Defining Transcriptional Networks Associated with Plant Salinity Tolerance

A thesis submitted in fulfilment of the requirement for the degree of

Doctor of Philosophy

By

Shanika Lakmini Amarasinghe

BSc Bioinformatics (Hons)

THE UNIVERSITY of ADELAIDE

School of Agriculture, Food & Wine

Faculty of Sciences

The University of Adelaide

March, 2018

Table of Contents

List of Figures

Chapter 2

Chapter 3

Chapter 5

Chapter 6

List of Tables

CHAPTER 2

CHAPTER 3

CHAPTER 5

List of Abbreviations

ABA	abscisic acid
ACC	1-Aminocyclopropane-1-carboxylic acid
ACS	1-aminocyclopropane-1-carboxylic acid synthases
AF	Adjacency function
AIC	Akaike Information Criterion
AIC2	Akaike Information Criterion 2
ALI	Activation loop insertion
APX	ascorbate peroxidase
AtCIPK16	*Arabidopsis thaliana* Calcineurin B-Like Protein Interacting Protein Kinase 16
AtCIPK25	*Arabidopsis thaliana* CIPK25
AtCIPK5	*Arabidopsis thaliana* CIPK5
BADH	*Betaine aldehyde dehydrogenase*
BIC	Bayesian Information Criterion
BLAST	Basic Local Alignment Sequence Toolkit
Ca^{2+}	Calcium ion
CaMLs	calmodulin-like proteins
CaMs	calmodulins
Cas9	the CRISPR associated protein 9
CAT	catalase
CAX	Cation exchangers
CBL	calcineurin B-like
cDNA	Complementary DNA
CDPKs	calcium-dependent protein kinases
CDS	Coding sequence
CICR	calcium-induced calcium releas
CLC	Chloride Channel
CPM	counts per million
CRISPR	Clustered regularly interspaced short palindromic repeats
DAVID	Database for Annotation, Visualization and Integrated Discovery
DEG	Differentially expressed genes
DEG_{INT}	DEG with a significant transgene dependent salt responsive effect

DEG_{SN}	DEG with significant effect of salt response in nulls
DEG_{ST}	DEG with significant effect of salt response in transgenics
DEG_{TC}	DEG with a significant transgene effect in controls
DEG_{TS}	DEG with significant transgene effect in salt
DREB	Dehydration-responsive element-binding
dTTP	Deoxythymidine triphosphate
dUTP	Deoxyuridine Triphosphate
ERF6	Ethylene response factor6
FDR	False discovery rate
FISL/NAF	NAF domain
FPKM	Fragments per kilobase per million sequenced reads
FV	fast-activating vacuolar
GATK	Genome Analysis Toolkit
GFP	Green fluorescent protein
GO	Gene ontology
GORK	guard cell outward rectifying K^+ channel
GPX	glutathione peroxidase
GRDC	Grain research development corporation
GST	glutathione-S-transferase
GTF	gene-transfer format
GWAS	Genome-Wide Association Studies
HAK	High-Affinity K^+ transporter
HKT	High Affinity K^+ transporter
InDel	Insertion/Deletion
JA	Jasmonic acid
KAT1	K^+ transporter of *Arabidopsis thaliana*
KEGG	Kyoto Encyclopaedia of Genes and Genomes
MAPK	Mitogen Activated Protein Kinase
MAS	Marker assisted selection
MDS	Multi-dimensional scaling
ML	Maximum Likelihood
MM	Modular membership
MP	Maximum Parsimony
MSA	Multiple sequence alignment

mt1D	*Mannitol-1-phosphate dehydrogenase*
NHX	*Na^+/H^+ EXCHANGER*
NJ	Neighbor-Joining
NLS	Nuclear localisation signal
NPF	Nitrate transporter 1/Peptide Transporter family
NVT	National variety trials
OGTR	Office of gene technology regulator
P5CS	*Δ1-pyrroline-5-carboxylate synthetase*
PCA	Principle Component Analysis
PCR	Polymerase chain reaction
POD	peroxidase
PP2C	2C-type protein phosphatase
PPi	Protein–phosphatase interaction domain
QTL	Quantitative trait locus
ROS	Reactive oxygen species
RPKM	Reads per kilobase of transcript per million
RT	Reverse transcriptase
SBH	single-directional best hit
SBS	Sequencing by synthesis
SD	Segmental duplication
SKOR	STELAR K^+ outward rectifier
SLAC1	slow type anion channel-associated 1
SLAH3	SLAC1 homologue
SNF1	Sucrose non-fermenting 1
SNPs	Single nucleotide polymorphisms
SnRK3	SNF1-related kinases group 3
SOD	superoxide dismutase
SOS	Salt overly Sensitive
SV	slow-activating vacuolar
TBR	*TRICHOME BIREFRINGENCE*
TMM	Trimmed mean of M-values
TPM	Transcripts per Kilobase million
TPP	*Trehalose-6-phosphate phosphatase*
TPS	*Trehalose-6-phosphate synthase*

UDGase	Uracil-DNA Glycosylase
UPGMA	Unweighted Pairwise Group of Multiple Alignments
UTR	Untranslated region
USAID	United States Agency for International Development
WGCNA	Weighted gene co-expression network analysis
WRA	Weed risk assessment
Y2H	Yeast 2 hybrid assay

Abstract

Salinity is a major issue for the sustainability of agriculture worldwide. Salinity causes an initial hyperosmotic stress and subsequently, secondary nutritional imbalance and oxidative stress through ion toxicity. Many studies focus on identifying genes and the molecular mechanisms involved in salinity tolerance. The identification of such genes may then be used in the development of more salt tolerant crops required for a sustainable global food production.

Calcineurin B-like protein interacting protein kinases (CIPKs) are key regulators of pre-transcriptional and post-translational responses to abiotic stress. *Arabidopsis thaliana CIPK16 (AtCIPK16*) was identified from a forward genetic screen as a candidate gene that mediates lower shoot salt accumulation and improves salinity tolerance in Arabidopsis and transgenic barley. However, relatively little is known about the pathways in which CIPK16s operate to affect salinity tolerance and even about the presence of orthologues in cereals.

A transcriptomic study was conducted using *Arabidopsis thaliana* plants subjected to salt stress. The experiment included overexpressing *AtCIPK16* and null transgenic plants that were salt stressed or controls. Our analysis characterizes the transcriptional landscape of *AtCIPK16* overexpression dependent salt responsiveness in Arabidopsis. These transgene-dependent salt responsive genes suggest an involvement of transcription factors and phytohormones, such as ethylene, jasmonic acid and auxin in downstream signaling pathways. Whether these transcription factors and possible hormone changes have an impact on the plants' physiological aspect needs to be experimentally determined.

Although enhanced salt tolerance has been demonstrated in transgenic barley plants overexpressing *AtCIPK16*, the presence of a CIPK16 orthologue in barley has not been established. The second part of the project therefore was involved with a molecular phylogenetic analysis of CIPK16 homologues in terrestrial plant species. We mined genome sequence databases, including monocot and dicot species, for CIPK16 homologues. The subsequent phylogenetic analysis revealed a clade containing AtCIPK16 along with two segmentally duplicated CIPKs: AtCIPK5 and AtCIPK25. We found no evidence for an AtCIPK16 orthologue in any monocots but instead found homologues which formed a group basal to the entire CIPK16, 5 and 25 clade. Our analyses also revealed that CIPK16s contain a unique inDel (MMPEGLGGRRG) and a putative nuclear localization signal (PPTKKKKKD). Whether these synapomorphic characters have a biological function will require further experimental validation.

We investigated the transcriptome of a subset of six barley cultivars with varying Na^+ accumulation in the leaf blade and sheath using the RNA-Seq data generated for the leaf blade, leaf sheath and root tissues from plants grown in saline conditions. Based on prior knowledge we specifically investigated genes involved in sodium transport and salt response and examined their expression and genetic variation (SNPs and indels) across the 6 accessions. Our results showed that allelic variations in *HvHKT1;5* may be one of the crucial factors in determining the level of Na^+ in the shoots of barley. We hypothesise that for high shoot Na^+ accumulating cultivars such as Alexis, Commander and Maritime genes such as *HvNHXes* (e.g. *HvNHX4*) may play a role in dealing with high levels of Na^+, through sequestrating Na^+ into the vacuole or K^+ homeostasis.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

I acknowledge that copyright of published works contained within this thesis resides with the copyright holder(s) of those works.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

S. L. AMARASINGHE

Signature: ______________________________ Date: 09/03/2018

List of Publications and Awards

- Amarasinghe S, Watson-Haigh NS, Gilliham M, Roy S, Baumann U. The evolutionary origin of CIPK16: A gene involved in enhanced salt tolerance. Mol. Phylogenet. Evol. 2016 Jul;100:135–47
- Trave Grant to attend BioInfoSummer 2014, Monash University, Melbourne, Australia (1st Dec – 5th Dec 2014)

Acknowledgements

Even though it is my name on the front cover, there are so many people behind the scenes that supported me to make it to the end. I'm truly in debt to all my supervisors, Dr. Ute Baumann, Dr. Nathan S. Watson-Haigh, Prof Stuart Roy and Prof Matthew Gilliham.

Ute, you are one of the brilliant professionals I have ever encountered. Your love to do science and your high standards not only directed my thoughts to see things from every angle, but also moulded my mindset to be a real scientist who appreciates real science. Not only that, you are kind and patient, most knowledgeable and confident. Sharing the same gender, seeing what you have achieved inspire me to be a better person every day. More than a supervisor, you have been like a parent to me.

Nathan, oh, I don't think I will ever meet such a bright person like you. There were times that I needed to give it a thought in order to go to the extent you have gone with your thinking. If you didn't say I wouldn't have believed that I'm your first PhD student. Your craving to do and know things perfectly, was challenging to me at times. But when I turn back, I realize your influence and support has been a major force in many of my achievements in this PhD. To that I am truly grateful. Your attitude is very contagious, and it is definitely worth catching.

Stu, honestly, you are a great teacher. You are so up-to-date, and your memory is outstanding. Yet, you are so humble and kind, and always look at things from a student's perspective before making decisions. I am truly blessed to have worked with such an outstanding academic this early in my career. The support you have given, and the critical thinking ability you possess is remarkable. On top of that the way that you could pass on that knowledge and wisdom to the younger generation with so much ease is incredible.

Matt, I admire how you want to strive for success by challenging yourself every day. It is amazing how persistent you are, and being this high in the academic ladder, and juggling so many things at once makes me wonder whether you are a superhuman. Your critical analysis of my work has lifted it to a level of impeccable quality. I thank you very much for that.

The bioinformatics group at the Waite campus, small but superior at their research is where I spent my whole PhD life. Niharika, Rad, Elena, Mario, Juan-Carlos and Paul have been nothing but great friends and great support throughout this journey. I have worked with several teams and this team is so far one of the best I could have wished for. You all have critically evaluated my work, provided various support with programming and even shared your codes and resources with me.

I would like to immensely thank the former Australian Centre for Plant Functional Genomics (ACPFG) for my PhD scholarship and the University of Adelaide for the full fee waiver-off and continuing my PhD scholarship. Without them I would not have been writing this acknowledgement to begin with. I also would like to thank our former education manager Dr. Monica Ogierman who has been a bliss even though if it was for a short period of time, who has always been a great personality and a great strength from day one. I always will remember the support given by Mrs. Ruth Harris, as our English teacher, in the first year of my PhD. Her advice and support from a native speaker's perspective was invaluable asset all our PhD students miss greatly even today. I have been supported for developing my English by Dr. Ronald Smernik with writing groups and personal communications and Dr Margret Cargill with her free workshops, from which I learnt so much. I would like to remember Dr. Darren Plett, my postgraduate coordinator Prof Kenneth Chalmers, my external advisor Dr. Andreas Schreiber and Mr. Lachlan Tailby who has been supporting me on various occasions.

I also want to remind my friends whom gave me moral support and sound advice when I was faced with tough times. Most importantly, they reminded me of the work-life balance and helped me to have fun along the way. Without them the life would have been so monotonous.

I could not thank enough or repay with my whole life for everything my parents have done for me and my new family. My mom is not only this superwoman who could manage everything, but also such a kind and noble person who would put aside all her priorities to help me complete my studies. She also was my nit-picky proof-reader of this thesis as well as my life. My dad has always been my strength and sacrificed his time and effort to be with us when we most needed him. My dear mom and dad, I worship you with my forehead on your feet.

Last but not the least, I would like to remind my husband Ashan and my dear son Chanu, who has given a new meaning to my life. My husband encouraged me to apply for the PhD knowing that if I am successful in the application, his whole life would turn upside down. But still, he has always been there with me through thick and thin, motivating me when I was down, caring when I was tired and creating beautiful figures for the manuscripts when I struggled. I don't know what I did when I didn't have you both in my life.

“Would you tell me, please, which way I ought to go from here?"

"That depends a good deal on where you want to get to," said the Cat.

"I don't much care where-" said Alice.

"Then it doesn't matter which way you go," said the Cat.

"- so long as I get somewhere," Alice added as an explanation.

"Oh, you're sure to do that," said the Cat, "if you only walk long enough.”

-Lewis Carroll, Alice in Wonderland

Chapter 1 General Introduction

Structure of this thesis

This thesis is presented as three papers. One manuscript has been published in *Molecular Phylogenetics and Evolution* (Amarasinghe et al., 2016), two are drafted as manuscripts for publication. In **Chapter 1 (Introduction),** a general introduction sets forth the context of the thesis, briefly identifying the research gaps and stating the specific objectives and techniques used to achieve the objectives of this research. **Chapter 2 (Literature Review)** aims to provide a comprehensive literature review setting the background to the research topic, pointing out the research gaps and giving an overview about the techniques used in the thesis research. **Chapter 3 (Molecular Components of the AtCIPK16 Mediated Salt Stress Response)** is prepared in a manuscript format that discusses the molecular mechanisms underlying the *AtCIPK16* conferred salinity tolerance in Arabidopsis. **Chapter 4 (The evolutionary origin of CIPK16: A gene involved in enhanced salt tolerance)** is a report that discusses the molecular evolutionary study of a protein kinase gene from Arabidopsis (*AtCIPK16*) that is previously identified to be linked to enhanced salt tolerance in Arabidopsis and barley (Roy et al. 2013). This chapter has already been published (Amarasinghe et al. 2016). **Chapter 5 (Evaluation of the molecular basis of varying Na^+ accumulation in barley cultivars under salt stress)** is prepared in a manuscript format that discusses the genetic variations and similarities amongst six barley cultivars with varying leaf Na^+ accumulation levels. In addition to the manuscript, chapters 3, 4 and 5 includes a link page that serves to connect the chapter to the broader hypotheses addressed by this thesis. **Chapter 6 (General discussion),** as the name implies discusses the findings of this thesis in “one picture” and covers the broader significance of the research reported in this thesis, while identifying drawbacks and suggests improvements for future work. To avoid addition of large data files generated in this study to the thesis, the supplementary materials for each chapter, are made available through FigShare. A link to each file set is given following the description of the supplementary materials as well as in the **Appendix**. This thesis is in agreement with the specification of thesis of the Adelaide Graduate Centre Higher Degree by Research, University of Adelaide, South Australia. This “thesis by publication” format might show some unavoidable repetition, especially in the Introduction and Materials and Methods sections, but this has been kept to a minimum.

Context of this thesis

Salinity is an abiotic stress that causes agriculture in Australia and all around the world substantial losses every year (Deinlein et al. 2014; Munns and Tester 2008). Finding solutions to mitigate the negative effects of high salinity on crops therefore, is an important requirement for sustainability of world food production. Salinity poses initial hyperosmotic stress followed by secondary nutritional imbalance and oxidative stress through ion toxicity (Munns and Tester 2008; Zhu 2001). Research endeavours to identify underlying molecular mechanisms that lead to salt tolerance with an ultimate goal of developing salt tolerant crops (Munns and Gilliham 2015)

Comprehensive studies on gene expression, gene regulatory networks and allelic variants could provide us an understanding of the underlying molecular elements and their mechanisms associated with salinity tolerance in cereals. It also may lay the foundation for advanced experiments such as gene editing or screening for the genes which boosts tissue tolerance, salt exclusion and activated salt tolerance in cereals (Ashraf and Wu 1994). The information generated by these efforts therefore, can be utilized in designing effective breeding strategies for salt tolerance (Munns and Tester 2008; Negrão et al. 2017).

Research Objectives

The overall scientific goal of this thesis was to understand several aspects of salinity tolerance mechanisms in plants such as Arabidopsis and barley through bioinformatics techniques such as molecular phylogenetics, transcriptomics, network and variant analysis. The specific objectives of this thesis were to a) identify the downstream regulatory network controlled by *AtCIPK16* in *Arabidopsis thaliana* b) perform a comprehensive evolutionary study of *CIPK16s* in grasses and c) evaluate salt tolerance mechanisms of *Hordeum vulgare* L. (barley).

References

Amarasinghe, S., Watson-Haigh, N.S., Gilliham, M., Roy, S., Baumann, U., 2016. The evolutionary origin of CIPK16: A gene involved in enhanced salt tolerance. Mol. Phylogenet. Evol. 100, 135–147. https://doi.org/10.1016/j.ympev.2016.03.031

Ashraf, M., Wu, L., 1994. Breeding for Salinity Tolerance in Plants. Crit. Rev. Plant Sci. 13, 17–42. https://doi.org/10.1080/07352689409701906

Deinlein, U., Stephan, A.B., Horie, T., Luo, W., Xu, G., Schroeder, J.I., 2014. Plant salt-tolerance mechanisms. Trends Plant Sci. 19, 371–379. https://doi.org/10.1016/j.tplants.2014.02.001

Munns, R., Gilliham, M., 2015. Salinity tolerance of crops – what is the cost? New Phytol. 208, 668–673. https://doi.org/10.1111/nph.13519

Munns, R., Tester, M., 2008. Mechanisms of Salinity Tolerance. Annu. Rev. Plant Biol. 59, 651–681. https://doi.org/10.1146/annurev.arplant.59.032607.092911

Negrão, S., Schmöckel, S.M., Tester, M., 2017. Evaluating physiological responses of plants to salinity stress. Ann. Bot. 119, 1–11. https://doi.org/10.1093/aob/mcw191

Roy, S.J., Huang, W., Wang, X.J., Evrard, A., Schmöckel, S.M., Zafar, Z.U., Tester, M., 2013. A novel protein kinase involved in Na+ exclusion revealed from positional cloning. Plant Cell Environ. 36, 553–568. https://doi.org/10.1111/j.1365-3040.2012.02595.x

Zhu, J.-K., 2001. Plant salt tolerance. Trends Plant Sci. 6, 66–71. https://doi.org/10.1016/S1360-1385(00)01838-0

Chapter 2 Literature Review

Plant Stress

Factors that negatively affect the growth and development of plants are classified as stresses. Stress can affect photosynthesis, protein synthesis, energy and lipid metabolism in plants and reduce their growth and productivity (Ashraf and Wu 1994; Balmer et al. 2013; Deng et al. 2013a; Ma 2004).

Stresses that plants experiences can be divided into two categories; biotic and abiotic. Biotic stress is caused by living organisms such as bacteria, viruses, fungal and herbivorous pests (Ma, 2004). Abiotic stress is caused by non-living factors of the environment that include extreme temperatures, low water availability or waterlogging, mineral deficiencies or toxicities, and high soil salt concentrations (Gorji et al., 2013).

High Salinity as a Major Abiotic Stress for Crops

Many crops are already grown in suboptimal conditions which prevent them from attaining their full yield potential as a result of exposure to environmental stress such as high salinity that can reduce their production (Rengasamy, 2010). It has been estimated that out of the 230 million hectares (ha) of land farmed by irrigated agriculture, 44 million ha are currently affected by salt (Munns and Tester, 2008; Rengasamy, 2006). One of the two main reasons for the reduction in growth and development of crops under salt stress is stomatal closure, which reduces carbon dioxide uptake, and inhibits cell division (Zhu, 2001). The second reason is the reduction of photosynthesis owing to reduced tillering and premature leaf senescence resulting from disrupted cellular metabolic processes (Chinnusamy et al., 2004; Roy et al., 2014). The inability for crops to reach their full potential will reduce the global food production and also the gross income of farmers around the world (Munns and Gilliham, 2015). In Australia, even though there is an increase in total wheat production, mainly due to increased extent of land brought under farming and introduction of cultivars with improved optimal yield (Richards et al., 2014; Robertson et al., 2016), since 1990, the majority of farms yielded less than 2 tonnes/ha due to environmental constraints like salinity and drought (Gilliham et al., 2017). Engineering plants to improve stress resilience therefore is essential in the development of sustainable agriculture for the future (Gilliham et al., 2017; Sofia et al., 2013; Tester and Langridge, 2010).

Under high soil salinity plants initially suffer osmotic stress followed by salt-specific ionic stress (Brini et al., 2012; de Oliveira et al., 2013; Munns, 2005). Osmotic stress is observed immediately after a plant is exposed to salt and it continues throughout the exposure (Carillo et al., 2011). Plants manifest rapid onset of responses in the ‘osmotic phase’ (immediately after exposure to salt) and it is a result of the effect of salt on water potential and not due to accumulation of Na^+/Cl^- ions in the shoot and hence described as "shoot salt accumulation independent effect" (Roy et al., 2014).

Prolonged exposure to salt stress makes plants experience salt-specific ionic stress that can occur through several days to weeks; that is known as the "ionic phase" (Carillo et al., 2011; Roy et al., 2014; Zhu, 2003). Severe ion toxicity takes place in plants if Na^+ concentrations in the cytosol are higher than 40 mM (Munns and Tester, 2008). This leads to early senescence of mature leaves, resulting in reduced photosynthetic capability and lower growth rates (Cramer and Nowak, 1992; Munns, 2005). Leaf senescence is a result of a disruption of a number of key metabolic processes including excess Na^+ disturbing protein synthesis and enzymatic actions (Hasegawa et al., 2000); nutrient imbalances caused by salt-mineral interactions (e.g. calcium (Ehret et al., 1990; Maas and Grieve, 1987), iron (Abbas et al., 2015; Yousfi et al., 2007), nitrate (Zheng et al., 2013)) as well as accumulation of Na^+ in the cell wall which results in desiccating the cell (Munns, 2005). Although Cl^- toxicity cannot be easily distinguishable from Na^+ toxicity there are evidence to believe that negative effect of Cl^- adds onto or interacts with Na^+ toxicity and causes leaf chlorophyll decline, leaf pH changes etc. (Li et al., 2017).

Mechanisms of Plant Salinity Tolerance

The ability of a plant to maintain growth in the initial osmotic stress (i.e. shoot ion independent stress) phase is still unknown. Plants exposed to salinity immediately show the activation of long distance signals in response to salt, but these are transient and will activate both osmotic tolerance and ionic tolerance mechanisms. (Batistič and Kudla, 2010; Choi et al., 2014; Gilroy et al., 2014; Mittler et al., 2011; Schmöckel et al., 2015). Only a few genes have been suggested as being important in maintaining plant growth (Al-Tamimi et al., 2016). Plants have two, not mutually exclusive, mechanisms to enhance shoot ion tolerance; (a) shoot ion exclusion, by using transport processes which minimise Na^+ and Cl^- accumulation in the shoot by either retaining salt at the base of the stem or root, there by directing excess salt away from immature leaves towards mature ones which are more tolerant (Maathuis, 2014; Munns, 2006; Munns and Tester, 2008; Shabala, 2013; Teakle and Tyerman, 2010); and (b) tissue tolerance, by accumulating toxic ions in cellular compartments, such as the vacuole or in intracellular spaces (Carillo et al., 2011; Munns et al., 2016; Munns and Tester, 2008; Roy et al., 2014).

Known Molecular Components of Plant Salt Tolerance Mechanisms

As there are many responses elicited within a plant to initially receive the "salt signal" and then ameliorate the toxic effects of salinity, the underlying molecular components of these responses are extremely diverse. These components are easy to be described under following categories:

(a) salt sensing and signaling (b) shoot ion independent tolerance and (c) shoot ion dependent tolerance (Figure 1).

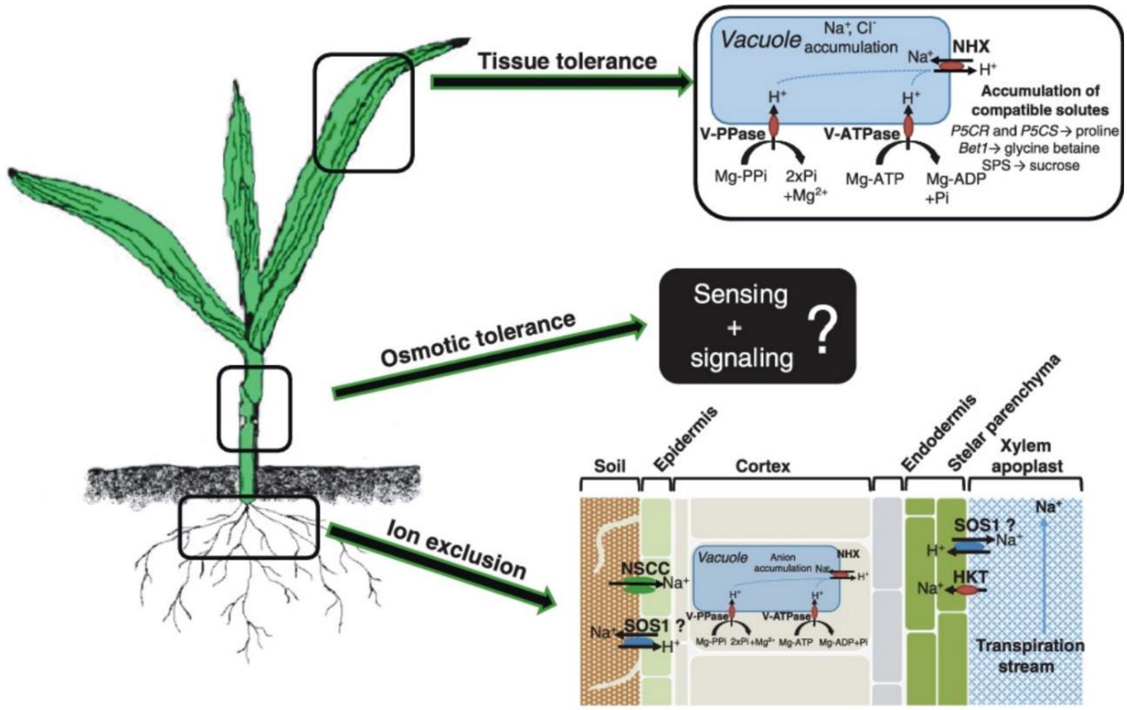


Figure 1 The three main mechanisms of salinity tolerance in a crop plant

Tissue tolerance, where high salt concentrations are found in leaves but are compartmentalized at the cellular and intracellular level (especially in the vacuole), a process involving ion transporters, proton pumps and synthesis of compatible solutes. Osmotic tolerance, which is related to minimizing the effects on the reduction of shoot growth, and may be related to as yet unknown sensing and signaling mechanisms. Ion exclusion, where Na^+ and Cl^- transport processes, predominantly in roots, prevent the accumulation of toxic concentrations of Na^+ and Cl^- within leaves. Mechanisms may include retrieval of Na^+ from the xylem, compartmentation of ions in vacuoles of cortical cells and/or efflux of ions back to the soil (reproduced from Roy et. al., 2014).

Salt Sensing and Signaling

It has been suggested that the rapid onset of salt stress responses is at least partly governed by initial salt sensing and long distance signaling (Gilroy et al., 2014; Maischak et al., 2010; Mittler et al., 2011; Roy et al., 2014). These signals have a rapid onset and last for a short time (second to minutes). The genes involved in these processes, therefore could be initial salt perceiving and sensing molecules, transcription factors, hormone related genes, MAPK pathway genes etc.

Ca^{2+} acts as a secondary messenger to transduce cellular responses to external stimuli (Shabala et al., 2015). In hyperosmotic and salt stress a single or biphasic Ca^{2+} elevation (20-60 s) takes place caused by the release of Ca^{2+} from the vacuole and extracellular stores (Pareek et al., 2010). Reduced hyperosmolality-induced calcium increase 1 (OSCA1) from Arabidopsis is a putative hyperosmotic sensor that changes the cytosolic free Ca^{2+} levels (Yuan et al., 2014). However, how OSCA1 detects osmotic stress is yet unclear (Zhu, 2016). Additionally, several other Ca^{2+} permeable channels such as cyclic nucleotide gated channels (CNGCs) and glutamate-receptor like channels (GLRs) may be involved in spawning stress related cytosolic Ca^{2+} signals (Swarbreck et al., 2013).

These calcium signals are perceived by sensor molecules containing helix-loop-helix EF-hand motifs that can bind to Ca^{2+} ions with high affinity (Kudla et al., 2010; Tuteja and Mahajan, 2007). These highly conserved EF-hands mostly exist in pairs and are 29 amino acids long. The loop region binds the Ca^{2+} ions (Tuteja and Mahajan, 2007). Sensor molecules with EF hand motifs fall into four major categories, of which calcineurin B-like proteins (CBL) is one (He et al., 2013). The other three are calcium-dependent protein kinases (CDPKs), calmodulins (CaMs), and calmodulin-like proteins (CAMLs) (Yu et al., 2007). CaMLs possess 1 to 6 EF-hand motifs (Luan et al., 2002). CaMs on the other hand are highly conserved, acidic small molecules with 2 EF-hand motifs (Luan et al., 2002; Zielinski, 1998). CDPKs contain four EF-hand motifs and a serine-threonine kinase domain that gets activated when the Ca^{2+} is bound and releases the protein from its auto inhibitory status (Cheng et al., 2002; Ludwig et al., 2004).

CBLs are usually 23-26 kD in size and contain four EF-hand motifs (Batistič and Kudla, 2010; Kolukisaoglu et al., 2004). CBL proteins can be divided into two groups according to the N-terminal domains: CBL proteins with a shorter (27-32 amino acid) N-terminal domain and CBL proteins with a longer (41-43 amino acid) N-terminal domain (Batistič and Kudla, 2009). Lipid modification by myristoylation and S-acylation by stearate and palmitate has been experimentally confirmed for CBL proteins. These modifications are considered to be important for determining the localization of the CBL-CIPK complexes (Batistic et al., 2008; Sanchez-Barrena et al., 2013). All CBL proteins share a reasonably well conserved central region encompassing four EF-hand Ca^{2+} binding sites that are arranged in strict spacing within the protein (Kolukisaoglu et al., 2004).

Selective interactions of CBL proteins with CIPKs (CBL Interacting Protein Kinases) are key for localization of CIPKs and activation of downstream target proteins (Batistic et al., 2008; Sanchez-Barrena et al., 2007). The CIPKs have been catalogued as SNF1 (Sucrose non-fermenting 1)-related kinases group 3 (SnRK3) proteins, according to their structural features and evolutionary associations (Hrabak et al., 2003). The general structure of all CIPK-type kinases includes a conserved N-terminal kinase domain, and a variable junction domain, which separates it from a unique C-terminal regulatory domain. While much of the regulatory domain sequence is divergent in these proteins, there exists a well conserved FISL/NAF domain, mediating the interaction with CBLs (Albrecht et al., 2001). Additionally, a conserved domain which mediates CIPK interaction with the 2C-type protein phosphatase (PP2C) group, via phosphorylation, has been discovered within the C-terminus of these kinases namely, protein–phosphatase interaction (PPI) domain (Ohta et al., 2003).

Most CBLs and CIPKs can interact with multiple CIPKs and CBLs, respectively (Batistic and Kudla, 2004; Batistič and Kudla, 2010; Gong et al., 2002; Guo et al., 2001). One such example is that, both *AtCBL4* and *AtCBL10* code for Calcineurin B-like (CBL) proteins that can interact with SOS2/AtCIPK24 to activate downstream targets (Qiu et al., 2002). This is a case where SOS2 shows alternative complex formation with either AtCBL4 or AtCBL10 because expression of *AtCBL4* is limited to root tissue and *AtCBL10* to shoot tissue (Guo et al., 2001). This concept of alternative complex formation by AtCIPK24 with either AtCBL4 or AtCBL10 makes it a dual functioning kinase. i.e. AtCBL4–AtCIPK24 complexes mediate Na^+ extrusion via the regulation of the H^+/Na^+ antiporter SOS1 at the plasma membrane, the formation of AtCBL10–AtCIPK24 is likely to result in Na^+ sequestration into the vacuole by regulation of unknown targets (Weinl and Kudla, 2009).

There are other CBLs and CIPKs known to be involved in plant salt response. A CIPK from Arabidopsis (*AtCIPK16*) identified by Roy et al (2013) is one such example. Overexpression of *AtCIPK16* in Arabidopsis and barley has conferred salt tolerance by Na^+ exclusion from shoots (Roy et al., 2013). There are many more CBLs and CIPKs identified to be involved in the regulation of ions such as Na^+ and K^+ in salt stress as shown in examples from Table 1.

Table 1 Examples of identified CBL proteins interacting with CIPKs in salinity tolerance At: Arabidopsis thaliana, Bd: Brachypodium distachyon, Bn:Brassica napus, Ca: Cicer arietinum, Gh: , Gossypium hirsutum* L. *Hb: Hordeum brevisubulatum, Md: Malus domestica, Nt: Nicotina tobaccum, Os:Oryza sativa Pt: Populus trichocarpa, Pe: Populus euphratica, Si: Setaria italica, Sl: Solanum lycopersicum, Ta: Triticum aestivum, Vv: Vitis Vinifera, Zm:Zea mays

CIPKs	Interacting CBLs If known	Reference for the interaction	Proposed function/s	Transgene Recipient (if applicable)	Reference for the proposed function/s

AtCIPK1	AtCBL1 AtCBL9	D'Angelo et al., 2006	Represents a convergence point for ABA-dependent and ABA-independent stress responses. Involved in salt stress	-	D'Angelo et al., 2006
AtCIPK3	AtCBL9	Pandey et al., 2008	Regulation of ABA response in seed germination. Involved in salt stress	-	Pandey et al., 2008
AtCIPK6	*Not known*	-	Required for development. Involved in salt stress and ABA.	-	Chen et al., 2013
AtCIPK9	AtCBL3	Liu et al., 2013	Regulates K^+ homeostasis under low-K^+ stress in Arabidopsis.	-	Liu et al., 2013
AtCIPK14	AtCBL2	Akaboshi et al., 2008	Responsible for the control of the salt and ABA responses.	-	Qin et al. 2008
AtCIPK16	AtCBL3 AtCBL4 AtCBL5	Lee et al., 2007	Overexpression in Arabidopsis and barley leads to enhanced salinity tolerance associated with reduced Na^+ accumulation in shoots	*Arabidopsis thaliana* (Arabidopsis) *Hordeum vulgare* L.(Barley)	Roy et al., 2013
AtCIPK21	AtCBL2 AtCBL3	Pandey et al., 2015	Loss-of-function mutant was hypersensitive to high salt and osmotic stress conditions	-	Pandey et al., 2015
AtCIPK23	AtCBL1	Xu et al., 2006	Serves as a positive regulator of the potassium transporter AKT1 by directly phosphorylating AKT1 in roots and in stomatal guard cells.	-	Cheong et al. 2007
	AtCBL9	Xu et al. 2006	Serves as a positive regulator of the potassium transporter AKT1 by directly phosphorylating AKT1 in roots and in stomatal guard cells. Involved in nitrate sensing.	-	Cheong et al. 2007

AtCIPK24/ SOS2	AtCBL4/SOS3	Guo et al., 2001; Halfter et al., 2000	Mediates Na^+ extrusion via the regulation of the H^+/Na^+ antiporter SOS1 at the plasma membrane in root tissue.	-	Liu et al. 2000
	AtCBL10	Guo et al., 2001; Halfter et al., 2000	Participates in Na^+ sequestration into the vacuole by regulation of unknown targets in shoot tissue.	-	Liu et al. 2000
BdCIPK31	BdCBL1 BdCBL2 BdCBL5	Luo et al., 2017	Overexpression functions in enhanced *NtSOS1* and *NtNHX2* expression, high Na^+ accumulation in shoots and reduced K^+ efflux in roots in tobacco plants	*Nicotiana tabacum (Tobacco)*	Luo et al., 2017
BnCIPK6	BnCBL1	Chen et al., 2012	Increased seedling growth through higher chlorophyll and proline content	-	Chen et al., 2012
CaCIPK25	*Not known*	-	Overexpression resulted in varied germination period and longer root length in salt stress	*N. tabacum*	Meena et al., 2015
GhCIPK6	*Not known*	-	Overexpression significantly enhanced the tolerance to salt, drought and ABA stresses	*A. thaliana*	He et al., 2013
HbCIPK2	*Not known*	-	Reduced shoot Na^+ accumulation and increased root K^+ accumulation	-	Li et al., 2012
MdCIPK6L	*Not known*	-	Overexpression enhanced the tolerance to salt, osmotic/drought and chilling stresses, but did not affect root growth in transgenic lines	*A. thaliana* *Malus domestica* (Apple)	Wang et al., 2012
MdCIPK24 –Like1 MdSOS2L1	*Not known*	-	Overexpression resulted in enhanced production of antioxidant metabolites	*M. domestica* *Solanum lycopersicum* (Tomato)	Hu et al., 2016

OsCIPK15	*Not known*	-	Overexpressed plants had significantly longer shoot and root length compared to wild type in 100mM salt	*Oryza sativa* L. ssp. *japonica* (rice)	Xiang et al., 2007
OsCIPK31	*Not known*	-	Involves in seed germination and seedling growth under abiotic stresses and induce the expression of several stress related genes *OsRAB21*, *OsDip1*, and *OsSalT*	*O. sativa* L. ssp. *japonica* (cv. Dongjin)	Piao et al., 2010
PtSOS2	*Not known*	-	Overexpressed plants have improved salt tolerance associated with low Na^+ accumulation levels	*Populus davidiana* × *Populus bolleana* (Poplar)	Yang et al., 2015
PeCIPK26	PeCBL1 PeCBL4/PeSOS3 PeCBL9 PeCBL10	Lv et al., 2014	Overexpression resulted in higher germination rate and lower Na^+ accumulation	Arabidopsis cipk2 mutant	Lv et al., 2014
SiCIPK24	SiCBL4	Zhang et al., 2017	Overexpression rescued salt hypersensitivity phenotype	Arabidopsis sos3-1 or sos2-1 mutant	Zhang et al., 2017
SlSOS2 SlCIPK24	SlSOS3 SlCBL4	Huertas et al., 2012	Increased salinity tolerances associated with higher Na^+ content in shoots in transgenic plants	Tomato	Huertas et al., 2012
TaCIPK14	*Not known*	-	Overexpression resulted in higher chlorophyll content, higher stress responsive gene expression, reduced Na^+ accumulation and longer root length	*N. tabacum*	Deng et al., 2013b
TaCIPK29	TaCBL2 TaCBL3 NtCBL2 NtCBL3 NtCAT1	Deng et al., 2013a	Overexpression resulted in higher germination rates, longer root length and better growth compared to controls	*N. tabacum*	Deng et al., 2013a
VvCIPK3	VvCBL2	Cuéllar et al., 2013	Activates a voltage-gated	-	Cuéllar et al., 2013

			inwardly rectifying K^+ channel VvK1.2		
VvCIPK4	VvCBL1	Cuéllar et al., 2013	Activates a voltage-gated inwardly rectifying K^+ channel VvK1.2	-	Cuéllar et al., 2013
ZmCIPK21	*Not known*	-	Overexpression leads to low accumulation of Na^+ and high accumulation of K^+	*A. thaliana*	Chen et al., 2014

Further, in response to alleviated Ca^{2+} concentration in the cytosol, there is rapid activation of a well-known group of proteins, Mitogen-Activated Protein Kinases (MAPK) (Colcombet and Hirt, 2008; Rodriguez et al., 2010; Zhu, 2002). A sequential MAPK circuit (i.e. MAPKKK →MAPKK→MAPK) that involves MEKK1, MAPKKK20, MAPKK2, interchangeable MAPK4/MAPK6, has being identified in Arabidopsis in hyperosmotic stress response (Moustafa et al., 2014 and references therein).

Reactive Oxygen Species (ROS) even if harmful when accumulated in large amounts, have been proposed to be involved in long distance stress signaling (Baxter et al., 2014; Mittler et al., 2011). For example, respiratory burst oxidase homologues (RBOHs), RBOHD and RBOHF that play a main role in ROS production have been shown to function in amalgamation to regulate seed germination, root elongation, stomatal closure and Na^+/K^+ homeostasis in Arabidopsis under salt stress (Ma et al. 2012).

Phytohormones such as abscisic acid (ABA) (Tuteja, 2007), jasmonic acid (JA) (Valenzuela et al. 2016; Wasternack and Hause 2013), auxin (Naser and Shani, 2016; Zhao et al., 2011), ethylene (Cao et al., 2007, 2008), gibberellic acid (GA) (Colebrook et al., 2014) and brassinosteroids (BR) (Fariduddin et al. 2014) have been known for playing an integrated pivotal role in salinity responses by facilitating long distance signaling (Kazan, 2015; Peleg and Blumwald, 2011; Santner and Estelle, 2009). Rapid gene expression alterations then occur by hormone based transcriptional factor induction or degradation through the ubiquitin–proteasome system (Santner and Estelle, 2010). ABA, one of the well-studied hormones in respect of salinity tolerance, is known to be involved in, stomatal closure probably by being synthesized as a response to ROS accumulation (Khokon et al., 2011; Mittler and Blumwald, 2015), reducing the rate of transpiration and water loss, which ultimately reduces plant growth, yet aids plant survival (Raghavendra et al., 2010; Ryu and Cho, 2015; Wilkinson and Davies, 2010) and biosynthesis of osmoprotectants by promoting the synthesis of their enzymes (Fujita et al. 2011). A recent study has shown that exogenous ABA reduced the net efflux of Na^+ from the xylem (Zhu et al. 2017).

As of yet, one important question remains unanswered. If salt stress signaling is a rapid transient process, how does a plant know that it is still in stress even after the signal has ceased, and continues to reduce its growth rate? Are there any other long term signaling cascades that we are yet unaware of? For example, existence of a secondary signaling network after the salt stress that affects growth has been reported in Arabidopsis (Geng et al., 2013). Therefore, comprehensive answers to above questions may lie in understanding the immediate next responses that occur in the osmotic phase, i.e. shoot ion independent phase.

Shoot Ion Independent Tolerance

Sparse information is available on the genes involved in osmotic stress tolerance. One reason for this might be the underdeveloped phenotyping methods to measure the plant growth and transpiration in this phase. However, it has been assumed that osmotic stress has a large influence on yield in low to moderate salinity conditions, especially in crops such as wheat (James et al., 2012; Munns et al., 2012). More recently a study on 24 barley cultivars revealed the variations of growth amongst the cultivars in the phase they describe to be showing shoot ion independent effects in high salinity (Tilbrook et al., 2017). In an ideal situation, if we could identify the growth inhibition related gene network and identify the allelic variations among the cultivars that may cause this, we may be able to develop a barley germplasm that has less yield penalty when faced with salinity. Al-Tamimi et al. (2016) uses an image-based, non-invasive, high-throughput phenotyping of shoot ion independent phase in rice that has the possibility to be extended similar studies of other crops. Through their study, Al-Tamimi et al. (2016) revealed loci influencing transpiration use efficiency on the chromosome 11 of rice.

Shoot Ion Dependent Tolerance

Shoot ion dependent tolerance takes effect when the salt accumulates in the photosynthetic apparatus of the plants, i.e. the leaf. The two main methods that have been identified so far in this context are ion exclusion and tissue tolerance.

Ion Exclusion

Ion exclusion from the shoots can be mainly achieved by (1) minimising net influx of salt into the root and (2) reducing ions in the transpiration stream (Munns and Tester, 2008). *SOS (Salt Overly Sensitive)* is one of the most discussed families with genes known to be involved in excluding salt from the cytosol (Ji et al., 2013). Several of the SOS family genes (SOS1-SOS4) are known to be involved in shoot Na^+ exclusion in many plant species (Apse et al. 1999; Shi et al. 2002b; Shi et al. 2002a). SOS1, initially was thought to be involved in xylem loading (Shi et al. 2002a; Shi et al. 2000) is a membrane bound transporter that is activated by the SOS2-SOS3 complex to efflux Na^+ from cells. SOS1 has been hypothesised to be important in the efflux of Na^+ from roots to the rhizosphere (Shabala et al. 2005). More information on SOS2 and 3 are included in the section on "Salt Sensing and Signaling". SOS4 has shown to be involved in Na^+ exclusion through pathways mediated by vitamin B6 (Shi et al. 2002b; Shi and Zhu 2002).

While it is important to remove excess Na^+ from the root cytosol, it is also important to restrict the entry of Na^+ into the cytosol. It has been suggested that unidirectional passive Na^+ influx can occur through voltage-independent non-selective cation channels (NSCCs) (Demidchik and Maathuis, 2007; Tyerman et al., 1997). Furthermore, it is likely that water channels (i.e. aquaporins) may contribute to passive influx of Na^+ into the root xylem (Byrt et al., 2017). Plants need therefore, to actively control the amount of Na^+ entering the root and possibly reduce the influx of Na^+ from the soil.

The high-affinity K^+ transporters (HKTs) play a crucial role in regulating the leaf Na^+ accumulation levels. Identification of an HKT from wheat (*Triticum aestivum*) named *HKT1 (TaHKT2;1)* initiated the characterization of many HKTs throughout the plant kingdom (Rubio et al., 1995; Schachtman and Schroeder, 1994). HKTs are categorised into class I and class II (Almeida et al., 2013; Horie et al., 2009; Platten et al., 2006). The class I HKTs arise from an S-G-G-G signature in the first pore of the protein and the class II HKTs have the G-G-G-G signature (Platten et al., 2006).

Two loci named *Nax1* and *Nax2* that were involved in reduced shoot Na^+ accumulation have been transferred from an ancestral wheat relative, *Triticum monococcum* to modern durum wheat (James et al., 2006). Candidate genes for *Nax1* and *Nax2* have been identified as *TmHKT1;4-A* (Huang et al., 2006) and *TmHKT1;5-A* (Byrt et al., 2007), respectively. Bread wheat, which is known to have greater ability to exclude Na^+ from plant leaves than durum wheat has a region on chromosome 4DL containing the major Na^+ exclusion locus named, *Kna1* (Byrt et al., 2007). The *Nax2* region on 5AL of durum wheat is homologous to *Kna1* (Byrt et al., 2007). *TmHKT1;5-A* expressing tetraploid durum wheat lines showed significantly reduced leaf Na^+ concentration and an increase in grain yield by 25% when grown under high salt compared to near-isogenic lines without the *Nax2* locus (Munns et al., 2012). More recently, a closely related gene to *TmHKT1;5-A* was identified from bread wheat (Byrt et al., 2014). Furthermore, the allelic variant *TaHKT1;5-D* has also been introgressed to create a synthetic hexaploid wheat which has shown increased salinity tolerance than its progenitor without the *D genome* (Yang et al.

2014). In rice, a quantitative trait locus (QTL) for K^+/Na^+ homeostasis under salt stress, *SKC1*, has been found to encode an HKT-type transporter, *OsHKT1;5* (Ren et al., 2005). Furthermore, a haplotype of rice *HKT1;5* in the wild relative has shown to be associated with high salinity tolerance (Mishra et al. 2016). Additionally, constitutive expression of Arabidopsis *HKT1;1* resulted in high Na^+ accumulation and growth penalties while cell-specific expression of the same gene led to reduction of root-shoot Na^+ in salt stress (Møller et al., 2009).

A class II HKT from barley (*HvHKT2;1*) has been identified as a Na^+ and K^+ co-transporter with low affinity for Na^+ (Mian et al., 2011). Similarly, a class II HKT from rice (OsHKT2;2) has K^+-Na^+ co-transport properties, with affinity to K^+ in higher extracellular Na^+ concentrations (Yao et al., 2010). Another rice HKT, OsHKT2;1 that mediates Na^+ influx has shown to be down regulated by the presence of external K^+ (Horie et al. 2007; Yao et al. 2010). A Tibetan wild cultivar contains unique alleles of *HvHKT1;2* and *HvHKT2;1* (Qiu et al. 2011). They primarily regulate Na^+ and K^+ transport under salt stress, respectively. However this paper fails to confirm whether there were more than two HKT family members in barley which could be involved in Na^+/K^+ homeostasis.

Not only cations such as Na^+ but also anions such as Cl^- accumulate in the cytosol in toxic levels due to salt stress (Li et al., 2017). Nitrate transporter 1/Peptide Transporter family proteins such as NPF2.4 and NPF2.5 have been shown to be, not solely, but in conjunction with other proteins, to be involved in Cl^- exclusion from shoots of Arabidopsis by regulating Cl^- loading into the xylem (Li et al., 2016, 2017). One of these other proteins could be SLAH1 - a homologue of slow type anion channel-associated 1 (SLAC1) that mediates Cl^- loading into the root xylem (Qiu et al., 2016).

Tissue Tolerance

Tissue tolerance is achieved mainly by (a) Na^+ sequestration into the vacuoles (b) production of compatible osmolites and (c) enzymatic and non-enzymatic ROS scavenging. Na^+ sequestration is mainly thought to be facilitated by intracellular antiporters such as *Na^+/H^+ EXCHANGER (NHX)* proteins (Bassil et al., 2012; Blumwald, 2000). Intracellular *NHX*s fall into two groups based on their location in the cell; *NHX1-4* belong to the vacuolar group and *NHX5-6* to the endosomal group (Bassil et al., 2012). Transgenic Arabidopsis overexpressing *NHX1* (*AtNHX1*) showed increased salt tolerance (Apse et al., 1999). *NHX2* was identified as a functionally redundant isoform of *NHX1* (Barragán et al., 2012). The double knockout mutant *Atnhx1 Atnhx2* had significantly reduced K^+/H^+ exchange in tonoplast vesicles compared to the wild type and also showed stored K^+ reduction in vacuoles from the leaf mesophyll, epidermal cells and stomata guard cells (Barragán et al., 2012). This indicates that *NHX1/2's* role could be to regulate K^+ rather than Na^+ homeostasis. Overexpression of *AtNHX1* homologues from many other plant species have given rise to salt tolerant phenotypes (Yamaguchi and Blumwald, 2005). Evidence has accumulated for the involvement of endosomal *NHX*s (*NHX5* and *6*) in protein trafficking, mainly to the vacuole (X et al., 2016). Their importance in plant growth and development, as well as salinity tolerance, has been proposed for several plant species (Bassil et al. 2011; Li et al. 2011). Moreover, it is mandatory for the sequestered Na^+ in the vacuole to remain in this compartment without leaking its way back to the cytosol. There are slow-activating vacuolar (SV) and fast-activating vacuolar (FV) non-selective cation channels that mediate Na^+ flux from vacuole to the cytosol (Hedrich and Marten, 2011). It has been shown, in the salt tolerant species, *Chenopodium quinoa*, that negative regulation of tonoplast SV and FV was needed to compartmentalise Na^+ in the vacuoles (Alatorre et al., 2013).

To transport Na^+ into the vacuole plant cells must first generate a proton gradient between the vacuole and the cytosol. Vacuolar H^+-ATPases (V-ATPases) and H^+-pyrophosphatases (V-PPases/ H^+-PPases) are the most prevalent proteins which use the energy from the breakdown of high energy containing phosphate molecules to pump protons across the tonoplast (Maeshima, 2001). The V-ATPases are composed of two subcomplexes, the peripheral V_1 with 8 subunits (VHA-A to H), and membrane integral V_o complex with six subunits (VHA-a, -c, -c, -c', -c'', -d, and -e) (Wani and Hossain, 2015). The number of VHA encoding genes varies among plants (Schumacher and Krebs, 2010). Numerous studies have reported an increase of V-ATPase in transcriptional, translational or post-translational levels in response to salt stress. For example, an increase of expression of several V-ATPase subunit coding genes has been shown in the halophytes *Mesembryanthemum crystallinum* (Dietz and Arbinger 1996; Golldack and Dietz 2001) and *Salicornia europaea* (Lv et al. 2012) in salt stress. Salt stress activates V-ATPases that in return drive the salt sequestration into organelles(Cotter et al. 2015). Furthermore, V-ATPase subunits B1 and B2 interactions with SOS2 *in vivo,* suggests that V-ATPases may be important in facilitating ion transport across cell membrane during salt stress (Batelli et al. 2007). A V-PPase, *AVP2* from Arabidopsis is known to be K^+ insensitive and Ca^{2+} hypersensitive (Drozdowicz et al., 2000; Schilling et al., 2017).

While cations are sequestrated in the vacuole by above mentioned ways, the Chloride channel (CLC) family is known for containing proteins with the functional capability to sequestrate excess Cl^- into the vacuole (Wei et al., 2016). Rice and citrus CLCs were highly expressed in salt stress (Diédhiou and Golldack, 2006; Wei et al., 2015) and the overexpression of soya bean CLC1

(GmCLC1), and maize CLC1 (ZmCLC1-d) in Arabidopsis leads to a sat tolerant phenotypes (Wang et al., 2015; Wei et al., 2016).

Keeping the shoot Na^+/K^+ ratio low is a well-known decisive factor responsible for a plant's ability to tolerate salt stress. Tonoplast bound K^+ selective channels such as two-pore K^+ channels (TPKs) and Kir-type KCO3 (Czempinski et al., 2002; Voelker et al., 2006) maintain potassium homeostasis within the cytosol. This would help to maintain a high cytosolic K^+/Na^+ ratio in salt stress. K^+ release channels such as SKOR and GORK are activated only when the net K^+ flux is directed outwards (Dreyer and Uozumi, 2011; Garcia-Mata et al., 2010). This is important in functions like stomatal closure that require K^+ efflux (Dreyer and Uozumi, 2011). High-Affinity K^+ transporters (HAK, e.g. AtHAK5) are crucial for K^+ uptake from even very low external K^+ concentrations (Bañuelos et al., 1995). The K^+ channel expressed in guard cells named KAT1 and AKT1 expressed in root epidermis regulate the K^+ in a cell by reducing its net efflux (Dreyer and Uozumi, 2011).

In order to balance the osmotic pressure between the cytosol and the ions within the vacuole the cell has to synthesis compatible solutes. Shoot tissue tolerance therefore, could also rely on the synthesis of compatible solutes, such as proline, glycine betaine and trehalose (Møller and Tester, 2007; Roy et al., 2014). A number of metabolites have been identified that accumulate during salt stress and contribute to the maintenance of cell growth under conditions of increased osmotic stress (Sairam and Tyagi, 2004; Shabala, 2013). These include carbohydrates (e.g. trehalose, sucrose, sorbitol, glycerol, mannitol, pinitol, arabinitol and other polyols), nitrogen compounds (e.g. proline, glycine betaine, glutamate, aspartate, glycine, choline, and putrescine) and organic acids (e.g. oxalates and malates) (Roy et al., 2014 and references therein). Overexpression of *trehalose-6-phosphate synthase (TPS)*, *trehalose-6-phosphate phosphatase (TPP)*, *tannitol-1-phosphate dehydrogenase (mt1D)*, *Δ1-pyrroline-5-carboxylate synthetase (P5CS)*, *betaine aldehyde dehydrogenase (BADH)*, *choline oxidase / dehydrogenase*, *ascorbate peroxidase*, *superoxide dismutase*, *catalase* and numerous other genes have shown improved tissue tolerance in various plants (Roy et al., 2014 and references therein) presumably through enhancing a plant to produce more compatible solutes.

Another issue with the accumulation of Na^+ and Cl^- in the cell is salt stress increases the generation of ROS within cells. While ROS can also act as signaling molecules, excessive accumulation of ROS can cause oxidative damage to membranes, proteins and nucleic acids and alter normal cellular metabolism (Miller et al., 2010; Mittler et al., 2011; Zhang et al., 2014). ROS-induced damage in plants is minimized by enzymatic reactions and non-enzymatic antioxidants. Among the antioxidant enzymes that are crucial in eliminating ROS are catalase

(CAT), peroxidase (POD), superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX) and glutathione-S-transferase (GST) (Ahmad and Rasool, 2014; Zhang et al., 2014). Ascorbic acid (AsA), α-tocopherols, carotenoids and phenolic compounds are among the non-enzymatic antioxidants (Ahmad et al., 2010). There is increasing evidence that ROS signaling is linked with the MAPK circuit that transfers stress signals from the receptor to the target molecules through its cascade even between tissues (Mittler et al., 2011). Many MAPK family proteins have also been showed to be involved in salinity tolerance mechanisms (Kiegerl et al. 2000; Miransari et al. 2013; Moustafa et al. 2014; Popescu et al. 2009; Wang et al. 2014).

Importance of Examining Salt Tolerance Mediated Pathways in Cereal Crops

The ultimate goal of salinity tolerance research is to develop crop germplasm that can withstand or even improve yield stability in high salt conditions (Roy et al., 2014; Shabala et al., 2015). From both forward (i.e. examining the genetic basis for a shown phenotypic trait) and reverse (i.e. examining the phenotypic effects of a particular sequence) genetics approaches a plethora of genes have been identified as being involved in salinity response (described in the previous section, Known Molecular Components of Plant Salt Tolerance). Additionally, we see that allelic diversity may affect the salt tolerant capabilities of a given species (Munoz-Amatriain et al., 2014). However, the translation of initial laboratory success stories of identifying novel genes and allelic variations involved in stress tolerance, to the commercial breeding programmes needs an intermediate step to identify the relay of actions which take place at the molecular level that lead to the desired phenotype.

In order to fully understand the effects of gene manipulation, it is necessary to comprehend the underlying molecular mechanisms that give rise to a complex trait such as salinity tolerance. Differential gene expression analysis and co-expression networks provide complimentary approaches to the analysis of changes in the transcriptomic profile. While the former concentrates on simple differences in expression for a contrast of interest, the latter aims to capture the complex expression relationships between pairs of genes even if they are not differentially expressed on their own (Kadarmideen et al., 2011). In addition to the analysis of transcript abundances, these data sets can also be analysed for variants in the coding regions, perhaps uncovering SNP or inDel differences between cultivars which might explain an observed phenotype.

Gene Expression Analysis (with RNA-Seq)

RNA-Seq uses high-throughput sequencing technologies to analyse the profile of the transcriptome of a biological sample at a particular point in time. RNA-Seq has become a commonly used tool in whole transcriptome studies due to its sensitivity, enabling the discovery and quantification of previously uncharacterized transcripts (Seyednasrollah et al., 2013; Wang et al., 2009). Advantages of RNA-Seq over the other available technologies such as microarray are;

1. RNA-Seq is not dependent on prior knowledge of transcripts and therefore is capable of discovering novel transcripts even in the absence of a complete genome sequence
2. RNA-Seq can help identify SNPs and other variations in the transcribed region,
3. RNA-Seq requires a smaller amount of RNA (Ozsolak and Milos, 2011; Wang et al., 2009).

Several factors have been shown to be important to successfully conduct an RNA-Seq experiment (Conesa et al. 2016; Li et al. 2015b; Quinn and McManus 2015); a) the experimental design: whether it is a 2 factor analysis (e.g. expression changes between two conditions) or more complicated with addition of other factors such as time, genotype, or tissue b) expected depth of sequencing: for example, transcriptome characterization and novel splice variant identification require more depth in sequencing than is required for a transcriptomic characterization of known genes coming from a species with a well-annotated reference, c) number of replicates: in order to properly justify the biological interpretation of the data, at least three replicates for an experimental condition is required to account for the existing biological variation. However it is worth noting that the confidence associated with the statistical analysis is directly proportional to the number of replicates (Conesa et al., 2016).

An RNA-Seq workflow contains the following major steps:

Sequencing

The current project will use the Illumina sequencing platform that uses a Sequencing-By-Synthesis (SBS) methodology (Bentley et al. 2008). Specifically we employed a workflow that utilised the TruSeq™ stranded RNA library preparation so we could identify the strand on which transcription took place. This is achieved by degrading the synthesized second strand before the PCR amplification step (Parkhomchuk et al., 2009). The polarity of the RNA is important when identifying novel genes (Zhao et al. 2015). The first step leading to the first cDNA strand synthesis starts with ligating a short primer complementary to the 3' end of the RNA (polyA tail), that is subsequently identified by the RTs to initiate the reverse transcription producing the first cDNA strand. The first strand is then used as a template for the polymerase to generate the second cDNA strand. The difference in the TruSeq protocol to the traditional approach is that, the second cDNA strand synthesis incorporates dUTPs instead of dTTPs. The addition of Y-shaped adapters is to make sure that the library is sequenced in the same direction, hence the orientation of the original RNA molecule is preserved (Figure 2).

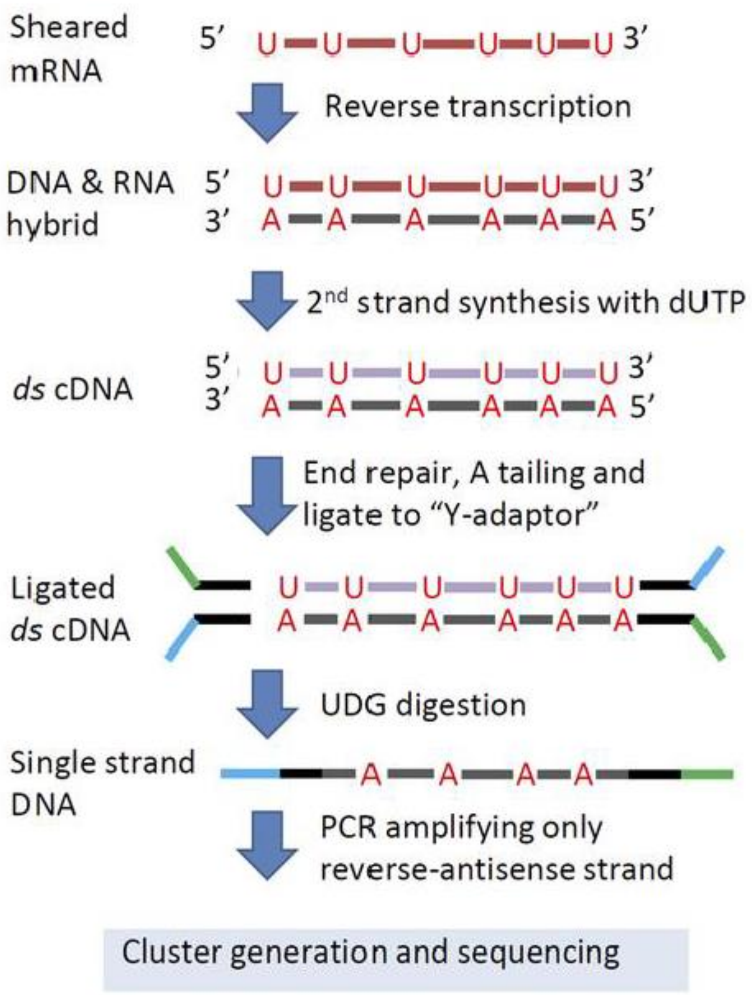


Figure 2 The dUTP method in TruSeq library preparation protocol

The difference in the TruSeq protocol lies in synthesizing of the second strand using dUTPs instead of dTTPs. After adding the Y-shaped adapters, UDGase treatment removes the strand containing Uridines. Blunt end DNA fragments are generated to which an A-base will get added. Subsequently a 3' end T-base overhang containing adapter is added to the A-tailed fragmented DNA. Additionally, a user-defined barcode can be added. The Y-shaped adapters make sure that the strands are sequenced in the same direction. The fragmented molecules are of different sizes, hence a gel size fractionation and extraction is performed to isolated DNA fragments with correct size for sequencing. (source: Wang et al. (2011)).

In the sample preparation that leads to the library creation, it needs to be taken into consideration that the RNA species in a cell are comprised of poly-adenylated messenger RNA (polyA RNA), non-adenylated RNA, ribosomal RNA (rRNA), and small and micro RNA. In order to confirm that the RNA which is being sequenced is of the RNA species of interest, protocols have been developed to remove unwanted RNA contaminations. Due to our necessity of wanting both polyA and non-adenylated RNA the method used in our study for purification is the RiboZero rRNA depletion kit from Illumina. RiboZero kit claims to remove the rRNA from even PolyA RNA fractions (Sooknanan et al., 2010).

The SBS method proprietary to Illumina creates sequencing templates through bridge amplification which are then used for sequencing through fluorescently labelled nucleotides of which the fluorescence is recorded (Figure 3). Difference in paired-end sequencing protocol to single-end protocol is that the process in Figure 3 is repeated with sequencing-by-synthesis occurring from the opposite adapter to the first round.

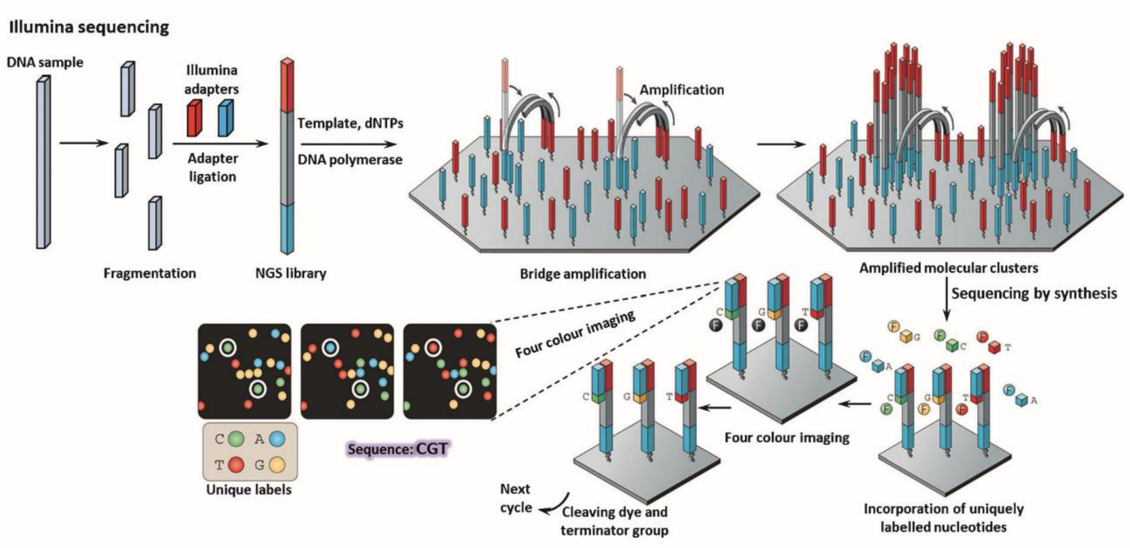


Figure 3 Sequencing-By-Synthesis approach from Illumina Technology

Sequencing libraries consist of single stranded and adapter ligated cDNA fragments. The cDNA loaded onto Illumina flow cells hybridizes with complementary oligo sequences attached to the base of the flow cells. In bridge amplification, successive rounds of complimentary strand synthesis and denaturing result in clusters of identical sequences. Next a mixture of all four individually labelled and 3'-blocked dNTPs are added which will compete and bind to the complementary nucleotide of the templates. After each addition, a light source excites the cluster, and fluoresce of attached labels is recorded (Adapted from Anandhakumar et al., 2015).

Quality Control

Quality control is critical for generating reproducible results through an RNA-Seq analysis. The quality control takes place at various stages of the analysis starting from the point of RNA isolation and library preparation for the sequencing (described above). Downstream quality control checks of the raw read files (FASTQ data) (Cock et al., 2010), after sequence alignment (Sequencing Alignment Map, SAM, or its corresponding Binary Alignment Map, BAM, files) and following the expression quantification, are important (Sheng et al., 2017).

The FASTQ format of the data has four lines representing each read; 1: identifier preceded by an "@" character, 2: sequence 3: a "+" character optionally followed by the identifier 4: base qualities. The information contained in the identifier includes machine, lane and flow cell identifiers. Tools such as FASTQC and NGSQC can calculate statistics for samples based on these values from the FASTQ files (Andrews, 2010; Dai et al., 2010). For example, information from the identifier line could be used to analyse the batch effects of the samples. Batch effects are those that are not caused by the variations of the samples *per se* but caused by the use of different instruments, human errors in handling samples, time of the sequence loading to the machine etc. Base qualities on the 4^{th} line are denoted by a Phred score; this is calculated by the formula $-10\log_{10} p$, where p is the probability of the base being incorrect. Phred score is given according to an ASCII table and the current scale in use is known as Phred +33 (ASCII 0–62). As a general rule, the base quality is expected to be above 30 to be acceptable (Phred 30 means that there is 10^{-3} chance for the base to be wrong; 99.99% accuracy and an error rate of 0.1%), and if the quality is below 20 (i.e. error rate above 1%), it is regarded as a low quality pass due to the signal being low than noise, etc (Cock et al., 2010). In current Illumina platforms the base quality drops at the first ~10 cycles, then increases, and may drop again towards the end, yet has not been reported to be impacting the overall alignment quality (Sheng et al., 2017). Trimming though tools such as Trimmomatic and FASTX-Toolkit Toolkit (Bolger et al., 2014; Gordon and Hannon, 2010) removes the bases with low quality, however the aligners have the ability to soft-clip (i.e. bases included in the alignment file yet marked as not part of the alignment) hence the trimming for base quality could be performed on the fly.

Another aspect that is being checked for is the GC content. Generally, the GC level of a monocot (e.g. barley) falls between 33.6% and 48.9% (Šmarda et al., 2014) and is higher than that of dicots (Li and Du, 2014). It is expected that the GC content of a sample should be around the same value as that of the reference genome, or else it hints at contamination by another species.

Quality control examines overrepresented sequences which could either represent genes with high expression, adapters, PCR artifacts or other contaminations. Trimming for adapters or small repetitive regions (k-mers) could be performed to remove these sequences. However, it has to be kept in mind that while trimming sequences increases the average read quality, over-trimming could alter the downstream differential expression results due to an increased chance of the trimmed read getting mapped to multiple locations (Williams et al., 2016).

