Evaluation of the effects of AtCIPK16 expression on the salt tolerance of barley and wheat

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List of Abbreviations

%	percentage
#	number
×	times
°C	degrees Celsius
®	registered trademark
$^{-1}$	per
-ve	negative
+ve	positive
µL	microliter(s)
µmoles	micromole(s)
µS	microSiemens
3'	three prime, of nucleic acid sequence
35S	promoter of cauliflower mosaic virus 35S
3D	three dimensional
5'	five prime, of nucleic acid sequence
aa	amino acid
ABA	abscisic acid
ABARES	Australian Bureau of Agricultural and Resource Economics and Sciences
ACPFG	Australian Centre for Plant Functional Genomics
AGRF	Australian Genome Research Facility
Agrobacterium	*Agrobacterium tumefaciens*
AKT	*Arabidopsis* potassium transporter
At	*Arabidopsis thaliana*
ANOVA	analysis of variance
AVP1	*Arabidopsis* vacuolar pyrophosphatase
Bay-0	*Arabidopsis* ecotype Bayreuth-0
BLAST	basic local alignment search tool
bp	base pairs, of nucleic acid
C-terminal	carboxyl (COOH)-terminal, of protein
Ca^{2+}	calcium ion
$CaCl_2$	calcium chloride
CaM	calmodulin
$CaSO_4$	calcium sulphate
Cat. No.	catalogue number
CBL	calcineurin B-like protein
cDNA	complimentary deoxyribonucleic acid

CDPK	calcium-dependent protein kinase
CIMMYT	International Maize and Wheat Improvement Centre (Centro Internacional de Mejoramiento de Maíz y Trigo)
CIPK	calcineurin B-like (CBL) interacting protein kinase
Cl^-	chloride ion
cm	centimetre
CML	calmodulin-like protein
CO_2	carbon dioxide
Col-0	*Arabidopsis* ecotype Columbia-0
CRCSLM	Cooperative Research Centre for Soil & Land Management
CRISPR/Cas	clustered regularly interspersed short palindromic repeats/CRISPR-associated
cv.	cultivar
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
DREB	dehydration-responsive element-binding
dS	deciSiemens
DTT	dithiothreitol
DW	dry weight
E.coli	*Escherichia coli*
EC	electrical conductivity
$EC_{1:5}$	electrical conductivity of a 1:5 soil to water solution
EC_a	apparent electrical conductivity
EC_e	electrical conductivity of a soil extract
EDTA	ethylenediaminetetraacetic acid
EF	elongation factor
EM	electromagnetic
ESP	exchangeable sodium percentage
FAO	Food and Agricultural Organization of the United Nations
FISH	fluorescence in situ hybridization
FW	fresh weight
g	grams(s)
g	gravity
GC	guanine-cytosine, nucleic acid content
gDNA	genomic deoxyribonucleic acid
GFP	green fluorescent protein
GM	genetically modified
GP	Golden Promise
GS	growth stage, of plant
H^+	hydrogen ion
H_2O	water
ha	hectare

HCL	hydrochloric acid
HF	high fidelity
HKT	high affinity potassium channel
hr	hour(s)
Hv	*Hordeum vulgare*
K	potassium
K^+	potassium ion
kb	kilobase pairs, of nucleic acid
kg	kilogram(s)
km	kilometre
km^2	square kilometre
L	litre
LB	luria betani (media or agar)
m	metre(s)
M	molar
min(s)	minute(s)
Mg	magnesium
$MgCl_2$	magnesium chloride
mL	millilitre(s)
mm	millimetre(s)
mM	millimolar
mRNA	messenger ribonucleic acid
mS	milliSiemens
n	sample size
N	nitrogen
N_2	nitrogen, gas
N-terminal	amino (NH_2)-terminal, of protein
Na^+	sodium ion
NaCl	sodium chloride
NAF	asparagine-alanine-phenylalanine motif (NAF in single amino acid code)
nd	not determined
ng	nanograms
NHX	Na^+/H^+ exchanger
NLWRA	National Land & Water Resources Audit
NSCC	non-selective cation channel
nt	line is not transgenic based on genotyping
OGTR	Office of the Gene Technology Regulator
Os	*Oryza sativa*
P	phosphorus
PIC	pre-initiation complex
PCR	polymerase chain reaction

PPC2	protein phosphatase 2C-type
PPI	protein-phosphate interaction
PVC	polyvinyl chloride
QTL	quantitative trait loci
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
S	sulphur
s.e.m	standard error of the mean
SDS	sodium dodecyl sulfate
s	second(s)
SnRK	SNF1 (sucrose non-fermenting 1)-related kinase subgroup
SOS	salt overly sensitive
T_1	progeny of the primary transformant containing transgene
T_2	progeny of T_1
T_3	progeny of T_2
T_4	progeny of T_3
T_5	progeny of T_4
Ta	*Triticum aestivum*
TBP	TATA-box binding protein(s)
TE	tris-EDTA
T_m	melting temperature, of primers
™	unregistered trademark
TGS	transgene silencing
TSS	transcription start site
U	unit(s)
Ubi	promoter of maize *Ubiquitin-1*
UTR	untranslated region, of nucleic acid
UV	ultraviolet
v/v	volume per volume
WA	Western Australia

Abstract

Soil salinity is a major constraint to crop production in Australia. This has prompted the need to produce salt tolerant cereal cultivars, through the understanding of genes involved in salt tolerance mechanisms and manipulating their expression levels. *Arabidopsis thaliana Calcineurin B-like Interacting Protein Kinase 16* (*AtCIPK16*) has been identified as a gene involved in sodium (Na^+) exclusion. Analysis of *AtCIPK16* alleles from Arabidopsis ecotypes suggests variances in expression are due to differences in the promoters. Experiments in Arabidopsis, barley and wheat (preliminary) have illustrated that *AtCIPK16* overexpression can enhance biomass production through increased Na^+ exclusion, although its full effect in barley and wheat has yet to be properly characterised in both greenhouse and field environments.

The first focus of this project evaluated the salt tolerance of *35S:AtCIPK16* barley (cv. Golden Promise) grown under low and high salinity field conditions in 2013 and 2014 at Kunjin, Western Australia. Comparisons between years were difficult due to waterlogging of the 2013 high salt site and the increased variability in plot establishment in 2014. *35S:AtCIPK16* barley lines had varying responses to high salt conditions depending on the annual rainfall. Results showed Na^+ and Cl^- exclusion in certain lines, although this correlated with decreased biomass and yield in high rainfall years. *AtCIPK16* expression also increased Na^+ and Cl^- exclusion in 2012 (a low rainfall year) which instead lead to increasing plant growth and yield.

The second focus of this project aimed to fully characterised the effects of the constitutive expression of *Ubi:AtCIPK16* in wheat (cv. Gladius). Despite conducting three hydroponic experiments, no definitive conclusions about the effects of *AtCIPK16* expression on wheat salt tolerance could be drawn. Although, one sibling transgenic line showed increased Na^+ and Cl^- exclusion from both root and shoot tissue accompanied by larger biomass under 200 mM salt stress. Despite this finding several factors hinder the analysis of data including the high number of null segregants, considerable variability between siblings of the same transformation event and minimal transgene expression.

The third focus of this project aimed to investigate expression differences between two *AtCIPK16* alleles from the Arabidopsis ecotypes Bay-0 and Shahdara. Since the only differences between the two alleles was a 10 base pair deletion in the Bay-0 promoter, it was hypothesised this deletion was the reason for the increased expression of *AtCIPK16* in Bay-0 as it forms a TATA box (TATATAA). The aim of this project was to alter the expression of each allele by: mutating the last A to a T, removing the TATA box in Bay-0, and mutating the T after the TATATA sequence to an A in Shahdara, forming a TATA box without the deletion. Through PCR mutagenesis the required point mutations were introduced into portions of the two promoter alleles, however due to technical difficulties and time constraints the point mutations were not introduced back into the full promoter constructs driving GFP. It was therefore unable to be determined if the point mutations to the TATA box would indeed affect *AtCIPK16* expression.

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.
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Chapter 1: Literature review

1.1 A global problem

By mid-century, food security will be one of the biggest global concerns as the world population undergoes dramatic change, with the rise of developing countries, urbanisation and population growth from 7.39 up to 13.5 billion people (FAO 2009b; United Nations Department of Economic and Social Affairs Population Division 2013). The UN has set a target of increasing crop production by 70 % by 2050, although recent estimates illustrate the need to increase production by over 100 % to meet the predicted increase in food demand (FAO 2009a; Tilman, et al. 2011). Only 10 – 20 % of the growth in production is thought to come from increases in arable land (FAO, 2009a). There is therefore a need to increase the yield per ha of crops, particularly of cereals such as wheat which supports over 2.5 billion people (CIMMYT 2012). Problematically the current rate of production increase is declining with cereal crop production projected to only increase by 0.75 % per annum, a fall from 2 % seen in previous decades (FAO 2009a) . This rate of production needs to increase to be able to meet the predicted demand of four billion tons of cereals annually by 2050 (FAO 2009a; Alexandratos & Bruinsma 2012). This global importance of cereal crops has resulted in huge research efforts towards increasing production and quality.

1.2 Salinity

1.2.1 Salt-affected soils

Soil salinity is one of the serious limitations to increasing crop production worldwide. Despite being a micronutrient, the primary ion involved in salt damage to plants is sodium (Na^+) with additional affects seen from chloride (Cl^-) (Tester & Davenport 2003; Broadley, et al. 2011; Tavakkoli, et al. 2011; Khare, et al. 2015). In Australia, soils are general classified as salt-affected when the electrical conductivity (EC_e) is over 4 dS/m (Abrol, et al. 1988; FAO, 2005). Depending on the exchangeable sodium percentage (ESP) and EC_e, salt-affected soils can be categorised as: saline (low ESP and high EC_e), sodic (ESP >6 and low EC_e) or saline-sodic (high EC_e and ESP) (Isbell 2002; Wicke, et al. 2011). Soil salinity can be inherent due to the accumulation of salts from meteorological and geological processes; or developed as secondary salinity associated with post-settlement variations in the landscape (CRCSLM 1999; NLWRA 2001; Rengasamy 2002; Rengasamy 2006). A variety of process associated with changes in vegetation, groundwater levels, crop management, soil structure

and irrigation practices contribute to soil salinization (Rengasamy 2002; Rengasamy 2006). In many parts of the world these processes interact and exacerbate the effects of soil salinization (Rengasamy & Olsson 1991). The most commonly advertised forms of salinity are dryland (seepage) salinity produced by vegetation changes leading to groundwater rises and transient salinity, a type of salinity that varies seasonally (CRCSLM 1999; NLWRA 2001; Rengasamy 2002; Rengasamy 2006).

1.2.2 Global salinity

In 2011 it was estimated that 1128 million ha of global land was salt affected, with the majority being affected slightly to moderately (Wicke, et al. 2011). A projected 45 million ha (20 %) of irrigated agricultural land and 2 % of the land under dryland agriculture worldwide are classified as salt-affected (Munns & Tester 2008). 580 million ha of land globally has been classed as sodic, with these soils covering much of the arid and semi-arid areas of the world (Rengasamy 2002). These figures and severities are expected to increase within the next few decades due to inappropriate irrigation practices, soil and land management as well as climate change (Rengasamy & Olsson 1991; Pitman & Läuchli 2002).

1.2.3 Salt-affected Australia

Australia's cropping and grain producing regions cover 26.8 million ha (outlined area on Fig. 1.1) with 80 % of the area either currently salt-affected or strongly likely to become salt-affected (Dent & Braaten 2000; Rengasamy 2006; ACLUMP 2010). In 2000 dryland salinity in Australia was projected to affect 5.7 million ha and rise to 17 million ha by 2050, of which 13.6 million ha will be agricultural land; while transient salinity currently affects 250 million ha across Australia (NLWRA 2001; Rengasamy 2002; Rengasamy 2010, Fig. 1.1). All forms of secondary salinity in Australia are estimated to have affected upwards of 840 km^2 and 1970 km^2 of irrigated and dryland agricultural soils respectively in total (Northcote & Skene 1972). With soil salinity already impacting severely on cereal crop production (CRCSLM 1999) and the effects projected to worsen, it is necessary to the long-term success of cropping to produce salt tolerant crops.

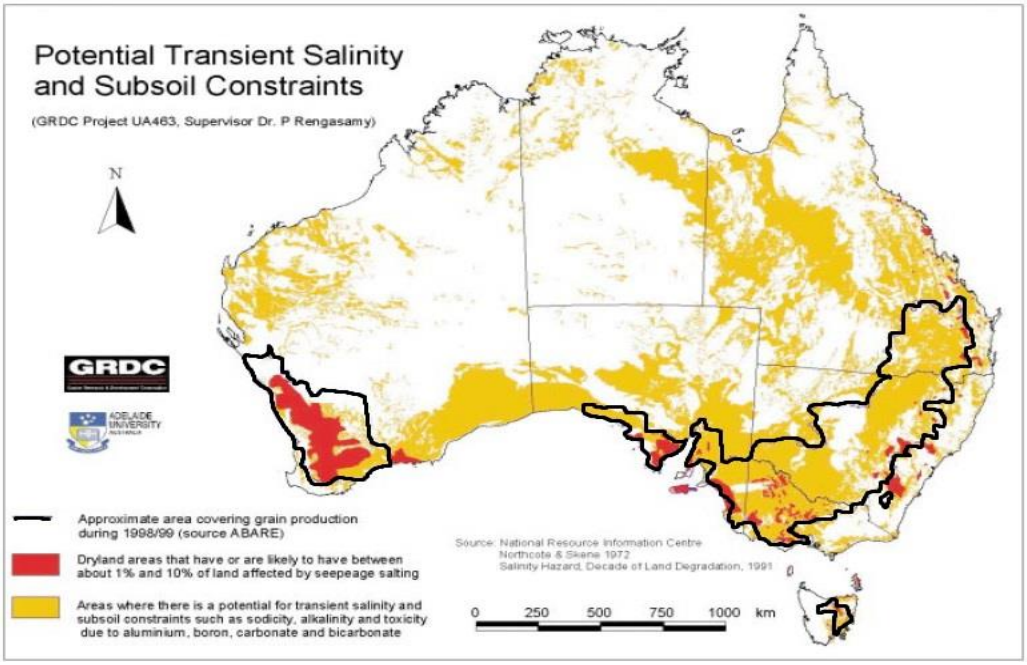


Figure 1.1: Map showing the regions of Australia affected or potentially affected by transient (yellow) and dryland (red) salinity. Area outlined in black is the main grain producing areas of Australia (Rengasamy 2002).

