R	Cyclin family protein	2H	TTGATCGGCACCAGCTCATA
HORVU2Hr1G111750.5F	Disease resistance protein	2H	TTTCAGTGGTGTGCATGTCC
HORVU2Hr1G111750.5R	Disease resistance protein	2H	ACTCGACGATACGTCCACAA
HORVU2Hr1G111760.1F	receptor-like protein kinase 4	2H	CTTCAGCTGTGCTGAGGTTC
HORVU2Hr1G111760.1R	receptor-like protein kinase 4	2H	GGTTATCACTACCGCAACGG
HORVU2Hr1G111780.3F	receptor-like protein kinase 4	2H	ACCAAGAAGATCGGGCAGAA
HORVU2Hr1G111780.3R	receptor-like protein kinase 4	2H	CGATCCAGTTGCAGTGACAG
HORVU2Hr1G111790.7F	receptor-like protein kinase 4	2H	GTGAAGGTCCTCAACAGCAC
HORVU2Hr1G111790.7R	receptor-like protein kinase 4	2H	GAGCCGTTCACCATGTACTC
HORVU2Hr1G111840F	Glutathione S	2H	TCATCGTGCAGTACCTCGAC
HORVU2Hr1G111840R	Glutathione S	2H	CCTTGATCCACGAGGCAAAG
HORVU2Hr1G111880F	Endoglucanase	2H	AACTCCTTCCTCCTGCTCAC
HORVU2Hr1G111880R	Endoglucanase	2H	GCAGCTTCTTCGGGTACTTG
HvGapdhF		U	AAGCATGAAGATACAGGGAGTGTG
HvGapdhR		U	AAATTTATTCTCGGAAGAGGTTGTACA

Supplementary Table 5.4 Gene specific markers used to amplify different regions of the sequences

Gene ID	Gene name	Prime ID	Sequence	Chromosome
HORVU2Hr1G111760.1	Receptor-like protein kinase 4	K1P1F	TGGCGTTTCCCGATCAAACT	2H
		K1P1R	CGCGATTTGTATGTGCTCTCC	2H
		K1P2F	TCGTCGAAACAAAGTCAACGAC	2H
		K1P2R	GGACTGACACCAAGGCAGAG	2H
		K1P3F	GAGGCCTCCTCTCTCATCGT	2H
		K1P3R	TCGCACAGACCACCGATACT	2H
		K1P4F	GAGACAGAGCTTGGGCATCG	2H
		K1P4R	GTGAGCAGGATGTTCGGTGG	2H
		K1P5F	GAGAACAGCTCGCTCGAGAA	2H
		K1P5R	TTTCAGCATTGCATCCGTCG	2H
		K1P6F	TTCAAGTACGTGTCGAGCGG	2H
		K1P6R	ATACATCAACGGCGGGCTTA	2H
HORVU2Hr1G111780.3	Receptor-like protein kinase 4	K2P1F	CACCTCTGACCATCGCCG	2H
		K2P1R	AGAATTCAAAAACTACTCGGCAG	2H
		K2P2F	TCTCCAATGACGCTGGACAT	2H
		K2P2R	CCTGGTCGCGATGTAGTGAA	2H
		K2P3F	AGCCAACTTTAGGGGCAATTTA	2H
		K2P3R	CTACCCAGAAGCCATCGACT	2H
		K2P4F	CTGATCGTCCATGGCTGGTT	2H
		K2P4R	CCGATCTCGATCACCACGTC	2H
		K2P5F	AACCCGTGTTCTTGCCTCG	2H
		K2P5R	TTGCTCGTACTTGTCCCAGAC	2H
		K2P6F	CCTCTATCACGACGGCGAC	2H
		K2P6R	TCGCATGTTTTAACACCGCT	2H
HORVU2Hr1G111790.7	Receptor-like protein kinase 4	K3P1F	ACCGACTTCGGGCAGTTTATT	2H
		K3P1R	GGCGGCTGCTACAAATCTCT	2H
		K3P2F	TGCGTCGGTGTTTTAGCGTA	2H
		K3P2R	ATGGCGATCGAGAAGCAAGT	2H
		K3P3F	CCACAGAGTTGACAACCGAGA	2H
		K3P3R	TGGAGCTTCTGGAACCCGAT	2H
		K3P4F	ATCAACCTCGTCCGCCTCTA	2H
		K3P4R	AACTCCTTGTCGTCCAACCA	2H
		K3P5F	AGTACATGGTGAACGGCTCG	2H
		K3P5R	TTCGGATGAACGGATCGGAC	2H
		K3P6F	TCGGGAGGACCTACCACATC	2H
		K3P6R	CAGCATCCGCACGACATTG	2H
HORVU2Hr1G111840	Glutathione S	GSTP1F	TGTCACATGTAAATCCACCCG	2H
		GSTP1R	CATGGTCGTCAGCTCGGC	2H
		GSTP2F	CCGAGCTGACGACCATGC	2H
		GSTP2R	GTACTCGTAGCCGGTGATCC	2H
		GSTP3F	GCGCAGAAGAAAAACCTCAGC	2H
		GSTP3R	CCACCAATTCGAGGCCTACTG	2H
		GSTP4F	AGAGCAGTGATCGAGCCAAC	2H
		GSTP4R	CACAGCCTCCGTGTGTAGAG	2H
		GSTP5F	GTCCACCATTGACGGGAGAG	2H
		GSTP5R	TGGATGTGGATGGACCCTGA	2H

Supplementary Table 5.5 Gene expression levels in reads per kilobase-pair per million mapped reads (RPKM) of five varieties at 24 and 48hrs after germination in DI water

	AC_Metcalfe	AC_Metcalfe	Morex (WA4655)	Morex (WA4655)	Harrington	Harrington	Stirling	Stirling	Bass	Bass	
Gene_ID	**24hrs**	**48hrs**	**24hrs**	**48hrs**	**24hrs**	**48hrs**	**24hrs**	**48hrs**	**24hrs**	**48hrs**	**Function descriptions**
HORVU2Hr1G111750	0	0	0	0	7	1	7	0	0	9	Disease resistance protein (DRP)
HORVU2Hr1G111760.1	4	36	2	2	5	2	17	20	4	0	Receptor-like protein kinase 4 (RLPK4 – 1)
HORVU2Hr1G111780.3	11	22	3	4	61	13	73	50	11	28	Receptor-like protein kinase 4 (RLPK4 – 2)
HORVU2Hr1G111790.7	1	3	0	3	1	3	1	1	1	2	Receptor-like protein kinase 4 (RLPK4 – 3)
HORVU2Hr1G111840.5	32	433	14	9	550	780	7	818	36	1046	Glutathione-S-transferase (GST) family protein
HORVU2Hr1G111880.1	3	0	4	0	0	0	3	2	2	0	Endoglucanase 3 (EDG3)

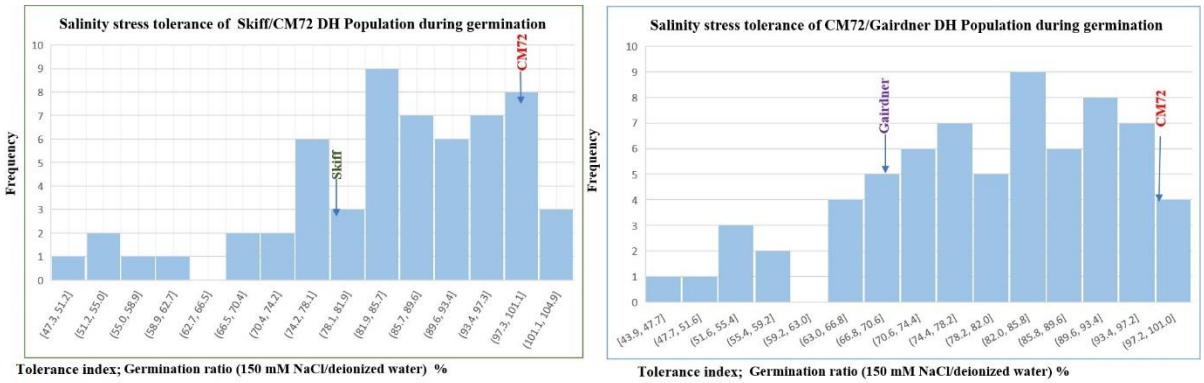
Supplementary Table 5.6 Expression prediction of nine genes in the 2H locus of salinity tolerance in barley

Gene ID	**Gene function name**	**Expression levels in fragments per kilobase million (FPKM) at different growth stages**														
		EMB	**ROO1**	**LEA**	**INF1**	**INF2**	**NOD**	**CAR5**	**CAR15**	**ETI**	**LEM**	**LOD**	**PAL**	**EPI**	**RAC**	**ROO2**	**SEN**
HORVU2Hr1G111740.1	Cyclin family protein	0.000	0.513	0.000	0.000	0.000	0.081	0.000	0.000	0.057	0.366	0.000	0.327	0.000	0.000	0.099	0.813
HORVU2Hr1G111750.5	Disease resistance protein	0.040	0.580	0.122	0.000	0.004	0.099	0.017	0.000	0.025	0.203	0.000	0.074	0.003	0.000	0.060	0.891
HORVU2Hr1G111760.1	Receptor-like protein kinase 4	0.718	8.464	7.264	0.000	0.000	0.086	0.036	0.000	0.215	0.004	0.000	0.000	0.351	0.004	0.244	13.977
HORVU2Hr1G111780.3	Receptor-like protein kinase 4	3.099	8.255	4.056	0.000	0.057	0.006	0.133	0.111	0.670	0.121	0.192	0.096	0.158	0.083	2.328	2.707
HORVU2Hr1G111790.7	Receptor-like protein kinase 4	0.000	0.037	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.065	0.000
HORVU2Hr1G111840.5	Glutathione S-transferase family protein	12.692	0.826	76.549	0.000	8.221	41.535	29.313	11.525	34.915	26.636	2.686	15.695	105.919	15.257	0.157	290.091
HORVU2Hr1G111880.1	Endoglucanase 3	0.000	0.033	0.000	0.000	0.000	0.013	0.000	0.113	0.000	0.000	0.000	0.000	0.000	0.000	0.056	0.000
HORVU2Hr1G111920.1	Undescribed protein	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
HORVU2Hr1G111930.1	Transmembrane emp24 domain-containing protein 3	0.000	0.000	0.000	0.000	0.000	0.045	2.674	0.939	0.026	0.325	1.300	0.525	0.105	1.933	0.000	0.207

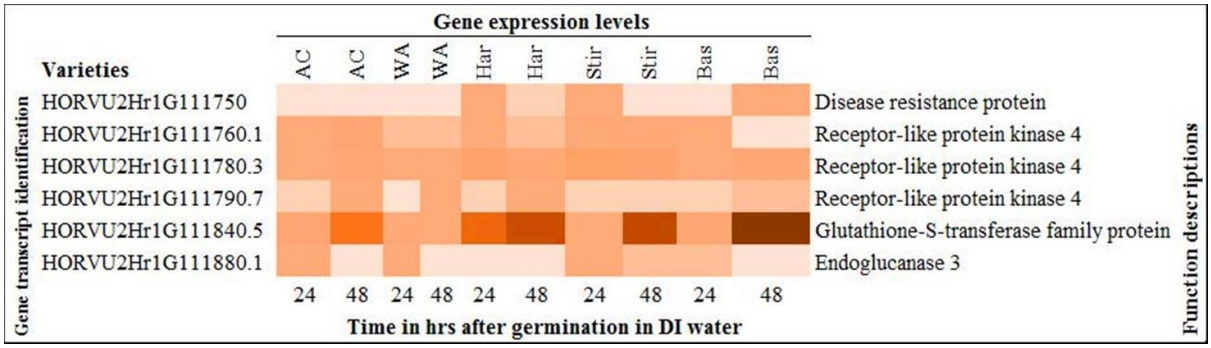
Supplementary Table 5.7 Phenotypic variations explained by the markers in whole DH populations and recombinant lines

Population	Group	Markers	% Explained
CM72/Gairdner DH population	1	InDel 74-48 and 74-49	11.13
	2	InDel 15-008, 15-009, 74-61 and 74-63	9.89
	3	InDel 15-001	10.69
	4	InDel 15-013, 74-67 and 74-68	10.17
	5	InDEl 13-44	9.47
	6	InDel 15-44,16-44, 17-44, 21-29, 20-44 , and 21-44	9.06
	7	InDel 74-26 and InDel 74-47	9.80
CM72/Gairdner DH recombinant	1	InDel 74-47, 74-48 and 74-49	1.84
	2	InDel 15-008, 15-009, 74-61 and 74-63	19.61
	3	InDel 15-001and 13-001	10.39
	4	Indel 13-010	18.67
	5	InDEl 13-44	7.69
	6	InDel 15-013, 74-67 and 74-68	16.55
	7	InDel 15-44,16-44, 17-44, 21-29, 20-44, and 21-44	5.54
Skiff/CM72 DH population	1	InDel 74-47 and 74-48	10.63
	2	InDel 74-56	14.52
	3	InDel 15-29, 18-44, 20-44 and 22-44	13.31
Skiff/CM72 DH recombinant	1	InDel 74-47 and 74-48	19.33
	2	InDel 74-56	20.03
	3	InDel 15-29, 18-44, 20-44 and 22-44	6.65

5.7.2 Supplementary figures

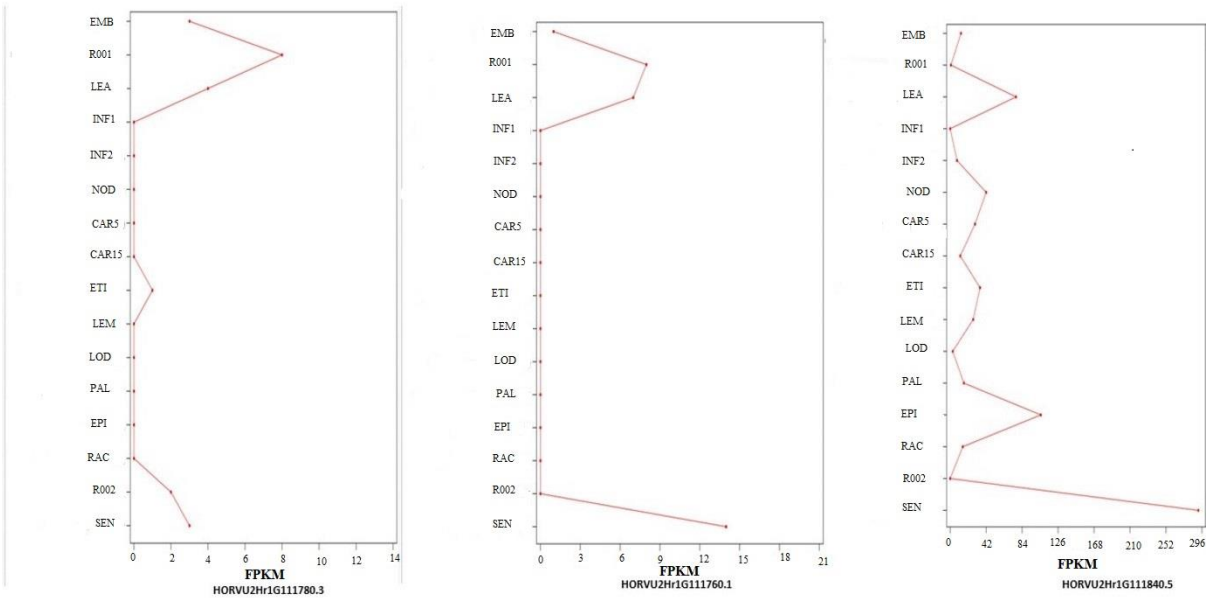


Supplementary Figure 5.1 Histograms of salinity tolerance index (%) for Skiff/CM72 and CM72/Gairdner DH populations during germination highlighting the position of the respective parents.