Quality checks at the alignment level can be retrieved through statistics output files from the alignment tools to understand how many reads were uniquely mapped, how many were discarded due to what reason (e.g. multimapping, ambiguity, etc.). Furthermore, the Picard suite of tools (“Picard Tools - By Broad Institute,”),a java-based program, can mark the duplicates which can be removed through SAMtools (Li et al., 2009).

Underlying the RNA-Seq method is the assumption that the level of expression in a particular experimental condition remains relatively similar. However, in some cases, there could be deviations from this assumption. In such cases, having more than 3 replicates gives the researcher the flexibility to remove the sample that is an outlier without compromising a statistical analysis. However, it is important to exercise caution when removing "outliers" as they may look like an outlier, but actually represent true biological variation. Particularly in experiments with low levels of replication. Therefore, such samples should be “flagged” and not simply removed. Methods for identification of potential “outliers” are principle component analyses (PCA) and multi-dimensional scaling (MDS) plots of sample data. Furthermore, clustering based on the expression values, and visualizing using density plots, heat maps or box plots can identify these outliers as well.

Alignment

For the species with a reference genome, reads can be directly mapped to that reference. An indicator of "good mapping" is the percentage of uniquely mapped reads (i.e. one read mapped to one genomic location) with minimum mismatch rate (ideally <2%). As a general rule, a sample with a percentage of mapped reads below ~70% for a well-annotated genome such as Arabidopsis, would be considered poor. In order to perform RNA-Seq read alignment, it is important to use a splice-aware read alignment tool. Such tools are capable of splice aligning a read which spans an exon-exon junction across the corresponding intron on the genomic sequence. The use of a read aligner which is not splice-aware will result in fewer reads aligning to the regions flanking introns. The possible incorrect alignments of non-gapped aligners are mentioned in Figure 4. Tools such as TopHat/HISAT2 (runs the Bowtie algorithm), STAR and

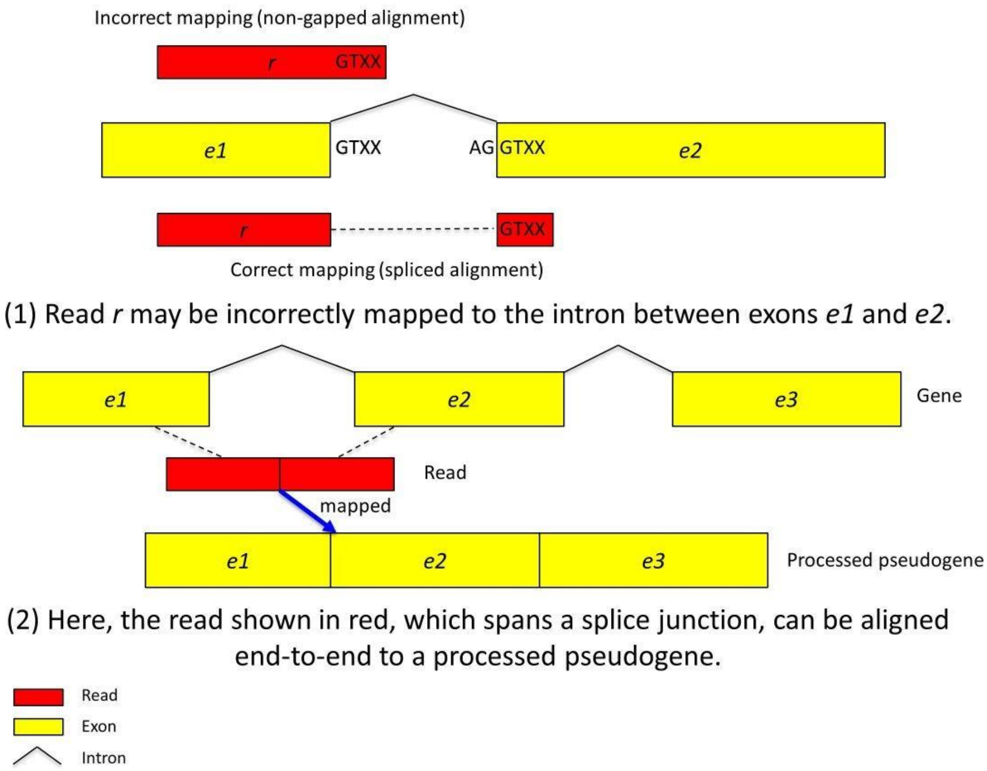


Figure 4 Two possible incorrect alignments of spliced reads

1) A read extending a few bases into the flanking exon can be aligned to the intron instead of the exon. 2) A read spanning multiple exons from genes with processed pseudogene copies can be aligned to the pseudogene copies instead of the gene from which it originates (Reproduced from Kim et al., 2013)

MapSplice are capable of splice-aligning reads (Dobin et al. 2013; Langmead and Salzberg 2012; Trapnell et al. 2009; Wang et al. 2010b). A range of developed read aligning algorithms and tools have been reviewed (Baruzzo et al., 2017; Ekre and Mante, 2016; Escalona et al., 2016; Li and Homer, 2010; Shang et al., 2014).

TopHat2 and STAR aligners were used in the current study because of the high accuracy in aligning reads to an incomplete reference and the power of speed on well annotated genomes, respectively TopHat2 first identifies the potential splice sites, then maps reads to known exons. The unmapped reads are then mapped to the exons considering the identified potential splice sites. Therefore, even though time consuming, TopHat2 is extremely sensitive when it comes to spliced reads (Kim et al., 2013). On the other hand, the aligner STAR was chosen for aligning reads to the well annotated Arabidopsis genome, since it is much faster. However, STAR has reduced sensitivity when it comes to novel spice junctions due to the fact that the reads are mapped independently of the other aligned reads (Dobin et al., 2013).

Feature Quantification

Feature quantification, gives a number on how many reads are mapped to a set of features (e.g. exons, transcripts, genes). I have employed the method featureCounts that is implemented in the *Rsubread* package in the R environment (Liao et al., 2013; R Core Team, 2014). The algorithm in featureCounts counts a read as a hit (i.e. mapped to the correct position hence counted as a read for that feature) if an overlap of the read to the reference is ≥1 base pair (bp). It also gives the user the flexibility to either include or exclude reads that have overlap across more than one feature (Liao et al., 2014).

Normalisation

The number of reads aligned to a gene is affected by the number of reads generated for a sample (library size) and the length of the transcript itself. Without normalizing read alignment counts to take these into consideration simple read counts are not directly comparable (Aanes et al. 2014; Dillies et al. 2013a). Several of the popular methods used for normalization are mentioned in Table 2 and discussed further below. Several others have been extensively compared and reviewed in Conesa et al., 2016; M.-A. Dillies et al., 2013; Li et al., 2015; Reddy, 2015; Wu et al., 2011.

Table 2 Popular methods of RNA-Seq data normalization that were considered in the project

Transformation method	Special features	References
Reads Per Kilobase of transcript per Million mapped reads (RPKM)	Within-sample normalization for sequencing depth before normalizing for the gene length	Mortazavi et al., 2008

	Performs poorly with diverse transcript distribution Developed for single-end sequences	
Fragments Per Kilobase of transcript per Million mapped reads (FPKM)	Derivative of RPKM, for paired-end reads. Has the same limitations as described above	Trapnell et al., 2013
Transcripts per Million (TPM)	A fractional measure of the abundance of a transcript among all sampled transcripts to yield a proportion between 0-1 and then multiplied by 1million to give transcripts per million	Li and Dewey, 2011
Trimmed Mean of M-values (TMM)	Weighted trimmed mean of the log expression ratios are used	Robinson et al., 2010
Counts Per Million (CPM)	Does not take the feature lengths into consideration. The log-CPM value introduces a prior value of 0.25 to avoid the log zero.	Law et al., 2016

In the RPKM and FPKM methods, the normalization algorithm takes the length of the transcript (l_g) and the depth of sampling (*N*) into consideration (Mortazavi et al., 2008).

$$\mu_g = \frac{r_g}{N l_g}$$

In the absence of any sampling biases, the normalized value (μ_g) of reads (r_g) for each gene *g* multiplied by 10^{-9} will result in a RPKM value. However, RNA-Seq counts are affected by biological variation and technical variation: i.e. batch effects (McIntyre et al., 2011). In their paper Wagner et al. (2012) showed that the inconsistency of RPKM values across samples is due to the fact that the total number of reads for a gene (r_g) is dependent on the sequencing run, but not on the biological variation, like the total RNA abundance would be. Wagner et. al (2012) suggested that the TPM method (Li and Dewey, 2011) that effectively normalizes only for library size to be better than RPKM in that sense. Even so, TPM method also takes the length of the transcripts into consideration as the sum of all counts per base. Hence, both TPM and RPKM are missing one fundamental criterion in differential expression analyses; RNA composition and complexity of a sample affect the total number of reads generated due to the finite sequencing real-estate on Illumina flow cells. Despite the fact that both sum of counts per base (as in TPM) and the length of all reads in a sample (as in RPKM) are not a constant across the samples RPKM/FPKM and TPM methods are still being used for comparing gene expression differences across samples. However, there is no clear requirement for normalization for the gene length. This is because the expression changes are measured for the same gene across the samples, hence the transcript length would be a constant for a statistical test.

Robinson et al., 2010 proposed the method TMM to overcome this issue of sample biases in RPKM and TPM and take the real biological variation across samples for normalization. The TMM approach uses a weighted trimmed mean of the log expression ratios. Another approach, CPM, can also be used for normalization because for example, CPM value for the i^{th} gene is the ratio between counts (X_i) and the number of sequenced fragments (N) multiplied by one million (Law et al., 2014), hence does not take the length normalization into account;

$$CPM_i = \frac{X_i}{N} \times 10^6$$

The TMM and CPM methods were employed in this study to normalize across samples, as these methods are sufficient in RNA-Seq experiments because the gene length biases are affected similarly for the same genes in different samples.

Differential Expression

Statistical approaches have been developed for detection of differentially expressed genes and are summarized in Table 3 and further explained below

Table 3 The Statistical approaches for identifying differentially expressed genes using normalized RNA-Seq data

Approach	**Features**	**Reference**
edgeR	Uses an empirical Bayes estimation and exact test based on binomial models to determine differential expression	Robinson et al., 2010
DESeq	Uses a binomial model that allows the differential gene expression analysis to be based on a dynamic range of data	Anders and Huber, 2010
BaySeq	Uses negative binomially distributed data and estimated posterior likelihoods of differential expression using a Bayesian method	Hardcastle and Kelly, 2010
NOIseq	A non-parametric tool that empirically models the noise distribution from the actual	Tarazona et al., 2011

	data that can adapt to the size of the dataset and control the false discoveries	
SAMseq	Non-parametric approach based tool that is believed to derive significant features better than the parametric models mentioned above	Li and Tibshirani, 2013
Cuffdiff2	A transcript based detection method that enables differential reports and uses a beta negative binomial model for controlling the variability and ambiguity	Trapnell et al., 2013
EBSeq	Estimating the posterior likelihoods of differential expression using a Bayesian method with an assumption that the data is distributed negative-binomially	Leng et al., 2013
Limma	Based on linear modelling Assigns weights to each observation through *voom* based on the mean-variance relationship prior to linear modeling of the observations	Law et al., 2014; Ritchie et al., 2015

Poisson distribution, that is used in PoissonSeq (Li et al. 2012a), can be used to model the read counts. Even though the Poisson distribution assumes the variance to be similar to the mean, it works well when modeling expression data from technical replicates (Marioni et al., 2008). Technical replicates are from the same biological sample sequenced across different lanes. However, expression data from various biological samples have a higher variance than their mean. Therefore, an over-dispersed Poisson model or a negative binomial distribution model (NB) is better at explaining gene expression data (Robinson and Smyth, 2008; Smyth, 2004). In the Bioconductor package edgeR, the association between the mean and the variance of the negative binomial is calculated through a single parameter that is estimated thorough a constant $\alpha_g$from all data;

$$\sigma_g^s = \mu_g + \alpha_g \mu_g^2$$

On the other hand, DESeq estimates the variance-mean dependence within each sample which makes it more flexible than edgeR.

Limma assumes a log-normal distribution of expression data and uses a generalised linear model to identify significantly differentially expressed genes (Smyth, 2004). Each gene has a vector of expression values (y_g) that is related to any coefficients of interest (i.e. hypotheses; β_g) through a design matrix (*X*).

$$E(y_g) = X\beta_g$$

The linear modelling allows sharing information across samples hence, analyses the entire experiment in integration. The ability to do simple pairwise comparisons, as well as more flexible hypotheses that include interactions between pairwise comparisons made it suitable for the current study with a multiple factorial design. Furthermore, independent studies from Soneson and Delorenzi, (2013), Seyednasrollah et al., (2013) and Schurch et al., (2015) show with the use of various simulated and real data sets that Limma (alongside edgeR and DESeq) has a minimum requirement of 3 biological replicates to detect differentially expressed genes for the threshold of 2 $\log_2$-fold change with lowest false positive rate. Additionally, the high quality of the available support and documentation as well as the high speed of the algorithm added to the suitability of Limma for this study.

Gene Co-expression analysis

A gene co-expression network is a graph containing nodes and edges, where genes are the nodes and correlation in expression are the edges (Civelek and Lusis, 2014; Zhang and Horvath, 2005) (Figure 5). Networks can identify potential genes that are likely to be the vital members of a biological process by acting in similar pathways and regulatory networks and having similar expression patterns (Civelek and Lusis, 2014; Lu et al., 2011).

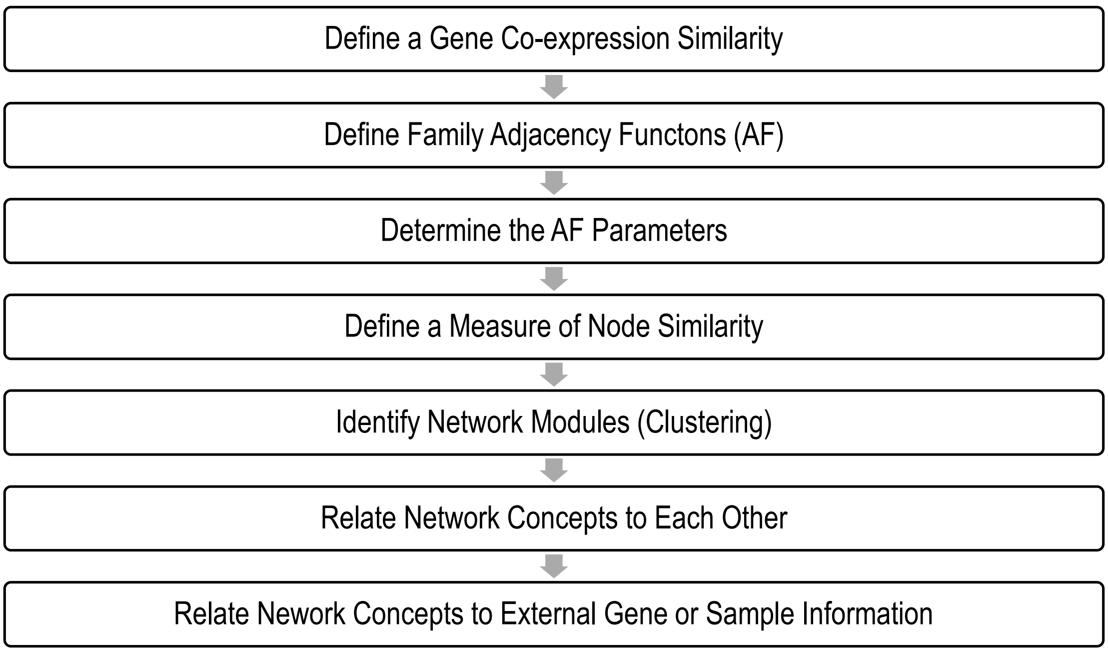


Figure 5 Flowchart of the process of gene co-expression analysis

(Reproduced from Zhang and Horvath, 2005)

Weighted gene co-expression network analysis (WGCNA), the method used in the present study for the gene co-expression analysis, describes the correlation patterns between gene expression profiles and assigns weights for each edge according to the strength of the correlation (Lu et al., 2011). Zhang and Horvath, (2005) proposed the workflow for WGCNA and it will be described below (Figure 5).

The first step of the network generation is to create a similarity matrix using the quantified and normalized expression value data. Statistical methods such as Pearson correlation can be used for this purpose (Galton, 1889; Pearson, 1920). There are other statistical methods that allow themeasure of correlation between data in gene co-expression analyses, and were used in examples shown in Table 4. The similarity matrix typically take values in the interval [-1, 1].

Table 4 Statistical methods for measuring correlation amongst observations

Statistical method	Special features	Reference
Pearson Correlation	Parametric measures the linear correlation between two variables	Pearson, 1920
Hoeffding method	Non-parametric The D statistic depends only on the ranks order of the observations	Hoeffding, 1948
Kendall method	Non-parametric Measures the strength of the dependence between two variables	Kendall, 1938
Theil-Sen method	Theil-Sen estimator is the median of the slopes determined by all pairs of sample points	Sen, 1968
Spearman method	Non-parametric Measures nonlinear monotonic relationship between two variables	Spearman, 1904
Weighted Rank method	gives weight to the distance between two ranks using a linear function of those ranks	Pinto da Costa and Soares, 2005

The next step is to convert the similarity matrix to an adjacency matrix (also known as a network) which must only contain values in the interval [0, 1]. Therefore, this conversion from an n x n similarity matrix ($A^{original}$) to an n x n adjacency matrix (A) is undertaken with the use of an Adjacency function (AF);

$$A = AF\left(A^{original}\right)$$

One such AF involves hard thresholding ($AF^{threshold}$) an assignment of either 1 or 0 is given if the original value surpasses some threshold value, thus deriving an unweighted network:

$$AF^{threshold}\left(A^{original}, \tau\right)_{ij} = \begin{cases} 1 \; if \; A_{ij}^{original} & \geq \tau \\ 0 \; if \; A_{ij}^{original} & < \tau \end{cases}$$

Hard thresholding has the issue of what to choose for the value for the threshold as edges falling just short of the threshold contribute nothing to the resulting network. This can be addressed by using soft thresholding, such as the power function (*AFpower*, *β*) for the transformation:

$$AF^{power}\left(A^{original}, \beta\right)_{ij} = \left|A_{ij}^{original}\right|^{\beta}$$

While hard thresholding results in an unweighted network, soft thresholding results in a weighted network (Horvath, 2011); which contains information on the weight of a connection, not just whether a connection exists or not. The topological overlap measure (TOM) is used to detect subsets of tightly interconnected nodes. This is done by measuring the degree of overlap between the neighbours for a pair of nodes. However, in the case of TOM, this is generalised to cover all neighbours, not just the first-step neighbours. The generalised TOM approach used in Zhang and Horvath (2005) has evolved from Ravasz et al. (2002) and is defined as;

$$t_{ij} = \begin{cases} \dfrac{l_{ij} + a_{ij}}{min\{k_i, k_j\} + 1 - a_{ij}}, & if \; i \neq j \\ 1, & if \; i = j \end{cases}$$

Where, a_{ij} is the adjacency matrix, $l_{ij} = \sum_u a_{iu}, a_{uj}$, $k_i = \sum_u a_{iu}$ and *u* is an index that runs across all nodes of the network.

One class of important nodes in a network, are those with the highest connectivity within a module (as measured by module membership). These are called hub genes. It is known that removal of a hub gene from a network causes the whole network topology to collapse (Figure 6) and are thus integral to the overall network structure. As such, hub genes are expected to be crucial players in understanding the biological mechanism underlying these modules.

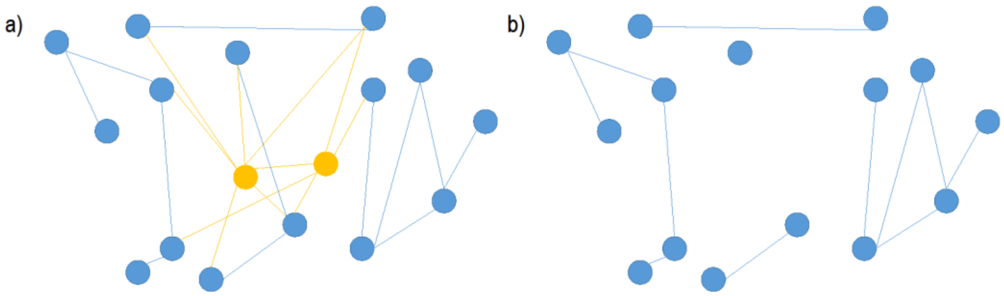


Figure 6 A hypothetical network topology to show the importance of a hub gene

The circles represent nodes (e.g. genes) and the connectors represent the edges (e.g. correlation between two nodes). The hub nodes and their edges are coloured in orange in a. These are the nodes that have the largest number of edge weights within the network.

Therefore, the presence of hub genes is mandatory for the presence of that network, and relating to the biological importance, the function of the hub genes can be assumed to be important in preserving the topology of the network.

Variant Analysis

Sequence variants caused by single nucleotide changes, a short insertion or deletion, can in some cases, be advantageous for the carrier (e.g. give rise to phenotypes with high Na^+ tolerating ability) over others of the same species (Saxena et al., 2014) and may explain phenotypic differences (Rafalski, 2002). Such variants, if associated with an advantageous trait can be used for marker-assisted selection in breeding programs (Telem et al., 2016).

Single Nucleotide Polymorphisms (SNPs) can occur in non-coding regions of the genome as well as in coding regions (Clevenger et al., 2015; Ganal et al., 2009). SNPs that are located in protein-coding regions may (non-synonymous SNP) or may not (synonymous SNP) give rise to a change of an amino acid in the encoded protein sequence. Thus non-synonymous SNPs can either be a) missense: can lead to altered protein function or activity or b) nonsense: result in a premature stop codon. Another common type of genetic variant is insertions and deletions (inDels) (Ajawatanawong and Baldauf, 2013). InDels in genes may cause reading frame shifts that lead to gene knockouts through premature translational stops or an altered amino acid sequence.

One process of identifying SNPs and short InDels is sequencing a genotype of interest, aligning the reads to a reference genome, and calling the variants. There are a range of tools for calling variants including Genome Analysis Toolkit (GATK) HaplotypeCaller and GATK UnifiedGenotyper, SAMtools mpileup/bcftool pipeline, VCFtools, FreeBayes and SNPSV (Danecek et al., 2011; Garrison and Marth, 2012; Li et al., 2009; Li, 2011; O'Fallon et al., 2013). Since most of the tools have been developed for and tested on human/mammalian but not on plant data (Cornish and Guda 2015; Deelen et al. 2015; Hwang et al. 2015; Liu et al. 2013b) (Olson et al., 2015), I decided to use the GATK HaplotypeCaller for variant calling based on available evaluations on accuracy shown with multi-sample analyses (Cornish and Guda 2015; Liu et al. 2013b; Pirooznia et al. 2014).

Variant annotations and effect predictions are feasible with tools such as ANNOVAR, VEP, CCED, CooVar, SNPEff and SNPSift (Cingolani et al. 2012; Kircher et al. 2014; McLaren et al. 2016; Vergara et al. 2012; Wang et al. 2010a). Similarly to the variant calling tools these have not been evaluated for plant genomes. Therefore, based on prior knowledge in working with, I chose SNPEff to predict the variant effects in the present study.

Molecular Phylogenetics

Molecular phylogenetics is a method to infer orthologues relationships among genes/proteins. Moreover, one can infer the functions for uncharacterised proteins based on their orthologues relationship to members of the same family that have functional evidence.

There are several steps in a phylogenetic analysis. They are: 1) Identification of homologous sequences; 2) Model selection and 3) Tree building.

Identifying Homologous Sequences

One of the first steps in phylogenetic analyses is often the identification of similar sequences to a given nucleotide or protein sequence (Altschul et al., 1990; Pei, 2008). Genes can become similar in sequence by convergence, rather than by divergence, and thus have no common ancestor and are therefore not homologous. Therefore, clues to support the divergence of a particular set of sequences from a common ancestor by the process of mutation and selection is important in homology studies (Reeck et al., 1987). Sequence alignment algorithms can be used for genomic data mining to identify homologous sequences (Henikoff and Henikoff 1992).

The alignment algorithms can be loosely categorized as, 1) pairwise local aligners 2) pairwise global aligners and 3) multiple sequence aligners. Pairwise local aligners are for associated fragments of sequences. Pairwise global aligners are for sets of sequences linked by mutual ancestry throughout their lengths. Multiple sequence aligners are for multiple members of sequence families and alignments made in database investigations to detect homology (Henikoff and Henikoff 1992). Some of the popular algorithms developed for this purpose include the Needlemann-Wunsch algorithm, Smith-Waterman algorithm, BLAST and FASTA (Polyanovsky et al., 2011).

Needleman and Wunsch (1970) and Smith and Waterman (1981) both developed alignment algorithms using dynamic programming to generate a global alignment. The dynamic programming algorithm is computationally intensive but is guaranteed to find the optimal alignment for a scoring function. Heuristic alignment algorithms such as BLAST can perform high speed local pairwise alignments but are not guaranteed to find the optimal solution (Altschul et al., 1990). The heuristic method FASTA is considered to be much more sensitive but slower than BLAST (Lipman and Pearson, 1985).

Progressive alignment techniques have been introduced for multiple sequence alignment to avoid the weaknesses of pairwise alignments which uses dynamic programming techniques (Pei, 2008). Progressive methods create multiple alignments by the use of a series of pairwise alignments or pre-aligned clusters. However, they do not promise an optimal solution and could be error-prone in the pairwise alignment step. Nevertheless, using a correct substitution scoring model these multiple sequence alignment can produce a reliable and fast result (Pei, 2008).

From the currently available tools, CLUSTALW, MAFFT, MUSCLE and TCoffee are considered to produce reasonably accurate multiple sequence alignments (Chenna et al., 2003; Edgar, 2004; Katoh et al., 2005; Notredame et al., 2000; Thompson et al., 1994). MUSCLE, the technique I have employed in the multiple sequence alignment of the sequences, involves initial pairwise sequence profile alignment, which is then used for progressive alignment and later for fine-tuning (Edgar, 2004).

Model Selection

Any method for inferring the homology of nucleotide or protein sequences directly or indirectly uses the evolutionary models of nucleotide or protein substitutions (Adachi and Hasegawa, 1996; Hasegawa et al., 1985; Kimura, 1980). Even though the mutation events occur at the nucleotide level, selective pressure primarily pertains on the protein level (Massingham and Goldman, 2005; Tourasse and Li, 2000). Therefore codon substitution models and amino acid substitution models are more reliable than the models applied to nucleotide sequences (Arenas, 2015).

Codon substitutions can be of two different types; a) synonymous (no changes to amino acids) and b) non-synonymous (altering amino acids). Therefore, the ratio between synonymous (dS) and non-synonymous (dN) substitutions (i.e. $\frac{dS}{dN}$ ratio) was used initially for identifying the selection pressure on a population. Existence of positive selection pressure caused by the substitutions on the gene that codes for altered proteins is implied by $\frac{dS}{dN} > 0$, while $\frac{dS}{dN} \leq 0$ implies that there is either neutral or negative selection pressure on the gene (Yang and Bielawski, 2000).

Proteins from different species tend to have varied amino acid substitutions, as do proteins with different functions (Miyazawa, 2013). Residues at different positions are exposed to different selective pressures. In distantly related sequences where non-synonymous substitutions are significant, it is important to evaluate selective pressures on amino acids and consider substitution models based on amino acids (Miyazawa, 2013). When multiple sequence alignments are inspected, some positions are more conserved than others, and some regions of a multiple alignments seem to be more tolerant to inDels than others (Henikoff and Henikoff 1992).

Unlike empirical models, mechanistic codon models take these issues into consideration. They attempt to explain the biology involved in protein evolution including things such as mutational biases in the DNA and the genetic code. These models usually separate mutational biases at the nucleotide level from selective constraints at the amino acid level and take features of sequence evolution into consideration (e.g. transition-transversion and base or codon occurrence bias) and make use of physical and chemical properties of amino acids to stipulate non-synonymous substitution rates (Yang et al., 1998). Therefore, mechanistic codon models perform better than the empirical models (Miyazawa, 2013). However, the matrices involved in codon substitution models are so large (61 x 61 excluding the stop codons), which makes the application of these models computationally extensive. A better alternative for codon substitution models therefore are the amino acid substitution models that would test a 20 x 20 matrix instead.

Amino acid substitution models can be broadly categorized into; 1) empirical models 2) parametric models (Henikoff and Henikoff 1992; Marti-Renom 2004). Empirical models are based on the fact that the likelihood of an amino acid A replacing the amino acid B is same as B replacing A. This is assumed on the basis that likelihood should depend on the product of the frequencies of occurrence of the two amino acids and on their chemical and physical similarity caused by change in amino acid frequencies over the evolutionary distance (Dayhoff et al., 1978). Therefore, the relative substitution rates between amino acids are fixed in those models, no matter which protein is analysed (Yang et al., 1998). Dayhoff (PAM) model and BLOSUM (BLOck SUbstitution Matrix) are two of the main empirical models used today (Dayhoff et al., 1978). Further improvements of the empirical models involved the work and the critical analysis done between Dayhoff models and maximum likelihood models (Adachi and Hasegawa, 1996; Jones et al., 1992; Müller et al., 2002).

Tree Building

A phylogenetic tree is a representation of the evolutionary history of a group of species of sequences considered, whereby leaves (external nodes/Operational Taxonomic Units/OTUs) of the tree represent the species or the sequences and internal nodes represent the ancestral states (Soltis and Soltis 2003). This information on biological diversity, structural classification and insight into evolution provide the clues for homology modelling and identification of novel proteins which are not yet characterized (Baum, 2008).

Choosing a Method for Phylogenetic Analysis

Distance-based methods are simple approaches to constructing phylogenetic trees (Saitou and Imanishi, 1989). Pairwise distances between all pairs of sequences of a multiple sequence alignment are calculated. Missing data or gaps are handled by either deleting them pairwise or completely or considering them as all possible bases. Some examples of distance-based methods are the Unweighted Pairwise Group of Multiple Alignments (UPGMA), Neighbor-Joining (NJ), Minimum Evolution and Fitch-Margoliash (Saitou and Imanishi, 1989). These methods can tolerate a large number of sequences since they are derived from pairwise distance calculations which are quick and easy to calculate (Saitou and Imanishi, 1989).

Character-based methods such as Maximum Parsimony (MP), Maximum Likelihood (ML) and the Bayesian probability technique are alternative techniques, which depend on the likelihood or probability models (Reddy, 2011; Zvelebil and Baum, 2008). A distance-based method computes pairwise distances according to some measure which discards the actual data and the fixed distances are used in the construction of trees. On the other hand, trees derived by way of a character-based method have been optimized according to the distribution of actual data patterns in relation to a specified character. Although these character based models are more robust, they are significantly more time consuming than the distance-based methods. However, erroneous phylogenetic relationships can be drawn from any of these methods if they are not understood and explored properly. Therefore, careful consideration should be given to select the appropriate model and backing the results with the previous literature findings is important (Reddy, 2011).

The tool MEGA encompasses the capability to generate phylogenetic trees using either several distance-based or character-based methods (Tamura et al., 2013). This tool was used in the project to compute the phylogenetic trees using suitable mechanistic codon substitution models.

Confidence in Phylogenetic Trees

Bootstrapping is a method to provide a level of confidence in the branching order of phylogenetic trees (Efron et al., 1996). In bootstrapping, random sampling of alignment positions is conducted with replacement to generate pseudo datasets (Figure 7). These pseudo datasets are used as input into the phylogenetic tree reconstruction method being used (Nei and Kumar, 2000). The topology of each bootstrap tree is then compared with the initial phylogenetic tree. Each internal

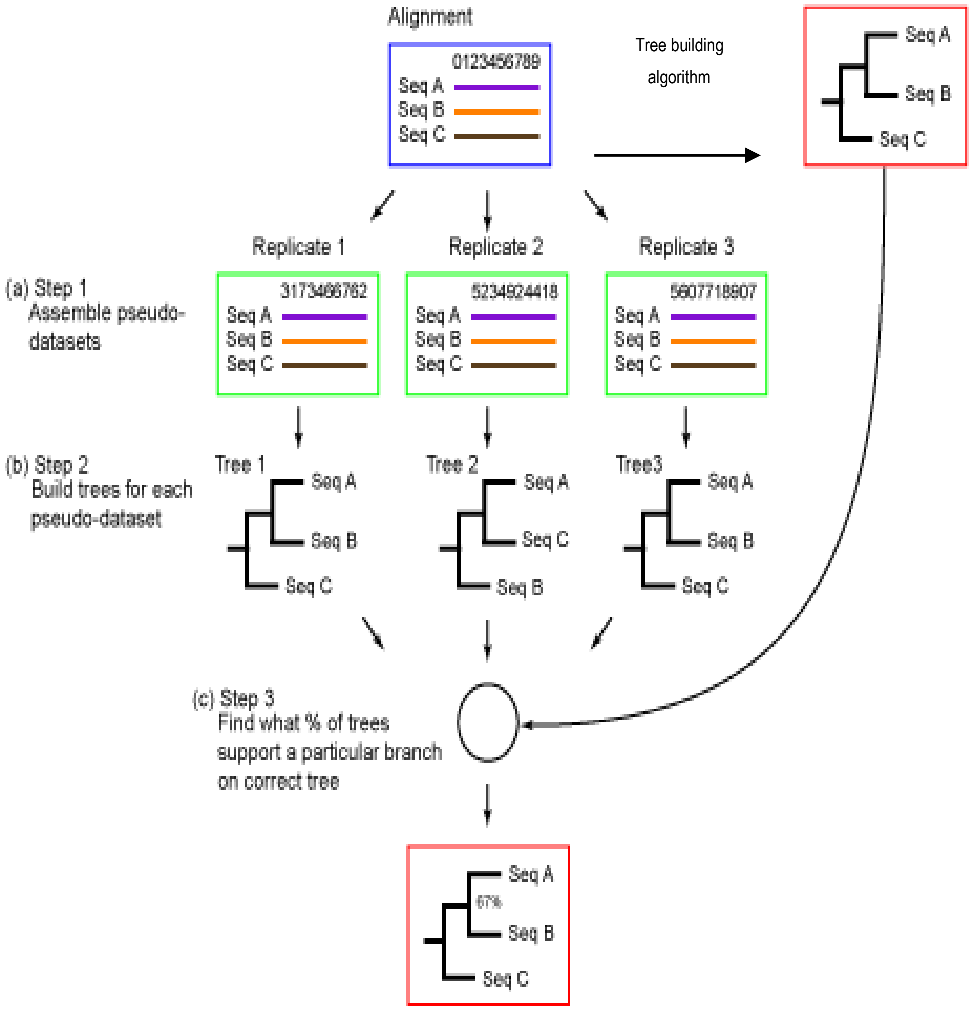


Figure 7 Steps in bootstrapping a phylogenetic tree

Reproduced from http://phylogenetictrees.com/images/seq_tree_4.gif

node that is different henceforth will be assigned a zero. A one is assigned to similar internal nodes. A percentage value (bootstrap value) will be calculated from the data generated in all bootstrapping iterations. As a general rule if the bootstrap value for a given internal node is ≥ 80%, the node is considered to be correct (Nei and Kumar, 2000). This can be applied to any phylogenetic reconstruction method (Felsenstein, 1985). As few as 100-200 bootstrapping replications can give reliable estimates (Efron et al., 1996).

Conclusion and Research Aims

The challenge for functional genomics in plant salinity tolerance related research now will be to develop sustainable and transferrable agronomically important crops with minimal manipulation to aid improvements of yield in areas with highly salinized soil (Atkinson and Urwin, 2012). Given that salinity is a complex trait, the plant responses to it involves a large array of genes. Investigation of global transcriptomes and variants of candidate genes of plants exposed to high salinity can provide a holistic view on how the plant genome is involved in salinity response and whether there are any noteworthy genotypic differences in this response that need further investigations.

Research in this thesis therefore, attempts to understand several aspects of salinity tolerance in the context of Arabidopsis and barley through RNA-Seq and phylogenetics. Being able to relate the genetic variation to phenotypic information through gene co-expression and genotypic variations will add another layer of information to this context. These findings will add to the overall body of knowledge on how to generate sustainable salt tolerant germplasm.

Specific research questions addressed in this thesis are;

1. What are the underlying molecular mechanisms of *AtCIPK16* overexpression conferred salinity tolerance in Arabidopsis?
2. What is the prevalence of CIPK16s in the terrestrial plant kingdom?
3. What are the main genetic and expression variations among the barley genotypes with varying Na^+ accumulation levels?

References

Aanes H, Winata C, Moen LF, Østrup O, Mathavan S, Collas P, et al. Normalization of RNA-Sequencing Data from Samples with Varying mRNA Levels. PLoS ONE. 2014 Feb 25;9(2):e89158.

Abbas G, Saqib M, Akhtar J, Haq MA ul. Interactive effects of salinity and iron deficiency on different rice genotypes. J. Plant Nutr. Soil Sci. 2015 Apr 1;178(2):306–11.

Adachi J, Hasegawa M. Model of amino acid substitution in proteins encoded by mitochondrial DNA. J. Mol. Evol. 1996 Apr 1;42(4):459–68.

Ahmad P, Jaleel CA, Salem MA, Nabi G, Sharma S. Roles of enzymatic and nonenzymatic antioxidants in plants during abiotic stress. Crit. Rev. Biotechnol. 2010 Sep 1;30(3):161–75.

Ahmad P, Rasool S. Emerging Technologies and Management of Crop Stress Tolerance: Volume 2 - A Sustainable Approach. Academic Press; 2014.

Ajawatanawong P, Baldauf SL. Evolution of protein indels in plants, animals and fungi. BMC Evol. Biol. 2013 Jul 4;13:140.

Akaboshi M, Hashimoto H, Ishida H, Saijo S, Koizumi N, Sato M, et al. The Crystal Structure of Plant-Specific Calcium-Binding Protein AtCBL2 in Complex with the Regulatory Domain of AtCIPK14. J. Mol. Biol. 2008 Mar 14;377(1):246–57.

Alatorre EB-, Shabala S, Chen Z, Pottosin II. Reduced tonoplast FV and SV channels activity is essential for conferring salinity tolerance in a facultative halophyte, Chenopodium quinoa. Plant Physiol. 2013 Jan 1;pp.113.216572.

Albrecht V, Ritz O, Linder S, Harter K, Kudla J. The NAF domain defines a novel protein-protein interaction module conserved in Ca2+-regulated kinases. EMBO J. 2001 Mar 1;20(5):1051–63.

Almeida P, Katschnig D, de Boer AH. HKT Transporters—State of the Art. Int. J. Mol. Sci. 2013 Oct 14;14(10):20359–85.

Al-Tamimi N, Brien C, Oakey H, Berger B, Saade S, Ho YS, et al. Salinity tolerance loci revealed in rice using high-throughput non-invasive phenotyping. Nat. Commun. 2016 Nov 17;7:13342.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J. Mol. Biol. 1990 Oct 5;215(3):403–10.

Anandhakumar C, Kizaki S, Bando T, Pandian GN, Sugiyama H. Advancing Small-Molecule-Based Chemical Biology with Next-Generation Sequencing Technologies. ChemBioChem. 2015 Jan 2;16(1):20–38.

Anders S, Huber W. Differential expression analysis for sequence count data. Genome Biol. 2010 Oct 27;11(10):R106.

Andrews S. FastQC: a quality control tool for high throughput sequence data. [Internet]. 2010 [cited 2015 Jan 7]. Available from: http://www.bioinformatics.babraham.ac.uk/projects/fastqc

Apse MP, Aharon GS, Snedden WA, Blumwald E. Salt Tolerance Conferred by Overexpression of a Vacuolar Na+/H+ Antiport in Arabidopsis. Science. 1999 Aug 20;285(5431):1256–8.

Arenas M. Trends in substitution models of molecular evolution. Front. Genet. [Internet]. 2015 [cited 2018 Feb 5];6. Available from: https://www.frontiersin.org/articles/10.3389/fgene.2015.00319/full#h3

Ashraf M, Wu L. Breeding for Salinity Tolerance in Plants. Crit. Rev. Plant Sci. 1994;13(1):17–42.

Atkinson NJ, Urwin PE. The interaction of plant biotic and abiotic stresses: from genes to the field. J. Exp. Bot. 2012 Jun 1;63(10):3523–43.

Balmer D, Flors V, Glauser G, Mauch-Mani B. Metabolomics of cereals under biotic stress: current knowledge and techniques. Front. Plant Sci. [Internet]. 2013 Apr 23 [cited 2014 Apr 22];4. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3632780/

Bañuelos MA, Klein RD, Alexander-Bowman SJ, Rodríguez-Navarro A. A potassium transporter of the yeast Schwanniomyces occidentalis homologous to the Kup system of Escherichia coli has a high concentrative capacity. EMBO J. 1995 Jul 3;14(13):3021–7.

Barragán V, Leidi EO, Andrés Z, Rubio L, De Luca A, Fernández JA, et al. Ion Exchangers NHX1 and NHX2 Mediate Active Potassium Uptake into Vacuoles to Regulate Cell Turgor and Stomatal Function in Arabidopsis[W][OA]. Plant Cell. 2012 Mar;24(3):1127–42.

Baruzzo G, Hayer KE, Kim EJ, Di Camillo B, FitzGerald GA, Grant GR. Simulation-based comprehensive benchmarking of RNA-seq aligners. Nat. Methods. 2017 Feb;14(2):135–9.

Bassil E, Coku A, Blumwald E. Cellular ion homeostasis: emerging roles of intracellular NHX Na+/H+ antiporters in plant growth and development. J. Exp. Bot. 2012 Oct 1;63(16):5727–40.

Bassil E, Ohto M, Esumi T, Tajima H, Zhu Z, Cagnac O, et al. The Arabidopsis Intracellular Na+/H+ Antiporters NHX5 and NHX6 Are Endosome Associated and Necessary for Plant Growth and Development. Plant Cell. 2011 Jan 1;23(1):224–39.

Batelli G, Verslues PE, Agius F, Qiu Q, Fujii H, Pan S, et al. SOS2 Promotes Salt Tolerance in Part by Interacting with the Vacuolar H+-ATPase and Upregulating Its Transport Activity. Mol. Cell. Biol. 2007 Nov;27(22):7781–90.

Batistic O, Kudla J. Integration and channeling of calcium signaling through the CBL calcium sensor/CIPK protein kinase network. Planta. 2004 Oct 1;219(6):915–24.

Batistič O, Kudla J. Plant calcineurin B-like proteins and their interacting protein kinases. Biochim. Biophys. Acta BBA - Mol. Cell Res. 2009 Jun 1;1793(6):985–92.

Batistič O, Kudla J. Calcium: Not Just Another Ion. In: Hell R, Mendel R-R, editors. Cell Biol. Met. Nutr. [Internet]. Springer Berlin Heidelberg; 2010 [cited 2016 Oct 18]. p. 17–54. Available from: http://link.springer.com/chapter/10.1007/978-3-642-10613-2_2

Batistic O, Sorek N, Schultke S, Yalovsky S, Kudla J. Dual Fatty Acyl Modification Determines the Localization and Plasma Membrane Targeting of CBL/CIPK Ca2+ Signaling Complexes in Arabidopsis. Plant Cell. 2008 May;20(5):1346–62.

Baum, D. Reading a phylogenetic tree: The meaning of monophyletic groups. Nat. Educ. 2008;1(1):190.

Baxter A, Mittler R, Suzuki N. ROS as key players in plant stress signalling. J. Exp. Bot. 2014 Mar 1;65(5):1229–40.

Bentley DR, Balasubramanian S, Swerdlow HP, Smith GP, Milton J, Brown CG, et al. Accurate whole human genome sequencing using reversible terminator chemistry. Nature. 2008 Nov 6;456(7218):53–9.

Blumwald E. Sodium transport and salt tolerance in plants. Curr. Opin. Cell Biol. 2000 Aug 1;12(4):431–4.

Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014 Aug 1;30(15):2114–20.

Brini F, &#xe7, al, Masmoudi K. Ion Transporters and Abiotic Stress Tolerance in Plants. ISRN Mol. Biol. 2012 Jun 3;2012:e927436.

Byrt CS, Platten JD, Spielmeyer W, James RA, Lagudah ES, Dennis ES, et al. HKT1;5-Like Cation Transporters Linked to Na+ Exclusion Loci in Wheat, Nax2 and Kna1. Plant Physiol. 2007 Apr;143(4):1918–28.

Byrt CS, Xu B, Krishnan M, Lightfoot DJ, Athman A, Jacobs AK, et al. The Na+ transporter, TaHKT1;5-D, limits shoot Na+ accumulation in bread wheat. Plant J. 2014 Nov 1;80(3):516–26.

Byrt CS, Zhao M, Kourghi M, Bose J, Henderson SW, Qiu J, et al. Non-selective cation channel activity of aquaporin AtPIP2;1 regulated by Ca2+ and pH. Plant Cell Environ. 2017 Jun 1;40(6):802–15.

Cao W-H, Liu J, He X-J, Mu R-L, Zhou H-L, Chen S-Y, et al. Modulation of Ethylene Responses Affects Plant Salt-Stress Responses. Plant Physiol. 2007 Feb 1;143(2):707–19.

Cao Y-R, Chen S-Y, Zhang J-S. Ethylene signaling regulates salt stress response. Plant Signal. Behav. 2008 Oct;3(10):761–3.

Carillo P, Grazia M, Pontecorvo G, Fuggi A, Woodrow P. Salinity Stress and Salt Tolerance. In: Shanker A, editor. Abiotic Stress Plants - Mech. Adapt. [Internet]. InTech; 2011 [cited 2014 Jul 28]. Available from: http://www.intechopen.com/books/abiotic-stress-in-plants-mechanisms-and-adaptations/salinity-stress-and-salt-tolerance

Chen L, Ren F, Zhou L, Wang Q-Q, Zhong H, Li X-B. The Brassica napus Calcineurin B-Like 1/CBL-interacting protein kinase 6 (CBL1/CIPK6) component is involved in the plant response to abiotic stress and ABA signalling. J. Exp. Bot. 2012;63(17):6211–6222.

Chen L, Wang Q-Q, Zhou L, Ren F, Li D-D, Li X-B. Arabidopsis CBL-interacting protein kinase (CIPK6) is involved in plant response to salt/osmotic stress and ABA. Mol. Biol. Rep. 2013 Aug 1;40(8):4759–67.

Chen X, Huang Q, Zhang F, Wang B, Wang J, Zheng J. ZmCIPK21, A Maize CBL-Interacting Kinase, Enhances Salt Stress Tolerance in Arabidopsis thaliana. Int. J. Mol. Sci. 2014 Aug 22;15(8):14819–34.

Cheng S-H, Willmann MR, Chen H-C, Sheen J. Calcium signaling through protein kinases. The Arabidopsis calcium-dependent protein kinase gene family. Plant Physiol. 2002 Jun;129(2):469–85.

Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, et al. Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res. 2003 Jul 1;31(13):3497–500.

Cheong YH, Pandey GK, Grant JJ, Batistic O, Li L, Kim B-G, et al. Two calcineurin B-like calcium sensors, interacting with protein kinase CIPK23, regulate leaf transpiration and root potassium uptake in Arabidopsis. Plant J. 2007 Oct 1;52(2):223–39.

Chinnusamy V, Schumaker K, Zhu J-K. Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. J. Exp. Bot. 2004 Jan 1;55(395):225–36.

Choi W-G, Toyota M, Kim S-H, Hilleary R, Gilroy S. Salt stress-induced Ca2+ waves are associated with rapid, long-distance root-to-shoot signaling in plants. Proc. Natl. Acad. Sci. 2014 Apr 29;111(17):6497–502.

Cingolani P, Platts A, Wang LL, Coon M, Nguyen T, Wang L, et al. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w^{1118}; iso-2; iso-3. Fly (Austin). 2012 Apr;6(2):80–92.

Civelek M, Lusis AJ. Systems genetics approaches to understand complex traits. Nat. Rev. Genet. 2014 Jan;15(1):34–48.

Clevenger J, Chavarro C, Pearl SA, Ozias-Akins P, Jackson SA. Single Nucleotide Polymorphism Identification in Polyploids: A Review, Example, and Recommendations. Mol. Plant. 2015 Jun;8(6):831–46.

Cock PJA, Fields CJ, Goto N, Heuer ML, Rice PM. The Sanger FASTQ file format for sequences with quality scores, and the Solexa/Illumina FASTQ variants. Nucleic Acids Res. 2010 Apr 1;38(6):1767–71.

Colcombet J, Hirt H. *Arabidopsis* MAPKs: a complex signalling network involved in multiple biological processes. Biochem. J. 2008 Jul 15;413(2):217–26.

Colebrook EH, Thomas SG, Phillips AL, Hedden P. The role of gibberellin signalling in plant responses to abiotic stress. J. Exp. Biol. 2014 Jan 1;217(1):67–75.

Conesa A, Madrigal P, Tarazona S, Gomez-Cabrero D, Cervera A, McPherson A, et al. A survey of best practices for RNA-seq data analysis. Genome Biol. 2016 Jan 26;17(1):13.

Cornish A, Guda C. A Comparison of Variant Calling Pipelines Using Genome in a Bottle as a Reference. BioMed Res. Int. [Internet]. 2015 [cited 2018 Feb 5];2015. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4619817/

Cotter K, Stransky L, McGuire C, Forgac M. Recent Insights into the Structure, Regulation, and Function of the V-ATPases. Trends Biochem. Sci. 2015 Oct 1;40(10):611–22.

Cramer GR, Nowak RS. Supplemental manganese improves the relative growth, net assimilation and photosynthetic rates of salt-stressed barley. Physiol. Plant. 1992 Apr 1;84(4):600–5.

Cuéllar T, Azeem F, Andrianteranagna M, Pascaud F, Verdeil J-L, Sentenac H, et al. Potassium transport in developing fleshy fruits: the grapevine inward K+ channel VvK1.2 is activated by CIPK–CBL complexes and induced in ripening berry flesh cells. Plant J. 2013 Mar 1;73(6):1006–18.

Czempinski K, Frachisse J-M, Maurel C, Barbier-Brygoo H, Mueller-Roeber B. Vacuolar membrane localization of the Arabidopsis'two-pore' K+ channel KCO1. Plant J. 2002 Mar 1;29(6):809–20.

Dai M, Thompson RC, Maher C, Contreras-Galindo R, Kaplan MH, Markovitz DM, et al. NGSQC: cross-platform quality analysis pipeline for deep sequencing data. BMC Genomics. 2010 Dec 2;11(4):S7.

Danecek P, Auton A, Abecasis G, Albers CA, Banks E, DePristo MA, et al. The variant call format and VCFtools. Bioinformatics. 2011 Aug 1;27(15):2156–8.

D'Angelo C, Weinl S, Batistic O, Pandey GK, Cheong YH, Schültke S, et al. Alternative complex formation of the Ca2+-regulated protein kinase CIPK1 controls abscisic acid-dependent and independent stress responses in Arabidopsis. Plant J. 2006 Dec 1;48(6):857–72.

Dayhoff MO, Schwartz RM, Orcutt BC. Chapter 22: A model of evolutionary change in proteins. Atlas Protein Seq. Struct. 1978. p. 345–352.

Deelen P, Zhernakova DV, de Haan M, van der Sijde M, Bonder MJ, Karjalainen J, et al. Calling genotypes from public RNA-sequencing data enables identification of genetic variants that affect gene-expression levels. Genome Med. 2015;7:30.

Demidchik V, Maathuis FJM. Physiological roles of nonselective cation channels in plants: from salt stress to signalling and development. New Phytol. 2007 Aug 1;175(3):387–404.

Deng X, Hu W, Wei S, Zhou S, Zhang F, Han J, et al. TaCIPK29, a CBL-Interacting Protein Kinase Gene from Wheat, Confers Salt Stress Tolerance in Transgenic Tobacco. PLoS ONE [Internet]. 2013a Jul 29 [cited 2014 Apr 17];8(7). Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3726728/

Deng X, Zhou S, Hu W, Feng J, Zhang F, Chen L, et al. Ectopic expression of wheat TaCIPK14, encoding a calcineurin B-like protein-interacting protein kinase, confers salinity and cold tolerance in tobacco. Physiol. Plant. 2013b Nov 1;149(3):367–77.

Diédhiou CJ, Golldack D. Salt-dependent regulation of chloride channel transcripts in rice. Plant Sci. 2006 Apr 1;170(4):793–800.

Dietz K-J, Arbinger B. cDNA sequence and expression of subunit E of the vacuolar H+-ATPase in the inducible Crassulacean acid metabolism plant Mesembryanthemum crystallinum. Biochim. Biophys. Acta BBA - Biomembr. 1996 Jun 11;1281(2):134–8.

Dillies M-A, Rau A, Aubert J, Hennequet-Antier C, Jeanmougin M, Servant N, et al. A comprehensive evaluation of normalization methods for Illumina high-throughput RNA sequencing data analysis. Brief. Bioinform. 2013a Nov;14(6):671–83.

Dillies M-A, Rau A, Aubert J, Hennequet-Antier C, Jeanmougin M, Servant N, et al. A comprehensive evaluation of normalization methods for Illumina high-throughput RNA sequencing data analysis. Brief. Bioinform. 2013b Nov 1;14(6):671–83.

Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. Bioinforma. Oxf. Engl. 2013 Jan 1;29(1):15–21.

Dreyer I, Uozumi N. Potassium channels in plant cells. FEBS J. 2011 Nov 1;278(22):4293–303.

Drozdowicz YM, Kissinger JC, Rea PA. AVP2, a Sequence-Divergent, K+-Insensitive H+-Translocating Inorganic Pyrophosphatase from Arabidopsis. Plant Physiol. 2000 May 1;123(1):353–62.

Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004 Mar 1;32(5):1792–7.

Efron B, Halloran E, Holmes S. Bootstrap confidence levels for phylogenetic trees. Proc. Natl. Acad. Sci. 1996 Nov 12;93(23):13429–13429.

Ehret DL, Redmann RE, Harvey BL, Cipywnyk A. Salinity-induced calcium deficiencies in wheat and barley. Plant Soil. 1990 Nov 1;128(2):143–51.

Ekre AR, Mante RV. Genome sequence alignment tools: A review. 2016 2nd Int. Conf. Adv. Electr. Electron. Inf. Commun. Bio-Inform. AEEICB. 2016. p. 677–81.

Escalona M, Rocha S, Posada D. A comparison of tools for the simulation of genomic next-generation sequencing data. Nat. Rev. Genet. 2016 Aug;17(8):459–69.

Fariduddin Q, Yusuf M, Ahmad I, Ahmad A. Brassinosteroids and their role in response of plants to abiotic stresses. Biol. Plant. 2014 Mar 1;58(1):9–17.

Felsenstein J. Confidence Limits on Phylogenies: An Approach Using the Bootstrap. Evolution. 1985 Jul 1;39(4):783–91.

Fujita Y, Fujita M, Shinozaki K, Yamaguchi-Shinozaki K. ABA-mediated transcriptional regulation in response to osmotic stress in plants. J. Plant Res. 2011 Jul 1;124(4):509–25.

Galton F. I. Co-relations and their measurement, chiefly from anthropometric data. Proc. R. Soc. Lond. 1889 Jan 1;45(273–279):135–45.

Ganal MW, Altmann T, Röder MS. SNP identification in crop plants. Curr. Opin. Plant Biol. 2009 Apr 1;12(2):211–7.

Garcia-Mata C, Wang J, Gajdanowicz P, Gonzalez W, Hills A, Donald N, et al. A Minimal Cysteine Motif Required to Activate the SKOR K+ Channel of Arabidopsis by the Reactive Oxygen Species H2O2. J. Biol. Chem. 2010 Sep 17;285(38):29286–94.

Garrison E, Marth G. Haplotype-based variant detection from short-read sequencing. ArXiv12073907 Q-Bio [Internet]. 2012 Jul 17; Available from: http://arxiv.org/abs/1207.3907

Geng Y, Wu R, Wee CW, Xie F, Wei X, Chan PMY, et al. A Spatio-Temporal Understanding of Growth Regulation during the Salt Stress Response in Arabidopsis. Plant Cell. 2013 Jun 1;25(6):2132–54.

Gilliham M, Able JA, Roy SJ. Translating knowledge about abiotic stress tolerance to breeding programmes. Plant J. 2017 Feb 1;n/a-n/a.

Gilroy S, Suzuki N, Miller G, Choi W-G, Toyota M, Devireddy AR, et al. A tidal wave of signals: calcium and ROS at the forefront of rapid systemic signaling. Trends Plant Sci. 2014 Oct;19(10):623–30.

Golldack D, Dietz K-J. Salt-Induced Expression of the Vacuolar H+-ATPase in the Common Ice Plant Is Developmentally Controlled and Tissue Specific. Plant Physiol. 2001 Apr 1;125(4):1643–54.

Gong D, Guo Y, Jagendorf AT, Zhu J-K. Biochemical Characterization of the Arabidopsis Protein Kinase SOS2 That Functions in Salt Tolerance. Plant Physiol. 2002 Sep;130(1):256–64.

Gordon A, Hannon GJ. Fastx-toolkit. FASTQ/A short-reads pre-processing tools (unpublished) [Internet]. 2010. Available from: http://hannonlab. cshl. edu/fastx_toolkit

Guo Y, Halfter U, Ishitani M, Zhu J-K. Molecular Characterization of Functional Domains in the Protein Kinase SOS2 That Is Required for Plant Salt Tolerance. Plant Cell. 2001 Jun;13(6):1383–400.

Halfter U, Ishitani M, Zhu J-K. The Arabidopsis SOS2 protein kinase physically interacts with and is activated by the calcium-binding protein SOS3. Proc. Natl. Acad. Sci. 2000 Mar 28;97(7):3735–40.

Hardcastle TJ, Kelly KA. baySeq: Empirical Bayesian methods for identifying differential expression in sequence count data. BMC Bioinformatics. 2010 Aug 10;11(1):422.

Hasegawa M, Kishino H, Yano T. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. 1985 Oct 1;22(2):160–74.

Hasegawa PM, Bressan RA, Zhu J-K, Bohnert HJ. Plant Cellular and Molecular Responses to High Salinity. Annu. Rev. Plant Physiol. Plant Mol. Biol. 2000;51(1):463–99.

He L, Yang X, Wang L, Zhu L, Zhou T, Deng J, et al. Molecular cloning and functional characterization of a novel cotton CBL-interacting protein kinase gene (GhCIPK6) reveals its involvement in multiple abiotic stress tolerance in transgenic plants. Biochem. Biophys. Res. Commun. 2013 May 31;435(2):209–15.

Hedrich R, Marten I. TPC1 – SV Channels Gain Shape. Mol. Plant. 2011 May;4(3):428–41.

Henikoff S, Henikoff JG. Amino acid substitution matrices from protein blocks. Proc. Natl. Acad. Sci. U. S. A. 1992 Nov 15;89(22):10915–9.

Hoeffding W. A Non-Parametric Test of Independence. Ann. Math. Stat. 1948;19(4):546–57.

Horie T, Costa A, Kim TH, Han MJ, Horie R, Leung H-Y, et al. Rice OsHKT2;1 transporter mediates large Na+ influx component into K+-starved roots for growth. EMBO J. 2007 Jun 20;26(12):3003–14.

Horie T, Hauser F, Schroeder JI. HKT transporter-mediated salinity resistance mechanisms in Arabidopsis and monocot crop plants. Trends Plant Sci. 2009 Dec;14(12):660–8.

Horvath S. Weighted Network Analysis [Internet]. New York, NY: Springer New York; 2011 [cited 2017 Dec 12]. Available from: http://link.springer.com/10.1007/978-1-4419-8819-5

Hrabak EM, Chan CWM, Gribskov M, Harper JF, Choi JH, Halford N, et al. The Arabidopsis CDPK-SnRK Superfamily of Protein Kinases. Plant Physiol. 2003 Jun;132(2):666–80.

Hu D-G, Ma Q-J, Sun C-H, Sun M-H, You C-X, Hao Y-J. Overexpression of MdSOS2L1, a CIPK protein kinase, increases the antioxidant metabolites to enhance salt tolerance in apple and tomato. Physiol. Plant. 2016 Feb 1;156(2):201–14.

Huang S, Spielmeyer W, Lagudah ES, James RA, Platten JD, Dennis ES, et al. A Sodium Transporter (HKT7) Is a Candidate for Nax1, a Gene for Salt Tolerance in Durum Wheat. Plant Physiol. 2006 Dec 1;142(4):1718–27.

Huertas R, Olías R, Eljakaoui Z, Gálvez FJ, Li J, De Morales PA, et al. Overexpression of SlSOS2 (SlCIPK24) confers salt tolerance to transgenic tomato. Plant Cell Environ. 2012 Aug 1;35(8):1467–82.

Hwang S, Kim E, Lee I, Marcotte EM. Systematic comparison of variant calling pipelines using gold standard personal exome variants. Sci. Rep. 2015 Dec 7;5:17875.

James RA, Blake C, Zwart AB, Hare RA, Rathjen AJ, Munns R. Impact of ancestral wheat sodium exclusion genes Nax1 and Nax2 on grain yield of durum wheat on saline soils. Funct. Plant Biol. 2012;39(7):609–18.

James RA, Davenport RJ, Munns R. Physiological Characterization of Two Genes for Na+ Exclusion in Durum Wheat, Nax1 and Nax2. Plant Physiol. 2006 Dec 1;142(4):1537–47.

Ji H, Pardo JM, Batelli G, Oosten MJV, Bressan RA, Li X. The Salt Overly Sensitive (SOS) Pathway: Established and Emerging Roles. Mol. Plant. 2013 Jan 25;sst017.

Jones DT, Taylor WR, Thornton JM. The rapid generation of mutation data matrices from protein sequences. Comput. Appl. Biosci. CABIOS. 1992 Jun 1;8(3):275–82.

Kadarmideen HN, Watson-Haigh NS, Andronicos NM. Systems biology of ovine intestinal parasite resistance: disease gene modules and biomarkers. Mol. Biosyst. 2011;7(1):235.

Katoh K, Kuma K, Toh H, Miyata T. MAFFT version 5: improvement in accuracy of multiple sequence alignment. Nucleic Acids Res. 2005 Jan 1;33(2):511–8.

Kazan K. Diverse roles of jasmonates and ethylene in abiotic stress tolerance. Trends Plant Sci. 2015 Apr 1;20(4):219–29.

Kendall MG. A New Measure of Rank Correlation. Biometrika. 1938;30(1/2):81–93.

Khokon MAR, Jahan MS, Rahman T, Hossain MA, Muroyama D, Minami I, et al. Allyl isothiocyanate (AITC) induces stomatal closure in Arabidopsis. Plant Cell Environ. 2011 Nov 1;34(11):1900–6.

Kiegerl S, Cardinale F, Siligan C, Gross A, Baudouin E, Liwosz A, et al. SIMKK, a Mitogen-Activated Protein Kinase (MAPK) Kinase, Is a Specific Activator of the Salt Stress–Induced MAPK, SIMK. Plant Cell. 2000 Nov 1;12(11):2247–58.

Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol. 2013 Apr 25;14:R36.

Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 1980 Jun 1;16(2):111–20.

Kircher M, Witten DM, Jain P, O'Roak BJ, Cooper GM, Shendure J. A general framework for estimating the relative pathogenicity of human genetic variants. Nat. Genet. 2014 Mar;46(3):310.

Kolukisaoglu Ü, Weinl S, Blazevic D, Batistic O, Kudla J. Calcium Sensors and Their Interacting Protein Kinases: Genomics of the Arabidopsis and Rice CBL-CIPK Signaling Networks. Plant Physiol. 2004 Jan 1;134(1):43–58.

Kudla J, Batistič O, Hashimoto K. Calcium Signals: The Lead Currency of Plant Information Processing. Plant Cell Online. 2010 Mar 1;22(3):541–63.

Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat. Methods. 2012 Mar 4;9(4):357–9.

Law CW, Alhamdoosh M, Su S, Smyth GK, Ritchie ME. RNA-seq analysis is easy as 1-2-3 with limma, Glimma and edgeR. F1000Research [Internet]. 2016 Nov 30 [cited 2017 Dec 7];5. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4937821/

Law CW, Chen Y, Shi W, Smyth GK. Voom: precision weights unlock linear model analysis tools for RNA-seq read counts. Genome Biol. 2014;15(2):R29.

Lee SC, Lan W-Z, Kim B-G, Li L, Cheong YH, Pandey GK, et al. A protein phosphorylation/dephosphorylation network regulates a plant potassium channel. Proc. Natl. Acad. Sci. 2007 Oct 2;104(40):15959–64.

Leng N, Dawson JA, Thomson JA, Ruotti V, Rissman AI, Smits BMG, et al. EBSeq: An empirical Bayes hierarchical model for inference in RNA-seq experiments. Bioinformatics. 2013 Feb 21;btt087.

Li B, Byrt C, Qiu J, Baumann U, Hrmova M, Evrard A, et al. Identification of a Stelar-Localized Transport Protein That Facilitates Root-to-Shoot Transfer of Chloride in Arabidopsis. Plant Physiol. 2016 Feb 1;170(2):1014–29.

Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics. 2011 Aug 4;12:323.

Li B, Tester M, Gilliham M. Chloride on the Move. Trends Plant Sci. 2017 Mar 1;22(3):236–48.

Li H. A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. Bioinformatics. 2011 Nov 1;27(21):2987–93.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009 Aug 15;25(16):2078–9.

Li H, Homer N. A survey of sequence alignment algorithms for next-generation sequencing. Brief. Bioinform. 2010 Sep;11(5):473–83.

Li J, Tibshirani R. Finding consistent patterns: a nonparametric approach for identifying differential expression in RNA-Seq data. Stat. Methods Med. Res. 2013 Oct;22(5):519–36.

Li J, Witten DM, Johnstone IM, Tibshirani R. Normalization, testing, and false discovery rate estimation for RNA-sequencing data. Biostat. Oxf. Engl. 2012a Jul;13(3):523–38.

Li M, Li Y, Li H, Wu G, Näsholm T. Overexpression of AtNHX5 improves tolerance to both salt and drought stress in Broussonetia papyrifera (L.) Vent. Tree Physiol. 2011 Mar 1;31(3):349–57.

Li P, Piao Y, Shon HS, Ryu KH. Comparing the normalization methods for the differential analysis of Illumina high-throughput RNA-Seq data. BMC Bioinformatics. 2015a Oct 28;16:347.

Li R, Zhang J, Wu G, Wang H, Chen Y, Wei J. HbCIPK2, a novel CBL-interacting protein kinase from halophyte Hordeum brevisubulatum, confers salt and osmotic stress tolerance. Plant Cell Environ. 2012b;35(9):1582–1600.

Li X, Nair A, Wang S, Wang L. Quality Control of RNA-Seq Experiments. RNA Bioinforma. [Internet]. Humana Press, New York, NY; 2015b [cited 2018 Feb 3]. p. 137–46. Available from: https://link.springer.com/protocol/10.1007/978-1-4939-2291-8_8

Li X-Q, Du D. Variation, Evolution, and Correlation Analysis of C+G Content and Genome or Chromosome Size in Different Kingdoms and Phyla. PLOS ONE. 2014 Feb 13;9(2):e88339.

Liao Y, Smyth GK, Shi W. The Subread aligner: fast, accurate and scalable read mapping by seed-and-vote. Nucleic Acids Res. 2013 May 1;41(10):e108–e108.

Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinforma. Oxf. Engl. 2014 Apr 1;30(7):923–30.

Lipman DJ, Pearson WR. Rapid and Sensitive Protein Similarity Searches. Science. 1985 Mar 22;227(4693):1435–41.

Liu J, Ishitani M, Halfter U, Kim C-S, Zhu J-K. The Arabidopsis thaliana SOS2 gene encodes a protein kinase that is required for salt tolerance. Proc. Natl. Acad. Sci. U. S. A. 2000 Mar 28;97(7):3730–4.

Liu J, Zhu J-K. A Calcium Sensor Homolog Required for Plant Salt Tolerance. Science. 1998 Jun 19;280(5371):1943–5.

Liu L-L, Ren H-M, Chen L-Q, Wang Y, Wu W-H. A Protein Kinase, Calcineurin B-Like Protein-Interacting Protein Kinase9, Interacts with Calcium Sensor Calcineurin B-Like Protein3 and Regulates Potassium Homeostasis under Low-Potassium Stress in Arabidopsis1[W][OA]. Plant Physiol. 2013a Jan;161(1):266–77.

Liu X, Han S, Wang Z, Gelernter J, Yang B-Z. Variant Callers for Next-Generation Sequencing Data: A Comparison Study. PLOS ONE. 2013b Sep 27;8(9):e75619.

Lu HH-S, Schölkopf B, Zhao H, editors. Handbook of Statistical Bioinformatics [Internet]. Berlin, Heidelberg: Springer Berlin Heidelberg; 2011 [cited 2014 Jun 17]. Available from: http://link.springer.com/10.1007/978-3-642-16345-6

Luan S, Kudla J, Rodriguez-Concepcion M, Yalovsky S, Gruissem W. Calmodulins and calcineurin B-like proteins: calcium sensors for specific signal response coupling in plants. Plant Cell. 2002;14 Suppl:S389-400.

Ludwig AA, Romeis T, Jones JDG. CDPK-mediated signalling pathways: specificity and cross-talk. J. Exp. Bot. 2004 Jan;55(395):181–8.

Luo Q, Wei Q, Wang R, Zhang Y, Zhang F, He Y, et al. BdCIPK31, a Calcineurin B-Like Protein-Interacting Protein Kinase, Regulates Plant Response to Drought and Salt Stress. Front. Plant Sci. [Internet]. 2017 Jul 7;8. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5500663/

Lv F, Zhang H, Xia X, Yin W. Expression profiling and functional characterization of a CBL-interacting protein kinase gene from Populus euphratica. Plant Cell Rep. 2014 May 1;33(5):807–18.