1.3 How salt affects plants

Salt affects plants by imposing two different types of stresses: shoot ion independent stresses (osmotic stress) and shoot ion dependent stresses. The effects of shoot ion independent stresses are seen within minutes to days after salt treatment, before Na^+ can accumulate in the shoot (Munns & Tester 2008; Roy, et al. 2014). Reductions in plant growth, leaf expansion rate, root biomass and tiller number are symptoms of ion independent stress in cereals (Munns, et al. 1995; Zeng & Shannon 2000; Munns & Tester 2008; Shelden, et al. 2013; Ul Haq, et al. 2014). Rapid stomatal closures, CO_2 assimilation reduction and increases in leaf temperature & reactive oxygen species (ROS) formation are all interconnected reactions involved in ion independent stress due to both the presence of soil Na^+ and its effects on water potential (Vaidyanathan, et al. 2003; James, et al. 2008; Sirault, et al. 2009; Khare, et al. 2015).

Longer term plant growth inhibition comes from shoot ion dependent stress, due to the accumulation of toxic levels of Na^+ in shoot tissues (Colmer, et al. 1995; Munns & Tester 2008). The inability to tolerate high concentrations of Na^+ results in the premature senescence of older leaves due to their longer transpiration time and inability to dilute incoming salt through cell expansion (Munns, et al. 1995; Colmer, et al. 1995; Munns 2002; Ul Haq, et al. 2014). This can also impact on the maintenance of growth in younger leaves by depriving the plant of carbon sources and further reducing photosynthetic rates (Munns 2002; Munns & Tester 2008). Plants which are salt tolerant are therefore

described as those that can maintain yield and biomass (or experience only small reductions) when grown under saline conditions (Munns 2002; Flowers 2004). Considerable variation in salt tolerance is seen between different plant species as well as between genotypes, cultivars and ecotypes of the same species, often due to the reliance on different components of salt tolerance (Munns & James 2003; Vaidyanathan, et al. 2003; James, et al. 2008; Munns & Tester 2008; Shelden, et al. 2013); with wheat and barley generally more salt tolerant than many crop species (Munns & Tester 2008).

1.3.1 Sodium toxicity

Na^+ has many detrimental effects on plant growth. Competition between K^+ and Na^+ results in cytosolic Na^+ toxicity, as Na^+ is able to compete with K^+ for protein binding sites and so disrupts normal cellular functions (Tester & Davenport 2003). Na^+ also prevents protein synthesis by interfering with ribosome formation (Hurkman & Tanaka 1987; Tester & Davenport 2003). As Na^+ has so many detrimental effects on growth, plants have developed tolerance mechanisms.

1.4 Salt tolerance mechanisms

1.4.1 Osmotic tolerance

The three main tolerance mechanisms of plants fall into two broad categories, those that mediate ionic stresses and those that affect "osmotic" stresses. Plants with osmotic tolerance are able to maintain leaf growth and keep stomatal apertures open (James, et al. 2008; Rajendran, et al. 2009). Mechanisms possibly involved in osmotic stress tolerance include the sensing of Na^+ and the long distance signalling of the stress, maybe involving ROS or calcium (Ca^{2+}) (Kiegle, et al. 2000; Mittler, et al. 2011; Roy, et al. 2014). Signalling may also involve hormones such as abscisic acid (ABA) or gibberellins (Zhu 2002; Munns & Tester 2008). Understanding the processes behind this tolerance mechanism is of great interest due to its involvement in mild salt stress and the impact it has on growth throughout the growing season (Roy, et al. 2014).

1.4.2 Ionic tolerance

Ionic tolerance involves the regulation of Na^+ transportation as well as tolerance to Na^+ accumulation in tissues. Studies have shown that above average Na^+ exclusion can be a vital salt tolerance mechanism for crops such as wheat (Munns & James 2003; Poustini & Siosemardeh 2004) and barley (Garthwaite, et al. 2005; Qiu, et al. 2010). The regulation of Na^+ transport starts in the rhizosphere, with the regulation of Na^+ influx and efflux to and from the soil. Na^+ is thought to enter through different transporters and channels, including non-selective cation channels (NSCC) and

members of the high-affinity K^+ transporter (HKT) family (Plett & Møller 2010). Several types of NSCCs have been proposed (Demidchik & Maathuis 2007), with reduced expression of these channels under salt conditions resulting in restricted Na^+ influx, contributing to the differences in cultivar salt tolerances, e.g. *OsCNGC1* (Senadheera, et al. 2009). Members of HKT subfamily 2 are also thought to be involved in Na^+ influx in rice (*OsHKT2;1*) (Horie, et al. 2007) and barley (*HvHKT2;1*) (Mian et al. 2011). This has been demonstrated in barley, where plants overexpressing *HvHKT2;1* show increased Na^+ influx into both root and shoot tissues (Mian, et al. 2011). Reductions in the expression of these genes may reduce the amount of Na^+ entering the root.

A second mechanism for reducing root Na^+ accumulation would be the excretion of accrued Na^+ through the salt overly sensitive (SOS) pathway and the plasma membrane Na^+/H^+ antiporter SOS1 (Shi, et al. 2000; Shi, et al. 2002; Qiu, et al. 2003). SOS pathway members operate in many plant tissues and are involved in several salt partitioning mechanisms, including Na^+ efflux into soil, xylem unloading and perhaps vacuolar sequestration (Olías, et al. 2009; Plett & Møller 2010; Ji, et al. 2013).

The exclusion of ions from the shoot involves a third mechanism, the removal of Na^+ from the transpiration stream and the retention of Na^+ in the root, preventing its mobilization to shoot tissue (Munns & Tester 2008; Roy, et al. 2014). Members of the HKT1;x family are involved in the unloading of Na^+ from the xylem (Ren, et al. 2005; Davenport, et al. 2007; Møller, et al. 2009; Plett, et al. 2010). The Na^+ remaining in the root would then be compartmentalised in cortical cell vacuoles or extruded to the soil.

This process is thought to involve tonoplast Na^+/H^+ antiporters (NHXs) and tonoplast H^+ pyrophosphatases (e.g. AVP1), which together enable the movement of Na^+ into the vacuole (Roy, et al. 2014). Which specific transporters are naturally involved in compartmentalisation are still to be confirmed as there is uncertainty about which ions NHXs transport (Bassil, et al. 2011; Barragán, et al. 2012) and alternative roles for *AVP1* have also been suggested (Ferjani, et al. 2011; Gaxiola, et al. 2012). NHXs and tonoplast H^+ pyrophosphatases have also been implicated in intracellular Na^+ sequestration in the shoot, removing Na^+ from the cytosol; a process that contributes to shoot tissue tolerance (Gaxiola, et al. 2001; Brini, et al. 2007). As well as vacuolar sequestration, shoot salt tolerance involves ROS detoxification and compatible solute synthesis (Vaidyanathan, et al. 2003; Sickler, et al. 2007; Lu, et al. 2007; Agarwal, et al. 2013; Khare, et al. 2015). While knowing how salt tolerance mechanisms work is important, understanding the signalling and protein activation processes behind is each mechanism is also vital.

1.5 CBLs and CIPKs

A recent review (Thoday-Kennedy, et al. 2015) on the role of CBLs and CIPKs in regulating the ionic responses of plants to abiotic stresses has been written and published (see Appendix 1). The following is a more detailed review of the literature focusing on AtCIPK16.

1.5.1 Ca^{2+} signalling in plants

Ca^{2+} is the most versatile ion in all eukaryotes with its involvement in many developmental and physiological processes. The use of various membrane channel and transporter proteins allow cells to form stimuli-specific "Ca^{2+} signatures", by storing and releasing Ca^{2+} from internal and external pools at different rates (Sanders, et al. 1999; Sanders, et al. 2002; Dodd, et al. 2010). These "Ca^{2+} signatures" are unique changes, spatio-temporally and cell-specifically, in cytosolic Ca^{2+} concentrations which relay specific stimulus information (Rudd & Franklin-Tong 2001; Knight & Knight 2001; Allen, et al. 2001; Ng & McAinsh 2003). These "signatures" form in response to various stimuli including drought, oxidative, salt and temperature stresses, as well as light and fungal infections (Shacklock, et al. 1992; Knight, et al. 1996; Ehrhardt, et al. 1996; Knight, et al. 1997; Gong, et al. 1998; Evans, et al. 2005; Ranf, et al. 2008; Schmöckel, et al. 2015). To decode incoming Ca^{2+} signals, cells possess a multitude of Ca^{2+} sensor proteins from four main protein families: the calcineurin B-like proteins (CBL), Ca^{2+}-dependent protein kinases (CDPK), calmodulins (CaM) and the calmodulin-like proteins (CML) (Batistič & Kudla 2012). These sensor proteins all bind Ca^{2+} through elongation factor (EF) hand domains and can be further divided into sensor relay or sensor responder proteins (Sanders, et al. 2002). Responder proteins undergo direct conformational alterations upon binding to Ca^{2+} that changes the activity and/or structure of the protein itself (Sanders, et al. 2002). CDPKs are a unique family of sensor responder proteins which undergo intramolecular activation of their kinase domains allowing different isoforms of CDPKs to themselves regulate specific signalling pathways (Sanders et al., 1999; 2002). Relay proteins, like CBLs, CaMs and CMLs, also undergo Ca^{2+}-dependent conformational changes, although lacking an effector domain the Ca^{2+} signal is transduced via target protein interactions (Luan, et al. 2002; Batistič & Kudla 2012). To allow further intracellular localisation of Ca^{2+} signals, many sensor proteins undergo differential post-translational modifications to assist in their anchorage to cellular membranes (Rodríguez-Concepción, et al. 1999; Martín & Busconi 2000; Rodríguez-Concepción, et al. 2000; Dong, et al. 2002; Li, et al. 2008; Batistič, et al. 2008; Batistič, et al. 2010). This sub-cellular Ca^{2+}-determined restriction of sensor protein locations also allows for a localised final response to the original Ca^{2+} signal.

1.5.2 Calcineurin B-like proteins (CBLs)

Originally identified in *Arabidopsis thaliana,* CBLs are so named due to their significant similarity to the yeast and animal proteins, calcineurin regulatory subunit B (CNB) and neuronal calcium sensor (NCS) (Liu & Zhu 1998; Kudla, et al. 1999). *AtCBL4* (Salt Overly Sensitive 3) was the first CBL identified (Liu & Zhu 1997; Halfter, et al. 2000) and therefore many Arabidopsis CBLs were originally identified as SOS3-like calcium binding proteins (ScaBP) (Guo, et al. 2001). Possessing four highly conserved, 12 amino acid (aa) long helix-loop-helix structural domains (EF-hand motifs) (Fig. 1.2), CBLs are able to bind Ca^{2+} through metal binding residues in each motif (Lewit-Bentley & Réty 2000; Kolukisaoglu, et al. 2004). These EF-hands are arranged with absolute conserved spacing (Fig. 1.2), ensuring that all CBLs are approximately the same size, with any size variations attributed to divergence in the C- and N-terminals, particularly the 5'-untranslated region (Kolukisaoglu, et al. 2004). This suggests a conserved 3D structure of all CBLs perhaps to ensure interacting ability with their target proteins calcineurin B-like interacting protein kinases (CIPKs) (Kolukisaoglu, et al. 2004). Sequence variation in EF-hands is seen within and between CBLs, likely leading to variable Ca^{2+} affinities for each domain (Kolukisaoglu, et al. 2004; Batistič & Kudla 2004). Interestingly, for most CBLs the conserved first EF-hand seems unable to bind Ca^{2+} through a number of variable mutations (Kolukisaoglu, et al. 2004). The arrangement of altered EF-hands with variable Ca^{2+} affinity within each CBL may play a part in their deciphering of different Ca^{2+} signatures. To add further complexity to CBL mediated Ca^{2+} signalling, Kolukisaoglu et al. (2004) also found several CBLs with alternative splice forms.

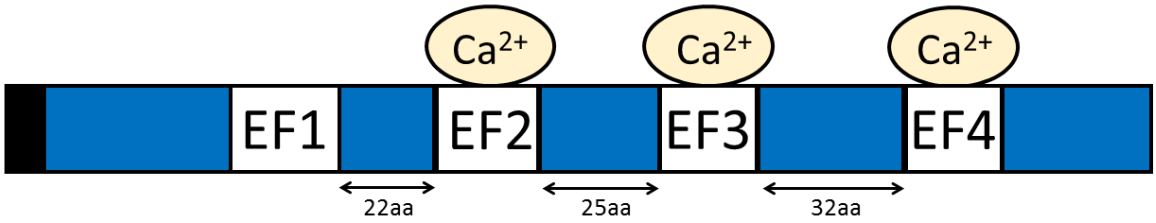


Figure 1.2: General structure of a calcineurin B-like protein (CBL). General CBL structure showing the four elongation factor domains (EF-hands) in white. The conserved spacing of the EF-hands is shown with the exact amino acid (aa) lengths. Black box represents N-terminal post-translational motif.