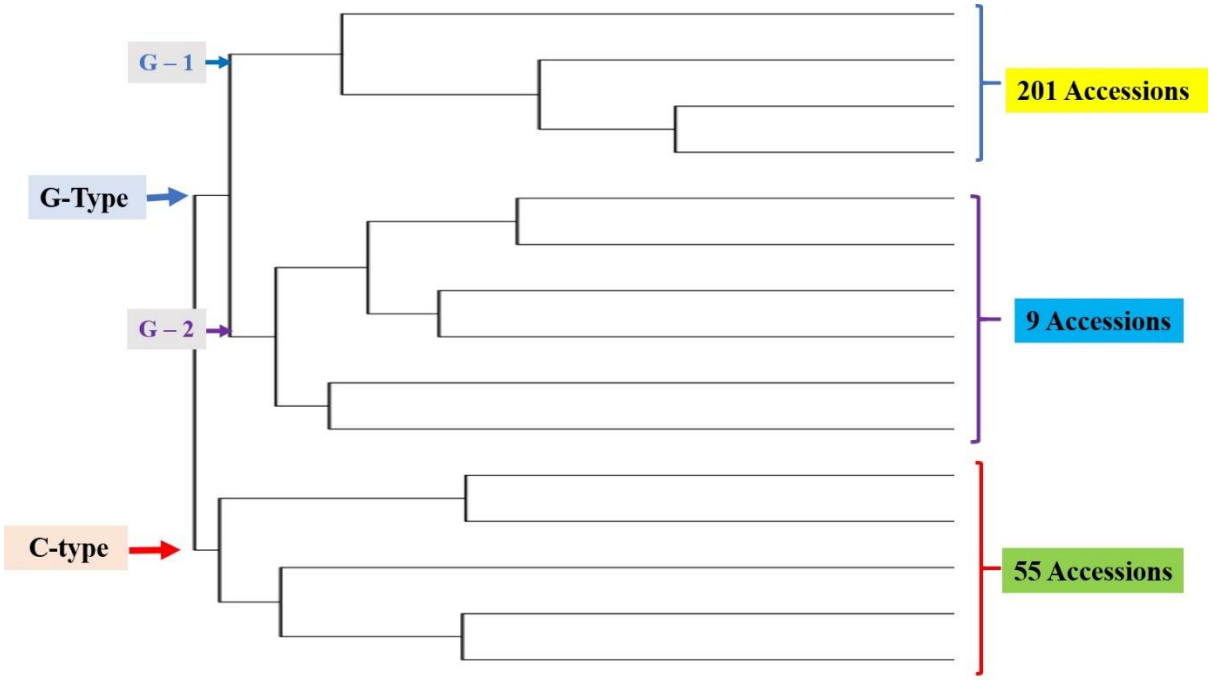
Supplementary Figure 5.2 Heatmap of gene expression Reads Per Kilo base-pair per Million mapped reads (RPKM) values for six salinity tolerance genes at germination identified in the fine mapped region based on germination data in DI water for five varieties (AC Metcalfe, Morex, Harrington, Stirling and Bass).

Dark brown indicates high expression while ligh brown is low and the actual values are found in supplementary data table 3.

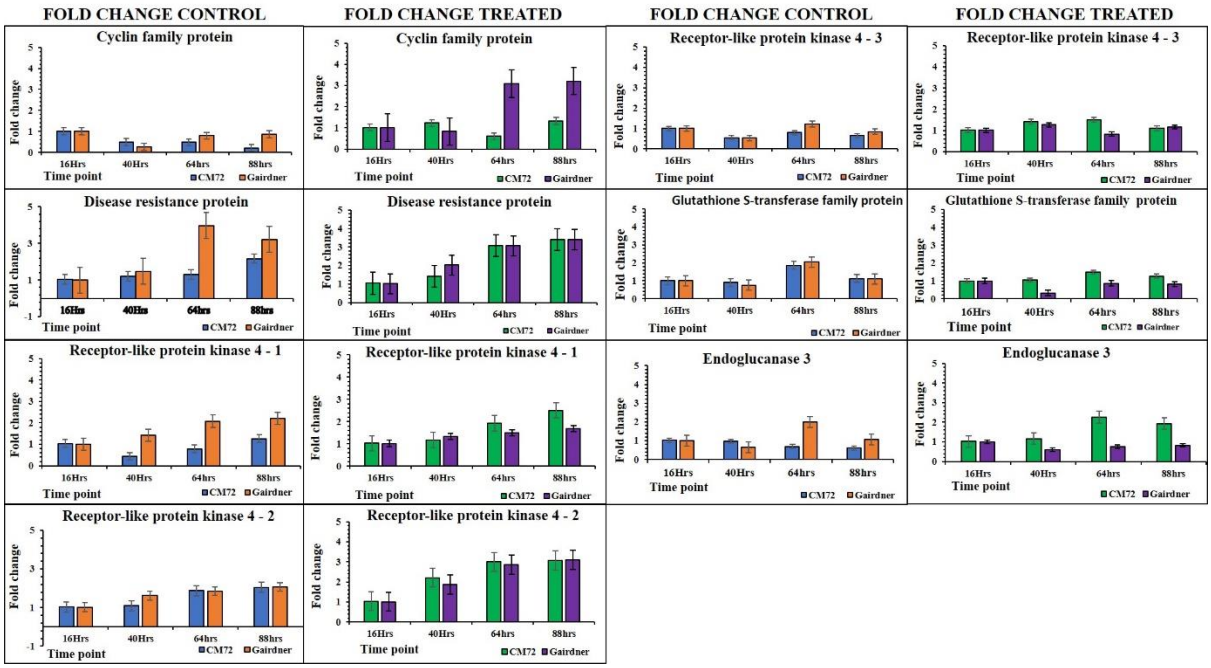


Supplementary Figure 5.3 Expression patterns of HORVU2Hr1G111760.1, HORVU2Hr1G111780.3, and HORVU2Hr1G111840.5 genes across various tissues and developmental stages (Colmsee *et al.* 2015).

The actual values at each stage are found in supplementary data table 1. EMB, 4-day-old embryo; ROO1, root from seedlings (10 cm shoot stage); LEA, shoot from seedling stage (10 cm shoot stage); INF1, young developing inflorescences (5 mm); INF2, developing inflorescences (1–1.5 mm); NOD, developing tillers, third internode (42 DAP); CAR5, developing grain (5 DAP); CAR15, developing grain (15 DAP); ETI, etiolated seedling dark con (10 DAP); LEM, inflorescences lemma (42 DAP); LOD, inflorescences lodicule (42 DAP); PAL, dissected inflorescences, palea (42 DAP); EPI, epidermal strips (28 DAP); RAC, inflorescences rachis (35 DAP); ROO2, roots (28 DAP), SEN, senescing leaves (56 DAP).

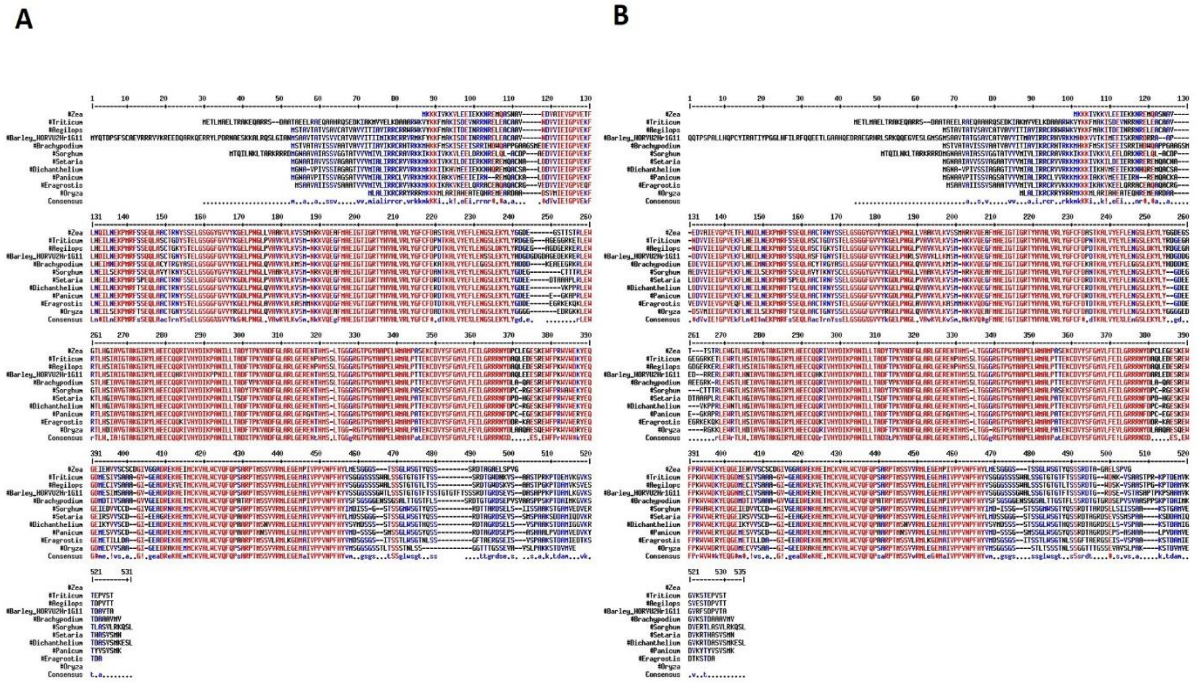


Supplementary Figure 5.4 A simplified dendrogram representation of genotypic cluster of two markers for salinity tolerance index at germination of 265 world accessions of barley. Where CM7 is C (81.38) with 55 varieties and Gairdner is G – type groups (78.02%) with 210 entries, G – type has 2 minor groups of 201 with mean tolerance index of 77.35% and 9 accessions with average of 78.69% and a standard deviation of ±1.23.

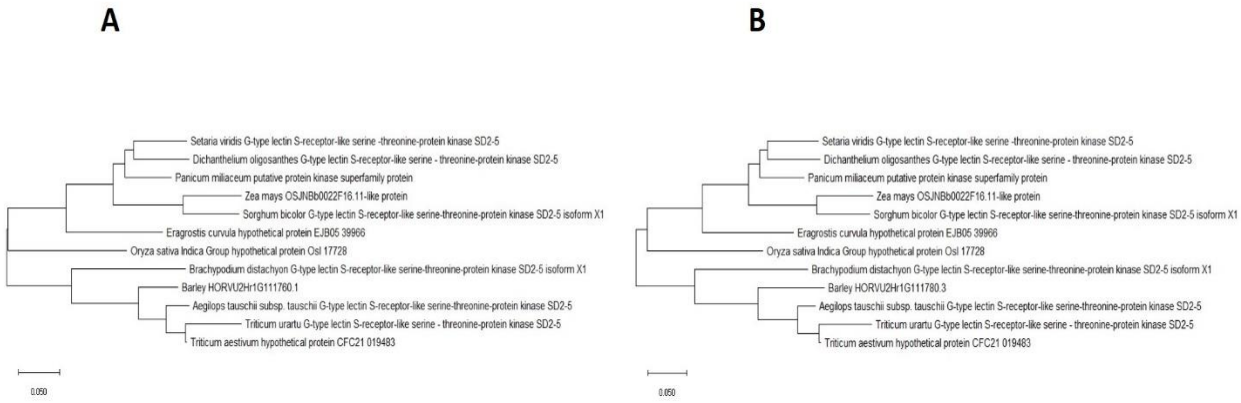


Supplementary Figure 5.5 Relative gene expression levels of seven genes in the embryo during germination of CM72 and Gairdner under deionized (DI) water (control) and 150mM NaCl (treated).

Cyclin family protein (HORVU2Hr1G111740.1), Disease resistance protein (HORVU2Hr1G111750.5), Receptor-like protein kinase 4 – 1 (HORVU2Hr1G111760.1), Receptor-like protein kinase 4 – 2 (HORVU2Hr1G111780.3), Receptor-like protein kinase 4 – 3 (HORVU2Hr1G111790.7), Glutathione-S-transferase family protein (HORVU2Hr1G111840.5), Endoglucanase 3 (HORVU2Hr1G111880.1). Expression of 7 genes were analysed by RT-qPCR using *HvGAPDH* as internal control under DI water and salinity stress for 16, 40, 64 and 88 hrs. Data was shown as means ± S.D. Error bars which are not overlapping differ significantly at $P \leq 0.05$.



Supplementary Figure 5.6 Protein sequence alignement of representatives from 10 different species including wheat, rice, maize and sorghum having a percentage resemblance of more than 70% from NCBI website for RLPK4 – 1 (HORVU2Hr1G111760.1) A, and RLPK4 – 2 (HORVU2Hr1G111780.3) **B**.



Supplementary Figure 5.7 Phylogenetic tree constructed using protein sequence of 10 representative hits of RLPK4 – 1 (HORVU2Hr1G111760.1) A and RLPK4 – 2 (HORVU2Hr1G111780.3) B from NCBI website by means of maximum likelihood (ML) in MEGA-X.

Supplementary Figure 5.8 Protein sequence alignment of all the hits above 70% from NCBI showing the conserved domains (red) of the genes.

CHAPTER SIX

SALINITY TOLERANCE IN BARLEY DURING GERMINATION—HOMOLOGS AND POTENTIAL GENES

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The contribution of the authors is as follows: **EM** performed literature search, data analysis, interpretation of information, and drafting the manuscript. **TA** and **YH** gave guidance on relevant literature search, information and data interpretation. **CL** conceived the projects idea. All authors revised the paper and approved the final version to be published.

6.1 Abstract

Salinity affects more than 6% of the world's total land area, causing massive losses in crop yield. Salinity inhibits plant growth and development through osmotic and ionic stresses; however, some plants exhibit adaptations through osmotic regulation, exclusion, and translocation of accumulated Na^+ or Cl^-. Currently, there are no practical, economically viable methods for managing salinity, so the best practice is to grow crops with improved tolerance. Germination is the stage in a plant's life cycle most adversely affected by salinity. Barley, the fourth most important cereal crop in the world, has outstanding salinity tolerance, relative to other cereal crops. Here, we review the genetics of salinity tolerance in barley during germination by summarizing reported quantitative trait loci (QTLs) and functional genes. The homologs of candidate genes for salinity tolerance in *Arabidopsis*, soybean, maize, wheat, and rice have been blasted and mapped on the barley reference genome. The genetic diversity of three reported functional gene families for salt tolerance during barley germination, namely dehydration-responsive element-binding (DREB) protein, somatic embryogenesis receptor-like kinase and aquaporin genes, is discussed. While all three gene families show great diversity in most plant species, the *DREB* gene family is more diverse in barley than in wheat and rice. Further to this review, a convenient method for screening for salinity tolerance at germination is needed, and the mechanisms of action of the genes involved in salt tolerance need to be identified, validated, and transferred to commercial cultivars for field production in saline soil.

6.2 Introduction

Salinity affects about 6% of the world's total land area, including 20% of arable land and 33% of irrigated land (Shrivastava and Kumar, 2015; Machado and Serralheiro, 2017; Kuang et al., 2019; Safdar et al., 2019), causing estimated yield losses of 20% (Ashraf and Harris, 2005; Pirasteh-Anosheh et al., 2016). Furthermore, land salinisation is increasing, with 10 million ha of agricultural land destroyed annually by salt accumulation (Pimentel et al., 2004) due to several factors including the use of contaminated irrigation water, intensive farming and poor drainage, and climate change (Machado and Serralheiro, 2017; Isayenkov, 2019). Without proper and sustainable control, salinity-affected areas will increase to more than 50% of the world's total arable land by 2050 (Ashraf, 2009; Anosheh et al., 2011; Jamil et al., 2011; Emam et al., 2013).