Lv S, Jiang P, Chen X, Fan P, Wang X, Li Y. Multiple compartmentalization of sodium conferred salt tolerance in Salicornia europaea. Plant Physiol. Biochem. 2012 Feb 1;51:47–52.

Ma JF. Role of silicon in enhancing the resistance of plants to biotic and abiotic stresses. Soil Sci. Plant Nutr. 2004;50(1):11–8.

Ma L, Zhang H, Sun L, Jiao Y, Zhang G, Miao C, et al. NADPH oxidase AtrbohD and AtrbohF function in ROS-dependent regulation of Na+/K+ homeostasis in Arabidopsis under salt stress. J. Exp. Bot. 2012 Jan 1;63(1):305–17.

Maas EV, Grieve CM. Sodium-induced calcium deficiency in salt-stressed corn. Plant Cell Environ. 1987 Sep 1;10(7):559–64.

Maathuis FJM. Sodium in plants: perception, signalling, and regulation of sodium fluxes. J. Exp. Bot. 2014 Mar 1;65(3):849–58.

Maeshima M. TONOPLAST TRANSPORTERS: Organization and Function. Annu. Rev. Plant Physiol. Plant Mol. Biol. 2001;52(1):469–97.

Maischak H, Zimmermann MR, Felle HH, Boland W, Mithöfer A. Alamethicin-induced electrical long distance signaling in plants. Plant Signal. Behav. 2010 Aug 1;5(8):988–90.

Marioni JC, Mason CE, Mane SM, Stephens M, Gilad Y. RNA-seq: An assessment of technical reproducibility and comparison with gene expression arrays. Genome Res. 2008 Sep 1;18(9):1509–17.

Marti-Renom MA. Alignment of protein sequences by their profiles. Protein Sci. 2004 Apr 1;13(4):1071–87.

Massingham T, Goldman N. Detecting Amino Acid Sites Under Positive Selection and Purifying Selection. Genetics. 2005 Mar;169(3):1753–62.

McIntyre LM, Lopiano KK, Morse AM, Amin V, Oberg AL, Young LJ, et al. RNA-seq: technical variability and sampling. BMC Genomics. 2011 Jun 6;12(1):293.

McLaren W, Gil L, Hunt SE, Riat HS, Ritchie GRS, Thormann A, et al. The Ensembl Variant Effect Predictor. Genome Biol. [Internet]. 2016 Jun 6 [cited 2018 Feb 5];17. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4893825/

Meena MK, Ghawana S, Dwivedi V, Roy A, Chattopadhyay D. Expression of chickpea CIPK25 enhances root growth and tolerance to dehydration and salt stress in transgenic tobacco. Front. Plant Sci. [Internet]. 2015 [cited 2018 Feb 20];6. Available from: https://www.frontiersin.org/articles/10.3389/fpls.2015.00683/full

Mian A, Oomen RJFJ, Isayenkov S, Sentenac H, Maathuis FJM, Véry A-A. Over-expression of an Na+- and K+-permeable HKT transporter in barley improves salt tolerance. Plant J. 2011 Nov 1;68(3):468–79.

Miller G, Suzuki N, Ciftci-Yilmaz S, Mittler R. Reactive oxygen species homeostasis and signalling during drought and salinity stresses. Plant Cell Environ. 2010 Apr 1;33(4):453–67.

Miransari M, Rangbar B, Khajeh K, Tehranchi MM, Azad RR, Nagafi F, et al. Salt Stress and MAPK Signaling in Plants. In: Ahmad P, Azooz MM, Prasad MNV, editors. Salt Stress Plants [Internet]. Springer New York; 2013 [cited 2017 Apr 3]. p. 157–73. Available from: http://link.springer.com/chapter/10.1007/978-1-4614-6108-1_7

Mishra S, Singh B, Panda K, Singh BP, Singh N, Misra P, et al. Association of SNP Haplotypes of HKT Family Genes with Salt Tolerance in Indian Wild Rice Germplasm. Rice. 2016 Mar 29;9(1):15.

Mittler R, Blumwald E. The Roles of ROS and ABA in Systemic Acquired Acclimation. Plant Cell. 2015 Jan 1;27(1):64–70.

Mittler R, Vanderauwera S, Suzuki N, Miller G, Tognetti VB, Vandepoele K, et al. ROS signaling: the new wave? Trends Plant Sci. 2011 Jun;16(6):300–9.

Miyazawa S. Superiority of a mechanistic codon substitution model even for protein sequences in Phylogenetic analysis. BMC Evol. Biol. 2013 Nov 21;13(1):257.

Møller IS, Gilliham M, Jha D, Mayo GM, Roy SJ, Coates JC, et al. Shoot Na+ Exclusion and Increased Salinity Tolerance Engineered by Cell Type–Specific Alteration of Na+ Transport in Arabidopsis. Plant Cell. 2009 Jul 1;21(7):2163–78.

Møller IS, Tester M. Salinity tolerance of Arabidopsis: a good model for cereals? Trends Plant Sci. 2007 Dec 1;12(12):534–40.

Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat. Methods. 2008 Jul;5(7):621–8.

Moustafa K, AbuQamar S, Jarrar M, Al-Rajab AJ, Trémouillaux-Guiller J. MAPK cascades and major abiotic stresses. Plant Cell Rep. 2014 Aug 1;33(8):1217–25.

Müller T, Spang R, Vingron M. Estimating Amino Acid Substitution Models: A Comparison of Dayhoff’s Estimator, the Resolvent Approach and a Maximum Likelihood Method. Mol. Biol. Evol. 2002 Jan 1;19(1):8–13.

Munns R. Genes and salt tolerance: bringing them together. New Phytol. 2005 Sep 1;167(3):645–63.

Munns R. Approaches to increasing the salt tolerance of wheat and other cereals. J. Exp. Bot. 2006 Mar 2;57(5):1025–43.

Munns R, Gilliham M. Salinity tolerance of crops – what is the cost? New Phytol. 2015 Nov 1;208(3):668–73.

Munns R, James RA, Gilliham M, Flowers TJ, Colmer TD. Tissue tolerance: an essential but elusive trait for salt-tolerant crops. Funct. Plant Biol. 2016 Dec 2;43(12):1103–13.

Munns R, James RA, Xu B, Athman A, Conn SJ, Jordans C, et al. Wheat grain yield on saline soils is improved by an ancestral Na^{+} transporter gene. Nat. Biotechnol. 2012 Apr;30(4):360.

Munns R, Tester M. Mechanisms of Salinity Tolerance. Annu. Rev. Plant Biol. 2008 Jun;59(1):651–81.

Munoz-Amatriain M, Cuesta-Marcos A, Hayes PM, Muehlbauer GJ. Barley genetic variation: implications for crop improvement. Brief. Funct. Genomics. 2014 Jul 1;13(4):341–50.

Naser V, Shani E. Auxin response under osmotic stress. Plant Mol. Biol. 2016 Aug 1;91(6):661–72.

Nei M, Kumar S. Molecular Evolution and Phylogenetics. 1 edition. Oxford; New York: Oxford University Press; 2000.

Notredame C, Higgins DG, Heringa J. T-coffee: a novel method for fast and accurate multiple sequence alignment. J. Mol. Biol. 2000 Sep 8;302(1):205–17.

O'Fallon BD, Wooderchak-Donahue W, Crockett DK. A support vector machine for identification of single-nucleotide polymorphisms from next-generation sequencing data. Bioinformatics. 2013 Jun 1;29(11):1361–6.

Ohta M, Guo Y, Halfter U, Zhu J-K. A novel domain in the protein kinase SOS2 mediates interaction with the protein phosphatase 2C ABI2. Proc. Natl. Acad. Sci. U. S. A. 2003 Sep 30;100(20):11771–6.

de Oliveira AB, Mendes Alencar NL, Gomes-Filho E. Comparison Between the Water and Salt Stress Effects on Plant Growth and Development. In: Akinci S, editor. Responses Org. Water Stress [Internet]. InTech; 2013 [cited 2014 Jun 16]. Available from: http://www.intechopen.com/books/responses-of-organisms-to-water-stress/comparison-between-the-water-and-salt-stress-effects-on-plant-growth-and-development

Olson ND, Lund SP, Colman RE, Foster JT, Sahl JW, Schupp JM, et al. Best practices for evaluating single nucleotide variant calling methods for microbial genomics. Front. Genet. 2015;6:235.

Ozsolak F, Milos PM. RNA sequencing: advances, challenges and opportunities. Nat. Rev. Genet. 2011 Feb;12(2):87.

Pandey GK, Grant JJ, Cheong YH, Kim B-G, Li LG, Luan S. Calcineurin-B-Like Protein CBL9 Interacts with Target Kinase CIPK3 in the Regulation of ABA Response in Seed Germination. Mol. Plant. 2008 Mar 1;1(2):238–48.

Pandey GK, Kanwar P, Singh A, Steinhorst L, Pandey A, Yadav AK, et al. CBL-interacting protein kinase, CIPK21, regulates osmotic and salt stress responses in Arabidopsis. Plant Physiol. 2015 Jan 1;pp.00623.2015.

Pareek A, Sopory SK, Bohnert HJ, editors. Abiotic Stress Adaptation in Plants [Internet]. Dordrecht: Springer Netherlands; 2010 [cited 2014 Sep 2]. Available from: http://link.springer.com/10.1007/978-90-481-3112-9

Parkhomchuk D, Borodina T, Amstislavskiy V, Banaru M, Hallen L, Krobitsch S, et al. Transcriptome analysis by strand-specific sequencing of complementary DNA. Nucleic Acids Res. 2009 Oct 1;37(18):e123–e123.

Pearson K. Notes on the History of Correlation. Biometrika. 1920;13(1):25–45.

Pei J. Multiple protein sequence alignment. Curr. Opin. Struct. Biol. 2008 Jun;18(3):382–6.

Peleg Z, Blumwald E. Hormone balance and abiotic stress tolerance in crop plants. Curr. Opin. Plant Biol. 2011 Jun 1;14(3):290–5.

Piao H, Xuan Y, Park SH, Je BI, Park SJ, Park SH, et al. OsCIPK31, a CBL-interacting protein kinase is involved in germination and seedling growth under abiotic stress conditions in rice plants. Mol. Cells. 2010;30(1):19–27.

Pinto da Costa J, Soares C. A Weighted Rank Measure of Correlation. Aust. N. Z. J. Stat. 2005 Dec 1;47(4):515–29.

Pirooznia M, Kramer M, Parla J, Goes FS, Potash JB, McCombie WR, et al. Validation and assessment of variant calling pipelines for next-generation sequencing. Hum. Genomics. 2014 Jul 30;8(1):14.

Platten JD, Cotsaftis O, Berthomieu P, Bohnert H, Davenport RJ, Fairbairn DJ, et al. Nomenclature for HKT transporters, key determinants of plant salinity tolerance. Trends Plant Sci. 2006;11(8):372–374.

Polyanovsky VO, Roytberg MA, Tumanyan VG. Comparative analysis of the quality of a global algorithm and a local algorithm for alignment of two sequences. Algorithms Mol. Biol. 2011 Oct 27;6(1):25.

Popescu SC, Popescu GV, Bachan S, Zhang Z, Gerstein M, Snyder M, et al. MAPK target networks in Arabidopsis thaliana revealed using functional protein microarrays. Genes Dev. 2009 Jan 1;23(1):80–92.

Qin Y, L i X, Guo M, Deng K, Lin J, Tang D, et al. Regulation of salt and ABA responses by CIPK14, a calcium sensor interacting protein kinase in Arabidopsis. Sci. China Ser. C Life Sci. Chin. Acad. Sci. 2008 May;51(5):391–401.

Qiu J, Henderson SW, Tester M, Roy SJ, Gilliham M. SLAH1, a homologue of the slow type anion channel SLAC1, modulates shoot Cl − accumulation and salt tolerance in Arabidopsis thaliana. J. Exp. Bot. 2016 Aug 1;67(15):4495–505.

Qiu L, Wu D, Ali S, Cai S, Dai F, Jin X, et al. Evaluation of salinity tolerance and analysis of allelic function of HvHKT1 and HvHKT2 in Tibetan wild barley. Theor. Appl. Genet. 2011 Mar 1;122(4):695–703.

Qiu Q-S, Guo Y, Dietrich MA, Schumaker KS, Zhu J-K. Regulation of SOS1, a plasma membrane Na+/H+ exchanger in Arabidopsis thaliana, by SOS2 and SOS3. Proc. Natl. Acad. Sci. U. S. A. 2002 Jun 11;99(12):8436–41.

Quinn EM, McManus R. Quality Control and Analysis of NGS RNA Sequencing Data. Celiac Dis. [Internet]. Humana Press, New York, NY; 2015 [cited 2018 Feb 3]. p. 217–32. Available from: https://link.springer.com/protocol/10.1007/978-1-4939-2839-2_18

R Core Team. R: A Language and Environment for Statistical Computing [Internet]. Vienna, Austria: R Foundation for Statistical Computing; 2014. Available from: http://www.R-project.org

Rafalski A. Applications of single nucleotide polymorphisms in crop genetics. Curr. Opin. Plant Biol. 2002 Apr 1;5(2):94–100.

Raghavendra AS, Gonugunta VK, Christmann A, Grill E. ABA perception and signalling. Trends Plant Sci. 2010 Jul 1;15(7):395–401.

Ravasz E, Somera AL, Mongru DA, Oltvai ZN, Barabási A-L. Hierarchical Organization of Modularity in Metabolic Networks. Science. 2002 Aug 30;297(5586):1551–5.

Reddy MBN. Basics for the Construction of Phylogenetic Trees. WebmedCentral Biol. [Internet]. 2011 [cited 2014 May 21];2(12). Available from: http://archives.webmedcentral.com:8080/xmlui/handle/20461690/1064

Reddy R. A Comparison of Methods: Normalizing High-Throughput RNA Sequencing Data. bioRxiv. 2015;026062.

Reeck GR, de Haën C, Teller DC, Doolittle RF, Fitch WM, Dickerson RE, et al. “Homology” in proteins and nucleic acids: A terminology muddle and a way out of it. Cell. 1987 Aug 28;50(5):667.

Ren Z-H, Gao J-P, Li L-G, Cai X-L, Huang W, Chao D-Y, et al. A rice quantitative trait locus for salt tolerance encodes a sodium transporter. Nat. Genet. 2005 Oct;37(10):1141–6.

Rengasamy P. World salinization with emphasis on Australia. J. Exp. Bot. 2006 Mar 1;57(5):1017–23.

Rengasamy P. Soil processes affecting crop production in salt-affected soils. Funct. Plant Biol. 2010 Jul 23;37(7):613–20.

Richards RA, Hunt JR, Kirkegaard JA, Passioura JB. Yield improvement and adaptation of wheat to water-limited environments in Australia—a case study. Crop Pasture Sci. 2014 Aug 28;65(7):676–89.

Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. 2015 Jan 20;gkv007.

Robertson M, Kirkegaard J, Rebetzke G, Llewellyn R, Wark T. Prospects for yield improvement in the Australian wheat industry: a perspective. Food Energy Secur. 2016 May 1;5(2):107–22.

Robinson M, Oshlack A, others. A scaling normalization method for differential expression analysis of RNA-seq data. Genome Biol. 2010a;11(3):R25.

Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinforma. Oxf. Engl. 2010b Jan 1;26(1):139–40.

Robinson MD, Smyth GK. Small-sample estimation of negative binomial dispersion, with applications to SAGE data. Biostatistics. 2008 Apr 1;9(2):321–32.

Rodriguez MCS, Petersen M, Mundy J. Mitogen-Activated Protein Kinase Signaling in Plants. Annu. Rev. Plant Biol. 2010;61(1):621–49.

Roy SJ, Huang W, Wang XJ, Evrard A, Schmöckel SM, Zafar ZU, et al. A novel protein kinase involved in Na+ exclusion revealed from positional cloning. Plant Cell Environ. 2013 Mar 1;36(3):553–68.

Roy SJ, Negrão S, Tester M. Salt resistant crop plants. Curr. Opin. Biotechnol. 2014 Apr;26:115–24.

Rubio F, Gassmann W, Schroeder JI. Sodium-Driven Potassium Uptake by the Plant Potassium Transporter HKT1 and Mutations Conferring Salt Tolerance. Science. 1995;270(5242):1660–3.

Ryu H, Cho Y-G. Plant hormones in salt stress tolerance. J. Plant Biol. 2015 Jun;58(3):147–55.

Sairam RK, Tyagi A. Physiology and molecular biology of salinity stress tolerance in plants. Curr. Sci.-BANGALORE-. 2004;86(3):407–421.

Saitou N, Imanishi T. Relative efficiencies of the Fitch-Margoliash, maximum-parsimony, maximum-likelihood, minimum-evolution, and neighbor-joining methods of phylogenetic tree construction in obtaining the correct tree. Mol Biol Evol. 1989;6(5):514–525.

Sanchez-Barrena MJ, Fujii H, Angulo I, Martinez-Ripoll M, Zhu J-K, Albert A. The Structure of the C-Terminal Domain of the Protein Kinase AtSOS2 Bound to the Calcium Sensor AtSOS3. Mol. Cell. 2007 May 11;26(3):427–35.

Sanchez-Barrena MJ, Martinez-Ripoll M, Albert A. Structural Biology of a Major Signaling Network that Regulates Plant Abiotic Stress: The CBL-CIPK Mediated Pathway. Int. J. Mol. Sci. 2013 Mar 12;14(3):5734–49.

Santner A, Estelle M. Recent advances and emerging trends in plant hormone signalling. Nature. 2009 Jun 25;459(7250):1071–8.

Santner A, Estelle M. The ubiquitin-proteasome system regulates plant hormone signaling. Plant J. 2010 Mar 1;61(6):1029–40.

Saxena RK, Edwards D, Varshney RK. Structural variations in plant genomes. Brief. Funct. Genomics. 2014 Jul;13(4):296–307.

Schachtman DP, Schroeder JI. Structure and transport mechanism of a high-affinity potassium uptake transporter from higher plants. Nature. 1994 Aug 25;370(6491):655–8.

Schilling RK, Tester M, Marschner P, Plett DC, Roy SJ. AVP1: One Protein, Many Roles. Trends Plant Sci. 2017 Feb 1;22(2):154–62.

Schmöckel SM, Garcia AF, Berger B, Tester M, Webb AAR, Roy SJ. Different NaCl-Induced Calcium Signatures in the Arabidopsis thaliana Ecotypes Col-0 and C24. PLoS ONE. 2015 Feb 27;10(2):e0117564.

Schumacher K, Krebs M. The V-ATPase: small cargo, large effects. Curr. Opin. Plant Biol. 2010 Dec;13(6):724–30.

Sen PK. Estimates of the Regression Coefficient Based on Kendall's Tau. J. Am. Stat. Assoc. 1968 Dec 1;63(324):1379–89.

Seyednasrollah F, Laiho A, Elo LL. Comparison of software packages for detecting differential expression in RNA-seq studies. Brief. Bioinform. 2013 Dec 2;bbt086.

Shabala L, Cuin TA, Newman IA, Shabala S. Salinity-induced ion flux patterns from the excised roots of <Emphasis Type="Italic">Arabidopsis sos</Emphasis> mutants. Planta. 2005 Dec 1;222(6):1041–50.

Shabala S. Learning from halophytes: physiological basis and strategies to improve abiotic stress tolerance in crops. Ann. Bot. 2013 Oct 1;mct205.

Shabala S, Wu H, Bose J. Salt stress sensing and early signalling events in plant roots: Current knowledge and hypothesis. Plant Sci. 2015 Dec;241:109–19.

Shang J, Zhu F, Vongsangnak W, Tang Y, Zhang W, Shen B. Evaluation and Comparison of Multiple Aligners for Next-Generation Sequencing Data Analysis [Internet]. BioMed Res. Int. 2014 [cited 2017 Dec 12]. Available from: https://www.hindawi.com/journals/bmri/2014/309650/

Sheng Q, Vickers K, Zhao S, Wang J, Samuels DC, Koues O, et al. Multi-perspective quality control of Illumina RNA sequencing data analysis. Brief. Funct. Genomics. 2017 Jul 1;16(4):194–204.

Shi H, Ishitani M, Kim C, Zhu J-K. The Arabidopsis thaliana salt tolerance gene SOS1 encodes a putative Na+/H+ antiporter. Proc. Natl. Acad. Sci. 2000 Jun 6;97(12):6896–901.

Shi H, Quintero FJ, Pardo JM, Zhu J-K. The Putative Plasma Membrane Na+/H+ Antiporter SOS1 Controls Long-Distance Na+ Transport in Plants. Plant Cell. 2002a Feb;14(2):465–77.

Shi H, Xiong L, Stevenson B, Lu T, Zhu J-K. The Arabidopsis *salt overly sensitive 4* Mutants Uncover a Critical Role for Vitamin B6 in Plant Salt Tolerance. Plant Cell Online. 2002b Mar 1;14(3):575–88.

Shi H, Zhu J-K. *SOS4*, A Pyridoxal Kinase Gene, Is Required for Root Hair Development in Arabidopsis. Plant Physiol. 2002 Jun 1;129(2):585–93.

Šmarda P, Bureš P, Horová L, Leitch IJ, Mucina L, Pacini E, et al. Ecological and evolutionary significance of genomic GC content diversity in monocots. Proc. Natl. Acad. Sci. U. S. A. 2014 Sep 30;111(39):E4096–102.

Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat. Appl. Genet. Mol. Biol. 2004;3:Article3.

Sofia A, de Almeida AM, da Silva AB, da Silva JM, Paula A, Santos D, et al. Abiotic Stress Responses in Plants: Unraveling the Complexity of Genes and Networks to Survive. In: Vahdati K, editor. Abiotic Stress - Plant Responses Appl. Agric. [Internet]. InTech; 2013 [cited 2014 Apr 22]. Available from: http://www.intechopen.com/books/abiotic-stress-plant-responses-and-applications-in-agriculture/abiotic-stress-responses-in-plants-unraveling-the-complexity-of-genes-and-networks-to-survive

Soltis DE, Soltis PS. The Role of Phylogenetics in Comparative Genetics. Plant Physiol. 2003 Aug;132(4):1790–800.

Sooknanan R, Pease J, Doyle K. Novel methods for rRNA removal and directional, ligation-free RNA-seq library preparation. Nat. Methods. 2010;7(10).

Spearman C. “General Intelligence,” Objectively Determined and Measured. Am. J. Psychol. 1904;15(2):201–92.

Swarbreck SM, Colaço R, Davies JM. Plant Calcium-Permeable Channels. Plant Physiol. 2013 Oct 1;163(2):514–22.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol. Biol. Evol. 2013 Dec 1;30(12):2725–9.

Tarazona S, García-Alcalde F, Dopazo J, Ferrer A, Conesa A. Differential expression in RNA-seq: A matter of depth. Genome Res. 2011 Sep 8;gr.124321.111.

Teakle NL, Tyerman SD. Mechanisms of Cl^- transport contributing to salt tolerance. Plant Cell Environ. 2010 Apr;33(4):566–89.

Telem RS, Wani SH, Singh NB, Sadhukhan R, Mandal N. Single Nucleotide Polymorphism (SNP) Marker for Abiotic Stress Tolerance in Crop Plants. Adv. Plant Breed. Strateg. Agron. Abiotic Biot. Stress Traits [Internet]. Springer, Cham; 2016 [cited 2017 Oct 17]. p. 327–43. Available from: https://link.springer.com/chapter/10.1007/978-3-319-22518-0_9

Tester M, Langridge P. Breeding Technologies to Increase Crop Production in a Changing World. Science. 2010 Feb 12;327(5967):818–22.

Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994 Nov 11;22(22):4673–80.

Tilbrook J, Schilling RK, Berger B, Garcia AF, Trittermann C, Coventry S, et al. Variation in shoot tolerance mechanisms not related to ion toxicity in barley. Funct. Plant Biol. 2017 Nov 29;44(12):1194–206.

Tourasse NJ, Li W-H. Selective Constraints, Amino Acid Composition, and the Rate of Protein Evolution. Mol. Biol. Evol. 2000 Apr 1;17(4):656–64.

Trapnell C, Hendrickson DG, Sauvageau M, Goff L, Rinn JL, Pachter L. Differential analysis of gene regulation at transcript resolution with RNA-seq. Nat. Biotechnol. 2013 Jan;31(1):46–53.

Trapnell C, Pachter L, Salzberg SL. TopHat: discovering splice junctions with RNA-Seq. Bioinformatics. 2009 May 1;25(9):1105–11.

Tuteja N. Abscisic Acid and Abiotic Stress Signaling. Plant Signal. Behav. 2007;2(3):135–8.

Tuteja N, Mahajan S. Calcium Signaling Network in Plants. Plant Signal. Behav. 2007;2(2):79–85.

Tyerman SD, Skerrett M, Garrill A, Findlay GP, Leigh RA. Pathways for the permeation of Na+ and Cl− into protoplasts derived from the cortex of wheat roots. J. Exp. Bot. 1997 Mar 1;48(Special_Issue):459–80.

Valenzuela CE, Acevedo-Acevedo O, Miranda GS, Vergara-Barros P, Holuigue L, Figueroa CR, et al. Salt stress response triggers activation of the jasmonate signaling pathway leading to inhibition of cell elongation in Arabidopsis primary root. J. Exp. Bot. 2016 May 23;erw202.

Vergara IA, Frech C, Chen N. CooVar: Co-occurring variant analyzer. BMC Res. Notes. 2012 Nov 1;5:615.

Voelker C, Schmidt D, Mueller-Roeber B, Czempinski K. Members of the Arabidopsis AtTPK/KCO family form homomeric vacuolar channels in planta. Plant J. 2006 Oct 1;48(2):296–306.

Wagner GP, Kin K, Lynch VJ. Measurement of mRNA abundance using RNA-seq data: RPKM measure is inconsistent among samples. Theory Biosci. 2012 Dec 1;131(4):281–5.

Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res. 2010a Sep;38(16):e164.

Wang K, Singh D, Zeng Z, Coleman SJ, Huang Y, Savich GL, et al. MapSplice: Accurate mapping of RNA-seq reads for splice junction discovery. Nucleic Acids Res. 2010b Oct 1;38(18):e178–e178.

Wang L, Liu Y, Cai G, Jiang S, Pan J, Li D. Ectopic expression of ZmSIMK1 leads to improved drought tolerance and activation of systematic acquired resistance in transgenic tobacco. J. Biotechnol. 2014 Feb 20;172:18–29.

Wang L, Si Y, Dedow LK, Shao Y, Liu P, Brutnell TP. A Low-Cost Library Construction Protocol and Data Analysis Pipeline for Illumina-Based Strand-Specific Multiplex RNA-Seq. PLOS ONE. 2011 Oct 19;6(10):e26426.

Wang R-K, Li L-L, Cao Z-H, Zhao Q, Li M, Zhang L-Y, et al. Molecular cloning and functional characterization of a novel apple MdCIPK6L gene reveals its involvement in multiple abiotic stress tolerance in transgenic plants. Plant Mol. Biol. 2012 May 1;79(1–2):123–35.

Wang S, Su SZ, Wu Y, Li SP, Shan XH, Liu HK, et al. Overexpression of maize chloride channel gene <Emphasis Type="Italic">ZmCLC-d</Emphasis> in <Emphasis Type="Italic">Arabidopsis thaliana</Emphasis> improved its stress resistance. Biol. Plant. 2015 Jan 1;59(1):55–64.

Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. Nat. Rev. Genet. 2009 Jan;10(1):57–63.

Wani SH, Hossain MA, editors. Managing salt tolerance in plants: molecular and genomic perspectives. Boca Raton London New York: CRC Press, Taylor & Francis Group, CRC Press is an imprint of the Taylor & Francis Group, an informa business; 2015.

Wasternack C, Hause B. Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in Annals of Botany. Ann. Bot. 2013 Jun 1;111(6):1021–58.

Wei P, Wang L, Liu A, Yu B, Lam H-M. GmCLC1 Confers Enhanced Salt Tolerance through Regulating Chloride Accumulation in Soybean. Front. Plant Sci. [Internet]. 2016 Jul 25 [cited 2017 Apr 5];7. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4959425/

Wei QJ, Gu QQ, Wang NN, Yang CQ, Peng SA. Molecular cloning and characterization of the chloride channel gene family in trifoliate orange. Biol. Plant. 2015 Dec 1;59(4):645–53.

Weinl S, Kudla J. The CBL–CIPK Ca2+-decoding signaling network: function and perspectives. New Phytol. 2009 Nov 1;184(3):517–28.

Wilkinson S, Davies WJ. Drought, ozone, ABA and ethylene: new insights from cell to plant to community. Plant Cell Environ. 2010 Apr 1;33(4):510–25.

Williams CR, Baccarella A, Parrish JZ, Kim CC. Trimming of sequence reads alters RNA-Seq gene expression estimates. BMC Bioinformatics. 2016;17:103.

Wu P-Y, Phan JH, Zhou F, Wang MD. Evaluation of Normalization Methods for RNA-Seq Gene Expression Estimation. IEEE Int. Conf. Bioinforma. Biomed. Workshop IEEE Int. Conf. Bioinforma. Biomed. 2011 Nov;2011:50–7.

X W, K E, T U, Qs Q. AtNHX5 and AtNHX6 Are Required for the Subcellular Localization of the SNARE Complex That Mediates the Trafficking of Seed Storage Proteins in Arabidopsis. PloS One PLoS ONE. 2016;11, 11(3, 3):e0151658–e0151658.

Xiang Y, Huang Y, Xiong L. Characterization of Stress-Responsive CIPK Genes in Rice for Stress Tolerance Improvement. Plant Physiol. 2007 Jul;144(3):1416–28.

Xu J, Li H-D, Chen L-Q, Wang Y, Liu L-L, He L, et al. A Protein Kinase, Interacting with Two Calcineurin B-like Proteins, Regulates K+ Transporter AKT1 in Arabidopsis. Cell. 2006 Jun 30;125(7):1347–60.

Yamaguchi T, Blumwald E. Developing salt-tolerant crop plants: challenges and opportunities. Trends Plant Sci. 2005 Dec;10(12):615–20.

Yang C, Zhao L, Zhang H, Yang Z, Wang H, Wen S, et al. Evolution of physiological responses to salt stress in hexaploid wheat. Proc. Natl. Acad. Sci. U. S. A. 2014 Aug 12;111(32):11882–7.

Yang Y, Tang R-J, Jiang C-M, Li B, Kang T, Liu H, et al. Overexpression of the PtSOS2 gene improves tolerance to salt stress in transgenic poplar plants. Plant Biotechnol. J. 2015 Sep 1;13(7):962–73.

Yang Z, Bielawski JP. Statistical methods for detecting molecular adaptation. Trends Ecol. Evol. 2000 Dec 1;15(12):496–503.

Yang Z, Nielsen R, Hasegawa M. Models of amino acid substitution and applications to mitochondrial protein evolution. Mol. Biol. Evol. 1998;15(12):1600–1611.

Yao X, Horie T, Xue S, Leung H-Y, Katsuhara M, Brodsky DE, et al. Differential Sodium and Potassium Transport Selectivities of the Rice OsHKT2;1 and OsHKT2;2 Transporters in Plant Cells. Plant Physiol. 2010 Jan;152(1):341–55.

Yousfi S, Wissal M, Mahmoudi H, Abdelly C, Gharsalli M. Effect of salt on physiological responses of barley to iron deficiency. Plant Physiol. Biochem. 2007 May 1;45(5):309–14.

Yu Y, Xia X, Yin W, Zhang H. Comparative genomic analysis of CIPK gene family in Arabidopsis and Populus. Plant Growth Regul. 2007 Jun 1;52(2):101–10.

Yuan F, Yang H, Xue Y, Kong D, Ye R, Li C, et al. OSCA1 mediates osmotic-stress-evoked Ca^{2+} increases vital for osmosensing in *Arabidopsis*. Nature. 2014 Aug 27;514(7522):367.

Zhang B, Horvath S. A general framework for weighted gene co-expression network analysis. Stat. Appl. Genet. Mol. Biol. [Internet]. 2005 [cited 2014 Jun 18];4(1). Available from: http://www.degruyter.com/view/j/sagmb.2005.4.1/sagmb.2005.4.1.1128/sagmb.2005.4.1.1128.xml

Zhang X, Yin H, Chen S, He J, Guo S. Changes in Antioxidant Enzyme Activity and Transcript Levels of Related Genes in *Limonium sinense* Kuntze Seedlings under NaCl Stress. J. Chem. 2014 Jun 19;2014:e749047.

Zhang Y, Linghu J, Wang D, Liu X, Yu A, Li F, et al. Foxtail Millet CBL4 (SiCBL4) Interacts with SiCIPK24, Modulates Plant Salt Stress Tolerance. Plant Mol. Biol. Report. 2017 Oct 18;1–13.

Zhao S, Zhang Y, Gordon W, Quan J, Xi H, Du S, et al. Comparison of stranded and non-stranded RNA-seq transcriptome profiling and investigation of gene overlap. BMC Genomics. 2015 Sep 3;16:675.

Zhao Y, Wang T, Zhang W, Li X. SOS3 mediates lateral root development under low salt stress through regulation of auxin redistribution and maxima in Arabidopsis. New Phytol. 2011 Mar 1;189(4):1122–34.

Zheng D, Han X, An Y, Guo H, Xia X, Yin W. The nitrate transporter NRT2.1 functions in the ethylene response to nitrate deficiency in Arabidopsis. Plant Cell Environ. 2013 Jul 1;36(7):1328–37.

Zhu J-K. Plant salt tolerance. Trends Plant Sci. 2001 Feb 1;6(2):66–71.

Zhu J-K. Salt and Drought Stress Signal Transduction in Plants. Annu. Rev. Plant Biol. 2002;53:247–73.

Zhu J-K. Regulation of ion homeostasis under salt stress. Curr. Opin. Plant Biol. 2003 Oct;6(5):441–5.

Zhu J-K. Abiotic Stress Signaling and Responses in Plants. Cell. 2016 Oct 6;167(2):313–24.

Zhu M, Zhou M, Shabala L, Shabala S. Physiological and molecular mechanisms mediating xylem Na+ loading in barley in the context of salinity stress tolerance. Plant Cell Environ. 2017 Jul 1;40(7):1009–20.

Zielinski RE. CALMODULIN AND CALMODULIN-BINDING PROTEINS IN PLANTS. Annu. Rev. Plant Physiol. Plant Mol. Biol. 1998 Jun;49:697–725.

Zvelebil MJ, Baum JO. Understanding Bioinformatics. Garland Science; 2008.

Picard Tools - By Broad Institute [Internet]. [cited 2018 Feb 20]. Available from: http://broadinstitute.github.io/picard/

Chapter 3 Molecular Components of the AtCIPK16 Mediated Salt Stress Response

Statement of Authorship

Title of Paper	Molecular Components of the *AtCIPK16* Mediated Salt Stress Response
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	**Amarasinghe, S., Watson-Haigh, N.S., Gilliham, M., Roy, S., and Baumann, U.,** This is an original research article on underlying molecular mechanisms in transgenic Arabidopsis plants with CIPK16 (*AtCIPK16*) overexpression. From a forward genetic approach the *CIPK16* gene from Arabidopsis (*AtCIPK16*) has been identified to be involved in enhanced Na^{+} tolerance both in Arabidopsis and barley. This investigation suggest the involvement of hormones such as ethylene, jasmonic acid and auxin in the initial phase of the salinity response in transgenic plants and the possibility of the early adaptation of transgenics to the new unfavourable conditions as a mode of salt tolerance. This article is closely related to the subject matter of this thesis.

Principal Author

Name of Principal Author (Candidate)	Shanika Lakmini Amarasinghe		
Contribution to the Paper	Conceived and designed the experiment, performed literature research, data analysis, critical interpretation and wrote the manuscript		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	01/02/2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Nathan S. Watson-Haigh		
Contribution to the Paper	Study concepts and design. Provided critical comments. Supervised the experiment. Edited the manuscript. I hereby certify that the statement of the contribution is accurate.		
Signature		Date	01/02/2018

Name of Co-Author	Matthew Gilliham		
Contribution to the Paper	Conceived the experiment. Provided comments and edited the manuscript. I hereby certify that the statement of the contribution is accurate.		
Signature		Date	01/02/2018

Name of Co-Author	Stuart J. Roy		
Contribution to the Paper	Conceived the experiment. Provided comments and edited the manuscript. I hereby certify that the statement of the contribution is accurate.		
Signature		Date	01/02/2018

Name of Co-Author	Ute Baumann		
Contribution to the Paper	Conceived Supervised the experiment. Provided critical comments. Edited the manuscript. I hereby certify that the statement of the contribution is accurate.		
Signature		Date	01/02/2018

Link to Chapter 3

A novel QTL for Na^+ exclusion has been discovered on chromosome 2 in a Bay-0 × Shahdara mapping population and by fine mapping, the protein kinase *AtCIPK16* has been exposed to underlie the QTL (Roy et al. 2013). In experiments using transgenic Arabidopsis constitutively overexpressing *AtCIPK16,* researchers have found a significant reduction of shoot Na^+ in plants grown in both soil and hydroponics. Strikingly, transgenic barley constitutively expressing *AtCIPK16* had decreased leaf Na^+ and increased salinity tolerance, providing opportunities for genetic engineering for salinity tolerance in crops (Roy et al. 2013). This indicates the existence of a common pathway between these two evolutionarily diverse plant species that encompass a similar molecular machinery for salt tolerance. However, we are still unaware of the underlying molecular mechanisms of the salinity tolerance elicited in *AtCIPK16* transgenic Arabidopsis.

Understanding the molecular mechanism of *AtCIPK16* mediated salt stress tolerance requires an understanding of the downstream targets, as well as the interactions of AtCIPK16 with other proteins in 3D-space and across a time course. The focus of this chapter is to fill the knowledge gap in our understanding of the downstream molecular components of *AtCIPK16* expression in salt stress. We assume that transgenic Arabidopsis lines show enhanced tolerance at least partly owing to changes in expression levels of downstream targets. We employed the gene expression analysis through RNA-Seq approach to investigate this assumption.

Differential gene expression analysis at different time points for different treatments (e.g. salt treated and non-salt treated) and tissues (e.g. roots and shoots) provided insight with respect to how the overexpression of *AtCIPK16* has changed the plants' transcriptome under saline conditions leading to its direct and indirect consequences. Moreover, we have used gene co-expression analysis, that would provide another layer of information by detecting groups of genes whose expression profiles are fundamentally alike for a biological condition (Kadarmideen et al. 2011). The chapter is formatted in a manuscript format according to the guidelines from *Springer Plant Cell Reports*.

References

Kadarmideen HN, Watson-Haigh NS, Andronicos NM. Systems biology of ovine intestinal parasite resistance: disease gene modules and biomarkers. Mol. Biosyst. 2011;7(1):235.

Roy SJ, Huang W, Wang XJ, Evrard A, Schmöckel SM, Zafar ZU, et al. A novel protein kinase involved in Na+ exclusion revealed from positional cloning. Plant Cell Environ. 2013 Mar 1;36(3):553–68.

Molecular Components of the AtCIPK16 Mediated Salt Stress Response

Running title: Transcriptome of AtCIPK16 mediated salt stress response

Shanika Amarasinghe[1,2], Nathan S. Watson-Haigh[1,2], Matthew Gilliham[2,3], Stuart Roy[1,2], Ute Baumann[1,2]

1. Australian Centre for Plant Functional Genomics, PMB 1, Glen Osmond, SA 5064, Australia

2. School of Agriculture, Food and Wine, Waite Campus, The University of Adelaide, PMB 1 Glen Osmond, SA 5064, Australia.

3. Australian Research Council Centre of Excellence in Plant Energy Biology, Department of Plant Science, School of Agriculture, Food and Wine, University of Adelaide, Glen Osmond SA 5064, Australia.

Correspondence:

Dr. Ute Baumann,
Plant Genomics Centre,
School of Agriculture, Food and Wine,
The University of Adelaide,
Urrbrae,
South Australia,
5064, AUSTRALIA.

Abstract

Soil salinity causes large productivity losses for agriculture worldwide. “Next-generation crops” that can tolerate salt stress are required for the sustainability of global food production. Previous research that attempted to uncover novel plant salinity tolerant capabilities has identified a protein kinase named AtCIPK16 to be involved in enhanced salinity stress response. A comparative transcriptomic study on Arabidopsis lines expressing *AtCIPK16* was conducted in the presence and absence of salt stress, using an RNA-Seq approach. Previously *AtCIPK16* overexpression has shown to be involved in enhanced salinity tolerance through high Na^+ exclusion and increased biomass in both Arabidopsis and barley. In this study, we provide evidence for a possible involvement of a transcription factor, AtTZF1, phytohormones and the ability to quickly reach a new homeostasis as components of the salinity tolerance response in transgenics. Furthermore, we suggest the possibility of both biotic and abiotic tolerance achieved by AtCIPK16 transgenics and propose a model for the salt tolerance pathway elicited through AtCIPK16.

Keywords

Arabidopsis thaliana, salinity tolerance, *AtCIPK16*, RNA-Seq, differential gene expression, gene co-expression, *AtTZF1*, phytohormones

Introduction

Soil salinity has adverse effects on global agricultural production (FAOSTAT, 2014; Rengasamy, 2006, 2010). An estimated 30% of the irrigated land and 6% of the world's total land is affected by salt, and these areas are increasing in size (Schroeder et al., 2013). Estimates put agricultural production losses at 12 billion USD per annum in the US alone (Munns and Gilliham, 2015; Shabala, 2013). Finding crops that can withstand high salinity therefore is a high-priority for achieving sustainable world food production. Salinity imposes two main limitations on plant growth and survival: (i) an initial hyperosmotic stress, and (ii) secondary nutritional imbalance, ionic and oxidative stress through accumulation of high concentrations of Na^+ and Cl^- (Roy et al., 2014). There is extensive research efforts toward understanding the molecular mechanisms that enable salt tolerance with the ultimate goal of developing more salt tolerant crops (Deinlein et al., 2014; Hanin et al., 2016; Munns and Gilliham, 2015; Roy et al., 2014).

Molecular mechanisms involved in salt tolerance in plants, including Arabidopsis, can be broadly classified into the following categories: a) transporters that can reduce influx, or increase efflux or compartmentalization of Na^+/Cl^- ions, or maintain K^+ homeostasis (e.g. SOS, NHX, HKT, AKT, HAK, KAT,CCC, SLAH1) (Bassil et al., 2012; Chen et al., 2007; Diédhiou and Golldack, 2006; Grabov, 2007; Hamamoto et al., 2015; Ji et al., 2013; Qiu et al., 2016; Wang et al., 2015); b) detoxifiers that can scavenge excessive reactive oxygen species (ROS) and alleviate negative effects of ROS (e.g. SOD, APX, AsA, CAT, GPX PrxR) (Baxter et al., 2014; Mittler et al., 2011); c) osmotic adjusters that can maintain low intracellular osmotic potential in plants under salt stress (for example proline, glycine betaine, free amino acids, sugars, polyamines and polyphenols) (Rosa et al., 2009); d) phytohormones that can facilitate a broad array of adaptive responses and long distance signalling (such as abscisic acid, indole acetic acid, cytokinins, gibberellic acid, salicylic acid, brassinosteroids, jasmonates, ethylene) (Fahad et al., 2015; Peleg and Blumwald, 2011; Ryu and Cho, 2015); and e) salt sensors including those that sense cytosolic Ca^{2+} changes resulting from changes in the cytosol due to salinity and communicate the effects to downstream activating proteins (CBLs and CIPKs, CDPKs, CaMs, CAMLs, etc.) (Shabala et al., 2015).

The involvement of CBL-CIPK complexes as signalling components in salt stress has been well established (Hashimoto et al. 2012; Luan 2009; Mao et al. 2016; Thoday-Kennedy et al. 2015). Arabidopsis CIPKs found to be involved in salinity tolerance mechanisms of plants include *CIPK1* (D'Angelo et al., 2006), *CIPK3* (Kim, 2003), *CIPK6* (Tripathi et al., 2009), *CIPK16* (Roy et al., 2013) and *CIPK24* (*SOS2)* (Liu et al., 2000). *AtCIPK16* from *Arabidopsis thaliana* (*At2g25090*) was identified from a forward genetic screen as a gene with a role in reducing Na^+ content in leaves during salt stress (Roy et al., 2013). Therefore *AtCIPK16* is a potential candidate for the genetic engineering of salinity tolerant crops. The knowledge on the mode of action of AtCIPK16 is still largely unknown, however previous studies have shown that AtCIPK16 may get directed to the nucleus (Huang, 2015) and has a nuclear localisation signal (NLS) (Amarasinghe et al., 2016).

The current study is an attempt to fill the gap in our understanding of *AtCIPK16* mediated salt stress tolerance in *A. thaliana.* Through an investigation of the transcriptomic responses in transgenic and null-transgenic plants, as well as a co-expression network analysis, we aimed to identify a set of genes, whose expression is influenced directly or indirectly by *AtCIPK16* overexpression. Our results suggest that the *AtCIPK16* mediated salt tolerance is mainly achieved through transcription factor modulation and phytohormone signalling. We propose a molecular pathway for at least a part of the *AtCIPK16* mediated salt tolerance mechanism for validation in future laboratory experiments.

Results

Determining presence and transgene expression level in *35S:AtCIPK16* expressing Arabidopsis and DNA binding properties of AtCIPK16

Presence of the transgene was determined by using primers designed to the transgene specific 3′ UTR region of the gene. As expected only transgenic plants contained the *AtCIPK16* transgene (S1). *AtCIPK16* transgene expression was higher in transgenic plants compared to native *AtCIPK16* expression in null segregants in the absence of salt stress, and after both 3 and 51 hours of salt stress (Figure 1a). The AtCIPK16 sequence was tested for DNA binding potential based on the postulation that AtCIPK16 activity is within the nucleus. It was identified that the protein region from A^{357} – G^{391} has the ability to bind to DNA (Figure 1b).

Differential Gene Expression

To determine differential gene expression between *AtCIPK16* over-expressing lines and null segregants, RNA was extracted from shoot and root material of 5 week old, hydroponically grown, plants exposed to either 0 or 75 mM NaCl for 3 or 51 hours. RNA-Seq analysis was performed to determine the plants’ gene expression profiles. On average, a mapping percentage of ~88% was reported across root and shoot material collected from both transgenic and null segregants for the 3 hour time point data and a mapping percentage of ~86% for 51 hour time point samples (S2).

A total of 21,974 and 21,160 genes were differentially expressed across the two tissues from 3 hours and 51 hours, respectively, in salt treated *AtCIPK16* transgenic plants compared to the null transgenics. In order to identify the differentially expressed genes in salt stressed plants with *AtCIPK16* overexpression several contrasts were tested (Figure 2) based on the differences in gene expression levels in the roots at 3 hours, shoots at 3 hours, roots at 51 hours and shoots at 51 hours. The number of genes which were up or down-regulated at each of the two time points and in each tissue for each line are shown in Figure 3.

Contrast 1: Transgenic Control Vs Null Control

In the comparison of transgenic control vs null control (transgene-effect in controls; TC) samples, 5 differentially expressed genes (DEG_{TC}) in the roots at 3 hrs ($DEG_{TC(3R)}$) and 160 in the shoots at 3 hrs ($DEG_{TC(3S)}$) were identified (Figure 3a, S4 worksheet 1-2). As expected, *AtCIPK16* (*AT2G25090*) was present in both $DEG_{TC(3R)}$ and $DEG_{TC(3S)}$ (Figure 4a). Most (150) of the genes in $DEG_{TC(3S)}$ had higher expression in transgenics.

At 51 hours, there was only 1 DEG_{TC} in roots (i.e. $DEG_{TC(51R)}$) (Figure 3a; S4 worksheet 3). In shoot controls at 51 hours there were 17 DEG_{TC} (i.e. $DEG_{TC(51S)}$) (Figure 3a; S4 worksheet 4). While there is 1 DEGs in common between the root and the shoot DEG_{TC} at 51 hours, it is not *AtCIPK16* but *AT1G47970* (Figure 4b).

Contrast 2: Transgenic Salt Vs Null Salt

In the comparison of transgenic vs null samples in presence of salt (transgene effect in salt: TS) (Figure 2) There were DEGs (DEG_{TS}) present for this contrast for both tissues at both time points (i.e. $DEG_{TS(3R)}$: 403, $DEG_{TS(3S)}$: 108, $DEG_{TS(51R)}$: 4, $DEG_{TS(51S)}$: 13) (Figure 3b; S4 worksheet 5, 6, 7 and 8). While there was a ~80 fold increase in the $DEG_{TS(3R)}$ compared to $DEG_{TC(3R)}$ (403 vs 5), the $DEG_{TS(3S)}$ remained more or less in the same range as $DEG_{Tc(3S)}$ (160 vs 108) (Figure 3 a and b). However, there were proportionally more down regulated genes in the $DEG_{TS(3S)}$. While only ~6% of the DEGs from $DEG_{TC(3S)}$ were down regulated (10/160), ~37% of $DEG_{TS(3S)}$ were down regulated (40/108). Clearly, in salt stress overexpression of *AtCIPK16* has reduced the expression of genes.

At 51 hours, very low number of DEGs were seen in salt stress in both roots and shoots similar to the observations in non-stressed conditions (4 $DEG_{TS(51R)}$ vs 1 $DEG_{TC(51R)}$ and 13 $DEG_{TS(51S)}$ vs 17 $DEG_{TC(51S)}$) (Figure 3 a and b). There were 10 genes in common between DEG_{TS} of root and shoots at 3 hours (Figure 4c) while there were none at 51 hours (Figure 4d).

Contrast 3: Transgenic Salt Vs Transgenic Control and Contrast 4: Null Salt Vs Null Control

At 3 hours, in both tissues the effect of salt (salt effect on transgenics: ST; Figure 2) elicited differential expression of more genes (DEG_{ST}) in *AtCIPK16* transgenics (i.e. $DEG_{ST(3R):}$ 1696 and $DEG_{ST(3S):}$ 572) compared to the null transgenics (salt effect on nulls: SN; Figure 2) ($DEG_{SN(3R):}$ 849 and $DEG_{SN(3S):}$ 439) (Figure 3 c and d). But at 51 hours it is the opposite; effect of salt elicited the differential expression of fewer genes in *AtCIPK16* transgenics (i.e. $DEG_{ST(51R):}$ 123 and $DEG_{ST(51S):}$ 135) compared to nulls ($DEG_{SN(51R):}$ 1043 and $DEG_{SN(51R):}$ 358) (Figure 3 c and d).

Contrast 5: Interaction between SN and ST

With the presence of DEGs in both ST and SN, genes with significantly different expression levels between these two contrasts were examined through linear modelling of data. The results of contrast 5 are differentially expressed genes due to the absolute effect of transgene in salt stress (INT) (i.e. DEG_{INT}) (Figure 2). Even though for 3 hours there were 231 DEG_{INT} in roots ($DEG_{INT(3R)}$) and 152 DEG_{INT} in shoots ($DEG_{INT(3S)}$), there were no DEG_{INT} for the 51 hours (Figure 3 e; S4 worksheet 9, 10, 11 and 12). Furthermore, there were 9 DEG_{INT} common to both roots and shoots at 3 hour time point (Figure 4e).

Investigating potential biological implications of *AtCIPK16* overexpression

Genes with a significant transgene-effect in controls (5 $DEG_{TC(3R)}$, 160 $DEG_{TC(3S)}$, 1 $DEG_{TC(51R)}$ and 17 $DEG_{TC(51S)}$) (Figure 3a) were further analysed through Gene Ontology (GO) studies and pathway analysis to understand potential biological consequences of *AtCIPK16* overexpression in the non-stressed conditions. GO analysis showed that $DEG_{TC(3S)}$ that are up-regulated were most enriched for response to chitin (p value = 3.47×10^{-92}) (S5; worksheet1 cells with yellow background colour). Perhaps not surprisingly, the corresponding Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways that $DEG_{TC(3S)}$ fell into include plant-pathogen interaction (S6, column B). Additionally, molecular functions such as transcription regulator activity was significant for the $DEG_{TC(3S)}$ that are up-regulated (p value = 7.51×10^{-09}) (S5; worksheet 1 cells with yellow background colour). Down-regulated $DEG_{TC(3S)}$ were enriched for the molecular function of negative regulation of RAS protein signal transduction (p value = 6.95×10^{-04}) and RHO-GTPase binding (p value = 6.95×10^{-04}) (S5, worksheet1 cells with blue background colour).

The significant GO terms found for up-regulated $DEG_{TS(3R)}$ were the biological process response to organic substance (p value = 1.59×10^{-42}) and the molecular function sequence specific DNA binding transcription factor activity (p value = 5.65×10^{-11}) (S5, worksheet 2 cells with yellow background colour). The up-regulated $DEG_{TS(3S)}$ were enriched for cell wall modification involved in abscission (p value = 7.28×10^{-04}) and indole-3-acetic acid amido synthetase activity (p value = 1.52×10^{-03}) (S5, worksheet 3 cells with yellow background colour). The down-regulated $DEG_{TS(3S)}$ were enriched for terms such as cellular response to iron starvation (p value = 7.03×10^{-07}) and iron ion binding (p value = 6.37×10^{-04}) (S5, worksheet3 cells with blue background colour). The molecular functions related to metal binding, interestingly had a focus on calcium ion binding in salt absent shoots (S5, worksheet1 cells with yellow background colour) while it is more DNA and Ferric ion binding for salt stressed roots (S5, worksheet2 cells with yellow background colour) and shoots (S5, worksheet3 cells with yellow background colour), respectively.

The transgene dependent salt responsiveness was investigated for the combined effect of both *AtCIPK16* overexpression and salt on the plant. $DEG_{INT(3R)}$ were enriched for response to chitin (p value = 3.08×10^{-26}) (S5, worksheet 4 biological process). The pathways the $DEG_{INT(3R)}$ fall in included "carbon metabolism", "Phenylpropanoid biosynthesis", "Glyoxylate and dicarboxylate metabolism" and "Galactose metabolism" (S6 column E). Response to carbohydrate stimulus (p value = 5.13×10^{-10}) was a GO category identified for the $DEG_{INT(3S)}$ (S5, worksheet 5 biological process). Peroxidases that are involved in the Phenylpropanoid biosynthesis pathway and genes involved in flavonoid biosynthesis were identified through pathway analysis (S6 column F). Furthermore, Calcium-binding EF-hand motif containing genes involved in plant-pathogen interaction were among the pathways which $DEG_{INT(3S)}$ were grouped into (S6, column F).

Next specific roles of DEGs were investigated in the following functional categories; a) transporters/channels, b) regulation of transcription, c) metal handling, d) enzyme families, e) hormone metabolism and f) signalling pathways (S7).

Transporters/Channels

More transporters were identified as $DEG_{TS(3R)}$ compared to transporter $DEG_{TS(3S)}$ and $DEG_{TC(3S)}$ (S7 worksheet 1, under Transport in S8 a, b, d and e). The transporter genes from the $DEG_{TC(3S)}$ included *SLAH3*. The transporter $DEG_{TS(3R)}$ included several *NRTs*, *CHX17*, *CNGC19*, *root hair specific 2* genes. Furthermore, there are transporters from $DEG_{TS(3R)}$ associated with JAZ proteins that are involved in ubiquitination leading to proteolysis, as well as SAUR protein coding genes involved in cell expansion through auxins (S9 a). Other pathways that $DEG_{TS(3R)}$ belonged to were: Phenylpropanoid biosynthesis and phenylalanine metabolism (S10 a) and "valine, leucine and isoleucine degradation related genes". While transporter $DEG_{TS(3S)}$ are directly or indirectly associated with cell wall biosynthesis, α-Linolenic acid metabolism and Pentose and glucuronate interconversions were associated with $DEG_{INT(3S)}$ (S10 a).

Regulation of Transcription and DNA/DNA Processing

The largest number of DEGs encoding transcription factors (TFs) belonged to $DEG_{TS(3R)}$ (S7 worksheet 2, under regulation of transcription in S8 d). Only five of these TFs were identified in pathways and they fell into plant hormone transduction and plant-pathogen interaction pathways (S10 b). The TFs from $DEG_{TS(3S)}$ were related to limonene and pinene degradation, ubiquitin mediated proteolysis, starch and sucrose metabolism and stilbenoid, diarylheptanoid and gingerol biosynthesis (S10 b). The TF genes from $DEG_{INT(3R)}$ and $DEG_{INT(3S)}$ are directly or indirectly involved in plant-pathogen interactions, starch and sucrose metabolism and plant hormone transduction (S10 b). The hormone signal transduction related genes from $DEG_{INT(3R)}$ are related to auxin, ABA and jasmonic acid (S9 b). RNA synthesis and processing genes were not in $DEG_{INT(3R)}$ while they were in $DEG_{TS(3R)}$ (under RNA synthesis and RNA processing in S8 d and h). $DEG_{TC(51S)}$ contained DNA replication and nucleotide excision repair pathway genes, which included the transcription factor NF-YB11 (*AT2G27470*) (S6 column J).

Metal Synthesis and Assimilation

There are metal related genes within both $DEG_{TS(3R)}$ and $DEG_{TS(3S)}$ which are not in $DEG_{TC(3R)}$ or $DEG_{TC(3S)}$ (under metal handling in S8 a, d and e). Moreover, DEG_{INT} at 3 hours contain metal handling genes that are iron (Fe) related (S7 worksheet 3). Pathways these metal binding DEG_{INT} directly or indirectly modulate include Porphyrin and chlorophyll metabolism (S10 c).

Enzyme Families

There were 'enzyme related' 1 $DEG_{TC(3R)}$ and 2 $DEG_{TC(3S)}$ (S7 worksheet 4, under enzyme families in S8 a). However, there are at least 12 'enzyme related' DEG_{TS} (S7 worksheet 4, under 'enzyme families' in S8 d and e). Enzyme related $DEG_{INT(3R)}$ were fewer compared to those from $DEG_{TS(3R)}$ but the number of enzyme related genes from $DEG_{INT(3S)}$ and $DEG_{TS(3S)}$ were more or less similar (S7 worksheet 4). 'Enzyme family' DEG_{TS} showed associations to genes that fell into pathways of ROS mediation, pathogen interactions and cell growth, and cell wall strengthening (S10 d). Phenylalanine metabolism and phenylpropanoid biosynthesis were seen to be pathways the differentially expressed enzymes of 3 hour DEG_{INT} grouped into (S10 d).

Hormone Metabolism

The hormone related $DEG_{TC(3S)}$ were directly or indirectly involved in ethylene, auxin and brassinosteroid metabolism (S8 j, S9 c). Additionally, 66 of the DEGs from this contrast have putative involvement in biotic stress which include the ethylene signalling related genes and ethylene-responsive element binding protein family genes (S8 q).

Within $DEG_{TS(3R)}$ there were genes that were either directly associated to or indirectly modulating genes related to gibberellin, ethylene, auxin, brassinosteroids and JA (S8 l, S9 d). Several genes encode products that are known to be involved in ethylene biosynthesis (*1-aminocyclopropane-1-carboxylic acid (acc) synthase 6; AT4G11280*) and JA biosynthesis (*allene oxide cyclase 2; AT3G25770, allene oxide synthase; AT5G42650*) were evident within $DEG_{TS(3R)}$ (S7 worksheet 5). The potential function of the proteins encoded by these genes mainly was ubiquitination mediated proteolysis (S9 d). However, it was observed that there are genes related to auxin metabolism that may also be related to ubiquitination related proteolysis or plant growth from $DEG_{TS(3S)}$ and $DEG_{INT(3R)}$ (S8 m, o and p; S9 e and f). Plant pathogen interactions were suggestive as a function of the proteins encoded by $DEG_{TC(3S)}$ and $DEG_{TS(3R)}$ (S10 f). A gene of which the product is regulated by ethylene and JA (CEJ1; AT3G50260) was differentially expressed as a $DEG_{INT(3S)}$ (S7 worksheet 5). Furthermore, the only hormone related gene that was differentially expressed in shoots $DEG_{TS(51S)}$ was GASA14 (*AT5G14920*) (S7 worksheet 5, S8 n).

Putative biotic stress related signaling pathways

Compared to the number of signalling related genes in putative biotic stress pathways from $DEG_{TC(3S)}$, there were fewer numbers in DEG_{TS} (S7 worksheet 6, under signalling in S8 q, r and s). Calcium signalling genes dominated the biotic stress related signalling pathway $DEG_{TC(3S)}$ (S7 worksheet 6). Additionally to the groups of genes in putative biotic pathways that were a $DEG_{TS(3S)}$, $DEG_{TS(3R)}$ had genes related to ROS mediation, signal recognition and propagation to the MAPK cascade and heat shock (S8 q, r and s). While $DEG_{TS(3S)}$ were involved in starch and sucrose metabolism, DEG_{INT} contained genes in phenylpropanoid biosynthesis in both roots and shoot (S10 g).

Narrowing Down on Potential Genes Involved in the AtCIPK16 Dependent Salt Response

A pairwise comparison of $DEG_{INT(3R)}$ with the $DEG_{TC(3R)}$ revealed that there are 187 genes out of the 231 genes that are only expressed in a transgene dependent manner in salt (S11 worksheet 1). Furthermore, out of the 152 $DEG_{INT(3S)}$, 120 are uniquely expressed as a transgene dependent salt response, compared to the transgene effect on non-stressed plants (S11 worksheet 2).

The GO terms such as response to ethylene activated signalling pathway, response to wounding and response to chitin were enriched for this subset of 187 genes from root at 3 hours (S11 worksheet 3). Functional clustering of these 187 genes in DAVID revealed the presence of 24 transcription factors, 10 ethylene responsive genes and 15 iron related genes (S11 worksheet 4).

The subset of 120 genes from shoot 3 hours were enriched for GO terms such as cellular response to iron starvation, response to chitin and iron ion homeostasis (S11 worksheet 3). The functional clustering in DAVID revealed that the 120 subset contains genes involved in 'nucleus', 'metal binding' and 'transcription regulation' (S11 worksheet 4).

Narrowing Down on Transcription Factors Putatively Controlled by AtCIPK16

AtCIPK16 was thought to be directly phosphorylating one or more transcription factors in the presence of salinity. To investigate if this was the case, transcription factors with a significant transgene effect only in salt responsiveness were identified; the TFs from DEG_{INT} were compared to TFs from $DEG_{TC.}$ Any genes that were common to these two sets were thought to be differentially expressed due to the transgene, yet not explicitly due to transgene effect in salinity. On the other hand, TF genes that were exclusively DEG_{INT} from both roots and shoots at 3 hours were considered as explicitly expressed due to transgene n presence of salt. There were 25 and 16 TFs that were thus, exclusive to $DEG_{INT(3R)}$ and $DEG_{INT(3S)}$, respectively (S7 worksheet 2; yellow background). Interestingly, there was only one such exclusive TF gene common to $DEG_{INT(3R)}$ and $DEG_{INT(3S)}$ (*AT2G25900*; *AtTZF1*) (S7 worksheet 2; yellow background, bold with black border).

It was previously shown that AtTZF1 acts as a transcription factor and binds ARE promoter domains in AU rich regions (Pomeranz et al., 2011; Qu et al., 2014). Therefore, in order to identify potential downstream transcriptional regulatory targets of AtTZF1 in *AtCIPK16* overexpression lines, the region 3000 bp upstream of the transcription start site of all root and shoot transgene dependent salt responsive genes was scanned for the ARE motif through the FIMO tool in MEME suite. In roots 14 such genes with 17 putative ARE promoter motifs were discovered (Table 2). In shoots 10 genes with 13 putative promoter ARE motifs were identified (Table 2).

Known DEGs with Potential Phosphorylation Ability with a Focus on MAPK Phosphorylated DEGs

Furthermore, the NetPhoS4.1 phosphorylation prediction server results showed that the above subset of 187 genes from $DEG_{INT(3R)}$ contained 181 genes that code for amino acid sequences containing multiple serine/threonine phosphorylation sites (S11 worksheet 5). Furthermore, NetPhoS4.1 server shows that, 109 out of 120 $DEG_{INT(3S)}$ could potentially be phosphorylated with a given score ≥ 0.9 (S11 worksheet 6).This observation was not surprising because the consensus sequence of a phosphorylation site is less than 20 amino acids long.

The ability of protein phosphorylation is best studied for the MAPK cascade in various stress conditions. Therefore, genes that are phosphorylated by various MPKs were identified. There were twelve and two $DEG_{INT(3R)}$ and $DEG_{INT(3S)}$, respectively, that are potential targets of the MAPK phosphorylation (S12). Majority of the identified substrates are phosphorylated by MPK6 (S12).

While the nine DEGs that are common between $DEG_{INT(3R)}$ and $DEG_{INT(3S)}$ are showing the ability to get phosphorylated (S11 worksheet 5 and 6), ZAT10 (AT1G27730) and ATCTH (AT2G25900) are also known to be substrates of the MAPK cascade (S12).

Co-expression Analysis

Roots

The WGCNA network analysis created 66 modules. Hub genes of a module are comprehended as the key drivers of that module which have highest connectivity to the module (i.e. most responsible for the intact network topology). In order to identify the effect of transgene dependent salt responsiveness on these modules (i.e. gene clusters), hub genes from each cluster were screened for $DEG_{INT(3R)}$. Out of the 86, 14 modules contained one or more $DEG_{INT(3R)}$ as hub genes

(S13 worksheet 1) and were selected for further investigations. The genes in each selected module are in S13 (worksheet 2). Hub genes from the modules are shown in S13 (worksheet 3) and the $DEG_{INT(3R)}$ are highlighted in yellow. Since there were no transgene dependent salt responsive genes in roots, no such analysis was performed for the 51 hour time-point.

To extend the network analysis further and retrieve biological relevance underlying the identified modules from 3 hours, functional enrichment analysis of genes in the selected 14 modules was performed (S13 worksheet 4, 5 and 6). The green module that contained 1026 genes was highly enriched for the biological process (BP) response to chitin (p value = 2.87×10^{-18}) (S13 worksheet 4). The darkgrey module was enriched for the term 'response to wounding' (p value = 3.81×10^{-05}) while pink module was enriched for 'defence response' (p value = 2.75×10^{-09}) (S13 worksheet 4). Interestingly, the lightsteelblue1 module was highly enriched for photosynthesis (p value = 1.89×10^{-61}). The other modules were enriched for the terms 'response to water deprivation', 'response to abscisic acid', 'response to absence of light', 'circadian rhythm', 'autophagy', 'rRNA modification', 'cell wall organisation', 'response to karrikin', 'oxidation reduction process' and 'syncytium formation' (S13 worksheet 4).

AtCIPK16 was found in the yellow module and co-clustered with *AtHKT1* (*AT4G10310)* (S13 worksheet 2) and *trehalose phosphate synthase 10* (*AT1G60140*). The yellow module is enriched for 'carbohydrate metabolic process' (p value = 0.002) and 'sodium ion transport' (p value = 0.002) (S13 worksheet 4). The KEGG pathways the yellow module genes fall into include starch and sucrose metabolism (S13 worksheet 5).

Shoots

There were 17 WGCNA modules for shoots. Out of these four modules contained transgene dependent salt responsive genes from shoot at 3 hours as hub genes (S13 worksheet 1). Again the analysis was restricted to the 3hr time-point since there were no transgene dependent salt responsive genes in shoots at 51 hours. The module genes and the respective module hub genes that were transgene dependent salt responsive are in S13 (worksheet 8 and 9, respectively).

The tan module was highest enriched for the term cellular response to iron starvation (S13 worksheet 10) and contained *bHLH43* (*POPEYE/PYE: AT3G47640*). The blue module, which also contained *AtCIPK16*, on the other hand was enriched for the term mRNA processing (p value = 4.81×10^{-19}) (S13 worksheet 10). Turquoise module was enriched for ribosome biogenesis (p value = 2.33×10^{-10}) while magenta was enriched for water deprivation (p value = 2.00×10^{-13}) (S13 worksheet 10).

Discussion

Plant transformation has the potential to be a fast, versatile method to improve plant traits with the ultimate goal of increasing or stabilising crop yield under adverse environmental conditions (Gilliham et al., 2017). It has been shown that *AtCIPK16* overexpression in Arabidopsis conferred enhanced salt tolerance (Roy et al., 2013). However, the underlying molecular mechanisms that govern the observed traits were unknown. It is important to identify the targets which are affected by AtCIPK16, to determine whether overexpression of *AtCIPK16* is not detrimental, but only beneficial to the plant in the long term. We attempted to reduce this disparity in knowledge using a transcriptomic approach.

The experiment was designed to study the transcriptome differences between the transgenics and null transgenics at two different time points that have possible early (3 hours after initial salt application) and late (51 hours after initial salt application) responses to salinity stress. Illumina sequencing was used to generate the transcriptomic data which were subsequently mapped and analysed to gauge the salt tolerance responses of *AtCIPK16* overexpression.

Effect of *AtCIPK16* transgene in salt stress

We are now able to provide *in-silico* evidence to support the assumption - AtCIPK16 may elicit its function within the root cell nucleus in the presence of salt stress and this function includes the manipulation of one or more transcription factors (TFs); a) we previously showed that AtCIPK16 possesses a putative nuclear localisation signal (Amarasinghe et al., 2016), b) here we show that AtCIPK16 has a putative DNA binding domain which may bind it to a DNA bound molecule, c) a GFP assay shows that AtCIPK16 is localised partially to the nucleus (Huang, 2015), d) there is minimal gene expression differences in control roots due to the which increases almost 4 fold in salt stressed roots and e) a large number of TFs are differentially expressed.

It is likely that a regulator is needed to release the AtCIPK16 from its auto-inhibitory status and direct towards the targets, however, RNA-Seq experiment cannot identify the potential regulators of AtCIPK16. Nonetheless, especially in roots it could possibly be that, these regulators are dormant until the plant is stressed. Lee et al. (2007) has suggested the possibility of CBL1 and CBL9 to be the interacting partners of CIPK16. More recently, the ability of other kinases, such as GRIK kinases, to release the auto-inhibitory state of SOS2 has been established (Barajas-Lopez et al., 2018). This implies that there could be an alternative interactome for CIPKs apart from the well-known CBLs to release it from its' auto-inhibitory form.