Many CBLs possess a conserved N-terminal where post-translational modifications occur for stable cellular membrane associations and allow accurate downstream signalling. These N-terminal sequences and modifications seem to be evolutionarily conserved, likely due to constraints on where CBLs can be targeted to as specific Ca^{2+} signal probably only occurs in specific cellular regions (Kleist, et al. 2014). To facilitate and stabilise protein-membrane attachments, CBLs can undergo dual or single myristoylation and S-acetylation of a glycine or cysteine residue respectively in the N-

terminus (Farazi, et al. 2001; Kolukisaoglu, et al. 2004; Huang & El-Husseini 2005; Batistič, et al. 2008; Zhou, et al. 2013). Other CBLs possess an N-terminal hydrophobic domain instead which likely directs membrane localisation and facilitates anchorage (Kim et al. 2007; Tang et al. 2012; 2014). This range of sequences and/or modifications results in CBLs being located to the plasma membrane, tonoplast, nucleus, cytoplasm and other internal membranes (Kim, et al. 2007; Batistič, et al. 2008; Batistič, et al. 2010; Zhang, et al. 2014; Tang, et al. 2014). Although the N-terminal of most CBLs is sufficient to direct targeting, Batistič et al. (2010) also found that full length CBLs can have different localisation patterns to just the N-terminal of specific CBLs fused to GFP. This indicates that for some CBLs cellular localisation is not exclusively determined by the N-terminal sequence or modifications. In many cases post-translational modifications are prerequisites for the function of the CBL-CIPK signalling pathways (Ishitani, et al. 2000; Batistič, et al. 2008; Held, et al. 2011).

1.5.3 Calcineurin B-like Interacting Proteins Kinases (CIPKs)

Originally identified by yeast two-hybrid studies of an Arabidopsis cDNA library, 26 CIPKs have now been identified in Arabidopsis (Shi, et al. 1999; Weinl & Kudla 2009), with another 410 having been identified to date in other algal and plant species. CIPKs belong to the CDPK-SnRK superfamily, classified as SNF1 (Sucrose non-fermenting 1)-related kinase subgroup 3 (SnRK3) proteins (Hrabak, et al. 2003; Weinl & Kudla 2009; Wang, et al. 2015a). CIPKs contain two conserved domains, the N-terminal serine/threonine kinase domain and the C-terminal regulatory domain connected by a junction domain involved in protein activation (Fig. 1.3) (Batistič & Kudla 2004). Within the kinase domain the 11 subdomains typical of serine/threonine kinases are conserved in all CIPKs along with an activation loop (Kolukisaoglu, et al. 2004; Batistič & Kudla 2004). The conserved C-terminal FISL/NAF domain is responsible for CBL binding (Albrecht, et al. 2001; Ye, et al. 2013). This domain allows the formation of hydrophobic bonds between CBLs and CIPKs, stabilising their binding (Sánchez-Barrena, et al. 2007). Variations around the asparagine-alanine-phenylalanine (NAF) motif likely accounts for the range of different CIPK-CBL complexes each CIPK can form (Albrecht, et al. 2001). The C-terminus is involved in auto-inhibition of CIPK activities, through the NAF domain binding to the kinase domain which structural analysis has confirmed is released upon CBL binding (Guo, et al. 2001; Sánchez-Barrena, et al. 2007; Chaves-Sanjuan, et al. 2014). The removal of the C-terminal of CIPK proteins causes the formation of a hyperactive CIPK protein, further illustrating the role of the C-terminus in regulating kinase activity (Guo, et al. 2001; Quintero et al. 2002). It has also been shown that proteins other than CBLs can interact at the NAF/FISL domain, such as NDPK2 which upon binding causes the inhibition of AtCIPK24 auto-phosphorylation (Verslues, et al. 2007).

SRK2D, a SnRK2 protein kinase known to regulate ABA signalling, has also been shown to interact with and be phosphorylated by AtCIPK3, AtCIPK9, AtCIPK23 and AtCIPK26, although the interaction mechanism is as yet unknown (Mogami, et al. 2015). The CIPK regulatory domain contains a 37 aa protein-phosphate interaction (PPI) domain next to the NAF domain (Fig. 1.3) which is involved in CIPK-PPC2 (protein phosphatase 2C-type) interactions, eg. ABA-insensitive 1 (ABI1), 2 (ABI2) and 5 (ABI5) (Ohta, et al. 2003; Lyzenga, et al. 2013; Zhou, et al. 2015b). These CIPK-PPC2 complexes are implicated in the regulation of Arabidopsis K^+ transporter 1 (AKT1) activity as well as early ABA signal transport (Lee, et al. 2007; Kudla, et al. 2010). The N-terminal sequence for CIPKs has also been implicated in determining which CBL binds to the protein, potentially blocking access of some CBLs binding which may otherwise have bound to the C- terminal of the protein (Kim, et al. 2000; Li, et al. 2009).

Figure 1.3: Overall structure of a CIPK showing the N-terminus serine/threonine kinase domain, with the activation loop (horizontal lines) and the C-terminus regulatory domain. In between the two is the junction domain which is also involved in kinase activation. The regulatory domain contains the NAF domain, responsible for CBL-CIPK interaction, and the PPI domain where protein phosphatases may bind.

The gene structure of CIPKs from all species can be divided into two subgroups, intron-rich and intron-poor, with evidence of intron loss and acquisition in all lineages (Hrabak, et al. 2003; Kolukisaoglu, et al. 2004; Ye, et al. 2013; Kleist, et al. 2014). Like CBLs, different alternatively spliced isoforms of some CIPKs have identified, often resulting in different C-termini and potentially changing auto-inhibition, CBL binding and other protein binding capabilities of the CIPK (Kolukisaoglu, et al. 2004; Imamura, et al. 2008; Chen, et al. 2011b). When CIPKs are not bound to a CBL they generally exhibit localisation to the nucleus and cytoplasm, however, this changes upon CBL binding and translocation, with CBL/CIPK complexes being observed on the plasma membrane, tonoplast and other cellular organelles (Cheong, et al. 2007; Kim, et al. 2007; Waadt, et al. 2008; Batistič, et al. 2010; Held, et al. 2011; Drerup, et al. 2013; Zhang, et al. 2013a).

Within the CIPK kinase domain, highly conserved amino acids are targets for phosphorylation by other protein kinases, with the conversion of these residues to aspartate resulting in CBL non-dependent hyperactivity (Gong, et al. 2002a; Gong, et al. 2002b; Gong, et al. 2002c; Gong, et al. 2002d; Batistič & Kudla 2004). These auto- and/or trans-phosphorylation sites indicate potential targets for regulation of CIPKs by other kinases and highlight probable points of cross-talk between cellular signalling and regulatory pathways (Kolukisaoglu, et al. 2004; Batistič & Kudla 2004). This complex regulation and specificity of CIPKs is a vital part of CIPK-CBL signalling.

1.5.4 CBL-CIPK signalling pathways

The CBL-CIPK signalling network is a vital part of the plant response system to abiotic stresses allowing plants to respond to changes in cytosolic Ca^{2+}. The CBL-CIPK interaction model proposes that upon the occurrence of a stress specific Ca^{2+} signature, Ca^{2+} is able to bind to specific CBLs allowing them to bind to and activate desired CIPKs resulting in the phosphorylation of the appropriate targeted proteins. In some cases CBL-CIPK interactions only occur in the presence of Ca^{2+} while it seems that other CBLs and CIPKs can bind independently of Ca^{2+}, with Ca^{2+} acting as an interaction fine tuner (Shi, et al. 1999; Halfter, et al. 2000; Sánchez-Barrena, et al. 2007; Akaboshi, et al. 2008; Mähs, et al. 2013; Lin, et al. 2014). This ability for some CBLs to bind CIPKs independently of Ca^{2+} while others need Ca^{2+} to interact may represent two different CBL-CIPK binding pathways (Kim 2012). One needs Ca^{2+} to induce CBL conformational changes before it can bind to a CIPK, blocking the CIPKs auto-inhibitory nature (e.g. AtCBL1-AtCIPK1); while the other pathway allows the CBL to bind to the CIPK independently of Ca^{2+} but needs Ca^{2+} present to allow the CIPK kinase to be fully functional (e.g. AtCBL4-AtCIPK24 or AtCBL2-AtCIPK14) (Shi, et al. 1999; Halfter, et al. 2000; Akaboshi, et al. 2008; Kim 2012). Once the CBL-CIPK interaction occurs, the tail sequence and/or post-translational modifications on the CBL direct the complex to the required site of activity (Quan, et al. 2007; Cheong, et al. 2007; Batistič, et al. 2008; Batistič, et al. 2010; Held, et al. 2011; Drerup, et al. 2013). The PPI domain in the CIPK may bind directly with membrane phospholipids (Sánchez-Barrena, et al. 2013), which may stabilise CBL-CIPK phosphorylation of membrane bound targets. Another critical component of CBL-CIPK interactions is the phosphorylation of CBLs by their interacting CIPKs, which is required for correct phosphorylation and regulation of a target protein by the CBL-CIPK complexes (Mahajan, et al. 2006; Lin, et al. 2009; Held, et al. 2011; Du, et al. 2011; Hashimoto, et al. 2012).

CBL-CIPK interactions have been identified in multiple species including brassica species (Kushwaha, et al. 2011; Chen, et al. 2012; Zhang, et al. 2014), cereals (Martínez-Atienza, et al. 2007; Zhao, et al. 2009; Kurusu, et al. 2010; Deng, et al. 2013a; Kanwar, et al. 2014; Sun, et al. 2015), fruits (Hu, et al. 2012; Huertas, et al. 2012; Cuéllar, et al. 2013; de la Torre, et al. 2013; Farani, et al. 2015), legumes (Mahajan, et al. 2006; Hamada, et al. 2009; Tominaga, et al. 2010; Meena, et al. 2015b) and poplar species (Tang, et al. 2010; Zhang, et al. 2013a; Tang, et al. 2014; Lv, et al. 2014). Transgenic experiments have shown that CBLs and CIPKs from one species can interact with those from another species, illustrating the conserved nature of CBL-CIPK interaction in plant species (Kim, et al. 2003b; Hwang, et al. 2005; Martínez-Atienza, et al. 2007; Tripathi, et al. 2009; Yoon, et al. 2009; Hu, et al. 2012; Wang, et al. 2012; Chen, et al. 2012; Deng, et al. 2013a; Meena, et al. 2015a).

Most CIPKs are capable of forming complexes with multiple CBLs, albeit not at the same time. This may result in different CBLs acting with the same CIPK simultaneously in the same cell or different tissues in response to the same stress (Xu, et al. 2006; D'Angelo, et al. 2006; Quan, et al. 2007; Cheong, et al. 2007; Kim, et al. 2007; Waadt, et al. 2008; Batistič, et al. 2010). Functional redundancy is also present in some CBL-CIPK pathways with some CBLs appearing to compensate for the lack of another. AtCBL2 and AtCBL3, as well as AtCBL1 and AtCBL9 were able to compensate for each other, as shown by the need to have double knockout mutants to produce noticeably abnormal phenotypes (Xu, et al. 2006; Cheong, et al. 2007; Tang, et al. 2012; Eckert, et al. 2014).

1.5.5 Examples of CBL-CIPK pathways

While hundreds of CBL and CIPK genes have been identified, the CBL-CIPK interactions and involvement in pathway regulation for most of these genes have yet to be reliably demonstrated (Thoday-Kennedy, et al. 2015). CBL-CIPK signalling has been implicated in plant responses to abiotic stresses such as cold, drought and heat (Cheong, et al. 2003; Kim, et al. 2003a; Albrecht, et al. 2003; Pandey, et al. 2004; Pandey, et al. 2008; Chen, et al. 2011a) as well as ionic abiotic stresses (reviewed in Thoday-Kennedy et al. 2015). As well as being involved in abiotic stress tolerance, many CBLs and CIPKs regulate pathways associated with general development and growth as well as biotic stress tolerance (Cheong, et al. 2003; Mahajan, et al. 2006; Kurusu, et al. 2010; Yan, et al. 2014; Zhou, et al. 2015a). Examples of known CBL-CIPK pathways include CBL1/CBL9 – CIPK23 complexes regulating AKT1 activity, CBL9 – CIPK23 regulation of NRT1.1 nitrate affinity and the Salt Overly Sensitive (SOS) pathway (reviewed in Thoday-Kennedy et al. 2015). The SOS pathway operates throughout the plant regulating Na^+ exclusion from the root, xylem and shoot, particularly in

response to a salt stress (Shi, et al. 2000; reviewed in Plett & Møller 2010 and Ji, et al. 2013). Under salt stress the plasma membrane Na^+/H^+ antiporter, SOS1, is activated when phosphorylated by CIPK24 (SOS2) bound to either CBL4 (SOS3) or CBL1, causing the efflux of Na^+ from plant cells (Qiu, et al. 2002; Quintero, et al. 2002; Kolukisaoglu, et al. 2004; Kim, et al. 2007). Particularly in the shoot, SOS2 through interactions with CBL10, is also involved in Na^+ sequestration by regulating a tonoplast Na^+/H^+ antiporter (Qiu, et al. 2004; Kim, et al. 2007). It has also been suggested that SOS2 is able to target other proteins associated with the plasma membrane, tonoplast and endosomes (Kolukisaoglu, et al. 2004; Quan, et al. 2007; Kim, et al. 2007; Huertas, et al. 2012). The SOS pathway and individual components has been identified in many plant species, and overexpression of these components has led to increased Na^+ efflux and sequestration, thus increased plant salt tolerance (reviewed in Thoday-Kennedy et al. 2015). While much of the role of the SOS pathway in salt tolerance has been resolved, many other CBLs and CIPKs have been found to respond to salt stress and deserve further investigation.