According to Pirasteh-Anosheh et al. (2016), plants experience four types of stress under saline conditions: (1) salinity reduces water uptake due to the low water potential of the soil which interferes with the osmotic gradient (Munns and Tester, 2008); (2) the absorbed salt reaches a level that causes severe cellular toxicity due to low sequestration of Na^+ into vacuoles (Nawaz, 2007); (3) the salt interacts with minerals causing nutrient imbalance and deficiency (Nawaz, 2007); and (4) salinity accelerates the production of active oxygen radicles, such as H_2O_2 (hydrogen peroxide), $\bullet O_2^-$ (superoxide), $^1O_2$ (singlet oxygen), and $\bullet OH^-$ (hydroxyl radicle), which can damage or even kill plants (Hernández et al., 2001). Greenway and Munns (1980) classified plants into two major categories based on their salinity tolerance, namely (1) halophytes and (2) glycophytes. Halophytes have an exceptional ability to produce heteromorphic seeds that have diverse dormancy and germination capacity under saline conditions (Liu RR et al., 2018). Seed germination in glycophytes is severely inhibited under salinity due to both osmotic stress and ionic toxicity stress, unlike in halophytes that are less affected by osmotic pressure (Romo and Haferkamp, 1987; Dodd and Donovan, 1999; Zhang et al., 2010). Halophytes can better regulate the ion-gate-controlled NaCl influx into seed cells (Glenn et al., 1999; Huang et al., 2018). However, salinity-tolerant glycophytes have a lower osmotic potential than sensitive glycophytes, enabling them to absorb more water from the soil during germination (Zhang et al., 2010).

Barley, the fourth most important cereal in the world (Schulte et al., 2009; Visioni et al., 2019), is a glycophyte, but its salinity tolerance varies among genotypes (Mano and Takeda, 1997; Flowers and Hajibagheri, 2001; Xue et al., 2009; Debez et al., 2019). Some barley genotypes

are able to thrive in saline conditions (Harlan, 1995; Shen et al., 2018). Salinity-tolerant barley genotypes exhibit halophytic features such as excluding Na^+ from uptake (Chen ZH et al., 2007) and accumulating Na^+ in tissues (Munns et al., 1988; Munns and Tester, 2008). Tolerant genotypes sequester Na^+ in their intracellular vacuoles, thereby maintaining high K^+/Na^+ levels in the cytosol while reducing damage from Na^+ toxicity (Shabala et al., 2010; Mian et al., 2011; Fu et al., 2018; Han et al., 2018; Ishikawa and Shabala, 2019). They can also synthesize compatible solutes in the cytoplasm to balance the osmotic potential of vacuolar Na^+ (Widodo et al., 2009). Salinity tolerance is controlled by multiple genes that are expressed differently during different growth phases (Qiu et al., 2011; Ahmed et al., 2013a). Germination, which determines seedling vigour and the plant population, is the most important growth stage, but is sensitive to salinity stress (Zhang et al., 2010; Bewley et al., 2013).

Several genes have association with enhanced salinity tolerance in barley (Wu et al., 2011) and are grouped into four classes based on their function (Walia et al., 2006; Wu et al., 2011; Yin et al., 2018). (1) Genes that enhance osmotic protection, such as *HvPIP2;5* (Alavilli et al., 2016), *HVA1* (Lal et al., 2008), *HvDREB1*, *HvCBF4*, *HvWRKY38* (Gürel et al., 2016), and reactive oxygen species (ROS)-scavenging genes that include osmoregulatory trehalose synthesis, mannitol-1-phosphate dehydrogenase (M1PD), and pyrroline-5-carboxylase synthetase (P5CS). (2) Genes controlling Na^+ and K^+ transport, such as the high-affinity potassium transporter (*HKT*) family (e.g. *HvHKT1;5* (Hazzouri et al., 2018; Huang et al., 2019), *HvHKT1;1* (Han et al., 2018), *HvHKT2;1* (Mian et al., 2011; Assaha et al., 2017), *HvHAK1* (Mangano et al., 2008), *HvHKT1*, *HvHKT2* (Qiu et al., 2011)), the Na^+/H^+ exchanger (*NHX*) family (*HvNax4* (Rivandi et al., 2011), and salt overly sensitive (SOS) engaged Na^+/H^+ antiporters (*HvSOS1* (*HvNHX7*), *HvSOS2* (*HvCIPK24*), *HvSOS3* (*HvCBL4*), *HvNHX1*, *HVA*) (Yousefirad et al., 2018; Wu et al., 2019). (3) Genes that produce regulatory proteins, such as the *CBF*/ *DREB* (C-repeat-binding protein/dehydration-responsive element-binding protein) family (e.g. *HvRAF* (Jung et al., 2007), *HvAP2*/*ERF* (ethylene response factor) (Guo et al., 2016), *HvDREB1* (Xu et al., 2009), *HvCBF4*, *HvWRKY38* (Gürel et al., 2016), *HvDRF1* (Xue and Loveridge, 2004)) in the signalling pathways of long distance and downstream gene expression. Salinity and drought stresses induce the expression of root abundant factor (*RAF*), *CBF3*, and *CBF4* from the *CBF*/*DREB* gene family in most plants. Twenty *CBF* genes have been identified in barley, which enhance tolerance to drought, salinity, and low temperature (Wu et al., 2011). (4) Genes that induce jasmonate (JA) biosynthesis, such as late embryogenesis abundant (LEA) protein genes (e.g. *HVA1* expressed in response to water and

salinity stresses, *HVA22* expressed in response to dehydration, extreme temperatures, abscisic acid (ABA) secretion, and salinity stress (al-Yassin and Khademian, 2015).

6.3 Seed germination process and roles of hormones

Seed germination requires optimum environmental factors, including water, oxygen, and temperature. Other environmental factors, such as light and nitrates, can also affect seed germination (Finch-Savage and Leubner-Metzger, 2006; Rajjou et al., 2012). The process of germination occurs in three main phases regulated by hormones, reactive nitrogen species, and ROS (Ma et al., 2017). The first phase involves the expression of genes that play a major role in cell wall metabolism. The second phase encompasses significant stimulation of hormonal and enzyme activity by genes involved in amino acid synthesis, starch metabolism, nucleic acid synthesis, protein synthesis and transport (Weitbrecht et al., 2011). The third phase involves the induction of genes for photosynthetic metabolism after radicle protrusion (Ma et al., 2017). Hormonal imbalance, caused by factors such as low temperature, drought, and salts (saline and sodic) that increase ground osmotic pressure (Bartels and Nelson, 1994), can affect the seed germination process (Lopez-Molina et al., 2001; Belin and Lopez-Molina, 2008).

Several hormones produced by plant and soil microorganisms such as bacteria play a role in inducing or breaking seed dormancy and thus contribute to the germination process (Bewley, 1997; Baskin and Baskin, 2001; Koornneef et al., 2002; Hoyle et al., 2015). Interestingly, as much as gene expression controls plant hormone activity, the reverse can be true with some hormones regulating gene expression (Miransari and Smith, 2014). For example, ABA usually induces dormancy at seed maturation and gibberellins (GAs) break dormancy during germination (Figure 6.1) (Bentsink and Koornneef, 2008; Hauvermale et al., 2012). When an environmental stress such as salinity occurs during germination, ABA is produced in the seeds (Weyers and Paterson, 2001) to upregulate transcription factors (TFs) such as *AB13* and *AB15* that stimulate genes encoding the osmotolerance protein and block the germination process (Fedoroff, 2002; Lopez-Molina et al., 2002; Graeber et al., 2010; Miransari and Smith, 2014). However, ABA is negatively regulated by ABA-INSENSITIVE1 (*ABI1*) and *ABI2* and the expression of the *HvABA8'OH-1* gene (Ma et al., 2017). GAs release seeds from dormancy by stimulating the production of hydrolases for the germination process (Miransari and Smith, 2014; Abido et al., 2019). The embryo synthesizes GA after imbibing water (Diaz-Mendoza et al., 2019). The GA binds to receptors to promote the breakdown of repressor of GA-like2

(RGL2), a DELL factor suppressing germination, the expression of several genes (*GAMYB*, *HvPTR*, *WRKY*, *PP2C*, *GATA*, and *HvKAO1*), and the production of essential proteins during germination in stress environments (Marrs, 1996; Sun and Gubler, 2004; Ma et al., 2017). The effect of GA is terminated by the expression of GA 2-oxidase 1 (*HvGA2ox*), which is responsible for its inactivation (Figure 1) (Ma et al., 2017).

During germination, the concentration of ethylene increases (Yang and Hoffman, 1984; Pennazio and Roggero, 1991; Petruzzelli et al., 2000), which reduces plant growth under saline conditions in the presence of ABA (Matilla, 2000; Rinaldi, 2000; Jalili et al., 2009). Auxins (indole-3-acetic acids (IAAs)) are essential for cell elongation, and radicle and embryo growth during germination (Popko et al., 2010; Hauvermale et al., 2012). Brassinosteroids (BRs) and IAA on the other hand stimulate the secretion of ethylene which works in conjunction with GAs to induce germination (Arora, 2005; Miransari and Smith, 2014). Auxins reduce seed sensitivity to ABA by overexpressing microRNAs (Liu et al., 2007) and interacting with GAs to counteract ABA suppression during germination (Chiwocha et al., 2005; Hentrich et al., 2013). Cytokinins interacting with ethylene enhance cell division and alleviate abiotic stresses like salinity during germination (Chiwocha et al., 2005; Subbiah and Reddy, 2010; Peleg and Blumwald, 2011; Miransari and Smith, 2014). BRs, in conjunction with GAs and ethylene, improve seed resistance to abiotic stress, enhance embryo growth out of the seed, and reduce the effects of ABA (Bajguz and Hayat, 2009; Miransari and Smith, 2014; Procházka et al., 2015) and salt stress (Vázquez et al., 2019) during germination. JAs are signalling molecules for plant defence against osmotic stress caused by salt (Kazan and Manners, 2012; Nguyen et al., 2019). They obstruct the production of two primary ABA biosynthesis genes (*TaNCED1* and *TaNCED2*) with acetylsalicylic acid (ASA) in stress germination in wheat (Xu et al., 2016).

6.4 Salinity versus sodicity

Saline soils predominantly contain sodium (Na^+) cations and chloride (Cl^-) anions and, in some cases, Ca^{2+}, Mg^{2+}, SO_4^{2-}, but not HCO_3^- or CO_3^{2-} (Abrol et al., 1988). Saline soils have good structure, an advantage for proper tillage and crop cultivation, and usually an electric conductivity extract (ECE) higher than 4 dS/m (40 mmol/L), an exchangeable water-soluble sodium of >15%, and a pH of <8.5 but >7. However, when the surface of the ground appears black, hard, and dry, the soil is sodic. In this case, the predominant cation is Na^+, but the primary anions are Cl^-, SO_4^{2-}, HCO_3^- and a small amount of CO_3^{2-}. Sodic soils have an exchangeable Na^+ of >15%, an ECE of >4 dS/m, and a pH of >8.5 (Abrol et al., 1988).

6.4.1 Causes and types of salinity

Soil salinity is caused by (1) natural or primary, and (2) secondary or human-induced events (Manchanda and Garg, 2008; Parihar et al., 2015) and is either (1) groundwater-associated (dryland salinity), (2) non- groundwater-associated (transient salinity), or (3) irrigation-associated (Ghassemi et al., 1995; Rengasamy, 2006; Majeed and Muhammad, 2019). Transient salinity fluctuates with soil depth and its effect on plant growth is dependent on rainfall. It occurs in semi-arid environments where there is insufficient rain to leach the soil. Clay layers below the topsoil hinder the movement of water and salts, leading to ion accumulation on the soil surface (Rengasamy, 2002, 2006). Most of Western Australia is characterized by a temperate climate. Ion accumulation in the topsoil following dry, hot summers can result in high salinity at sowing, which affects seed germination. These ions can be leached only by rainfall or irrigation. Dry-seeding or seeding with the first rain increases the likelihood that germinating seeds will be affected by salinity stress.

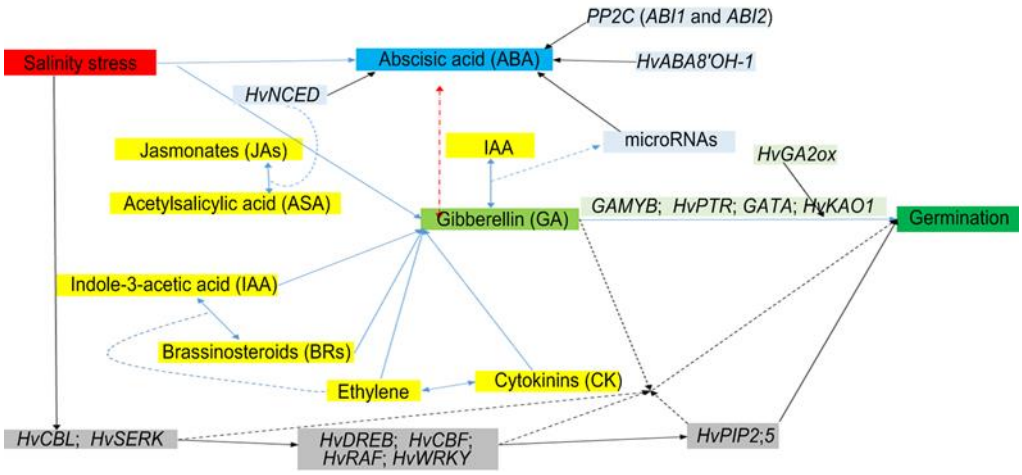

Figure 6.1 Interactions among the hormones and gene regulatory pathways in barley during germination under salinity stress.
Blue lines indicate hormonal regulation, while black lines are for genes and transcription factor expression. Red double-arrowed dotted line shows the negative interaction between ABA and GA during germination, while blue double-arrowed line shows the positive interaction among the hormones. Blue dotted lines show the stimulation outcome of the hormonal interactions, while black dotted lines show the regulatory interactions of genes. *GAMYB*: GA-induced Myb (myeloblastosis)-like protein; *HvPTR*: barley scutellar peptide transporter; *HvKAO1*: barley kaurenoic acid oxidase 1; *HvGA2ox*: barley GA 2-oxidase 1; *HvNCED*: barley nine-*cis*-epoxycarotenoid dioxygenase; *PP2C*: protein phosphatase type 2C; *ABI1*: ABA-insensitive 1; *HvABA8'OH-1*: barley ABA 8' hydroxylase; *HvCBL*: barley calcineurin B-like protein; *HvSERK*: barley somatic embryogenesis receptor-like kinase; *HvCBF*: barley C-repeat-binding protein; *HvDREB*: barley dehydration-responsive element-binding protein; *HvRAF*: barley root abundant factor; *HvPIP2;5*: barley aquaporin 2;5.