Possible Downstream Activation of AtTZF1

Among the TFs differentially expressed, we identified one CCCH zinc finger (*AtTZF1*) that stands out as being the only upregulated TF in both roots and shoots at 3 hours exclusive to the transgene dependent salt responsiveness. A previous study has revealed that Arabidopsis plants overexpressing At*TZF1* show enhanced salinity tolerance compared to the wild type due to less shoot Na^+ accumulation, increased chlorophyll content and increased growth (Han et al. 2014). *AtCIPK16* transgenics also do show reduced Na^+ accumulation and increased biomass (Roy et al., 2013). Increased chlorophyll content can also be directly related to the increased growth (Wieckowski, 1963). We propose AtTZF1 as a potential downstream master regulator of AtCIPK16 mediated salt stress tolerance and suggest knockout or knockdown lines to investigate this contention. The ability of C3H zinc fingers to be post translationally phosphorylated and enhance their activity has been shown and suggested previously for plants and mammals (Bogamuwa and Jang, 2016; Brooks and Blackshear, 2013; Maldonado-Bonilla et al., 2014; Taylor et al., 1995). It was identified that a Serine after the zinc finger can be phosphorylated and enhance the activity of the TF (Cziferszky et al., 2002). It would be interesting therefore, to know whether AtTZF1 enhances its activity through phosphorylation, and if so, could AtCIPK16 phosphorylate AtTZF1 as well. Furthermore, it was shown in this study that there are 14 and 10 genes from roots and shoots respectively that could be transcriptionally regulated by AtTZF1 in presence of AtCIPK16. This is an exciting path for further investigations due to the fact, that manipulation of a fine-tuned TF that can control many downstream targets is a desirable feature in developing crops that can tolerate a highly complex trait such as salt stress, with no detrimental consequences (Zhou et al., 2007).

Potential Regulation through Phytohormones

It was evident from the functional categorisation that hormone metabolism related genes, mainly those related to ethylene biosynthesis (e.g. 1-Aminocyclopropane-1-carboxylic acid synthase 6; ACC synthase 6/ACS6) (Wang et al., 2002), jasmonic acid (JA) biosynthesis and cross talk with ethylene (e.g. Allene Oxide Synthase/AOS, AtERF1, CEJ1 and AtMYC2) (Cheng et al., 2013; Park et al., 2002; Vogel et al., 2012; Wasternack and Hause, 2013; Zhao et al., 2014) and auxin regulation (e.g. SAUR genes) (Ren and Gray, 2015), were differentially expressed in the transgenic salt stressed transcriptome, especially at 3 hours. It could mean that phytohormone regulation is an important aspect of AtCIPK16 mediated salt stress tolerance. There is also a possibility that while AtCIPK16 affects the transcription of these genes downstream, the phosphorylation also could enhance their activity post-translationally. Salt stress was shown to enhance ethylene production (Cao et al. 2007; Cao et al. 2006). In turn, ethylene biosynthesis and signalling has been shown to reduce salt sensitivity (Cela et al. 2011; Tao et al. 2015).Could it be that the downstream activity of AtCIPK16 under salt stress enhances the ethylene biosynthesis at least partly owing to increased ACS6 gene expression? If so, higher accumulation rates of ethylene may inhibit the negative effect of ethylene receptors on salinity caused growth arrest. *AtCIPK16* overexpression in *ACS6 knockouts* can firmly link the function of ACS6 to salinity tolerant *AtCIPK16* overexpressing phenotypes.

Inhibition of primary root growth and promotion of lateral root growth owing to redistributed auxin from shoots to roots as a response to ethylene synthesis has been suggested to be important in tolerating low salinity stress in Arabidopsis (Zhao et al., 2011) and more recently in barley (Witzel et al., 2018). The involvement of a protein coded by another *CIPK* gene (*SOS2*; *AtCIPK24*) in the process of auxin redistribution that contributes to lateral growth development in mild salinity stress has been reported previously (Zhao et al., 2011). Data on lateral root length and number could provide answer to the query on whether AtCIPK16 mediated salt tolerance cause a similar morphological change.

A zinc finger protein named ZFP5 has been shown to integrate ethylene with other phytohormones to control root hair development in Arabidopsis (An et al. 2012). In the present study, ZFP5 is co-expressed and a direct neighbour of AtZAT10 in the green module of roots. It is possible that AtZAT10 may be involved in modulating the activity of ZFP5. If so, the downstream effect of *AtCIPK16* overexpression may direct the ethylene signalling cascade towards ZFP5 through AtZAT10 that can cause morphological effects such as root hair growth. Plant root hair growth is observed in salinity stress which enables rapid influx and efflux of ions (An et al. 2012; Gilroy and Jones 2000). It has been suggested that root hairs show preferential expression of certain K^+ channels involved in K^+ uptake (Ivashikina et al., 2001). Increase of K^+ increases the K^+/Na^+ ratio hence provides a protective function against the toxicity of Na^+ within the cytosol (Maathuis and Amtmann, 1999). This could be investigated further in transgenic lines by measurements of K+ in the roots in control and salt stressed conditions.

Our study identified multiple MPK substrates differentially expressed (S12) (Meng et al. 2013; Meng and Zhang 2013; Nguyen et al. 2012; Vogel et al. 2012) yet MPKs were not differentially expressed. One of these substrates,MKK9, acts as a negative regulator of salinity tolerance (Alzwiy and Morris, 2007). Therefore, whether the molecular machinery activated by the *AtCIPK16* overexpression can rescue the negative effects of MKK9 on salinity tolerance remains to be answered.

Potential Fe Deficiency Mitigation

Transgene dependent salt responsive gene from shoots at 3 hours, *bHLH43* (*POPEYE/PYE*: *AT3G47640*), that is also a hub gene in the shoot tan module, has been identified as a crucial gene in maintaining Fe homeostasis when Fe availability is low (Long et al., 2010). While, we maintained a pH of 5.5 throughout the experiment, and replaced the nutrient solution to ensure there was no mineral deficiency (refer to materials and methods), salinity has shown to reduce Fe uptake leading to Fe deficiency (Rabhi et al., 2007). Therefore, the differential expression of the *POPEYE* could be related to Fe deficiency. A recent study shows an involvement of another transcription factor that regulated Fe deficiency, *Femu2,* in the protective function against salt stress in *Chlamydomonas reinhardtii* (Li et al., 2017). Interestingly, in this same study they observed the down regulation of a *CIPK23* homologue, which is from the same family as *CIPK16*, after *Femu2* silencing in *C. reinhardtii.* Several other research have shown the cross-talk between Fe homeostasis and salinity response in plants and fungi (Abbas et al., 2015; Li et al., 2016; Purohit et al., 2016). Furthermore, the involvement of ethylene in up regulating many Fe regulating genes as well as promotion of ethylene biosynthesis by Fe deficiency has been discussed (García et al., 2010). This warrants further investigation into the specific role of the POPEYE in *AtCIPK16* overexpression through *POPEYE* knockout lines.

Possible Regulation of Carbohydrate Synthesis

Carbohydrates such as proline, glycine betaine and trehalose may play a role as osmoprotectants in salinity stress (Delauney and Verma, 1993; Li et al., 2011; Wani et al., 2013). Carbohydrate metabolism in the presence of *AtCIPK16* in salinity was observed in the differential expression and gene co-expression analysis, especially in roots. It is therefore important to validate the possible protective function elicited through carbohydrates such as Trehalose in *AtCIPK16* overexpression to further zoom in on *AtCIPK16* overexpression mediated salinity tolerance.

Transgenics Adapt to New Conditions Faster than the Wild type

There could be several possible reasons for not seeing any DEG_{INT} at 51 hours; a) *AtCIPK16* mediated salinity tolerance has already reached homeostasis by 51 hours, while it still has not reached homeostasis in the null transgenics; this could explain why we see reduced number of DEGs as an effect of salt in transgenics compared to the nulls at 51 hours, in contrast to 3 hours. Rapid adjustment to new conditions may explain the high salinity tolerance of halophytes, such as mangroves, and this may be an important mechanism for improved salt tolerance (Krishnamurthy et al., 2017; Liang et al., 2012; Zhu, 2001). b) it is a consequence of low number of replicates that reduces the statistical power (current study uses only four). However, it has been noted that the minimum number of replicates needed for most RNA-Seq studies is three (Conesa et al., 2016). On the other hand, it has also been suggested by many that the osmotic phase of salinity tolerance, which must have a rapid onset to counteract the immediate reduction in plant growth, requires rapid root-to-shoot signalling once salt has been detected at the roots (Batistič and Kudla, 2010; Gilroy et al., 2014; Roy et al., 2011, 2014; Shabala et al., 2016). It is plausible that overexpression of *AtCIPK16* may be responsible for the rapid induction of its downstream stress tolerant pathway that aides in the rapid adjustment to the new stressful condition.

Is AtCIPK16 a Potential Intermediate between Abiotic and Biotic Stress Responses?

In nature, plants are exposed to various concurrent abiotic and biotic stresses. Abiotic stresses have been shown to affect the tolerance of biotic stresses negatively as well as positively. Cross-tolerance is a term used to define the phenomenon of abiotic stress augmenting plant pathogen resistance (Ayres, 1984). An example for this is barley, *Hordeum vulgare*, grown in saline water exhibiting enhanced tolerance to the barley powdery mildew fungus (Wiese et al., 2004) while, pre-treatment of Arabidopsis with chitin, a key component of the fungal cell wall, was shown to improve salt tolerance (Brotman et al., 2012). Recently, the identified interaction of a chitin receptor CERK1 with the Na^{+} induced Ca^{2+} channel ANN1, was shown to function both in fungal attack and salt stress tolerance (Espinoza et al., 2017). Seeing transgene dependent salt responsive genes involved in putative biotic stress pathways poses the question whether the *AtCIPK16* mediated molecular mechanism also confers tolerance to biotic stresses. This is plausible due to the fact that *AtCIPK16* overexpression, leads to an increased abundance of the respective CIP kinase which can phosphorylate multiple targets, hence could potentially activate more than one pathway or signal transduction cascade and lead to cross-tolerance. Transgene dependent salt responsive genes being enriched for chitin response in both roots and shoots would support our speculation on *AtCIPK16's* overexpression also activating biotic stress tolerance pathways. There is previous evidence and suggestions on the involvement of CIPKs such as CIPK11 (Xie et al., 2010), CIPK25 (Huibers et al., 2009) and CIPK26 (Drerup et al., 2013) in biotic stresses. Further investigation of the involvement of *AtCIPK16* in cross-tolerance would be required therefore.

We found an abundance of DEGs implicated in the phenylpropanoid biosynthesis pathway in salinity stressed roots and shoots. Phenylpropanoids are considered antimicrobial compounds that were shown to increase resistance to viral and bacterial infections or function as signalling molecules in biotic stress responses (Naoumkina et al. 2010). Phenylpropanoid biosynthesis, however, can lead to lignin formation which increases the rigidity of plant cell wall and stalls the plants development (Gall et al., 2015) thereby reducing its biomass. Plants with *AtCIPK16* overexpression however had higher biomass than that wild type plants grown under salinity stress (Roy et al., 2013). Therefore, it is still unclear whether the phenylpropanoid biosynthesis is detrimental or favourable in this particular situation.

Caution should be taken, however, not to over analyse these results, as it first needs to be investigated whether the observation of differentially regulated biotic stress genes are due to a real pathogen infection, and not due to the presence of the transgene. This can be investigated by an independent test on RNA from another set of plants. There is also a possibility for what we observe in shoots in control conditions to be not biotic stress related, but due to actual unintended effects due to the ubiquitous overexpression of root stellar specific *AtCIPK16*. This assumption can be investigated using cell specific promoters in future transgenic studies.(Cellini et al., 2004).

The Proposed Model of Salinity Response in *AtCIPK16* Transgenics

We propose a molecular pathway of *AtCIPK16* mediated salt stress tolerance (Figure 5). Salt stress signals may be sensed by "sensor molecules" owing to the salt stress related changes in cytosolic Ca^{2+} levels in root cells. These sensor molecules can then interact with/phosphorylate AtCIPK16 to release it from the auto-inhibitory state (Barajas-Lopez et al., 2018; Sanchez-Barrena et al., 2007). The active form of AtCIPK16 could phosphorylate multiple downstream targets including ACS6 which in return could enhance the ethylene biosynthesis. Elevated levels of ethylene could overrule ethylene receptor induced arrest of root growth. Furthermore, ethylene can promote auxin redistribution to promote lateral root and root hair growth which may involve ZAT10/12 and AtZFP5 (An et al. 2012; Ivanchenko et al. 2008; Zhao et al. 2011). A possible increase in root surface area could result in elevated uptake of K^+ thereby creating a favourable K^+/Na^+ ratio (Cellier et al., 2004). Furthermore, carbohydrates such as trehalose may be synthesised in the roots, possibly as osmoprotective molecules. AtCIPK16 may even enhance the activity of AtTZF1 through phosphorylation which leads to regulation of multiple downstream targets of AtTZF1. Through enhancing the expression of downstream targets such as *ERF104* in roots, AtTZF1 might aid the enhancement of ethylene production. Additionally, AtCIPK16 may phosphorylate and enhance activity of *AOC* which leads to enhanced biosynthesis of JA that can elicit salinity tolerance responses such as inhibition of primary root growth, as well as potential root-shoot signalling. Salt stress signalling to shoots could activate Fe accumulation and suppress the inhibition of photosynthetic systems which in return may promote plant shoot growth. Auxin in shoots may also promote cell growth that increases biomass of AtCIPK16 transgenics in salinity.

Conclusion

We now have reasons to believe that *AtCIPK16* mediated salinity tolerance is achieved through the activity of a host of TFs synchronised with the regulation of phytohormones, mainly ethylene and jasmonic acid. Modulating TFs and phytohormone mediated responses may well be a crucial aspect in generating salt tolerant germplasm. However, this can be an audacious task, given that both these components are involved in all aspects of a plant's life cycle including its response towards environmental cues. Yet, their importance is re-established by this study. The large overlap of putative functionality of differentially expressed gene products with biotic stress responses shows that *AtCIPK16* overexpression may have the ability to elicit tolerance to multiple abiotic and biotic stresses which is also an important trait towards developing a field-ready salt tolerant plant. However, the importance of *AtCIPK16* as a genetic tool for engineering salt tolerance in crops such as barley need further investigation. These investigations should shed light on the stability of the transgene in propagation through generations, its ability to be fine-tuned by using cell specific promoters therefore eliminating any negative effects of *AtCIPK16* overexpression.

Experimental Procedures

Experimental Design

This study has a 2 (genotype: null, transgenic) by 2 (tissue: root, shoot) by 2 (treatment: control, salt treated) by 2 (time: 3 hr, 51 hr) factorial design. To ensure a minimum number of 4 biological replicates for the RNASeq analysis six *A. thaliana* replicate plants were sampled for each experimental group of which 4 were sent for sequencing. The final experimental design is summarised in Table 1. For the 3 hour time point there were two technical replicates per biological sample hence represented as a multiple of 2.

Transformation of *AtCIPK16,* T2 seed germination, Plant material, growth conditions, salt treatment and sampling

Transgenic *35S:AtCIPK16* overexpressing *Arabidopsis thaliana*, Col-0, were previously generated as described in Roy et al. 2013. The plant growth in hydroponics was conducted according to Jha et al. (2010). Seeds of T_2 *35S:AtCIPK16* plants were soaked in 70% ethanol for 2 minutes followed by 4-5 rinses in sterile milli-Q water for surface sterilisation. Subsequently the seeds were planted in 1.5 mL microfuge tubes containing half-strength *Arabidopsis* nutrient solution (Arteca and Arteca 2000) and 0.8% Bacto agar. After vernalisation for 2 days at 4°C the seeds were transferred to a growth room with controlled light (10 hr light/14 hr photoperiod, an irradiance of 70 mmol $m^{-2}s^{-1}$) and constant temperature of 21 °C. After the emergence of the seedling roots, the plants were transferred to a hydroponic tank containing full-strength *Arabidopsis* nutrient solution. The pH in the hydroponic solution was maintained at 5.7. After 5 weeks of growth in hydroponics, salt stress was applied to half the plants by the addition of 75 mM NaCl. Calcium activity in the growth medium was maintained at 0.3 mM by the addition of the correct amount of $CaCl_2$, as calculated using Visual Minteq Version 2.3 (KTH, Department of Land and Water Resources Engineering, Stockholm, Sweden). Shoot and root tissues were removed after 3 hours and 51 hours of salt stress for RNA extraction and were immediately frozen in liquid nitrogen. The time point 51 hours was selected, rather than 48 hours (2 days) after stress treatment so that the plants were sampled at the same time of day as the 3 hour time point, to ensure that effects of the circadian or diurnal rhythm on gene expression is minimal.

RNA isolation, library preparation and Illumina sequencing

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), following the protocol described by Chomczynski (1993). TruSeq™ stranded RNA sample preparation was utilized with dUTP second strand marking protocol for cDNA library preparation. Ribo-Zero kit (Epicentre, an Illumina company, Madison, WI) was used to remove rRNA from the libraries.

Illumina Sequencing was carried out to collect 100bp paired-end (2 * 100bp). Aim was to get a read depth of 15 Mill read pairs per library.

RNA-Seq data pre-processing

Raw data was examined by the program FASTQC for read quality, detection of adapter contaminations and presence of overrepresented sequences (Andrews, 2010). Next, a Java based in-house *k-mer* counting algorithm was used to confirm the presence/absence of the transgene in each sample by counting reads belonging to the UTRs of the transgene and the wild type respectively. The complete gene of *AtCIPK16*, including the intron and 49 bp of the 3′UTR, had previously been inserted behind the 35S promoter of Cauliflower Mosaic virus (CaMV) and in front of a 3′ UTR from of the nopaline synthase (nos) terminator sequence in the pTOOL2 transformation vector (Roy et al., 2013). *Arabidopsis thaliana*, Col-0, were previously transformed with the construct, using *Agrobacterium* transformation and single insert lines were grown on for further studies (Roy et al., 2013). To distinguish expression of the transgene from the endogenous *AtCIPK16* in Col-0 plants, an k-*mer* counting script was supplied with these sequences (35S promoter, nos terminator, and region in between the *AtCIPK16* exon 1 and the *35S* promoter) to count reads belonging to the expressed transgene from the FASTQ files Furthermore, regions 20 kb upstream and downstream of the *AtCIPK16* gene obtained from the TAIR database (https://www.arabidopsis.org/) were used to count the reads expressed from the endogenous wild-type *AtCIPK16*.

The Reads with length spanning ≥ 70 bp after quality trimming were used for further processing. The Arabidopsis reference genome TAIR10, and gene model annotation files were downloaded from the TAIR ftp site (http://www.tair.org). Read alignment to the reference genome was performed using the splice aligner STAR (version 2.4.1c) (Dobin et al., 2013). There are two steps in mapping using the STAR aligner. 1; Building a genome index for the reference genome (FASTA sequences): *A. thaliana* GFF file was used with an overhang of 100 (i.e. max *readLength* -1) for creating the index. The chloroplast and mitochondrial genomes were excluded when creating these index files. 2; Mapping the reads to the genome: the paired-end reads were mapped to the reference with no mismatches allowed (both reference and the samples were from Col-0 cultivar), with a maximum intron size of 2000 and a maximum gap of 2000 between two mates. Alignment results were output in Sequence/Binary Alignment Map (SAM/BAM) format sorted by the chromosome coordinates. Alignments with non-canonical junctions were filtered out. A simple shell/bash script was used to count mapped, multi-mapped hence eliminated, and unmapped read percentages from the BAM files. The package ggplot2 from Bioconductor was used to create plots for this experiment and one such plot was to identify the number of counts mapped to the *AtCIPK16* (*AT2G25090*) in order to see whether any samples have visibly high or low reads.

Quantification of gene expression level and identification of Differentially Expressed Genes (DEGs)

Read counting for the transcripts was done using the *featureCounts()* function of the *Rsubread* package (Liao et al., 2014) implemented in the R environment (http://www.R-project.org). *DGEList()* function from *DESeq* R library was used to calculate the counts per million (CPM) for each experimental group based on the count matrix, and the *calcNormFactors()* function was applied to estimate normalization factors (Anders and Huber, 2010). Data from each time point (i.e. 3 hr, 51 hr) and each tissue were analysed separately resulting in 4 groups (i.e. Root_3Hr, Shoot_3Hr, Root_51Hr and Shoot_51Hr). Transcripts with CPM values of less than 100 in 75% of the samples or more were filtered out. Out of 64, 60 samples at the 3 hour time point were analysed. A sample from root and the corresponding shoot sample (of which each had 2 technical replicates; Table 1) from the 3 hour time point had to be removed due to a large variation in *AtCIPK16* expression compared to other samples (S3).

T-statistics for mean expression values for each gene was determined using the LIMMA (Linear Models for Microarray Data) package implemented in the R software environment (Ritchie et al., 2015; Smyth, 2004). The read count data fed into the Limma linear model fitting were transformed using *Voom* with quantile normalization followed by group-means parameterization and robust eBayes (Law et al., 2014; Smyth, 2004). The contrast matrix was created with the final goal of; identifying the differentially expressed genes in salt stress that is due to the definite effect of the transgene (Figure 2). This contrast matrix was used on the 4 groups of expression value data separately. P-values for multiple testing were corrected according to Benjamini and Hochberg 1995. Differentially expressed genes are those with a FDR-adjusted P value of ≤0.05 and ≥2-fold change in expression relative to the control.

Regulatory network construction using WGCNA

Weighted Gene Co-expression Network Analysis (WGCNA) enables the detection of modules of genes with high expression value correlation to one another (Langfelder and Horvath, 2008). Briefly, WGCNA assigns a connection weight between pairs of genes within the network based on a biologically motivated criterion and attempts to identify relevant modules by applying a soft threshold to correlations between pairs of genes within a network. R software environment was used for all WGCNA analysis (Langfelder and Horvath, 2008; Zhang and Horvath, 2005). After confirming that there were no outliers in the tissue-separated samples optimization of the soft threshold values was performed. Signed co-expression network was constructed using the automatic one-step network construction method (function *cuttreeDynamic()*) with following settings; a signed type of network, an unsigned type of topological overlap matrix (TOM), correlations of the network raised to a soft thresholding power β (roots:10; shoots: 5), correlation measures with option '*bicor*', deepSplit value of 2, a minimum module size of 20. The first principle component of a module (module eigengene) value was calculated and used to test the association of modules with salt response in the null and transgenic genotypes. Total network connectivity (kTotal), and module membership (MM), were calculated for each of the DEGs. Modules for further analysis were selected if one or more of their hub genes (genes with modular membership ≥ 0.9) was among the transgene dependent salt-responsive gene list of the respective tissue.

Functional analysis

GO term enrichment was performed using the Plant Gene Set Enrichment Analysis Toolkit (PlantGSEA) and the DAVID online web server (http://david.abcc.ncifcrf.gov/) (Dennis et al., 2003; Yi et al., 2013). Up and down regulated DEGs from each contrast were used for GO and pathway enrichment analysis, and a False Discovery Ratio (FDR) corrected p value ≤ 0.05 was selected as the threshold level of significance to determine the enrichment in the gene set. MapMan stand-alone software allowed the assignment of DEGs into regulatory pathways (Thimm et al., 2004). Additionally, Kyoto Encyclopaedia of Genes and Genomes (KEGG) was used to identify higher order functional information related to the DEGs (Kanehisa et al., 2017). ATTED-II (http://atted.jp) gene co-expression database was mined to identify additional yet relevant genes that may be co-expressed with the DEGs (Aoki et al., 2016).

In order to identify genes that are exclusively differentially expressed only in transgene dependent manner in presence of salt, the DEGs from the transgene dependent salt responsive gene list was compared in a pairwise manner to the DEGs from that of transgene effect in controls for a given tissue at a particular time point.

Phosphorylation targets were identified using the NetPhoS4.1 online server (Blom et al., 1999). MPK substrates were identified by comparing the DEGs to known substrates recorded in the literature (Meng et al. 2013; Nguyen et al. 2012; Popescu et al. 2009; Vogel et al. 2012). Promoter analysis for the ARE motif; regions 3000 bp upstream from the transcription start site (TSS) of all transgene dependent salt responsive genes of root and shoot at 3 hours were downloaded from the TAIR database (https://www.arabidopsis.org/). The motif pattern ATTTATTTATTT{A|T] was searched against the downloaded sequences through the FIMO tool (http://meme-suite.org/tools/fimo) in the MEME suite (Grant et al., 2011). The p-value threshold was set to 0.01. DNA binding domains and amino acid properties were identified from protein sequences using the consensus of results obtained through several freely available online tools with the use of their default settings; DP-BIND (Hwang et al., 2007), BINDN (Wang and Brown, 2006), NetSurfP (Petersen et al. 2009), PredictProtein (Rost et al., 2004), paircoil2 (McDonnell et al., 2006) and pepinfo (Li et al., 2015).

Acknowledgements

The authors would like to thank Dr. Radoslaw Suchecki for the *k-mer* counting algorithm that is used in this study. Authors also wish to thank Mr. Ashan Hettiarachchige for creating the final high resolution images according to the guidelines from the journal.

Tables and Figures

Table 1 Experimental design for the current study

There were 4 biological replicates per condition per experimental group for both time points. The 3 hour samples were sequenced in two technical replicates per biological sample hence indicated as a multiple of 2.

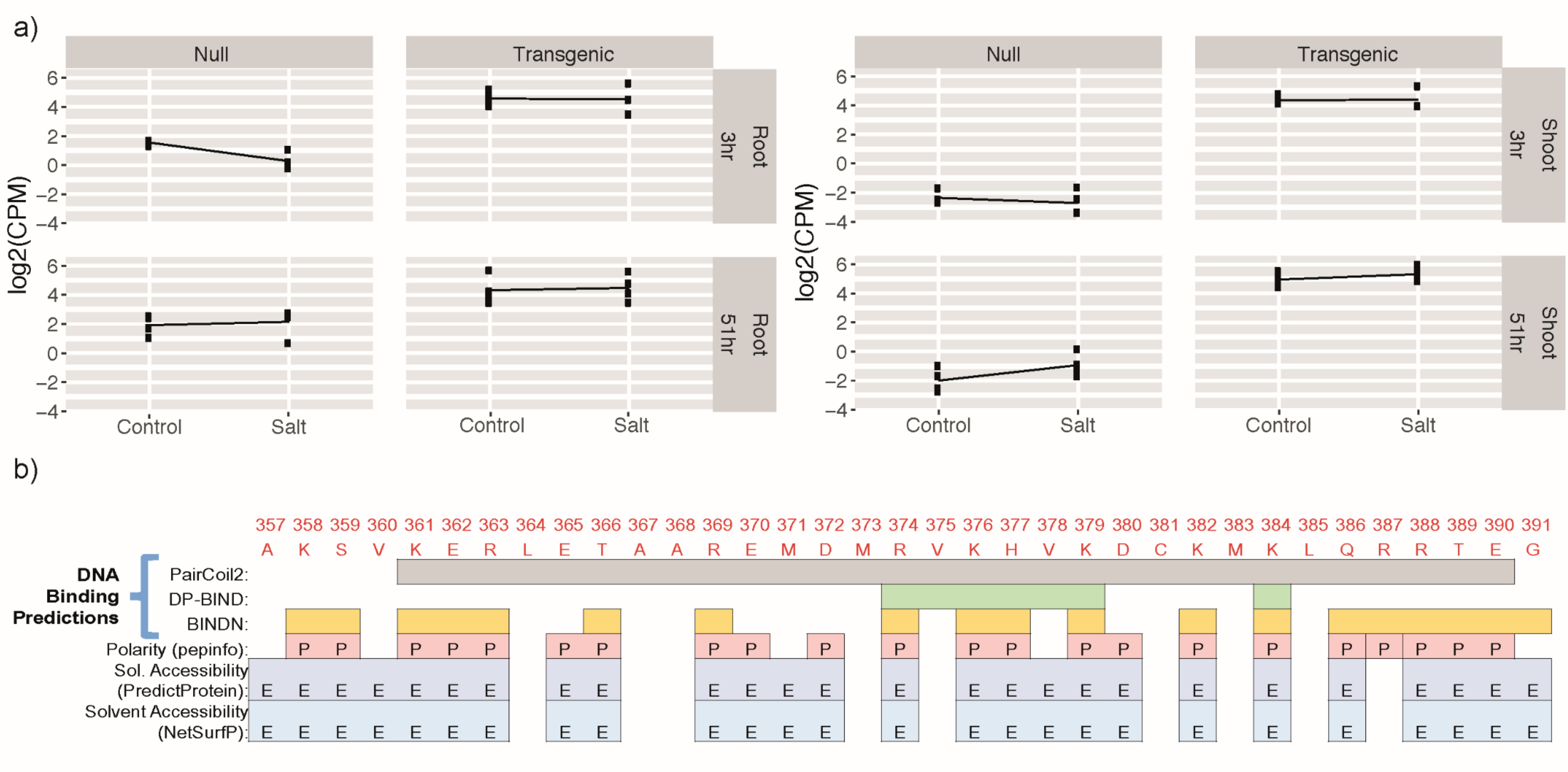
Genotype	Time	3Hr		51Hr	
	Treatment	Root	Shoot	Root	Shoot
Null lines	Control	4×2	4×2	4	4
	75 Mm Salt treated	4×2	4×2	4	4
Transgenic lines	Control	4×2	4×2	4	4
	75 Mm Salt treated	4×2	4×2	4	4
Total number of samples		32	32	16	16

Table 2 The genes that are putatively regulated by transcriptional activity of AtTZF1 from roots and shoots at 3 hours

The DNA binding motif of AtTZF1 ATTTATTTATTT[T|A] (Pomeranz et al., 2011; Qu et al., 2014), was scanned on the 3,000 bp upstream from the transcription start site (TSS) of all transgene dependent salt responsive genes from roots and shoots at 3 hours (sequences were retrieved using bulk sequence retrieval option from TAIR portal; https://www.arabidopsis.org/tools/bulk/index.jsp and scanned through FIMO from MEME suite; http://meme-suite.org/tools/fimo). The genes with one or more positive hits with a p value ≤ 0.01 are reported here. The gene ID and the descriptions are in the first two columns. The start and the stop site of the identified motif are in the 3rd and 4th columns, respectively. The strand the motif is predicted on is in the 5th columns. FIMO assigned score, p-value and FDR corrected p-value (q-value are in the 6th 7th and 8th columns, respectively. The matched motif is displayed in 9th column.

Gene ID	Gene description	start	stop	strand	score	p-value	q-value	matched sequence
AT1G12540	basic helix-loop-helix (bHLH) DNA-binding superfamily protein;(source:Araport11)	-1465	-1453	-	21.9827	5.04E-08	0.00404	ATTTATTTATTTA
AT1G43160	RELATED TO AP2 6 (RAP2.6)	-1217	-1205	-	21.9827	5.04E-08	0.00404	ATTTATTTATTTA
AT1G57990	PURINE PERMEASE 18 (PUP18)	-1467	-1455	+	21.9769	1.01E-07	0.00444	ATTTATTTATTTT
AT1G63057	transmembrane protein;(source:Araport11)	-1265	-1253	+	21.9827	5.04E-08	0.00404	ATTTATTTATTTA
	transmembrane protein;(source:Araport11)	-1261	-1249	+	21.9769	1.01E-07	0.00444	ATTTATTTATTTT
AT1G64950	CYTOCHROME P450, FAMILY 89, SUBFAMILY A, POLYPEPTIDE 5 (CYP89A5)	-56	-44	+	21.9769	1.01E-07	0.00444	ATTTATTTATTTT
AT2G04110	pseudogene of expressed protein;(source:Araport11)	-653	-641	-	21.9827	5.04E-08	0.00404	ATTTATTTATTTA
AT2G38240	JASMONATE-INDUCED OXYGENASE4 (JOX4)	-873	-861	+	21.9827	5.04E-08	0.00404	ATTTATTTATTTA
	JASMONATE-INDUCED OXYGENASE4 (JOX4)	-877	-865	+	21.9827	5.04E-08	0.00404	ATTTATTTATTTA
AT3G59480	FRUCTOKINASE 7 (FRK7)	-1573	-1561	-	21.9827	5.04E-08	0.00404	ATTTATTTATTTA
	FRUCTOKINASE 7 (FRK7)	-1577	-1565	-	21.9769	1.01E-07	0.00444	ATTTATTTATTTT
AT4G22690	CYTOCHROME P450, FAMILY 706, SUBFAMILY A, POLYPEPTIDE 1 (CYP706A1)	-1970	-1958	+	21.9769	1.01E-07	0.00444	ATTTATTTATTTT
AT4G29780	nuclease;(source:Araport11)	-1659	-1647	+	21.9827	5.04E-08	0.00404	ATTTATTTATTTA
AT4G39640	GAMMA-GLUTAMYL TRANSPEPTIDASE 1 (GGT1)	-518	-506	-	21.9827	5.04E-08	0.00404	ATTTATTTATTTA

AT5G13080	WRKY DNA-BINDING PROTEIN 75 (WRKY75)	-1966	-1954	+	21.9769	1.01E-07	0.00444	ATTTATTTATTTT
AT5G56550	OXIDATIVE STRESS 3 (OXS4)	-29	-17	+	21.9769	1.01E-07	0.00444	ATTTATTTATTTT
AT5G61600	ETHYLENE RESPONSE FACTOR 104 (ERF104)	-1664	-1652	+	21.9769	1.01E-07	0.00444	ATTTATTTATTTT
Shoot | 3Hr								
AT1G47370	RESPONSE TO THE BACTERIAL TYPE III EFFECTOR PROTEIN HOPBA1 (RBA1)	-588	-576	-	21.9769	1.01E-07	0.00464	ATTTATTTATTTT
	RESPONSE TO THE BACTERIAL TYPE III EFFECTOR PROTEIN HOPBA1 (RBA1)	-877	-865	-	21.9769	1.01E-07	0.00464	ATTTATTTATTTT
AT1G73325	Kunitz family trypsin and protease inhibitor protein;(source:Araport11)	-2763	-2751	-	21.9827	5.04E-08	0.00464	ATTTATTTATTTA
AT2G07042	other_RNA;(source:Araport11)	-924	-912	-	21.9769	1.01E-07	0.00464	ATTTATTTATTTT
AT2G21140	PROLINE-RICH PROTEIN 2 (PRP2)	-1392	-1380	+	21.9769	1.01E-07	0.00464	ATTTATTTATTTT
AT3G16720	TOXICOS EN LEVADURA 2 (ATL2)	-317	-305	+	21.9827	5.04E-08	0.00464	ATTTATTTATTTA
	TOXICOS EN LEVADURA 2 (ATL2)	-313	-301	+	21.9769	1.01E-07	0.00464	ATTTATTTATTTT
AT3G29000	Calcium-binding EF-hand family protein;(source:Araport11)	-2278	-2266	-	21.9769	1.01E-07	0.00464	ATTTATTTATTTT
AT4G18440	L-Aspartase-like family protein;(source:Araport11)	-1181	-1169	+	21.9769	1.01E-07	0.00464	ATTTATTTATTTT
AT4G20860	(ATBBE22)	-336	-324	+	21.9769	1.01E-07	0.00464	ATTTATTTATTTT
AT5G03150	JACKDAW (JKD)	-1717	-1705	-	21.9769	1.01E-07	0.00464	ATTTATTTATTTT
AT5G04340	ZINC FINGER OF ARABIDOPSIS THALIANA 6 (ZAT6)	-868	-856	+	21.9827	5.04E-08	0.00464	ATTTATTTATTTA



a)
Null
Transgenic
Null
Transgenic
log2(CPM)
6
4
2
0
−2
−4
Root 3hr
Root 51hr
Shoot 3hr
Shoot 51hr
Control
Salt
b)
357 358 359 360 361 362 363 364 365 366 367 368 369 370 371 372 373 374 375 376 377 378 379 380 381 382 383 384 385 386 387 388 389 390 391
A K S V K E R L E T A A R E M D M R V K H V K D C K M K L Q R R T E G
DNA Binding Predictions
PairCoil2:
DP-BIND:
BINDN:
Polarity (pepinfo):
Sol. Accessibility (PredictProtein):
Solvent Accessibility (NetSurfP):
P
E

Figure 1 *AtCIPK16* gene expression in the current study and putative DNA binding domain of AtCIPK16

a) The expression values are measured in counts per million (CPM) and displayed as log_2(CPM) for clarity (y axis). Each dot represent a sample and is coloured according to the experimental condition. Expression values are separated by genotype (i.e. null, transgenic: on top) and tissue-time point (i.e. root 3hr, shoot 3hr, root 51 hr and shoot 51hr: right) and treatment (i.e. control, salt: bottom). A black solid line connects the mean expression value from the two treatments in each group of samples. A gene is considered expressed if the mean of log expression value is above 0 for a given experimental condition; b) Identified region of AtCIPK16 protein with DNA binding affinity: the amino acid residue numbers and the respective residues are mentioned in the first and second rows, respectively; residues in the putative region with DNA binding ability are shown in red. The server result summary for each residue is shown below the respective amino acid. Row 3-5 show the predicted DNA binding affinity by three independent online tools (i.e. PairCoil2; grey, DP-BIND; green and BINDN; yellow); 6th row shows the polarity prediction for the region using pepinfo server (denoted with a P in red background); 7th and 8th rows show the solvent accessibility predicted by two independent servers (PredictProtein and NetSurfP, respectively; denoted by E in blue background).

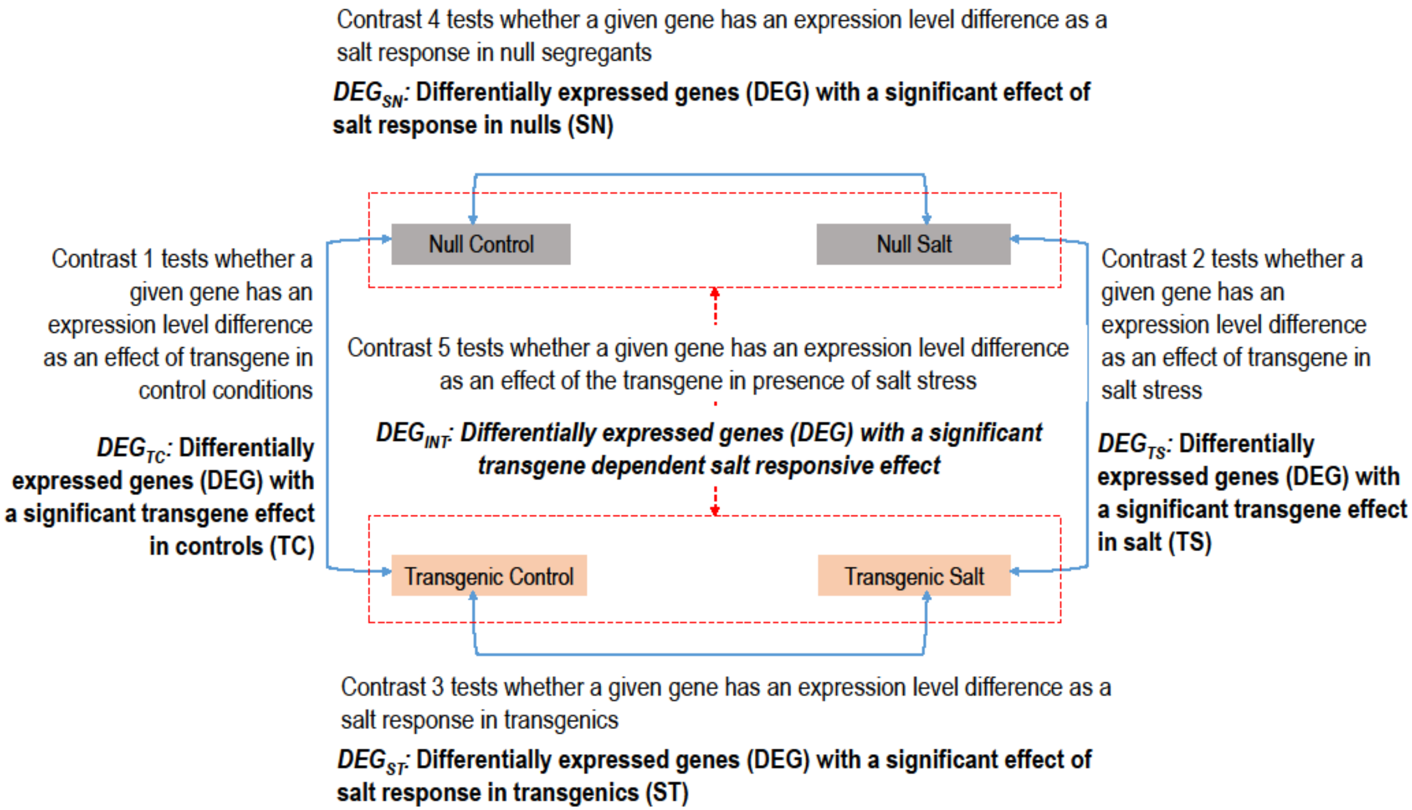


Figure 2 Contrasts tested in the current analysis

The experimental groups compared by Limma (Ritchie et al, 2015) to test each contrast are shown by blue two headed arrows. The red dashed boxes and two headed arrow denote the contrast to test the interaction term. The term that defines the differentially expressed genes for each contrast is mentioned below each hypothesis in ***bold italics***

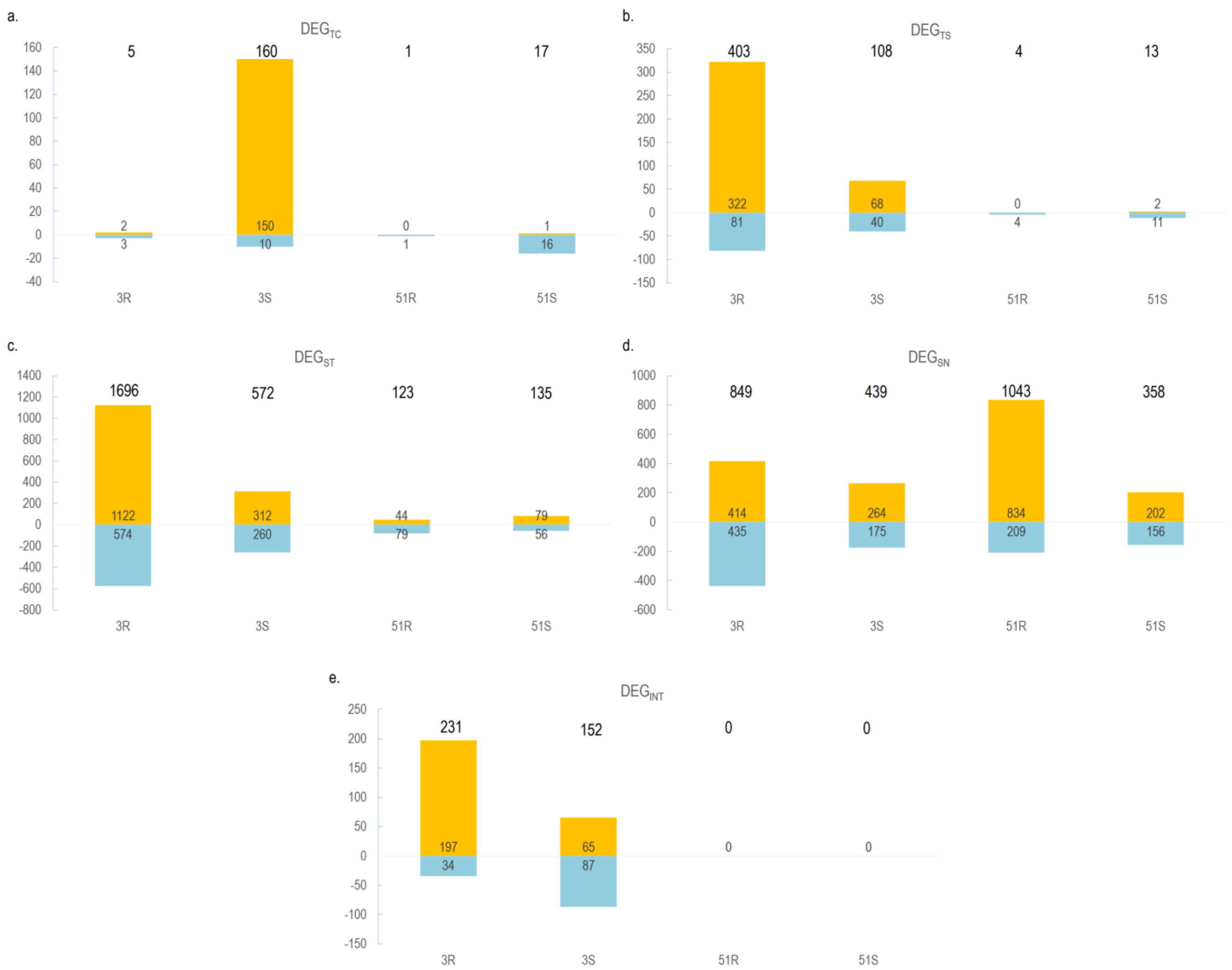


Figure 3 Number of genes differentially identified

The number of differentially expressed genes (DEG) for each test shown in Figure 2 are shown for; a) transgene effect in controls (DEG_{TC}); b) transgene effect in salt (DEG_{TS}); c) salt effect in transgenics (DEG_{ST}); d) salt effect in nulls (DEG_{SN}) and e) transgene dependent salt responsiveness (DEG_{INT}). Y axis displays the number of DEG; x axis represent the experimental group (3R: 3 hr root; 3S: 3 hr shoot; 51R: 51 hr root; 51S: 51 hr shoot) through Limma analysis (Ritchie et al, 2015). Yellow denoted the upregulated genes and blue denoted the down regulated genes. The total number of DEGs are shown on top of each bar. Individual numbers for up and down regulated genes are shown along the y axis on yellow and blue bars

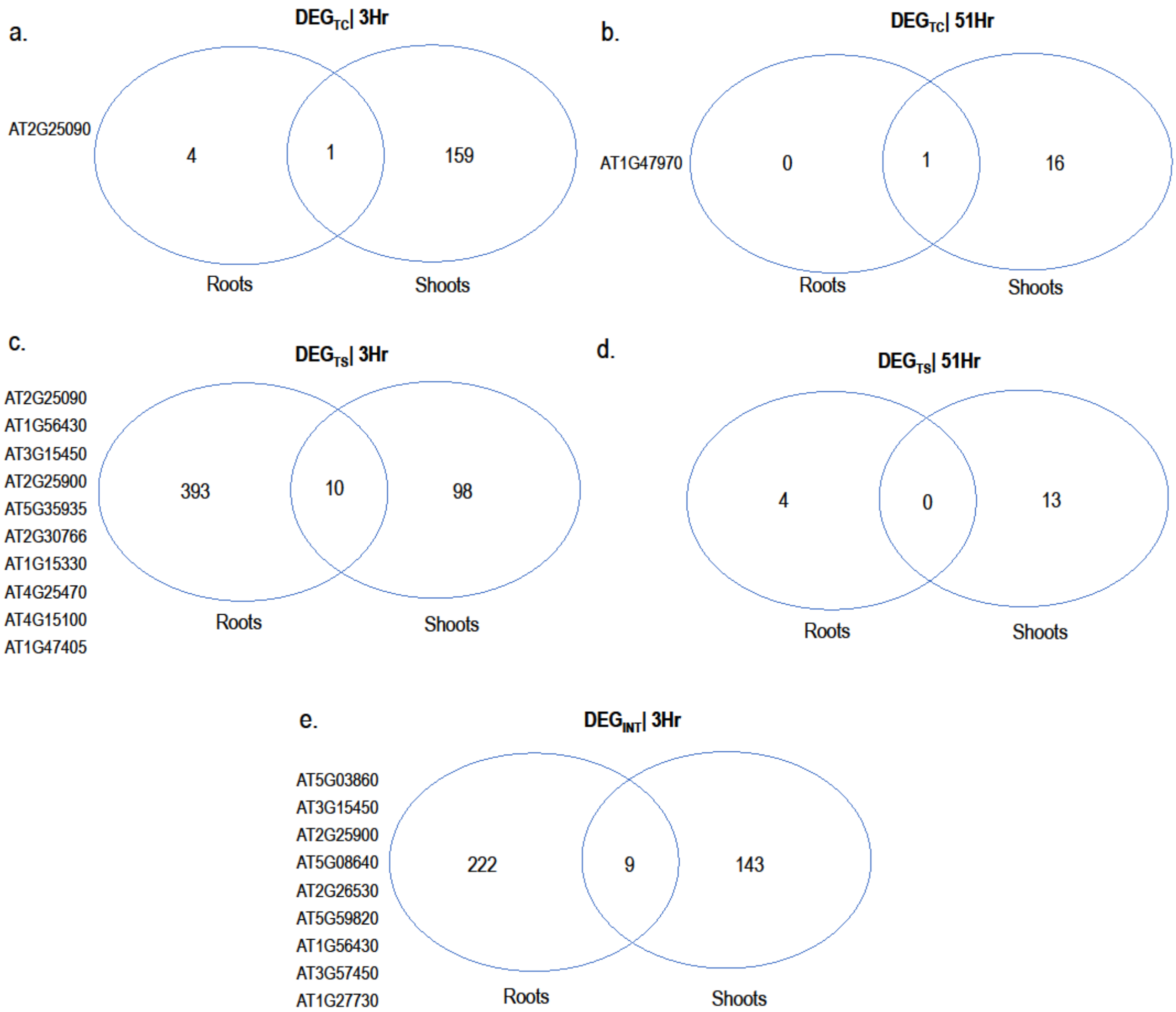


Figure 4 Comparison of the DEGs

Overlap of differentially expressed genes (DEG) in roots and shoots. The comparisons that were tested (Figure 2) are given at the top and the genes which overlap between the two tissues for that treatment are listed on the left side of each venn diagram. DEG_{TC}: DEG from transgenic controls; DEG_{TS}: DEG from transgenics in salt; DEG_{TC}: DEG from transgene dependent salt responsiveness

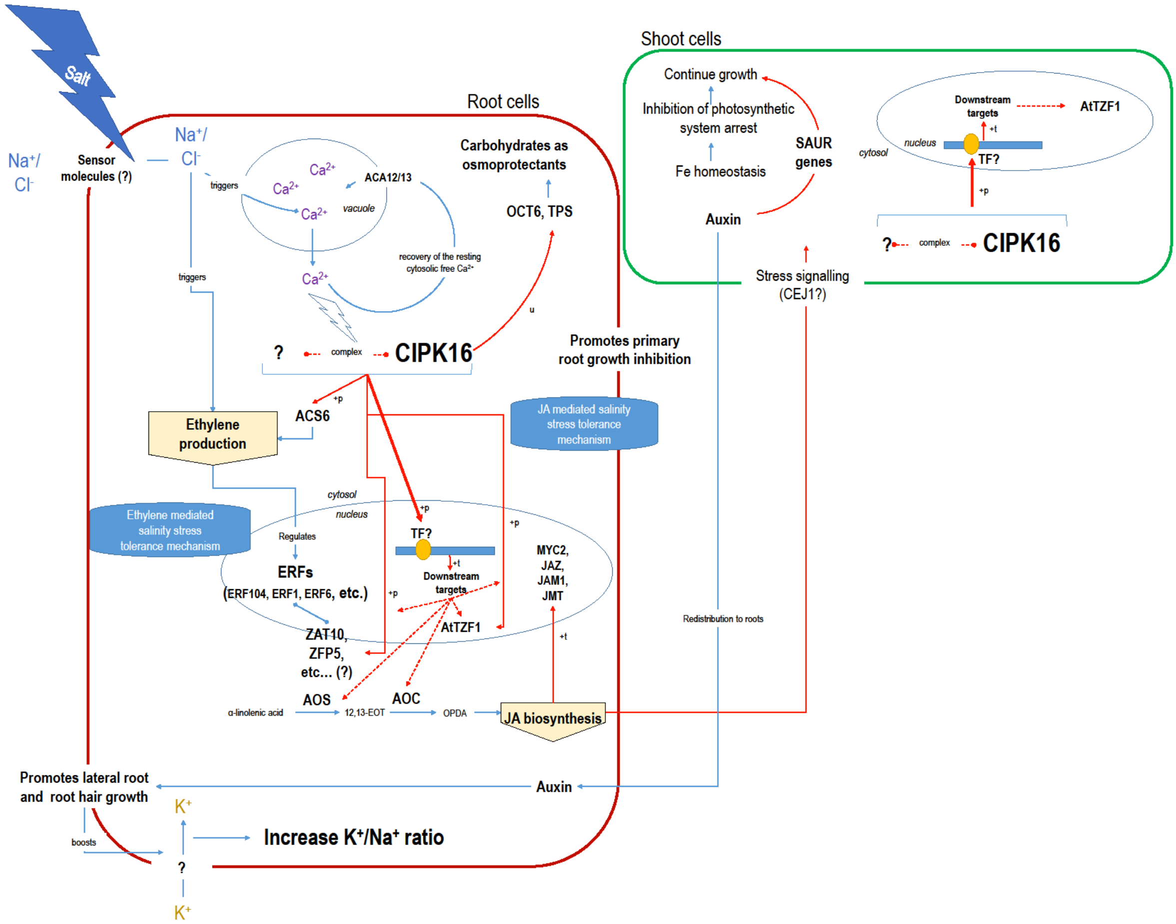


Figure 5 The proposed model for *AtCIPK16* mediated salinity tolerance mechanism in Arabidopsis

The model proposes the involvement of ethylene and JA in the *AtCIPK16* overexpression mediated salinity tolerance. Blue arrows depict currently known knowledge and red arrows depict the proposed AtCIPK16 related pathways. If a potential method of regulation is known for the proposed pathways they are shown next to the arrow (+p: phosphorylated for enhanced activity; +t: enhanced by transcriptional regulation; u: unknown method of regulation). The arrow heads represent the direction of regulation. Double pointed arrows are when the direction of regulation is uncertain.

Supplementary Material

Available at https://doi.org/10.4225/55/5aa11470444b0

S1: Summary of *k-mer* baiting step to confirm the presence of the transgene in samples

a) Construct architecture of the *AtCIPK16* transgene; b) the sequence in between the *35S* promoter and the *AtCIPK16* exon 1; c) *35S* promoter sequence that was used to bait the 5′ UTR region of the transgene; d) *NOS terminator* sequence that was used to bait the 3′ UTR region of the transgene; e) wild type *AtCIPK16* 5′ UTR region; f) wild type *AtCIPK16* 3′ UTR region; g) baiting results from the 3 hour time point; h) baiting results from the 51 hour time point; for g and h the columns from left to right represent the following: *column1*: name of the fastq file, *column2*: number of baits for the transgene 3′ UTR region, *column3*: number of baits for the transgene 5′ UTR region, *column4*: number of baits for the transgene 5′ UTR region between exon1 and the *35S* promoter, *column5*: number of baits for the wild type 3′ UTR region, *column6*: number of baits for the transgene 5′ UTR region, *column7-9*: experimental conditions of each sample. Rows of g and h are coloured for green shades to represent the shoot samples and brown shades to represent root samples.

S2: Percentages of mapped, multi-mapped and unmapped reads from the samples using STAR aligner

S3: The plot to show the justification of the removal of a root and a shoot sample

a) Mapped raw counts for *AtCIPK16* (*AT2G25090*) of the 3 hour samples, b) Normalised counts mapped to *AtCIPK16.* The blue semi-transparent bars indicate the samples that were removed based on their visibly high amount of normalised reads mapped to *AtCIPK16*.

S4: Differentially expressed genes (DEGs) resulted from the hypothesis testing shown in Figure 1

Where applicable the yellow colour represents up regulated genes and blue colour represents down regulated genes

S5: GO enrichment analysis of the DEGs performed through PlantGSEA online web server (http://structuralbiology.cau.edu.cn/PlantGSEA/index.php)

Where applicable the yellow colour represents GO terms enriched by up regulated genes and blue colour represents GO terms enriched by down regulated genes

S6: Results of Kyoto Encyclopedia of Genes and Genomes (KEGG) information mining for the DEGs using the "Search and Color Pathway" option (http://www.kegg.jp/)

S7: Selected MapMan categories that the DEGs fall into

S8: MapMan pathway analysis of the DEGs

a-i: cell function overview; j-p: regulation overview; q-u: putative biotic stress pathways

S9: S9: KEGG Pathways of DEG subsets that are discussed in Mapman categories and their associated genes identified through ATTED-II.

The pathways are auto generated through the search and colour pathway option in Kyoto Encyclopaedia of Genes and Genomes (KEGG) server (www.genome.jp/kegg/) The input genes for each section are the up regulated DEGs for a), c), d) and e). and all DEGs for b) and f). The associated genes that are included in the list are retrieved through the NetworkDrawer tool with default options (Platform is automatic, CoEx option add many genes and PPI option add a few genes) of ATTED-II server (atted.jp/). Rectangular boxes with green colour background represent genes in the pathway, arrows represent a molecular interaction or a relationship. The red framed green boxes with red letters show the genes that are in the input lists, each group of DEGs used to mine the pathways in each sub figure are mentioned below the respective sub figure a)-f), empty circles represent chemical molecules, rectangles with rounded edges shows the ink from the current pathway to another pathway, doubled lines represent the plasma membrane, the dashed grey lines are shown when a direct association between two molecules are unknown.

S10: KEGG pathways enriched for the DEG subsets of selected MapMan categories and their associated genes identified through ATTED-II

S11: Novel genes involved in the AtCIPK16 dependent salt responsiveness

The gene lists identified from both roots and shoots, their GO enrichment and functional categorisation through DAVID online web server and the novel genes that have the ability to get phosphorylated that are identified through NetPhos3.1b online server

S12: Identified novel AtCIPK16 transgene dependent salt responsive genes that are also putative MAPK substrates

S13: Summary of the WGCNA analysis of DEGs

Interesting modules were selected if one or more transgene dependent salt responsive DEGs from the respective tissue (i.e. root or shoot) are hub genes of the said module. The GO enrichment was performed for each selected module through DAVID online tool (https://david.ncifcrf.gov/). The pathway analysis was cone using search and Color pathway option in Kyoto Encyclopedia of Genes and Genomes (KEGG) (www.genome.jp/kegg/)

References

Abbas G, Saqib M, Akhtar J, Haq MA ul. Interactive effects of salinity and iron deficiency on different rice genotypes. J. Plant Nutr. Soil Sci. 2015 Apr 1;178(2):306–11.

Alzwiy IA, Morris PC. A mutation in the Arabidopsis MAP kinase kinase 9 gene results in enhanced seedling stress tolerance. Plant Sci. 2007 Sep 1;173(3):302–8.

Amarasinghe S, Watson-Haigh NS, Gilliham M, Roy S, Baumann U. The evolutionary origin of CIPK16: A gene involved in enhanced salt tolerance. Mol. Phylogenet. Evol. 2016 Jul;100:135–47.

An L, Zhou Z, Sun L, Yan A, Xi W, Yu N, et al. A zinc finger protein gene ZFP5 integrates phytohormone signaling to control root hair development in Arabidopsis. Plant J. 2012 Nov 1;72(3):474–90.

Anders S, Huber W. Differential expression analysis for sequence count data. Genome Biol. 2010 Oct 27;11(10):R106.

Andrews S. FastQC: a quality control tool for high throughput sequence data. [Internet]. 2010 [cited 2015 Jan 7]. Available from: http://www.bioinformatics.babraham.ac.uk/projects/fastqc

Aoki Y, Okamura Y, Tadaka S, Kinoshita K, Obayashi T. ATTED-II in 2016: A Plant Coexpression Database Towards Lineage-Specific Coexpression. Plant Cell Physiol. 2016 Jan;57(1):e5.

Arteca RN, Arteca JM. A novel method for growing Arabidopsis thaliana plants hydroponically. Physiol. Plant. 2000 Feb 1;108(2):188–93.

Ayres PG. The Interaction Between Environmental Stress Injury and Biotic Disease Physiology. Annu. Rev. Phytopathol. 1984 Sep 1;22(1):53–75.

Barajas-Lopez J de D, Moreno JR, Gamez-Arjona FM, Pardo JM, Punkkinen M, Zhu J-K, et al. Upstream kinases of plant SnRKs are involved in salt stress tolerance. Plant J. 2018 Jan 1;93(1):107–18.

Bassil E, Coku A, Blumwald E. Cellular ion homeostasis: emerging roles of intracellular NHX Na+/H+ antiporters in plant growth and development. J. Exp. Bot. 2012 Oct 1;63(16):5727–40.

Batistič O, Kudla J. Calcium: Not Just Another Ion. In: Hell R, Mendel R-R, editors. Cell Biol. Met. Nutr. [Internet]. Springer Berlin Heidelberg; 2010 [cited 2016 Oct 18]. p. 17–54. Available from: http://link.springer.com/chapter/10.1007/978-3-642-10613-2_2

Baxter A, Mittler R, Suzuki N. ROS as key players in plant stress signalling. J. Exp. Bot. 2014 Mar 1;65(5):1229–40.

Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. J. R. Stat. Soc. Ser. B Methodol. 1995;57(1):289–300.

Blom N, Gammeltoft S, Brunak S. Sequence and structure-based prediction of eukaryotic protein phosphorylation sites1 1Edited by F. E. Cohen. J. Mol. Biol. 1999 Dec 17;294(5):1351–62.

Bogamuwa S, Jang J-C. Plant Tandem CCCH Zinc Finger Proteins Interact with ABA, Drought, and Stress Response Regulators in Processing-Bodies and Stress Granules. PLOS ONE. 2016 Mar 15;11(3):e0151574.

Brooks SA, Blackshear PJ. Tristetraprolin (TTP): Interactions with mRNA and proteins, and current thoughts on mechanisms of action. Biochim. Biophys. Acta. 2013;1829(0):666–79.

Brotman Y, Landau U, Pnini S, Lisec J, Balazadeh S, Mueller-Roeber B, et al. The LysM Receptor-Like Kinase LysM RLK1 Is Required to Activate Defense and Abiotic-Stress Responses Induced by Overexpression of Fungal Chitinases in Arabidopsis Plants. Mol. Plant. 2012 Sep 1;5(5):1113–24.

Cao W-H, Liu J, He X-J, Mu R-L, Zhou H-L, Chen S-Y, et al. Modulation of Ethylene Responses Affects Plant Salt-Stress Responses. Plant Physiol. 2007 Feb 1;143(2):707–19.

Cao W-H, Liu J, Zhou Q-Y, Cao Y-R, Zheng S-F, Du B-X, et al. Expression of tobacco ethylene receptor NTHK1 alters plant responses to salt stress. Plant Cell Environ. 2006 Jul 1;29(7):1210–9.

Cela J, Chang C, Munné-Bosch S. Accumulation of γ- Rather than α-Tocopherol Alters Ethylene Signaling Gene Expression in the vte4 Mutant of Arabidopsis thaliana. Plant Cell Physiol. 2011 Aug 1;52(8):1389–400.

Cellier F, Conéjéro G, Ricaud L, Luu DT, Lepetit M, Gosti F, et al. Characterization of AtCHX17, a member of the cation/H+ exchangers, CHX family, from Arabidopsis thaliana suggests a role in K+ homeostasis. Plant J. 2004 Sep 1;39(6):834–46.

Cellini F, Chesson A, Colquhoun I, Constable A, Davies HV, Engel KH, et al. Unintended effects and their detection in genetically modified crops. Food Chem. Toxicol. 2004 Jul 1;42(7):1089–125.

Chen Z, Pottosin II, Cuin TA, Fuglsang AT, Tester M, Jha D, et al. Root Plasma Membrane Transporters Controlling K+/Na+ Homeostasis in Salt-Stressed Barley. Plant Physiol. 2007 Dec 1;145(4):1714–25.

Cheng M-C, Liao P-M, Kuo W-W, Lin T-P. The Arabidopsis ETHYLENE RESPONSE FACTOR1 Regulates Abiotic Stress-Responsive Gene Expression by Binding to Different cis-Acting Elements in Response to Different Stress Signals. PLANT Physiol. 2013 Jul 1;162(3):1566–82.

Conesa A, Madrigal P, Tarazona S, Gomez-Cabrero D, Cervera A, McPherson A, et al. A survey of best practices for RNA-seq data analysis. Genome Biol. 2016 Jan 26;17(1):13.

Cziferszky A, Mach RL, Kubicek CP. Phosphorylation Positively Regulates DNA Binding of the Carbon Catabolite Repressor Cre1 of Hypocrea jecorina(Trichoderma reesei). J. Biol. Chem. 2002 Apr 26;277(17):14688–94.

D’Angelo C, Weinl S, Batistic O, Pandey GK, Cheong YH, Schültke S, et al. Alternative complex formation of the Ca2+-regulated protein kinase CIPK1 controls abscisic acid-dependent and independent stress responses in Arabidopsis. Plant J. 2006 Dec 1;48(6):857–72.

Deinlein U, Stephan AB, Horie T, Luo W, Xu G, Schroeder JI. Plant salt-tolerance mechanisms. Trends Plant Sci. 2014 Jun;19(6):371–9.

Delauney AJ, Verma DPS. Proline biosynthesis and osmoregulation in plants. Plant J. 1993 Aug 1;4(2):215–23.

Dennis G, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, et al. DAVID: Database for Annotation, Visualization, and Integrated Discovery. Genome Biol. 2003 Apr 3;4(5):P3.

Diédhiou CJ, Golldack D. Salt-dependent regulation of chloride channel transcripts in rice. Plant Sci. 2006 Apr 1;170(4):793–800.

Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: ultrafast universal RNA-seq aligner. Bioinforma. Oxf. Engl. 2013 Jan 1;29(1):15–21.

Drerup MM, Schlücking K, Hashimoto K, Manishankar P, Steinhorst L, Kuchitsu K, et al. The Calcineurin B-Like Calcium Sensors CBL1 and CBL9 Together with Their Interacting Protein Kinase CIPK26 Regulate the Arabidopsis NADPH Oxidase RBOHF. Mol. Plant. 2013 Mar 1;6(2):559–69.

Espinoza C, Liang Y, Stacey G. Chitin receptor CERK1 links salt stress and chitin-triggered innate immunity in Arabidopsis. Plant J. 2017 Mar 1;89(5):984–95.

Fahad S, Hussain S, Matloob A, Khan FA, Khaliq A, Saud S, et al. Phytohormones and plant responses to salinity stress: a review. Plant Growth Regul. 2015 Mar 1;75(2):391–404.

FAOSTAT. Food and Agriculture Organization fo the United Nations [Internet]. Food Agric. Organ. Fo U. N. 2014 [cited 2014 Apr 22]. Available from: http://www.fao.org/economic/ess/en/#.U1XSWvmSymw

Gall HL, Philippe F, Domon J-M, Gillet F, Pelloux J, Rayon C. Cell Wall Metabolism in Response to Abiotic Stress. Plants. 2015 Feb 16;4(1):112–66.

García MJ, Lucena C, Romera FJ, Alcántara E, Pérez-Vicente R. Ethylene and nitric oxide involvement in the up-regulation of key genes related to iron acquisition and homeostasis in Arabidopsis. J. Exp. Bot. 2010 Sep 1;61(14):3885–99.

Gilliham M, Able JA, Roy SJ. Translating knowledge about abiotic stress tolerance to breeding programmes. Plant J. 2017 Feb 1;n/a-n/a.

Gilroy S, Jones DL. Through form to function: root hair development and nutrient uptake. Trends Plant Sci. 2000 Feb 1;5(2):56–60.

Gilroy S, Suzuki N, Miller G, Choi W-G, Toyota M, Devireddy AR, et al. A tidal wave of signals: calcium and ROS at the forefront of rapid systemic signaling. Trends Plant Sci. 2014 Oct;19(10):623–30.

Grabov A. Plant KT/KUP/HAK Potassium Transporters: Single Family – Multiple Functions. Ann. Bot. 2007 Jun 1;99(6):1035–41.

Grant CE, Bailey TL, Noble WS. FIMO: scanning for occurrences of a given motif. Bioinformatics. 2011 Apr 1;27(7):1017–8.

Hamamoto S, Horie T, Hauser F, Deinlein U, Schroeder JI, Uozumi N. HKT transporters mediate salt stress resistance in plants: from structure and function to the field. Curr. Opin. Biotechnol. 2015 Apr;32:113–20.

Han G, Wang M, Yuan F, Sui N, Song J, Wang B. The CCCH zinc finger protein gene AtZFP1 improves salt resistance in Arabidopsis thaliana. Plant Mol. Biol. 2014 Oct;86(3):237–53.

Hanin M, Ebel C, Ngom M, Laplaze L, Masmoudi K. New Insights on Plant Salt Tolerance Mechanisms and Their Potential Use for Breeding. Front. Plant Sci. [Internet]. 2016 Nov 29 [cited 2017 Apr 4];7. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC5126725/

Hashimoto K, Eckert C, Anschutz U, Scholz M, Held K, Waadt R, et al. Phosphorylation of Calcineurin B-like (CBL) Calcium Sensor Proteins by Their CBL-interacting Protein Kinases (CIPKs) Is Required for Full Activity of CBL-CIPK Complexes toward Their Target Proteins. J. Biol. Chem. 2012 Mar 9;287(11):7956–68.

Huang W. Role of AtCIPK16 in Arabidopsis abiotic tolerance [Internet] [PhD Thesis]. The University of Adelaide; 2015 [cited 2018 Feb 5]. Available from: https://digital.library.adelaide.edu.au/dspace/bitstream/2440/103616/1/01front.pdf

Huibers RP, de Jong M, Dekter RW, Van den Ackerveken G. Disease-Specific Expression of Host Genes During Downy Mildew Infection of Arabidopsis. Mol. Plant. Microbe Interact. 2009 Aug 5;22(9):1104–15.