1.6 AtCIPK16

1.6.1 *Arabidopsis thaliana Calcineurin B-like Interacting Protein Kinase 16*

Through Quantitative Trait Locus (QTL) mapping for shoot Na^+ exclusion in a Bay-0 × Shahdara Arabidopsis recombinant inbred line (RIL) mapping population, a novel QTL associated with this trait was identified (Roy, et al. 2013). Fine mapping of this QTL on chromosome 2 narrowed the region down to 41 genes from which *AtCIPK16* was identified as the most likely candidate gene (Roy, et al. 2013). Analysis showed the positive allele came Bay-0, with the Bay-0 allele lines having a 47 % reduction in shoot Na^+ accumulation compared to those with the Shahdara allele (Roy, et al. 2013). Sequence analysis of both alleles of *AtCIPK16* revealed no coding sequence differences, instead the only significant variation was a 10 bp deletion in the promoter region, 22 bp 5' of the start of transcription and 65 bp 5' of the start codon in the Bay-0 allele (Roy, et al. 2013). This resulted in the formation of a TATA box in the Bay-0 promoter by the addition of an A to the existing TATATA sequence found in both alleles (Fig. 1.4). The formation of a TATA box, which is known to naturally enhance gene expression through increased recruitment of RNA polymerases, may explain the differences in gene expression seen between the two alleles (Nikolov & Burley 1994; Roy, et al. 2013). *AtCIPK16* transcript was only detected in root tissue, with the expression of the Bay-0 allele considerably higher than the Shahdara allele under both control and salt conditions (Roy, et al. 2013). Promoter-GFP fusions showed that the Bay-0 allele promoter drove increased GFP fluorescence under salt conditions, and thus likely drives higher *AtCIPK16* expression under these conditions (Roy,

et al. 2013). The expression pattern of *AtCIPK16* in root stellar tissue infers that AtCIPK16 may be involved in regulating Na^+ transport through xylem unloading (Roy, et al. 2013). *AtCIPK16* overexpression in Arabidopsis and barley caused increased salt tolerance through larger biomass and decreased in Na^+ accumulation for both hydroponic and soil grown plants; while knockdown Arabidopsis lines showed increased Na^+ accumulation (Roy, et al. 2013). Preliminary experiments with field grown transgenic *AtCIPK16* expressing barley and hydroponically grown *AtCIPK16* expressing wheat also suggest that under high salt conditions these plant have increased salt tolerance through reduced shoot Na^+ accumulation (Roy, et al. unpublished).

Elucidating the targets and interacting CBLs of AtCIPK16 is still ongoing work. AtCBL1 and AtCBL9 have repeatedly been shown to complex with AtCIPK16, with possible interactions also shown for AtCBL2, AtCBL3, AtCBL4/SOS3, AtCBL5 and AtCBL10 (Kolukisaoglu, et al. 2004; Lee, et al. 2007; Huang 2015). This suggests that AtCIPK16 may be involved in the response to many different stresses. AtCIPK16 may be involved in K^+ uptake through the regulation of the voltage-gated K^+ channel AKT1 as it can bind to the AKT1 ankyrin repeat domain (Lee, et al. 2007). Bimolecular fluorescence complementation (BiFC) experiments have shown many of the AtCBLx-AtCIPK16 complexes are targeted to the nucleus as well as a few to the cytosol and plasma membrane, which along with a nuclear localization sequence in the junction domain, suggests that AtCIPK16 may have a novel role in gene expression regulation under stress (Huang 2015; Amarasinghe in press).

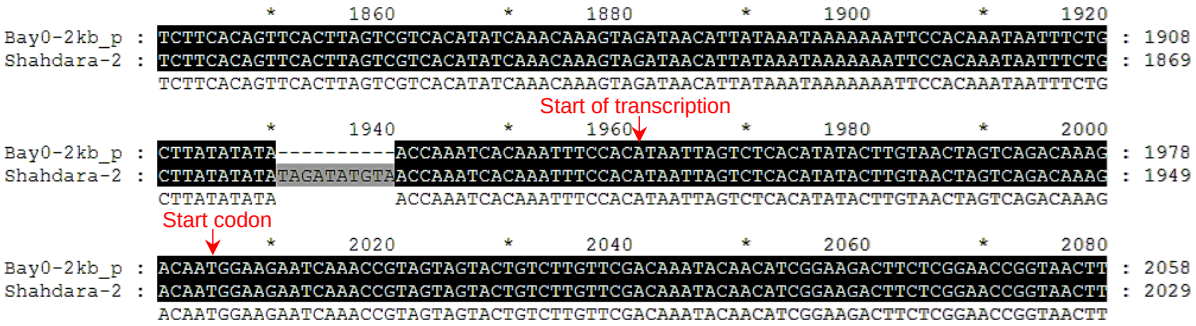


Figure 1.4: Sequence alignment of the region of interest of the *AtCIPK16* promoter and gene. The alignment of both Bay-0 and Shahdara alleles shows the deletion in the promoter of the Bay-0 *AtCIPK16* gene results in the addition of a TATA box element. The TATATA sequence is present in both alleles with the deletion adding an A to the end of the Bay-0 sequence forming the TATA box, instead of the T present in the Shahdara allele. The start of transcription and the start codon are marked. (sourced from Roy, et al. 2013)

1.6.2 Other *CIPK16*s

Sequence analysis of CIPKs has revealed that AtCIPK16 forms its own clade, with AtCIPK5 and AtCIPK25 being the closest related CIPKs to AtCIPK16 (Wang, et al. 2015a). Analysis has also shown that CIPK16, is unique to the Brassicales (Amarasinghe in press). This gene arose after the divergence of the last common ancestor of the Brassicales from the rest of the dicots, where a whole genome duplication event occurred giving rise to *AtCIPK16* (Amarasinghe in press). This means that while genes labelled CIPK16s (which share some sequence similarity to *AtCIPK16*) have now been identified in brachypodium, rice, maize, sorghum and wheat, no orthologs of *AtCIPK16* will ever be identified in cereal crops (Kolukisaoglu, et al. 2004; Zhao, et al. 2009; Li, et al. 2010; Chen, et al. 2011b; Sun, et al. 2015; Wang, et al. 2015a). It therefore should be noted that genes such as *OsCIPK16* and *ZmCIPK16* were annotated so because they were usually the sixteenth CIPK to be identified in that species. They are therefore not orthologs of *AtCIPK16* and should not be expected to perform the same cellular functions.

1.7 Research Aims

AtCIPK16 has been suggested as a possible target for manipulation to improve the salt tolerance of important crop species. Preliminary work in Arabidopsis and barley has demonstrated that the expression of *AtCIPK16* in transgenic plants may indeed improve salt tolerance through the maintenance of biomass and decreased shoot Na^+ accumulation (Roy, et al. 2013; Roy, et al. unpublished). The aim of this project is to further characterise the effects of *AtCIPK16* expression on salt tolerance in wheat and barley and to investigate the effect of the TATA box in native *AtCIPK16* gene expression. These aims will be met by the following objectives:

1. To further characterise the effects of *35S:AtCIPK16* expression on field grown barley (cv. Golden Promise) in 2013 and 2014
2. To evaluate the salt tolerance and Na^+ exclusion capacity of *Ubi:AtCIPK16* wheat (cv. Gladius) in hydroponic systems
3. To demonstrate, via editing of the AtCIPK16 promoter, that the presence/absence of a TATA box causes differences in gene expression between Arabidopsis ecotypes

Chapter 2: Evaluation of *35S:AtCIPK16* Golden Promise barley lines under field conditions in 2013 & 2014

2.1 Introduction

Australia is one of the ten highest producers of barley worldwide, with over $2199 million worth exported in 2014 (ABARES 2014; http://faostat.fao.org/). Barley is considered one of the more salt tolerant crops grown, with yield penalties not occurring until soil electrical conductivity (EC_e) is in excess of 8 dS/m and 50 % yield reductions not reached until soil EC_e exceeds 18 dS/m (Maas & Hoffman 1977; Richards, et al. 1987; Chesworth 2008; Munns & Tester 2008). With less Na^+ exclusion than other crops, barley relies on tissue tolerance mechanisms such as vacuolar sequestration to cope with Na^+ influxes into shoot tissue, particularly under salt stress conditions (Munns, et al. 1995; Munns, et al. 2002; Garthwaite, et al. 2005; James, et al. 2006b; Roy, et al. 2014). Despite these tolerance mechanisms there may be room to improve the overall salt tolerance of barley through the manipulation of other mechanisms such as Na^+ exclusion. One candidate gene for enhancing Na^+ exclusion in plants via transgenic methods is *AtCIPK16* (Roy, et al. 2013). *AtCIPK16* was identified in an Arabidopsis mapping population, where overexpression resulted in increased salt tolerance via decreased shoot Na^+ accumulation (Roy, et al. 2013). Hydroponic experiments with transgenic *35S:AtCIPK16* barley lines have shown results similar to those seen in Arabidopsis (Roy, et al. 2013). Transgenic plants had approximately 30 % more biomass compared to non-transgenic plants with a corresponding drop of 10 % in leaf Na^+ concentration under 300 mM salt stress (Roy, et al. 2013). A small scale preliminary field trial with these lines under non-saline and saline field conditions was conducted in 2012, which showed promising results for increased salt tolerance (Roy, et al. unpublished, see Appendix 2). This chapter will outline the work conducted to further evaluate the effects on *AtCIPK16* expression in barley on Na^+ accumulation, biomass, yield and overall salt tolerance, over the course of two field trial seasons, 2013 and 2014.

2.2 Materials and Methods

2.2.1 Environmental characterisation of field trial site

The field trial site (83 m length × 32 m wide) was located in the locality of Kunjin, near Corrigin in the wheat belt of Western Australia (Longitude: 117.734118, Latitiude: -32.340058). The long-term average annual rainfall for this area was 372 mm, with a mean maximum temperature of 24°C (Weather Station 010536, Corrigin WA, http://www.bom.gov.au/climate/). Rainfall and temperature data for 2013 and 2014 had been collected from http://www.bom.gov.au/climate/ for analysis. Soil at the site was comprised of 90 % sand, 5 % clay and 5 % silt and was therefore classified as a sandy soil (Schilling, et al. 2014). An electromagnetic (EM) map of the trial site (Fig. 2.1) showed a gradient in the electrical conductivity ranging from a high salinity (blue) over EC_e 30 dS/m to a low salinity (red) under EC_e 3.5 dS/m (Schilling, et al. 2014). The low salt trial site was located at the southern end of field trial site with EC_e ranging from approximately 2 – 6 dS/m. The high salt trial site was located at the northern end of field trial site with EC_e ranging from approximately 15 – 27 dS/m. Both trial sites were of equal size, 15.5 – 17.5 m length × 11 – 13.5 m width, with the high salt site was separated from the low salt site by 4 – 8 m; measurements varied due to changes in whole trial size between years.

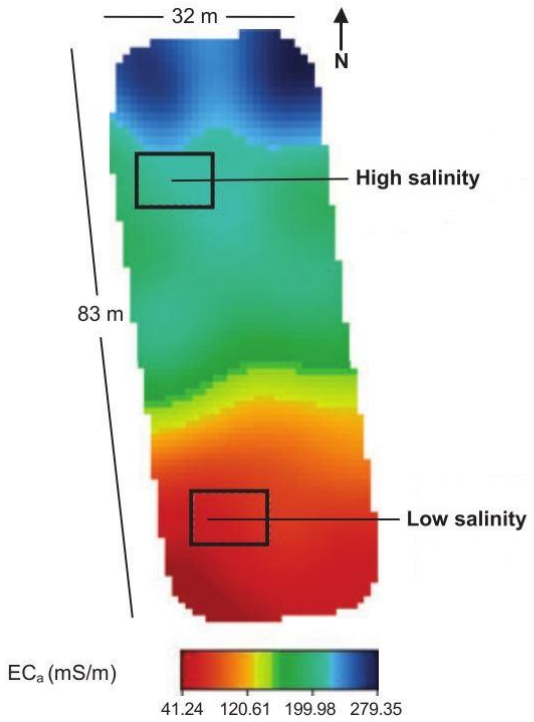


Figure 2.1: EM38 map of the field trial site in Kunjin, WA (83 m length × 32 m width) showing the apparent electrical conductivity (EC_a). Red indicates low EC and blue high EC. Black rectangles indicate the location of the saline and non-saline plots in the field trial site. (sourced from Schilling, et al. 2014)

2.2.2 Plant material

Evaluation of salt tolerance was carried out on three independently transformed transgenic *35S:AtCIPK16* lines, generated as described in Roy, et al. (2013), and wildtype barley (cv. Golden Promise) which had previously been grown at this site. These lines were previously characterised and found to be expressing a single copy of the AtCIPK16 transgene (Roy, et al. 2013). For each field trial, seed produced in the previous year was used to sow the following year's trial plots. Seed from the highest yielding low salt plot for each line was used to sow all plots (low and high salinity) for that line in the next year. T_4 seed, produced in 2012, was used in 2013 and T_5 seed generated during the 2013 field trial was sown in 2014.

2.2.3 Field trial of transgenic barley

Each year the field trial design was completely randomised with three plots (2 m length × 1.2 m width) per line in both the low salt site and the high salt site. In accordance with GM field trial regulations the field trial sites for each year switched locations within in the field area. In 2012 and 2014 the trial sites were on the eastern side of the area, while in 2013 the sites were on the western side of the area. Plots were sown in June 2013 and 2014 at a sowing rate of 160 plants per plot, using a no till system (Kalyx Australia, Perth, Western Australia). Standard agricultural practices were used each year including the application of fertilisers (Table 2.1) as well as herbicides, fungicides and insecticides (Table 2.2). In late September 2013 and 2014, shoot and leaf tissue were sampled, as well as tiller number counted, for 3 or 6 plants per plot (depending on plot establishment). A 4 cm of green leaf blade was collected for genotyping and a fully emerged (youngest emerged blade or flag leaf -1) leaf was taken for ion measurements. Shoot material and ion leaves were dried for 2 days at 70°C in an oven (Contherm Scientific Ltd, Wellington, New Zealand) and weighed. In September 2014 photographs of each plot was taken to compare establishment and plant density. In early December 2013 and 2014 grain was sampled from 3 or 6 different plants per plot (depending on plot establishment) and the number of grain heads, number of grains and grain weight per plant as well as 100 grain weight was recorded. Grain yield per plot was provided by Kaylx Australia after final harvest in December.

Sampling and analysis of material from the 2012 field trial was conducted by Stuart Roy, and the data kindly provided for comparison. Sampling of the 2013 field trial was done prior to this project and the analysis was conducted as part of this project. Sampling and analysis of the 2014 field trial was conducted as part of this study.

Table 2.1: Fertilisers applied during 2013 and 2014 field at Kunjin, WA. GS= growth stage according to Zadocks score.