6.4.2 Effect of salinity on germination

Salinity affects the seed germination process by altering water imbibition, changing enzymatic activities causing ionic toxicity, interfering with protein metabolism, causing hormonal

imbalances, and reducing the possibility of seeds using their reserves, all of which delay and reduce the number of sprouting seeds (Läuchli and Grattan, 2007; Bordi, 2010; Munns et al., 2012; Parihar et al., 2015; Debez et al., 2019). Saline conditions create an external osmotic stress that reduces water availability and uptake (Bernstein, 1963; Bliss et al., 1986; Sayar et al., 2010; Sabagh et al., 2019) and increases the toxic absorption of ions (Hampson and Simpson, 1990), which decreases seed germination (Dodd and Donovan, 1999; Luan et al., 2014; Narsing Rao et al., 2019; Polash et al., 2019).

6.4.3 Salinity tolerance mechanisms

Plants overcome salinity stress through various mechanisms that involve osmotic adjustment, Na^+ exclusion, and tissue tolerance (Munns and Tester, 2008; Liang et al., 2018; Miransari and Smith, 2019). These encompass biochemical, physiological, and molecular mechanisms of variable complexity (Han et al., 2015; Pirasteh-Anosheh et al., 2016; Kumari et al., 2019). During germination, plants become saline-tolerant by (1) excluding salts from seed cells while maintaining high osmotic potential using organic solutes, or (2) accumulating salt ions in seed cells to increase osmotic potential while putting mechanisms in place to mitigate toxicity (Zhang et al., 2010). Seeds of salt-tolerant barley genotypes take up sodium to increase osmotic potential to absorb water during germination under salinity stress (Zhang et al., 2010), while minimising K^+ losses (al-Karaki, 2001). The vacuole is a vital cell organelle for compound deposition during osmotic stress regulation, and its size is a measure of salinity tolerance (Lauchli and Epstein, 1990; Volkmar et al., 1998; Yarra, 2019; Yarra and Kirti, 2019).

6.4.3.1 Indicators of salinity tolerance

Salinity tolerance indicators in plants can be divided into three main groups: (1) agronomic/morphological, (2) physiological, and (3) biochemical.

6.4.3.1.1 Agronomic/morphological indicators

Salinity tolerance is expressed externally through visible morphological/agronomic traits as a result of several physio-chemical processes that take place inside the plant. They are a reflection of genetic and physiological mechanisms influenced by the environmental effect on the plant that confer salinity tolerance (Ashraf and Harris, 2004). They occur at the whole plant or organ level and are mostly physically visible. These indicators are easy to measure and include germination percentage, yield, survival rate/percentage, plant height, leaf area, leaf injury, relative growth rate, and relative growth reduction. Distinguishing between tolerant and non-tolerant plants using agronomic indicators can be subjective.

6.4.3.1.2 Physiological indicators

Like the other indicator groups, physiological indicators are the result of processes that take place inside plant tissues, organs, cells, and organelles when exposed to salinity stress. They include the transportation of excess ions to the vacuole or sequestering them in older tissues. This group of indicators is measured by traits that include relative growth rate, germination speed, ion homeostasis, photosynthesis, transpiration, and senescence (Negrão et al., 2017). They provide more objective information than morphological indicators when combined with knowledge of the genetic model of salinity tolerance (Ashraf and Harris, 2004). Sodium ions are transported and compartmentalized in the vacuoles using two types of H^+ pumps (V-ATPase and H^+-ATPase) and vacuolar pyrophosphatase (V-PPase). The pumps are facilitated by SOS pathways with three types of proteins (SOS_1, SOS_2, and SOS_3). SOS_1 is essential for regulating Na^+ efflux at the cellular level and enhancing Na^+ transportation in the organelles and tissues. The SOS_2 can not only interact with SOS_3 and subsequently activate SOS1, but also increases transport activity of proteins such as NHX (Gupta and Huang, 2014). Electron transport chains in mitochondria can overflow, deregulate, or become disrupted by salinity stress leading to the accumulation of toxic compounds. Antioxidant enzymes and nonenzymatic compounds are essential for detoxifying ROS, which include helicase proteins, catalase (CAT), peroxidase, polyphenol, flavonoid, ascorbate, and glutathione (Gupta and Huang, 2014).

6.4.3.1.3 Biochemical indicators

Biochemical indicators are chemical solutes that build up inside plants in response to salt stress (Ashraf and Harris, 2004). The accumulation of low molecular weight organic solutes, inorganic ions, compatible osmolytes, soluble sugars, soluble proteins, amino acids and amides, quaternary ammonium compounds, polyamines (PAs), polyols, antioxidants, and ATPases is associated with tolerance to salinity stress. Secretion of biochemical compounds occurs during healthy growth and germination; hence it is not easy to tell whether a chemical is a reaction or tolerance to salinity stress, or an adaptive plant mechanism (Ashraf and Harris, 2004). Plant compatible osmolytes are organic compounds synthesized by various plant species which accumulate in variable amounts and are soluble in cells and do not disturb cellular activities at high or low concentrations (Hanin et al., 2016). These compounds, including proline, glycine betaine, sugars, and polyols, increase within the cell in response to salinity stress, and are sustained by permanent synthesis and/or degradation. Accumulation of these compounds is proportional to the surrounding osmolarity, and they function to shield the cell structure and

maintain osmotic balance through constant water entry (Hasegawa et al., 2000; Mansour and Ali, 2017; Alhasnawi, 2019).

The secretion of PAs has a diverse function in healthy plant growth, including seed germination, and is critical for abiotic stress adaptation, including salinity. Diamine putrescine (PUT), tetra-amine spermine (SPM), and triamine spermidine (SPD) are some typical PAs found in plants (Shu et al., 2012) and whose increase has been associated with salinity stress (Gupta and Huang, 2014). Nitric oxide (NO) regulates several plant growth and developmental activities, stress signalling molecules and stress responses, including salinity and the activation of antioxidant enzymes (superoxide dismutase (SOD), CAT, guaiacol peroxidase (GPX), ascorbate peroxidase (APX), and glutathione reductase (GR)), and triggers the expression of various redox-regulated genes that enhance germination and root growth under salinity stress (Gupta and Huang, 2014). Salinity stress mitigation by NO is due to its antioxidant functions, modulation of the ROS detoxification system (Mishra et al., 2011) associated with an increase in antioxidant enzymes, such as SOD, CAT, GPX, APX, and GR (Zhao et al., 2004; Ali et al., 2018), and suppression of malondialdehyde (MDA) production during lipid peroxidation (Nalousi et al., 2012). NO offers a force for Na^+/H^+ exchange, providing the H^+ gradient to stimulate H^+-ATPase (H^+-PPase) and contributing to K^+ and Na^+ homeostasis (Zhang et al., 2006; Gupta and Huang, 2014).

Other biochemical indicators include the production of several hormones in response to salinity stress, including ABA in roots and shoots, which mitigates the antagonistic effect of salinity stress on physiological processes (Popova et al., 1995; Jaschke et al., 1997). ABA accumulation triggers the expression of salinity stress tolerance enhancing genes in cultivated barley (*HVP1*, *HVP10*, and *HvVHA-A*) and wheat (*MAPK4-like*, *TIP 1*, and *GLP 1*) (Keskin et al., 2010). Salicylic acid (SA) concentration, along with the SA biosynthetic enzyme, increases under salinity stress in rice seedlings (Jayakannan et al., 2013). Application of BR promotes antioxidant enzyme (SOD, POX, APX, and GPX) activity and the accumulation of salinity stress mitigating nonenzymatic antioxidant compounds, such as tocopherol, ascorbate, and reduced glutathione (Ashraf et al., 2010; el-Mashad and Mohamed, 2012; Gupta and Huang, 2014).

6.4.3.2 Salinity stress signalling pathways and molecules

The response to salinity stress in plants involves three types of signalling pathway: (1) ionic and osmotic stress signalling (homeostasis) essential for the re-establishment of cellular

homeostasis, (2) detoxification signalling that regulates and repairs damage, and (3) cell division and expansion signalling (Zhu, 2001). Homeostatic signalling governs detoxification leading to tolerance by reducing the response of growth inhibitors (Zhu, 2002). The signalling information flows in a way that ionic (Na^+), osmotic (turgor pressure change), and detoxification are stress input, while salinity-induced damage, control, and repair are output (response) (Xiong and Zhu, 2001; Zhu, 2002). The outcomes of osmotic signalling include gene expression or activation of osmolyte biosynthesis enzymes and the water and osmolyte transport system (Zhu, 2001). Detoxification signalling changes induced by salt include phospholipid hydrolysis, changes in the expression of LEA/dehydrin-type genes, molecular chaperones, and proteinase to remove denatured proteins, and the activation of enzymes for the generation and removal of ROS and other detoxification proteins (Zhu, 2001, 2002).

Signalling molecules are crucial components for salinity stress tolerance in plants, and they determine downstream actions involving protein phosphorylation, dephosphorylation, phospholipid metabolism, and calcium ion (Ca^{2+}) sensing (Agarwal et al., 2013). Ca^{2+} signalling is one of the first responses to salt stress and is essential for ion homeostasis (Zhu, 2003; Reddy and Reddy, 2004). It is sensed by calcineurin B-like proteins (CBLs) that are involved in the salt stress indication transduction pathway and govern the influx and efflux of Na^+. CBLs increase germination under salinity stress in tobacco (Pardo et al., 1998), *Arabidopsis* (Liu and Zhu, 1998), and maize (Wang MY et al., 2007). Other molecules include mitogen-activated protein kinase (SAPK) (Diédhiou et al., 2008), CBL-interacting protein kinase (CIPK), nucleoside diphosphate kinase (NDPK), and mitogen-activated protein (MAP) kinase (MPK) (Moon et al., 2003), whose overexpression enhances salinity tolerance capacity by increasing seed germination. Initiation of the MPK cascade is the second response to decode external salinity stress into cellular responses (Teige et al., 2004). This includes signalling modules that phosphorylate particular serine/threonine residues on amide protein substrates that regulate several cellular processes (MAPK Group et al., 2002). Overexpression of MAP in rice (Xiong and Yang, 2003; Jeong et al., 2006) and maize MAP in *Arabidopsis* (Kong et al., 2011) enhances salinity tolerance and activates transcriptional factors controlling downstream genes such as *COR47*, *RD29A*, *P5CS2* (Wurzinger et al., 2011), and *CDPK* (Capiati et al., 2006).

6.4.3.3 Salinity stress tolerance: transcriptional regulation and gene expression

Salt stress prompts the induction of various genes that fall into two broad groups: (1) single function genes that facilitate the production of protective metabolites, and (2) regulatory genes that control the expression of downstream genes (Agarwal et al., 2013). Single function genes promote the release of compounds such as transporters/channel proteins, osmolytes, lipid biosynthesis genes, antioxidative enzymes, and PAs, while *DREB, bZIP*, *NAC*, and *MYC/MYB* (myelocytomatosis/myeloblastosis) are regulatory genes (Shinozaki and Yamaguchi-Shinozaki, 2007; Agarwal and Jha, 2010). Many of these genes from both groups play different roles in enhancing the adaptation process, and are further categorized into functional groups that include: (1) senescence-associated genes (SAGs), (2) ion transport or homeostasis genes (*SOS*, *HKT*, *AtNHX1*, and H^+*-ATPase*), (3) molecular chaperones (*HSP* genes), and (4) dehydration-related TFs (*DREB*) (Hasegawa et al., 2000; Liu et al., 2000; Shi et al., 2000; Yen et al., 2000). Under osmotic pressure from salinity stress, plants synthesize novel proteins that increase the expression of genes, such as osmotic regulation (OR) genes, at the cell level to aid their adaptation. The OR genes are categorized into nine groups, namely LEA genes, osmolyte biosynthesis genes, transporter genes, OR genes that encode regulatory proteins, photosynthetic genes, OR genes encoding proteins involved in protein synthesis processes and degradation, heat shock protein genes, osmotins, and other protein genes (Zhu et al., 1997; Turan et al., 2012). Other OR genes include those encoding RNA-binding proteins, putative lipid transfer, *RD29A*, *RD29B*, *Kin1*, and *Kin2*. Considerable variation among factors such as plant tissue type, age, and developmental stage also affects the expression of OR genes (Zhu et al., 1997).

Transcription factors (TFs), including *bZIP*, *WRKY*, *AP2*, *NAC*, C_2H_2 zinc finger gene, and *DREB*, are the most important regulators of gene expression under salinity stress (Gupta and Huang, 2014). They interact with diverse *cis*-elements in the promoter regions of many downstream genes and modify their expression. There are many different types of TFs in the plant kingdom (Shiu et al., 2005) with more than 50 families distinguished by their DNA-binding domains (Riechmann et al., 2000). Several TFs have been identified in *Arabidopsis*, including a basic-leucine zipper, *AP2/ERF* (APETALA2/ethylene-responsive factor), *MYC*, *HD-ZIP* (homeodomain-leucine zipper), *MYB*, and different classes of zinc finger domains (Shinozaki and Yamaguchi-Shinozaki, 2000). The expression of a given number of genes occurs through TF interactions with non-DNA-binding proteins and/or *cis*-regulatory elements (Grotewold, 2008). On the same transduction pathways, ABA, SA,

ethylene, BR, and JA regulate TF expression (Agarwal et al., 2006; Seo et al., 2008; Agarwal and Jha, 2010; Gürel et al., 2016). The four classes of TFs are: (1) ABA-dependent (CBF/DREB proteins), (2) ABA-independent (MYC, MYB, ABA-responsive element-binding protein (AREB), ABA-binding factor protein (ABF)) (Agarwal and Jha, 2010; Liu SW et al., 2018), (3) ABA-dependent and -independent (no apical meristem, ATAF1,2 and cup-shaped cotyledon (NAC)) (Agarwal et al., 2013; Joshi et al., 2016), and (4) TFs that do not belong to any of the first three groups, including homeodomain TFs osmotically responsive gene 9 (HOS9) and an R2R3-type MYB protein(HOS10) (Zhu et al., 2004, 2005).

6.5 Genetics of salinity tolerance at germination in barley

6.5.1 QTLs for salinity tolerance in barley and the homologs genes from *Arabidopsis*, Soybean, maize, wheat and rice at the germination stage

At the germination stage, salinity tolerance is controlled by various loci (Mano and Takeda, 1997), indicating that it is polygenic. Angessa et al. (2017) reported transgressive phenotypic segregation for germination percentage in a doubled haploid (DH) population developed from salinity-tolerant genotype CM72 and the sensitive commercial Australian barley cultivar Gairdner. Using 150 and 300 mmol/L NaCl, Angessa et al. (2017) mapped two stable quantitative trait loci (QTLs) to chromosome 2H, close to the sodium concentration QTL reported by Xue et al. (2009). A third QTL reported by Angessa et al. (2017) at 300 mmol/L NaCl was mapped to chromosome 5H, but slightly distant from the potassium concentration-linked QTL of Xue et al. (2009). Mano and Takeda (1997) reported QTLs controlling ABA response on chromosomes 2H, 3H, 1H, and 5H in Steptoe/Morex DH lines, and 2H and 5H in Harrington/TR306 DH lines. In both crosses, chromosome 5H was very close to the position of the salinity tolerance QTL. A QTL analysis of the Oregon Wolf Barley mapping population (DOM×REC) by Witzel et al. (2010) at the germination stage identified two chromosomal regions on 5H, one on 7H, and one on 2H associated with the salt stress response. A single chromosomal region on 5H, responsible for 42% of the variation in the phenotype, was constant across all NaCl concentrations (Table 6.1).