Hwang S, Gou Z, Kuznetsov IB. DP-Bind: a web server for sequence-based prediction of DNA-binding residues in DNA-binding proteins. Bioinforma. Oxf. Engl. 2007 Mar 1;23(5):634–6.

Ivanchenko MG, Muday GK, Dubrovsky JG. Ethylene–auxin interactions regulate lateral root initiation and emergence in Arabidopsis thaliana. Plant J. 2008 Jul 1;55(2):335–47.

Ivashikina N, Becker D, Ache P, Meyerhoff O, Felle HH, Hedrich R. K+ channel profile and electrical properties of Arabidopsis root hairs. FEBS Lett. 2001 Nov 23;508(3):463–9.

Jha D, Shirley N, Tester M, Roy SJ. Variation in salinity tolerance and shoot sodium accumulation in Arabidopsis ecotypes linked to differences in the natural expression levels of transporters involved in sodium transport. Plant Cell Environ. 2010 May 1;33(5):793–804.

Ji H, Pardo JM, Batelli G, Oosten MJV, Bressan RA, Li X. The Salt Overly Sensitive (SOS) Pathway: Established and Emerging Roles. Mol. Plant. 2013 Jan 25;sst017.

Kanehisa M, Furumichi M, Tanabe M, Sato Y, Morishima K. KEGG: new perspectives on genomes, pathways, diseases and drugs. Nucleic Acids Res. 2017 Jan 4;45(D1):D353–61.

Kim K-N. CIPK3, a Calcium Sensor-Associated Protein Kinase That Regulates Abscisic Acid and Cold Signal Transduction in Arabidopsis. PLANT CELL ONLINE. 2003 Feb 1;15(2):411–23.

Krishnamurthy P, Mohanty B, Wijaya E, Lee D-Y, Lim T-M, Lin Q, et al. Transcriptomics analysis of salt stress tolerance in the roots of the mangrove Avicennia officinalis. Sci. Rep. 2017 Aug 30;7(1):10031.

Langfelder P, Horvath S. WGCNA: an R package for weighted correlation network analysis. BMC Bioinformatics. 2008 Dec 29;9(1):559.

Law CW, Chen Y, Shi W, Smyth GK. Voom: precision weights unlock linear model analysis tools for RNA-seq read counts. Genome Biol. 2014;15(2):R29.

Lee SC, Lan W-Z, Kim B-G, Li L, Cheong YH, Pandey GK, et al. A protein phosphorylation/dephosphorylation network regulates a plant potassium channel. Proc. Natl. Acad. Sci. 2007 Oct 2;104(40):15959–64.

Li H-W, Zang B-S, Deng X-W, Wang X-P. Overexpression of the trehalose-6-phosphate synthase gene OsTPS1 enhances abiotic stress tolerance in rice. Planta. 2011 Nov 1;234(5):1007–18.

Li Q, Yang A, Zhang W-H. Efficient acquisition of iron confers greater tolerance to saline-alkaline stress in rice (Oryza sativa L.). J. Exp. Bot. 2016 Dec;67(22):6431–44.

Li W, A C, M U, T G, H M, S S, et al. The EMBL-EBI bioinformatics web and programmatic tools framework. Nucleic Acids Res. 2015 2015;43(W1):W580-4.

Li Y, Fei X, Wu X, Deng X. Iron deficiency response gene Femu2 plays a positive role in protecting Chlamydomonas reinhardtii against salt stress. Biochim. Biophys. Acta BBA - Gen. Subj. 2017 Jan 1;1861(1, Part A):3345–54.

Liang S, Fang L, Zhou R, Tang T, Deng S, Dong S, et al. Transcriptional Homeostasis of a Mangrove Species, Ceriops tagal, in Saline Environments, as Revealed by Microarray Analysis. PLOS ONE. 2012 May 4;7(5):e36499.

Liao Y, Smyth GK, Shi W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinforma. Oxf. Engl. 2014 Apr 1;30(7):923–30.

Liu J, Ishitani M, Halfter U, Kim C-S, Zhu J-K. The Arabidopsis thaliana SOS2 gene encodes a protein kinase that is required for salt tolerance. Proc. Natl. Acad. Sci. U. S. A. 2000 Mar 28;97(7):3730–4.

Long TA, Tsukagoshi H, Busch W, Lahner B, Salt DE, Benfey PN. The bHLH Transcription Factor POPEYE Regulates Response to Iron Deficiency in Arabidopsis Roots. Plant Cell. 2010 Jul 1;22(7):2219–36.

Luan S. The CBL–CIPK network in plant calcium signaling. Trends Plant Sci. 2009 Jan;14(1):37–42.

Maathuis FJ, Amtmann A. K+ nutrition and Na+ toxicity: the basis of cellular K+/Na+ ratios. Ann. Bot. 1999;84(2):123–133.

Maldonado-Bonilla LD, Eschen-Lippold L, Gago-Zachert S, Tabassum N, Bauer N, Scheel D, et al. The Arabidopsis Tandem Zinc Finger 9 Protein Binds RNA and Mediates Pathogen-Associated Molecular Pattern-Triggered Immune Responses. Plant Cell Physiol. 2014 Feb 1;55(2):412–25.

Mao J, Manik SMN, Shi S, Chao J, Jin Y, Wang Q, et al. Mechanisms and Physiological Roles of the CBL-CIPK Networking System in Arabidopsis thaliana. Genes. 2016 Sep 8;7(9):62.

McDonnell AV, Jiang T, Keating AE, Berger B. Paircoil2: improved prediction of coiled coils from sequence. Bioinformatics. 2006 Feb 1;22(3):356–8.

Meng X, Xu J, He Y, Yang K-Y, Mordorski B, Liu Y, et al. Phosphorylation of an ERF Transcription Factor by *Arabidopsis* MPK3/MPK6 Regulates Plant Defense Gene Induction and Fungal Resistance. Plant Cell Online. 2013 Mar 1;25(3):1126–42.

Meng X, Zhang S. MAPK Cascades in Plant Disease Resistance Signaling. Annu. Rev. Phytopathol. 2013;51(1):245–66.

Mittler R, Vanderauwera S, Suzuki N, Miller G, Tognetti VB, Vandepoele K, et al. ROS signaling: the new wave? Trends Plant Sci. 2011 Jun;16(6):300–9.

Munns R, Gilliham M. Salinity tolerance of crops – what is the cost? New Phytol. 2015 Nov 1;208(3):668–73.

Naoumkina MA, Zhao Q, Gallego-Giraldo L, Dai X, Zhao PX, Dixon RA. Genome-wide analysis of phenylpropanoid defence pathways. Mol. Plant Pathol. 2010 Nov 1;11(6):829–46.

Nguyen XC, Kim SH, Lee K, Kim KE, Liu X-M, Han HJ, et al. Identification of a C2H2-type zinc finger transcription factor (ZAT10) from Arabidopsis as a substrate of MAP kinase. Plant Cell Rep. 2012 Apr 1;31(4):737–45.

Park J-H, Halitschke R, Kim HB, Baldwin IT, Feldmann KA, Feyereisen R. A knock-out mutation in allene oxide synthase results in male sterility and defective wound signal transduction in Arabidopsis due to a block in jasmonic acid biosynthesis. Plant J. 2002 Jul 1;31(1):1–12.

Peleg Z, Blumwald E. Hormone balance and abiotic stress tolerance in crop plants. Curr. Opin. Plant Biol. 2011 Jun 1;14(3):290–5.

Petersen B, Petersen TN, Andersen P, Nielsen M, Lundegaard C. A generic method for assignment of reliability scores applied to solvent accessibility predictions. BMC Struct. Biol. 2009 Jul 31;9(1):51.

Pomeranz M, Zhang L, Finer J, Jang J-C. Can AtTZF1 act as a transcriptional activator or repressor in plants? Plant Signal. Behav. 2011 May;6(5):719–22.

Popescu SC, Popescu GV, Bachan S, Zhang Z, Gerstein M, Snyder M, et al. MAPK target networks in Arabidopsis thaliana revealed using functional protein microarrays. Genes Dev. 2009 Jan 1;23(1):80–92.

Purohit D, Sankararamasubramanian HM, Pal AK, Parida AK. Identification and characterization of a novel iron deficiency and salt stress responsive transcription factor IDEF1 in <Emphasis Type="Italic">Porteresia coarctata</Emphasis>. Biol. Plant. 2016 Sep 1;60(3):469–81.

Qiu J, Henderson SW, Tester M, Roy SJ, Gilliham M. SLAH1, a homologue of the slow type anion channel SLAC1, modulates shoot Cl − accumulation and salt tolerance in Arabidopsis thaliana. J. Exp. Bot. 2016 Aug 1;67(15):4495–505.

Qu J, Kang SG, Wang W, Musier-Forsyth K, Jang J-C. Arabidopsis thaliana tandem zinc finger 1 (AtTZF1) protein in RNA binding and decay. Plant J. Cell Mol. Biol. 2014 May;78(3):452–67.

Rabhi M, Barhoumi Z, Ksouri R, Abdelly C, Gharsalli M. Interactive effects of salinity and iron deficiency in Medicago ciliaris. C. R. Biol. 2007 Nov 1;330(11):779–88.

Ren H, Gray WM. SAUR Proteins as Effectors of Hormonal and Environmental Signals in Plant Growth. Mol. Plant. 2015 Aug 3;8(8):1153–64.

Rengasamy P. World salinization with emphasis on Australia. J. Exp. Bot. 2006 Mar 1;57(5):1017–23.

Rengasamy P. Soil processes affecting crop production in salt-affected soils. Funct. Plant Biol. 2010 Jul 23;37(7):613–20.

Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. 2015 Jan 20;gkv007.

Rosa M, Prado C, Podazza G, Interdonato R, González JA, Hilal M, et al. Soluble sugars—Metabolism, sensing and abiotic stress. Plant Signal. Behav. 2009 May;4(5):388–93.

Rost B, Yachdav G, Liu J. The PredictProtein server. Nucleic Acids Res. 2004 Jul 1;32(suppl 2):W321–6.

Roy SJ, Huang W, Wang XJ, Evrard A, Schmöckel SM, Zafar ZU, et al. A novel protein kinase involved in Na+ exclusion revealed from positional cloning. Plant Cell Environ. 2013 Mar 1;36(3):553–68.

Roy SJ, Negrão S, Tester M. Salt resistant crop plants. Curr. Opin. Biotechnol. 2014 Apr;26:115–24.

Roy SJ, Tucker EJ, Tester M. Genetic analysis of abiotic stress tolerance in crops. Curr. Opin. Plant Biol. 2011 Jun;14(3):232–9.

Ryu H, Cho Y-G. Plant hormones in salt stress tolerance. J. Plant Biol. 2015 Jun;58(3):147–55.

Sanchez-Barrena MJ, Fujii H, Angulo I, Martinez-Ripoll M, Zhu J-K, Albert A. The Structure of the C-Terminal Domain of the Protein Kinase AtSOS2 Bound to the Calcium Sensor AtSOS3. Mol. Cell. 2007 May 11;26(3):427–35.

Schroeder JI, Delhaize E, Frommer WB, Guerinot ML, Harrison MJ, Herrera-Estrella L, et al. Using membrane transporters to improve crops for sustainable food production. Nature. 2013 May 2;497(7447):60–6.

Shabala S. Learning from halophytes: physiological basis and strategies to improve abiotic stress tolerance in crops. Ann. Bot. 2013 Oct 1;mct205.

Shabala S, White RG, Djordjevic MA, Ruan Y-L, Mathesius U. Root-to-shoot signalling: integration of diverse molecules, pathways and functions. Funct. Plant Biol. 2016;43(2):87.

Shabala S, Wu H, Bose J. Salt stress sensing and early signalling events in plant roots: Current knowledge and hypothesis. Plant Sci. 2015 Dec;241:109–19.

Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat. Appl. Genet. Mol. Biol. 2004;3:Article3.

Tao J-J, Chen H-W, Ma B, Zhang W-K, Chen S-Y, Zhang J-S. The Role of Ethylene in Plants Under Salinity Stress. Plant Nutr. 2015;1059.

Taylor GA, Thompson MJ, Lai WS, Blackshear PJ. Phosphorylation of Tristetraprolin, a Potential Zinc Finger Transcription Factor, by Mitogen Stimulation in Intact Cells and by Mitogen-activated Protein Kinase in Vitro. J. Biol. Chem. 1995 Jun 2;270(22):13341–7.

Thimm O, Bläsing O, Gibon Y, Nagel A, Meyer S, Krüger P, et al. MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. Plant J. Cell Mol. Biol. 2004 Mar;37(6):914–39.

Thoday-Kennedy EL, Jacobs AK, Roy SJ. The role of the CBL–CIPK calcium signalling network in regulating ion transport in response to abiotic stress. Plant Growth Regul. 2015 May;76(1):3–12.

Tripathi V, Parasuraman B, Laxmi A, Chattopadhyay D. CIPK6, a CBL-interacting protein kinase is required for development and salt tolerance in plants. Plant J. 2009 Jun 1;58(5):778–90.

Vogel MO, Gomez-Perez D, Probst N, Dietz K-J. Combinatorial Signal Integration by APETALA2/Ethylene Response Factor (ERF)-Transcription Factors and the Involvement of AP2-2 in Starvation Response. Int. J. Mol. Sci. 2012 May 16;13(5):5933–51.

Wang KL-C, Li H, Ecker JR. Ethylene Biosynthesis and Signaling Networks. Plant Cell. 2002 May 1;14(suppl 1):S131–51.

Wang L, Brown SJ. BindN: a web-based tool for efficient prediction of DNA and RNA binding sites in amino acid sequences. Nucleic Acids Res. 2006 Jul 1;34(Web Server issue):W243-248.

Wang S, Su SZ, Wu Y, Li SP, Shan XH, Liu HK, et al. Overexpression of maize chloride channel gene <Emphasis Type="Italic">ZmCLC-d</Emphasis> in <Emphasis Type="Italic">Arabidopsis thaliana</Emphasis> improved its stress resistance. Biol. Plant. 2015 Jan 1;59(1):55–64.

Wani SH, Singh NB, Haribhushan A, Mir JI. Compatible Solute Engineering in Plants for Abiotic Stress Tolerance - Role of Glycine Betaine. Curr. Genomics. 2013 May;14(3):157–65.

Wasternack C, Hause B. Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in Annals of Botany. Ann. Bot. 2013 Jun 1;111(6):1021–58.

Wieckowski S. The Relation Between the Growth of the Leaf and the Synthesis of Chlorophylls in Phaseolus Vulgaris. Photochem. Photobiol. 1963 Apr 1;2(2):199–205.

Wiese J, Kranz T, Schubert S. Induction of Pathogen Resistance in Barley by Abiotic Stress. Plant Biol. 2004 Sep 1;6(5):529–36.

Witzel K, Matros A, Møller ALB, Ramireddy E, Finnie C, Peukert M, et al. Plasma membrane proteome analysis identifies a role of barley Membrane Steroid Binding Protein in root architecture response to salinity. Plant Cell Environ. 2018;pre-print.

Xie C, Zhou X, Deng X, Guo Y. PKS5, a SNF1-related kinase, interacts with and phosphorylates NPR1, and modulates expression of WRKY38 and WRKY62. J. Genet. Genomics. 2010 Jun 1;37(6):359–69.

Yi X, Du Z, Su Z. PlantGSEA: a gene set enrichment analysis toolkit for plant community. Nucleic Acids Res. 2013 Jul;41(Web Server issue):W98-103.

Zhang B, Horvath S. A general framework for weighted gene co-expression network analysis. Stat. Appl. Genet. Mol. Biol. [Internet]. 2005 [cited 2014 Jun 18];4(1). Available from: http://www.degruyter.com/view/j/sagmb.2005.4.1/sagmb.2005.4.1.1128/sagmb.2005.4.1.1128.xml

Zhao Y, Dong W, Zhang N, Ai X, Wang M, Huang Z, et al. A Wheat Allene Oxide Cyclase Gene Enhances Salinity Tolerance via Jasmonate Signaling1[C][W]. Plant Physiol. 2014 Feb;164(2):1068–76.

Zhao Y, Wang T, Zhang W, Li X. SOS3 mediates lateral root development under low salt stress through regulation of auxin redistribution and maxima in Arabidopsis. New Phytol. 2011 Mar 1;189(4):1122–34.

Zhou F, Sosa J, Feldmann KA. High throughput approaches for the identification of salt tolerance genes in plants. Adv. Mol. Breed. Drought Salt Toler. Crops [Internet]. 1st ed. Springer Netherlands; 2007 [cited 2018 Feb 6]. p. 359–79. Available from: https://arizona.pure.elsevier.com/en/publications/high-throughput-approaches-for-the-identification-of-salt-toleran

Zhu J-K. Plant salt tolerance. Trends Plant Sci. 2001 Feb 1;6(2):66–71.

Chapter 4 The Evolutionary Origin of CIPK16: A Gene Involved In Enhanced Salt Tolerance

Statement of Authorship

Title of Paper	The evolutionary origin of CIPK16: A gene involved in enhanced salt tolerance
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Amarasinghe S, Watson-Haigh NS, Gilliham M, Roy S, Baumann U. The evolutionary origin of CIPK16: A gene involved in enhanced salt tolerance. Mol. Phylogenet. Evol. 2016 Jul;100:135–47. This is an original research article on my investigation on the evolution and current pervasiveness of CIPK16. From a forward genetic approach the *CIPK16* gene from Arabidopsis (*AtCIPK16*) has been identified to be involved in enhanced Na^+ tolerance both in Arabidopsis and barley. This is the first of the studied on the prevalence of CIPK16 across the plant kingdom that lays the foundation to better understanding its mode of action in plants. This article is closely related to the subject matter of this thesis

Principal Author

Name of Principal Author (Candidate)	Shanika Lakmini Amarasinghe		
Contribution to the Paper	Conceived the study and designed the study. Performed literature research, Data analysis. Critical interpretation and wrote the manuscript.		
Overall percentage (%)	80%		
Certification:	This paper reports on an original study I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	01/01/2016

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Nathan S. Watson-Haigh		
Contribution to the Paper	Conceived the study. Study concepts and design. Provided support with the variant analysis. Supervised the study. Edited the manuscript. I hereby certify that the statement of the contribution is accurate.		
Signature		Date	01/01/2016

Name of Co-Author	Matthew Gilliham		
Contribution to the Paper	Conceived the study. Provided comments and edited the manuscript. I hereby certify that the statement of the contribution is accurate.		
Signature		Date	01/01/2016

Name of Co-Author	Stuart J. Roy		
Contribution to the Paper	Conceived the study. Provided comments and edited the manuscript. I hereby certify that the statement of the contribution is accurate.		
Signature		Date	01/01/2016

Name of Co-Author	Ute Baumann		
Contribution to the Paper	Conceived and supervised the study. Provided comments and edited the manuscript. I hereby certify that the statement of the contribution is accurate.		
Signature		Date	01/01/2016

Link to Chapter 4

The molecular components underlying the *AtCIPK16* mediated salt tolerance as we show in **Chapter 3** are largely transcription factors, and they are related to phytohormone regulation in the presence of salinity stress. However, what we were unaware of is how prevalent the CIPK16 within the terrestrial plants. Investigating how CIPK16s evolved through time will enable us to gain an understanding of its functional importance in different species. Through this study we also attempted to distinguish a protein in barley that can potentially function similar to AtCIPK16 in salt stress. This chapter has been published as follows: Amarasinghe, S., Watson-Haigh, N.S., Gilliham, M., Roy, S., Baumann, U., 2016. The evolutionary origin of CIPK16: A gene involved in enhanced salt tolerance. Mol. Phylogenet. Evol. 100, 135–147. https://doi.org/10.1016/j.ympev.2016.03.031. Sequence similarity testing with the *Arabidopsis thaliana* sequence as a reference was used to identify *CIPK* homologues in monocots and dicots. Together with information on domain and intron structure, an in-depth phylogenetic analysis has been performed. The findings of this study suggested that CIPKs contained unique characters that define them and were confined to a very specific group of dicots called *core Brassicales*. According to the presented model of evolution of CIPK16s in the terrestrial plant kingdom, it is likely that an AtCIPK16 orthologue is not present in the monocot species barley.

Molecular Phylogenetics and Evolution 100 (2016) 135–147

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

The evolutionary origin of CIPK16: A gene involved in enhanced salt tolerance

Shanika Amarasinghe [a], Nathan S. Watson-Haigh [a], Matthew Gilliham [b], Stuart Roy [a], Ute Baumann [a,*]

[a] Australian Centre for Plant Functional Genomics, PMB 1, Glen Osmond, SA 5064, Australia
[b] Australian Research Council Centre of Excellence in Plant Energy Biology, Department of Plant Science, School of Agriculture, Food and Wine, University of Adelaide, Glen Osmond SA 5064, Australia

ARTICLE INFO

Article history:
Received 6 January 2016
Revised 30 March 2016
Accepted 31 March 2016
Available online 1 April 2016

Keywords:
CIPK16
Arabidopsis thaliana
Barley
Salt tolerance
Phylogenetic analysis

ABSTRACT

Calcineurin B-like protein interacting protein kinases (CIPKs) are key regulators of pre-transcriptional and post-translational responses to abiotic stress. *Arabidopsis thaliana* CIPK16 (*AtCIPK16*) was identified from a forward genetic screen as a gene that mediates lower shoot salt accumulation and improved salinity tolerance in Arabidopsis and transgenic barley. Here, we aimed to gain an understanding of the evolution of *AtCIPK16*, and orthologues of *CIPK16* in other plant species including barley, by conducting a phylogenetic analysis of terrestrial plant species. The resulting protein sequence based phylogenetic trees revealed a single clade that included AtCIPK16 along with two segmentally duplicated CIPKs, AtCIPK5 and AtCIPK25. No monocots had proteins that fell into this clade; instead the most closely related monocot proteins formed a group basal to the entire *CIPK16*, *5* and *25* clade. We also found that *AtCIPK16* contains a core *Brassicales* specific indel and a putative nuclear localisation signal, which are synapomorphic characters of *CIPK16* genes. In addition, we present a model that proposes the evolution of *CIPK16*, *5* and *25* clade.

© 2016 Elsevier Inc. All rights reserved.

1. Introduction

Salinity in soil impacts negatively on crop growth and is a significant limiting factor for agriculture, particularly in arid and semi-arid regions, with an estimated cost of US$27 billion due to lost crop production per year (Munns and Gilliham, 2015; Qadir et al., 2014). It has been estimated that on land irrigated for agriculture, which produces 40% of the world's calories, one-fifth of soils are salt-affected (FAOSTAT, 2014). The extent of this salt-affected irrigated agricultural land has been forecasted to increase by 4% every year (FAOSTAT, 2014; Pimentel et al., 2004). Crops with increased tolerance to salt, which provide higher yields under saline soil conditions, are needed to sustain future global food production (Munns and Gilliham, 2015). To this end, aspects of plant responses to salinity have to be understood before they can be manipulated by molecular assisted breeding or transgenesis (Roy et al., 2014).

Plants retain only around 5% of the water that they take up in transpiration, thus salt concentration in the transpiration stream needs to be in the order of 1/20th of that in the soil to avoid the accumulation of salt in leaves to concentrations above that in the soil (Munns, 2005). As a result, all plants have developed mechanisms to exclude salt to a large degree: halophytes exclude ~92–95% of the salt in the soil solution and most crop plants exclude 96–99% (Munns, 2005; Munns and Tester, 2008). Plants achieve this by either minimising the entry of salt into the leaves (i.e. the trait of shoot ion exclusion) or by tolerating the accumulation of salt in leaves by reducing the concentration of salt in the cytoplasm (i.e. the trait of tissue tolerance) by compartmentalizing of the salt in the cells of leaf sheath or leaf cell vacuole (Munns, 2005; Munns and Gilliham, 2015; Munns and Tester, 2008; Plett and Møller, 2010; Shabala, 2009). Both ion exclusion and tissue tolerance demand high amounts of energy for osmotic adjustment within the cytosol via organic solutes (Adem et al., 2014; Munns and Gilliham, 2015; Plett and Møller, 2010; Shabala, 2013). Wheat and rice have lower Na^+ and Cl^- concentrations in their leaves than the external solution as a consequence of ion exclusion mechanisms (Roy et al., 2014). Salt tolerant non-halophytes such as barley, exclude less salt from leaves more clearly exhibit the trait of tissue tolerance (Colmer et al., 2005; Munns and Gilliham, 2015; Shabala, 2013).

Abbreviations: [CIPK16/5/25], CIPK16, 5 and 25 clade; NCBs, none core *Brassicales* dicots; ALI, activation loop indel in CIPK16; JDNLS, junction domain nuclear localisation signal in CIPK16.

* Corresponding author at: Australian Centre for Plant Functional Genomics, The University of Adelaide, School of Agriculture, Food and Wine, Glen Osmond, South Australia 5064, Australia.
E-mail address: ute.baumann@adelaide.edu.au (U. Baumann).

http://dx.doi.org/10.1016/j.ympev.2016.03.031
1055-7903/© 2016 Elsevier Inc. All rights reserved.

In instances when plants are unable to eliminate the negative effects of salinity, they initially suffer due to the buildup of osmotic stress followed by salt-specific ionic stress (Munns, 2005; Munns and Tester, 2008; Rajendran et al., 2009). Immediately after exposure to salinity but before ions accumulate in the plant's shoot, plant growth rate is reduced (Rajendran et al., 2009; Tavakkoli et al., 2010). Over time as ions accumulate in the shoot, ion toxicity reduced plant growth rate even further. Reducing the severity of salinity stress in plants therefore, needs early detection of salinity stress and the activation of stress signalling mechanisms.

Many aspects of stress signalling are facilitated through secondary messengers such as calcium ions (Ca^{2+}) (Batistic et al., 2011). A 20–60 s long single or biphasic Ca^{2+} elevation in the cytosol is one of the initial cellular responses of a plant to high salinity (Choi et al., 2014; Tracy et al., 2008). The sensor molecules capturing these signals fall into four major categories, namely Calcineurin B-Like (CBL) proteins, calcium-dependent protein kinases (CDPKs), calmodulins (CaMs), and calmodulin-like proteins (CAMLs) (Weinl and Kudla, 2009). Amongst these, CBLs selectively interact with one or more protein kinases from the group named CBL interacting protein kinases (CIPKs) (Batistic and Kudla, 2004; Kim et al., 2000). The CIPKs have been catalogued as SNF1 (Sucrose non-fermenting 1)-related kinases and group 3 (SnRK3) proteins, according to their structural features and evolutionary associations (Hrabak et al., 2003). The general structure of all CIPK-type kinases includes a conserved N-terminal kinase domain, and a variable junction domain, which separates it from a unique C-terminal regulatory domain (Sanchez-Barrena et al., 2007; Weinl and Kudla, 2009). In common with many kinases, an activation loop lies between two conserved tri-peptide motifs (DFG…APE) in the kinase domain, which needs to be phosphorylated for the kinase to be activated (Nolen et al., 2004). While much of the regulatory domain sequence is divergent in these proteins, there exists a well conserved FISL/NAF domain, which mediates the interaction with CBLs (Albrecht et al., 2001). Additionally, a conserved C-terminal protein–phosphatase interaction (PPi) domain mediates CIPK interaction with the 2C-type protein phosphatase (PP2C) group, via phosphorylation (Ohta et al., 2003; Sanchez-Barrena et al., 2013).

A *CIPK* from the model dicot *Arabidopsis thaliana*, named *AtCIPK16* is associated with Na^+ exclusion in plants (Roy et al., 2013). Transgenic Arabidopsis constitutively overexpressing *AtCIPK16* showed a significant reduction of shoot Na^+ in plants grown in elevated salt in both soil and hydroponics (Roy et al., 2013). Moreover, transgenic barley constitutively expressing *AtCIPK16* also exhibited decreased leaf Na^+ and increased salinity tolerance. This implies that *AtCIPK16* can be used as a tool in genetic engineering to improve salinity tolerance in crops. In Roy et al. (2013), *AtCIPK16* was identified using a Bay-0 × Shahdara mapping population. The Bay-0 accession allele of *AtCIPK16* contained a TATA box 65 bp upstream of the start codon and had higher gene expression under salt stress compared to Shahdara (which contained no TATA box in this region) (Roy et al., 2013). However, our understanding of the underlying mechanism of *CIPK16* mediated salt tolerance is still incomplete.

A study on how widespread the *CIPK16*-associated salinity tolerance mechanism is in the plant world could be an initial step in understanding the functional network associated with *CIPK16*. It also may lay the foundation for further experiments such as screening or editing the genes that boost salt tolerance in plants. A phylogenetic study on the prevalence of *CIPK16* in the plant kingdom would facilitate the discovery of *AtCIPK16* orthologues in crops important for global food production. Orthologues, by definition, would have a common ancestor and tend to have similar functionality (Fulton et al., 2006; Wu et al., 2006). Several phylogenetic analyses on *CIPKs* from different plant species have been conducted (Thoday-Kennedy et al., 2015 and references therein). For instance, a previous phylogenetic study on 25 *A. thaliana* CIPKs revealed that *AtCIPK16* resides in close proximity to two other segmentally duplicated *CIPKs*, namely *AtCIPK5* and *AtCIPK25* (Kolukisaoglu et al., 2004). However, to our knowledge, a phylogenetic analysis detailing the prevalence of *CIPK16* across the plant kingdom has not been conducted so far.

The aim of the current study is to discover the origin of *CIPK16* and its closest relatives, *CIPK5* and *CIPK25*, using phylogenetic approaches, *in silico* protein analysis and known evolutionary relationships between terrestrial plants (Fig. 1) (Chase, 2004; Kagale et al., 2014b; Soltis et al., 2011).

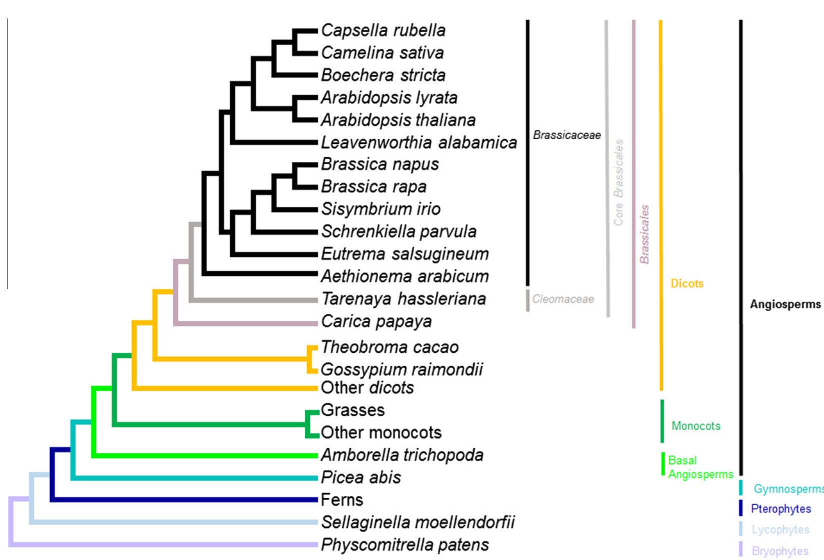


Fig. 1. A cladogram showing a summary of known relationships (Chase, 2004; Kagale et al., 2014b; Soltis et al., 2011) amongst the species used in this study together with higher level taxonomic designations to which we commonly refer.

2. Materials and methods

2.1. Molecular phylogenetics of CIPK16

2.1.1. Sequence retrieval

Protein and nucleotide sequences were retrieved from the sources detailed in Table 1. Species were selected based on the availability of the full genomic sequences (Cheng et al., 2013; Michael and Jackson, 2013). *Brassicaceae* species were targeted as they are closely related to *A. thaliana*. Sequences were retrieved through one of the following methods: (1) sequence similarity to *A. thaliana* CIPK gene/protein sequences; and, (2) keyword searches. Sequences retrieved by sequence similarity were performed either via the BLAST tool linked to online databases or by locally indexed databases using the NCBI BLAST+ tool (V 2.2.29). All blastn, blastp, tblastn, tblastx options were used in BLAST querying with an expectation value (*e*-value) $\leqslant 1 \times 10^{-5}$. Default settings were used for querying with complete sequences. Settings were changed for queries with partial protein sequence in order to increase sensitivity; the short query option was deselected, the expect threshold was changed to 5 million, word size was changed to 2, and the compositional adjustments setting was set to "no adjustments". Sequence retrieval by keyword searches used the terms "cipk", "cbl interacting protein kinase", "cbl interacting" and "calcineurin b like". A fasta formatted sequence file for all the sequences used in this study is in the supplementary materials (S1).

2.1.2. Sequence alignment

Protein multiple sequence alignments (MSAs) were generated using MUSCLE (default settings) implemented in Jalview (Edgar, 2004; Waterhouse et al., 2009). Manual alignment was carried

Table 1
Species and resources used from which protein and nucleotide sequences were identified for this study.

Sequence acquired species	Web resource	References	Sequence access method	Identification method
Arabidopsis thaliana	TAIR (http://www.arabidopsis.org/)	Lamesch et al. (2012)	Online from TAIR10	BLAST (blastn, blastp), keyword search
Arabis alpina *Boechera stricta*	NCBI (www.ncbi.nlm.nih.gov/)	Coordinators (2013)	Online	BLAST (blastn, blastp, tblastn, tblastx)
Leavensworthia alabamica	CoGe (https://genomevolution.org/CoGe/)	Lyons and Freeling (2008)	FTP download	BLAST (blastn, blastp, tblastn, tblastx)
Aethionema arabicum	CoGe (https://genomevolution.org/CoGe/)	Haudry et al. (2013)	FTP download	BLAST (blastn, blastp, tblastn, tblastx)
A. lyrata	Phytozome (www.phytozome.net/)	Nordberg et al. (2014)	Online	BLAST (blastn, blastp, tblastn, tblastx)
Raphanus sativus	Raphanus sativus Genome Database (http://radish.kazusa.or.jp/)	Kitashiba et al. (2014)	Online	BLAST (blastn, blastp, tblastn)
Capsella rubella	Phytozome (www.phytozome.net/)	Goodstein et al. (2012)	Online	BLAST (blastn, blastp, tblastn, tblastx)
Schrenkiella parvula	thellungiella.org (http://thellungiella.org/)	Dassanayake et al. (2011)	FTP download	BLAST (blastn, blastp, tblastn, tblastx)
Eutrema salsugineum	thellungiella.org (http://thellungiella.org/)	Yang et al. (2013)	Online	BLAST (blastn, blastp, tblastn, tblastx)
Sisymbrium irio	Brassicadb (http://brassicadb.org/brad/)	Haudry et al. (2013)	FTP download	BLAST (blastn, blastp, tblastn, tblastx)
Brassica rapa (v 1.5) *Brassica napus (v 1.0)*	Brassicadb (http://brassicadb.org/brad/)	Cheng et al. (2011)	Online	BLAST (blastn, blastp, tblastn, tblastx)
Brassica oleraceae (v 1.0)	Brassicadb (http://brassicadb.org/brad/)	Cheng et al. (2011)	FTP download	BLAST (blastn, blastp, tblastn, tblastx)
Camelina sativa	Camelinadb (http://www.camelinadb.ca/)	Kagale et al. (2014a)	FTP download	BLAST (blastn, blastp, tblastn, tblastx)
Tarenaya hassleriana	CoGe (https://genomevolution.org/CoGe/)	Cheng et al. (2013)	Online	BLAST (blastn, blastp, tblastx)
Carica papaya	NCBI (www.ncbi.nlm.nih.gov/)	Coordinators (2013)	Online	BLAST (blastn, blastp, tblastn, tblastx)
Theobroma cacao	Cacao Genome Database (www.cacaogenomedb.org/)	Argout et al. (2011)	FTP download	BLAST (blastn, blastp, tblastn, tblastx)
Gossypium raimondii	Phytozome (www.phytozome.net/)	Goodstein et al. (2012)	Online	BLAST (blastn, blastp, tblastn, tblastx)
Vitis vinifera	Genoscope (www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/)	Jaillon et al. (2007)	Online	BLAST (blastn, blastp, tblastn, tblastx)
Musa acuminate malaccensis	Banana Genome Hub (banana-genome.cirad.fr/)	Droc et al. (2013)	Online	BLAST (blastn, blastp, tblastn, tblastx)
Fragaria vesca *Malus x domestica* *Prunus persica* *Pyrus communis*	Genome Database for Rosaceae (www.rosaceae.org/)	Jung et al. (2014)	Online	BLAST (blastn, blastp, tblastn, tblastx)
Brachypodium distachyon	Brachypodium database moved to Phytozome	Goodstein et al. (2012)	Online	BLAST (blastn, blastp, tblastn, tblastx)
Oryza sativa	Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/)	Ouyang et al. (2007)	Online	BLAST (blastn, blastp, tblastn, tblastx)
Triticum aestivum	IWGSC (www.wheatgenome.org/)	International Wheat Genome Sequencing Consortium (IWGSC) (2014)	Online	BLAST (blastn, blastp, tblastn, tblastx)
Hordeum vulgare	BARLEX from IPK (www.ipk-gatersleben.de/en/)	Colmsee et al. (2015)	Online	BLAST (blastn, blastp, tblastn, tblastx)
Hordeum vulgare	MIPS (http://mips.helmholtz-muenchen.de/plant/barley/)	Nussbaumer et al. (2013)	Online	BLAST (blastn, blastp, tblastn, tblastx)
Amborella trichopoda	http://www.amborella.org/	Albert et al. (2013)	FTP download	BLAST (blastn, blastp, tblastn, tblastx)
Picea abies	The cogenie.org (http://congenie.org/)	Nystedt et al. (2013)	Online	BLAST (blastn, blastp, tblastn, tblastx)
Generic	UniProt (www.uniprot.org/)	Consortium (2015)	Online	BLAST (blastn, blastp, tblastn, tblastx), keyword search
Generic	PlantGDB (www.plantgdb.org/)	Duvick et al. (2008)	Online	BLAST (blastn, blastp, tblastn, tblastx)
Generic	EnsamblePlants (plants.ensembl.org/)	Cunningham et al. (2015)	Online	BLAST (blastn, blastp, tblastn, tblastx)

Fig. 2. Molecular phylogenetic analysis for Brassicales–Malvales CIPKs used in this study by Maximum Likelihood method (summarised view). The evolutionary history was inferred by using the Maximum Likelihood method based on the Le_Gascuel_2008 model (Le and Gascuel, 2008). The tree with the highest log likelihood (−65950.5450) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbour-Joining method to a matrix of pairwise distances estimated using a JTT model. A discrete Gamma distribution was used to model evolutionary rate differences amongst sites (5 categories (+G, parameter = 1.0539)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 408 amino acid sequences. There were a total of 698 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013). The CIPK16, 5 and 25 clade from the intron less group is shown expanded. The other CIPK nodes are collapsed down and named for clarity of presentation. The fully expanded tree is available as supplementary materials (S14). Sequences from KC310466.1, AAU87882.1, AAU87884.1, KC991147.1, AGO32663.1, KC991149.1 and AEX07321.2 were included in the analyses as the most closely related non-CIPK-type protein kinases.

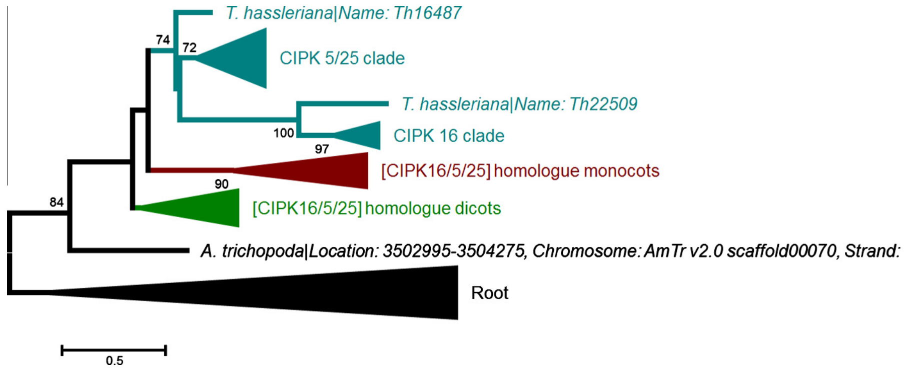


Fig. 3. Summary of the molecular phylogenetic analysis for CIPK16/5/25 group CIPKs used in this study by Maximum Likelihood method (summarised view). The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992). The tree with the highest log likelihood (−32815.7688) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using a JTT model. A discrete Gamma distribution was used to model evolutionary rate differences amongst sites (5 categories (+G, parameter = 0.9058)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 113 amino acid sequences. There were a total of 504 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013). The nodes are collapsed down and named for clarity of presentation. The tree is rooted on other *A. thaliana* CIPKs. The fully expanded tree is available as supplementary materials (S15).

out to improve the MSAs. Duplicates (defined by 99% identity or above) were removed from the multiple sequence alignments via the remove duplicates option in Jalview. Nucleotide alignments corresponding to the protein MSA were generated using Dialign 2.0 implemented in the RevTrans2.0 server, in order to correctly align DNA codons with corresponding amino acid residues (Morgenstern, 1999; Wernersson and Pedersen, 2003). Sequences were validated to be functional CIPK sequences by screening for the DF(G/D)L, APE motifs in the N-terminal kinase domain and the (N/T)AF motif in the C-terminal regulatory domain via a custom Perl script. Partial sequences and sequences without any of the DF(G/D)L, APE and (N/T)AF motifs were therefore excluded from the final refined alignment files. However, these sequences were not discarded but separated and manually examined.

Additionally, SeqFIRE and GBlock tools were employed to identify the conserved regions of the alignments, which encompass important domains (Ajawatanawong et al., 2012; Talavera and Castresana, 2007). The "Conserved Block Module – single alignment mode" from SeqFire accepted protein MSAs in FASTA format. The default parameter settings were used for the SeqFire tool. The online Gblock server was used to extract the well-aligned and conserved sequence blocks from the MSAs. FASTA formatted protein sequences were input with all the options for "less stringent selection" enabled.

2.1.3. Phylogenetic tree computation

The refined alignment files were used for this step. The phylogenetic analysis was conducted in such a way that, initially, bi-species trees were created using 26 CIPKs of *A. thaliana* as the reference. After examining the 137 trees developed this way, sequences were sequentially joined with the 26 *A. thaliana* sequences to generate the final tree including all 47 terrestrial species used in our analyses (the known evolutionary relationships amongst these species are shown in Fig. 1).

MODELGENERATOR v. 0.85 was used to determine the best substitution model for each dataset (with and without outgroups) (Keane et al., 2006). We hypothesised that unknown substitution rate variations exist in the genes of our data sets. Therefore, we used the gamma distribution for modelling the rate variation (5 categories) (Yang, 1996; Yang and Rannala, 2012). The best model fit for the phylogenetic tree creation was based on Corrected Akaike Information Criterion (AICc), Akaike Information Criterion 2 (AIC2) and Bayesian Information Criterion (BIC) (S2).

Phylogenetic trees were generated using MEGA 6.06 software using a Maximum Likelihood approach (Mount, 2008; Tamura et al., 2013). To estimate how well the nodes of the ML tree were supported, 10,000 bootstrap trees were generated (Felsenstein, 1985). The DOLLOP program from the PHYLIP package implemented in T-REX (http://www.trex.uqam.ca/index.php?action=phylip&app=dollop) was used to determine the minimum gene set for ancestral nodes of the phylogenetic tree (Boc et al., 2012; Felsenstein, 1996). The generated parsimony tree (Newick format) was used as the input to Ancestor v 1.1 in order to predict the ancestral sequences (Diallo et al., 2010). These ancestral sequences were used as queries for further BLAST searches.

2.2. Identification of unique sequence features

The Prosite (http://prosite.expasy.org/) and Pfam (http://pfam.xfam.org/) web resources were used to extract known important residues, motifs and domains of AtCIPK16 and its homologues (Finn et al., 2014; Sigrist et al., 2013). CIPK homologous sequences were examined using ScanProsite available through Prosite (v. 20.124) with the option "high sensitivity". We queried the Pfam database (v.27.0) using protein sequences with the default *e*-value threshold of 1×10^{-6} (Finn et al., 2014).

To identify potential nuclear localisation signals (NLS) within AtCIPK16 and its homologues, we submitted protein sequences to cNLS Mapper (http://nls-mapper.iab.keio.ac.jp/) in FASTA format (Kosugi et al., 2009). The following parameters were used; a cut-off score of 2.0; long bipartite NLSs were searched in the entire region of the proteins. Structural (e.g. secondary structure) and biochemical (e.g. solvent accessibility, subcellular localisation) features were predicted using PredictProtein and NetSurfP (Petersen et al., 2009; Rost et al., 2004). Default parameters were used.

2.3. Intron–exon architecture analysis of CIPK16 orthologues

To visualise and compare the intron–exon structure of CIPK16 orthologues we used GSDraw, available in PIECE (http://wheat.pw.usda.gov/piece/) (Wang et al., 2013). The input files contained the genomic nucleotide sequences and the cDNA sequences (S3). PIECE is a comparative genomics database named for Plant Intron and Exon Comparison and Evolution studies.

2.4. AtCIPK16 diversity amongst A. thaliana accessions

VCF files and BAM files were obtained from http://1001genomes.org for the purpose of identifying SNPs within the vicinity of *AtCIPK16* (at2g25090.1) ± 2500 bp (up to but not including any neighbouring genes). For the identification of SNPs, we used VCF files for 696 accessions made available under the GMINordborg2011, MPICWang2013 and Salk projects. SNPeff was used to predict the effect (e.g. synonymous, non-synonymous, start codon gain/loss, stop codon gain/loss and frameshifts) of the identified SNPs (Cingolani et al., 2012).

Roy et al., 2013 have previously reported a 10 bp deletion in the promoter region of *AtCIPK16* in Bay-0. For this reason, we examined accessions for which BAM files were available since VCF files are typically generated by SNP identification pipelines that ignore indel information. Furthermore, we restricted the selection of BAM files to those accessions for which reads had been mapped to the reference Col-0 (i.e. Shahdara, Bay-0, Sakata, Ri-0, Oy-0, Jea, blh-1 and Alc-0 under the JGIHazelWood 2008/11 projects). BAM files were visualised in IGV (Robinson et al. 2011) and alignments padded with gaps to reduce mismatches and achieve perfect gapped alignments.

3. Results

3.1. Molecular phylogenetics of CIPK 16, 5 and 25 protein sequences

Computation of phylogenetic trees allowed us to predict evolutionary relationships between genes. In the first instance, we computed phylogenetic trees for CIPK families from different *Brassicaceae* and *Cleome* species; the evolutionary relationships between these species is shown in Fig. 1. These species include *C. sativa*, *C. rubella*, *A. alpina*, *B. stricta*, *B. oleraceae*, *E. salsugineum*, *S. parvula*, *L. alabamica*, *A. arabicum* and *T. hassleriana*. The individual unrooted protein sequence derived phylogenetic trees for these species are provided in supplementary materials (S4–S13). The summary of the gene/protein tree for all studied *Brassicales* species is shown in Fig. 2 and the fully expanded tree is in S14.

The number of representatives for the CIPK16, 5 and 25 clade ([CIPK16/5/25]) varies amongst *Brassicales* species (Fig. 2). We were able to identify a complete or a partial sequence in all core *Brassicales* (*Brassicaceae* and *Cleomaceae*), which clustered with AtCIPK16 (Fig. 1), with the exception of *L. alabamica*, Within the *Brassicaceae* we were also able to identify orthologues for both

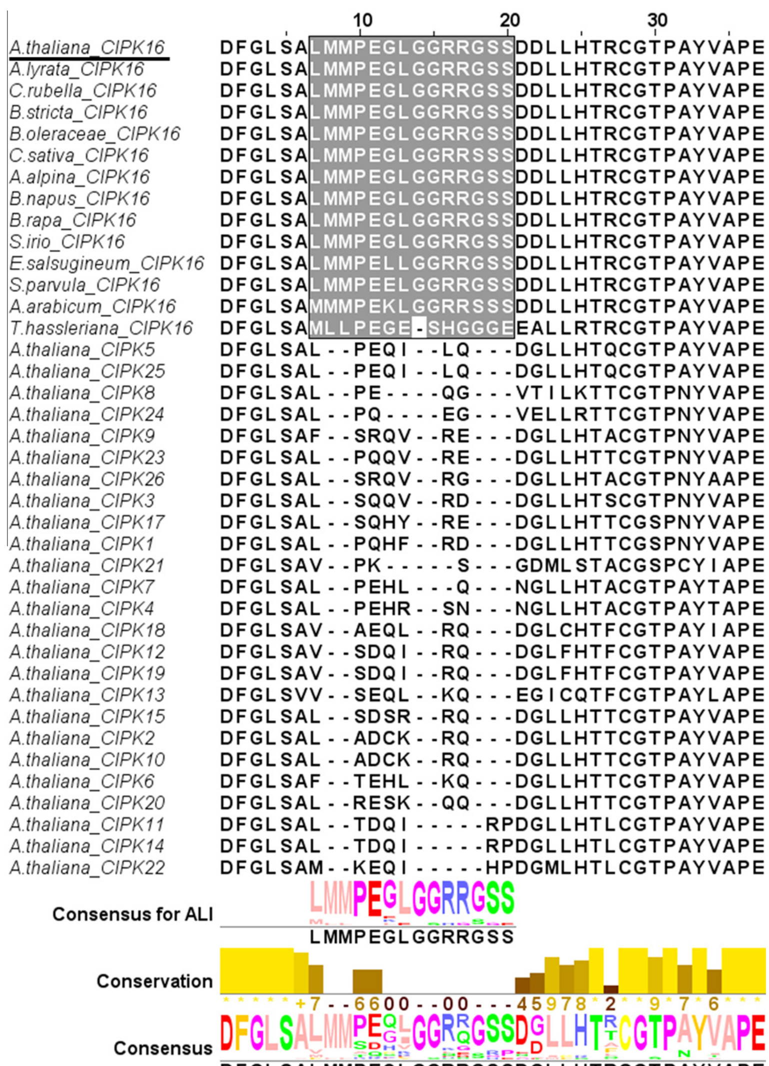


Fig. 4. Multiple sequence alignment (MSA) of the activation loop domain of CIPK proteins. The alignment was developed from the complete sequences of CIPK proteins using MUSCLE algorithm incorporated in Jalview application. The MSA showing the indel (ALI) of proteins in CIPK16 clade (indicated by the shaded box). Other *A. thaliana* CIPKs (AtCIPKs) were used to support the fact that ALI is only present in the CIPK16 clade proteins. ALI lies between the conserved regions of the activation loop (i.e. between DFGLSAL and SSDDLLHTRCGTPAYVAPE). For easy referencing in this text we would number the activation loop from D1FGLSAL…AYVAPE37. AtCIPK16 is underlined for clarity. The conservation histogram and normalised consensus logo is shown beneath the MSA.

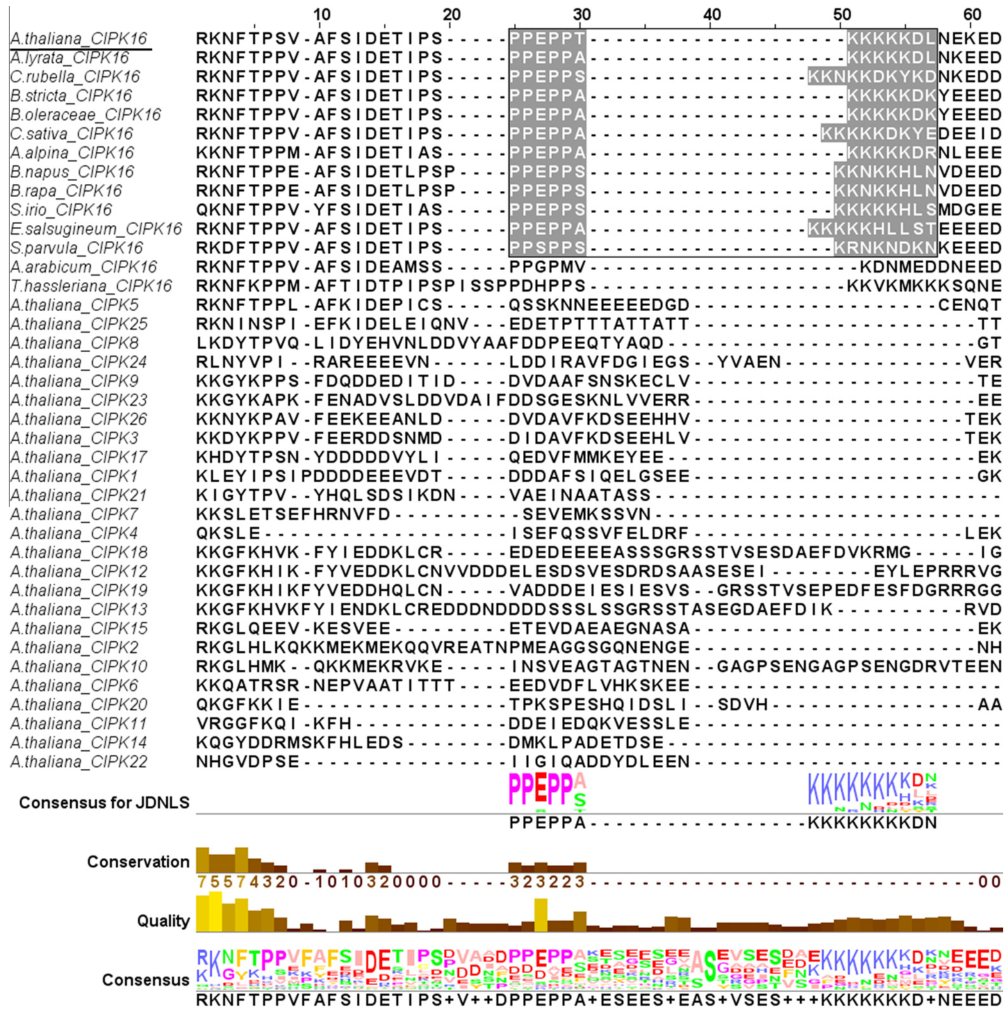


Fig. 5. Multiple sequence alignment (MSA) of the junction domain of CIPK proteins. The alignment was developed from the complete sequences of CIPK proteins using MUSCLE algorithm incorporated in Jalview application. The nuclear localization signal (NLS) unique to CIPK16 junction domain is shown in the shaded box (JDNLS). The NLS was predicted by the cNLS Mapper (http://nls-mapper.iab.keio.ac.jp/) (Kosugi et al., 2009). CIPK16 orthologues from *A. arabicum* and *T. hassleriana* did not predict a NLS in this region. Other *A. thaliana* CIPKs were used to show the variability within this region. JDNLS lies in the junction domain in middle of kinase domain and the regulatory domain. AtCIPK16 is underlined for clarity. The consensus logo for the NLS in CIPK16s, conservation histogram and normalised consensus logo is shown beneath the MSA.

AtCIPK5 and AtCIPK25. However, the only homologous sequence we identified in *T. hassleriana*, (a single representative of *Cleomaceae*) was placed at the base of the CIPK5/CIPK25 clade. For dicot species outside the core *Brassicales* (*C. papaya*, *T. cacao* and *G. raimondii*), and monocots, we found only homologues which form groups basal to [CIPK16/5/25] (Figs. 2 and 3). We could not identify any AtCIPK16, 5 or 25 orthologues in "non-core *Brassicales*" dicots (NCBs) (Fig. 3). A fully expanded tree for Fig. 3 is available as a supplementary figure (S15). The basal angiosperm *Amborella trichopoda* is the most distant species to *A. thaliana* that possesses a gene that clusters in the basal group for [CIPK16/5/25] (Fig. 3). We were unable to identify close homologues to [CIPK16/5/25] in terrestrial plant species outside of angiosperms (data not shown).

3.2. Unique characteristics of CIPK16s

Comparing MSAs and the computed phylogenetic trees revealed unique regions of CIPK16 orthologues. One such significant character is a unique indel (MMPEGLGGRRG) that exists in the activation loop of the kinase domain of CIPK16 orthologues (ALI) (Fig. 4). ALI-CIPK16 was not present in any other gene we studied. Additionally, it was not present in any sequence in any of the sequence databases we used for our study (Table 1). ALI lies between the conserved regions of the activation loop. A fragment 100% identical to ALI was present in the manually curated database of *B. oleraceae* scaffolds (Scaffold000171 FRAGMENT 1092155:1092254). This sequence was only partial and did not contain the C terminal NAF domain and the PPi domain (S16).

Another distinguishing feature is a putative nuclear localization signal in the junction domain of CIPK16 orthologues (JDNLS). According to cNLS server predictions, AtCIPK16 has monopartite and bipartite nuclear localization signals (NLS) with the sequence spanning from 300 to 308 ($PPTKKKKKD_{308}$) (Fig. 5). A score of 6.5 assigned by the server for this signal suggests that AtCIPK16 can be partially located to the nucleus. Proteins from other CIPK clades did not possess an NLS in the junction domain (Fig. 5). However, all CIPKs possessed a bipartite signal (a score equal to or less than 5.5) with a tendency to be directed to the cytoplasm.

3.3. Intron–exon architecture of CIPK16 orthologues

The intron–exon study conducted on *AtCIPK16* orthologues from members of the *Brassicaceae* and *Cleomaceae* shows that they all possess two exons separated by an intron (Fig. 6). Exon 1 length varies amongst species from 692 to 709 nucleotides, whereas Exon 2 length varies from 685 to 742 nucleotides. The indels in exon 1 and exon 2 were analysed separately by a multiple sequence alignment of the DNA sequences (S16). We see the presence of many

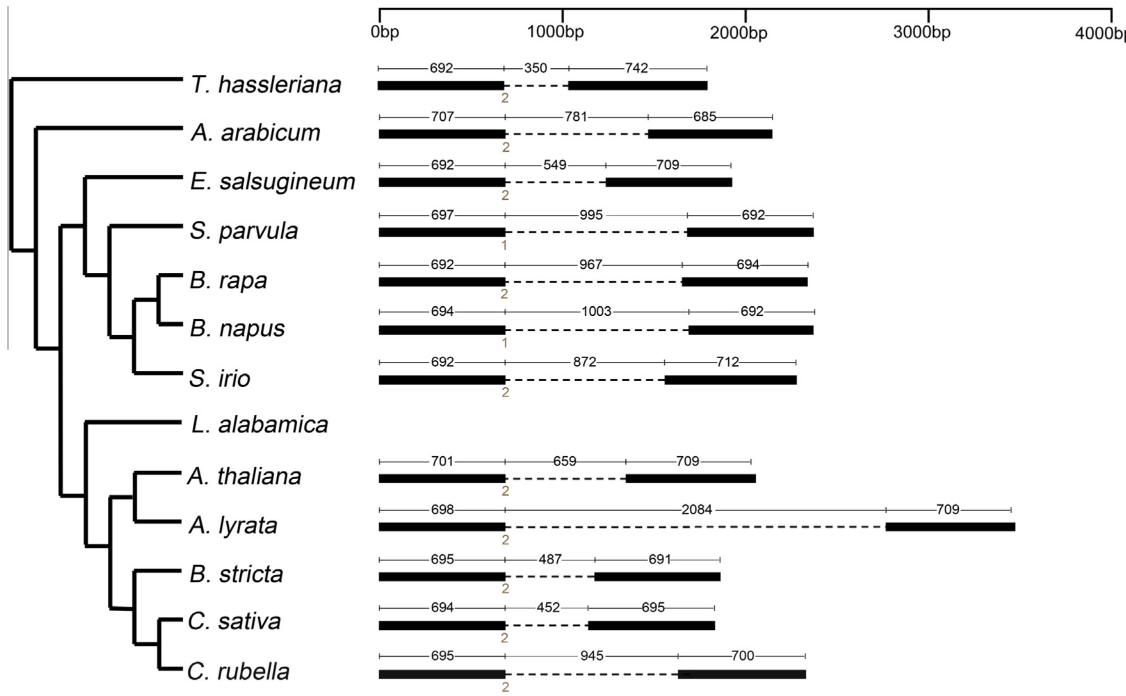


Fig. 6. Intron–exon analysis of CIPK16 clade proteins using PIECE web tool (Wang et al., 2013): The species names are shown in a cladogram with the respective intron–exon architecture of the CIPK proteins. A base pair scale is shown on top. The exons are shown as solid black bars connected by dashed lines representing the introns. The length of each exon and intron are shown above each region. The phase of the intron is shown below the start of the intron. We deliberately left the intron–exon structure for *L. alabamica* blank as we were unable to find a *CIPK16* orthologue for this species.

transitions and transversions compared to the consensus sequence within both exons of the analysed species (S16). The intron lengths of the *AtCIPK16* orthologues are quite variable (Fig. 6). They vary from 350 bp in *T. hassleriana* to 2048 bp in *A. lyrata*. All introns except the ones from *B. napus*, *S. parvula*, and *C. sativa* are phase 2 introns (i.e. they interrupt the reading frame of a gene by inserting a sequence between the second and third nucleotide of a codon). *B. napus*, *S. parvula*, and *C. sativa* contain phase 1 introns (i.e. they interrupt the reading frame of a gene by inserting a sequence between the first and second nucleotide of a codon). *AtCIPK5* and *AtCIPK25* orthologues are intron-less and therefore are not shown.

3.4. AtCIPK16 diversity amongst A. thaliana accessions

From the analysis of VCF files from 696 *A. thaliana* accessions, we identified 359 positions harbouring SNPs within the vicinity of *AtCIPK16*. Of these, 195 (54.3%) were upstream, 4 (1.1%) in the 5′-UTR, 17 (4.7%) in the CDS of exon 1, 59 (16.4%) in the intron, 22 (6.1%) in the CDS of exon 2, 10 (2.8%) in the 3′-UTR and 52 (14.5%). Twenty-two of the 39 SNPs that fell within the coding region are silent (synonymous) while 17 cause a change in an amino acid (non-synonymous) (S17).

Of the 8 accessions for which we had access to BAM files, we identified two (Bay-0 and blh-1) which contained a 10 bp deletion within the promoter region (65 bases upstream of the ATG) of *AtCIPK16* compared to the Col-0 reference (Fig. 7). This deletion has previously been reported only in Bay-0 (Roy et al., 2013), and results in the creation of a TATA box.

4. Discussion

AtCIPK16 promotes sodium exclusion and salt tolerance (Roy et al., 2013). Understanding the pervasiveness of *CIPK16* in the plant kingdom would lay the foundation to better understanding its mode of action in plants. Already identified CIPKs from *A. thaliana*, predicted ancestral versions of the AtCIPKs and keywords were used to mine for CIPK sequences from the plant sequence databases. We carried out a molecular phylogenetic analysis of the multigene CIPK family in terrestrial plants to investigate potential processes in evolution that may have given rise to the modern day CIPK proteins (Soltis and Soltis, 2003). Additional *in silico* protein analysis approaches were used to identify unique regions in primary protein structures, intron–exon architecture and variation within the sequences of *AtCIPK16* in different accessions of Arabidopsis to strengthen the phylogenetic inferences.

In order to generate the phylogenetic trees, we gathered protein sequences from all fully sequenced species to minimise the impact of missing data and evolutionary pressure on domain identification in AtCIPK16 orthologues and misinterpretation of the analysis (Haudry et al., 2013; Kagale et al., 2014b).

4.1. Synapomorphic characters define core Brassicales restricted CIPK16s

Comparison of the phylogenetic data and MSAs show that the CIPK protein sequences and nucleotide sequences of *Brassicaceae* CIPK16 orthologues have a highly conserved synapomorphic character (Figs. 4 and 5). Indel ALI is one of these, although this sequence lacks one amino acid in the *Cleomaceae* species *T. hassleriana* and is slightly dissimilar to those of the *Brassicaceae* species (Fig. 4). It is noteworthy that this unique insertion was not found in any other dicot or monocot species that has been fully sequenced. Therefore, we hypothesise that ALI can be used as a unique of CIPK16 orthologues within the *Brassicales*. The partial sequence we discovered in *B. oleraceae* supports this hypothesis, although given that ambiguity of partial sequences tends to introduce false relationships amongst species in a phylogenetic analysis, the *B. oleraceae* sequence was excluded when generating phylogenetic trees.

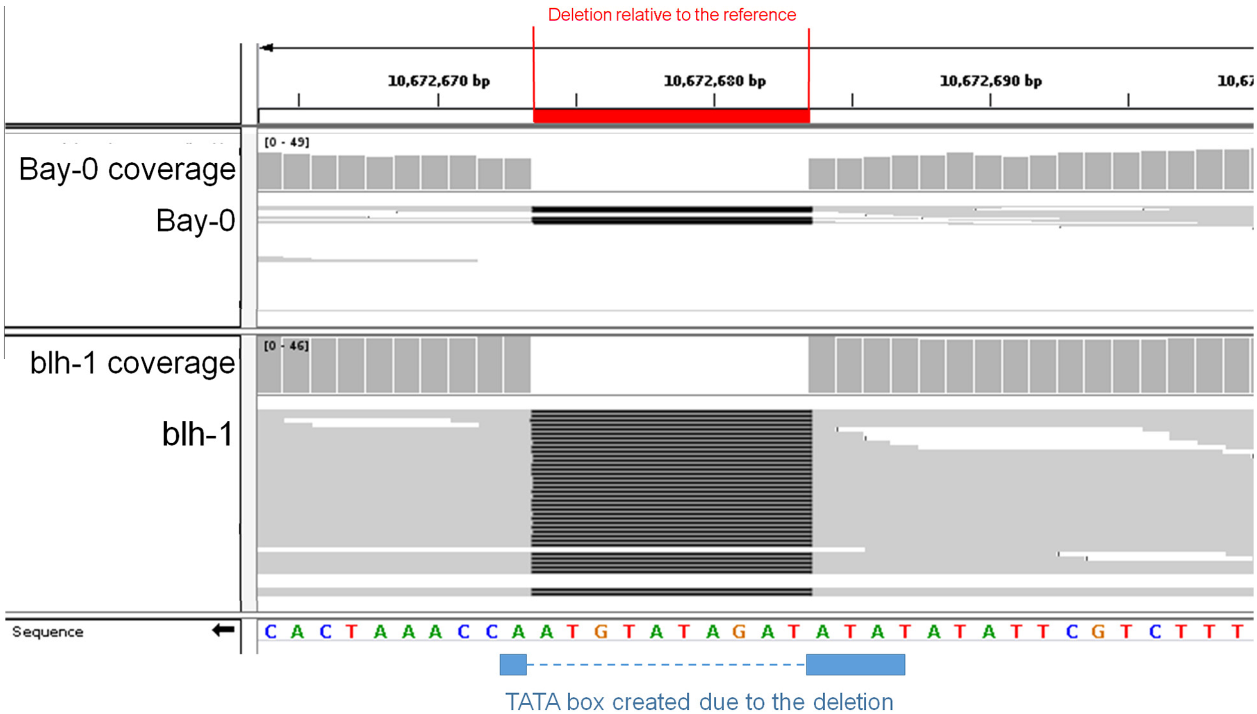


Fig. 7. An IGV (Robinson et al. 2011) screenshot of the promoter region of AtCIPK16 previously shown to contain a deletion in Bay-0 (Roy et al., 2013). Read alignments for both Bay-0 and blh-1 accessions are shown together with their corresponding coverage tracks and indicate both accessions contain a conserved 10 bp deletion (location indicated above the chromosomal co-ordinates) relative to the Col-0 reference (mismatching bases not shown). The read alignments of the original BAM files were modified slightly to pad the alignments of reads spanning the deletion. The drop in coverage seen in Bay-0 is indicative of a non-gapped alignment tool being used to generate the BAM file. The BAM file of blh-1 already contained gapped read alignments, indicating a gapped aligner was used to generate the BAM file. The effect of the deletion in these two accessions is the creation of a TATA box (location indicated by bars, below the sequence track, spanning the deletion).

The other important highly conserved character noted was the junction domain nuclear localisation signal (JDNLS) (Fig. 5). It is present in all CIPK16 orthologues from *Brassicaceae* except that of the basal species *A. arabicum*. This raises the question of its functional importance for the localization of a CIPK16 orthologue in the cell. However, this requires further experimental validation.

It is clear from our study that *CIPK16* is a lineage-specific gene for core *Brassicales*. The consistency in intron–exon studies supports the CIPK16 orthologues (Fig. 6). The most parsimonious explanation for *CIPK16s* to be core *Brassicales* specific is that *CIPK16* arose as a result of a gene duplication event after the speciation of this group of plants. Genes that are duplicated can evolve through the acquisition of new or specialised functions at the expression or protein level (neofunctionalization), the retention of ancestral functionality or to escape from adaptive conflict (EAC) (Blanc and Wolfe, 2004; Des Marais and Rausher, 2008; Moghe et al., 2014). The identification of non-synonymous SNPs (S17) amongst the 696 accessions we analysed warrant closer examination to ascertain whether they are associated with higher or lower tolerance to salt. Interestingly, we see that Bay-0 and blh-1 accessions share a common TATA box positioned 65 bp upstream of *CIPK16* (Fig. 7). This is important as Bay-0 has shown higher *CIPK16* gene expression in response to salt stress compared to Shahdara in a previous study (Roy et al., 2013). Whether the presence of the TATA box confers a similar increase in CIPK16 expression in blh-1 needs to be experimentally determined.

Prior research on *Brassicaceae* gene evolution revealed that the majority of lineage-specific genes from *A. thaliana* are stress responsive (Donoghue et al., 2011). AtCIPK16 has been shown to interact with *shaker-type* K^+ channels in *A. thaliana* (AKT1), which keeps the cellular Na^+/K^+ ratio low under low K^+ stress and confers salt tolerance when overexpressed (Lee et al., 2007; Roy et al., 2013). Apart from AKT1, AtCIPK16 has shown interactions with CBL1, CBL9 and protein phosphatase 2C type proteins (Lan et al., 2011; Lee et al., 2007). Moreover, there is experimental evidence showing AtCIPK5, one of AtCIPK16's closest relatives, interacts with CBL1, CBL3, CBL4 and CBL9 (Kim et al., 2000; Kolukisaoglu et al., 2004; Schlücking et al., 2013). However, very little, if anything is known about the functionality of AtCIPK16s other closest relative AtCIPK25. There is evidence on [CIPK16/5/25] homologues' from species such as Chickpea and rice being responsive to plant abiotic stress (Meena et al., 2015; Yoon et al., 2009). Nevertheless, we believe that CIPK16, 5 and 25 and identified homologues of [CIPK16/5/25] should be further pursued to analyse their function in order to help us understand the drivers of *CIPK16* evolution.