	Crop Stage	**Product**	**Rate**	**Placement**
2013	At sowing	Gusto Gold (10.2N:13.1P:12K:7.2S) (Summit Fertilisers)	100 kg ha^{-1}	With seed
		Urea	100 kg ha^{-1}	Topdressed & Incorporated by sowing
	GS30	Urea	60 kg ha^{-1}	Topdressed
2014	At sowing	Gusto Gold (10.2N:13.1P:12K:7.2S) (Summit Fertilisers)	100 kg ha^{-1}	Banded
		Urea	100 kg ha^{-1}	Topdressed

Table 2.2: Herbicides, fungicides and insecticides applied during 2013 and 2014 field trials at Kunjin, WA. GS= growth stage according to Zadocks score.

	Crop Stage	**Product**	**Brand**	**Rate**
2013	At sowing	Roundup® Attack™	Nufarm	2 L ha^{-1}
		Boxer Gold®	Syngenta	2.5 L ha^{-1}
		Chlorpyrifos/ Lorsban®	Dow AgroScience	2.5 L ha^{-1}
	GS30	Axial®	Syngenta	500 mL ha^{-1}
		Velocity®	Bayer	1 L ha^{-1}
		Lontrel™	Dow AgroScience	120 g ha^{-1}
		Hasten™	Vicchem	1% v/v
	GS32	Prosaro®	Bayer	300 mL ha^{-1}
		Alphacypermethrin/ Astound®	Nufarm	300 mL ha^{-1}
		Chlorpyrifos/ Lorsban®	Dow AgroScience	300 mL ha^{-1}
2014	At sowing	Boxer Gold®	Syngenta	2.5 L ha^{-1}
		Chlorpyrifos/ Lorsban®	Dow AgroScience	1 L ha^{-1}
	Post-emergence	Chlorpyrifos/ Lorsban®	Dow AgroScience	500 mL ha^{-1}
		Talstar®	FMC	400 mL ha^{-1}
		Alphacypermethrin/ Astound®	Nufarm	400 mL ha^{-1}
	GS32	Tilt® Xtra	Syngenta	500 mL ha^{-1}
		Alphacypermethrin/ Astound®	Nufarm	300 mL ha^{-1}

2.2.4 DNA extraction and genotyping analysis

Genomic DNA (gDNA) was extracted from leaf tissue using a modified Edwards, et al. 1991 protocol. Leaves were ground in 2 mL tubes with 3 small steel ball bearings using a vortex and a small pre-chilled needle. Ball bearings were removed before 600 µL of extraction buffer (0.1 M Tris-HCl (pH 7.5), 0.05 M EDTA (pH 8), 1.25 % SDS) was added to ground tissue, shaken and incubated at 65°C for 45 mins. Samples were then cooled in a 4°C fridge for 15 mins before 300 µL of chilled 6 M ammonium acetate was added. Samples were again incubated at 4°C for 15 mins than centrifuged for 15 mins at 2300 *g*. Volumes of 600 µL of supernatant were transferred to new tubes containing 360 µL of isopropanol, which were mixed and incubated at room temperature for 30 mins to allow DNA to precipitate. To pellet DNA, samples were centrifuged at 2300 *g* for 15 mins with the resulting supernatant discarded. Pellets were then cleansed by added 200 µL of 70 % (v/v) ethanol and centrifuging for 2 mins at 2300 *g*. Supernatant was discarded and samples re-centrifuged at 2300 *g* for 2 mins, before any further supernatant was discarded. Samples were left to air dry after which they were resuspended in 35 µL of R40 (40 ng/mL RNase A in 1× TE buffer). To quantify DNA concentration, 1 µL aliquots of extracted DNA were loaded on a spectrophotometer (Nanodrop ND-100, Thermo Fisher Scientific, MA, USA). Finally, DNA samples were stored at -20°C.

In each plant the presence or absence of the *HvVRT2* vernalisation gene (GenBank DQ201168) was used as a control to check the success of the extractions. *HvVRT2* was amplified using PCR and the *HvVRT2* specific forward primer 5' - CCG AAT GTA CTG CCG TCA TCA CAG - 3' and reverse primer 5' - TGG CAG AGG AAA ATA TGC GCT TGA - 3' which amplified a 280 bp fragment. The PCR conditions used to amplify *HvVRT2* were as follows: an initial denaturation at 94°C for 2 mins, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s and extension at 68°C for 30 s, followed by a final extension at 68°C for 5 mins. The presence or absence in each plant of the transgene *AtCIPK16* (At2g25090) was identified using primers specific to *AtCIPK16* which bind in a unique region and amplify a short 230 bp fragment: forward primer 5' - ACT CTC AAG ATT GCT TGT GCC G - 3' and reverse primer 5' - TGA TGT GAT GAA TTG GAA GGC G - 3'. The PCR conditions used to amplify *AtCIPK16* were as follows: an initial denaturation at 94°C for 2 mins, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 30 s and extension at 68°C for 30 s, followed by a final extension at 68°C for 5 mins. All PCRs were performed with either OneTaq® or CrimsonTaq® (New England Biolabs) in 25 µL reaction volumes. Each reaction contained 1 µL of extracted gDNA, 5 µL 5× OneTaq standard reaction buffer/ 5× CrimsonTaq (with Mg) reaction buffer, 0.5 µL 10 mM dNTPs, 0.625 U OneTaq/ CrimsonTaq DNA polymerase, 5 µL 10 mM forward

primer and 5 µL 10 mM reverse primer for the appropriate gene. PCR products were visualised using gel electrophoresis with a 1.5 % agarose gel containing 5 µL/100 mL SYBRsafe® stain (Invitrogen).

2.2.5 Soil analysis of field trial plots

Soil was collected from the top 10 cm using a hollow stainless steel pipe (N Schilling Inc, Culburra, Australia) from each plot. A CyberScan PC 510 meter (Eutech Instruments, Thermo Fisher Scientific Inc., Waltham, Ma, USA) was used to measure electrical conductivity (EC) and pH in a 1:5 (soil: water) extract, after samples had been shaken on an orbital shaker for 1 hr and left to settle for 30 mins. $EC_{1:5}$ was converted to EC_e using the formula EC_e (dS/m) = ($EC_{1:5}$ (µS/cm) × 22.7) / 1000 (Slavich & Petterson 1993). The Na^+, K^+ and Cl^- concentrations of the soil extracts were determined using a flame photometer (Model 420, Sherwood Scientific, Cambridge, UK) and a chloride analyser (Model 926, Sherwood Scientific, Cambridge, UK), as per manufacturer's instructions.

2.2.6 Ion analysis of leaf tissue

The fully expanded (youngest emerged blade or flag – 1) leaf from each plant was oven dried for 2 days at 70°C and subsequently weighed. Leaves were then digested in 1 % (v/v) nitric acid at 80°C for 4 hr in a 54-well HotBlock (Environmental Express, Mount Pleasant, SC, USA). The Na^+ and K^+ concentrations of the digested leaves were determined using a Sherwood 420 flame photometer (Sherwood Scientific, Cambridge, UK). A chloride analyser (Model 926, Sherwood Scientific, Cambridge, UK) was used to measure the Cl^- concentrations of the digested leaves.

2.3 Results

2.3.1 Environmental characterisation of field trial site

The total rainfall at Corrigin in 2013 was 444 mm, with 250 mm falling within the growing season (June to December) which was well above the long term average. January and July had extremely high total rainfall due to extreme rainfall events, as illustrated in Fig. 2.2A. July was of particular interest, as 112 mm of rain was received, 60 % of which occurred on one day (12th July 2013), which was three weeks after sowing. This rainfall event resulted in the waterlogging of the high salt site, which prevented plant establishment in most plots. In the 2013 growing season, mean maximum temperatures differed little from the long term average except in the latter months where temperatures were on average 2°C warmer (Fig. 2.2B). 2014 had above average rainfall, with the area receiving 398 mm in total and 247 mm throughout the growing season, although the rainfall was more evenly spread across all months, except October (when grain filling occurred) which received above average rainfall (Fig. 2.2A). The mean maximum temperature during the growing season was slightly higher (2 – 3°C) than the average especially between August and November.

In 2013 soil analysis revealed few differences between plots with minimal variation in soil EC_e or pH (Sup. Fig. 2.2), similar to the soil pH results in 2014 (Sup. Fig. 2.3E). Soil analysis showed a wide range of EC_e, Na^+, K^+ and Cl^- values across the trial site in 2014, with considerable variation within both the low and high salt trial sites, which resulted in the data being analysed based on soil EC_e rather than site location (Sup. Fig. 2.3).

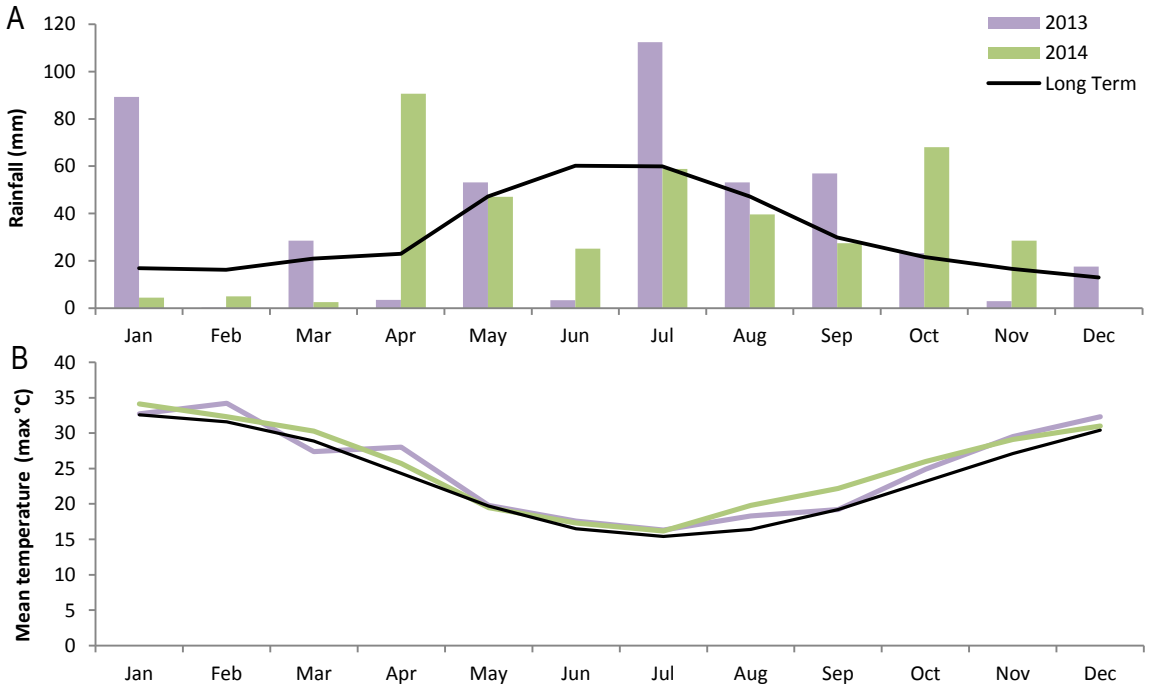


Figure 2.2: Average rainfall (mm) (A) and maximum temperature (°C) (B) at Corrigin, Western Australia for the year 2013 (purple) and 2014 (green). Black represents the long term last 65 year average. Rainfall for each month is the total monthly rainfall. Temperature for each month is the mean maximum daily temperature. Data from weather station 010536 (http://www.bom.gov.au/climate/).

2.3.2 Transgenic *AtCIPK16* barley show variations in plant growth

Presence of the native *HvVRT2* gene was confirmed in gDNA samples from plants in each transgenic line, null segregants and wildtype plants using PCR (Fig. 2.3A). PCR analysis also confirmed the presence of the *AtCIPK16* transgene in transgenic plants from all three transgenics lines, and the absence of the transgene in the null segregant and wildtype plants (Fig. 2.3B).

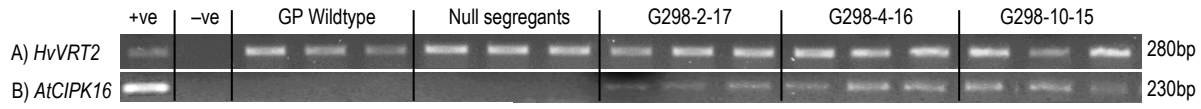


Figure 2.3: Electrophoresis gel showing presence of the native *HvVRT2* gene (A) and the *AtCIPK16* transgene (B) in extracted gDNA from wildtype, null segregant and three *AtCIPK16* expressing barley lines grown at Kunjin, WA. Each gel includes a positive control (+ve), a water negative control (-ve) and three replicates from each line.

While variation in plot establishment and plant growth is to be expected in field trials, 2014 saw a much wider array of plot establishment in both the low salt and high salt trial sites. In previous years, most plots across the whole trial site showed plot densities ranging across the first three digital images for either low or high salt in Fig. 2.4. In 2014 however plots in both the low and high salt trial

areas showed a range of plant density from mostly established plots to plots containing less than ten plants (Fig. 2.4).

Under low soil EC_e conditions (<8 dS/m), *AtCIPK16* expression had no significant effect on the biomass or tiller number production in either 2013 or 2014 (Fig. 2.5), except for line G298-4-16 in 2013 which had 45 % more biomass than any other line (Fig. 2.5A). Plants in 2014 not only produced on average 10 % more biomass, but also produced nearly 50 % more tillers than those lines in 2013 (Fig. 2.5). Growth on soils with EC >8 dS/m affected the biomass and tillering of all plants in 2014, except for line G298-4-16 which had significantly increased biomass production (Fig. 2.5B & D). It should be noted that this data point represented two plants, one of which followed a similar trend to other transgenic lines and one plant which skewed the data in its' growth response to salt stress. While not significant, G298-10-15 increased (40 %) biomass and tiller number (35 %) compared to wildtype and null segregants plants. G298-2-17 had the same low plant growth and tiller number as wildtype and null segregant plant. Under high salt *AtCIPK16* expression in barley had varying results on the biomass production of barley plants.