Protein sequences of functional salinity tolerance genes during germination in *Arabidopsis*, soybean, maize, wheat, and rice were extracted from the National Center for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/protein) (Benson et al., 2013). The protein sequences in FAST formats were blasted on BARLEX (https://apex.ipk-gatersleben.de/apex/f?p=284:10) to identify homologous genes from the barley genome with

the highest percentage match depending on length and expression levels in tissues at different growth stages (Colmsee et al., 2015). There were 63 functionally characterized genes (*Arabidopsis* (13), maize (4), rice (12), soybean (7), and wheat (27)) for salinity tolerance at the germination stage (Table 6.2). Overall, 65 homolog genes were identified in barley, with the percentage match ranging from 100% (between wheat gene *TaPLDα* (phospholipase D) and barley homolog gene HORVU1Hr1G048970.4 (phospholipase D P2)) to 30% (between rice gene *OsOPT10* (oligopeptide transporter family homolog) and barley's HORVU6Hr1G067430.2(oligopeptide transporter 4)) (Table 2). Generally, the wheat genes had the highest match with barley homologs, averaging 90.93%, followed by maize (83.00%), rice (67.58%), soybean (59.43%), and *Arabidopsis* (57.94%). Apart from homolog connections, gene expression patterns in tissues can suggest the function of particular genes. For instance, the expression levels of the 65 barley homologs for the first three development stages (4-d embryo (EMB), root from seedlings (ROO1; 10 cm shoot stage) and shoot from seedling stage (LEA; 10 cm shoot stage)) were more than zero for all except HORVU1Hr 1G080820.6 (CBL protein 8). The aquaporin-like superfamily protein (HORVU2Hr1G096360.13) had the highest expression levels in the EMB, ROO1, and LEA stages at 1305.90, 1094.39, and 1133.08 fragments per million kb, respectively (Supplementary Table 6.1) (The International Barley Genome Sequencing Consortium, 2012; Colmsee et al., 2015; Mascher et al., 2017).

6.5.2 Barley salinity tolerance characterized transcriptional factors and genes during germination

A highly positive correlation has been reported between salinity stress and polyethylene glycol treatments, indicating that salt stress in germinating seed is mostly osmotic (Mano et al., 1996). Therefore, osmoprotection is the likely early response in barley geminating seeds (Walia et al., 2006).

6.5.2.1 6PGDH and Glc/RibDH

Salinity tolerant lines at germination in a mapping population containing 94 DH lines of DOM/ REC expressed a higher level of 6-phosphogluconate dehydrogenase (6PGDH) and glucose/ribitol dehydrogenase (Glc/RibDH) in mature seeds (Witzel et al., 2010). Overexpression of the two enzymes in yeast enhanced the growth of transformed cells in saline media (Witzel et al., 2010). Cytosolic 6PGDH is an enzyme participating in the pentose phosphate pathway, which provides reduced nicotinamide adenine dinucleotide phosphate

(NADPH) as an electron donor in the reductive biosynthetic process and is important for the ascorbate–glutathione synthesis phase in the plant antioxidant protection system (Corpas et al., 1998). This protein concentration is increased under salinity stress in olive (Valderrama et al., 2006) and rice (Huang et al., 2003; Hou et al., 2007). Transcripts and protein Glc/RibDH are short alcohol dehydrogenases (Jornvall et al., 1984) found in developing barley embryos, whose level of transcription reduces with germination (Alexander et al., 1994), and which catalyse the oxidation of d-glucose to synthesize sugars for osmoprotective functions (Witzel et al., 2010).

Table 6.1 Reported QTLs for salinity tolerance in barley during germination

Trait at germination	Salt levels	Mapping population	Analysis type	QTL number and location	% explained	Reference
Germination %	150 mM NaCl	103-DH of CM72/Gairdner	Bi-Parent	1(2H)	21.8	Angessa *et al.*, 2017
	300 mM NaCl	103-DH of CM72/Gairdner	Bi-Parent	1(2H)	16.1	Angessa *et al.*, 2017
	300 mM NaCl	103-DH of CM72/Gairdner	Bi-Parent	1(5H)	10.0	Angessa *et al.*, 2017
Germination speed	250 mM NaCl	149-DH of Steptoe/Morex	Bi-Parent	2(2H), 3(3H), and 7(5H)	7.6 – 20.3	Mano and Takeda, 1997
	300 mM NaCl	146-DH of Harrington/TR306	Bi-Parent	1(7H), 6(6H), and 7(5H)	8.7 – 15.3	Mano and Takeda, 1997
Salt tolerance	250 mM NaCl	149-DH of Steptoe/Morex	Bi-Parent	4 (4H), 6(6H), and 7(5H)	6.8 – 46.7	Mano and Takeda, 1997
	300 mM NaCl	146-DH of Harrington/TR306	Bi-Parent	5(1H) and 7(5H)	17.4 – 41.1	Mano and Takeda, 1997
	1.5%	94-DH of DOM/REC	Bi-Parent	1(5H) and 1(7H)	42	Witzel *et al.*, 2010
	2.0%	94-DH of DOM/REC	Bi-Parent	1(5H) and 1(7H)	42	Witzel *et al.*, 2010
	2.5%	94-DH of DOM/REC	Bi-Parent	1(5H) and 1(7H)	-	Witzel *et al.*, 2010
	1.5%	94-DH of DOM/REC	Bi-Parent	1(2H)	-	Witzel *et al.*, 2010
	1.5–2.5%	94-DH of DOM/REC	Bi-Parent	2(5H) and 1(7H) 1(2H)	-	Witzel *et al.*, 2010

* 1%=0.01 g/mL

Table 6.2 Homologs of candidate functional salinity genes in barley during the germination of Arabidopsis, maize, rice, soybean, and wheat

Gene source	Gene ID	Transgenic host	Homolog ID in barley	Similarity	Gene name in barley	Chromosome	Location	Reference
A. thaliana L.	AtRZFP	Arabidopsis	HORVU5Hr1G001400.5	52	RING finger protein 5	5	4181876-4184642	Zang *et al.*, 2015
A. thaliana L.			HORVU4Hr1G003600.1	51	|RING finger protein 5	4	7870080-7872190	
A. thaliana L.	AtNHX1	Maize/wheat	HORVU2Hr1G021020.4	69	Sodium hydrogen exchanger 2	2	59025629-59030226	Ali, 2000; Xue *et al.*, 2004
A. thaliana L.			HORVU4Hr1G033760.1	69	Sodium hydrogen exchanger 2	4	238472323-238482634	
A. thaliana L.			HORVU7Hr1G046030.1	62	Sodium hydrogen) exchanger 3	7	148709554-148713307	
A. thaliana L.	AtSOS1	Arabidopsis	HORVU3Hr1G003150.1	58	Sodium/hydrogen exchanger 7	3	8165119-8185196	Wang *et al.*, 2007b
A. thaliana L.	AtMYBL	Arabidopsis	HORVU5Hr1G077100.1	56	myb domain protein 3r-3	5	552718664-552726337	Zhang *et al.*, 2010
A. thaliana L.	RAP2.6L	Arabidopsis	HORVU2Hr1G071270.4	60	Ethylene-responsive transcription factor 1	2	507785971-507787466	Krishnaswamy *et al.*, 2011
A. thaliana L.	AtERF96	Arabidopsis	HORVU4Hr1G000960.1	59	Ethylene-responsive transcription factor 14	4	1356682-1357123	Wang *et al.*, 2017
A. thaliana L.	AtCYSa	Arabidopsis	HORVU1Hr1G067870.2	56	Cysteine proteinase inhibitor	1	479563716-479564368	Zhang *et al.*, 2008
A. thaliana L.	AtCYSb	Arabidopsis	HORVU3Hr1G038190.1	66	Cysteine proteinase inhibitor 12	3	219953557-219956556	Zhang *et al.*, 2008
A. thaliana L.	AtNHX1	Cotton	HORVU2Hr1G021020.4	69	Sodium hydrogen exchanger 2	2	59025629-59030226	Cheng *et al.*, 2018b
A. thaliana L.	AtPP2-B11	Arabidopsis	HORVU6Hr1G090000.1	42	F-box protein PP2-B1	6	571135647-571137859	Jia *et al.*, 2015
A. thaliana L.	AnnAt8	Arabidopsis	HORVU6Hr1G074440.1	50	Annexin 7	6	514097241-514103959	Yadav *et al.*, 2016
A. thaliana L.	AtDIF1	Arabidopsis	HORVU5Hr1G079500.1	42	Sister chromatid cohesion 1 protein 4	1	557145143-557147352|	Gao *et al.*, 2017
A. thaliana L.	NF-YA1 - 9	Arabidopsis	HORVU5Hr1G007890.11	66	Nuclear transcription factor Y subunit A-10	5	16694492-16699091	Mu *et al.*, 2013
Maize (*Z. mays)*	ZmCBL4	Arabidopsis	HORVU1Hr1G080820.6	72	Calcineurin B-like protein 8	1	526318770-526320885|	Wang *et al.*, 2007a
Maize (*Z. mays)*	ZmMKK4	Arabidopsis	HORVU0Hr1G038850.2	88	Protein kinase superfamily protein	0	247582088-247583184	Kong *et al.*, 2011
Maize (*Z. mays)*	ZmMPK5	Tobacco	HORVU7Hr1G023760.3	89	Mitogen-activated protein kinase 3	7	37300015-37306217	Zhang *et al.*, 2014
Rice (*O. sativa*)	OSISAP1	Tobacco	HORVU5Hr1G072920.1	70	Zinc finger A20 and AN1 domain-containing stress-associated protein 1	5	536933399-536936238	Mukhopadhyay *et al.*, 2004
Rice (*O. sativa*)	OsDREB2A	Soybean	HORVU6Hr1G050520.2	78	Ethylene-responsive transcription factor 4	6	306973236-306973949	Zhang *et al.*, 2013
Rice (*O. sativa*)	OsiSAP8	Tobacco/rice	HORVU2Hr1G053670.4	43	Zinc finger A20 and AN1 domain-containing stress-associated protein 9|	2	328713964-328714708	Kanneganti and Gupta, 2008
Rice (*O. sativa*)	OsMYB48-1	Rice	HORVU1Hr1G008810.3	82	myb domain protein 59	1	19369578-19370912	Xiong *et al.*, 2014
Rice (*O. sativa*)	OsSAP11	Arabidopsis	HORVU7Hr1G050270.3	66	Zinc finger A20 and AN1 domain-containing stress-associated protein 11	7	180473211-180476075	Giri *et al.*, 2011
Rice (*O. sativa*)	OsRLCK253	Arabidopsis	HORVU6Hr1G061280.1	69	Protein kinase superfamily protein	6	410507581-410509354	Giri *et al.*, 2011
Rice (*O. sativa*)	OsGSTU4	Arabidopsis	HORVU6Hr1G026810.3	71	Glutathione S-transferase family protein	6	103574244-103575543	Sharma *et al.*, 2014
Rice (*O. sativa*)	OsDREB1D	Arabidopsis	HORVU5Hr1G080420.1	71	C-repeat-binding factor 4	5	560569797-560571193	Zhang *et al.*, 2009
Rice (*O. sativa*)	OsNAC9	Rice	HORVU7Hr1G060310.1	75	NAC domain protein	7	276430981-276436993	Redillas *et al.*, 2012
Rice (*O. sativa*)	OsZFP179	Rice	HORVU5Hr1G055970.1	85	RING/U-box superfamily protein	5	438292337-438301123	Sun *et al.*, 2010
Rice (*O. sativa*)	OsOPT10	Arabidopsis/rice	HORVU6Hr1G067430.2	30	Oligopeptide transporter 4	6	467362064-467373338	Jung *et al.*, 2010
Rice (*O. sativa*)	OsMYB3R-2	Arabidopsis	HORVU3Hr1G086270.3	71	myb domain protein 3r-5	3	616465321-616471572	Dai *et al.*, 2007
Soybean (*G. max*)	GmbZIP44	Arabidopsis	HORVU5Hr1G023000.1	43	Basic-leucine zipper transcription factor family protein	5	119817528-119818004	Liao *et al.*, 2008
Soybean (*G. max*)	GmbZIP62	Arabidopsis	HORVU6Hr1G031330.2	49	Basic-leucine zipper transcription factor family protein	6	132098841-132101167	Liao *et al.*, 2008
Soybean (*G. max*)	GmbZIP78	Arabidopsis	HORVU2Hr1G055230.4	61	Basic-leucine zipper 8	2	342114231-342120751	Liao *et al.*, 2008
Soybean (*G. max*)	GmDREB2	Arabidopsis/tobacco	HORVU4Hr1G015350.1	66	Ethylene-responsive transcription factor 2	4	59331360-59332535	Chen *et al.*, 2007a
Soybean (*G. max*)	GmDREB1	Wheat	HORVU7Hr1G026940.1	67	Ethylene-responsive transcription factor 5	7	47357790-47359063	Jiang *et al.*, 2014
Soybean (*G. max*)	GmNARK	Arabidopsis	HORVU5Hr1G098840.2	54	Leucine-rich receptor-like protein kinase family protein	5	608904210-608907733	Cheng *et al.*, 2018a
Soybean (*G. max*)	GmFDL19	Soybean	HORVU2Hr1G110500.1	76	ABSCISIC ACID-INSENSITIVE 5-like protein 5	2	720057018-720058296	Li *et al.*, 2017a
Wheat (*T. aestivum*)	TaNAC29	Arabidopsis	HORVU2Hr1G017470.1	93	NAC domain protein	2	42672748-42675686	Huang *et al.*, 2015
Wheat (*T. aestivum*)	TaSC	Arabidopsis	HORVU5Hr1G067740.1	99	Transmembrane protein 50A	5	514305842-514309764	Huang *et al.*, 2012
Wheat (*T. aestivum*)	TaNAC2D	Arabidopsis	HORVU5Hr1G111590.2	95	NAC domain protein	5	636772274-636774317	Huang and Wang, 2016