4.2. The evolution of CIPK16, 5 and 25 clade

From our analysis, we are able to propose an evolutionary model for *CIPK16* (Fig. 8). We considered the whole *CIPK 16, 5* and *25* clade in explaining the evolution of *CIPK16* as well as sister taxa (Kolukisaoglu et al., 2004). It has been shown that a recent paleopolyploidization event (At-α) took place, which was restricted to *Brassicaceae* (Barker et al., 2010; Schranz and Mitchell-Olds, 2006). To support this fact, our study shows that segmental duplication (SD) of intron-less CIPKs in *Brassicaceae* are confined to that group. This includes the SD, which gave rise to *CIPK5* and *CIPK25*. We could not find evidence that *Cleomaceae* experienced an independent genome duplication (Cs-α) as suggested previously (Schranz and Mitchell-Olds, 2006). However, our results indicate that *CIPK16* existed before the speciation of *Cleomaceae* and therefore before Cs-α. It can be assumed that the WGD event that took place 124.6 ± 2.57 Mya (At-β) gave rise to the ancestral version of the *CIPK16, 5* and *25* clade from a single ancestral state (Fig. 8). This is consistent with previous work, which states that the paleopolyploidization event At-β is shared

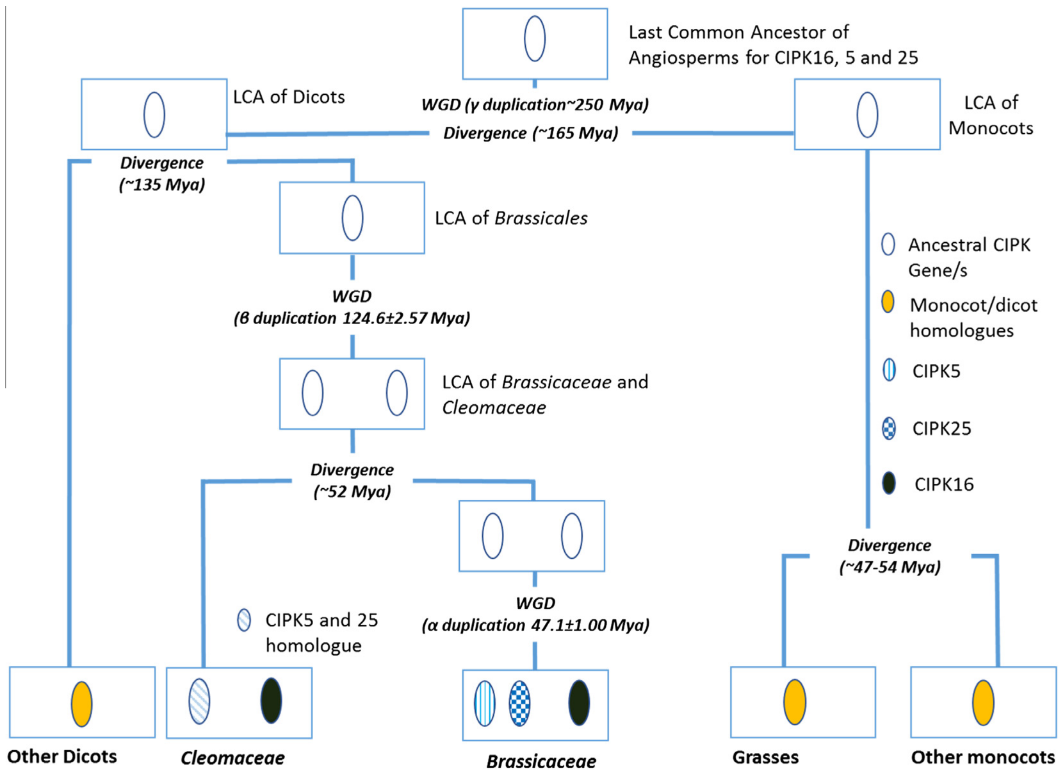


Fig. 8. Proposed evolutionary model of CIPK 16, 5 and 25. The model of evolution of CIPK16, 5 and 25 from their last common ancestor in angiosperms. Each oval represents a gene. The rectangular box represents a group of plants with a common ancestor. The ovals with no fill colour represents inferred ancestral states. The coloured ovals represent the present day proteins from different groups of terrestrial plants mentioned below each group. Previously recorded evolutionary milestones are mentioned appropriately. A cladogram shows the known evolutionary relationships amongst the groups.

between *Brassicaceae* and *Cleomaceae* (Barker et al., 2010). According to this hypothesis, and supported by our study, the ancestral version of *CIPK16* and *CIPK5* and *25* therefore had to evolve after the rise of non-core *Brassicales* species. This agrees with the previous work which showed species of *Carica* do not share At-β (Barker et al., 2010; Kagale et al., 2014b) (Fig. 8).

The fact that NCBs and monocots have no CIPK16, CIPK5 or CIPK25 orthologues suggests they must possess an ancestral version of [CIPK16/5/25] or the gene itself has been made redundant by evolution (Pérez-Pérez et al., 2009). The most basal species in our phylogenetic analysis to contain a sequence that clusters with [CIPK16/5/25] is the angiosperm *A. trichopoda.* This suggests that the earliest ancestor of [CIPK16/5/25] evolved after the diversification of angiosperms.

4.3. Continuing CIPK16 research for salinity stress

It is clear from our phylogenetic analysis that AtCIPK16 does not have a clear orthologue in important crops such as barley or wheat. However, our finding that the last common ancestor of [CIPK16/5/25] gave rise to *CIPK16* after the divergence of dicots and monocots (more specifically after the diversification of core *Brassicales*, a subgroup of dicots), and the previous finding that overexpressing *AtCIPK16* confers salt tolerance in the monocot barley, poses further questions (Roy et al., 2013). Do the conserved elements ALI and the JDNLS have functional importance in CIPK16s? Would it be possible that the functionality of CIPK16, 5 and 25 result from functional partitioning of the ancestral genes due to selective pressure? If so, are the functionalities of CIPK16, 5 and 25 still retained in seemingly ancestral versions we see in NCBs and monocots? This study therefore, highlights the necessity to explore the functionality of *AtCIPK16* in *A. thaliana* and cereals such as barley.

Author and contributors

Study concepts/study design or data acquisition or data analysis/interpretation: all authors; manuscript drafting or manuscript revision for important intellectual content: all authors; manuscript final version approval: all authors; literature research: SA; phylogenetic and *in silico* analysis: SA and NWH; and manuscript editing: all authors. Agreement to be accountable for all aspects of the work: all authors.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgements

Authors would like to thank the Australian Centre for Plant Functional Genomics for funding the research of this project. Authors would also like to thank Mr. Ashan Hettiarachchige for generating the final high-resolution images according to the manuscript guidelines.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2016.03.031.

References

Adem, G.D., Roy, S.J., Zhou, M., Bowman, J.P., Shabala, S., 2014. Evaluating contribution of ionic, osmotic and oxidative stress components towards salinity tolerance in barley. BMC Plant Biol. 14, 113. http://dx.doi.org/10.1186/1471-2229-14-113.

Ajawatanawong, P., Atkinson, G.C., Watson-Haigh, N.S., MacKenzie, B., Baldauf, S.L., 2012. SeqFIRE: a web application for automated extraction of indel regions and conserved blocks from protein multiple sequence alignments. Nucleic Acids Res., gks561 http://dx.doi.org/10.1093/nar/gks561.

Albert, V.A., Barbazuk, W.B., dePamphilis, C.W., Der, J.P., Leebens-Mack, J., Ma, H., Palmer, J.D., Rounsley, S., Sankoff, D., Schuster, S.C., Soltis, D.E., Soltis, P.S., Wessler, S.R., Wing, R.A., Albert, V.A., Ammiraju, J.S.S., Barbazuk, W.B., Chamala, S., Chanderbali, A.S., dePamphilis, C.W., Der, J.P., Determann, R., Leebens-Mack, J., Ma, H., Ralph, P., Rounsley, S., Schuster, S.C., Soltis, D.E., Soltis, P.S., Talag, J., Tomsho, L., Walts, B., Wanke, S., Wing, R.A., Albert, V.A., Barbazuk, W.B., Chamala, S., Chanderbali, A.S., Chang, T.-H., Determann, R., Lan, T., Soltis, D.E., Soltis, P.S., Arikit, S., Axtell, M.J., Ayyampalayam, S., Barbazuk, W.B., Burnette, J. M., Chamala, S., Paoli, E.D., dePamphilis, C.W., Der, J.P., Estill, J.C., Farrell, N.P., Harkess, A., Jiao, Y., Leebens-Mack, J., Liu, K., Mei, W., Meyers, B.C., Shahid, S., Wafula, E., Walts, B., Wessler, S.R., Zhai, J., Zhang, X., Albert, V.A., Carretero-Paulet, L., dePamphilis, C.W., Der, J.P., Jiao, Y., Leebens-Mack, J., Lyons, E., Sankoff, D., Tang, H., Wafula, E., Zheng, C., Albert, V.A., Altman, N.S., Barbazuk, W.B., Carretero-Paulet, L., dePamphilis, C.W., Der, J.P., Estill, J.C., Jiao, Y., Leebens-Mack, J., Liu, K., Mei, W., Wafula, E., Altman, N.S., Arikit, S., Axtell, M. J., Chamala, S., Chanderbali, A.S., Chen, F., Chen, J.-Q., Chiang, V., Paoli, E.D., dePamphilis, C.W., Der, J.P., Determann, R., Fogliani, B., Guo, C., Harholt, J., Harkess, A., Job, C., Job, D., Kim, S., Kong, H., Leebens-Mack, J., Li, G., Li, L., Liu, J., Ma, H., Meyers, B.C., Park, J., Qi, X., Rajjou, L., Burtet-Sarramegna, V., Sederoff, R., Shahid, S., Soltis, D.E., Soltis, P.S., Sun, Y.-H., Ulvskov, P., Villegente, M., Xue, J.-Y., Yeh, T.-F., Yu, X., Zhai, J., Acosta, J.J., Albert, V.A., Barbazuk, W.B., Bruenn, R.A., Chamala, S., de Kochko, A., dePamphilis, C.W., Der, J.P., Herrera-Estrella, L.R., Ibarra-Laclette, E., Kirst, M., Leebens-Mack, J., Pissis, S.P., Poncet, V., Schuster, S. C., Soltis, D.E., Soltis, P.S., Tomsho, L., 2013. The Amborella genome and the evolution of flowering plants. Science 342, 1241089. http://dx.doi.org/10.1126/science.1241089.

Albrecht, V., Ritz, O., Linder, S., Harter, K., Kudla, J., 2001. The NAF domain defines a novel protein–protein interaction module conserved in Ca^{2+}-regulated kinases. EMBO J. 20, 1051–1063. http://dx.doi.org/10.1093/emboj/20.5.1051.

Argout, X., Salse, J., Aury, J.-M., Guiltinan, M.J., Droc, G., Gouzy, J., Allegre, M., Chaparro, C., Legavre, T., Maximova, S.N., Abrouk, M., Murat, F., Fouet, O., Poulain, J., Ruiz, M., Roguet, Y., Rodier-Goud, M., Barbosa-Neto, J.F., Sabot, F., Kudrna, D., Ammiraju, J.S.S., Schuster, S.C., Carlson, J.E., Sallet, E., Schiex, T., Dievart, A., Kramer, M., Gelley, L., Shi, Z., Bérard, A., Viot, C., Boccara, M., Risterucci, A.M., Guignon, V., Sabau, X., Axtell, M.J., Ma, Z., Zhang, Y., Brown, S., Bourge, M., Golser, W., Song, X., Clement, D., Rivallan, R., Tahi, M., Akaza, J.M., Pitollat, B., Gramacho, K., D'Hont, A., Brunel, D., Infante, D., Kebe, I., Costet, P., Wing, R., McCombie, W.R., Guiderdoni, E., Quetier, F., Panaud, O., Wincker, P., Bocs, S., Lanaud, C., 2011. The genome of Theobroma cacao. Nat. Genet. 43, 101–108. http://dx.doi.org/10.1038/ng.736.

Barker, M.S., Vogel, H., Schranz, M.E., 2010. Paleopolyploidy in the Brassicales: analyses of the Cleome transcriptome elucidate the history of genome duplications in Arabidopsis and other Brassicales. Genome Biol. Evol. 1, 391–399. http://dx.doi.org/10.1093/gbe/evp040.

Batistic, O., Kim, K.-N., Kleist, T., Kudla, J., Luan, S., 2011. The CBL–CIPK network for decoding calcium signals in plants. In: Luan, S. (Ed.), Coding and Decoding of Calcium Signals in Plants. Springer, Berlin, Heidelberg, pp. 235–258.

Batistic, O., Kudla, J., 2004. Integration and channeling of calcium signaling through the CBL calcium sensor/CIPK protein kinase network. Planta 219, 915–924. http://dx.doi.org/10.1007/s00425-004-1333-3.

Blanc, G., Wolfe, K.H., 2004. Functional divergence of duplicated genes formed by polyploidy during arabidopsis evolution. Plant Cell 16, 1679–1691. http://dx.doi.org/10.1105/tpc.021410.

Boc, A., Diallo, A.B., Makarenkov, V., 2012. T-REX: a web server for inferring, validating and visualizing phylogenetic trees and networks. Nucleic Acids Res. 40, W573–W579. http://dx.doi.org/10.1093/nar/gks485.

Chase, M.W., 2004. Monocot relationships: an overview. Am. J. Bot. 91, 1645–1655. http://dx.doi.org/10.3732/ajb.91.10.1645.

Cheng, F., Liu, S., Wu, J., Fang, L., Sun, S., Liu, B., Li, P., Hua, W., Wang, X., 2011. BRAD, the genetics and genomics database for Brassica plants. BMC Plant Biol. 11, 136. http://dx.doi.org/10.1186/1471-2229-11-136.

Cheng, S., van den Bergh, E., Zeng, P., Zhong, X., Xu, J., Liu, X., Hofberger, J., de Bruijn, S., Bhide, A.S., Kuelahoglu, C., Bian, C., Chen, J., Fan, G., Kaufmann, K., Hall, J.C., Becker, A., Brautigam, A., Weber, A.P.M., Shi, C., Zheng, Z., Li, W., Lv, M., Tao, Y., Wang, J., Zou, H., Quan, Z., Hibberd, J.M., Zhang, G., Zhu, X.-G., Xu, X., Schranz, M. E., 2013. The *Tarenaya hassleriana* genome provides insight into reproductive trait and genome evolution of crucifers[W][OPEN]. Plant Cell 25, 2813–2830. http://dx.doi.org/10.1105/tpc.113.113480.

Choi, W.-G., Toyota, M., Kim, S.-H., Hilleary, R., Gilroy, S., 2014. Salt stress-induced Ca^{2+} waves are associated with rapid, long-distance root-to-shoot signaling in plants. Proc. Natl. Acad. Sci. 111, 6497–6502. http://dx.doi.org/10.1073/pnas.1319955111.

Cingolani, P., Platts, A., Wang, L.L., Coon, M., Nguyen, T., Wang, L., Land, S.J., Lu, X., Ruden, D.M., 2012. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w 1118; iso-2; iso-3. Fly (Austin) 6, 80–92. http://dx.doi.org/10.4161/fly.19695.

Colmer, T.D., Munns, R., Flowers, T.J., 2005. Improving salt tolerance of wheat and barley: future prospects. Aust. J. Exp. Agric. 45, 1425–1443. http://dx.doi.org/10.1071/EA04162.

Colmsee, C., Beier, S., Himmelbach, A., Schmutzer, T., Stein, N., Scholz, U., Mascher, M., 2015. BARLEX – the barley draft genome explorer. Mol. Plant 8, 964–966. http://dx.doi.org/10.1016/j.molp.2015.03.009.

Consortium, T.U., 2015. UniProt: a hub for protein information. Nucleic Acids Res. 43, D204–D212. http://dx.doi.org/10.1093/nar/gku989.

Coordinators, N.R., 2013. Database resources of the national center for biotechnology information. Nucleic Acids Res. 41, D8–D20. http://dx.doi.org/10.1093/nar/gks1189.

Cunningham, F., Amode, M.R., Barrell, D., Beal, K., Billis, K., Brent, S., Carvalho-Silva, D., Clapham, P., Coates, G., Fitzgerald, S., Gil, L., Girón, C.G., Gordon, L., Hourlier, T., Hunt, S.E., Janacek, S.H., Johnson, N., Juettemann, T., Kähäri, A.K., Keenan, S., Martin, F.J., Maurel, T., McLaren, W., Murphy, D.N., Nag, R., Overduin, B., Parker, A., Patricio, M., Perry, E., Pignatelli, M., Riat, H.S., Sheppard, D., Taylor, K., Thormann, A., Vullo, A., Wilder, S.P., Zadissa, A., Aken, B.L., Birney, E., Harrow, J., Kinsella, R., Muffato, M., Ruffier, M., Searle, S.M.J., Spudich, G., Trevanion, S.J., Yates, A., Zerbino, D.R., Flicek, P., 2015. Ensembl 2015. Nucleic Acids Res. 43, D662–D669. http://dx.doi.org/10.1093/nar/gku1010.

Dassanayake, M., Oh, D.-H., Haas, J.S., Hernandez, A., Hong, H., Ali, S., Yun, D.-J., Bressan, R.A., Zhu, J.-K., Bohnert, H.J., Cheeseman, J.M., 2011. The genome of the extremophile crucifer *Thellungiella parvula*. Nat. Genet. 43, 913–918. http://dx.doi.org/10.1038/ng.889.

Des Marais, D.L., Rausher, M.D., 2008. Escape from adaptive conflict after duplication in an anthocyanin pathway gene. Nature 454, 762–765. http://dx.doi.org/10.1038/nature07092.

Diallo, A.B., Makarenkov, V., Blanchette, M., 2010. Ancestors 1.0: a web server for ancestral sequence reconstruction. Bioinformatics 26, 130–131. http://dx.doi.org/10.1093/bioinformatics/btp600, Oxford Univ Press.

Donoghue, M.T., Keshavaiah, C., Swamidatta, S.H., Spillane, C., 2011. Evolutionary origins of Brassicaceae specific genes in *Arabidopsis thaliana*. BMC Evol. Biol. 11, 47. http://dx.doi.org/10.1186/1471-2148-11-47.

Droc, G., Larivière, D., Guignon, V., Yahiaoui, N., This, D., Garsmeur, O., Dereeper, A., Hamelin, C., Argout, X., Dufayard, J.-F., Lengelle, J., Baurens, F.-C., Cenci, A., Pitollat, B., D'Hont, A., Ruiz, M., Rouard, M., Bocs, S., 2013. The banana genome hub. Database 2013, bat035. http://dx.doi.org/10.1093/database/bat035.

Duvick, J., Fu, A., Muppirala, U., Sabharwal, M., Wilkerson, M.D., Lawrence, C.J., Lushbough, C., Brendel, V., 2008. PlantGDB: a resource for comparative plant genomics. Nucleic Acids Res. 36, D959–D965. http://dx.doi.org/10.1093/nar/gkm1041.

Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32, 1792–1797. http://dx.doi.org/10.1093/nar/gkh340.

FAOSTAT, 2014. Food and Agriculture Organization of the United Nations [WWW Document]. Food Agric. Organ. Fo U. N. URL: <http://www.fao.org/economic/ess/en/#.U1XSWvmSymw> (accessed 4.22.14).

Felsenstein, J., 1996. Inferring phylogenies from protein sequences by parsimony, distance, and likelihood methods. Methods Enzymol. 266, 418–427. http://dx.doi.org/10.1016/S0076-6879(96)66026-1.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791. http://dx.doi.org/10.2307/2408678.

Finn, R.D., Bateman, A., Clements, J., Coggill, P., Eberhardt, R.Y., Eddy, S.R., Heger, A., Hetherington, K., Holm, L., Mistry, J., Sonnhammer, E.L.L., Tate, J., Punta, M., 2014. Pfam: the protein families database. Nucleic Acids Res. 42, D222–D230. http://dx.doi.org/10.1093/nar/gkt1223.

Fulton, D.L., Li, Y.Y., Laird, M.R., Horsman, B.G., Roche, F.M., Brinkman, F.S., 2006. Improving the specificity of high-throughput ortholog prediction. BMC Bioinformatics 7, 270. http://dx.doi.org/10.1186/1471-2105-7-270.

Goodstein, D.M., Shu, S., Howson, R., Neupane, R., Hayes, R.D., Fazo, J., Mitros, T., Dirks, W., Hellsten, U., Putnam, N., Rokhsar, D.S., 2012. Phytozome: a comparative platform for green plant genomics. Nucleic Acids Res. 40, D1178–D1186. http://dx.doi.org/10.1093/nar/gkr944.

Haudry, A., Platts, A.E., Vello, E., Hoen, D.R., Leclercq, M., Williamson, R.J., Forczek, E., Joly-Lopez, Z., Steffen, J.G., Hazzouri, K.M., Dewar, K., Stinchcombe, J.R., Schoen, D.J., Wang, X., Schmutz, J., Town, C.D., Edger, P.P., Pires, J.C., Schumaker, K.S., Jarvis, D.E., Mandáková, T., Lysak, M.A., van den Bergh, E., Schranz, M.E., Harrison, P.M., Moses, A.M., Bureau, T.E., Wright, S.I., Blanchette, M., 2013. An atlas of over 90,000 conserved noncoding sequences provides insight into crucifer regulatory regions. Nat. Genet. 45, 891–898. http://dx.doi.org/10.1038/ng.2684.

Hrabak, E.M., Chan, C.W.M., Gribskov, M., Harper, J.F., Choi, J.H., Halford, N., Kudla, J., Luan, S., Nimmo, H.G., Sussman, M.R., Thomas, M., Walker-Simmons, K., Zhu, J.-K., Harmon, A.C., 2003. The Arabidopsis CDPK-SnRK superfamily of protein kinases. Plant Physiol. 132, 666–680. http://dx.doi.org/10.1104/pp.102.011999.

International Wheat Genome Sequencing Consortium (IWGSC), 2014. A chromosome-based draft sequence of the hexaploid bread wheat (*Triticum aestivum*) genome. Science 345, 1251788. http://dx.doi.org/10.1126/science.1251788.

Jaillon, O., Aury, J.-M., Noel, B., Policriti, A., Clepet, C., Casagrande, A., Choisne, N., Aubourg, S., Vitulo, N., Jubin, C., Vezzi, A., Legeai, F., Hugueney, P., Dasilva, C., Horner, D., Mica, E., Jublot, D., Poulain, J., Bruyère, C., Billault, A., Segurens, B., Gouyvenoux, M., Ugarte, E., Cattonaro, F., Anthouard, V., Vico, V., Del Fabbro, C., Alaux, M., Di Gaspero, G., Dumas, V., Felice, N., Paillard, S., Juman, I., Moroldo, M., Scalabrin, S., Canaguier, A., Le Clainche, I., Malacrida, G., Durand, E., Pesole, G., Laucou, V., Chatelet, P., Merdinoglu, D., Delledonne, M., Pezzotti, M., Lecharny, A., Scarpelli, C., Artiguenave, F., Pè, M.E., Valle, G., Morgante, M., Caboche, M., Adam-Blondon, A.-F., Weissenbach, J., Quétier, F., Wincker, P., 2007. The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. Nature 449, 463–467. http://dx.doi.org/10.1038/nature06148.

Jones, D.T., Taylor, W.R., Thornton, J.M., 1992. The rapid generation of mutation data matrices from protein sequences. Comput. Appl. Biosci. 8, 275–282. http://dx.doi.org/10.1093/bioinformatics/8.3.275.

Jung, S., Ficklin, S.P., Lee, T., Cheng, C.-H., Blenda, A., Zheng, P., Yu, J., Bombarely, A., Cho, I., Ru, S., Evans, K., Peace, C., Abbott, A.G., Mueller, L.A., Olmstead, M.A., Main, D., 2014. The genome database for rosaceae (GDR): year 10 update. Nucleic Acids Res. 42, D1237–D1244. http://dx.doi.org/10.1093/nar/gkt1012.

Kagale, S., Koh, C., Nixon, J., Bollina, V., Clarke, W.E., Tuteja, R., Spillane, C., Robinson, S.J., Links, M.G., Clarke, C., Higgins, E.E., Huebert, T., Sharpe, A.G., Parkin, I.A.P., 2014a. The emerging biofuel crop *Camelina sativa* retains a highly undifferentiated hexaploid genome structure. Nat. Commun. 5, 3706. http://dx.doi.org/10.1038/ncomms4706.

Kagale, S., Robinson, S.J., Nixon, J., Xiao, R., Huebert, T., Condie, J., Kessler, D., Clarke, W.E., Edger, P.P., Links, M.G., Sharpe, A.G., Parkin, I.A.P., 2014b. Polyploid evolution of the Brassicaceae during the Cenozoic era. Plant Cell 26, 2777–2791. http://dx.doi.org/10.1105/tpc.114.126391.

Keane, T.M., Creevey, C.J., Pentony, M.M., Naughton, T.J., Mclnerney, J.O., 2006. Assessment of methods for amino acid matrix selection and their use on empirical data shows that ad hoc assumptions for choice of matrix are not justified. BMC Evol. Biol. 6, 29. http://dx.doi.org/10.1186/1471-2148-6-29.

Kim, K.-N., Cheong, Y.H., Gupta, R., Luan, S., 2000. Interaction specificity of arabidopsis calcineurin B-like calcium sensors and their target kinases. Plant Physiol. 124, 1844–1853. http://dx.doi.org/10.1104/pp.124.4.1844.

Kitashiba, H., Li, F., Hirakawa, H., Kawanabe, T., Zou, Z., Hasegawa, Y., Tonosaki, K., Shirasawa, S., Fukushima, A., Yokoi, S., Takahata, Y., Kakizaki, T., Ishida, M., Okamoto, S., Sakamoto, K., Shirasawa, K., Tabata, S., Nishio, T., 2014. Draft sequences of the radish (*Raphanus sativus* L.) genome. DNA Res., dsu014 http://dx.doi.org/10.1093/dnares/dsu014.

Kolukisaoglu, Ü., Weinl, S., Blazevic, D., Batistic, O., Kudla, J., 2004. Calcium sensors and their interacting protein kinases: genomics of the arabidopsis and rice CBL-CIPK signaling networks. Plant Physiol. 134, 43–58. http://dx.doi.org/10.1104/pp.103.033068.

Kosugi, S., Hasebe, M., Tomita, M., Yanagawa, H., 2009. Systematic identification of cell cycle-dependent yeast nucleocytoplasmic shuttling proteins by prediction of composite motifs. Proc. Natl. Acad. Sci. USA 106, 10171–10176. http://dx.doi.org/10.1073/pnas.0900604106.

Lamesch, P., Berardini, T.Z., Li, D., Swarbreck, D., Wilks, C., Sasidharan, R., Muller, R., Dreher, K., Alexander, D.L., Garcia-Hernandez, M., Karthikeyan, A.S., Lee, C.H., Nelson, W.D., Ploetz, L., Singh, S., Wensel, A., Huala, E., 2012. The Arabidopsis Information Resource (TAIR): improved gene annotation and new tools. Nucleic Acids Res. 40, D1202–D1210. http://dx.doi.org/10.1093/nar/gkr1090.

Lan, W.-Z., Lee, S.-C., Che, Y.-F., Jiang, Y.-Q., Luan, S., 2011. Mechanistic analysis of AKT1 regulation by the CBL–CIPK–PP2CA interactions. Mol. Plant 4, 527–536. http://dx.doi.org/10.1093/mp/ssr031.

Le, S.Q., Gascuel, O., 2008. An improved general amino acid replacement matrix. Mol. Biol. Evol. 25, 1307–1320. http://dx.doi.org/10.1093/molbev/msn067.

Lee, S.C., Lan, W.-Z., Kim, B.-G., Li, L., Cheong, Y.H., Pandey, G.K., Lu, G., Buchanan, B. B., Luan, S., 2007. A protein phosphorylation/dephosphorylation network regulates a plant potassium channel. Proc. Natl. Acad. Sci. 104, 15959–15964. http://dx.doi.org/10.1073/pnas.0707912104.

Lyons, E., Freeling, M., 2008. How to usefully compare homologous plant genes and chromosomes as DNA sequences. Plant J. 53, 661–673. http://dx.doi.org/10.1111/j.1365-313X.2007.03326.x.

Meena, M.K., Ghawana, S., Dwivedi, V., Roy, A., Chattopadhyay, D., 2015. Expression of chickpea CIPK25 enhances root growth and tolerance to dehydration and salt stress in transgenic tobacco. Plant Physiol. 683. http://dx.doi.org/10.3389/fpls.2015.00683.

Michael, T.P., Jackson, S., 2013. The first 50 plant genomes. Plant Genome 6. http://dx.doi.org/10.3835/plantgenome2013.03.0001in.

Moghe, G.D., Hufnagel, D.E., Tang, H., Xiao, Y., Dworkin, I., Town, C.D., Conner, J.K., Shiu, S.-H., 2014. Consequences of whole-genome triplication as revealed by comparative genomic analyses of the wild radish *Raphanus raphanistrum* and three other Brassicaceae species. Plant Cell. http://dx.doi.org/10.1105/tpc.114.124297, Online tpc.114.124297.

Morgenstern, B., 1999. DIALIGN 2: improvement of the segment-to-segment approach to multiple sequence alignment. Bioinformatics 15, 211–218. http://dx.doi.org/10.1093/bioinformatics/15.3.211, Oxford Univ Press.

Mount, D.W., 2008. The maximum likelihood approach for phylogenetic prediction. Cold Spring Harb. Protoc., pdbtop34. http://dx.doi.org/10.1101/pdb.top34.

Munns, R., 2005. Genes and salt tolerance: bringing them together. New Phytol. 167, 645–663. http://dx.doi.org/10.1111/j.1469-8137.2005.01487.x.

Munns, R., Tester, M., 2008. Mechanisms of salinity tolerance. Annu. Rev. Plant Biol. 59, 651–681. http://dx.doi.org/10.1146/annurev.arplant.59.032607.092911.

Munns, R., Gilliham, M., 2015. Salinity tolerance of crops – what is the cost? New Phytol. 668–673. http://dx.doi.org/10.1111/nph.13519.

Nolen, B., Taylor, S., Ghosh, G., 2004. Regulation of protein kinases: controlling activity through activation segment conformation. Mol. Cell 15, 661–675. http://dx.doi.org/10.1016/j.molcel.2004.08.024.

Nordberg, H., Cantor, M., Dusheyko, S., Hua, S., Poliakov, A., Shabalov, I., Smirnova, T., Grigoriev, I.V., Dubchak, I., 2014. The genome portal of the Department of Energy Joint Genome Institute: 2014 updates. Nucleic Acids Res. 42, D26–D31. http://dx.doi.org/10.1093/nar/gkt1069.

Nussbaumer, T., Martis, M.M., Roessner, S.K., Pfeifer, M., Bader, K.C., Sharma, S., Gundlach, H., Spannagl, M., 2013. MIPS PlantsDB: a database framework for comparative plant genome research. Nucleic Acids Res. 41, D1144–D1151. http://dx.doi.org/10.1093/nar/gks1153.

Nystedt, B., Street, N.R., Wetterbom, A., Zuccolo, A., Lin, Y.-C., Scofield, D.G., Vezzi, F., Delhomme, N., Giacomello, S., Alexeyenko, A., Vicedomini, R., Sahlin, K., Sherwood, E., Elfstrand, M., Gramzow, L., Holmberg, K., Hällman, J., Keech, O., Klasson, L., Koriabine, M., Kucukoglu, M., Käller, M., Luthman, J., Lysholm, F., Niittylä, T., Olson, Å., Rilakovic, N., Ritland, C., Rosselló, J.A., Sena, J., Svensson, T., Talavera-López, C., Theißen, G., Tuominen, H., Vanneste, K., Wu, Z.-Q., Zhang, B., Zerbe, P., Arvestad, L., Bhalerao, R., Bohlmann, J., Bousquet, J., Garcia Gil, R., Hvidsten, T.R., de Jong, P., MacKay, J., Morgante, M., Ritland, K., Sundberg, B., Lee Thompson, S., Van de Peer, Y., Andersson, B., Nilsson, O., Ingvarsson, P.K., Lundeberg, J., Jansson, S., 2013. The Norway spruce genome sequence and conifer genome evolution. Nature 497, 579–584. http://dx.doi.org/10.1038/nature12211.

Ohta, M., Guo, Y., Halfter, U., Zhu, J.-K., 2003. A novel domain in the protein kinase SOS2 mediates interaction with the protein phosphatase 2C ABI2. Proc. Natl. Acad. Sci. USA 100, 11771–11776. http://dx.doi.org/10.1073/pnas.2034853100.

Ouyang, S., Zhu, W., Hamilton, J., Lin, H., Campbell, M., Childs, K., Thibaud-Nissen, F., Malek, R.L., Lee, Y., Zheng, L., Orvis, J., Haas, B., Wortman, J., Buell, C.R., 2007. The TIGR Rice Genome Annotation Resource: improvements and new features. Nucleic Acids Res. 35, D883–D887. http://dx.doi.org/10.1093/nar/gkl976.

Pérez-Pérez, J.M., Candela, H., Micol, J.L., 2009. Understanding synergy in genetic interactions. Trends Genet. 25, 368–376. http://dx.doi.org/10.1016/j.tig.2009.06.004.

Petersen, B., Petersen, T.N., Andersen, P., Nielsen, M., Lundegaard, C., 2009. A generic method for assignment of reliability scores applied to solvent accessibility predictions. BMC Struct. Biol. 9, 51. http://dx.doi.org/10.1186/1472-6807-9-51.

Pimentel, D., Berger, B., Filiberto, D., Newton, M., Wolfe, B., Karabinakis, E., Clark, S., Poon, E., Abbett, E., Nandagopal, S., 2004. Water resources: agricultural and environmental issues. Bioscience 54, 909. http://dx.doi.org/10.1641/0006-3568(2004) 054[0909:WRAAEI]2.0.CO;2.

Plett, D., Møller, I.S., 2010. Na^+ transport in glycophytic plants: what we know and would like to know. Plant, Cell Environ. 33, 612–626. http://dx.doi.org/10.1111/j.1365-3040.2009.02086.x.

Qadir, M., Quillérou, E., Nangia, V., Murtaza, G., Singh, M., Thomas, R.j., Drechsel, P., Noble, A.d., 2014. Economics of salt-induced land degradation and restoration. Nat. Resour. Forum 38, 282–295. http://dx.doi.org/10.1111/1477-8947.12054.

Rajendran, K., Tester, M., Roy, S.J., 2009. Quantifying the three main components of salinity tolerance in cereals. Plant, Cell Environ. 32, 237–249. http://dx.doi.org/10.1111/j.1365-3040.2008.01916.x.

Robinson, J.T., Thorvaldsdóttir, H., Winckler, W., Guttman, M., Lander, E.S., Getz, G., Mesirov, J.P., 2011. Integrative genomics viewer. Nat. Biotechnol. 29, 24–26. http://dx.doi.org/10.1038/nbt.1754.

Rost, B., Yachdav, G., Liu, J., 2004. The predictprotein server. Nucleic Acids Res. 32, W321–W326. http://dx.doi.org/10.1093/nar/gkh377.

Roy, S.J., Huang, W., Wang, X.J., Evrard, A., Schmöckel, S.M., Zafar, Z.U., Tester, M., 2013. A novel protein kinase involved in Na^+ exclusion revealed from positional cloning. Plant, Cell Environ. 36, 553–568. http://dx.doi.org/10.1111/j.1365-3040.2012.02595.x.

Roy, S.J., Negrão, S., Tester, M., 2014. Salt resistant crop plants. Curr. Opin. Biotechnol, Food Biotechnol. – Plant Biotechnol. 26, 115–124. http://dx.doi.org/10.1016/j.copbio.2013.12.004.

Sanchez-Barrena, M.J., Fujii, H., Angulo, I., Martinez-Ripoll, M., Zhu, J.-K., Albert, A., 2007. The structure of the C-terminal domain of the protein kinase AtSOS2 bound to the calcium sensor AtSOS3. Mol. Cell 26, 427–435. http://dx.doi.org/10.1016/j.molcel.2007.04.013.

Sanchez-Barrena, M.J., Martinez-Ripoll, M., Albert, A., 2013. Structural biology of a major signaling network that regulates plant abiotic stress: the CBL-CIPK mediated pathway. Int. J. Mol. Sci. 14, 5734–5749. http://dx.doi.org/10.3390/ijms14035734.

Schlücking, K., Edel, K.H., Köster, P., Drerup, M.M., Eckert, C., Steinhorst, L., Waadt, R., Batistič, O., Kudla, J., 2013. A new β-estradiol-inducible vector set that facilitates easy construction and efficient expression of transgenes reveals CBL3-dependent cytoplasm to tonoplast translocation of CIPK5. Mol. Plant 6, 1814–1829. http://dx.doi.org/10.1093/mp/sst065.

Schranz, M.E., Mitchell-Olds, T., 2006. Independent ancient polyploidy events in the sister families Brassicaceae and Cleomaceae. Plant Cell 18, 1152–1165. http://dx.doi.org/10.1105/tpc.106.041111.

Shabala, S., 2009. Salinity and programmed cell death: unravelling mechanisms for ion specific signalling. J. Exp. Bot. 60, 709–712. http://dx.doi.org/10.1093/jxb/erp013.

Shabala, S., 2013. Learning from halophytes: physiological basis and strategies to improve abiotic stress tolerance in crops. Ann. Bot., mct205 http://dx.doi.org/10.1093/aob/mct205.

Sigrist, C.J.A., de Castro, E., Cerutti, L., Cuche, B.A., Hulo, N., Bridge, A., Bougueleret, L., Xenarios, I., 2013. New and continuing developments at PROSITE. Nucleic Acids Res. 41, D344–D347. http://dx.doi.org/10.1093/nar/gks1067.

Soltis, D.E., Smith, S.A., Cellinese, N., Wurdack, K.J., Tank, D.C., Brockington, S.F., Refulio-Rodriguez, N.F., Walker, J.B., Moore, M.J., Carlsward, B.S., Bell, C.D., Latvis, M., Crawley, S., Black, C., Diouf, D., Xi, Z., Rushworth, C.A., Gitzendanner, M.A., Sytsma, K.J., Qiu, Y.-L., Hilu, K.W., Davis, C.C., Sanderson, M.J., Beaman, R.S., Olmstead, R.G., Judd, W.S., Donoghue, M.J., Soltis, P.S., . Angiosperm phylogeny: 17 genes, 640 taxa. Am. J. Bot. 98, 704–730. http://dx.doi.org/10.3732/ajb.1000404.

Soltis, D.E., Soltis, P.S., 2003. The role of phylogenetics in comparative genetics. Plant Physiol. 132, 1790–1800. http://dx.doi.org/10.1104/pp.103.022509.

Talavera, G., Castresana, J., 2007. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. Syst. Biol. 56, 564–577. http://dx.doi.org/10.1080/10635150701472164.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729. http://dx.doi.org/10.1093/molbev/mst197.

Tavakkoli, E., Rengasamy, P., McDonald, G.K., 2010. The response of barley to salinity stress differs between hydroponic and soil systems. Funct. Plant Biol. 37, 621. http://dx.doi.org/10.1071/FP09202.

Thoday-Kennedy, Jacobs, A.K., Roy, S.J., 2015. The role of the CBL–CIPK calcium signalling network in regulating ion transport in response to abiotic stress. Plant Growth Regul. 76, 3–12. http://dx.doi.org/10.1007/s10725-015-0034-1.

Tracy, F.E., Gilliham, M., Dodd, A.N., Webb, A.a.R., Tester, M., 2008. NaCl-induced changes in cytosolic free Ca^{2+} in *Arabidopsis thaliana* are heterogeneous and modified by external ionic composition. Plant, Cell Environ. 31, 1063–1073. http://dx.doi.org/10.1111/j.1365-3040.2008.01817.x.

Wang, Y., You, F.M., Lazo, G.R., Luo, M.-C., Thilmony, R., Gordon, S., Kianian, S.F., Gu, Y.Q., 2013. PIECE: a database for plant gene structure comparison and evolution. Nucleic Acids Res. 41, D1159–D1166. http://dx.doi.org/10.1093/nar/gks1109.

Waterhouse, A.M., Procter, J.B., Martin, D.M.A., Clamp, M., Barton, G.J., 2009. Jalview Version 2—a multiple sequence alignment editor and analysis workbench. Bioinformatics 25, 1189–1191. http://dx.doi.org/10.1093/bioinformatics/btp033.

Weinl, S., Kudla, J., 2009. The CBL–CIPK Ca^{2+}-decoding signaling network: function and perspectives. New Phytol. 184, 517–528. http://dx.doi.org/10.1111/j.1469-8137.2009.02938.x.

Wernersson, R., Pedersen, A.G., 2003. RevTrans: multiple alignment of coding DNA from aligned amino acid sequences. Nucleic Acids Res. 31, 3537–3539. http://dx.doi.org/10.1093/nar/gkg609.

Wu, F., Mueller, L.A., Crouzillat, D., Pétiard, V., Tanksley, S.D., 2006. Combining bioinformatics and phylogenetics to identify large sets of single-copy orthologous genes (COSII) for comparative, evolutionary and systematic studies: a test case in the euasterid plant clade. Genetics 174, 1407–1420. http://dx.doi.org/10.1534/genetics.106.062455.

Yang, R., Jarvis, D.E., Chen, H., Beilstein, M.A., Grimwood, J., Jenkins, J., Shu, S., Prochnik, S., Xin, M., Ma, C., Schmutz, J., Wing, R.A., Mitchell-Olds, T., Schumaker, Wang, X., 2013. The reference genome of the halophytic plant *Eutrema salsugineum*. Front. Plant Sci. 4. http://dx.doi.org/10.3389/fpls.2013.00046.

Yang, Z., 1996. Among-site rate variation and its impact on phylogenetic analyses. Trends Ecol. Evol. 11, 367–372. http://dx.doi.org/10.1016/0169-5347(96)10041-0.

Yang, Z., Rannala, B., 2012. Molecular phylogenetics: principles and practice. Nat. Rev. Genet. 13, 303–314. http://dx.doi.org/10.1038/nrg3186.

Yoon, S., Park, J., Ryu, M., Yoon, I.S., Kim, K.-N., 2009. Calcineurin B-like proteins in rice can bind with calcium ion and associate with the Arabidopsis CIPK family members. Plant Sci. 177, 577–583. http://dx.doi.org/10.1016/j.plantsci.2009.08.011.

Glossary

CIPK: Calcineurin B-like protein interacting protein kinase
AtCIPK16: *Arabidopsis thaliana* CIPK16
AtCIPK5: *Arabidopsis thaliana* CIPK5
AtCIPK25: *Arabidopsis thaliana* CIPK25
Indel: Insertion/Deletion
Ca^{2+}: Calcium ion
CBL: Calcineurin B-Like
CDPK: Calcium-dependent protein kinase
CaM: Calmodulin
CAML: Calmodulin like
SNF1: Sucrose non-fermenting 1
SnRK3: SNF1-related kinases group 3
FISL/NAF: NAF domain
PPi: Protein–phosphatase interaction domain
PP2C: 2C-type protein phosphatase
MSA: Multiple sequence alignment
AIC: Akaike Information Criterion
AIC2: Akaike Information Criterion 2
BIC: Bayesian Information Criterion
ML: Maximum Likelihood
NLS: Nuclear localisation signal
cDNA: Complimentary DNA
SD: Segmental duplication

The supplementary materials for this chapter are available at: https://doi.org/10.4225/55/5aa085b5c4991

Chapter 5 Investigating Genetic Variations of Contrasting Na^+ Accumulation in Barley Genotypes under Salt Stress

Statement of Authorship

Title of Paper	Evaluation of the molecular basis of varying Na^+ accumulation in barley cultivars under salt stress
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	**Amarasinghe, S., Watson-Haigh, N.S., Byrt, C.S., Roy, S., Gilliham, M., and Baumann, U.,** This is an original research article that discusses the genetic variations and similarities amongst six barley cultivars with varying leaf Na^+ accumulation levels. Out findings suggest that allelic variations in *HvHKT1;5* may be one of the crucial factors in determining the level of Na^+ in the shoots of barley. We hypothesise that for high shoot Na^+ accumulating cultivars such as Alexis to successfully deal with high levels of Na^+, genes such as *HvNHXes* (e.g. *HvNHX4*) may play a role in sequestrating Na^+ into the vacuole or K^+ homeostasis. This article is closely related to the subject matter of this thesis.

Principal Author

Name of Principal Author (Candidate)	Shanika Lakmini Amarasinghe		
Contribution to the Paper	Conceived and designed the RNA-Seq analysis. Performed literature research, data analysis and critical interpretation of data. Wrote the manuscript.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	01/02/2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Nathan S. Watson-Haigh		
Contribution to the Paper	Provided critical feedback on the RNA-Seq and variant analysis. Supervised the experiment. Edited the manuscript. I hereby certify that the statement of the contribution is accurate.		
Signature		Date	01/02/2018

Name of Co-Author	Caitlin S. Byrt		
Contribution to the Paper	Conceived and designed the experiment. Provided feedback and edited the manuscript. I hereby certify that the statement of the contribution is accurate.		
Signature		Date	01/02/2018

Name of Co-Author	Stuart J. Roy		
Contribution to the Paper	Conceived and designed the experiment. Provided feedback and edited the manuscript. I hereby certify that the statement of the contribution is accurate.		
Signature		Date	01/02/2018

Name of Co-Author	Matthew Gilliham		
Contribution to the Paper	Conceived and designed the experiment. Provided feedback and edited the manuscript. I hereby certify that the statement of the contribution is accurate.		
Signature		Date	01/02/2018

Name of Co-Author	Ute Baumann		
Contribution to the Paper	Provided critical feedback on the RNA-Seq and variant analysis. Supervised the experiment. Edited the manuscript. I hereby certify that the statement of the contribution is accurate.		
Signature		Date	01/02/2018

Link to Chapter 5

Barley is amongst the most important crop plants in the world today. It is the fourth most abundant cereal in area and tonnage harvested after wheat, maize and rice (Beier et al. 2017). The study by Roy et al. (2013) showed that transgenic barley overexpressing *AtCIPK16* conferred salt tolerance in both glasshouse and field conditions. As we presume if it is due to the existence of a common molecular machinery that can be mediated by AtCIPK16 in both transgenic Arabidopsis and barley, a comparative transcriptomic analysis could discover common molecular components among the transgenic Arabidopsis and barley.

We had therefore, planned to investigate the transcriptome of barley transgenics overexpressing *AtCIPK16* as the next step of the project. Unfortunately, the transgene was silenced in the T5 generation. Transgene silencing is a process through which, transgene expression is inactivated translationally or post-translationally after its' integration into a genome (Marenkova and Deineko 2010). Initially thought to be anecdotal, researchers subsequently realized that transgene silencing is similar to natural epigenetic behaviours, and occurs more frequently (Matzke and Matzke 2004). Not only the transgene, but also the host genes can be silenced after the introduction of a transgene due to their sequence identity to the transgene (Vaucheret et al. 1998).

Several reasons for transgene silencing have been suggested and the frequently discussed are: a) position effects: as integration of the transgene to the host genome is a random process, b) homology: when multiple copies of a particular sequence are present in a genome, the pairing interaction between the homologous or complimentary sequences can result in gene silencing (Kooter et al. 1999; Milot and Ellis 2005; Vaucheret et al. 1998), c) GC content bias in monocot codon usage; the genome of the monocots have a strong bias towards high GC content compared to the dicots that have a bias towards AT content (Batard et al. 2000). Therefore, when an AT rich sequence is introduced to a monocot the plant's innate immunity may identify it as a foreign molecule and attempt to eliminate the activity of the gene that leads to gene silencing (Rajeevkumar et al. 2015).

We have taken much care to control positional effects by generating multiple independent transformation events and selected transgenics that had single copy integrations and different insertion sites. Therefore, the gene silencing in barley could not have taken place due to both these reasons. However, a complete CDS codon reengineering approach could be used in future barley retransformation approaches. Furthermore, the vector construct should be made with more tissue-specific promoters to understand whether the effect of the promoter is detrimental and causes the transgene to be silenced. However, a re-transformation experiment would have been beyond the scope of my PhD.

We could nevertheless, learn much from studying the variations within barley genome itself to understand how barley could tolerate high levels of salinity (Beier et al. 2017; E.y and G.j 1977; Jenks et al. 2009; Maas and Hoffman 1977). Studies based on barley's salt stress tolerance variation are presented but are not limited to the examples in Table 1.

Table 1 Example studies on barley salinity tolerance

Type of Experiment	**Barley varieties under study**	**Main findings**	**Reference**
GWAS	2671 genotypes	SNPs on barley *HKT1;5* that are correlated with salt tolerance that is related to high Na^+ accumulation in roots and sheath	Hazzouri et al., 2018
Physiological study	TX9425 and ZUG293 (salt tolerant cultivars) and Franklin and Gairdner (salt sensitive cultivars	Higher in the residual transpiration rate in salt tolerant cultivars	Hasanuzzaman et al., 2017
Plant imaging and physiological measurements	Twenty-four commercial and landrace barley lines (*Hordeum vulgare* L. ssp *vulgare* and *H. vulgare* L. ssp *spontaneum*)	Shoot-ion-independent tolerance, ion exclusion and ion tolerance are needed cumulatively for the complete salt tolerant phenotype	Tilbrook et al., 2017
De-novo assembly and transcriptomic study on roots	Sahara (salt tolerant) and Clipper (salt sensitive)	There are differences between the transcripts related to sugar-mediated signaling, cell wall metabolism and defense response of the root meristem, elongation and maturation zones, respectively	Hill et al., 2016
Physiological study	Forty-seven barley and forty-five wheat (25 bread wheat, *Triticum aestivum*; and 20 durum wheat, *Triticum turgidum* spp. *durum*) genotypes contrasting in their salinity tolerance	Barley has more Na^+ accumulation capacity in the leaf mesophyll vacuoles that leads to more tissue tolerance capacity compared to wheat	Wu et al., 2015

De-novo assembly and transcriptomic study on leaf	Wild barley (*H. spontaneum*)	Involvement of Ethylene, flavonoids, ROS, and kinases	Bahieldin et al., 2015
Proteomics of the root (MALDI-TOF/TOF mass spectrometry)	DH14 (salt sensitive) and DH187 (salt tolerant)	Enhanced salinity tolerance of DH187 is due to mainly the signal transduction activity increase that subsequently affects the accumulation of stress protective proteins and changes in the cell wall structure	Mostek et al., 2015
Proteomics (two-dimensional gel electrophoresis and mass spectrometry)	Morex (salt tolerant) and Steptoe (salt sensitive)	Detoxification pathway and terpenoid biosynthesis proteins were detected as early responses to salinity	Witzel et al., 2014
Metabolite analysis	Tibetan wild barley XZ16 (*H. spontaneum*) and cultivated barley CM72 (*H. vulgare*)	Higher chlorophyll content and higher contents of compatible solutes than cultivated barley in wild barley, an assumption of cultivated barley enhancing its salt tolerance mainly through increasing glycolysis and energy consumption	Wu et al., 2013
GWAS	192 spring barley accessions	QTL on 6H and 4H associated with salt tolerance	Long et al. 2013
Proteomics (MALDI-TOF-TOF MS technique)	Afzal (salt-tolerant) and Line 527 (salt-sensitive) barley cultivars	Eighteen proteins have been found to respond differently between these two cultivars	Fatehi et al., 2012
Physiological study	TX9425 (salt-tolerant) and Franklin (salt-sensitive) barley cultivars	Interaction between polyamines and ROS in the roots that causes differences in the cytosolic K^+ homeostasis as a contributor of sensitivity to salinity in barley	Velarde-Buendía et al., 2012
Proteomics	Afzal (salt tolerant) and L-527 (salt sensitive)	Differences in proteins involved in stress defense, metabolism, protein synthesis and Photosynthesis among the two genotypes	Rasoulnia et al., 2011
HvHKT2;1 overexpression and expressed in *Xenopus* oocyte	Golden Promise	*HvHKT2;1* is predominantly expressed in the root cortex, Over-expression of *HvHKT2;1* led to enhanced Na^+ uptake, higher Na^+ concentrations in the xylem sap, and enhanced translocation of Na^+ to leaves	Mian et al., 2011
Association analysis and gene	Wild barley and CM72	Salt tolerance of Tibetan wild barley is mainly due to superior	Qiu et al., 2011

expression assay of *HvHKT1*(*HvHKT2;1*) and *HvHKT2* (*HvHKT1;2*)		Na^+ exclusion and better maintenance of tissue K^+ concentration	
Physiological study	Barque73, Clipper, Sahara, and Tadmor	The four genotypes had different independent Na^+ and Cl^- tolerant mechanisms Cl^- was mainly responsible for the photosynthetic inhibition Osmotic potentials in salt stress are different among soil and hydroponically grown plants	Tavakkoli et al., 2011
Physiological study	Seeds of two varieties: 'Cask' and 'County' (Cropmark Seeds Ltd, Christchurch, New Zealand)	Na^+ can act as an osmotic regulator and allow barley seeds to take up more water and germinate more rapidly in salinity	Zhang et al., 2010
Biophysical and physiological techniques	CM72 and Numar (salt tolerant), Gairdner and ZUG403 (salt sensitive)	Salt tolerant traits in barley constitute of tissue tolerance pin leaves and maintaining high xylem K^+ and Na^+ concentrations	Shabala et al., 2010
Metabolomics	Sahara (salt tolerant) and Clipper (salt sensitive)	Sahara has more leaf protectant metabolites compared to Clipper	(Widodo et al. 2009)
QTL mapping	A segregating DH population of 93 lines, developed by anther culture of the F1 hybrid between CM72 (salt-tolerant) and Gairdner (salt sensitive)	13 QTLs which associated with salt stress and accumulation of Na^+ in barley shoots, region of the 4H chromosome flanked by bPb-1278 and bPb-8437 is important in salt tolerance	Xue et al., 2009
Proteomics	Morex (salt tolerant) and Steptoe (salt sensitive)	Proteins involved in ROS scavenging were more abundant in Morex and proteins involved in iron uptake were highly expressed in Steptoe	Witzel et al., 2009
Physiological study	Salt-tolerant Numar and ZUG293, and salt-sensitive Gairdner and ZUG403	ROS-induced K^+ efflux is evident in salt-tolerant cultivars	Chen et al., 2007
Physiological study	6 varieties of barley including Melusine + ISABON3 (high	The stomatal conductance, vigorous growth, osmotic potential were some of the	Katerji et al., 2006

	salinity tolerant variety from Afghanistan)	traits that differed in ISABON3 compared to Melusine	

According to the examples given in Table 1, we can understand that barley contains a wide inter-cultivar variation in salt tolerance/response mechanisms. For example, differences in the metabolome, tissue tolerance, and photosynthetic capabilities may be responsible for differences among the cultivars in salt tolerant responses. These differences are likely to be governed by the underlying molecular mechanisms or allelic variations. Therefore, barley presents us with an excellent crop germplasm to study the salinity tolerance.

The study in this chapter used RNA-Seq data from multiple barley cultivars to analyse the natural variations and transcriptomic differences underlying the differences in tissue tolerance of barley. Aim of this chapter was to improve the knowledge base on the inter-cultivar differences in barley tissue tolerance (Negrão et al. 2017; Roy et al. 2011). We report novel allelic variations on transporter genes that may be responsible for the varying leaf sheath Na^+ accumulation levels in the studied cultivars. This chapter is formatted to be submitted to *BMC Plant Biology.*

References

Bahieldin A, Atef A, Sabir JSM, Gadalla NO, Edris S, Alzohairy AM, et al. RNA-Seq analysis of the wild barley (H. spontaneum) leaf transcriptome under salt stress. C. R. Biol. 2015 May;338(5):285–97.

Batard Y, Hehn A, Nedelkina S, Schalk M, Pallett K, Schaller H, et al. Increasing Expression of P450 and P450-Reductase Proteins from Monocots in Heterologous Systems. Arch. Biochem. Biophys. 2000 Jul;379(1):161–9.

Beier S, Himmelbach A, Colmsee C, Zhang X-Q, Barrero RA, Zhang Q, et al. Construction of a map-based reference genome sequence for barley, *Hordeum vulgare* L. Sci. Data. 2017 Apr 27;4:sdata201744.

Chen Z, Zhou M, Newman IA, Mendham NJ, Zhang G, Shabala S. Potassium and sodium relations in salinised barley tissues as a basis of differential salt tolerance. Funct. Plant Biol. 2007;34(2):150–62.

E.y M, G.j H. CROP SALT TOLERANCE CURRENT ASSESSMENT. 1977 Jan 1;103(2):115–34.

Fatehi F, Hosseinzadeh A, Alizadeh H, Brimavandi T, Struik PC. The proteome response of salt-resistant and salt-sensitive barley genotypes to long-term salinity stress. Mol. Biol. Rep. 2012 May;39(5):6387–97.

Hasanuzzaman M, Davies NW, Shabala L, Zhou M, Brodribb TJ, Shabala S. Residual transpiration as a component of salinity stress tolerance mechanism: a case study for barley. BMC Plant Biol. 2017 Jun 19;17:107.

Hazzouri K, Pauli D, Blake T, Shahid M, Khraiwesh B, Salehi-Ashtiani K, et al. Mapping of HKT1;5 gene in barley using GWAS approach and its implication in salt tolerance mechanism. Front. Plant Sci. [Internet]. 2018 [cited 2018 Feb 11];9. Available from: https://www.frontiersin.org/articles/10.3389/fpls.2018.00156/abstract

Hill CB, Cassin A, Keeble-Gagnère G, Doblin MS, Bacic A, Roessner U. De novo transcriptome assembly and analysis of differentially expressed genes of two barley genotypes reveal root-zone-specific responses to salt exposure. Sci. Rep. [Internet]. 2016 Aug 16 [cited 2017 Apr 11];6. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4985707/

Jenks MA, Hasegawa PM, Jain SM. Advances in Molecular Breeding Toward Drought and Salt Tolerant Crops. Springer Science & Business Media; 2009.

Katerji N, van Hoorn JW, Hamdy A, Mastrorilli M, Fares C, Ceccarelli S, et al. Classification and salt tolerance analysis of barley varieties. Agric. Water Manag. 2006 Sep 16;85(1–2):184–92.

Kooter JM, Matzke MA, Meyer P. Listening to the silent genes: transgene silencing, gene regulation and pathogen control. Trends Plant Sci. 1999;4(9):340–347.

Long NV, Dolstra O, Malosetti M, Kilian B, Graner A, Visser RGF, et al. Association mapping of salt tolerance in barley (<Emphasis Type="Italic">Hordeum vulgare</Emphasis> L.). Theor. Appl. Genet. 2013 Sep 1;126(9):2335–51.

Maas EV, Hoffman GJ. Crop salt tolerance : evaluation of existing data. Manag. Saline Water Irrig. Proc. Int. Salin. Conf. [Internet]. 1977 [cited 2017 Sep 15]; Available from: http://agris.fao.org/agris-search/search.do?recordID=US201302482113

Marenkova TV, Deineko EV. Transcriptional gene silencing in plants. Russ. J. Genet. 2010 May 1;46(5):511–20.

Matzke MA, Matzke AJM. Planting the Seeds of a New Paradigm. PLOS Biol. 2004 May 11;2(5):e133.

Mian A, Oomen RJFJ, Isayenkov S, Sentenac H, Maathuis FJM, Véry A-A. Over-expression of an Na+- and K+-permeable HKT transporter in barley improves salt tolerance. Plant J. 2011 Nov 1;68(3):468–79.

Milot E, Ellis J. Transgene Silencing. Encycl. Ref. Genomics Proteomics Mol. Med. [Internet]. Springer, Berlin, Heidelberg; 2005 [cited 2017 Dec 13]. p. 1896–9. Available from: https://link.springer.com/referenceworkentry/10.1007/3-540-29623-9_2940

Mostek A, Börner A, Badowiec A, Weidner S. Alterations in root proteome of salt-sensitive and tolerant barley lines under salt stress conditions. J. Plant Physiol. 2015 Feb 1;174:166–76.

Negrão S, Schmöckel SM, Tester M. Evaluating physiological responses of plants to salinity stress. Ann. Bot. 2017 Jan 1;119(1):1–11.

Qiu L, Wu D, Ali S, Cai S, Dai F, Jin X, et al. Evaluation of salinity tolerance and analysis of allelic function of HvHKT1 and HvHKT2 in Tibetan wild barley. Theor. Appl. Genet. 2011 Mar 1;122(4):695–703.

Rajeevkumar S, Anunanthini P, Sathishkumar R. Epigenetic silencing in transgenic plants. Front. Plant Sci. [Internet]. 2015 Sep 10 [cited 2017 Dec 21];6. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4564723/

Rasoulnia A, Bihamta MR, Peyghambari SA, Alizadeh H, Rahnama A. Proteomic response of barley leaves to salinity. Mol. Biol. Rep. 2011 Nov 1;38(8):5055–63.

Roy SJ, Huang W, Wang XJ, Evrard A, Schmöckel SM, Zafar ZU, et al. A novel protein kinase involved in Na+ exclusion revealed from positional cloning. Plant Cell Environ. 2013 Mar 1;36(3):553–68.

Roy SJ, Tucker EJ, Tester M. Genetic analysis of abiotic stress tolerance in crops. Curr. Opin. Plant Biol. 2011 Jun;14(3):232–9.

Shabala S, Shabala S, Cuin TA, Pang J, Percey W, Chen Z, et al. Xylem ionic relations and salinity tolerance in barley. Plant J. 2010 Mar 1;61(5):839–53.

Tavakkoli E, Fatehi F, Coventry S, Rengasamy P, McDonald GK. Additive effects of Na+ and Cl– ions on barley growth under salinity stress. J. Exp. Bot. 2011 Mar;62(6):2189–203.

Tilbrook J, Schilling RK, Berger B, Garcia AF, Trittermann C, Coventry S, et al. Variation in shoot tolerance mechanisms not related to ion toxicity in barley. Funct. Plant Biol. 2017 Nov 29;44(12):1194–206.

Vaucheret H, Béclin C, Elmayan T, Feuerbach F, Godon C, Morel J-B, et al. Transgene-induced gene silencing in plants. Plant J. 1998 Dec 1;16(6):651–9.

Velarde-Buendía AM, Shabala S, Cvikrova M, Dobrovinskaya O, Pottosin I. Salt-sensitive and salt-tolerant barley varieties differ in the extent of potentiation of the ROS-induced K+ efflux by polyamines. Plant Physiol. Biochem. 2012 Dec;61:18–23.

Widodo, Patterson JH, Newbigin E, Tester M, Bacic A, Roessner U. Metabolic responses to salt stress of barley (Hordeum vulgare L.) cultivars, Sahara and Clipper, which differ in salinity tolerance. J. Exp. Bot. 2009 Oct 1;60(14):4089–103.

Witzel K, Matros A, Strickert M, Kaspar S, Peukert M, Mühling KH, et al. Salinity Stress in Roots of Contrasting Barley Genotypes Reveals Time-Distinct and Genotype-Specific Patterns for Defined Proteins. Mol. Plant. 2014 Feb;7(2):336–55.

Witzel K, Weidner A, Surabhi G-K, Börner A, Mock H-P. Salt stress-induced alterations in the root proteome of barley genotypes with contrasting response towards salinity. J. Exp. Bot. 2009 Aug 1;60(12):3545–57.

Wu D, Cai S, Chen M, Ye L, Chen Z, Zhang H, et al. Tissue Metabolic Responses to Salt Stress in Wild and Cultivated Barley. PLOS ONE. 2013 Jan 31;8(1):e55431.

Wu H, Shabala L, Zhou M, Stefano G, Pandolfi C, Mancuso S, et al. Developing and validating a high-throughput assay for salinity tissue tolerance in wheat and barley. Planta. 2015 May 20;1–11.

Xue D, Huang Y, Zhang X, Wei K, Westcott S, Li C, et al. Identification of QTLs associated with salinity tolerance at late growth stage in barley. Euphytica. 2009 Sep 1;169(2):187–96.

Zhang H, Irving LJ, McGill C, Matthew C, Zhou D, Kemp P. The effects of salinity and osmotic stress on barley germination rate: sodium as an osmotic regulator. Ann. Bot. 2010 Dec;106(6):1027–35.

Investigating Genetic Variations of Contrasting Na^+ Accumulation in Barley Genotypes under Salt Stress

Running title: Transcriptomics of tissue tolerance variations in barley

Shanika Amarasinghe[1,2], Nathan S. Watson-Haigh[1,2], Caitlin Byrt[2,3], Stuart Roy[1,2], Matthew Gilliham[2,3], Ute Baumann[1,2]

1. Australian Centre for Plant Functional Genomics, PMB 1, Glen Osmond, SA 5064, Australia

2. School of Agriculture, Food and Wine, Waite Campus, The University of Adelaide, PMB 1 Glen Osmond, SA 5064, Australia.

3. Australian Research Council Centre of Excellence in Plant Energy Biology, Department of Plant Science, School of Agriculture, Food and Wine, University of Adelaide, Glen Osmond SA 5064, Australia.

Correspondence:

Dr. Ute Baumann,
Plant Genomics Centre,
School of Agriculture, Food and Wine,
The University of Adelaide,
Urrbrae,
South Australia,
5064, AUSTRALIA.

Abstract

Soil salinity causes large productivity losses for agriculture worldwide. Barley, one of the most important crops, is identified as salt tolerant compared to other staple crops such as wheat and rice. Identification of the genes and allelic variations underlying various salt tolerance mechanisms in barley will be a practical contribution towards the development of barley lines with greater salinity tolerance. We sequenced the RNA from six barley genotypes with varying leaf sheath Na^+ accumulation levels in salt stress and examined differential gene expression, variant analysis and gene co-regulatory networks to link the phenotypic characteristics to the underlying molecular components. We identified novel alleles on barley *HKT1;5* that could potentially be responsible for the high sheath Na^+ accumulation. Furthermore, through statistical modelling of gene expression levels a NHX was recognized as a candidate for high sheath Na^+ tolerance ability. Through co-expression networks, we discovered subtle expression pattern variations of genes amongst the six genotypes related to terpenoid, phenylpropanoid and flavonoid metabolism. These variations have provided us with candidate genes of interest for future characterisation of genetic mechanisms that contribute to salt stress tolerance in barley.

Keywords

Hordeum vulgare, salinity tolerance, Na^+ accumulation, allelic variation, *HKT1;5, NHX*

Introduction

Salinity is a major abiotic stress that causes productivity loss for agriculture worldwide [1–4]. This is exacerbated by the changes in rainfall patterns associated with global warming and by human practices, such as irrigation and clearing of vegetation [5, 6]. Surplus NaCl in soil hinders water extraction by plant roots and leads to accumulation of Na^+ and Cl^- within the plant [7, 8]. These osmotic and ionic stresses come at a cost in terms of plant energy use, reducing cell, tissue and plant growth rates [8–13] and increasing senescence, through damage to metabolic processes and ion imbalances [14–17].

Plants have various mechanisms to mitigate salinity stress, including mechanisms for detecting and signalling salt stress [18–22], maintenance of cell and tissue expansion [23, 24], exclusion of toxic sodium (Na^+) and chloride (Cl^-) ions from the shoot [8, 25–27], accumulation of ions in vacuoles [28–30], maintenance of K^+ homeostasis [31–34] and synthesis of compatible solutes [29, 35]. These mechanisms are typically grouped into three categories; a) shoot ion exclusion b) shoot ion tissue tolerance and c) osmotic tolerance [7, 8]. However, the salt tolerant capabilities of a plant may vary largely with genetic traits, the environmental factors and the development stage [36]. The genes and gene networks involved in salinity tolerance mechanisms are of interest as this information can be used to develop crop germplasm that produces higher yields in salt affected soil [18]. Once these genes are known, assessment of genotypic variation in plant salt tolerance mechanisms will identify the best alleles of important genes which can be used to improve crops.

Barley, the fourth most important crop in the world, is considered a salt-tolerant crop, relative to other cultivated cereals, as it can grow on soils with an EC_e of 8 dS/m with little reduction in growth, and still maintain 50% of its yield potential at 18 dS/m [37, 38]. On the other hand, the closest crop relative of barley, bread wheat, can tolerate only up to 6 dS/m in optimal conditions and can maintain 50% of the yield potential at 13 dS/m [39]. Relative to bread wheat, barley tends to accumulate Na^+ and Cl^- in leaf blades, combining ion accumulation in plant vacuoles and maintenance of cytosolic K^+ homeostasis, with the synthesis of compatible solutes that assist with ion homeostasis [5, 33, 40–45].