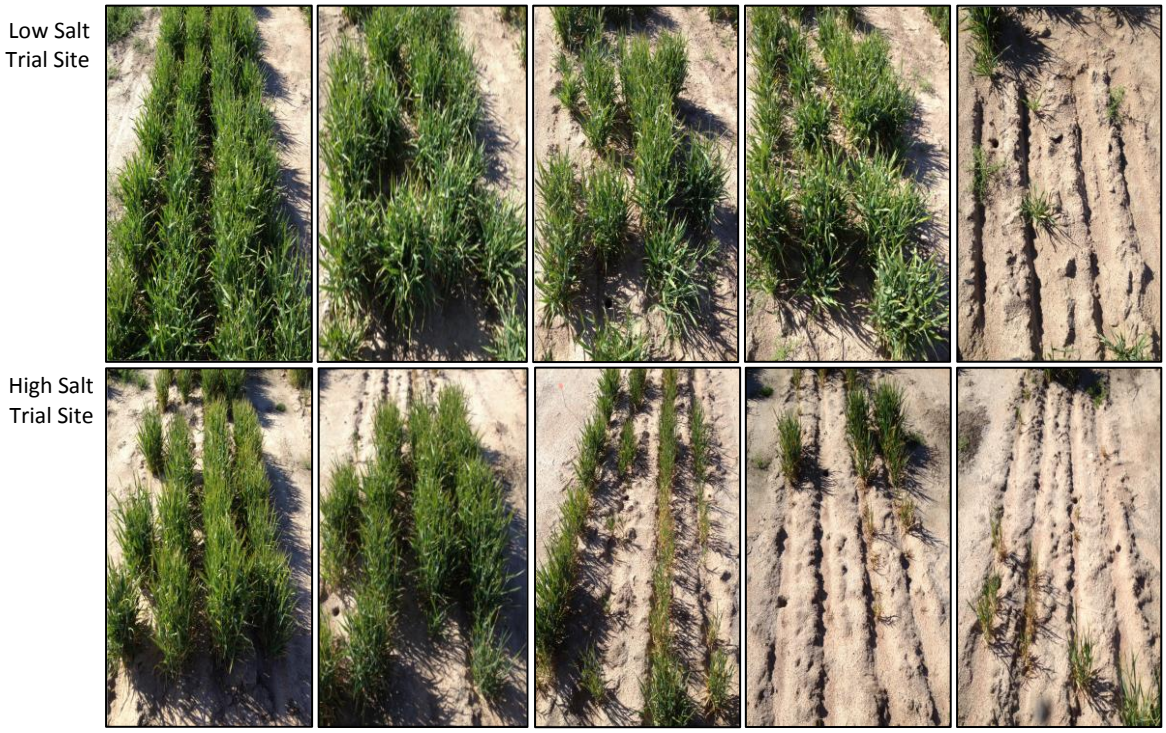


Figure 2.4: Digital images of wildtype and transgenic *AtCIPK16* expressing barley plots displaying the range of plant densities in both low and high salt trial sites at Kunjin, Western Australia in 2014. Digital images were taken in late September 2014 and are representative of the range of establishment seen in both low and high salt plots.

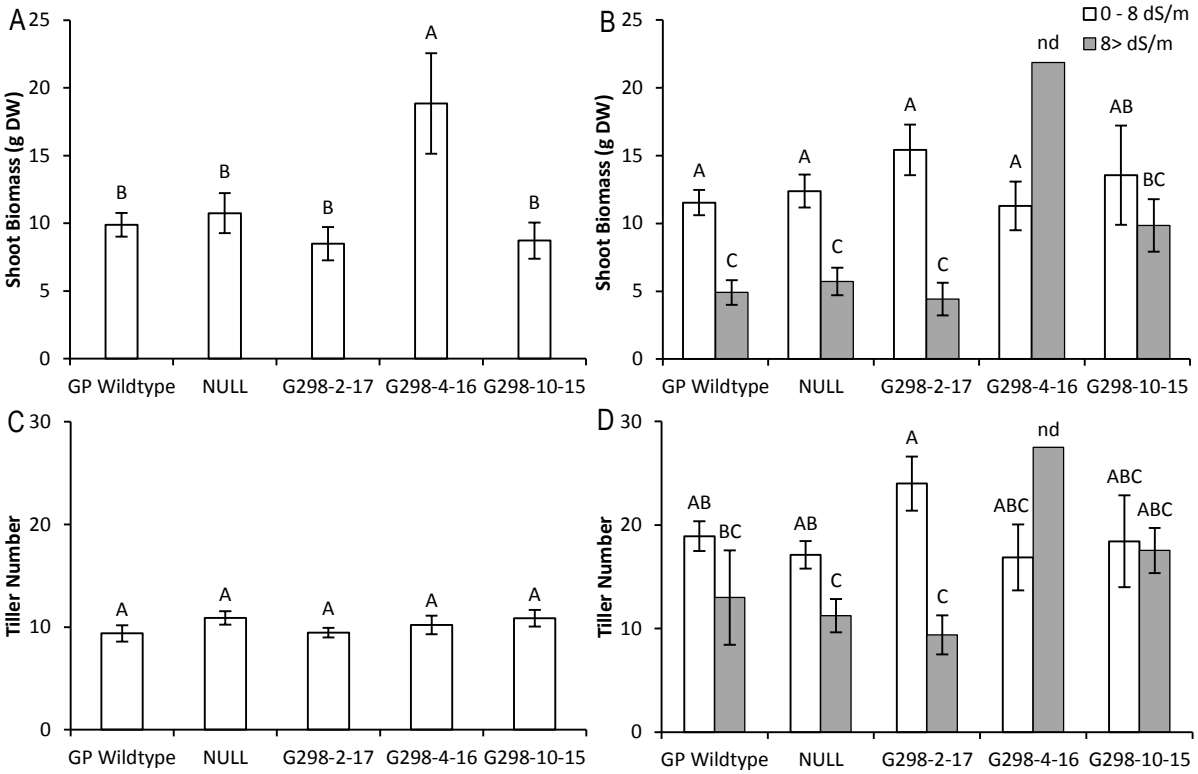


Figure 2.5: Shoot biomass and tiller number per plant of wildtype, null segregant and transgenic *AtCIPK16* expressing barley grown at Kunjin, Western Australia. Shoot biomass (A & B) and tiller number (C & D) of wildtype (cv. Golden Promise), null segregant and three independent *AtCIPK16* expressing transgenic barley lines grown in 2013 (A & C) and 2014 (B & D) in low salt (EC_e 0 – 8 dS/m) (white bars) and in high salt (EC_e >8 dS/m) (grey bars). Values are mean ± s.e.m (n = 5 – 28, except for high salt G298-4-16 where n = 2). Significant differences (one-way ANOVA, Tukey-Kramer, $P \leq 0.05$) indicated by letters. nd = not determined due to low number of replicates.

2.3.3 Transgenic *AtCIPK16* expressing barley lines show possible Na^+ exclusion

Expression of *AtCIPK16* had no effects on ion content (Na^+, K^+, Cl^- and Na^+/K^+ ratio) in transgenic barley lines compared to wildtype or null segregant plants, when grown in soils with EC_e <8 dS/m (Fig. 2.6). When plants were grown in soils with EC_e >8 dS/m, two transgenic lines (G298-2-17 and G298-10-15) and one plant from G298-4-16 showed a significant or nearly significant reduction plants and the same plant from G298-4-16 also had reduced Cl^- concentration (22 %) compared to wildtype and null segregant plants, although these differences were not significant (Fig. 2.6H). Few differences in K^+ concentration were seen between all lines under both salt conditions in 2014, with those differences attributed to the significantly low K^+ concentration in G298-2-17 under low salt conditions (Fig. 2.6D). Under high salt conditions, transgenic lines G298-2-17 and G298-10-15 had a slightly higher (18 %) Na^+/K^+ ratio due to the lower amount of Na^+ accumulated (Fig. 2.6F). Plants in 2013 accumulated slightly more (7 – 14 %) of all ions than plants of the same lines in 2014. Under high salt transgenic lines G298-2-17 and G298-10-15 showed possible Na^+ and Cl^- exclusion.

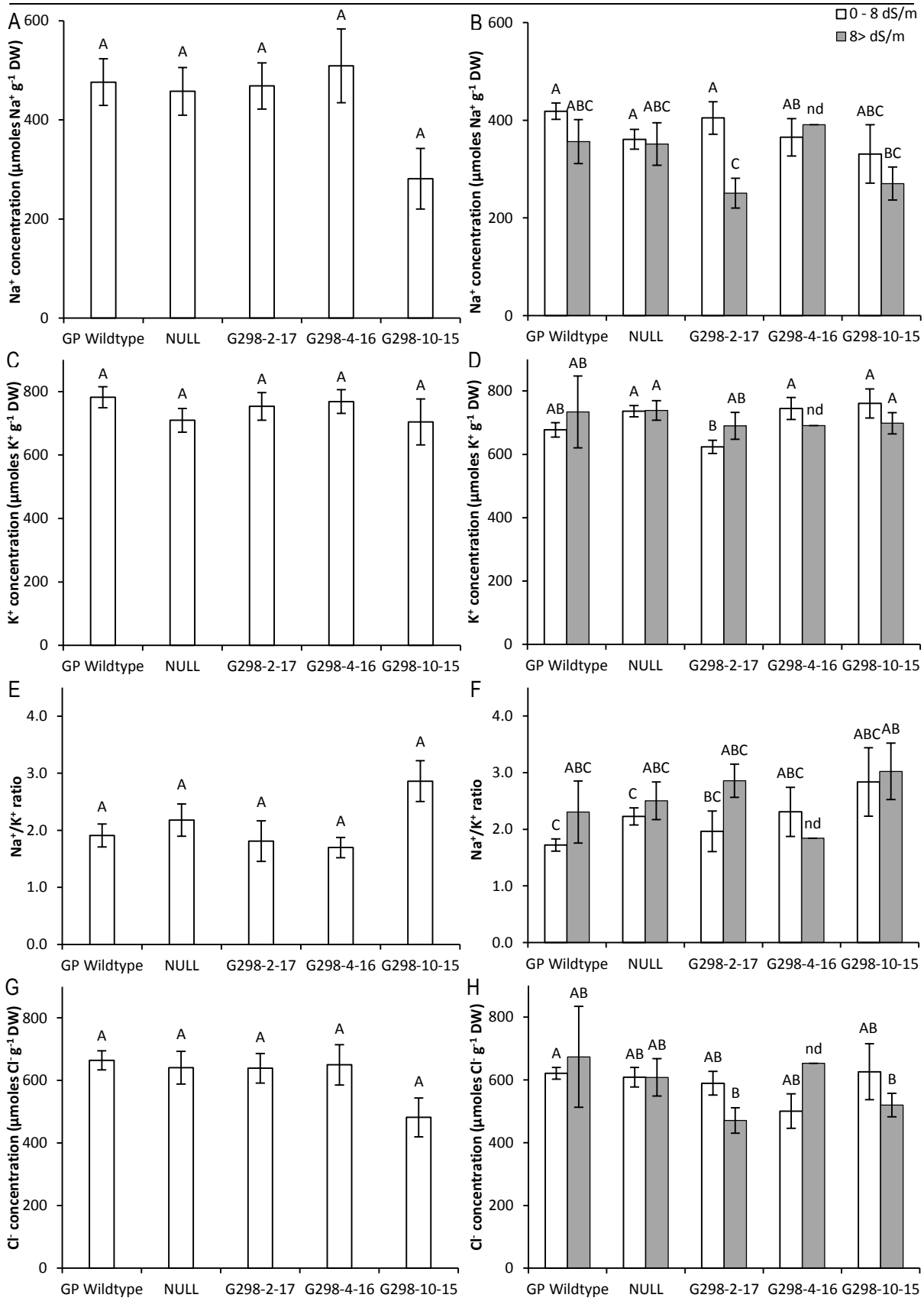


Figure 2.6: Na^+, K^+ and Cl^- concentration (µmoles ion g^{-1} DW) and Na^+/K^+ ratio of wildtype, null segregant and transgenic *AtCIPK16* barley grown at Kunjin, WA. (A & B) Na^+ concentration, (C & D) K^+ concentration, (E & F) Na^+/K^+ ratio and (G & H) Cl^- concentration of wildtype (cv. Golden Promise), null segregant and three independent transgenic *AtCIPK16* lines grown in 2013 (A, C, E & G) and 2014 (B, D, F & H) in low salt (EC_e 0 – 8 dS/m) (white bars) and in high salt (EC_e >8 dS/m) (grey bars). Values are mean ± s.e.m (n = 5 – 28, except high salt G298-4-16 n = 2). Significant differences (one-way ANOVA, Tukey-Kramer, $P \leq 0.05$) indicated by letters. nd = not determined.

2.3.4 Expression of *AtCIPK16* in barley does not improve yield

Yield parameters sampled in December for all lines varied between years as well as salinity levels. In 2013 and 2014, under low salt, the expression of *AtCIPK16* had no effect on grain number and grain weight per plant or 100 grain weight (Fig. 2.7). All transgenic lines grown in EC_e <8 dS/m soils in 2014 had decreased (13 – 30 %) grain number and grain weight per plant, although these decreases were not significant compared to wildtype plants (Fig. 2.7F). No differences in 100 grain weight were seen between all lines under low salt or high salt except for the line G298-10-15 which did suffer a significant decrease (21 %) in grain quality under high salt conditions. When grown on EC_e >8 dS/m soils, plants from one transgenic line (G298-2-17) had a large but not significant decrease (70 %) in grain number and grain weight per plant compared to wildtype plants, while the G298-10-15 line performed the same as wildtype (Fig. 2.7B & D). In 2014 G298-4-16 had increased grain number and grain weight per plant compared to wildtype (51 %) and other transgenic lines (70 %).

Overall, in both 2013 and 2014 there were no differences between transgenic lines and wildtype under low salt, although plot yields in 2014 were reduced by 50 % compared to those in 2013 (Fig. 2.8). All lines had significant decreases in grain weight between low and high salt conditions, except for G298-4-16 which did not suffer such a dramatic yield decrease (Fig. 2.8B). Despite variance, the grain weight per plot for all three transgenic lines was not significantly different from wildtype plants. The expression of *AtCIPK16* in barley lines under both low and high salt conditions either had no or a small negative effect on yield parameters.