Wheat (*T. aestivum*)	TaARGOS	Arabidopsis	HORVU4Hr1G043960.1	88	Eukaryotic aspartyl protease family protein	4	351442597-351444197	Zhao *et al.*, 2017
Wheat (*T. aestivum*)	TaCRT1	Tobacco	HORVU3Hr1G089830.2	98	Calreticulin 3	3	630039636-630043684	Xiang *et al.*, 2015
Wheat (*T. aestivum*)	TaSRG	Arabidopsis/rice	HORVU2Hr1G072420.11	97	Family of unknown function (DUF662)	2	519528221-519529717	He *et al.*, 2011
Wheat (*T. aestivum*)	TaSTRG	Tobacco	HORVU4Hr1G063430.1	90	Salt-tolerant-related protein	4	531361349-53136272	Kavas *et al.*, 2016
Wheat (*T. aestivum*)	DHN-5	Arabidopsis	HORVU5Hr1G103460.2	79	Dehydrin Rab15	5	616115076-616116086	Brini *et al.*, 2007
Wheat (*T. aestivum*)	TaAQP8	Tobacco	HORVU2Hr1G096360.13	91	Aquaporin-like superfamily protein	2	674156225-674158128	Hu *et al.*, 2012
Wheat (*T. aestivum*)	TaWRKY10	Tobacco	HORVU2Hr1G109330.1	87	WRKY family transcription factor	2	716404110-716405772	Wang *et al.*, 2013
Wheat (*T. aestivum*)	TaDi19A	Arabidopsis	HORVU3Hr1G061690.1	97	Protein DEHYDRATION-INDUCED 19 homolog 3	3	469768135-469771916	Li *et al.*, 2010
Wheat (*T. aestivum*)	TaWD40D	Arabidopsis	HORVU0Hr1G016450.1	99	Transducin/WD40 repeat-like superfamily protein	0	88366914-88373289	Kong *et al.*, 2015
Wheat (*T. aestivum*)	TaAIDFa	Arabidopsis	HORVU5Hr1G105980.4	72	Histone-lysine N-methyltransferase NSD3	5	623061366-623067662	Xu *et al.*, 2008
Wheat (*T. aestivum*)	TaCIPK29	Tobacco	HORVU2Hr1G018340.3	92	Protein kinase family protein	2	46669169-46672779	Deng *et al.*, 2013
Wheat (*T. aestivum*)	TaERF3	Wheat	HORVU2Hr1G098330.1	85	Ethylene-responsive transcription factor 1	2	682511166-682511937	Rong *et al.*, 2014
Wheat (*T. aestivum*)	TaPUB1	Tobacco	HORVU4Hr1G083960.3	95	Pre-mRNA-processing factor 19	3	627004064-627010259	Zhang *et al.*, 2017
Wheat (*T. aestivum*)	TaWRKY44	Tobacco	HORVU4Hr1G048400.12	92	WRKY DNA-binding protein 3	4	390705028-390708928	Wang *et al.*, 2015
Wheat (*T. aestivum*)	TaVB	Arabidopsis	HORVU4Hr1G033760.1	97	Sodium hydrogen exchanger 2	4	238472323-238482634	Wang *et al.*, 2011
Wheat (*T. aestivum*)	TaSRHP	Arabidopsis	HORVU7Hr1G037410.4	90	Protein of unknown function	7	89779039-89780525	Hou *et al.*, 2013
Wheat (*T. aestivum*)	TaNHXS1	Tobacco	HORVU2Hr1G021020.4	70	Sodium hydrogen exchanger 2	2	59025629-59030226	Gouiaa *et al.*, 2012
Wheat (*T. aestivum*)	TaVP1	Tobacco	HORVU7Hr1G031480.1	97	K(+)-insensitive pyrophosphate-energised proton pump	7	63936492-63936858	Gouiaa *et al.*, 2012
Wheat (*T. aestivum*)			HORVU7Hr1G114250.2	88	Pyrophosphate-energised vacuolar membrane proton pump	7	639161697-639164555	Gouiaa *et al.*, 2012
Wheat (*T. aestivum*)	TaPLDα	Arabidopsis	HORVU1Hr1G048970.4	100	Phospholipase D P2	1	363556495-363563036	Wang *et al.*, 2014
Wheat (*T. aestivum*)	TaOPR1	Arabidopsis	HORVU1Hr1G001850.3	82	12-oxophytodienoate reductase 2	1	4131150-4132543	Dong *et al.*, 2013
Wheat (*T. aestivum*)	TaNF-YA10-1	Arabidopsis	HORVU6Hr1G081080.12	89	Nuclear transcription factor Y subunit A-5	6	544207949-544211747	Ma *et al.*, 2015
Wheat (*T. aestivum*)	TaPI4KIIγ	Arabidopsis	HORVU2Hr1G118320.7	94	Phosphatidylinositol 3- and 4-kinase family protein	2	742201795-742204374	Liu *et al.*, 2013

6.5.2.2 Dehydrins

Dehydrins (DHNs), a subfamily of LEA proteins from *Hordeum vulgare* (*aba2*), enhanced seed germination in transgenic lines of *Arabidopsis* under salinity stress, relative to the wild type (Calestani et al., 2015). Similar protein-coding genes have been identified in wheat (*Dhn5*) and maize (*Rab17*) with comparable results when overexpressed in transgenic *Arabidopsis* plants (Figueras et al., 2004; Brini et al., 2011). DHNs are thought to be involved in protecting the embryo through seed desiccation and rehydration (Skriver and Mundy, 1990; Blackman et al., 1991; Tunnacliffe and Wise, 2007) by increasing their contents during the last phase of seed development, and in dormant embryos. DHN content decreases when imbibition and germination begin as seeds lose their capacity to withstand desiccation (Blackman et al., 1991; Han et al., 1997). DHNs also facilitate water uptake during seed germination on media with low osmotic potential (Hara, 2010), and may act as a hydration buffer inside cells in the presence of sugars (Walters et al., 1997; Hara, 2010). Moreover, they are likely to play a role in cellular detoxification because they contain lipids and metal-binding capacity that can prevent lipid peroxidation (Cheng et al., 2002; Krüger et al., 2002; Alsheikh et al., 2003; Koag et al., 2003, 2009).

6.5.2.3 CBLs

Rice transgenic lines with CBL protein 8 (*HsCBL8*) transferred from wild barley homolog to *HvCBL8* of cultivated barley improved seed germination, reduced Na^+ uptake, adjusted K^+ concentration in tissues, provided in vivo water protection of the plasma membrane, and accumulated more proline under salt stress (Guo et al., 2016). The build-up of compatible osmolytes, like proline, is linked to stress tolerance in plants (Ahmed et al., 2013b; Mekawy et al., 2015). Phylogenetic analysis of *HsCBL8* showed that it encodes proteins of the CBL group modified with only N-myristoylation or S-acylation (Batistič et al., 2012). Such proteins are involved in relationships with the transmembrane (TM) helix (Kleist et al., 2014) including *HvCBL8*. Reports about other CBLs from different plant species reflected similar trends to that of *HsCBL8*, such as transgenic *Arabidopsis* having *35S-AtCBL5* (Cheong et al., 2010) and poplar harbouring *35S-PeCBL10* (Li DD et al., 2013). *OsCBL2* is likely to be involved in GA-signalling that facilitates the vacuolation of aleurone cells (Hwang et al., 2005), and *AtCBL1* is involved in upregulation of GA (Li ZY et al., 2013) and downregulation of ABA (Pandey et al., 2008) during seed germination.

6.5.2.4 SERKs

Three orthologs of somatic embryogenesis receptor-like kinase (SERK) genes (*HvSERK1*/*2*/*3*) isolated from barley were induced in microspore-derived embryogenic callus under salt stress (Li et al., 2016), indicating their protective role for developing embryos during salinity stress. SERKs are a subfamily of the leucine-rich repeat receptor-like kinase II group (LRR-RLKII) with proline-rich SPP motifs between the LRRs and the TM domain (Hecht et al., 2001). These genes are present during somatic embryogenesis in plants before declining in later stages (Schmidt et al., 1997; Somleva et al., 2000; Nolan et al., 2003; Singla et al., 2008; Li, 2010). They have been characterized in many plants, including maize (Baudino et al., 2001), wheat (Singla et al., 2008), soybean (Yang et al., 2011), rice (Singla et al., 2009), cotton (Pandey and Chaudhary, 2014), and *Arabidopsis* (Hecht et al., 2001). In rice (*Oryza sativa* L.), BRs reverse the inhibitory effect of salinity during germination (Anuradha and Rao, 2001), and in *Arabidopsis* are reportedly signalled by *SERK1*, *SERK3*, and *SERK4* (Albrecht et al., 2008), while ABA stimulates *OsSERK1* during stress (Hu et al., 2005).

6.5.2.5 DREBs

A gene that encodes DREB proteins in *H. vulgare* (*HvDREB1*) is induced by exogenous ABA to enhance germination and early root growth in *Arabidopsis* plants under salinity stress (Xue et al., 2004). TF *HvDREB1* is a member of the AP2 group of the *DREB* subfamily that is vital for regulating responses to various stresses (Agarwal et al., 2006; Xu et al., 2008b) and reportedly improves salt, drought, and cold responses in transgenic plants (Oh et al., 2007). Overexpressing rice *OsDREB1A* in *Arabidopsis* enhanced tolerance to drought and freezing stresses (Dubouzet et al., 2003), and barley's *HvCBF4* in rice transgenes increased drought, salt, and cold stress tolerance (Oh et al., 2007). DREB proteins isolated from several plants including rice, maize, soybean, and wheat are involved in several signal transduction pathways during abiotic stress (Dubouzet et al., 2003; Agarwal et al., 2006). Of particular interest are those belonging to the A-2 group that respond to dehydration and salinity stress (Liu et al., 1998; Dubouzet et al., 2003) and regulate ABA-independent gene expression in target plants under stress (Chinnusamy et al., 2006; Nakashima and Yamaguchi-Shinozaki, 2006). It is possible that *HvDREB1* may take part in both ABA-independent and -dependent pathways concurrently (Xue et al., 2004).

6.5.2.6 ERFs

Barley ERF-type TF *HvRAF* improved seed germination and root growth under salinity stress but was not induced by ABA treatment in transgenic *Arabidopsis* plants (Jung et al., 2007). TF *HvRAF* is a member of the *AP2* group in the *ERF* family (Sakuma et al., 2002; Gutterson and Reuber, 2004), which is involved in regulating both biotic and abiotic stress-responsive genes in plants (Lee et al., 2004; Yi et al., 2004). However, the response of *Arabidopsis* transgenic lines with *HvRAF* was specific to salinity stress, where it acted as a regulator for ABA-independent signalling in root growth and seed germination (Jung et al., 2007). Similar ABA regulation responses to salinity have been documented in *Arabidopsis* for *DREB2A* and *DREB2B* (Chinnusamy et al., 2005). A tolerance response in terms of root growth and seed germination was observed when *CaERFLP1* and *JERF1* from pepper and tomato, respectively, were overexpressed in transgenic tobacco plants in response to salt stress (Lee et al., 2004; Zhang et al., 2004).

6.5.2.7 Aquaporin genes

Overexpression of a barley aquaporin gene *HvPIP2;5* in yeast enhanced salt and osmotic stress tolerance, and transgenic *Arabidopsis* with the gene showed better seed germination and root growth than the wild type under salinity stress (Alavilli et al., 2016). Aquaporins form part of the major intrinsic proteins (MIPs) found in living organisms that enable the transportation of water and small nonpolar molecules across living membranes (Zardoya et al., 2002; Maurel et al., 2015). In plants, all but the GlpF-like intrinsic proteins show water-specific channel movement. Hence, the collective name for MIPs in plants is aquaporins (Maurel et al., 2008), even though they can transport additional small molecules such as CO_2 and ammonia inside cells (Uehlein et al., 2003; Jahn et al., 2004). There are five groups of aquaporins in plant genomes, reflective of their diverse physiological roles. Of importance are the plasma membrane intrinsic proteins (PIPs) that are further subdivided into PIP1 and PIP2, each with several isoforms (Javot et al., 2003; Postaire et al., 2010). Overexpression of *PIP* genes from various plants (*O. sativa*, *Nicotiana tabacum*, *Vicia faba*, *Arabidopsis*, and *Triticum aestivum*) enhanced dehydration stress tolerance in their respective transgenic lines (Alavilli et al., 2016).

6.5.3 Diversity of barley salinity tolerance genes at germination

In this review, we have compared studies to determine whether any of the eight genes (Table 6.3) that have been reported and functionally characterized for salinity tolerance in barley at germination are unique or similar to their homologs from wheat and rice. All the sequences

producing a significant match, based on length and total relationship hits, were downloaded and recorded. The following total significant homolog hits from barley, wheat, and rice were recorded: dehydrins (17), dehydrogenase/reductase SDR family member 4 (20), 6PGDH, (29), ERFs (31), CBL protein (72), DREB protein (164), SERKs (215), and aquaporin gene (227).

Three genes families—DREB protein, SERKs and aquaporin gene—were considered for further analysis based on their high numbers of hits. The proteins from the three genes were blasted on the Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) to download matched homologs (>30%) in 40 different plant species. Mega software (https://www.megasoftware.net) was used to align the 40 species' sequences, and a phylogenetic tree was constructed to estimate the evolutionary distances between the genes using MEGA-X software (https://www.megasoftware.net) (Kumar et al., 2018). Gene alignments showed three distinct regions across the sequences of the gene families. The start and end sections of the sequences were more divergent than the middle segments. The phylogenetic tree of the DREB proteins divided the genes into two major groups that were further divided into two subgroups (Figure 6.2a). The groups were distinguished by the presence or absence of genes from wheat, barley, and rice. Among the four sub-groups, barley genes appeared three times in two categories while genes from the three cereals were missing in two sub-groups (Figure 6.2a). The phylogenetic tree shows that DREB proteins are no more diverse in barley than in wheat or rice. The SERK phylogenetic tree (Figure 6.2b) was similar to that of the DREB proteins, but with more hits. However, the four subgroups contained genes from the three cereals with five hits for barley and wheat, and three hits for rice. The aquaporin gene phylogenetic tree differed from the other two trees in which the genes were first divided into three major groups, two small and one major, containing representative genes from the three cereals (Figure 6.3).

6.6 Conclusions and future prospects

Among the most destructive abiotic stresses, salinity causes massive yield losses in crops in arid, semi-arid, coastal regions, and humid and sub-humid landscapes. Thus, more effort is needed to increase crop yields in these areas to produce enough food for the increasing global population. Growing salt-adapted cultivars requires knowledge of the donating characters at different growth stages. It involves the use of many disciplines to identify and functionally characterize the genes contributing to tolerance, and then to transfer them to commercially acceptable cultivars. Barley is one of the hardiest crops that can grow in saline environments, but its germination is severely affected by salinity stress. Development of cultivars that can

acclimatise to salinity at this stage is essential in regions like Western Australia that experience hot and dry summers and increasing salt levels in the topsoil before sowing in autumn. The identification and characterization of salinity tolerance genes, enzymes, and compounds during germination in barley have been ongoing, and some transgenic "salt-tolerant" plants have been developed. However, the seeds of these genotypes have had little success in commercial production because they are not equipped with holistic genes or the mechanisms required for successful germination and growth under saline field conditions.

This review summarized the agronomic/morphological, physiological, and biochemical traits related to salinity stress. The best trait or combination of traits needs to be identified which can be used to accurately screen for salinity stress tolerance at germination to identify and characterize novel genes. Homolog salinity tolerance genes in barley during germination have been reported, but they need to be validated in barley and other transgenic plants that carry them. Functional characterization by blending genetic, agronomic, biochemical, and physiological indicators can facilitate proof of identity of the genes, leading to the development of barley cultivars with improved salt tolerance at germination and better performance in the field.

Table 6.3 Barley salinity tolerance functional transcriptional factors and genes at germination

Reported functional barley gene	Reported gene ID	Gene ID in barley	Gene name in barley	Chromosome	Location	Reference
6-phosphogluconate dehydrogenase	6PGDH	HORVU7Hr1G006160.4	6-phosphogluconate dehydrogenase, decarboxylating 1	7	8000958-8002650	Witzel *et al.*, 2010
Glucose/ribitol dehydrogenase	Glc/RibDH	HORVU1Hr1G018140.5	Dehydrogenase/reductase SDR family member 4	1	65592292-65593858	Witzel *et al.*, 2010
Dehydrins	Dhnaba2	HORVU7Hr1G099800.5	Dihydroneopterin aldolase	7	602554874-602555971	Calestani *et al.*, 2015
Calcineurin B-like proteins 8	HvCBL8	HORVU1Hr1G064470.4	Calcineurin B-like protein 1	1	461521906-461524442	Guo *et al.*, 2016
Somatic embryogenesis receptor-like kinases	HvSERK1/2/3	HORVU7Hr1G068990.2	Receptor-like protein kinase 4	7	366099333-366114129	Li *et al.*, 2017b
Dehydration-responsive element binding protein	HvDREB1	HORVU3Hr1G017950.4	Chromodomain-helicase-DNA-binding protein 5|	3	46482481-46494788	Xue *et al.*, 2004
Ethylene response factor (ERF)	HvRAF	HORVU4Hr1G077310.1	Ethylene-responsive transcription factor 1|	4	603804858-603809470	Jung *et al.*, 2007
Aquaporin gene	HvPIP2	HORVU2Hr1G089940.1	Aquaporin-like superfamily protein	2	640763978-640768942	Alavilli *et al.*, 2016

The protein sequences of identified functional barley genes retrieved from BARLEX (https://apex.ipk-gatersleben.de/apex/f?p=284:10) and blasted on barley (https://webblast.ipk-gatersleben.de/barley_ibsc/viroblast.php), wheat (https://urgi.versailles.inra.fr/blast_iwgsc/?dbgroup=wheat_iwgsc_refseq_v1_chromosomes&program=blastn), and rice (https://www.plantgdb.org/OsGDB) genome explorers.