A previous study of shoot ion tissue accumulation, that focussed on 50 salt stressed barley genotypes revealed differences in leaf blade and sheath Na^+ accumulation (James et al. in prep.). Preferential sequestration of Na^+ in the leaf sheath over the leaf blade may enhance salinity tolerance by keeping Na^+ away from the photosynthetically active leaf blade [46]. Variation was observed in terms of total tissue Na^+ concentration as well as differences in the sheath:blade (S:B) Na^+ ratio. These data were used to select six barley genotypes which differed for tissue Na^+ accumulation: Three of the genotypes (Beecher, Fleet and Sloop) showed similar levels of Na^+ in their sheath and leaf blade tissues, whereas the other three genotypes (Alexis, Commander and Maritime) accumulated relatively more Na^+ in their sheath than in their leaf blade, thus giving them a higher S:B Na^+ ratio. In addition, Alexis accumulated at least twice as much total Na^+ in the leaf blade than the other five genotypes (James et al. in prep.). These data indicate that it is likely that the different genotypes vary in their salt tolerance mechanisms. For example, Na^+ leaf exclusion may contribute to the salt tolerance of the low Na^+ accumulating lines, higher than to Alexis. Identification of the genes involved in these different mechanisms will help guide future research towards the development of barley lines with greater salinity tolerance, through marker assisted selection to incorporate the best allele of the relevant genes.

The objective of the current study was to test which genes may be relevant. To achieve this we analysed the transcriptome of leaf blade, leaf sheath and root tissues from six barley accessions using RNA-Seq data. This analysis revealed allelic variations in barley HKTs that we suggest may be linked to the differences observed in the Na^+ levels in the shoots of different genotypes. We observed genotypic differences in the regulation of NHXs, which we hypothesise may play a role in Na^+ sequestration in higher sheath Na^+ accumulating genotypes. In addition, we detected transcript differences that we expect would influence the biosynthesis of flavonoids and terpenoids, K^+ homeostasis and cell wall strengthening, and these differences have provided us with candidate genes of interest for future characterisation of genetic mechanisms that contribute to salt stress tolerance in barley.

Methods

Plant Growth and Stress Treatment

Six barley (*Hordeum vulgare* L.) genotypes, Beecher (Australia), Commander (Australia), Fleet (Australia), Sloop (Australia), Alexis (Germany) and Maritime (Australia) have differing capacity to partition Na^+ into leaf sheaths (James et al. unpublished data). Plants were grown in a supported hydroponics set up using 40-L trays and quartz gravel as described previously [47]. Seeds were germinated on filter paper in Petri dishes over 2 d at 4°C, and seedlings were planted into individual hydroponic pots. At approximately 5 d after emergence of the first leaf, 25 mM NaCl was added twice daily to a final concentration of 200 mM NaCl. Supplemental Ca^{2+} was added as $CaCl_2$ to give a final Na^+: Ca^{2+} of 15:1. Plants were grown in a controlled environment chamber with a 10 h photoperiod and a photosynthetic photon flux density of 1000 μmol m^{-2} s^{-1} at 20°C during the day and 10°C during the night. Plant tissues (leaf blade, leaf sheath and root) were sampled 18 days after the commencement of NaCl treatment for measurement of tissue ion (Na^+ and K^+) concentration and gene expression. Blade and sheath tissues were sampled from leaves three (L3) and four (L4). Roots were washed twice in a cold solution of 10 mM $Ca(NO_3)_2$ for 10 – 15 s, blotted on absorbent paper to remove excess solution, divided in half vertically (half for ion analysis and half for RNA extractions) and weighed before proceeding with subsequent steps.

Physiological Traits of Stressed Plants

The leaf blade and sheath samples for ion determination were dried at 70°C for 2 days. Samples were then weighed, digested in 500 mM HNO_3 at 80°C for 1.5 h and analysed for Na^+ and K^+ using an Inductively Coupled Plasma – Atomic Emission Spectrometer (Vista Pro, Varian, Melbourne, Australia) following the protocol in [48]. For each genotype Na^+ concentrations and K^+ concentrations of L3 blade and L3 sheath were recorded.

RNA-Seq library construction, Illumina sequencing and Mapping

Gene expression in the third leaf blade, third leaf sheath and root of the six barley genotypes exposed to 200 mM NaCl was determined by RNA-Seq. Tissue samples were immediately frozen in liquid N_2 and stored at -80°C; four biological replicates per tissue were taken. RNA was extracted using Zymo research Direct-zol™ RNA MiniPrep kit (California, USA) following the manufacturer's suggested protocol, including the in-column DNase I digestion. RNA was quantified using a NanoDrop spectrophotometer (Agilent Technologies, Palo Alto, Calif) and quality was checked by visualisation of a sample of RNA by gel electrophoresis and on an Agilent 2100 Bioanalyzer (Adelaide Microarray Centre, Institute of Medical and Veterinary Science). RNA-seq libraries were prepared from total RNA using the TruSeq Stranded Total RNA with Ribo-Zero Plant kit according to the manufacturer's instructions (TruSeq, San Diego, CA, USA). Sequencing runs were performed on a HiSeq1500 (San Diego, CA, USA), generating paired-end reads with a length of 125 base pairs (bp).

The Illumina sequencing of the RNA-Seq libraries resulted in 72 sequence files in FASTQ format. The read quality was checked using FastQC [49]. FASTX-Toolkit v0.0.14 fastx_clipper was used to remove adapter sequences [50]. Read pairs were retained if both reads were ≥ 70 base pairs (bp) (S1). Reads were aligned to the IBSC 2016 Morex reference genome [51] using TopHat v2.1.1 [52], enabling stranded alignment, allowing up to 2bp mismatches, 5 gaps, 5000 bp of intron length and 250bp of inner distance of mates and no multi-mapping. The resulting read alignments (binary alignment/map; BAM files) were indexed using SAMtools v1.4.1 [53]. A total of 3.16 billion reads (65 % of the total reads) were aligned to the IBSC 2016 Morex reference which contains 39,734 annotated genes (S2). A quality checking step was performed on BAM files to calculate the number of mapped reads, unmapped reads (filtered BAM files on -F 4 vs –f 4, respectively) and contamination levels by counting reads mapped to plant rRNA, chloroplast and mitochondrial genome sequences using SAMtools v1.4.1[53] (S2). We eliminated one sample (Maritime sheath L2E) with low percentage of mapped reads compared to others, which is highlighted in S2 in red, from all further analyses.

Identifying Genotype Specific Genes

Aligned reads were normalised (CPM: counts per million) and fitted with a linear model in order to identify highly and lowly expressed genes. Feature counting (genes as features) was performed on BAM files using the *RSubread* package in R [54]. The counts were separated into the three tissues (blade, sheath and root) and normalised using calcNormFactors() function from *edgeR* package [55, 56]. Genes with ≤10 CPM were removed prior to fitting the linear model using *Limma* to identify genes that were differentially expressed [57]. The genes were considered differentially expressed if they exhibited a difference of at least two-fold change with a FDR (BH method) adjusted p value ≤ 0.05.

We define a gene as having “genotype specific gene expression” if that gene’s expression is: a) significantly higher in one genotype compared to all others, which we refer to as “highly expressed”; or b) is significantly lower in one genotype compared to all others, which we call “lowly expressed”. The comparisons were performed in a pairwise manner. The “highly” and “lowly” sets of genes identified for each genotype were subjected to further analyses.

Identifying Genotype Specific Salt-related Homologous

A subset of genotype specific genes were identified from a reciprocal BLAST using salt related Arabidopsis sequences that were candidates for possibly being involved in the salt response in barley based on previous studies in Arabidopsis. *Arabidopsis thaliana* genes were selected from TAIR which had been annotated with various salt-related terms [58]. The TAIR database was queried with keywords “salt”, “sodium ion transmembrane transporter activity”, “response to osmotic stress”, “sodium ion transport”, “sodium ion homeostasis”, “cyclic nucleotide binding“, “cation channel activity”, “cation transmembrane transport”, “antiporter activity”, “potassium ion transport” and ”anion channel activity” [58]. These genes were then used to identify the putative homologues in barley using a reciprocal BLAST approach [59]. For reciprocal hits to be valid, we required at least 60% of query coverage and an e-value of 1×10^{-100}. The list of putative barley homologous were then compared with list of genotype specific genes and common genes between both lists were identified as genotype specific salt related homologous.

Assigning Common Names through Molecular Phylogenetics

A phylogenetic analysis was performed on putative major candidate gene families involved in Na^+ transport (i.e. *HKT*, *NHX* and *SOS*) [60]. A BLAST search (tBLASTn) was carried out on the whole barley genome to identify loci that contained putative orthologues of above said candidate genes (>60% of query coverage and an e-value of $1x10^{-100}$). Sequences were then manually curated through the following procedure; 1) Alignment refinement through Exonerate v 2.4 [61]; 2) use the alignment coordinates produced by Exonerate to define the exon structure of the gene and extract the CDS corresponding to the aligned portion of the query protein sequence; 3) confirm the structure using the RNA-Seq read alignments. Where the defined CDS was still missing the 5′ and/or 3′ end, we extended the 5′ and 3′ ends of the CDS to an in-frame start or stop codon respectively, which most closely matched that of the query protein sequence. Phylogenetic trees were generated using MEGA 6.06 software using Juke Cantor amino acid substitution model using a Maximum Likelihood approach [62, 63]. To determine how well the nodes of the ML tree were supported, 10,000 bootstrap trees were generated [64]. The common names were then assigned based on the occurrence of the genes within the clades of the phylogenetic trees.

Variant Discovery

Variant calling for genes of interest was performed for each genotype using all BAM files associated with that genotype. BAM files of the four replicates per genotype were merged and one pileup file was created for each genotype using SAMtools mpileup function [65]. The resulting six pileup files were then used to create a VCF file using SAMtools along with the IBSC 2016 Morex reference sequence. The VCF file was annotated using SNPEff tool that predicts the effect of each variant [66]. An in-house variant calling Java based tool, merutensils.jar (Suchecki et al. in prep.), was used to count the number of reads supporting each variant and confirm the obtained results. The following parameters to merutensils.jar in order to predict a variant with confidence; the coverage of a variant per genotype ≥ 5 reads, at least 1 variant needs to be present in at least in 1 sample and the maximum error per allele to be 5%.

Co-expressed Genes

In order to identify gene clusters that may work in concert, as well as be linked to ion accumulation traits of interest (i.e. modules), we performed a weighted gene co-expression network analysis (WGCNA) using genes with normalised reads (CPM values). In a gene co-expression network, nodes denote the genes and the edges between the nodes represent a significant association between them. The R package Weighted Gene Co-expression Network Analysis (WGCNA) v1.49 [67] allowed us to associate modules that are co-expressed based on the correlation of their expression patterns, to particular salt traits of interest. Signed co-expression networks were constructed using the automatic one-step network construction method (function *cuttreeDynamic()*) with the following settings; a signed type of network, an unsigned type of topological overlap matrix (TOM), correlations of the network raised to a soft thresholding power β (blade: 9, sheath: 9, roots: 8), correlation measures with option '*bicor*', deepSplit value of 2, a minimum module size of 20.

It was assumed that the expression of a particular module that is highly associated with a trait of interest is mainly governed by that particular trait and may not be solely the genotypic differences. We used ion ratios as traits to gain a holistic picture of how the tissues behave in presence of Na^+ (i.e. S:B Na^+ ratio; S:B K^+ ratio, blade K^+:Na^+ ratio and sheath K^+:Na^+ ratio). The first principle component of a module (module Eigen gene) value was calculated and used to test the association of modules with the above mentioned traits.

Module gene significance (GS, the correlation between gene expression and physiological traits), total network connectivity (kTotal), and module membership (MM) were calculated for each gene in the modules for the three tissues. Genes within each module were then ranked using the absolute value of MM, in order to identify hub genes as the top 30 genes with highest MM. Hub genes are the ones that have the highest connectivity and play a major role for the existence of that network. Next genotype specific genes that are also hub genes were identified by the overlap of genotype specific gene lists and hub genes of selected modules. Genotype-specific genes which are also hub genes are biologically interesting as they represent those genes which play a major role in the structure/topology of the said network in a genotype specific manner.

Functional Annotation

In order to predict the putative biological importance of the expressed genes, gene ontology (GO) enrichment, functional categorisation, and pathway analysis were used. GO enrichment analysis was performed using AgriGO (http://bioinfo.cau.edu.cn/agriGO/analysis.php) with Fisher's exact test and false discovery rate (FDR) correction [68]. GO annotations for barley genes were transferred from Arabidopsis and rice through blastx (>60% of query coverage and an e-value of $1x10^{-100}$). The *p*- values for each overrepresented annotation was calculated using the hypergeometric distribution. The terms were considered significant if the calculated FDR corrected p value ≤ 0.05.Mapman bins were obtained using the terms associated with the rice and Arabidopsis homologs of the gene lists [69]. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were assigned to the barley CDS sequences using the online KEGG Automatic Annotation Server (KAAS) using single-directional best hit (SBH) method (http://www.genome.jp/kegg/kaas) [70–72]. Transmembrane regions were predicted using InterPro web tool [73].

Results

Variations in leaf Na^+ and K^+ content among barley genotypes

To determine the extent to which different barley genotypes vary in their accumulation of Na^+ and K^+ in leaf sheath and blade tissues in saline conditions, six commercial barley genotypes (Alexis, Beecher, Commander, Maritime, Fleet and Sloop) were grown in 200 mM NaCl. These six genotypes were selected based on previous data indicating that they differ in their accumulation of Na^+ and K^+ in leaf tissues (James et al. in prep.). Alexis had the significantly high Na^+ in both sheath and blade compared to the other five genotypes (Figure 1 a-b) (p value ≤ 0.05; TukeyHSD test). Alexis similarly had the lowest K^+ in both blade and sheath but the difference was only significant in the sheath (Figure 1d). The root Na^+ concentration was highest in Beecher and Maritime, yet they were not significantly different from the four other genotypes (Figure 1e). However, the root K^+ levels were significantly higher in Beecher and Maritime compared Commander and Sloop (p value ≤ 0.05; TukeyHSD test) (Figure 1f). Differences in the accumulation of Na^+ and K^+ in the genotypes can be represented by plotting the Na^+ sheath/blade (S:B) ratio against the K^+ sheath/blade ratio, and this analysis indicated that the genotypes separate into three clusters. Alexis was assigned to group i, having the highest S:B Na^+ ratio and the lowest S:B K^+ ratio (Figure 1g); Beecher and Sloop was assigned to group ii, as they accumulated more K^+ in the sheath than in the leaf blade and more Na^+ in the leaf blade than in the sheath therefore having the highest S:B K^+ ratio to lowest S:B Na^+ ratio amongst all genotypes (Figure 1g); and Maritime, Fleet and Commander were assigned to group iii, as they accumulated more Na^+ and K^+ in the leaf sheath than in the leaf blade but had a higher S:B Na^+ ratio than S:B K^+ ratio (Figure 1g). The raw data used for the generation of Figure 1 is included in S3.

Genotype Specific Genes

The tissue specific RNA-Seq data for the six genotypes were analysed in order to identify candidate genes that displayed expression patterns consistent with a possible role in contributing to the observed tissue ion accumulation phenotypes. The number of genes with ≥ 10 normalised reads for each tissue was 16,914 (blade), 20,765 (sheath) and 22,831 (root). Of these we were interested in categorising genes that had a genotype-specific level of expression, to narrow down the number of genes that can be responsible for the genotype-specific tissue ion accumulation phenotypes (Figure 1). The number of genotype specific genes from the three tissues are shown in Table 1 with full lists available in S4.

Using the KEGG pathway information we identified highly expressed genotype specific genes in the leaf blades of Alexis that encode proteins which are involved in ethylene formation; and we observed peroxidase related genotype specific genes that were relatively lowly expressed in Alexis (S5). The GO analysis revealed that both highly expressed and lowly expressed Alexis specific genes from leaf sheath and blade tissues were enriched GO term "protein amino acid phosphorylation". It was also noted that lowly expressed Alexis specific genes in roots were enriched for transcription.

The GO analysis revealed a trend in Maritime leaf blade and sheath where highly expressed Maritime-specific genes were enriched for ones which encode proteins involved in protein amino acid phosphorylation and the same GO enrichment was also seen for the lowly expressed Maritime-specific genes as well. Highly expressed Maritime-roots were enriched for cutin, suberin and wax biosynthesis related genes (S5).

The pathway analysis for Beecher leaf blade genes indicated the high expression of genes encoding proteins which regulate nucleotide and nucleoside binding processes. Beecher leaf blade and leaf sheath both had lowly expressed genes encoding proteins which regulate protein amino acid phosphorylation (S5). Beecher sheath tissues had highly expressed genes encoding proteins which regulate oxygen binding processes. Highly expressed genes in Beecher roots were associated with endocytosis and protein processing in the endoplasmic reticulum and lowly expressed genes were associated with sugar metabolism, transport and MAPK signalling (S5). Furthermore, highly expressed genes from Sloop sheaths were associated with phagosome function and cell wall biogenesis while lowly expressed genes were associated with transmembrane transporter activity and transferase activity. We also observed that highly expressed genes from roots of all genotypes were enriched for protein modification and protein amino acid phosphorylation GO terms.

Genotype Specific Salt-related Genes

The TAIR databases were mined and retrieved 1,635 unique loci that are linked to salinity stress and salinity tolerance mechanisms in Arabidopsis, and then used a reciprocal BLAST approach to identify 609 putative barley homologues using the 1,635 Arabidopsis sequences as query sequences (S6).

Through this procedure 609 genes were identified and subsequently used as query sequences to identify the subsets of genotype specific genes of each tissue that can putatively be involved in salinity tolerance mechanisms. The number of genotype specific salt related genes for the three tissues are listed in Table 1, and a summary of the genes is included in S4. To compare these salt tolerance mechanism associated genes in the six genotypes, the subset of genotype specific salt responsive genes were further analysed. In particular, the genes associated with ion transport that have previously been linked to plant salinity tolerance were investigated (Figure 2, S7).

There were 21, 38 and 27 genotype specific genes that code for putative transporter proteins from blade, sheath and root, respectively (Figure 2 a, b and c respectively). In Alexis relatively high expression for several ABC transporter genes was observed in all three tissues. Additionally, a sodium-proton exchanger (*HORVU2Hr1G021020*) was revealed as a highly expressed genotype specific gene in Alexis sheath (Figure 2). Several putative potassium channel coding genes were relatively highly expressed in Beecher. A K^+/H^+ antiporter was identified as being a highly expressed genotype specific salt responsive gene for all three tissues of Beecher (*HORVU7Hr1G008600*), and this gene contains a KefB/KefC domain (retrieved from BLASTP through NCBI). Both Beecher and Sloop had relatively high expression of aquaporin like super family proteins in sheath tissue.

Genotypic Variation of Transporters Known To Be Linked To Salinity Tolerance

NHX genes

NHXs (Na^+/H^+ antiporters) promote Na^+ sequestration in the vacuole which allows the cell to use Na^+ as a cheap vacuole osmoticum and may as a consequence reduce the toxicity of the excess Na^+ towards the cytosol [29, 74, 75]. Recently it was also suggested that NHXs may preferentially transport K^+ over Na^+ [5, 76, 77]. The relevance of NHXs in the context of studied genotypes and whether their expression patterns and variants can be related to salt response variations among the genotypes was therefore, of interest. Using the new barley genome reference data, 6 full-length *NHX* family genes were revealed (S8). A phylogenetic tree using identified barley NHX protein sequences and other known NHXs was generated to identify the evolutionary relationship between them (S9).

With the exception of one *NHX* gene (*HvNHX3; HORVU7Hr1G046030*), all other *NHX* genes were expressed in at least one of the three tissues for all genotypes (Figure3). *HvNHX2* (*HORVU1Hr1G020360*) was highly expressed in blade and sheath compared to roots (Figure 3). *HvNHX4* (*HORVU2Hr1G021020*) was particularly highly expressed in Alexis sheath compared to expression in other five genotypes and in Alexis blade compared to Beecher, Commander and Maritime. *HvNHX5;1* (*HORVU7Hr1G049400*) was highly expressed in roots of all genotypes compared to blade and sheath tissues, while the *HvNHX5;2* (*HORVU5Hr1G072440*) was expressed in Alexis only in sheath tissue. *HvNHX6* (*HORVU5Hr1G053720*) expression was highest in roots relative to other tissues, and expression levels were similar for all genotypes. SNPs were predicted for the four genes *HvNHX4, HvNHX5;1, HvNHX5;2*, and *HvNHX6* within the coding region, however, none of them were non-synonymous (S10, Figure 4 a-d) *HvNHX2*, *HvNHX3* and *HvNHX5;1* were identical in all genotypes and also were 100% similar to the reference Morex sequence (Figure 4 e-f).

HKT genes

We identified five full-length barley *HKT* genes (*HvHKTs*) (S11) through sequence similarity to known rice and Arabidopsis HKTs. A phylogenetic tree using manually curated HKT protein sequences from barley was generated including known HKTs form other species to identify the evolutionary relationship between them (S9). All *HvHKTs* except *HvHKT1;4* were significantly expressed in at least one sample in at least one tissue. *HvHKT1;5* (*HORVU4Hr1G087960*) was expressed only in roots, *HvHKT1;2* (*HORVU2Hr1G100440*) expression was higher in sheath and blade compared to roots (Figure 5), *HvHKT2;1* (*HORVU0Hr1G022090*) was expressed in only roots (Figure 5).Alexis had low expression of *HvHKT1;5* when compared to Beecher, Commander, Fleet and Maritime. Furthermore *HvHKT1;3* (*HORVU6Hr1G031360*) was highly expressed in Alexis blade compared to Commander, Fleet and Sloop and in Alexis sheath compared to Beecher, Commander and Sloop. *HvHKT1;3* was highly expressed in Maritime sheath compared to Sloop sheath (Figure 5).

We observed that the coding sequence for *HvHKT1;5* in Alexis is identical to that of the reference Morex sequence (S13). The *HvHKT1;5* genes for the other five genotypes differed in sequence to both Alexis and the Morex reference sequence but were similar to each other (Figure 6a). Allelic variants cause six predicted amino acid differences in Morex/Alexis relative to the other genotypes (Figure 6a). *HvHKT1;2* has the highest number of missense variants (7) relative to the reference sequence, and these differences were observed in Alexis, Beecher and Fleet (Figure 6b). The coding sequences of Commander and Maritime *HvHKT1;2s* are identical to the reference Morex sequence. For *HvHKT1;3* there is 1 variant (G>A) that causes the amino acid change Asp>Asn in Maritime and Beecher relative to Morex (Figure 6c). For *HvHKT1;4* there were 6 non-synonymous variants (Figure 6d). *HvHKT2;1* has 3 non-synonymous SNPs that causes amino acid changes in Maritime relative to Morex sequence (Figure 6e). The identified HvHKT amino acid sequences were aligned with known HKT sequences to identify putative pore forming residues of each HKT. This revealed that the first three residues of pore A were conserved among all the HKTs while the HKT1 group had the TVSSM[A|Q|S|S][A|T] signature (Figure 6f). ANCGF signature from pore B was conserved in HvHKT1;2, HvHKT1;3 and HvHKT1;5. Pore C contained R[H|Q][T|A|S]GEXX architecture for all the HvHKTs. GNVG[F|Y|L|]S[T|L|M] was the architecture for the pore D (Figure 6f).

Co-expression analysis of expressed genes

In order to identify gene clusters that may work in concert, as well as linked to ion accumulation traits of interest, a weighted gene co-expression network analysis (WGCNA) was performed. Co-expression networks can identify genes that are potentially important members of a biological process by acting in similar regulatory pathways.

The analysis identified 52, 48 and 52 modules for blade, sheath and root, respectively. A co-expression network module represents a group of genes which are tightly connected to each other based on correlation, as measured by the Pearson correlation coefficient. The hypothesis being that the genes within a module are under some common regulatory control. These modules are referred to by various colours (Figure 7). The modules were then correlated with traits of interest (see Methods) based on leaf sheath and blade ion content measurements. This was to identify the modules that were positively and negatively correlated with these traits (i.e. modules with highest absolute correlation) (Figure 7 a, b, c). We also looked at the Eigen gene for the modules. Eigen gene is the first principle component of the expression profiles of the genes making up a module. It provides a means to summarise the gene expression profiles of all the constituent genes in a given module.

Blade

The modules from blade that had the highest positive or negative correlation to the selected traits were darkolivegreen, coral4, darkorange2, orangered1, yellow2, darkgrey and pink4 (Figure 7a). Darkolivegreen and coral4 modules were positively and negatively correlated to S:B Na^+ ratio, respectively (Figure 7a). Darkolivegreen module was highly expressed in Maritime (Figure 7 d i). Coral4 module was highly expressed in Beecher (Figure 7 d ii). Darkorange2 and orangered1 modules were positively and negatively correlated to S:B K^+ ratio, respectively (Figure 7a). Darkorange2 module was highly expressed in Beecher, Fleet and Maritime and lowly expressed in Alexis (Figure 7 d iii). Furthermore, orangered1 module was highly expressed in Alexis and lowly expressed in Commander Fleet and Maritime (Figure 7 d iv). Darkorangered2 and yellow2 modules were positively and negatively correlated to blade K^+:Na^+ ratio, respectively (Figure 7a). Yellow2 module was highly expressed in Alexis (Figure 7 d v). Darkgrey and pink4 modules were positively and negatively correlated to sheath K^+:Na^+ ratio, respectively (Figure5a). Darkgrey module was highly expressed in Beecher and Sloop, while lowly expressed in Alexis (Figure 7 d vi). Pink4 module was highly expressed in Alexis (Figure 7 d vii).All blade modules except pink4 were enriched for the GO term small molecule metabolic process catalytic activity or nucleotide binding (S14). Pink4 module was enriched mainly for glycoprotein biosynthetic process and protein transporter activity. KEGG pathways revealed that all blade modules contain metabolic pathway related genes (S14). According to Mapman all of the above mentioned modules except darkolivegreen, yellow2 and pink4 contain phenylpropanoid metabolism related genes (S15). Coral4, darkorange2 and darkgrey modules show genes involved in terpenoid metabolism. All except darkolivegreen and pink4 contain Flavonoid metabolism related genes (S15).

In the overlap of hub genes from the blade modules with genotype-specific genes, we saw that genotype specific genes from Beecher blade were dominating the hub genes from coral4 module, which includes a K^+/H^+ antiporter (*HORVU7Hr1G008600*) (S16). There was a hub gene from the darkorange2 module (*HORVU7Hr1G100570: glutathione synthetase 2*) that is highly expressed in Maritime while lowly expressed in Alexis (S16).

Sheath

The modules from sheath that had the highest positive or negative correlation to the selected traits were firebrick2, navajowhite3, firebrick and yellow4 (Figure 7b). Firebrick2 and navajowhite3 modules were positively and negatively correlated with S:B Na^+ ratio, respectively (Figure 7b). Firebrick2 module was highly expressed in Alexis and lowly expressed in Beecher and Sloop (Figure 7 e i). Navajowhite3 module was highly expressed in Sloop (Figure 7 e ii). Firebrick and yellow4 modules were positively and negatively correlated respectively, with several traits (S:B K^+ ratio, blade K^+:Na^+ ratio and sheath K^+:Na^+ ratio) (Figure 7b). Firebrick module was lowly expressed in Alexis (Figure 7 e iii) whereas yellow4 module was highly expressed in Alexis (Figure 7 e iv).The firebrick2 module was enriched for terms involved in epigenetic modifications such as histone methylation, nucleobase-containing small molecule metabolic process, and negative regulation of gene expression (S14). While genes from the navajowhite3 module were enriched for cell wall biosynthesis and catalytic activity related GO terms, yellow4 genes were enriched for defence and stress responses (S14). All four sheath modules contain phenylpropanoid and flavonoid metabolism related genes (S15). Navajowhite3 and firebrick modules contain wax related genes. Firebrick and yellow4 modules include terpenoid metabolism related genes (S15).

A hub gene from the navajowhite3 module (*HORVU3Hr1G058810; TRICHOME BIREFRINGENCE-LIKE 38*) is a highly expressed gene from Sloop. Interestingly, except one hub gene from firebrick module, all the genes from both firebrick and yellow4 modules were lowly and highly expressed genes in Alexis, respectively (S16).

Root

The modules from root that had the highest positive or negative correlation to the selected traits were darkgoldenrod4, paleturquoise4, blueviolet and coral4 (Figure 7c). Darkgoldenrod4 and paleturquoise4 modules were positively and negatively correlated with S:B Na^+ ratio, respectively (Figure 7c). Darkgoldenrod4 module was highly expressed in Alexis, Commander, Fleet and Maritime, while lowly expressed in Beecher and Sloop (Figure 7 f i). Paleturquoise4 module was highly expressed in all except Commander and Maritime (Figure 7 f ii). Blueviolet and coral4 modules were positively and negatively correlated respectively, with several traits (S:B K^+ ratio, blade K^+:Na^+ ratio and sheath K^+:Na^+ ratio) (Figure 7 c). Blueviolet module was lowly expressed in Alexis (Figure 7 f iii). In contrast, coral4 module was highly expressed in Alexis (Figure 7 f iv).The darkgoldenrod4 module was enriched for metabolic processes such as tetrapyrrole metabolic process (S14). The paleturquoise4 module was enriched for terms related to stress responses. The blueviolet module was enriched for terms associated with root morphogenesis and growth (S14). Similarly to blade and sheath, Mapman shows that all root modules also contain phenylpropanoid and flavonoid metabolism related genes (S15). Interestingly, however, except for one gene from darkgoldenrod4 module, no other genes from root modules are categorised as being involved in terpenoid metabolism (S15).

Many unknown or undescribed genes were identified as hub genes for the darkgoldenrod4 module (S16). However, we see a NRT1/ PTR FAMILY 4.3 protein coding gene (*HORVU2Hr1G085260*), which is a highly expressed gene from the roots of Alexis and a lowly expressed gene from Sloop root be a hub gene in this module (S16). There was a high affinity nitrate transporter coding gene (*HORVU6Hr1G005600*) and several Glutathione S-transferase family protein coding genes as hub genes of the paleturquoise4 module (S16).

Discussion

Regulation of tissue ion content is an important component of salinity tolerance. In particular, maintaining high leaf blade K^+/Na^+ ratio is necessary, and retention of Na^+ in roots or even in sheath may be beneficial to avoid accumulation in leaf blades. We compared the molecular machinery of six barley genotypes with varying sheath and blade Na^+ accumulation levels. In particular, we were interested in transcriptomics and genetic variations in genes that are known to be influential in controlling ion transport. Analysis of tissue specific transcript differences between genotypes varying in leaf ion accumulation has revealed possible candidate genes implicated in this trait.

Alexis Possesses Specific Genetic Variations in *HvHKT1;5*

HKT1s are well known for mediating Na^+ unloading from the xylem to reduce excessive Na^+ accumulation in leaves [26, 78, 79]. Here we observed that there were differences in the sequence of *HvHKT1;5* within the genotypes. The *HvHKT1;5* sequence in Alexis was identical to the Morex reference genome (IBSC 2016), however, all the other five genotypes were different with non-synonymous variants that gave rise to 6 amino acid substitutions (Figure 6a). Alexis is a higher leaf Na^+ accumulating phenotype, compared to the other barleys in this study. It has been shown that Morex is also a genotype with high leaf Na^+ accumulation ability [80]. Therefore, it would be of interest to test whether the differences in the Alexis/Morex HvHKT1;5 protein alter the capacity to retrieve Na^+ from the transpiration stream, relative to genotypes with differing HvHKT1;5 sequence. The amino acid sequences which make up the pore regions of the gene, and therefore determine which ions are transported by the protein, were similar between the barley genotypes, but they are different to those in the same region in the well characterised wheat HKT1;5 transporters [81, 82]. Interestingly, a recent Genome-Wide Association Study (GWAS) on the region with *HvHKT1;5* reports polymorphisms related to salt tolerance, yet not finding any polymorphisms on the coding region of *HvHKT1;5* contradicts our findings [83]. The genotypes used in Hazzouri et. al (2018) for a follow-up study from GWAS are currently unavailable but could be different to ours [84]. Therefore, heterologous expression assays, such as expressing the two different alleles of the gene identified in this study in yeast or *Xenopus* oocytes, may reveal whether the transport and/or regulation properties of the two proteins differ, and this could shed light on how such subtle variations affect HvHKT1;5 activity. This encourages researchers to expand these findings to a large diversity panel through techniques such as KASP™ genotyping.

HvNHXs are Candidates Implicated in High shoot Na^+ Accumulation

The *NHX* gene families have been previously reported as being of interest as candidates for mediating Na^+ sequestration in the vacuole or regulating cellular pH and/or regulating K^+ [85–90]. Higher expression levels of *HvNHX4* were observed in the leaf blade and especially sheath of Alexis compared to the other genotypes, and these tissues of Alexis accumulated significantly more Na^+ than the other genotypes tested. Based on these results, we speculate that HvNHX4 could have a role in tissues with a high Na^+ content. Based on previous characterisation of these proteins, this role might be in sequestrating Na^+ into the vacuole or in maintenance of K^+ homeostasis [85, 88, 91–93].

In addition to different *HvNHX4* regulation, Alexis also had higher *HvNHX5;2* expression in the sheath compared to the other 5 genotypes (Figure 3). These two sequences are evolutionarily quite distant (S9). Co-expression analysis revealed that another *NHX* (*HvNHX2*) is a hub gene from a module (yellow2) that is negatively correlated with blade K^+:Na^+ ratio and the genes in this module tend to have higher expression in Alexis compared to the other genotypes (Figure 7 e Iv, S15). Na^+ sequestration in older leaves as a means of salt tolerance is well established, especially in salt tolerance halophytes [30]. Previous studies have raised the possibility that Na^+ sequestration in the vacuole is important for more salt tolerant barley genotypes such as Morex and K305, when compared to less salt tolerant genotypes such as Steptoe and genotype I743 [80, 99]. The functionality of *HvNHX4* and its role in salinity tolerance needs to be investigated further, along with the differences in the protein function between the alleles of Alexis, Commander and Maritime.

Inter-Genotype Transcript Co-expression Patterns in Saline Conditions

The co-expression modules for comprehensive analysis were selected based on their highest and lowest correlation to the measured physiological traits. Genes with differing expression profiles between genotypes were related to their putative functionality in an attempt to understand the differing mechanisms of salinity tolerance that might be present in the six genotypes of interest.

Halophytic species that are well adapted to salinity environments accumulate high levels of antioxidants such as polyphenols [100]. With this in mind we analysed the expression of genes involved in related metabolic pathways (S15). Genes relating to regulating secondary metabolism, particularly phenylpropanoid and flavonoid metabolism related genes were found to be present in all three tissues. phenylpropanoids and flavonoids have been reported to be oxidised by peroxidase and are involved in reactive oxygen species (ROS) scavenging phenolic/AsA/POD system [101]. The accumulation of Na^+ inside a cell leads to formation of ROS that damages the cell through plasma membrane lipid peroxidation and protein and DNA degradation [102, 103]. Synthesis of phenolic compounds such as phenylpropanoids and flavonoids in salt stressed plants is therefore, a likely mechanism employed by plants to remove ROS that are generated by excessive cytosolic Na^+ [104, 105].

We observed that terpenoid metabolism genes were mostly restricted to the leaf tissues. Terpenoids are the most abundant chemical in plants that are known to be involved in normal growth and development, as well as being involved in abiotic and biotic stress responses [106]. Halophytic mangroves have been found to produce high concentrations of terpenoids under salinity stress [107]. Contrary to our findings, a previous proteomic study on salinity stressed roots of two barley genotypes Steptoe and Morex, reported that the involvement of terpenoid biosynthesis proteins is part of the early response to salinity in the roots of these two genotypes [80]. However, there could be various reasons for this contradiction including differences in the timing of sampling (10 vs 18 days) and dissimilarities in the experimental conditions (final exposed salt concentrations of 150 mM vs 200 mM, proteomic study vs a transcriptomic study, etc). However, terpenoids were shown to be involved in other stress responses, such as in cotton plants under herbivore attack, and in roots of salt tolerant mangroves [107, 108].

Sheath navajowhite3 module was annotated as being involved in wax synthesis and cell wall organisation and biogenesis (S14) [109]. Eigen-gene from this module indicates that genes from navajowhite3 module tend to have higher expression in Sloop and Beecher. A gene coding for a *TRICHOME BIREFRINGENCE (TBR)* gene family is a key driver (i.e. hub gene) of the module that is also highly expressed in the sheath tissue of Sloop (S16). Although not conclusive, TBR family proteins have being suggested to be involved as "bridging proteins" that bind various polysaccharides in a cell wall [110]. The role of cell wall and extracellular changes in barley tolerance to salt stress is a key area for future research [111].

Epigenetic regulation of the gene expression and plants' ability to memorise the stress responses via histone modification has been shown previously [112–114]. In the current study, a sheath module that had positive association to sheath:blade Na^+ in Alexis, Commander and Maritime show GO enrichment related to epigenetic modifications. Interestingly, these three genotypes accumulate more Na^+ in leaf sheath compared to Beecher and Sloop (Figure 1). Could there be a mechanism in Alexis, Commander and Maritime to "train" the tissue to tolerate the salt stress [113]? This is a question that needs further validation.

Conclusion

Tissue specific gene expression variations in six barley genotypes varying in shoot Na^+ accumulation levels was assessed in this study. Our results indicate that allelic variations in *HvHKT1;5* may be a key factors in determining the level of Na^+ that accumulates in the shoots of barley. We hypothesise that in high shoot Na^+ accumulating genotypes such as Alexis, successful adaptation to excess Na^+ is likely to involve genes such as *HvNHXs* and that in particular, higher expression of *HvNHX4* may play a role in sequestrating Na^+ into the vacuole or K^+ homeostasis in Alexis.

We suggest that the expression of genes involved in terpenoid, phenylpropanoid and flavonoid metabolism in response to salt stress is of interest in relation to further understanding how barley tolerates the accumulation of Na^+. We also identify genes of interest in relation to cell wall modification and wax synthesis, particularly in the genotype Sloop, which are of interest in relation to future studies of mechanisms for tolerating salt stress. Enrichment of genes related to epigenetic modifications that may possibly aid plants to memorise the stress experience were evident in Alexis, Commander and Maritime, that accumulate more sheath Na^+ in the sheath than the other studied genotypes.

Acknowledgements

We thank the GRDC for funding this research through UA000145 to R.J., S.R., M.G., C.S.B., and J.Q., and through a Science and Innovation Award for C.S.B. This research was also supported by the Australian Research Council in the form of CE140100008 for M.G., C.S.B., and J.Q., and DE150100837 for C.S.B. We also thank Carol Blake (CSIRO Plant Industry) for help with plant sampling; Oliver Berkowitz and Cynthia Liu from the ARC Centre of Excellence in Plant Energy Biology, for their contribution to generating the RNAseq data; and Asmini Athman (University of Adelaide) for assistance with arranging RNA quality control analysis.

Tables and Figures

Table 1 Genotype Specific genes

Total number of genotype specific genes highly (H) and lowly (L) expressed in three tissues of Na^{+} stressed barley genotypes. The number of genes annotated as being putatively involved in a salt response are in parenthesis.

Genotype	Level of expression	Blade	Sheath	Root
Alexis	**H**	88 (37)	90 (21)	104 (32)
	L	107 (16)	106 (16)	108 (21)
Beecher	**H**	92 (25)	206 (60)	173 (43)
	L	140 (38)	207 (44)	218 (62)
Commander	**H**	23 (3)	38 (10)	15 (3)
	L	46 (18)	44 (15)	33 (9)
Fleet	**H**	37 (8)	53 (13)	71 (9)
	L	39 (10)	65 (13)	83 (21)
Maritime	**H**	81 (27)	107 (38)	76 (27)
	L	76 (26)	117 (34)	87 (16)
Sloop	**H**	9 (0)	159 (57)	56 (16)
	L	28 (4)	106 (25)	62 (11)

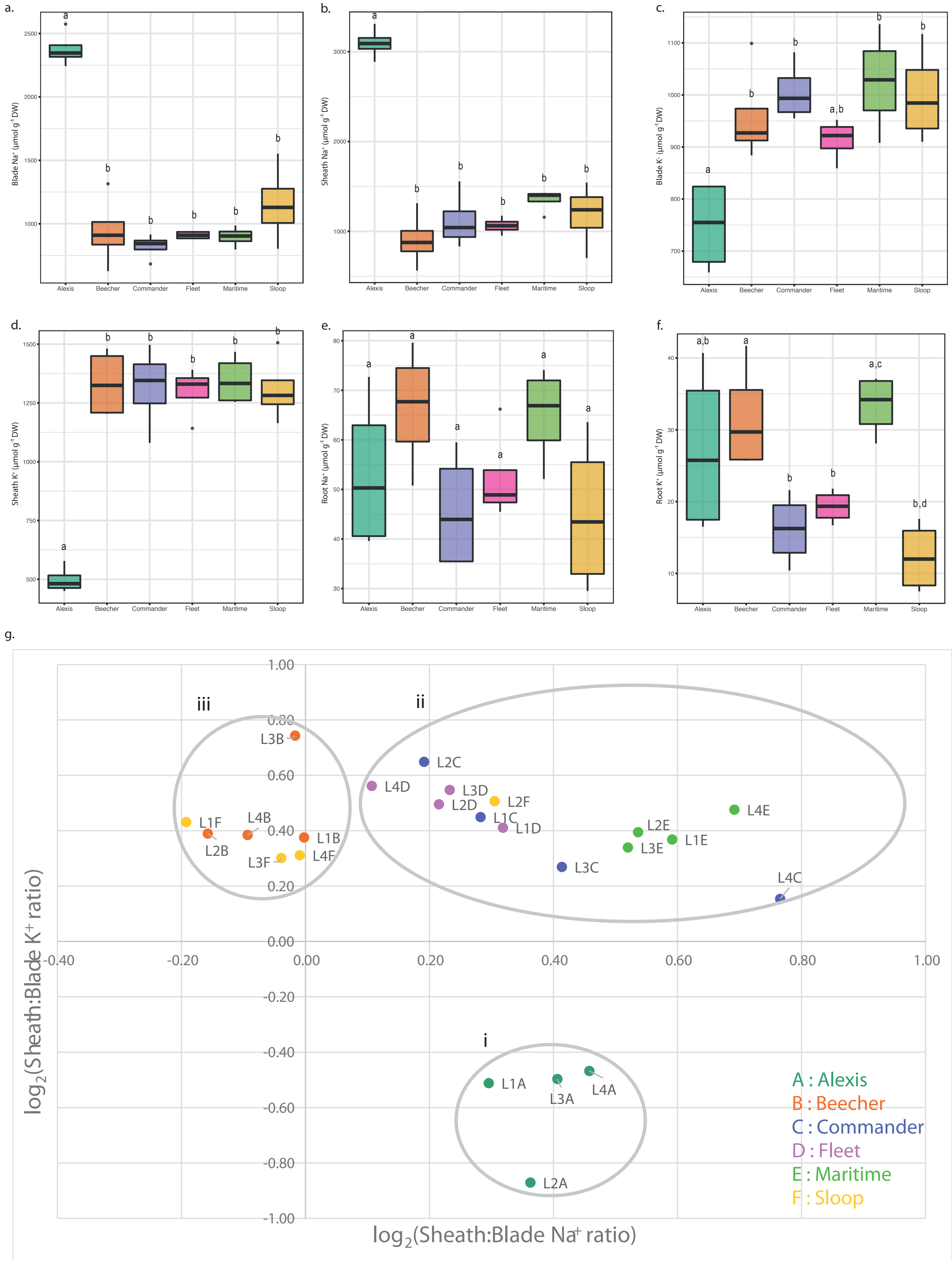

a.
Blade Na+ (µmol g-1 DW)
b.
Sheath Na+ (µmol g-1 DW)
c.
Blade K+ (µmol g-1 DW)
d.
Sheath K+ (µmol g-1 DW)
e.
Root Na+ (µmol g-1 DW)
f.
Root K+ (µmol g-1 DW)
Alexis
Beecher
Commander
Fleet
Maritime
Sloop
g.
iii
ii
i
L3B
L2C
L4D
L3D
L2D
L2F
L1C
L1D
L4E
L2E
L1E
L3E
L3C
L4C
L1F
L2B
L4B
L1B
L4F
L3F
L1A
L3A
L4A
L2A
log2(Sheath:Blade K+ ratio)
log2(Sheath:Blade Na+ ratio)
A : Alexis
B : Beecher
C : Commander
D : Fleet
E : Maritime
F : Sloop

Figure 1 Physiological traits' relationship to the genotypes

a. Na^+ concentration in leaf blade; b. Na^+ concentration in leaf sheath; c. K^+ concentration in blade; d. K^+ concentration in sheath; e. Na^+ concentration in root; f. K^+ concentration in root for the studied six genotypes. All ion measurements are in μmol/g^{-1} DW; mean values of genotypes which share the same letter are not significantly different ($p > 0.05$) from each other; g. log_2 ratio of sheath:blade Na^+ vs sheath:blade K^+ for the studied genotypes. The grey circles show the three distinct genotype clusters comprised of i. Alexis, ii. Beecher and Sloop, iii. Maritime, Commander and Fleet.


a.
HORVU7Hr1G085680 | ABC transporter ATP–binding protein NatA 2.08 0.12 −0.01 0.72 0.64 −0.51
HORVU5Hr1G022260 | ABC transporter B family member 4 1.34 −5.82 −5.55 −5.81 −5.56 −4.12
HORVU5Hr1G125010 | ABC transporter G family member 3 1.29 −0.19 −0.13 −0.15 −0.47 0.25
HORVU3Hr1G013630 | Folate–biopterin transporter 1.76 −1.04 −0.36 0.14 0.31 0.38
HORVU7Hr1G038300 | Transmembrane amino acid transporter family protein 3.69 1.95 1.68 2.2 2.37 2.12
HORVU5Hr1G114000 | nodulin MtN21 /EamA–like transporter family protein 1.31 −5.18 1.84 1.72 1.41 0.55
HORVU0Hr1G006740 | phosphate transporter 2 1.89 0.41 2.12 2.37 1.94 1.98
HORVU2Hr1G036590 | sugar transporter 9 2.6 −0.04 1.73 1.44 1.72 1.36
HORVU4Hr1G067450 | sugar transporter protein 7 1.75 0.18 2.14 1.74 3.04 1.7
HORVU7Hr1G003880 | Vacuolar iron transporter (VIT) family protein 0.1 −5.82 0.65 −0.37 0.54 −0.28
HORVU6Hr1G084900 | EamA–like transporter family protein 0.61 4.09 1.68 0.48 1.55 0.86
HORVU5Hr1G053550 | Transmembrane amino acid transporter family protein −1.71 0.33 −1.87 −1.1 −1.25 −0.99
HORVU1Hr1G047100 | Aquaporin–like superfamily protein 0.5 2.78 1.37 0.57 0.51 1.51
HORVU4Hr1G079230 | Aquaporin–like superfamily protein 3.2 4.89 2.26 2.89 3.2 3.22
HORVU7Hr1G008600 | K(+)/H(+) antiporter 1 −5.49 2.87 −5.55 −5.77 −5.87 −5.56
HORVU2Hr1G127500 | Magnesium transporter NIPA2 2.37 1.51 0.45 2.08 2.68 1.65
HORVU2Hr1G019010 | ABC transporter G family member 15 0.54 1.93 1.03 −0.03 −2.23 0.3
HORVU6Hr1G001480 | Transmembrane amino acid transporter family protein 3.33 3.32 3.45 3.73 1.32 2.85
HORVU5Hr1G117080 | phosphate transporter 1;4 −0.55 −3.16 −1.37 −3.14 1.44 −2.71
HORVU2Hr1G094110 | Mechanosensitive ion channel protein 1.56 1.76 1.71 1.42 1.24 2.79
Alexis Beecher Commander Fleet Maritime Sloop
b.
HORVU5Hr1G022260 | ABC transporter B family member 4 2.93 −3.25 −2.27 −2.44 −2.97 −3.68
HORVU4Hr1G081330 | Boron transporter 4 5.72 2.25 2.81 2.76 3.37 1.89
HORVU5Hr1G093090 | cationic amino acid transporter 5 2.19 0.94 0.95 1.13 0.64 0.95
HORVU3Hr1G018700 | sugar transporter 1 0.99 −2.01 −1.52 −0.2 −0.83 −1.2
HORVU3Hr1G018740 | sugar transporter 1 1.97 −0.35 0.11 0.48 0.39 −0.07
HORVU7Hr1G056530 | ABC transporter G family member 45 1.17 −0.45 1.9 2 2.4 1.58
HORVU0Hr1G016830 | sugar transporter 6 0.7 −4.26 0.05 0.94 1.04 −0.17
HORVU7Hr1G003880 | Vacuolar iron transporter (VIT) family protein 3.59 −3.6 3.8 2.93 3.7 3.79
HORVU6Hr1G084900 | EamA–like transporter family protein 3.48 6.94 3.25 2.75 3.09 3.28
HORVU3Hr1G082870 | Magnesium transporter MRS2–E 0.75 2.58 0.78 1.13 0.6 1.04
HORVU1Hr1G065250 | Potassium channel AKT2 −0.26 1.81 −0.01 0.2 0.55 0.63
HORVU3Hr1G055740 | S–type anion channel SLAH2 3.02 4.04 2.97 2.86 3.06 2.73
HORVU3Hr1G068140 | sulfate transporter 3;1 2.05 3.58 2.16 1.15 1.72 1.19
HORVU3Hr1G055450 | Transmembrane amino acid transporter family protein 2.39 3.73 2.35 2.45 2.48 2.65
HORVU7Hr1G008600 | K(+)/H(+) antiporter 1 −4.74 1.45 −4.03 −5.64 −2.12 −4
HORVU6Hr1G000440 | Bidirectional sugar transporter N3 2.59 2.31 2.49 3.21 0.55 2.57
HORVU5Hr1G077680 | High affinity cationic amino acid transporter 1 2.27 2 1.37 2.39 −0.88 1
HORVU6Hr1G005570 | high affinity nitrate transporter 2.6 4.25 4.91 4.21 4.96 1.84 4.88
HORVU7Hr1G025110 | ABC transporter C family member 10 1.88 −2.21 1.1 −0.69 4.61 2.25
HORVU6Hr1G071960 | Nucleotide/sugar transporter family protein 0.21 0.08 −0.49 −2.05 0.18 −0.29
HORVU3Hr1G061190 | Organic cation/carnitine transporter 4 0.34 0.61 0.38 −4.43 −0.36 0.24
HORVU6Hr1G084290 | sugar transporter protein 7 4.59 3.33 4.74 1.66 4.64 4.68
HORVU3Hr1G013170 | Bidirectional sugar transporter N3 1.26 0.12 0.85 2.88 0.15 0.5
HORVU3Hr1G104090 | sulfate transporter 3;2 0.74 1.61 −3.39 2.74 −4.34 −5.66
HORVU2Hr1G061870 | ABC transporter B family member 23, mitochondrial 2.67 2.42 2.64 2.44 2.81 0.35
HORVU7Hr1G088600 | ABC transporter G family member 42 2.89 3.71 2.9 2.45 2.72 0.13
Alexis Beecher Commander Fleet Maritime Sloop
c.
HORVU5Hr1G022260 | ABC transporter B family member 4 2.71 −5.39 −5.36 −5.72 −4.24 −5.49
HORVU7Hr1G045290 | aluminum–activated malate transporter 9 2.93 0.25 0.81 0.55 0.48 1.29
HORVU4Hr1G019930 | Auxin transporter–like protein 3 1.49 −1.97 −1.3 −1.53 −0.54 −1.83
HORVU7Hr1G086180 | Mechanosensitive ion channel protein 1.55 0.44 0.47 −0.14 −0.41 0.52
HORVU2Hr1G021020 | sodium hydrogen exchanger 2 4.63 2.07 2.91 3.46 3.48 3.05
HORVU7Hr1G056530 | ABC transporter G family member 45 1.25 0.18 1.93 1.53 2.09 1.42
HORVU3Hr1G023220 | chloride channel C 4.45 3.31 4.58 4.67 4.5 4.55
HORVU6Hr1G079040 | cyclic nucleotide gated channel 19 4.11 3.05 4.19 4.19 4.42 4.13
HORVU3Hr1G002710 | nodulin MtN21 /EamA–like transporter family protein 5.26 3.81 4.81 4.94 5.78 5
HORVU5Hr1G114000 | nodulin MtN21 /EamA–like transporter family protein 1.71 −6.22 2.03 2.18 2.46 1.05
HORVU0Hr1G006740 | phosphate transporter 2 3.06 1.86 3.19 3.25 3.41 2.99
HORVU7Hr1G003880 | Vacuolar iron transporter (VIT) family protein 4.46 −4.98 3.52 2.33 2.48 2.05
HORVU7Hr1G097270 | Zinc transporter 7 3.18 0.27 2.95 2.62 2.2 2.32
HORVU6Hr1G084900 | EamA–like transporter family protein 3.12 7.67 4.02 3.32 3.79 4.22
HORVU2Hr1G121200 | nodulin MtN21 /EamA–like transporter family protein −0.35 0.99 −0.98 −1.45 −0.87 −1.11
HORVU3Hr1G045630 | Transmembrane amino acid transporter family protein 0.25 2.63 1.44 1.21 1.19 0.7
HORVU3Hr1G088360 | Transmembrane amino acid transporter family protein 0.58 2.84 1.55 1.6 1.48 1.12
HORVU5Hr1G053550 | Transmembrane amino acid transporter family protein 0.07 2.48 −0.03 0.28 0.42 0.21
HORVU4Hr1G085820 | UDP–galactose transporter 2 2.6 4.33 3.06 2.27 1.13 2.98
HORVU2Hr1G066920 | Voltage–gated potassium channel subunit beta–3 6.71 7.98 6.39 6.65 6.71 6.08
HORVU1Hr1G047100 | Aquaporin–like superfamily protein 0.84 3.88 2.08 1.66 1.1 2.2
HORVU7Hr1G008600 | K(+)/H(+) antiporter 1 −5.45 3.77 −4.85 −4.58 −5.71 −6.22
HORVU7Hr1G040970 | Potassium channel SKOR 0.21 −1.67 −3.2 −1.63 −1.56 −0.46
HORVU2Hr1G019010 | ABC transporter G family member 15 4.38 6.03 5.48 4.97 2.85 5.01
HORVU2Hr1G018920 | ABC transporter G family member 15 3.06 4.83 4.22 3.7 1.59 3.76
HORVU6Hr1G064740 | Transmembrane amino acid transporter family protein 4.71 4.4 5.15 4.48 3.22 5.35
HORVU6Hr1G001480 | Transmembrane amino acid transporter family protein 5.02 5.94 5.92 5.54 3.22 5.46
HORVU2Hr1G108340 | Organic cation/carnitine transporter 4 −0.42 −1.61 −1.18 −0.85 0.69 −1.81
HORVU2Hr1G117120 | EamA–like transporter family protein 1.82 1.09 1.76 −0.37 0.63 0.96
HORVU6Hr1G083720 | Transmembrane amino acid transporter family protein 5.15 4.99 5.16 3.9 5.42 5.3
HORVU7Hr1G085680 | ABC transporter ATP–binding protein NatA 0.85 −0.06 0.52 0.48 1.23 −1.47
HORVU2Hr1G061870 | ABC transporter B family member 23, mitochondrial 2.74 2.61 2.83 2.61 2.96 −1.04
HORVU5Hr1G110180 | phosphate transporter 1;7 6.75 5.57 6.13 5.45 6.71 4.29
HORVU5Hr1G110220 | phosphate transporter 1;7 6.24 5.1 5.48 4.83 6.19 3.79
HORVU2Hr1G064160 | sugar transporter 1 0.29 0.49 0.61 0.46 1.28 −1.18
HORVU3Hr1G031680 | Aquaporin–like superfamily protein −1.42 −1.87 −1.09 −1.19 −1.42 0.68
HORVU1Hr1G070460 | Zinc transporter 5 0.18 0.66 0.5 0.78 −0.02 1.79
Alexis Beecher Commander Fleet Maritime Sloop

Figure 2 Expression heat map of the expressed genotype specific transporter genes

The expression values are measured in CPM (counts per million). The mean values of the four independent replicates are shown per genotype (X axis). The mean log expression values for a gene per genotype is shown in each tile. The transporter genes are along the Y axis. The HORVU id and the common name retrieved from the IBSC2016 annotations are included as identifiers of the genes. The colour gradient is across each gene (row). The highest expression value per gene is coloured khaki and the lowest is coloured steel blue, colour white denotes no expression. The genes are assigned to tissues a. blade, b. sheath and c. root. The mean expression pattern of these genes across all tissues are in S7.

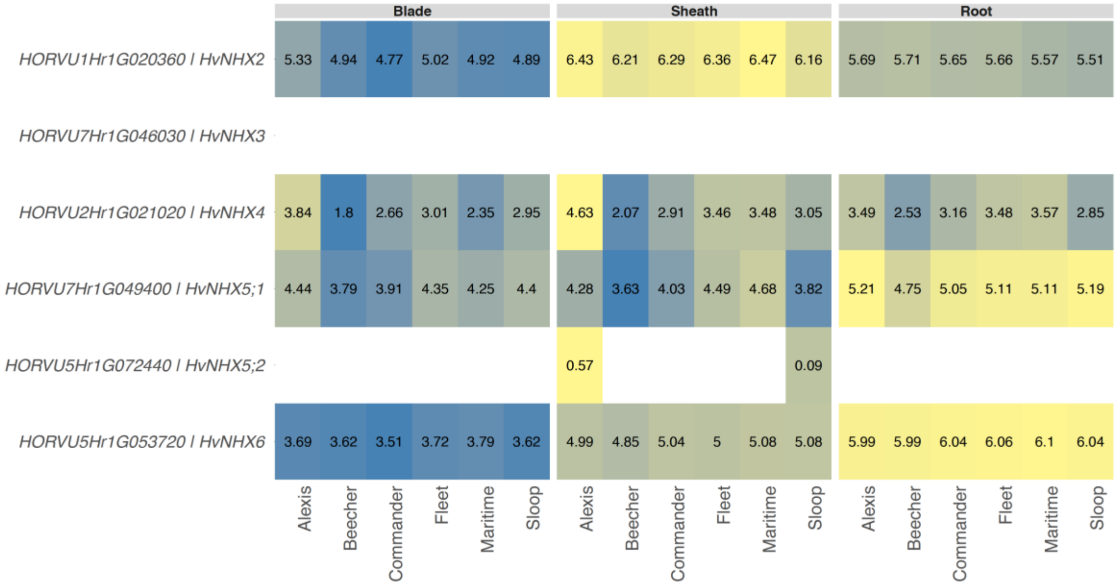


Figure 3 Expression heatmaps of identified *HvNHX* genes

The expression values are plotted as heatmaps. The genes are assogned to tissues blade, sheath and root (top). The mean expression values of the four independent replicates are shown per genotype (columns). Each row represent the expression values for a particular transporter gene. If a particular gene is not expressed in a tissue, the row is coloured white. The HORVU id and the common name retrieved from the IBSC2016 annotations are included as identifiers of the genes. The log mean expression values for a gene per genotype is shown in each tile (counts per million, CPM). The colour gradient is across each gene (row). The highest expression value per gene is coloured khaki and the lowest is coloured steel blue, colour white denotes no expression.

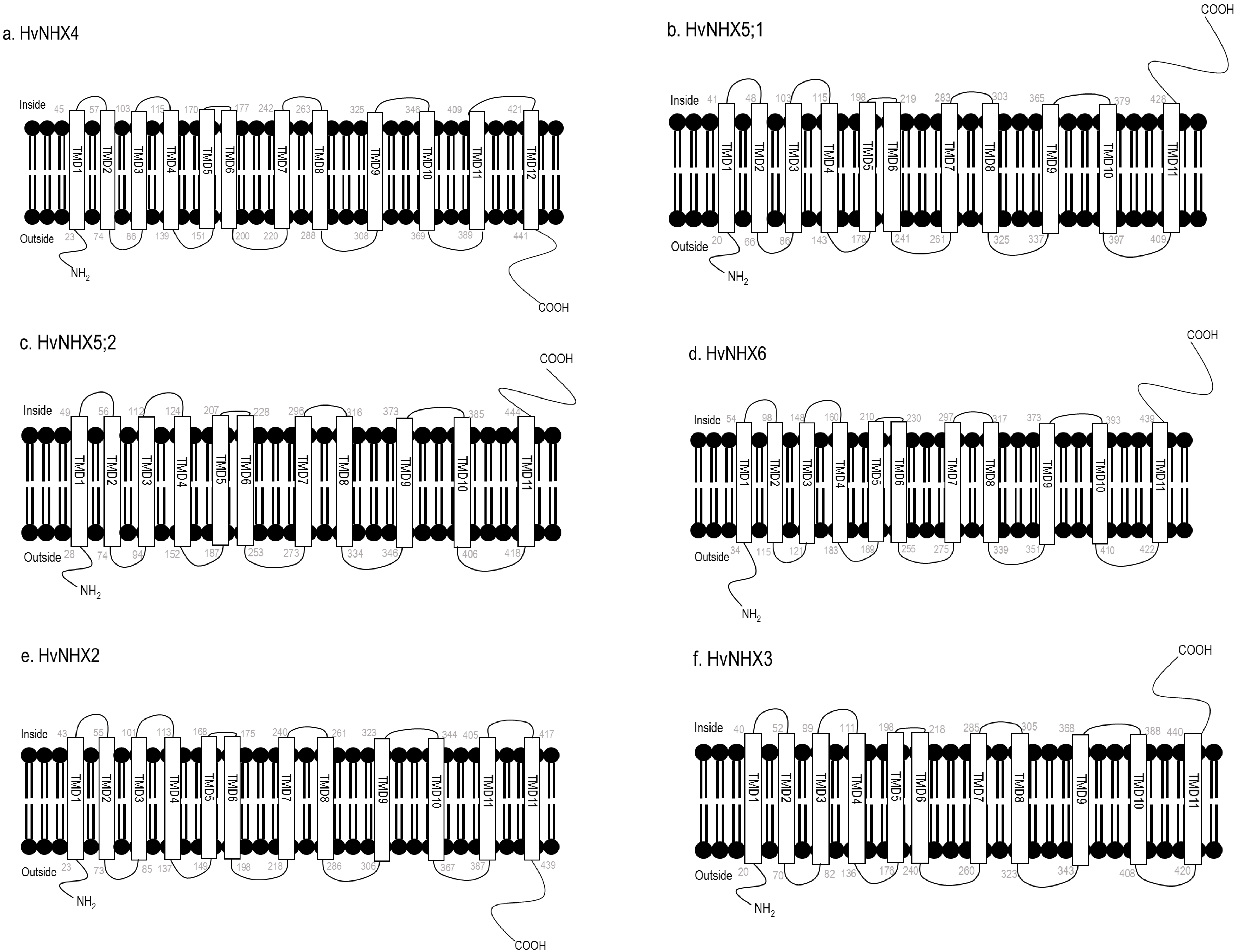

a. HvNHX4
b. HvNHX5;1
c. HvNHX5;2
d. HvNHX6
e. HvNHX2
f. HvNHX3
Inside
Outside
NH_2
COOH

Figure 4 Schematic drawing depicting transmembrane domains of HvNHXs and locations of amino acid substitutions resulted through identified non-synonymous Single Nucleotide Polymorphisms (SNPs)

The predicted domains for the HvNHX proteins are based on InterPro predictions. a-i the hypothetical models of the HvNHXs. The red numbered stars on the structures indicate the location of the allelic variations caused by the non-synonymous SNPs relative to the reference Morex sequence (IBSC 2016).The allelic variation corresponding to each numbered star is at the bottom of each figure. The genotype/s with the variation is/are shown below each allelic variation. TMD: Transmebrane domain

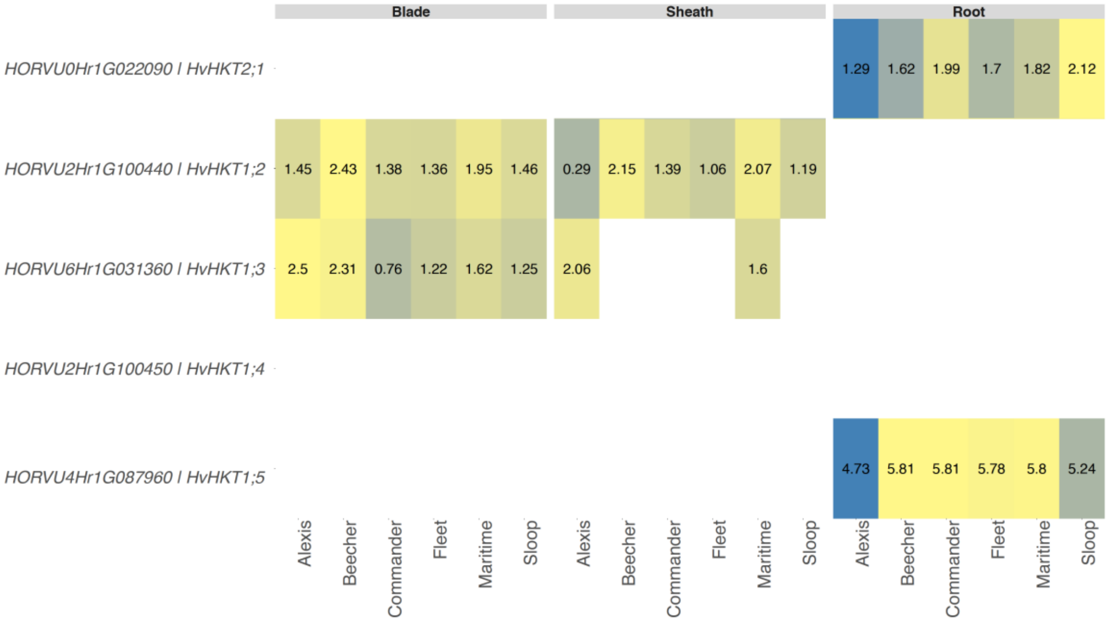
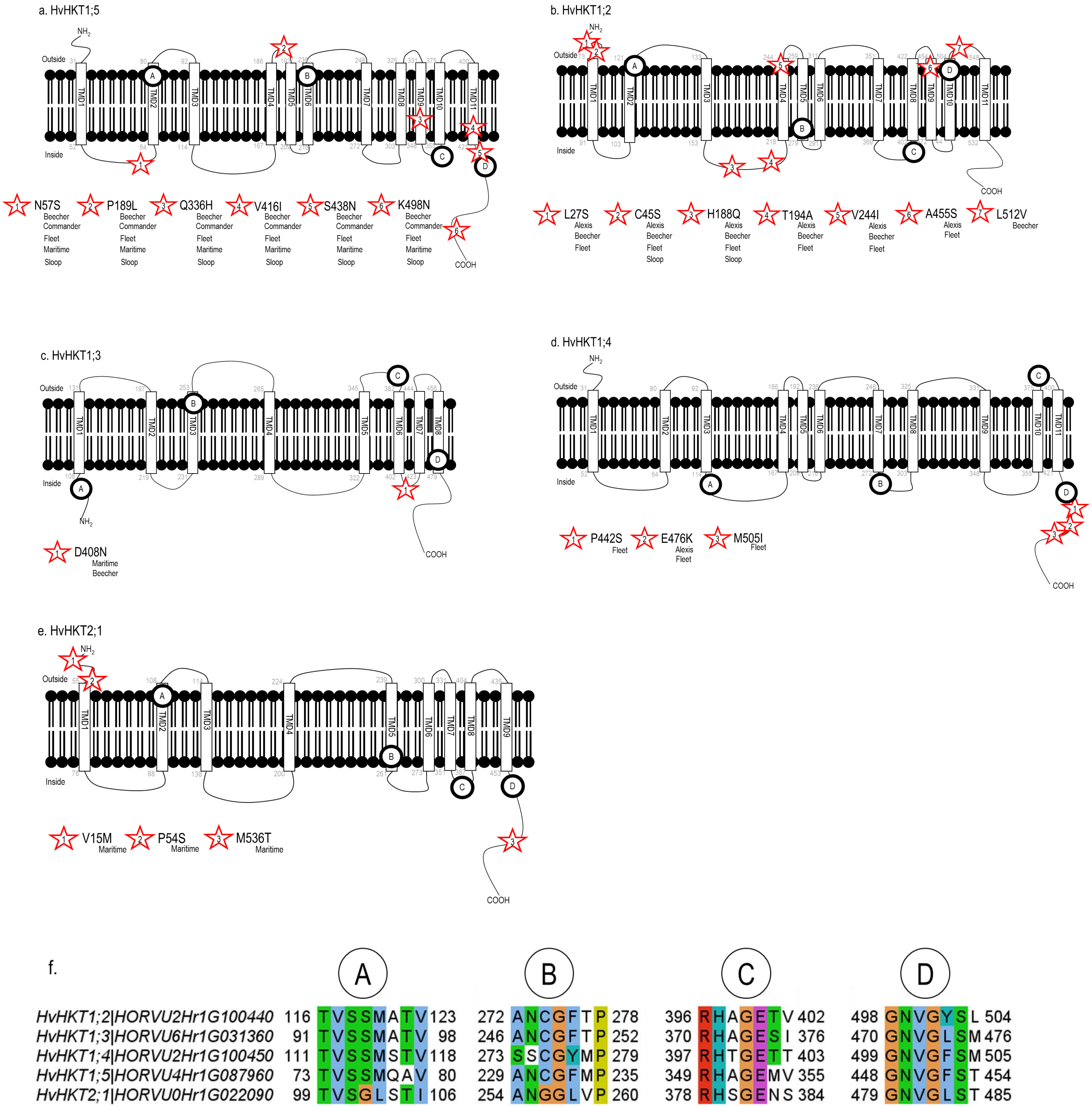


Figure 5 Expression heatmaps of identified *HvHKT* genes

The expression values are plotted as heatmaps. The genes are assigned to tissues blade, sheath and root (top). The mean expression values of the four independent replicates are shown per genotype (columns). Each row represent the expression values for a particular transporter gene. If a particular gene is not expressed in a tissue, the row is coloured white. The HORVU id and the common name retrieved from the IBSC2016 annotations are included as identifiers of the genes. The log mean expression values for a gene per genotype is shown in each tile (counts per million, CPM). The colour gradient is across each gene (row). The highest expression value per gene is coloured khaki and the lowest is coloured steel blue, colour white denotes no expression.



a. HvHKT1;5
N57S Beecher Commander Fleet Maritime Sloop
P189L Beecher Commander Fleet Maritime Sloop
Q336H Beecher Commander Fleet Maritime Sloop
V416I Beecher Commander Fleet Maritime Sloop
S438N Beecher Commander Fleet Maritime Sloop
K498N Beecher Commander Fleet Maritime Sloop
b. HvHKT1;2
L27S Alexis Beecher Fleet
C45S Alexis Beecher Fleet Sloop
H188Q Alexis Beecher Fleet Sloop
T194A Alexis Beecher Fleet
V244I Alexis Beecher Fleet
A455S Alexis Fleet
L512V Beecher
c. HvHKT1;3
D408N Maritime Beecher
d. HvHKT1;4
P442S Fleet
E476K Alexis Fleet
M505I Fleet
e. HvHKT2;1
V15M Maritime
P54S Maritime
M536T Maritime
Outside
Inside
NH2
COOH
f.
A
B
C
D
HvHKT1;2|HORVU2Hr1G100440 116 TVSSMATV 123 272 ANCGFTP 278 396 RHAGETV 402 498 GNVGYSL 504
HvHKT1;3|HORVU6Hr1G031360 91 TVSSMATV 98 246 ANCGFTP 252 370 RHAGESI 376 470 GNVGLSM 476
HvHKT1;4|HORVU2Hr1G100450 111 TVSSMSTV 118 273 SSCGYMP 279 397 RHTGETT 403 499 GNVGFSM 505
HvHKT1;5|HORVU4Hr1G087960 73 TVSSMQAV 80 229 ANCGFMP 235 349 RHAGEMV 355 448 GNVGFST 454
HvHKT2;1|HORVU0Hr1G022090 99 TVSGLSTI 106 254 ANGGLVP 260 378 RHSGENS 384 479 GNVGLST 485

Figure 6 Schematic drawing depicting transmembrane domains of HvHKTs and the locations of amino acid substitutions resulted through non-synonymous Single Nucleotide Polymorphisms (SNPs)

The predicted domains for the HvHKT proteins are based on InterPro predictions. a-e the hypothetical models of the HvHKTs with full sequence. The red numbered stars on the structures indicate the location of the allelic variations caused by the non-synonymous SNPs relative to the reference Morex sequence (IBSC 2016).The allelic variation corresponding to each numbered star is at the bottom of each figure. The genotype/s with the variation is/are shown below each allelic variation. f. The multiple sequence alignment (MUSCLE with default settings) of barley HvHKT pore region amino acid sequences to known HKTs. Corresponding sequence regions that are likely to be in pore forming areas are denoted by circles with A, B C and D. The residues are coloured based on CLUSTAL color scheme. TMD: Transmembrane domain.


a
b
c
d
e
f
Blade darkolivegreen (418)
Blade coral4 (418)
Blade darkorange2 (978)
Blade orangered1 (418)
Blade yellow2 (134)
Blade darkgrey (704)
Blade pink4 (158)
Sheath firebrick2 (2404)
Sheath navajowhite3 (2263)
Sheath firebrick (142)
Sheath yellow4 (87)
Root darkgoldenrod4 (515)
Root paleturquoise4 (37)
Root blueviolet (600)
Root coral4 (308)

Figure 7 Relationship between co-expression modules of interest and ion ratios, and their Eigen-gene expression profiles

The modules were selected based on their correlation to the selected traits. Modules that had highest positive and negative correlation to the ion ratios were selected as "interesting" modules. Module-trait relationship are shown for the selected modules in the a. blade b. sheath and c. root tissues. Traits (ion ratios) are as follows; SB_Na: sheath:blade ratio of Na^+, SB_K: sheath:blade ratio of K^+, blade_K_Na: K^+:Na^+ ratio of the blade, sheath_K_Na: K^+:Na^+ ratio of the sheath. The scatterplot matrices have a diagonal histogram of all the variables. The correlation coefficients are in the upper part of the matrix and a loess curves for each plot in the scatterplot matrix are shown in the lower part of the matrix. Red stars denote the level of significance; 0.05(.), 0.01(*), 0.001(**), 0(***). The Eigen-gene patterns of the selected modules for each tissue are beneath each tissue scatterplot d(i-vii) : blade modules, e(i-iv): sheath modules, f(i-iv): root modules. The top row shows the heat map of the genes in the module and the bar graph below shows the Eigen-gene pattern of the module (y axis; Eigen-gene expression, x axis; genotype).