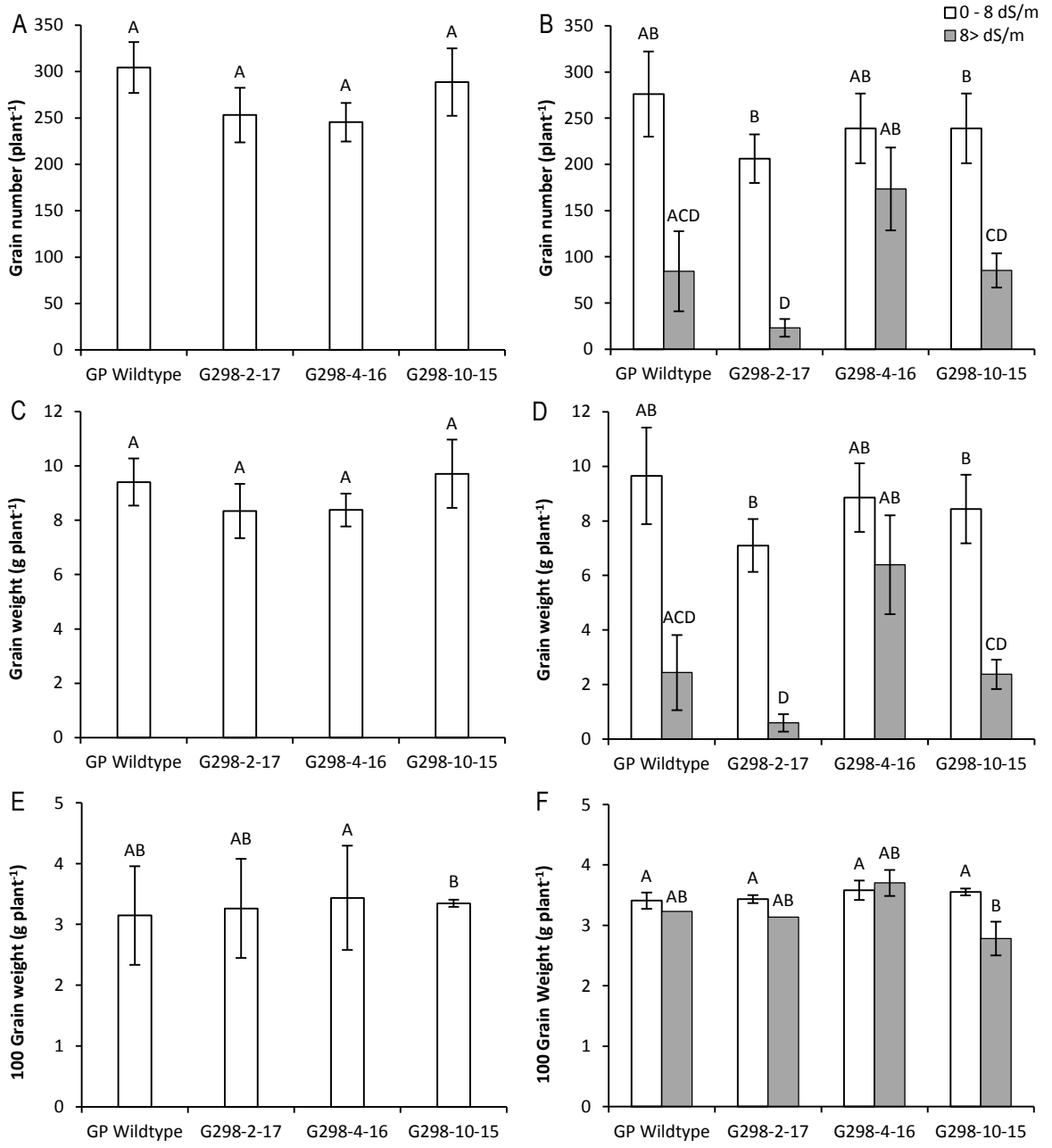


Figure 2.7: Grain yield per plants parameters of wildtype and transgenic *AtCIPK16* expressing barley grown at Kunjin, Western Australia. (A & B)Grain number (per plant), (C & D) grain weight (per plant) and (E & F) 100 grain weight of wildtype (cv. Golden Promise) and three independent *AtCIPK16* expressing transgenic barley lines grown in 2013 (A, C & E) and 2014 (B, D & F) in low salt (EC_e 0 – 8 dS/m) (white bars) and in high salt (EC_e >8 dS/m) (grey bars). Values are mean ± s.e.m (n = 6 – 27, for grain number and grain weight) (n = 15 – 17 for 100 grain weight in 2013 and n = 6 – 22 for 100 grain weight in 2014 except for wildtype high salt n = 1 and G298-2-17 high salt n = 2). Significant differences (one-way ANOVA, Tukey-Kramer, $P \leq 0.05$) indicated by letters.

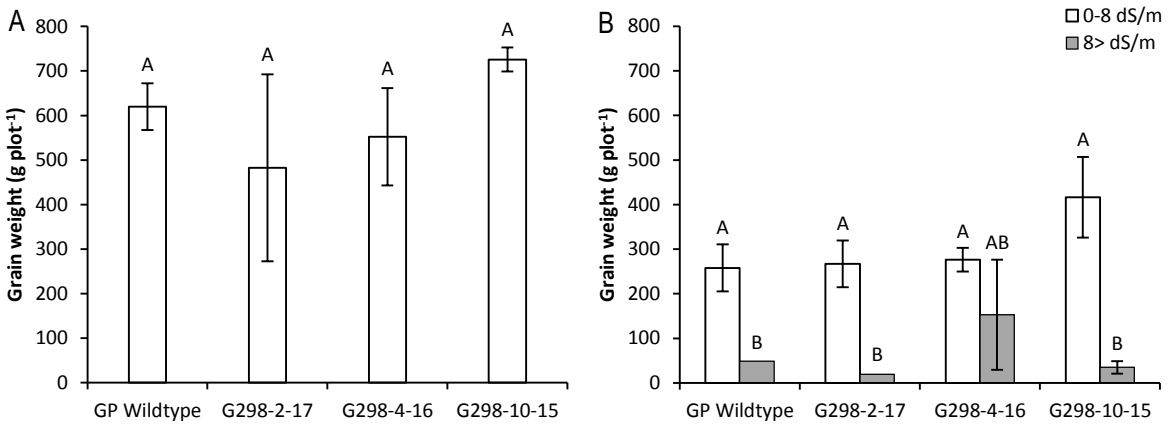


Figure 2.8: Grain yield per plot for wildtype and transgenic *AtCIPK16* expressing barley lines grown at Kunjin, Western Australia. Grain weight (per plot) (A & B) of wildtype (cv. Golden Promise) and three independent *AtCIPK16* expressing transgenic barley lines grown in 2013 (A) and 2014 (B) in low salt (EC_e 0 – 8 dS/m) (white bars) and in high salt (EC_e >8 dS/m) (grey bars). Values are mean ± s.e.m (n = 15 – 17 in 2013 and n = 6 – 22 in 2014 except for wildtype high salt n = 1 and G298-2-17 high salt n = 2). Significant differences (one-way ANOVA, Tukey-Kramer, $P \leq 0.05$) indicated by letters.

2.4 Discussion

2.4.1 Transgenic *AtCIPK16* barley has increased Na^+ and Cl^- exclusion

In recent years more focus has been given to identifying and understanding CIPKs from agriculturally relevant crops (Li, et al. 2010; Chen, et al. 2011b; Deng, et al. 2013a; Cuéllar, et al. 2013; He, et al. 2013; Deng, et al. 2013b; Zhang, et al. 2014; Farani, et al. 2015; Meena, et al. 2015a; Chen, et al. 2015) and producing transgenic crops expressing *CIPK*s, with most of these yet to progress past glasshouse or laboratory-based experimental systems (Xiang, et al. 2007; Wang, et al. 2010; Wang, et al. 2012; Huertas, et al. 2012; Li, et al. 2014b). The expression of *AtCIPK16* has previously been shown to improve the growth and Na^+ exclusion capacity of Arabidopsis and barley under saline glasshouse conditions (Roy, et al. 2013). To further understand the role of *AtCIPK16* in plant salt tolerance and its possible use in improving the salt tolerance of crop species, it is important to validate glasshouse-based findings in saline field trial conditions.

In this study, the results of the saline field trial in 2013 and 2014 show that barley expressing *AtCIPK16* growing in EC_e <8 dS/m soils have few differences in growth, ion concentrations or yield parameters from wildtype or null segregants (Fig. 2.5, 2.6, 2.7, 2.8). The consistency of these results across years does suggest that constitutively expressing *AtCIPK16* does not have any detrimental effects on plant growth and development under control or low salt conditions; consistent with results seen in glasshouse grown transgenic Arabidopsis and barley (Roy, et al. 2013). Unlike other ions measured, K^+ showed no differences between low and high salt growth conditions for all lines (Fig. 2.7D), although it was hypothesized that *AtCIPK16* might be involved with regulation of K^+ transport (Lee, et al. 2007). Unlike low salt conditions, when plants were subjected to soils with EC_e >8 dS/m significant differences between lines were established for shoot Na^+ and Cl^- concentrations. Two transgenic barley lines expressing *AtCIPK16* (G298-2-17 and G298-10-15) had decreased levels of Na^+ and Cl^- in shoot tissues (Fig. 2.7B and Fig. 2.7H). Due to the wide variation in values expected in field grown plants, many of these differences were not significant, although the decreases in ion concentrations (30 % for Na^+ and 22 % for Cl^-) are large enough to be of interest for further research. In 2012 transgenic line G298-10-15 also demonstrated Na^+ exclusion, with a decrease in shoot Na^+ concentration by 30 % (Sup. Fig. 2.6A). In 2014, G298-2-17 and G298-10-15 had a slightly increased Na^+/K^+ ratio due to Na^+ exclusion, a measure often associated with increased salt tolerance (Maathuis & Amtmann 1999; James, et al. 2006b).

An interesting result to note is not only the exclusion of Na^+ from the shoot but the accompanying smaller exclusion of Cl^-. This result suggests that AtCIPK16, which is known to regulate Na^+ and possibly K^+ transport (Lee, et al. 2007; Roy, et al. 2013), is also able to influence Cl^- transport. Although most of the Cl^- transport mechanisms have yet to be elucidated in plants, much of the movement and regulation of Cl^- is thought to occur independently to that of Na^+ (Teakle & Tyerman 2010; Tavakkoli, et al. 2011). This may be why, despite Na^+ and Cl^- often occurring in similar concentrations in soil (see Sup. Fig. 2.3), barley under salt stress often accumulates up to twice as much Cl^- as Na^+ (Fig. 2.7; Benes et al. 1996; Tavakkoli et al. 2011; Tavakkoli et al. 2012). Due to the high salt trial site in 2013 being flooded, this Cl^- exclusion phenotype has only been seen in one year of field trials, and therefore needs to be repeated before any solid conclusions can be drawn. Possible hypotheses for the Cl^- exclusion include the following. *AtCIPK16* is naturally expressed in root stelar tissue (Roy, et al. 2013) and the ectopic expression in other tissues under the control of a constitutive promoter (35S) could cause *AtCIPK16* to regulate pathways and targets it would not usually be involved in. Being a transgene from Arabidopsis target genes/proteins may be different in barley especially as AtCIPK16 is thought to localise to the nucleus and be involve in gene regulation (Huang 2015). With Cl^- being equally detrimental to plant growth (Teakle & Tyerman 2010), the understanding of how the expression of *AtCIPK16*, a gene associated with Na^+ exclusion, can effect shoot Cl^- accumulation is vital not only understanding the role of *AtCIPK16* in salt tolerance but the possible manipulation of both Na^+ and Cl^- exclusion.

2.4.2 Na^+ and Cl^- exclusion does not translate to improved biomass or yield in transgenic *AtCIPK16* lines

In a previous glasshouse-based hydroponic experiment, transgenic *AtCIPK16* barley lines not only had increased Na^+ exclusion capacity but also increased relative growth (Roy, et al. 2013). In this study under growth conditions EC_e >8 dS/m, two transgenic lines, G298-2-17 and G298-10-15, had increased Na^+ and Cl^- exclusion, although only G298-10-15 also demonstrated increased growth, with 50 % more biomass and 25 % more tiller (not significant) than wildtype or null segregants (Fig. 2.6B & D). Line G298-4-16, which consists of two plants, also had significantly greater biomass parameters with large variation between the two plants, as one plant behaved for all parameters (biomass, ion concentration and yield) with the same trend as those from G298-10-15; while the other plants had very large biomass, but still accumulated large amounts of ions. Under low salt conditions in 2013, this line also produced a couple of very large plants which kept accumulating ions. In 2012, under high salt, the opposite trend was seen, with G298-2-17 producing more biomass under high salt than

wildtype and G298-10-15 performing the same as wildtype (Sup. Fig. 2.5). The two transgenic lines in 2014 demonstrating exclusion had equal or decreased grain yield per plot compared to wildtype (Fig. 2.9B), which was due to the same trend in yield parameters grain number and grain weight per plant (Fig. 2.8B & D) rather than grain quality (100 grain weight; Fig. 2.8F). Whereas in 2012, G298-10-15 which demonstrated Na^+ exclusion did show increased grain parameters per plant and therefore some increase in yield per plot (Sup. Fig. 2.7 & 2.8).

Barley is known to store and use ions such as K^+ and Na^+ as ionic osmoticum, used for increasing cell turgor pressure and promoting plant growth, as part of normal growth mechanisms (Blumwald, et al. 2000; Adem, et al. 2015). It is therefore possible that increasing ion exclusion decreases the amount of ions available in shoot tissue to be used as growth promoting osmoticum, therefore explaining the lack of or negative effect of exclusion on plant growth and yield. Increased shoot Na^+ concentration, via the overexpression of *HvHKT1;2*, has been shown to increase barley salt tolerance in hydroponic experiments (Mian, et al. 2011). This suggests that perhaps increasing Na^+ exclusion is not the best mechanism for improving barley salt tolerance.