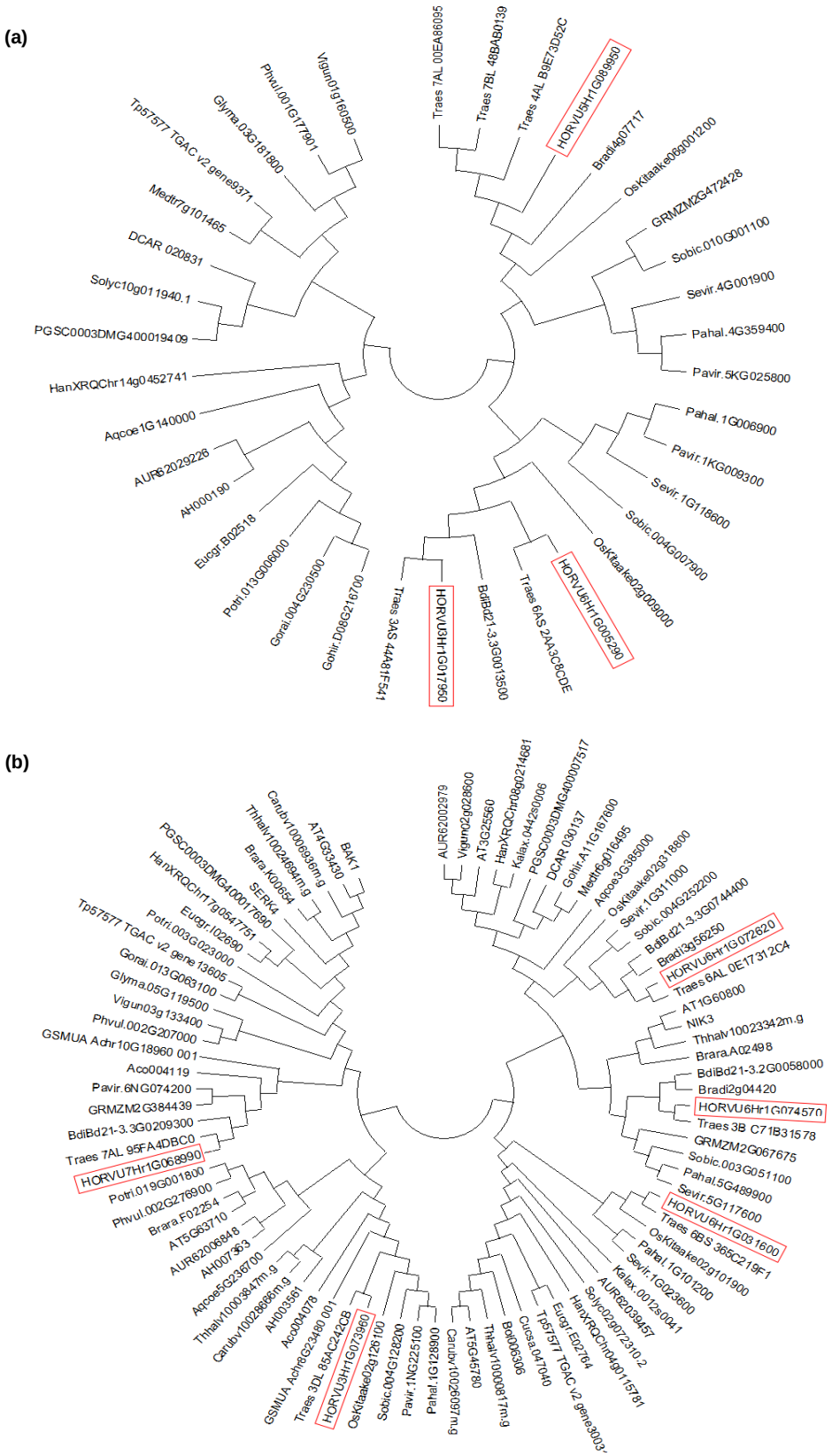

Figure 6.2 Phylogenetic analyses of barley homolog genes. (a) Dehydration-responsive element binding proteins. (b) Somatic embryogenesis receptor-like kinases. The unrooted phylogenetic trees of 37 (a) and 92 (b) domains comprising four (a) and five (b) domains, respectively, from barley were constructed using MEGA-X.

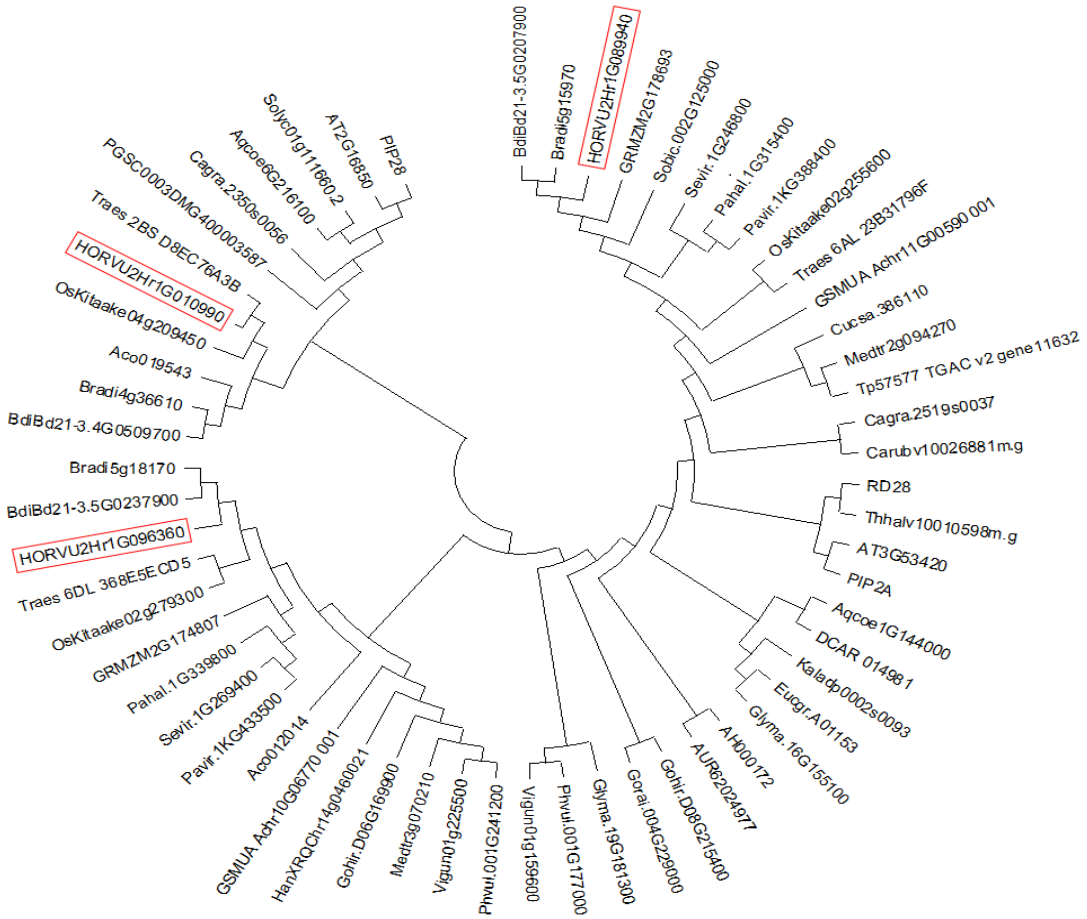

Figure 6.3 Phylogenetic analysis of barley homolog aquaporin genes. The unrooted phylogenetic tree of 74 domains comprising five domains from barley was constructed using MEGA-X

6.7 References

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6.8 Supplementary Material

6.8.1 Supplementary Tables

Supplementary Table 6.1 Barley salinity tolerance at germination:6 expression levels of homolog genes in different tissues and growth stages

Gene ID Name	EMB	ROO1	LEA	INF1	INF2	NOD	CAR5	CAR15	ETI	LEM	LOD	PAL	EPI	RAC	ROO2	SEN
HORVU5Hr1G001400.5	10.680	14.404	7.802	0.000	11.193	18.191	9.868	13.869	12.653	14.722	16.153	15.285	13.788	13.834	12.451	12.275
HORVU4Hr1G003600.1	30.763	102.072	61.556	0.000	16.424	77.195	28.363	155.646	56.569	103.083	66.746	81.768	39.401	52.113	49.135	76.174
HORVU2Hr1G021020.4	5.865	8.743	16.482	0.000	23.953	49.791	11.115	12.231	17.568	42.308	13.567	22.734	65.941	25.827	5.471	10.384
HORVU4Hr1G033760.1	4.918	9.283	14.821	0.000	4.223	11.698	8.135	15.964	6.677	10.937	9.418	8.983	16.004	8.956	6.230	23.503
HORVU7Hr1G046030.1	0.046	0.222	0.000	0.000	0.000	0.000	0.057	66.352	0.000	0.000	0.000	0.000	0.000	0.000	0.018	0.000
HORVU3Hr1G003150.1	45.481	32.857	27.306	0.000	38.285	37.098	43.078	54.442	89.815	58.011	36.436	50.987	102.837	39.454	111.369	60.351
HORVU5Hr1G077100.1	4.571	2.438	4.918	0.000	13.294	2.308	7.512	5.642	5.385	2.886	3.916	3.515	4.382	4.769	3.050	5.480
HORVU2Hr1G071270.4	2.470	5.637	2.622	0.000	0.657	0.192	1.216	3.577	6.440	0.410	1.182	0.275	1.068	0.083	2.570	0.709
HORVU4Hr1G000960.1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.885	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
HORVU1Hr1G067870.2	14.589	24.977	27.054	0.000	0.372	53.653	0.643	0.891	7.446	41.422	17.376	42.971	21.689	9.756	32.480	27.501
HORVU3Hr1G038190.1	74.543	55.893	67.299	0.000	22.959	115.939	72.663	56.305	61.287	114.177	139.238	86.273	112.404	106.974	94.642	76.913
HORVU2Hr1G021020.4	5.865	8.743	16.482	0.000	23.953	49.791	11.115	12.231	17.568	42.308	13.567	22.734	65.941	25.827	5.471	10.384
HORVU6Hr1G090000.1	30.784	25.630	32.180	0.000	20.807	50.918	33.745	41.372	26.647	32.392	33.975	36.520	30.638	32.921	18.951	60.294
HORVU6Hr1G074440.1	437.068	191.863	85.617	0.000	7.204	368.521	94.214	64.423	83.429	224.582	166.375	255.581	112.586	301.458	474.771	298.980
HORVU5Hr1G079500.1	3.181	1.054	0.632	0.000	7.812	0.024	3.364	1.142	0.008	0.067	0.624	0.047	0.005	0.895	1.383	0.000
HORVU5Hr1G007890.11	4.432	9.268	13.979	0.000	9.550	4.070	1.895	1.944	5.038	0.434	0.285	0.407	12.920	1.837	1.659	9.847
HORVU1Hr1G080820.6	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.614	0.031	1.064	0.874	0.893	0.081	0.041	1.093	0.000
HORVU0Hr1G038850.2	19.199	19.750	13.556	0.000	12.608	26.856	13.125	11.510	17.096	24.306	15.520	18.604	26.172	22.711	33.540	5.996
HORVU7Hr1G023760.3	87.343	57.420	76.815	0.000	51.800	140.740	62.120	51.157	89.136	80.849	116.690	101.581	67.666	81.541	82.810	72.686
HORVU5Hr1G072920.1	15.455	38.394	31.020	0.000	1.353	98.134	10.959	13.977	19.531	149.770	62.784	106.249	56.509	71.726	64.894	25.757
HORVU6Hr1G050520.2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.145	0.000	1.348	0.000	0.085	0.065	0.000
HORVU2Hr1G053670.4	32.578	51.812	65.166	0.000	9.988	79.027	30.092	33.406	40.503	57.825	48.245	52.142	80.105	37.604	62.634	82.534
HORVU1Hr1G008810.3	124.703	172.780	67.796	0.000	6.863	235.389	17.893	24.202	40.322	5.188	2.739	3.304	170.440	13.835	47.918	186.628
HORVU7Hr1G050270.3	163.787	251.773	195.037	0.000	7.625	377.512	75.134	52.844	188.993	587.204	424.828	404.746	384.064	299.260	337.110	161.542
HORVU6Hr1G061280.1	2.446	2.131	2.093	0.000	1.537	6.318	0.292	0.419	2.243	2.599	0.923	3.776	1.479	1.225	4.977	0.221
HORVU6Hr1G026810.3	158.784	100.159	3.340	0.000	0.016	0.354	0.025	1.354	1.086	2.559	1.074	3.692	0.855	5.554	323.722	0.024
HORVU5Hr1G080420.1	0.147	0.694	0.765	0.000	0.000	7.558	0.386	0.036	0.028	18.062	10.116	18.710	5.343	10.909	4.669	2.098
HORVU7Hr1G060310.1	36.751	36.749	52.256	0.000	58.892	33.422	58.869	56.231	39.444	38.463	38.683	33.350	37.365	34.613	27.814	54.473
HORVU5Hr1G055970.1	8.250	9.373	22.101	0.000	5.726	22.906	7.226	7.511	16.362	24.191	15.243	19.269	22.911	10.817	15.116	25.052
HORVU6Hr1G067430.2	23.124	16.497	13.382	0.000	6.231	27.254	22.851	3.635	58.177	86.899	76.859	117.001	49.011	39.750	45.580	8.388
HORVU3Hr1G086270.3	9.901	4.950	13.650	0.000	10.814	13.134	14.588	22.856	15.575	16.792	18.269	16.588	17.669	14.923	10.999	13.714
HORVU5Hr1G023000.1	0.448	0.620	0.000	0.000	0.315	1.317	0.580	0.630	2.387	3.850	9.250	8.864	3.487	7.819	10.358	2.770
HORVU6Hr1G031330.2	7.361	25.649	9.114	0.000	5.831	26.607	14.771	17.556	12.112	65.397	85.644	46.985	27.076	35.399	29.288	4.162
HORVU2Hr1G055230.4	6.230	5.410	6.863	0.000	13.917	6.727	7.818	6.302	6.749	8.227	11.806	9.283	13.641	10.581	9.412	12.630
HORVU4Hr1G015350.1	1.066	1.503	1.149	0.000	0.000	0.018	0.000	0.000	1.935	0.000	0.190	0.019	1.016	0.287	4.243	0.000
HORVU7Hr1G026940.1	4.104	16.037	5.933	0.000	3.653	9.355	5.954	17.738	13.805	25.598	16.963	12.849	35.786	8.854	5.398	11.565