Supplementary Material

(Available at https://doi.org/10.4225/55/5aa116ab810bd)

S1: Shell script used for length filtering of the FastQ data

S2: Mapping statistics

S3: Physiological data measured in the present study

S4: Information on the identified genotype specific genes and salt responsive genotype specific genes.

S5. Results of Kyoto Encyclopedia of Genes and Genomes (KEGG) information mining for the Genotype specific genes (gsGs) in each tissue using the KAAS portal option (http://www.kegg.jp/)

S6: FASTA sequences of the 609 barley genes identified as salt responsive

The genes were identified through a reciprocal BLAST salt related Arabidopsis sequences. The Arabidopsis sequences were mined using a keyword search in TAIR (www.tair.org/). The sequences of the Arabidopsis genes were used for reciprocal BLAST on the barley genome (refer to materials and methods).

S7: Expression heat map of the all expressed genotype specific transporter genes

The expression values are measured in CPM (counts per million). The mean values of the four independent replicates are shown per genotype (x axis). The mean log expression values for a gene per genotype is shown in each tile. The transporter genes are along the y axis. The HORVU id and the common name retrieved from the IBSC2016 annotations are included as identifiers of the genes. The homologs from Arabidopsis for each transporter is shown within brackets. The colour gradient is across each gene (row). The highest expression value per gene is coloured khaki and the lowest is coloured steel blue. The genes are separated to the tissues blade, sheath and root. In instances where the gene was not expressed, it is shown as a white space.

S8: Barley NHXs (HvNHXs) identified in this study

S9: Phylogenetic tree showing the relationship between the protein sequences of identified HvNHXs and NHXs previously identified.

The amino acid sequences of identified NHXs and previously known NHXs were used in this analysis. The evolutionary history was inferred by using the Maximum Likelihood method based on the Whelan And Goldman model [113]. The tree with the highest log likelihood (-15347.7036) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using a JTT model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.3046)). The tree is drawn to scale, with branch lengths me101asured in the number of substitutions per site. The analysis involved 17 amino acid sequences. There were a total of 1313 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [62].

S10: Identified single nucleotide polymorphisms (SNPs) on HvNHXs and their locations on the amino acid sequence

S11: Barley HKTs (HvHKTs) identified in this study

S12: Phylogenetic tree showing the relationship between the protein sequences of identified HvHKTs and previously identified plant HKTs.

Identified and previously known HKT amino acid sequences were used in this analysis. The evolutionary history was inferred by using the Maximum Likelihood method based on the Whelan And Goldman model [113]. The tree with the highest log likelihood (-23564.9681) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using a JTT model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.6315)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 32 amino acid sequences. There were a total of 1268 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [62].

S13: Identified single nucleotide polymorphisms (SNPs) on HvHKTs and their locations on the amino acid sequence, genomic sequence and the CDS sequence.

S14: Results of Kyoto Encyclopedia of Genes and Genomes (KEGG) information mining for the selected modules (gene clusters) of each tissue through the weighted gene-co-expression analysis (WGCNA) in each tissue using the KAAS portal option (http://www.kegg.jp/).

S15: Hub genes of the selected modules of weighted gene co-expression analysis (WGCNA).

S16 MapMan allocated secondary metabolism categories for WGCNA modules

a- g: blade modules; a. darkolivegreen, b. coral4, c. darkorange2, d. orangered1, e. yellow2, f. darkgrey, g. pink4. h-k: sheath modules; h: firebrick2, i: navajowhite3, j: firebrick, k: yellow4. l-o: root modules; l: darkgoldenrod4, m: paleturquoise4, n: blueviolet, o: coral4.

References

1. Rengasamy P. World salinization with emphasis on Australia. J Exp Bot. 2006;57:1017–23.

2. FAOSTAT. Food and Agriculture Organization fo the United Nations. Food and Agriculture Organization fo the United Nations. 2014. http://www.fao.org/economic/ess/en/#.U1XSWvmSymw. Accessed 22 Apr 2014.

3. Rozema J, Flowers T. Crops for a Salinized World. Science. 2008;322:1478–80.

4. Rengasamy P. Soil processes affecting crop production in salt-affected soils. Funct Plant Biol. 2010;37:613–20.

5. Adem GD, Roy SJ, Zhou M, Bowman JP, Shabala S. Evaluating contribution of ionic, osmotic and oxidative stress components towards salinity tolerance in barley. BMC Plant Biol. 2014;14:113.

6. Tester M, Langridge P. Breeding Technologies to Increase Crop Production in a Changing World. Science. 2010;327:818–22.

7. Roy SJ, Negrão S, Tester M. Salt resistant crop plants. Curr Opin Biotechnol. 2014;26:115–24.

8. Munns R, Tester M. Mechanisms of Salinity Tolerance. Annu Rev Plant Biol. 2008;59:651–81.

9. Yeo AR, Lee λ-S, Izard P, Boursier PJ, Flowers TJ. Short- and Long-Term Effects of Salinity on Leaf Growth in Rice (Oryza sativa L.). J Exp Bot. 1991;42:881–9.

10. Passioura JB, Munns R. Rapid environmental changes that affect leaf water status induce transient surges or pauses in leaf expansion rate. 2000. https://publications.csiro.au/rpr/pub?list=BRO&pid=procite:0f3c6345-aa19-4ef9-aeba-e170939ba5c7. Accessed 21 Nov 2017.

11. Colmer TD, Munns R, Flowers TJ. Improving salt tolerance of wheat and barley: future prospects. Aust J Exp Agric. 2005;45:1425–43.

12. Munns R, Gilliham M. Salinity tolerance of crops – what is the cost? New Phytol. 2015;208:668–73.

13. Rahnama A, Munns R, Poustini K, Watt M. A screening method to identify genetic variation in root growth response to a salinity gradient. J Exp Bot. 2011;62:69–77.

14. Wyn Jones RG, Brady CJ, Speirs J. Ionic and osmotic relations in plant cells. London: Academic Press; 1979. http://trove.nla.gov.au/version/28372474.

15. Bhandal IS, Malik CP. Potassium Estimation, Uptake, and Its Role in the Physiology and Metabolism of Flowering Plants. In: Bourne GH, Jeon KW, Friedlander M, editors. International Review of Cytology. Academic Press; 1988. p. 205–54. doi:10.1016/S0074-7696(08)61851-3.

16. Blaha G, Stelzl U, Spahn CMT, Agrawal RK, Frank J, Nierhaus KH. [19] Preparation of functional ribosomal complexes and effect of buffer conditions on tRNA positions observed by cryoelectron microscopy. In: Methods in Enzymology. Academic Press; 2000. p. 292–309. doi:10.1016/S0076-6879(00)17021-1.

17. Jacoby RP, Che-Othman MH, Millar AH, Taylor NL. Analysis of the sodium chloride-dependent respiratory kinetics of wheat mitochondria reveals differential effects on phosphorylating and non-phosphorylating electron transport pathways. Plant Cell Environ. 2016;39:823–33.

18. Shabala S, Wu H, Bose J. Salt stress sensing and early signalling events in plant roots: Current knowledge and hypothesis. Plant Sci. 2015;241:109–19.

19. Mittler R, Vanderauwera S, Suzuki N, Miller G, Tognetti VB, Vandepoele K, et al. ROS signaling: the new wave? Trends Plant Sci. 2011;16:300–9.

20. Gilroy S, Suzuki N, Miller G, Choi W-G, Toyota M, Devireddy AR, et al. A tidal wave of signals: calcium and ROS at the forefront of rapid systemic signaling. Trends Plant Sci. 2014;19:623–30.

21. Kudla J, Batistič O, Hashimoto K. Calcium Signals: The Lead Currency of Plant Information Processing. Plant Cell Online. 2010;22:541–63.

22. Choi W-G, Toyota M, Kim S-H, Hilleary R, Gilroy S. Salt stress-induced Ca2+ waves are associated with rapid, long-distance root-to-shoot signaling in plants. Proc Natl Acad Sci. 2014;111:6497–502.

23. Munns R, Termaat A. Whole-Plant Responses to Salinity. Funct Plant Biol. 1986;13:143–60.

24. Rajendran K, Tester M, Roy SJ. Quantifying the three main components of salinity tolerance in cereals. Plant Cell Environ. 2009;32:237–49.

25. Plett D, Møller IS. Na+ transport in glycophytic plants: what we know and would like to know. Plant Cell Environ. 2010;33:612–26.

26. Munns R. Approaches to increasing the salt tolerance of wheat and other cereals. J Exp Bot. 2006;57:1025–43.

27. Teakle NL, Tyerman SD. Mechanisms of Cl^- transport contributing to salt tolerance. Plant Cell Environ. 2010;33:566–89.

28. Tester M, Davenport R. Na+ Tolerance and Na+ Transport in Higher Plants. Ann Bot. 2003;91:503–27.

29. Shabala S. Learning from halophytes: physiological basis and strategies to improve abiotic stress tolerance in crops. Ann Bot. 2013;:mct205.

30. Flowers TJ, Colmer TD. Salinity tolerance in halophytes*. New Phytol. 2008;179:945–63.

31. Shabala S, Pottosin I. Regulation of potassium transport in plants under hostile conditions: implications for abiotic and biotic stress tolerance. Physiol Plant. 2014;151:257–79.

32. Chen Z, Newman I, Zhou M, Mendham N, Zhang G, Shabala S. Screening plants for salt tolerance by measuring K+ flux: a case study for barley. Plant Cell Environ. 2005;28:1230–46.

33. Chen Z, Zhou M, Newman IA, Mendham NJ, Zhang G, Shabala S. Potassium and sodium relations in salinised barley tissues as a basis of differential salt tolerance. Funct Plant Biol. 2007;34:150–62.

34. Chen Z, Shabala S, Mendham N, Newman I, Zhang G, Zhou M. Combining Ability of Salinity Tolerance on the Basis of NaCl-Induced K Flux from Roots of Barley. Crop Sci. 2008;48:1382–8.

35. Bohnert HJ, Nelson DE, Jensen RG. Adaptations to Environmental Stresses. Plant Cell. 1995;7:1099–111.

36. Carillo P, Grazia M, Pontecorvo G, Fuggi A, Woodrow P. Salinity Stress and Salt Tolerance. In: Shanker A, editor. Abiotic Stress in Plants - Mechanisms and Adaptations. InTech; 2011. http://www.intechopen.com/books/abiotic-stress-in-plants-mechanisms-and-adaptations/salinity-stress-and-salt-tolerance. Accessed 28 Jul 2014.

37. E.y M, G.j H. CROP SALT TOLERANCE CURRENT ASSESSMENT. 1977;103:115–34.

38. Greenway H, Munns R. Mechanisms of Salt Tolerance in Nonhalophytes. Annu Rev Plant Physiol. 1980;31:149–90.

39. Ayers RS, Westcot DW. Water quality for agriculture. Rome: Food and Agriculture Organization; 1985.

40. Munns R, James RA, Gilliham M, Flowers TJ, Colmer TD. Tissue tolerance: an essential but elusive trait for salt-tolerant crops. Funct Plant Biol. 2016;43:1103–13.

41. Widodo, Patterson JH, Newbigin E, Tester M, Bacic A, Roessner U. Metabolic responses to salt stress of barley (Hordeum vulgare L.) cultivars, Sahara and Clipper, which differ in salinity tolerance. J Exp Bot. 2009;60:4089–103.

42. Velarde-Buendía AM, Shabala S, Cvikrova M, Dobrovinskaya O, Pottosin I. Salt-sensitive and salt-tolerant barley varieties differ in the extent of potentiation of the ROS-induced K+ efflux by polyamines. Plant Physiol Biochem. 2012;61:18–23.

43. Rasoulnia A, Bihamta MR, Peyghambari SA, Alizadeh H, Rahnama A. Proteomic response of barley leaves to salinity. Mol Biol Rep. 2011;38:5055–63.

44. Zhu M, Zhou M, Shabala L, Shabala S. Linking osmotic adjustment and stomatal characteristics with salinity stress tolerance in contrasting barley accessions. Funct Plant Biol. 2014. doi:10.1071/FP14209.

45. Zhu M, Zhou M, Shabala L, Shabala S. Physiological and molecular mechanisms mediating xylem Na+ loading in barley in the context of salinity stress tolerance. Plant Cell Environ. 2017;40:1009–20.

46. Huang S, Spielmeyer W, Lagudah ES, James RA, Platten JD, Dennis ES, et al. A Sodium Transporter (HKT7) Is a Candidate for Nax1, a Gene for Salt Tolerance in Durum Wheat. Plant Physiol. 2006;142:1718–27.

47. James RA, Caemmerer S von, Condon AG (Tony), Zwart AB, Munns R. Genetic variation in tolerance to the osmotic stress componentof salinity stress in durum wheat. Funct Plant Biol. 2008;35:111–23.

48. Byrt CS, Platten JD, Spielmeyer W, James RA, Lagudah ES, Dennis ES, et al. HKT1;5-Like Cation Transporters Linked to Na+ Exclusion Loci in Wheat, Nax2 and Kna1. Plant Physiol. 2007;143:1918–28.

49. Andrews S. FastQC: a quality control tool for high throughput sequence data. 2010. http://www.bioinformatics.babraham.ac.uk/projects/fastqc. Accessed 7 Jan 2015.

50. Gordon A, Hannon GJ. Fastx-toolkit. FASTQ/A short-reads pre-processing tools (unpublished). 2010. http://hannonlab. cshl. edu/fastx_toolkit.

51. Beier S, Himmelbach A, Colmsee C, Zhang X-Q, Barrero RA, Zhang Q, et al. Construction of a map-based reference genome sequence for barley, *Hordeum vulgare* L. Sci Data. 2017;4:sdata201744.

52. Trapnell C, Pachter L, Salzberg SL. TopHat: discovering splice junctions with RNA-Seq. Bioinformatics. 2009;25:1105–11.

53. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009;25:2078–9.

54. Liao Y, Smyth GK, Shi W. The Subread aligner: fast, accurate and scalable read mapping by seed-and-vote. Nucleic Acids Res. 2013;41:e108–e108.

55. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinforma Oxf Engl. 2010;26:139–40.

56. McCarthy DJ, Chen Y, Smyth GK. Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. Nucleic Acids Res. 2012;40:4288–97.

57. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. 2015;:gkv007.

58. Lamesch P, Berardini TZ, Li D, Swarbreck D, Wilks C, Sasidharan R, et al. The Arabidopsis Information Resource (TAIR): improved gene annotation and new tools. Nucleic Acids Res. 2012;40 Database issue:D1202–10.

59. McGinnis S, Madden TL. BLAST: at the core of a powerful and diverse set of sequence analysis tools. Nucleic Acids Res. 2004;32 suppl 2:W20–5.

60. Ismail AM, Horie T. Genomics, Physiology, and Molecular Breeding Approaches for Improving Salt Tolerance. Annu Rev Plant Biol. 2017;68:null.

61. Slater GS, Birney E. Automated generation of heuristics for biological sequence comparison. BMC Bioinformatics. 2005;6:31–31.

62. Mount DW. The Maximum Likelihood Approach for Phylogenetic Prediction. Cold Spring Harb Protoc. 2008;2008:pdb.top34.

63. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol Biol Evol. 2013;30:2725–9.

64. Felsenstein J. Confidence Limits on Phylogenies: An Approach Using the Bootstrap. Evolution. 1985;39:783–91.

65. Li H. A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. Bioinformatics. 2011;27:2987–93.

66. Cingolani P, Platts A, Wang LL, Coon M, Nguyen T, Wang L, et al. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w ^{1118} ; iso-2; iso-3. Fly (Austin). 2012;6:80–92.

67. Langfelder P, Horvath S. WGCNA: an R package for weighted correlation network analysis. BMC Bioinformatics. 2008;9:559.

68. Tian T, Liu Y, Yan H, You Q, Yi X, Du Z, et al. agriGO v2.0: a GO analysis toolkit for the agricultural community, 2017 update. Nucleic Acids Res. 2017;45:W122–9.

69. Thimm O, Bläsing O, Gibon Y, Nagel A, Meyer S, Krüger P, et al. MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. Plant J Cell Mol Biol. 2004;37:914–39.

70. Moriya Y, Itoh M, Okuda S, Yoshizawa AC, Kanehisa M. KAAS: an automatic genome annotation and pathway reconstruction server. Nucleic Acids Res. 2007;35 suppl_2:W182–5.

71. Kanehisa M, Goto S. KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic Acids Res. 2000;28:27–30.

72. Kanehisa M, Furumichi M, Tanabe M, Sato Y, Morishima K. KEGG: new perspectives on genomes, pathways, diseases and drugs. Nucleic Acids Res. 2017;45:D353–61.

73. Finn RD, Attwood TK, Babbitt PC, Bateman A, Bork P, Bridge AJ, et al. InterPro in 2017—beyond protein family and domain annotations. Nucleic Acids Res. 2017;45:D190–9.

74. Blumwald E. Sodium transport and salt tolerance in plants. Curr Opin Cell Biol. 2000;12:431–4.

75. Shabala S, Bose J, Hedrich R. Salt bladders: do they matter? Trends Plant Sci. 2014;19:687–91.

76. Bassil E, Ohto M, Esumi T, Tajima H, Zhu Z, Cagnac O, et al. The Arabidopsis Intracellular Na+/H+ Antiporters NHX5 and NHX6 Are Endosome Associated and Necessary for Plant Growth and Development. Plant Cell. 2011;23:224–39.

77. Adem GD, Roy SJ, Plett DC, Zhou M, Bowman JP, Shabala S. Expressing AtNHX1 in barley (Hordium vulgare L.) does not improve plant performance under saline conditions. Plant Growth Regul. 2015;77:289–97.

78. Horie T, Hauser F, Schroeder JI. HKT transporter-mediated salinity resistance mechanisms in Arabidopsis and monocot crop plants. Trends Plant Sci. 2009;14:660–8.

79. Pardo JM, Cubero B, Leidi EO, Quintero FJ. Alkali cation exchangers: roles in cellular homeostasis and stress tolerance. J Exp Bot. 2006;57:1181–99.

80. Witzel K, Matros A, Strickert M, Kaspar S, Peukert M, Mühling KH, et al. Salinity Stress in Roots of Contrasting Barley Genotypes Reveals Time-Distinct and Genotype-Specific Patterns for Defined Proteins. Mol Plant. 2014;7:336–55.

81. Xu B, Waters S, Byrt CS, Plett D, Tyerman SD, Tester M, et al. Structural variations in wheat HKT1;5 underpin differences in Na+ transport capacity. Cell Mol Life Sci. 2017. doi:10.1007/s00018-017-2716-5.

82. Munns R, James RA, Xu B, Athman A, Conn SJ, Jordans C, et al. Wheat grain yield on saline soils is improved by an ancestral Na+ transporter gene. Nat Biotechnol. 2012;30:360–4.

83. Hazzouri K, Pauli D, Blake T, Shahid M, Khraiwesh B, Salehi-Ashtiani K, et al. Mapping of HKT1;5 gene in barley using GWAS approach and its implication in salt tolerance mechanism. Front Plant Sci. 2018;9. doi:10.3389/fpls.2018.00156.

84. Korte A, Farlow A. The advantages and limitations of trait analysis with GWAS: a review. Plant Methods. 2013;9:29.

85. Xu Y, Zhou Y, Hong S, Xia Z, Cui D, Guo J, et al. Functional Characterization of a Wheat NHX Antiporter Gene TaNHX2 That Encodes a K+/H+ Exchanger. PLOS ONE. 2013;8:e78098.

86. Huertas R, Rubio L, Cagnac O, García-Sánchez MJ, Alché JDD, Venema K, et al. The K+/H+ antiporter LeNHX2 increases salt tolerance by improving K+ homeostasis in transgenic tomato. Plant Cell Environ. 2013;36:2135–49.

87. Yuan H-J, Ma Q, Wu G-Q, Wang P, Hu J, Wang S-M. ZxNHX controls Na+ and K+ homeostasis at the whole-plant level in Zygophyllum xanthoxylum through feedback regulation of the expression of genes involved in their transport. Ann Bot. 2015;115:495–507.

88. Barragán V, Leidi EO, Andrés Z, Rubio L, De Luca A, Fernández JA, et al. Ion Exchangers NHX1 and NHX2 Mediate Active Potassium Uptake into Vacuoles to Regulate Cell Turgor and Stomatal Function in Arabidopsis[W][OA]. Plant Cell. 2012;24:1127–42.

89. Jiang X, Leidi EO, Pardo JM. How do vacuolar NHX exchangers function in plant salt tolerance? Plant Signal Behav. 2010;5:792–5.

90. Sottosanto JB, Saranga Y, Blumwald E. Impact of AtNHX1, a vacuolar Na+/H+ antiporter, upon gene expression during short- and long-term salt stress in Arabidopsis thaliana. BMC Plant Biol. 2007;7:18.

91. Apse MP, Aharon GS, Snedden WA, Blumwald E. Salt Tolerance Conferred by Overexpression of a Vacuolar Na+/H+ Antiport in Arabidopsis. Science. 1999;285:1256–8.

92. Leidi EO, Saiz JF. Is salinity tolerance related to Na accumulation in Upland cotton (Gossypium hirsutum) seedlings? Plant Soil. 1997;190:67–75.

93. Peng Z, He S, Sun J, Pan Z, Gong W, Lu Y, et al. Na^+ compartmentalization related to salinity stress tolerance in upland cotton (*Gossypium hirsutum*) seedlings. Sci Rep. 2016;6:srep34548.

94. Ligaba A, Katsuhara M. Insights into the salt tolerance mechanism in barley (<Emphasis Type="Italic">Hordeum vulgare</Emphasis>) from comparisons of cultivars that differ in salt sensitivity. J Plant Res. 2010;123:105–18.

95. Reginato MA, Castagna A, Furlán A, Castro S, Ranieri A, Luna V. Physiological responses of a halophytic shrub to salt stress by Na2SO4 and NaCl: oxidative damage and the role of polyphenols in antioxidant protection. AoB Plants. 2014;6. doi:10.1093/aobpla/plu042.

96. Sharma P, Jha AB, Dubey RS, Pessarakli M. Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions. Journal of Botany. 2012. doi:10.1155/2012/217037.

97. Gupta B, Huang B. Mechanism of Salinity Tolerance in Plants: Physiological, Biochemical, and Molecular Characterization. Int J Genomics. 2014;2014:e701596.

98. Wang J, Li B, Meng Y, Ma X, Lai Y, Si E, et al. Transcriptomic profiling of the salt-stress response in the halophyte Halogeton glomeratus. BMC Genomics. 2015;16:169.

99. Rossi L, Borghi M, Francini A, Lin X, Xie D-Y, Sebastiani L. Salt stress induces differential regulation of the phenylpropanoid pathway in Olea europaea cultivars Frantoio (salt-tolerant) and Leccino (salt-sensitive). J Plant Physiol. 2016;204:8–15.

100. Agati G, Azzarello E, Pollastri S, Tattini M. Flavonoids as antioxidants in plants: Location and functional significance. Plant Sci. 2012;196:67–76.

101. Tholl D. Biosynthesis and Biological Functions of Terpenoids in Plants. In: Biotechnology of Isoprenoids. Springer, Cham; 2015. p. 63–106. doi:10.1007/10_2014_295.

102. Basyuni M, Baba S, Inafuku M, Iwasaki H, Kinjo K, Oku H. Expression of terpenoid synthase mRNA and terpenoid content in salt stressed mangrove. J Plant Physiol. 2009;166:1786–800.

103. Opitz S, Kunert G, Gershenzon J. Increased Terpenoid Accumulation in Cotton (Gossypium hirsutum) Foliage is a General Wound Response. J Chem Ecol. 2008;34:508–22.

104. Tenhaken R. Cell wall remodeling under abiotic stress. Front Plant Sci. 2015;5. doi:10.3389/fpls.2014.00771.

105. Bischoff V, Selbig J, Scheible WR. Involvement of TBL/DUF231 proteins into cell wall biology., Involvement of TBL/DUF231 proteins into cell wall biology. Plant Signal Behav Plant Signal Behav. 2010;5, 5:1057, 1057–9.

106. Byrt CS, Munns R, Burton RA, Gilliham M, Wege S. Root cell wall solutions for crop plants in saline soils. Plant Sci. 2018. doi:10.1016/j.plantsci.2017.12.012.

107. Kim J-M, Sasaki T, Ueda M, Sako K, Seki M. Chromatin changes in response to drought, salinity, heat, and cold stresses in plants. Front Plant Sci. 2015;6. doi:10.3389/fpls.2015.00114.

108. Ding Y, Fromm M, Avramova Z. Multiple exposures to drought "train" transcriptional responses in *Arabidopsis*. Nat Commun. 2012;3:ncomms1732.

109. Li H, Yan S, Zhao L, Tan J, Zhang Q, Gao F, et al. Histone acetylation associated up-regulation of the cell wall related genes is involved in salt stress induced maize root swelling. BMC Plant Biol. 2014;14:105.

Chapter 6 General Discussion

Review of Thesis Aims

Salinisation is a challenge to global agriculture and affects some parts of the world more extensively than others, including Australia (Martinez Beltran and Licona Manzur, 2005; Rengasamy, 2006). Approximately 69% of the area used for agriculture in Australia is susceptible to high salinity (Rengasamy, 2002). The ability of crop plants to survive and perform in high salinity is therefore, important to improve yield stability and overall contribution to global food security as well as financial stability of farmers (FAOSTAT, 2014; Gilliham et al., 2017; Takeda and Matsuoka, 2008). The generation of transgenic material is currently been used as a pre-breeding strategy to explore the use of unknown gene networks that underlie high salinity tolerant crop phenotypes. One such attempt has revealed that overexpression of a gene coding for a kinase in Arabidopsis, *AtCIPK16,* leads to enhanced salinity tolerant phenotypes in both Arabidopsis and barley (Roy et al., 2013). The knowledge available on the downstream molecular mechanism mediated by AtCIPK16 as well as the ubiquity of CIPK16s is scarce. The potential natural genetic variations underlying salinity tolerance related traits in elite salt tolerant cultivars are beneficial for pre-breeding strategies that could be exploited to enhance salinity tolerance in related salt-sensitive cultivars (Negrão et al., 2017).

The aim of this PhD project therefore was to generate knowledge to answer the following:

1. What are the underlying molecular mechanisms of *AtCIPK16* overexpression conferred salinity tolerance in Arabidopsis? **(Chapter 3)**
2. What is the prevalence of CIPK16s in the terrestrial plant kingdom? **(Chapter 4)**
3. What are the main genetic variations among the barley genotypes with varying tissue tolerance levels? **(Chapter 5)**

Main experimental findings are specific to each chapter but will be briefly mentioned here. This chapter mainly discusses the importance of outcomes from this PhD project and suggests consideration for future research directions.

Summary of the Main Findings

Findings presented in Chapter 3 demonstrated that the salinity tolerance elicited through the expression of *AtCIPK16* (Roy et al., 2013) is related to activation of multiple transcription factors, several of which are involved in phytohormone regulation One of the conspicuous transcription factors is a CCCH Zinc finger AtTZF1 that has previously been shown to be involved in alleviating salinity shock responses when over-expressed (Han et al., 2014).

With transgenic plants exhibiting enhanced salt tolerance (Roy et al., 2013), an approach was taken to find the orthologs of *AtCIPK16* in other plants species, particularly crop species, as a first step to identifying a non-GM approach to enhancing salt tolerance of crops by manipulating native *CIPK16* expression. CIPKs like AtCIPK16s are confined to a very specific group of dicots called the *core Brassicales* (Amarasinghe et al., 2016; Chapter 4). Unique characteristics of CIPK16s (NLS and ALI) were identified only in them. These synapomorphic characters can be used to screen for potential CIPK16 orthologues in any genome sequence. We assume that the function of AtCIPK16 is at least partially dependent on the synapomorphic characters that are unique to CIPK16s. For monocots on the other hand, orthologues to CIPK16 were absent. Nevertheless, in monocots one homologue to AtCIPK16 and two other segmentally duplicated gene paralogues of AtCIPK16; AtCIPK5 and AtCIPK25 was present.

Chapter 5 includes a study on transcriptome of six barley cultivars, namely Alexis (Germany), Beecher, Commander, Maritime, Fleet and Sloop (Australia) which show varying capacity for sheath leaf Na^+ accumulation. Our study presents *in silico* evidence on the potential role of barley *HKT1;5* allelic variations on the ability of leaf sheath to accumulate Na^+.

Implications of the Main Findings

Salinity tolerance is a complex trait that associates with tolerance to other stresses caused by extreme temperatures, dehydration, deficiency of important minerals as well as biotic stress (Suzuki et al., 2014; Wang et al., 2003; Wani et al., 2016). Complex traits tend to be influenced by many genes simultaneously (Ismail and Horie, 2017; Roy et al., 2014; Yamaguchi and Blumwald, 2005). This would most likely account for only a few successful salt stress-tolerant commercial crops having been developed, despite more than 20 years of research on individual gene manipulation in salinity stress tolerance (Bhatnagar-Mathur et al., 2007; Møller et al., 2009). Furthermore, reduced genetic diversity through domestication of elite cultivars has made them more susceptible to drastic environmental conditions than their wild relatives. Efficient alternative methods to incorporate the desired traits into crops therefore is imperative to breed salinity tolerant crops.

The primary objective of crop breeding for salinity tolerance is to reduce the yield penalty that is caused by the high toxicity of salt. Selection of crop plants directly from the field for salinity tolerance is impractical due to variability in environmental factors. Identification of quantitative trait loci (QTL) for breeding programmes is carried out by plant performance typically under controlled environments, in relation to properties such as low tissue ion content, high K^+/Na^+ ratio, high water potential, high water-use efficiency, high chlorophyll content, high sugar content etc. that enhance crop production in salinity. Marker assisted selection (MAS) enables the introgression of these QTLs linked to markers into an appropriate genetic background (Ashraf and Foolad, 2013; Collard and Mackill, 2008). *AtCIPK16* was identified underlying a QTL on the chromosome 2 of Arabidopsis through fine mapping, and has the potential to be used to improve crops 1) through a genetic modification approach and 2) finding the crop equivalent of the Arabidopsis gene and identifying the best allele for it. The former approach of translating the QTL linked to a marker to the field requires confirmation of the benefits of *AtCIPK16* transgenics, and any detrimental effects the transgene has either directly or indirectly on growth. The latter approach requires the characterisation of crops with different alleles of the native CIPK16 gene.

The findings from the current study are important in the context of understanding the biological mechanisms underlying the conferred salt tolerance. Findings presented in Chapter 3 show that there are numerous pathways in action in transgenics to tolerate salt stress. This host of genes and their associated biological significance undoubtedly provide valuable resources for researchers and breeders to understand the molecular basis of AtCIPK16 mediated salinity tolerance. Transcription factors such as AtTZF1 that were revealed through the present study may play an important role downstream of AtCIPK16 mediated salinity tolerance in Arabidopsis hence deserve further investigation.

We have established that AtCIPK16 has no equivalent in monocots (Chapter 4). However, we cannot ignore the fact that transgenic barley expressing *AtCIPK16* had enhanced salt tolerance due to high shoot Na^+ exclusion, similar to transgenic *AtCIPK16* Arabidopsis. Barley attain its salt tolerance ability mainly owing to efficient tissue tolerant mechanisms. However, showing salt tolerant phenotypes mainly due to high Na^+ exclusion in transgenic barley implies that barley possesses the components of regulatory pathways that can be activated to exclude Na^+. If the synapomorphic characters do not define the function of AtCIPK16 as we hypothesise and propose to test through a) deleted NLS, b) deleted ALI, c) deleted NLS+ALI (Figure 1a), another hypothesis that can be tested is whether the barley homologue to AtCIPK16, AtCIPK5 and AtCIPK25, namely HvCIPK5, is able to confer salt tolerance (Figure 1b). If transgenic barley with constitutively expressed *HvCIPK5* also could confer salt tolerance, it enables us to refine the expression of *HvCIPK5* using specific promoters (e.g. stress induced, tissue-specific) in future experiments. Gene networks of transgenics with Constitutive *HvCIPK5* expression could be directly compared to those of transgenic *35S::AtCIPK16* transgenics to identify common pathways activated in both systems. Furthermore, similar phenotype to *35S::AtCIPK16* elicited by *35S::HvCIPK5* would suggest a possible functional redundancy amongst AtCIPK16, AtCIPK5 that could be tested through transformation of plants with *AtCIPK5*.

a)

Hypothesis: AtCIPK16 has a monopartite Nuclear localisation signal (NLS) in the C-terminal that is important in salt tolerance
Test: Overexpression of AtCIPK16 in Arabidopsis with no NLS in salt stress

Hypothesis: Activation Loop Insertion of AtCIPK16 (ALI) is important for salt tolerance
Test: Overexpression of AtCIPK16 in Arabidopsis with no ALI in salt stress

Hypothesis: Both ALI and NLS is important for salt tolerance
Test: Overexpression of AtCIPK16 in Arabidopsis with no ALI in salt stress

b)

Hypothesis: Barley paralogue of [CIPK16/5/25; *HvCIPK5*] overexpression also leads to salt tolerance
Test: Constitutive overexpression of HvCIPK5 in salt stressed Arabidopsis and barley

c)

Hypothesis: AtTZF1 is a downstream target of AtCIPK16 mediated salinity tolerance
Test:
•Y2H assay for AtCIPK16 interaction with AtTZF1
•AtTZF1 mutation in a transgenic AtCIPK16 background
•Comparison of the regulatory networks from AtTZF1 transgenics and AtCIPK16 transgenics in identical salt stress conditions

Figure 1 Hypotheses put forward related to AtCIPK16, based on the current study

A way to identify whether the downstream pathway regulated by CIPK16 is conserved across dicots and monocots would be to conduct a comparative transcriptomic study and determine if similar genes were differentially regulated in Arabidopsis and barley, when compared to non-GM controls. A study was designed to examine the transcriptome of transgenic expressing AtCIPK16 and determine if the gene was activating similar pathways in barley as it did in Arabidopsis, however, it was found that the transgene was being silenced in offspring from the barley plants used in Roy et al. (2013). While this is unfortunately a common occurrence in transgenic plants, it would require new *AtCIPK16* expressing barley to be generated. This is planned to take place in near future, however, it is out of the scope of my PhD study. Findings of this subsequent transgenic barley transcriptomic study would potentially reveal any overlaps of the regulatory networks with Arabidopsis expressing *AtCIPK16* and whether the downstream network include orthologues of any of the identified candidate transcription factors (TFs).

TFs are an excellent source for gene manipulation because they can regulate a range of genes potentially involved in numerous pathways which are regulated under stress conditions (Wang et al., 2016). However, there could be unwanted downstream effects that are associated with gene targets of altered TF activity. For example, it has been shown previously that transgenic wheat and barley plants constitutively overexpressing wheat TFs, TaDREB2 and TaDREB3, caused development and yield penalties despite being drought tolerant (Morran et al., 2011). Altering TF binding affinity to their targets and their cell/tissue-specific expression may allow further fine-tuning of its downstream function to elicit the advantageous and minimise the potentially damaging traits. An alternative approach would be to control the expression of the TF using a salt stress-specific promoter. The use of a drought-inducible promoter made the wheat plants from Morran et. al. (2011) more drought tolerant, and eliminated the undesired negative effects associated with growth and yield.

If the AtTZF1 is indeed involved in salinity tolerance mediated by AtCIPK16, overexpression of *AtCIPK16* will not confer salt tolerance in *Attzf1* knockout/knockdown mutants (Figure 1c). Y2H interaction assays would be a possible method to determine whether AtTZF1 is a direct interactor of AtCIPK16. If this is the case, finding the AtTZF1 orthologue from barley (HvTZF1) could lead to investigations on using *HvTZF1* as a potential genetic tool for enhancing salinity tolerance (Lata et al., 2011; Seo et al., 2012; Wang et al., 2016). When we conducted a quick BLAST search (tblastn with default settings) using the AtTZF1 protein on the current barley genome (IBSC, 2016) the top hit (*HORVU3Hr1G019510*) obtained showed low protein similarity (~60%) and sequence identity (~60%) to AtTZF1. This raises the question whether more of the machinery of the AtCIPK16 elicited salt tolerance mechanism is missing in barley. One needs to consider though that transcription factors tend not to be highly conserved across distant species. Therefore, a comprehensive phylogenetic analysis on zinc finger proteins would be required to confirm the indicated absence of AtTZF1 orthologue/s in barley.

If any conserved downstream regulatory pathways are identified among transgenic Arabidopsis and barley, the next important aspect will be to identify whether the involved key components of transgenic barley regulatory network are involved in native barley salinity tolerance. Since barley is considered naturally a salt tolerant crop in which, multiple processes with variations, are involved in salinity tolerance (Chapter 5), it is possible that more pathways are existent that contribute to salt tolerance which are yet to be explored and discovered. In a circumstance where comparative studies reveal that overexpression of *AtCIPK16* in barley gives rise to novel pathways that do not exist naturally in barley, one could further explore the key components of the particular networks that may or may not be part of the identified transgenic Arabidopsis network. The gene clusters from the comparative regulatory networks of barley and Arabidopsis can be further analysed through evolutionary genomics such as phylogenetics, to reveal the extent to which conservation of variability of the differentially expressed genes exist across species (Ruprecht et al., 2017b, 2017a; Schaefer et al., 2017). Further exploration of key downstream drivers of transgenic *AtCIPK16* barley is possible by using approaches such as gene overexpression, knockout lines and knockdown lines.

It is noteworthy that, even though transgenic *AtCIPK16* barley lines performed well under salinity stress conditions, there are some field data to suggest that *35S:CIPK16* wheat and barley have poor yield in absence of salt (SJ Roy, unpublished data). We also observed a considerable amount of unwanted *AtCIPK16* expression in the shoots of transgenic Arabidopsis under non-stressed conditions. Therefore, there is a need of fine-tuning AtCIPK16 expression suggested by the results of the current study. Native *AtCIPK16* has root stellar cell specific expression, so negative effects could be diminished by cell specific expression of *AtCIPK16* under a stress-induced promoter regulation in barley. A United States Agency for International Development (USAID, USA) funded research project "Abiotic stress tolerant bio-engineered cereals (AID-OAA-A-12-000013) has developed lines of wheat and rice expressing *AtCIPK16* under the control of salt inducible promoters – these plants are currently being phenotyped to determine if they have improved salt tolerance and no detrimental phenotype in control conditions.

As barley is considered a salt-tolerant crop, examining its natural genetic variations within the well-known genes involved in salinity tolerance is an alternative method to identifying transcriptomic changes. Possible functional implications of the allelic variations are presented in Chapter 5 (e.g. on *HvHKT1;5* and *HvNHX4*). They may provide plant breeders an opportunity to improve plants with enhanced capacity to tolerate salinity. If these alleles are related to high tissue tolerance, these variations could be introgressed into more salt-sensitive cultivars. Such an experiment has already been conducted for wheat (Munns et al., 2012) where the *Nax2* locus containing the wild wheat *Triticum monococcum* HKT1;5 (*TmHKT1;5-A*) was introgressed to modern day durum wheat that lacks this locus. This led to increase in grain yield by 25% in salinized field at least partly owing to the enhanced ability of shoot Na^+ exclusion. Similar studies where tolerance alleles have been introduced in to crops from near wild relatives have been done for crops subjected to other stresses such as flooding, drought, boron toxicity, etc. to improve their survival under these stresses (Mickelbart et al., 2015). Such breeding strategies are ideal to make malting cultivars such as Commander or high protein containing cultivars such as Gairdner adaptable to high salinity as much as Alexis and Morex are (Grains Research and Development Corporation, 2010; Tilbrook et al., 2017).

Another use of transcriptomics was demonstrated in this thesis, and that is the use of such studies to aid in the deregulation of GM crops. Prior to a genetically modified crop being released for farmers to use, the crop has to be proven to have no deleterious effects on the environment or on those organisms that would consume or interact with the crop. In Australia, the Office of Gene Technology Regulator (OGTR; www.ogtr.gov.au) assesses GM plants for a number of different parameters, such as the plant's fitness (in regards to parameters not targeted by modification), weediness, invasiveness and effects on non-target organisms (Warwick et al., 2009). While these evaluations have to determined experimentally, the use of transcriptomics can assist in clarifying whether the plant may have unforeseen advantages which could enhance its weediness, resistance to pathogens and pests, invasiveness and effect on the animals which consume them by uncovering if there are alterations in known genes in these pathways. Herbicide resistance can occur through changes in regulatory pathways such as shikimate pathways (Funke et al., 2006). Proteins involved in the shikimate pathway could either produce phenylproponoids, the most common and beneficial secondary metabolites in plants or tocopherols (vitamin E) that would confer herbicide resistance (Rippert et al., 2004). Results from *AtCIPK16* transgenics indicate the presence of genes that are related to phenylpropanoid metabolism. These could be used to suggest that those involved in the deregulation of GMOs should focus on establishing whether *AtCIPK16* expressing plants may have altered herbicide resistance. Surveys such as Australia Weed Risk Assessment (WRA) could be used to further evaluate the traits of invasiveness. Potential toxicity that is harmful for humans or animals is another undesirable trait that OGTR framework would assess in transgenics. There are possibilities that transgene may promote the uptake of toxic substances such as arsenic (As) or production of substances such as cyanides through altered pathways. By examining the transcriptome of the transgenic plants, the expression of known genes involved in the uptake of toxic ions could be determined and if so, measurements of ion concentrations in the shoot could be performed to determine whether or not the plants were indeed accumulating too much toxic ions. In this study there was no evidence to support that production of toxins or allergens that could be harmful to humans or animals through the introduction of the transgene. The cost of deregulation of one transgenic event is in the range of $10M-$50M. Being able to speed up the deregulation of a crop using transcriptomics to identify areas to focus on would reduce the incurred time and cost could be considerably beneficial to make the translation of research findings to agricultural application efficiently. This could be tested on lines currently going through the deregulation process and results could be presented to the OGTR for consideration in future deregulation events.

Future Work for Salinity Research in Crops

In this study it was seen that after 3 hours of initial salt application, there was an array of genes that were differentially expressed dependent on the presence of both the transgene AtCIPK16 and salinity stress. However, this effect has completely diminished by the second time point that was sampled to evaluate the late salt stress response. Additionally, the number of genes differentially expressed due to salt stress in transgenics were less in the late response compared to the late response in wild type and the early response in transgenics. It indicates that the transgenics have the capacity to adapt quickly to the new homeostasis. This needs to be validated by sampling, at least one more time point in between 3 hours and 51 hours. In order to study the diurnal effects of salt application, that have a large impact on the regulation of stress related genes of the plants (Grundy et al., 2015), another time point 48 hours later from the new sampling point, which then can be compared to study variation of gene expression due to circadian rhythms. Based on the results we also assume that it is possible to see the osmotic tolerance effects if the plants were sampled immediately after (30 minutes to 1 hour) exposure to salt (Brinker et al., 2010), again which needs to have a sample 48 hours later to complement the diurnal effects. Experiments can be designed to observe the temporal changes and tested through differential gene expression analysis. The analysis requires a multifactorial design. For example, a design matrix with 2 (treatment: salt, control) $\times$ 2 (genotype: transgenic, wildtype) $\times$ 6 (time: 1 hr, 3 hr, 30 hr, 49 hr, 51 hr, 78hr) could be used.

In the scenario where the *HvCIPK5* transgenics confer salinity tolerance, there are novel approaches that can increase the level of expression without exogeneous promoter enhancers. It is appropriate to use the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and the CRISPR associated protein 9 (Cas9) system for RNA guided transcriptional activation of *HvCIPK5* which will mimic the overexpression phenotypes without a foreign promoter driving its expression (Park et al., 2017; Perez-Pinera et al., 2013; Russa and Qi, 2015; Waltz, 2016). This has been adopted recently for Arabidopsis, to enhance the expression of two genes namely, *AVP1* and *PAP1* (Park et al., 2017). They have modified the CRISPR/Cas9 system through the addition of p65 transactivating subunit of NF-kappa B and a heat-shock factor 1 (HSF) activation domain to VP64 (tetramer of VP16) activation domain bound dCas9 (deactivated Cas9 Endonuclease). This alternative method can be employed to study expression of *AtCIPK16* in barley, but the use of a cell specific activation domain to VP64-dCas9 is important in this respect.

A successful retransformation should lead to *AtCIPK16* being tested in multiple adaptable genotypes for the Australian climate, such as Commander and Maritime, as well as for other globally cultivated barley varieties. Furthermore, it could be tested for the ability to confer salinity tolerance through transgenesis in a variety of other important crop species such as wheat and maize.

Transcriptomics studies however are inadequate in exemplifying the post-translational modifications that occur *in vivo* which are concealed from the transcriptome level (Haider and Pal, 2013; Mittler et al., 1998). Due to the uncertainty associated with the mode of action of AtCIPK16, if it is phosphorylation, it could be identified through Multiplex Substrate Profiling by Mass Spectrometry (MSP-MS) (O'Donoghue et al., 2012). There are also studies which have adopted an integrated approach to use both proteomics and transcriptomics that produce better information than using the two types of methods separately. (Batista et al., 2017; Hahne et al., 2010; Kohler et al., 2015; Kosová et al., 2011, 2013; Zhang et al., 2018). Mass spectrometry (MS) analysis such as Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometric (LC/ESI-MS/MS) study is one of the ways of conduct a proteomic study in presence of salinity (Passamani et al., 2017).

It is also required to functionally characterise the two novel barley *HKT1;5* and *HvNHX4* alleles discovered through this study using heterologous expression in *Xenopus* oocytes to examine ion specificity and transport activity (Liu and Luan, 2001) and in *Saccharomyces cerevisiae* to examine the level of salt tolerance associated with the two alleles (Figure 2a) (Henderson et al., 2018).

a)

Hypothesis: Amino acid substitutions in HvHKT1;5 from Alexis and Morex contribute towards altered leaf sheath Na^+ accumulation in barley
Test:
•Ion specificity examination and transporter activity measurements for the two types of alleles ($HvHKT1;5_{Alexis|Morex}$ and $HvHKT1;5_{Other}$) through heterologous expression in *Xenopus* oocytes
•Expression of the two alleles in *Saccharomyces cerevisiae* to examine the level of salt tolerance they confer

b)

Hypothesis: Substitutions in HvNHX4 in Alexis contribute towards altered leaf sheath Na^+ accumulation in barley
Test:
•Ion specificity examination and transporter activity measurements for the two types of alleles ($HvNHX4_{Alexis}$ and $HvNHX4_{Other}$) through heterologous expression in *Xenopus* oocytes
•Expression of the two alleles in *Saccharomyces cerevisiae* to examine the level of salt they accumulate

Figure 2 Hypothesis put forward based on allelic variations observed in barley

Furthermore, *HvNHX4* possesses an allelic variation on the N-terminal of the CDS that leads to an amino acid change in the high leaf sheath Na^+ accumulating variety (i.e. Alexis) that could also be characterised by the above methods (Figure 2b). If the identified *HvHKT1;5* and *HvNHX4* alleles could be characterised as being involved in high Na^+ accumulation tolerance, the 3D modelling of the proteins coded by the alleles could be useful to determine whether any structural changes are caused by them. It would also be needed to know whether the presence of both the alleles, and enhanced expression of *HvNHX4*, as seen in our study are required for enhanced tissue tolerance. After these validations, these variants should need to be further interrogated for the stability under various genetic backgrounds, as well as under non-stressed conditions.

HvHKT1;5 and *HvNHX4* alleles thereafter could be further tested through KASP™ genotyping assays (LGC, UK) on an available proprietary barley diversity panel (SJ Roy, unpublished data). If these alleles are verified as strong candidates for tissue tolerance in barley, they would provide breeders with a strong marker for MAS. Furthermore, allele-specific PCR assays can be developed to facilitate the selection of elite *HvHKT1;5* and *HvNHX4* alleles in marker assisted trait introgression and breeding to less tissue tolerant cultivars. CRISPR/Cas9 systems could be employed to introduce certain mutations on the coding sequence, which has been previously successful in such research with wheat (Li et al., 2017; Shan et al., 2014).

Concluding Remarks

Findings of this PhD project improves the current knowledge on the genetic basis of salt tolerance in crops through identifying molecular components of salinity tolerance in Arabidopsis and barley. Identification of these components was possible because of the global perspective of the transcriptome that is enabled by techniques such as RNA-Seq. Methods that are focussed on one-gene-at-a-time approach to elucidate the salt stress tolerance need to be reconsidered in the light of knowledge on synergistic action of more than one gene or even more than one pathway that is responsible for salinity tolerance in plants, a concept that this study also supports.

It has to be emphasised that unquestionably, salinity tolerance based research is a timely requirement for the world with the decrease of arable land and increase of food demand. Cooperation of research that involves plant molecular and cell biology, transcriptomics and genetic variation studies with conventional plant breeding strategies undoubtedly will speed up the development of salinity tolerance in crops.

References

Amarasinghe, S., Watson-Haigh, N.S., Gilliham, M., Roy, S., Baumann, U., 2016. The evolutionary origin of CIPK16: A gene involved in enhanced salt tolerance. Mol. Phylogenet. Evol. 100, 135–147. https://doi.org/10.1016/j.ympev.2016.03.031

Ashraf, M., Foolad, M.R., 2013. Crop breeding for salt tolerance in the era of molecular markers and marker-assisted selection. Plant Breed. 132, 10–20. https://doi.org/10.1111/pbr.12000

Batista, R., Fonseca, C., Planchon, S., Negrão, S., Renaut, J., Oliveira, M.M., 2017. Environmental stress is the major cause of transcriptomic and proteomic changes in GM and non-GM plants. Sci. Rep. 7, 10624. https://doi.org/10.1038/s41598-017-09646-8

Bhatnagar-Mathur, P., Vadez, V., Sharma, K.K., 2007. Transgenic approaches for abiotic stress tolerance in plants: retrospect and prospects. Plant Cell Rep. 27, 411–424. https://doi.org/10.1007/s00299-007-0474-9

Brinker, M., Brosché, M., Vinocur, B., Abo-Ogiala, A., Fayyaz, P., Janz, D., Ottow, E.A., Cullmann, A.D., Saborowski, J., Kangasjärvi, J., Altman, A., Polle, A., 2010. Linking the Salt Transcriptome with Physiological Responses of a Salt-Resistant Populus Species as a Strategy to Identify Genes Important for Stress Acclimation. Plant Physiol. 154, 1697–1709. https://doi.org/10.1104/pp.110.164152

Collard, B.C.., Mackill, D.J., 2008. Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. Philos. Trans. R. Soc. B Biol. Sci. 363, 557–572. https://doi.org/10.1098/rstb.2007.2170

FAOSTAT, 2014. Food and Agriculture Organization fo the United Nations [WWW Document]. Food Agric. Organ. Fo U. N. URL http://www.fao.org/economic/ess/en/#.U1XSWvmSymw (accessed 4.22.14).

Funke, T., Han, H., Healy-Fried, M.L., Fischer, M., Schönbrunn, E., 2006. Molecular basis for the herbicide resistance of Roundup Ready crops. Proc. Natl. Acad. Sci. 103, 13010–13015. https://doi.org/10.1073/pnas.0603638103

Gilliham, M., Able, J.A., Roy, S.J., 2017. Translating knowledge about abiotic stress tolerance to breeding programmes. Plant J. 90, 898–917. https://doi.org/10.1111/tpj.13456

Grains Research and Development Corporation, n.d. BARLEY VARIETIES AND AGRONOMY - CENTRAL NSW 2010 [WWW Document]. Grains Res. Dev. Corp. URL https://grdc.com.au/resources-and-publications/grdc-update-papers/tab-content/grdc-update-papers/2010/09/barley-varieties-and-agronomy-central-nsw-2010 (accessed 2.26.18).

Grundy, J., Stoker, C., Carré, I.A., 2015. Circadian regulation of abiotic stress tolerance in plants. Front. Plant Sci. 6. https://doi.org/10.3389/fpls.2015.00648

Hahne, H., Mäder, U., Otto, A., Bonn, F., Steil, L., Bremer, E., Hecker, M., Becher, D., 2010. A Comprehensive Proteomics and Transcriptomics Analysis of Bacillus subtilis Salt Stress Adaptation. J. Bacteriol. 192, 870–882. https://doi.org/10.1128/JB.01106-09

Haider, S., Pal, R., 2013. Integrated Analysis of Transcriptomic and Proteomic Data. Curr. Genomics 14, 91–110. https://doi.org/10.2174/1389202911314020003

Han, G., Wang, M., Yuan, F., Sui, N., Song, J., Wang, B., 2014. The CCCH zinc finger protein gene AtZFP1 improves salt resistance in Arabidopsis thaliana. Plant Mol. Biol. 86, 237–253. https://doi.org/10.1007/s11103-014-0226-5

Henderson, S.W., Dunlevy, J.D., Wu, Y., Blackmore, D.H., Walker, R.R., Edwards, E.J., Gilliham, M., Walker, A.R., 2018. Functional differences in transport properties of natural HKT1;1 variants influence shoot Na+ exclusion in grapevine rootstocks. New Phytol. 217, 1113–1127. https://doi.org/10.1111/nph.14888

Ismail, A.M., Horie, T., 2017. Genomics, Physiology, and Molecular Breeding Approaches for Improving Salt Tolerance. Annu. Rev. Plant Biol. 68, null. https://doi.org/10.1146/annurev-arplant-042916-040936

Jayaraman, D., Richards, A.L., Westphall, M.S., Coon, J.J., Ané, J.-M., 2017. Identification of the phosphorylation targets of symbiotic receptor-like kinases using a high-throughput multiplexed assay for kinase specificity. Plant J. 90, 1196–1207. https://doi.org/10.1111/tpj.13529

Kohler, C., Lourenço, R.F., Bernhardt, J., Albrecht, D., Schüler, J., Hecker, M., Gomes, S.L., 2015. A comprehensive genomic, transcriptomic and proteomic analysis of a hyperosmotic stress sensitive α-proteobacterium. BMC Microbiol. 15, 71. https://doi.org/10.1186/s12866-015-0404-x
Kosová, K., Práil, I., Vítámvás, P., 2013. Protein Contribution to Plant Salinity Response and Tolerance Acquisition. Int. J. Mol. Sci. 14, 6757–6789. https://doi.org/10.3390/ijms14046757
Kosová, K., Vítámvás, P., Prášil, I.T., Renaut, J., 2011. Plant proteome changes under abiotic stress — Contribution of proteomics studies to understanding plant stress response. J. Proteomics, Plant Proteomics in Europe 74, 1301–1322. https://doi.org/10.1016/j.jprot.2011.02.006
Lata, C., Yadav, A., Prasad, M., 2011. Role of Plant Transcription Factors in Abiotic Stress Tolerance. https://doi.org/10.5772/23172
Li, J., Sun, Y., Du, J., Zhao, Y., Xia, L., 2017. Generation of targeted point mutations in rice by a modified CRISPR/Cas9 system. Mol. Plant 10, 526–529.
Liu, K., Luan, S., 2001. Internal Aluminum Block of Plant Inward K+ Channels. Plant Cell 13, 1453–1466. https://doi.org/10.1105/TPC.010016
Martinez Beltran, J., Licona Manzur, C., 2005. Overview of salinity problems in the world and FAO strategies to address the problem [WWW Document]. Int. Salin. Forum Manag. Saline Soils Water. URL https://eurekamag.com/research/012/808/012808515.php (accessed 1.16.18).
Meyer, N.O., O'Donoghue, A.J., Schulze-Gahmen, U., Ravalin, M., Moss, S.M., Winter, M.B., Knudsen, G.M., Craik, C.S., 2017. Multiplex Substrate Profiling by Mass Spectrometry for Kinases as a Method for Revealing Quantitative Substrate Motifs. Anal. Chem. 89, 4550–4558. https://doi.org/10.1021/acs.analchem.6b05002
Mickelbart, M.V., Hasegawa, P.M., Bailey-Serres, J., 2015. Genetic mechanisms of abiotic stress tolerance that translate to crop yield stability. Nat. Rev. Genet. 16, 237–251. https://doi.org/10.1038/nrg3901
Mittler, R., Feng, X., Cohen, M., 1998. Post-Transcriptional Suppression of Cytosolic Ascorbate Peroxidase Expression during Pathogen-Induced Programmed Cell Death in Tobacco. Plant Cell 10, 461–473. https://doi.org/10.1105/tpc.10.3.461
Møller, I.S., Gilliham, M., Jha, D., Mayo, G.M., Roy, S.J., Coates, J.C., Haseloff, J., Tester, M., 2009. Shoot Na+ Exclusion and Increased Salinity Tolerance Engineered by Cell Type–Specific Alteration of Na+ Transport in Arabidopsis. Plant Cell 21, 2163–2178. https://doi.org/10.1105/tpc.108.064568
Morran, S., Eini, O., Pyvovarenko, T., Parent, B., Singh, R., Ismagul, A., Eliby, S., Shirley, N., Langridge, P., Lopato, S., 2011. Improvement of stress tolerance of wheat and barley by modulation of expression of DREB/CBF factors. Plant Biotechnol. J. 9, 230–249. https://doi.org/10.1111/j.1467-7652.2010.00547.x
Munns, R., James, R.A., Xu, B., Athman, A., Conn, S.J., Jordans, C., Byrt, C.S., Hare, R.A., Tyerman, S.D., Tester, M., Plett, D., Gilliham, M., 2012. Wheat grain yield on saline soils is improved by an ancestral Na^+ transporter gene. Nat. Biotechnol. 30, 360. https://doi.org/10.1038/nbt.2120
Negrão, S., Schmöckel, S.M., Tester, M., 2017. Evaluating physiological responses of plants to salinity stress. Ann. Bot. 119, 1–11. https://doi.org/10.1093/aob/mcw191
O'Donoghue, A.J., Eroy-Reveles, A.A., Knudsen, G.M., Ingram, J., Zhou, M., Statnekov, J.B., Greninger, A.L., Hostetter, D.R., Qu, G., Maltby, D.A., Anderson, M.O., DeRisi, J.L., McKerrow, J.H., Burlingame, A.L., Craik, C.S., 2012. Global identification of peptidase specificity by multiplex substrate profiling. Nat. Methods 9, 1095–1100. https://doi.org/10.1038/nmeth.2182
Park, J.-J., Dempewolf, E., Zhang, W., Wang, Z.-Y., 2017. RNA-guided transcriptional activation via CRISPR/dCas9 mimics overexpression phenotypes in Arabidopsis. PLoS ONE 12. https://doi.org/10.1371/journal.pone.0179410
Passamani, L.Z., Barbosa, R.R., Reis, R.S., Heringer, A.S., Rangel, P.L., Santa-Catarina, C., Grativol, C., Veiga, C.F.M., Souza-Filho, G.A., Silveira, V., 2017. Salt stress induces changes in the proteomic profile of micropropagated sugarcane shoots. PLOS ONE 12, e0176076. https://doi.org/10.1371/journal.pone.0176076
Perez-Pinera, P., Kocak, D.D., Vockley, C.M., Adler, A.F., Kabadi, A.M., Polstein, L.R., Thakore, P.I., Glass, K.A., Ousterout, D.G., Leong, K.W., Guilak, F., Crawford, G.E., Reddy, T.E., Gersbach,

C.A., 2013. RNA-guided gene activation by CRISPR-Cas9-based transcription factors. Nat. Methods 10, 973–976. https://doi.org/10.1038/nmeth.2600
Rengasamy, P., 2006. World salinization with emphasis on Australia. J. Exp. Bot. 57, 1017–1023. https://doi.org/10.1093/jxb/erj108
Rengasamy, P., 2002. Transient salinity and subsoil constraints to dryland farming in Australian sodic soils: an overview. Aust. J. Exp. Agric. 42, 351–361.
Rippert, P., Scimemi, C., Dubald, M., Matringe, M., 2004. Engineering Plant Shikimate Pathway for Production of Tocotrienol and Improving Herbicide Resistance. Plant Physiol. 134, 92–100. https://doi.org/10.1104/pp.103.032441
Roy, S.J., Huang, W., Wang, X.J., Evrard, A., Schmöckel, S.M., Zafar, Z.U., Tester, M., 2013. A novel protein kinase involved in Na+ exclusion revealed from positional cloning. Plant Cell Environ. 36, 553–568. https://doi.org/10.1111/j.1365-3040.2012.02595.x
Roy, S.J., Negrão, S., Tester, M., 2014. Salt resistant crop plants. Curr. Opin. Biotechnol., Food biotechnology - Plant Biotech 26, 115–124. https://doi.org/10.1016/j.copbio.2013.12.004
Ruprecht, C., Proost, S., Hernandez-Coronado, M., Ortiz-Ramirez, C., Lang, D., Rensing, S.A., Becker, J.D., Vandepoele, K., Mutwil, M., 2017a. Phylogenomic analysis of gene co-expression networks reveals the evolution of functional modules. Plant J. 90, 447–465. https://doi.org/10.1111/tpj.13502
Ruprecht, C., Vaid, N., Proost, S., Persson, S., Mutwil, M., 2017b. Beyond Genomics: Studying Evolution with Gene Coexpression Networks. Trends Plant Sci. 22, 298–307. https://doi.org/10.1016/j.tplants.2016.12.011
Russa, M.F.L., Qi, L.S., 2015. The New State of the Art: Cas9 for Gene Activation and Repression. Mol. Cell. Biol. 35, 3800–3809. https://doi.org/10.1128/MCB.00512-15
Schaefer, R.J., Michno, J.-M., Myers, C.L., 2017. Unraveling gene function in agricultural species using gene co-expression networks. Biochim. Biophys. Acta BBA - Gene Regul. Mech., Plant Gene Regulatory Mechanisms and Networks 1860, 53–63. https://doi.org/10.1016/j.bbagrm.2016.07.016
Seo, P.J., Jung, J.-H., Park, C.-M., 2012. Transcription Factors: Improving Abiotic Stress Tolerance in Plants, in: Tuteja, N., Gill, S.S., Tiburcio, A.F., Tuteja, R. (Eds.), Improving Crop Resistance to Abiotic Stress. Wiley-VCH Verlag GmbH & Co. KGaA, pp. 451–479. https://doi.org/10.1002/9783527632930.ch20
Shan, Q., Wang, Y., Li, J., Gao, C., 2014. Genome editing in rice and wheat using the CRISPR/Cas system. Nat. Protoc. 9, 2395–2410. https://doi.org/10.1038/nprot.2014.157
Suzuki, N., Rivero, R.M., Shulaev, V., Blumwald, E., Mittler, R., 2014. Abiotic and biotic stress combinations. New Phytol. 203, 32–43. https://doi.org/10.1111/nph.12797
Takeda, S., Matsuoka, M., 2008. Genetic approaches to crop improvement: responding to environmental and population changes. Nat. Rev. Genet. 9, 444. https://doi.org/10.1038/nrg2342
Tilbrook, J., Schilling, R.K., Berger, B., Garcia, A.F., Trittermann, C., Coventry, S., Rabie, H., Brien, C., Nguyen, M., Tester, M., Roy, S.J., 2017. Variation in shoot tolerance mechanisms not related to ion toxicity in barley. Funct. Plant Biol. 44, 1194–1206. https://doi.org/10.1071/FP17049
Waltz, E., 2016. CRISPR-edited crops free to enter market, skip regulation [WWW Document]. Nat. Biotechnol. https://doi.org/10.1038/nbt0616-582
Wang, Hongyan, Wang, Honglei, Shao, H., Tang, X., 2016. Recent Advances in Utilizing Transcription Factors to Improve Plant Abiotic Stress Tolerance by Transgenic Technology. Front. Plant Sci. 7. https://doi.org/10.3389/fpls.2016.00067
Wang, W., Vinocur, B., Altman, A., 2003. Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta 218, 1–14. https://doi.org/10.1007/s00425-003-1105-5
Wani, S.H., Kumar, V., Shriram, V., Sah, S.K., 2016. Phytohormones and their metabolic engineering for abiotic stress tolerance in crop plants. Crop J. 4, 162–176. https://doi.org/10.1016/j.cj.2016.01.010

Warwick, S.I., Beckie, H.J., Hall, L.M., 2009. Gene Flow, Invasiveness, and Ecological Impact of Genetically Modified Crops. Ann. N. Y. Acad. Sci. 1168, 72–99. https://doi.org/10.1111/j.1749-6632.2009.04576.x

Yamaguchi, T., Blumwald, E., 2005. Developing salt-tolerant crop plants: challenges and opportunities. Trends Plant Sci., Trends in Plant Science 10th Anniversary IssueFeeding the World: Plant Biotechnology Milestones 10, 615–620. https://doi.org/10.1016/j.tplants.2005.10.002

Zhang, A., Han, D., Wang, Y., Mu, H., Zhang, T., Yan, X., Pang, Q., 2018. Transcriptomic and proteomic feature of salt stress-regulated network in Jerusalem artichoke (Helianthus tuberosus L.) root based on de novo assembly sequencing analysis. Planta 247, 715–732. https://doi.org/10.1007/s00425-017-2818-1

Appendix: Availability of Supplementary Materials

The attached CD-ROM/DVD-ROM contains the supplementary data and figures for chapter 3, 4 and 5.

The chapter 3 contains 13 supplementary files. Both chapter 4 and chapter 5 contain 16 supplementary files each.

Due to the large amount of information within these files we retained from attaching them to the main text. However, for ease of access the supplementary materials were uploaded to FigShare and the links for each supplementary material set are denoted as appropriate in each chapter. For easy reference, they are repeated here.

Supplementary materials for chapter 3: https://doi.org/10.4225/55/5aa11470444b0

Supplementary materials for chapter 4: https://doi.org/10.4225/55/5aa085b5c4991

Supplementary materials for chapter 5: https://doi.org/10.4225/55/5aa116ab810bd