HORVU5Hr1G098840.2	16.495	10.496	14.728	0.000	67.579	12.383	35.897	92.127	8.308	11.539	14.556	18.488	6.481	20.820	9.483	3.149
HORVU2Hr1G110500.1	0.194	0.663	0.356	0.000	3.067	0.056	0.056	0.000	0.000	0.026	0.000	0.000	0.000	0.140	0.000	0.000
HORVU2Hr1G017470.1	3.485	3.693	18.670	0.000	0.230	0.267	1.697	4.318	11.276	6.886	27.301	5.591	28.974	0.743	1.725	27.690
HORVU5Hr1G067740.1	35.053	51.292	31.758	0.000	24.958	91.764	39.765	41.532	27.832	35.870	35.853	33.871	36.455	27.990	30.610	31.890
HORVU5Hr1G111590.2	33.123	30.729	83.335	0.000	30.250	132.291	15.399	32.295	55.521	438.717	201.047	345.189	194.401	204.532	164.808	50.149
HORVU4Hr1G043960.1	0.040	0.669	0.229	0.000	0.000	0.079	0.064	0.000	1.696	0.234	0.005	0.133	0.037	0.026	5.827	1.264
HORVU3Hr1G089830.2	118.325	89.799	289.048	0.000	39.878	157.062	18.702	12.965	199.228	106.743	3.637	41.640	288.295	53.122	181.437	549.716
HORVU2Hr1G072420.11	343.113	270.364	271.324	0.000	159.501	1837.740	262.717	975.653	284.725	415.538	350.302	523.468	165.620	372.102	306.266	291.913
HORVU4Hr1G063430.1	9.955	7.578	1.639	0.000	0.071	4.027	0.073	0.000	0.697	3.925	0.038	6.969	49.963	0.376	10.047	0.023
HORVU5Hr1G103460.2	6.458	29.776	11.277	0.000	0.574	3.562	0.382	21.231	0.761	161.759	32.728	34.437	13.746	87.748	41.292	2.207
HORVU2Hr1G096360.13	1305.897	1094.393	1133.081	0.000	131.139	1345.771	332.071	385.016	968.914	673.944	306.989	266.549	985.271	223.498	761.565	519.520
HORVU2Hr1G109330.1	0.141	0.284	0.123	0.000	0.231	0.684	0.030	0.044	0.080	0.238	0.158	0.133	0.183	0.091	0.402	1.123
HORVU3Hr1G061690.1	48.794	62.843	45.098	0.000	29.136	78.961	34.661	33.447	51.351	210.042	196.468	162.354	77.911	109.782	196.922	60.249
HORVU0Hr1G016450.1	14.357	16.710	16.052	0.000	8.369	15.613	12.984	16.628	10.717	15.443	18.637	14.167	12.470	13.130	29.153	15.141
HORVU5Hr1G105980.4	5.479	2.302	6.885	0.000	26.930	4.141	11.748	30.363	5.386	3.474	3.578	4.516	3.851	5.298	2.562	2.578
HORVU2Hr1G018340.3	23.059	41.801	24.761	0.000	17.530	160.163	14.221	0.962	17.282	158.751	85.071	161.561	23.544	66.317	13.017	3.815
HORVU2Hr1G098330.1	8.725	6.405	3.141	0.000	0.821	5.969	2.289	2.792	4.627	7.848	5.509	9.187	12.559	5.925	22.035	2.240
HORVU4Hr1G083960.3	9.297	7.680	11.406	0.000	17.361	15.883	14.857	19.413	14.752	12.873	17.803	14.617	19.110	16.650	16.564	22.046
HORVU4Hr1G048400.12	3.898	4.304	8.871	0.000	6.339	4.481	4.851	5.833	6.433	5.216	3.717	4.167	8.565	3.670	2.911	9.656
HORVU4Hr1G033760.1	4.918	9.283	14.821	0.000	4.223	11.698	8.135	15.964	6.677	10.937	9.418	8.983	16.004	8.956	6.230	23.503
HORVU7Hr1G037410.4	32.307	38.709	23.649	0.000	2.140	37.617	8.714	3.027	7.397	5.382	22.307	8.920	21.984	16.544	3.267	11.805
HORVU2Hr1G021020.4	5.865	8.743	16.482	0.000	23.953	49.791	11.115	12.231	17.568	42.308	13.567	22.734	65.941	25.827	5.471	10.384
HORVU7Hr1G031480.1	21.229	0.000	0.861	0.000	3.726	0.771	11.053	11.048	1.899	2.573	1.541	6.985	0.462	4.640	3.145	1.495
HORVU7Hr1G114250.2	243.945	66.406	62.404	0.000	38.657	110.696	135.010	41.202	27.212	31.682	65.250	120.775	40.350	44.664	81.259	13.588
HORVU1Hr1G048970.4	17.997	11.681	14.857	0.000	40.463	13.225	24.224	43.706	21.663	17.492	19.210	16.170	11.748	18.225	22.661	11.527
HORVU1Hr1G001850.3	130.689	130.618	1.422	0.128	0.032	0.002	0.022	0.108	0.144	0.026	0.018	0.040	1.164	0.055	131.255	0.300
HORVU6Hr1G081080.12	19.004	60.501	45.919	0.000	3.804	10.843	2.101	8.576	4.440	4.737	0.799	4.400	8.388	3.914	3.296	23.952
HORVU2Hr1G118320.7	41.806	38.715	60.416	0.000	45.389	129.032	43.690	22.130	69.421	60.849	49.445	48.148	38.077	33.915	18.727	49.211
HORVU3Hr1G087400.11	58.173	41.012	42.255	0.000	31.090	70.851	60.548	32.221	38.367	87.259	170.988	123.430	40.110	119.649	99.634	51.733

Key: *EMB – 4-day embryo, ROO1 – root from seedlings (10 cm shoot stage), LEA – shoot from seedling stage (10 cm shoot stage), INF1 – young developing inflorescences (5mm), INF2 – developing inflorescences (1 – 1.5 mm), NOD – developing tillers, third internode (42DAP), CAR5 – developing grain (5DAP), CAR15 – developing grain (15DAP), ETI – etiolated seedling dark con (10DAP), LEM – inflorescences lemma (42DAP), LOD – inflorescences lodicule (42DAP), PAL – dissected inflorescences, palea (42DAP), EPI – epidermal strips (28DAP), RAC – inflorescences rachis (35DAP) ROO2 – roots (28DAP), and SEN - senescing leaves (56DAP) (Colmsee et al., 2015)*

CHAPTER SEVEN
GENERAL DISCUSSION

7.1 Overview

Climate change and human activities causes agitations to the environment that encourages soil salinisation process (Ait-El-Mokhtar *et al.,* 2020). Salinisation of arable land is a global problem limiting crop production through osmotic stress and ionic toxicity which can further cause oxidative stress and nutrient imbalance (Zhu *et al.,* 2020). Therefore, cultivating crops with increased salinity stress tolerance is of principal standing in the world's food production. Salinity tolerance is quantitative in nature at the same time mutual at both genetic and physiological levels and it affects about 8% of genes expression (Tester and Davenport 2003). The first steps in achieving tolerance in crops is to understand the basic mechanisms of salt stress response. Barley shows a wide phenotypic variability to salinity stress because of its broad genetic diversity, natural ecological plasticity and stability in divergent habitats. At the same time, erosion and loss of alleles occasioned by domestication, selection and breeding programmes comparable to all other crops (Ebrahim *et al.,* 2020). Being the fourth most important crop in the world, barley is comparatively more tolerant to salt even though there is a significant reduction in growth and yield caused by salinity stress (Allel *et al.,* 2019). The adaptation to salinity stress in barley varies at different growth stages with germination and early growth phases being the most sensitive (Mwando *et al.,* 2020; Angesa *et al.,* 2017).

This study was based on the underpinning that germination is the first phase of barley seed exposure to salinity stress, the most important stage in the whole life cycle since it gives seedling vigour, determines seedling population and ultimate yield and is the most sensitive. Specifically, barley is a Mediterranean field crop that is sown in autumn in Australia after hot and dry summer which increases topsoil salinity concentration because of evapotranspiration. Hence, growing varieties that can survive high salinity during germination is paramount before rain and/or irrigation leaches the salts down and the crop becomes more tolerant at later vegetative growth periods. The broad objective of the current study was to understand the genetic tolerance at germination stage and survival of seedling after sprouting under salinity stress. This enabled a comprehensive understanding and identifications of genetic factors controlling salinity tolerance at germination and early seedling stage. The findings of this study thereafter will facilitated the designing of sound breeding programs to accelerate development of tolerant barley varieties. The above broad objective was achieved through 5 specific

objectives that are summarised in chapter one and they form contents of chapters' 3, 4, 5 and 6 of the theses.

7.2 Summary of methods and main findings

To detect the genetic loci and candidate genes linked with mechanisms of salinity tolerance in barley during germination and early seedling growth stages, we assumed a forward genetics method. Here, four barley populations comprising a diversity panel of 350 accession from across the globe, 2 double haploid (DH) populations (CM72/Gairdner and Skiff/CM72) and CM72/Gairdner/*Spartacus CL were phenotyped (Chapters 3, 4 and 5). Seed of individual germplasms were germinated in the laboratory and seedlings grown in glasshouse in nutrient solution under controlled (deionised water or nutrient solution only) and treated (salinity stress or nutrient solution with salt) at 20 ^{0}C. This allowed us to record distinct developmental traits at germination and early seedling stages of barley and the impact of salinity on them. Our finding indicated that salinity stress had a negative impact on all the reported phenotype traits.

A genome-wide association analysis (GWAS) of a Worldwide collection of 350 diverse barley accessions, was done using ~24,000 genetic markers, where 52 of them were significantly associations with salinity tolerance during germination. Nineteen loci across all 7 barley chromosomes were contained in the detected markers and 4 genes belonging to 4 family functions underlying them predicted (Chapter 3). The probable 4 genes associated with the following markers, *Piriformospora indica*-insensitive protein 2 (L1H018492689), lipase 1 (L7H212035410), protein kinase superfamily protein (C1H556900757), and heat shock protein 21 (D7H085710245) were given high confidence of possible candidates as indicated by their – log10 (P) and % R^2 values, and because they were very close to or inside the most significant markers. Literature search about the 4 identified genes indicated that they have been reported to be involved in enhancing salinity and abiotic stresses in barley and other species (Xu L. *et al.*, 2018; Baltruschat *et al.*, 2008; Vahabi *et al.*, 2016; Jogawat *et al.*, 2016; Shehzad *et al.*, 2019; Chaudhary *et al.*, 2019; Naranjo *et al.*, 2006). The finding in the chapter (3) will be a source of new genetic constituents and resources for improving barley salt tolerance at germination in future. The information will be used by breeders in developing varieties through genomic and marker-assisted selection (MAS) and it will open avenues for further validation and functional characterization of the genes (Chapter 3).

Quantitative trait locus for vigour and survival characters of barley seedlings germinating under salinity stress were mapped using 103 DH lines of CM72/Gairdner population with 350 DArT

and 84 SSR markers (Chapter 4). The phenotypic response of seedling survival traits under salinity stress were further validated using a selected diverse panel of 85 barley germplasms that ware previous selected from a diverse accession of 350 barley lines used in GWAS analysis of chapter 3. Linkage map analysis mapped 13 QTLs in the DH population on chromosomes 1H, 3H and 4H associated with seedling survival traits under salinity stress. The genetic hotspots regions likely to contain candidate genes on 1H (1) and 3H (2) were detected for more than one seedling salinity survival trait. The regions will form important location on barley genome that can be further validated and fine mapped leading to identification of candidate genes (Chapter 4).

The study further validated and fine – mapped a major QTL for salinity tolerance during germination that was previously mapped on the short arm of chromosome 2H with CM72/Gairdner doubled haploid population (Angesa *et al.,* 2017). This was done using DH populations of (CM72/Gairdner and Skiff/CM72), F_2 and F_3 of CM72/Gairdner/*Spartacus CL and newly designed InDel markers. Phenotype-genotype comparative analysis of the recombinant lines allowed for the narrowing down of the region to ~ 0.341 Mb containing nine candidate genes belonging to six functional families (Chapter 5). Real-time qPCR enabled us to predict 2 Receptors'-like protein kinase 4 (RLPK4) HORVU2Hr1G111760.1 and HORVU2Hr1G111780.3 as candidates' genes for enhanced germination under salinity stress because of their upregulated expression in CM72 (salt-tolerance variety). Full-length sequence analysis of the 2 RLPK4 identified several insertion/deletion polymorphisms within the 3rd exon of the genes between CM72 and Gairdner, which may be associated with the different tolerance. At the same time, research finding by Nanda *et al.,* (2019) recorded a combination of 2 receptor like kinases that are responsive to salt and osmotic stress in *Arabidopsis* seeds working together synergistically to regulate the timing of germination. To facilitate selection of the locus, 2 molecular markers were designed from the InDels (one of the InDel markers was inside the first receptor-like protein kinase 4 family gene) and validated using 265 diverse barley accessions. The designed markers will be used for pyramiding of chromosome 2H locus for salt-tolerant at germination in barley varieties. While the identified genes will form a foundation for further validation and understanding of the mechanisms by which salinity tolerance at germination is enhanced. The finding in this chapter and further studies will facilitate genetic improvement of plant for saline soils (Chapter 5).

A Lliterature search was done and found 7 genes to have been functionally characterised for salinity stress tolerance in barley at germination stage and 21 QTLs so far reported. The diversity of the genes was explored in 40 different plant species and three of them; dehydration-responsive element-binding (DREB) protein, somatic embryogenesis receptor-like kinase and aquaporin genes, were deliberated. The three gene families displayed great multiplicity in most plant species, however, *DREB* gene family was additional diverse in barley. At the same time, 63 candidate genes that have been functionally characterised for salinity tolerance during germination in *Arabidopsis*, soybean, maize, wheat, and rice were blasted and mapped on barley reference genome to identify their homologs. Sixty-five homolog genes were found in barley genome with a percentage match ranging from 100% (amid wheat gene *TaPLDα* (phospholipase D) and barley homolog gene HORVU1Hr1G048970.4 (phospholipase D P2) to 30% (between rice gene *OsOPT10* (oligopeptide transporter family homolog) and barley's HORVU6Hr1G067430.2 (oligopeptide transporter 4). The reported homolog genes will be a basis for validation and functional characterization in barley and other transgenic plants to enhance development of varieties with improved salt tolerance at germination and better field performance (Chapter 6).

7.3 References

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CHAPTER EIGHT

CONCLUDING REMARKS AND RECOMMENDATIONS

The findings in this thesis has demonstrated important variations in salinity stress tolerance among different barley genotypes at germination and early seedling stages. Salinity stress negatively affected germination and early seedling traits dissimilarly in different barley accessions. The identifications of several QTLs for salinity stress tolerance on all chromosomes of barley genome confirms the polygenic nature of the trait in the two developmental stages. Whereas, the location of the QTLs in different regions on barley genome for salinity tolerance during germination and those of early seedling survival traits reinforces the fact that salinity stress tolerance at the two phases is controlled by diverse genes. The mapping of new QTLs in this study, will form a basis for determining the genetic foundation of salinity tolerance in unmapped barley varieties through physiological and molecular methods. The identified or proposed possible candidate genes for salinity tolerance at germination and early seedling stage with specific markers associated with them will allow for further validation and functional characterisation. Nevertheless, this is just the tip of the iceberg, we need to fully comprehend the physiological, biochemical and molecular mechanisms employed by barley to survive salt stress at the two stages. At the same time, to breed new barley varieties that can endure climate change and tolerate harsh salinity conditions at germination and early seedling phases there is need to do several additional studies. Overexpression, knockdown and cloning of the reported genes among others will lead to understanding of the mechanism(s) underlying salinity tolerance in barley at the two phases. Most importantly, the identified markers will be utilised by the breeders to develop barley lines carrying the important loci for salinity tolerance both at germination and early seedling survival stage. This will eventually lead to increased barley yields in saline prone areas of the world because of the released new varieties that can grow in soils with increased salinity.