Despite being further advanced generations of the same transgenic lines used to originally screen *AtCIPK16* expressing barley, the results of this study are inconsistent with those seen in the Roy, et al. (2013) hydroponic experiment. Barley cultivars have previously been shown to respond differently to salt stress in soil-based experimental systems compared to hydroponic systems, often with results seen in soil experiments exacerbated in hydroponics (Tavakkoli, et al. 2010; Tavakkoli, et al. 2012). Hydroponic systems provide a constant supply of oxygenated water, a constant level of salt stress and therefore a homogenous growth environment throughout an experiment, compared to the highly variable, complex heterogeneous growth conditions experienced by plants during field trials. In field based experiments, differences in soil texture and structure can affects nutrient availability, soil water holding capacity and matric potential (Tavakkoli, et al. 2010), all of which are not concerns in hydroponic experiments. Hydroponic systems also do not account for variations seen during growing seasons, such as the accumulation or depletion of nutrients in the rhizosphere (Tavakkoli, et al. 2010), or the variation in salinity due root systems depths or groundwater fluctuations. Differences between the types of experimental systems used may account for the inconsistencies between these studies, although it should be noted that hydroponic systems have generally been showed to be the more sensitive experimental system (Tavakkoli, et al. 2010; Tavakkoli, et al. 2012), or underlying issues in the soil may have masked the effects during this study. It should also be noted that the previous study

was conducted on barley plants grown for in total 44 days (Roy, et al. 2013), whereas tissue analysis in this study was conducted on plants over three months old and grain analysis on plants over five months old. It is therefore highly likely that *AtCIPK16* is able to improve the salt tolerance of young barley plants, but it may be that the constitutive expression of *AtCIPK16* throughout the vegetative, flowering and maturity stages of barley has detrimental effects seen here in this study. There is little evidence in literature to show that salt tolerance in early growth stages under laboratory experimental conditions, e.g. hydroponic systems, correlates with salt tolerance in the later stages of growth and reproduction for plants, which may by supported by the results of this study (Tavakkoli, et al. 2012).

2.4.3 Variation in results between years linked to environmental factors

Differences in growth, ion concentrations and yield for all lines have been noted between 2013 and 2014, as well as for 2012. In 2013 an extreme rainfall event, occurring three weeks after sowing, caused waterlogging of the high salt site preventing sampling from this trial area (Fig. 2.2A). This rainfall event was not thought to waterlog the low salt trial area but rather provide more than adequate rainfall during germination and early growth, supplemented by slightly above average rainfall during the rest of the growing season. In 2014 rainfall was also slightly about average throughout the growing season, although more even distributed. Despite receiving more rainfall in 2013, most lines including wildtype produced less biomass than in 2014, although the 2013 plants accumulated 10 – 20 % more ions (Fig. 2.6). It has been demonstrated that under low oxygen conditions in maize roots, the mechanisms which restrict the amount of Na^+ able to reach shoot tissue malfunctions, causing the increased concentration of Na^+ in the shoot, as well as decreased biomass (Drew & Läuchli 1985). Although the results obtained in this study in 2013 does support the idea of the plants suffering a slight waterlogging at the start of their growth period, the measurements in this study were taken after the possible waterlogging when the soil had start to dry out. It should also not be ruled out that there were underlying soil problems, such as micronutrient deficiencies, the presence of soil pathogens, etc. which had a negative impacts on plant growth. Despite having lower biomass in 2013 than 2014, grain yield per plant parameters varied little between the two years, although grain yield per plot in 2013 was double that seen in 2014, likely due to issue with plot density in 2014 (Fig. 2.4). 2014 saw an increase in the variability of plot establishment and density in low and high salt trial sites, prompting analysis of data to be by underlying plot soil EC rather than plot location in the trial. In 2013 most plots were non-saline with EC_e values under 4 dS/m and pH averaging 5.5 (Sup. Fig. 2.2), while in 2014, the EC_e was more varied with plots grouped between 0 – 8 dS/m exhibiting EC_e from 2 – 6 dS/m and plot grouped as >8 dS/m having an EC_e from 10 – 16 dS/m (Sup Fig. 2.3A). Some of the variation in

plot density was explained by high EC, soil Na^+ or soil Cl^-, although there were some plots with low plant density which did not correlate to any measured soil trait (Sup. Fig. 2.3A). These variations may be explained by underlying soil characteristics not measured and made analysis of data, particularly grain per plot, more difficult.

A smaller scale preliminary field trial with these transgenic lines was conducted at Kunjin, WA in 2012 and the data kindly provided for comparison by Dr. Stuart Roy. 2012 was a hot and dry year, with well below average rainfall especially in July, August, September and October as well as higher than average mean maximum temperatures in these months (Sup. Fig. 2.4). Soil EC_e values for most plots were considerably lower than in 2013 and 2014, with only a few plots grouped as EC_e >8 dS/m although the range of EC_e varied from 9 – 36 dS/m (Sup Fig. 2.1). Due to the lack of rainfall, all lines under both salt conditions suffered a 50 % growth penalty compared to 2013 and 2014 (Sup. Fig. 2.5). G298-2-17 had increased biomass compared to wildtype under EC_e >8 dS/m conditions but this line showed only a minimal Na^+ exclusion phenotype, instead G298-10-15 which showed a strong Na^+ exclusion phenotype produced the same amount of biomass as wildtype, similar to trends seen in 2014 (Sup. Fig. 2.5 & 2.6A). It should be noted that under low salt conditions plants only accumulate approximately 100 µmoles Na^+ g^{-1} DW instead of 450 µmoles Na^+ g^{-1} DW seen in 2013 and 2014, which was likely due to the lack transpirational demand in these plants limiting the uptake of Na^+ through the water column. Unlike in 2014, under 2012 high salt conditions, transgenic *AtCIPK16* expressing barley lines had increased grain number and grain weight per plant as well as grain weight per plot (Sup. Fig. 2.7 & 2.8). It should be noted that one wildtype plot and the G298-2-17 plot under high salt had EC_e values >29 dS/m which would greatly affect the yield of any crop even one as salt tolerant as barley (Sup. Fig. 2.1). Issues in plot establishment due to high variability in soil salinity within individual plots in 2012 (Schilling 2014) hindered the analysis of the yield per plot data and the comparisons between years of different rainfall conditions. Plants in 2012 produced 70 % less grain than in wetter years on a per plant and a per plot basis especially for plots high salt trial area likely due to the lack of rainfall, although these results indicate that under drier conditions the expression of *AtCIPK16* may enhance grain yield due to Na^+ exclusion.

Depending on rainfall (drought status), salinity can have varying effects on plant growth. Several studies have shown that the impacts of salinity on barley growth and yield are more severe under combined salt-drought stress than salt stress only (Katerji, et al. 2009; Ahmed, et al. 2013). Plants use more energy to take up the same volume of water if the water is saline rather than non-saline and are

also unable to extract water at lower soil water potentials if water is saline (Rengasamy 2006). Salinity therefore has more of an impact on plant growth in drier years, reflecting the differences in growth and yield seen in this study between 2012, 2013 and 2014. Under high salt, well-watered conditions Na^+ exclusion may not be an as important tolerance mechanism as tolerance mechanisms, where Na^+, K^+ and Cl^- can be used as osmoticum to promote growth (Blumwald, et al. 2000; Tavakkoli, et al. 2012; Adem, et al. 2015). Instead under high salt, dry conditions Na^+ exclusion may be a much more important mechanism, as plants lack the water to dilute incoming Na^+ with cell expansion and growth.

2.5 Conclusions & Future directions

We hypothesis that under well-watered saline conditions, the expression of *AtCIPK16* in barley drives Na^+ exclusion decreasing the available pool of Na^+ for use as osmoticum to drive growth, thus effecting growth and yield. Under dry saline conditions, the expression of *AtCIPK16* in barley increases Na^+ and Cl^- exclusion which increases plant growth and therefore yield. To further test these hypotheses additional field trials need to be conducted in locations variable in rainfall and soil salinity over several years. Since the main effect of *AtCIPK16* expression is hypothesised to be under dry conditions (drought) it may be interesting to investigate the field salinity tolerance of *AtCIPK16* expressing barley under the control of a drought specific promoter. As the function of AtCIPK16 is currently unknown, but hypothesised to act in the nucleus, perhaps like a transcription factor, it would be preferable to limit the expression window to minimise any negative impacts like those seen under well-watered salt stress conditions. A similar approach has been taken with DREB transcription factors, which when constitutively expressed have increases stress tolerance with large negative side effects, which are mitigated when the genes are controlled by a stress inducible promoter (Kasuga, et al. 2004; Morran, et al. 2011; Kovalchuk, et al. 2013).

Chapter 3: Characterisation of *Ubi:AtCIPK16* wheat lines in hydroponic experiments

3.1 Introduction

Bread wheat is classified as moderately salt tolerant when compared to tolerant crops like barley. It is unable to tolerate high Na^+ concentrations in the shoot due to poorer tissue tolerance mechanisms but uses strong shoot Na^+ exclusion mechanisms (Maas & Hoffman 1977; Tester & Davenport 2003; Munns & Tester 2008). Several genes which are known to encode proteins involved in Na^+ exclusion pathways in wheat species have been identified, such as *TaCIPK24* (*SOS2*), *TdSOS1, Kna1* (*TaHKT1;5*), *Nax1* (*TmHKT1;4-A2*) and *Nax2* (*TmHKT1;5-A*) (Dvořák, et al. 1994; James, et al. 2006a; Byrt, et al. 2007; Feki, et al. 2011; Sun, et al. 2015). *Kna1*, *Nax1* and *Nax2* are all loci which have been associated with the retrieval of Na^+ from the transpiration stream and thus increased shoot Na^+ exclusion (Dvořák, et al. 1994; James, et al. 2006a; Byrt, et al. 2007). The candidate genes for these loci are all members of the *HKT1;x* gene family (Dvořák, et al. 1994; James, et al. 2006a; Byrt, et al. 2007). Incorporation of *Nax1* and/or *Nax2* alleles into bread wheat caused an increase in the capacity of bread wheat to exclude Na^+ from the leaf blade, despite these lines then possessing two Na^+ transporting HKTs, *Nax2* and *Kna1* (James, et al. 2011). This study illustrated that while bread wheat already possesses strong Na^+ exclusion pathways it is possible to improve of this tolerance mechanism through the introduction of new genes, whether by crossing or genetic modification.

One plausible gene for increasing the Na^+ exclusion capacity of plants is *AtCIPK16*, a protein kinase found in Arabidopsis (Roy, et al. 2013). Improved salt tolerance has been demonstrated in transgenic Arabidopsis and barley expressing *AtCIPK16* (Roy, et al. 2013). In hydroponic experiments with both species, transgenic plants produced increased biomass accompanied by reduced shoot Na^+ accumulation (Roy, et al. 2013). With promising results seen in barley, bread wheat *Ubi:AtCIPK16* lines were generated by the ACPFG wheat transformation group and preliminarily tested using hydroponic systems (Roy, et al. unpublished). Under 200 mM NaCl stress, transgenic wheat had up to 60 % more biomass than null segregants and a 45 % decrease in shoot Na^+ concentration (Roy, et al. unpublished). This chapter will outline the work conducted to further evaluate the potential Na^+ exclusion capacity and salt tolerance of *AtCIPK16* expressing wheat under glasshouse conditions.

3.2 Materials and Methods

3.2.1 Plant material

For this study, bread wheat (cv. Gladius) seed, biolistically transformed to express the transgene *AtCIPK16* (At2g25090) under the control of the maize *ubiquitin 1* promoter (DQ141598) was kindly provided by Dr. Stuart Roy. T_3 seed from three independent transformation events, designated CIPK16-1, CIPK16-2 and CIPK16-3, as well as a null segregant line from the second transformation event (CIPK16-2) (used as a control line), was used in each experiment. Due to issues with seed number and quality, within each transformation event sibling lines were used where necessary.

3.2.2 Growth conditions

To assess the effects of *AtCIPK16* expression on the salt tolerance of bread wheat, supported hydroponic experiments were conducted in glasshouses at Urrbrae, South Australia throughout 2014 and 2015. Plants were grown under natural light conditions with limited temperature control (approximately 15°C minimum and 25°C maximum temperatures) in three experiments: a preliminary experiment (Hydroponics #1) in autumn 2014 (March 21st to April 24th), a large scale experiment (Hydroponics #2) in late spring 2014 (October 31st to December 5th) and a follow up experiment (Hydroponics #3) in late winter 2015 (July 17th to August 21st). Seeds were UV sterilised before being germinated at 24°C on moist filter paper (Whatman, Maidstone, United Kingdom) in 80 mm petri dishes (Corning, New York, USA). After three to five days (depending on the season), the seedlings most uniform in growth were transplanted into two (three for large scale experiment) 80 L flood-drain hydroponic systems (Genc, et al. 2007; Shavrukov, et al. 2012). Each system contained two 20 L growth tanks mounted above a 120L barrel, which each held 42 PVC tubes (40 mm × 280 mm) containing polycarbonate plastic beads (Plastics Granulated Services, Adelaide, Australia) into which the seeds were transplanted. Ten replicates from each transgenic and null line were placed into each growth tanks in a randomised layout. All hydroponic systems were filled with 80 L of rainwater and standard ACPFG growth solution (Table 3.1). To prevent depletion of nutrients, growth solutions were changed every 8 days and pH adjusted with 3.2 % (v/v) HCL to maintain pH 6.5 – 7.5. At the emergence of the third leaf for the majority of plants 150 mM or 200 mM of NaCl was added to the treatment system/s in twice daily 25 mM increments, until the desired concentration was reached (3 days for 150 mM NaCl and 4 days for 200 mM NaCl). To enable Ca^{2+} activity to remain the same between control (0 mM) and salt treatments, 0.43 mM $CaCl_2$ was added with each 25 mM NaCl application. When nutrient solutions were changed after full salt treatment had been reached, the 150 or 200 mM NaCl was immediately added to maintain the treatment level.

After 23 days of salt treatment the number of tillers on the plant was recorded and the roots washed in 10 mM $CaSO_4$ to remove excess NaCl and blotted dry. In hydroponic experiments #2 and #3 roots and shoots were separated and fresh weights recorded, with the 4th leaf then taken for later ion analysis. As plants from hydroponics #1 were grown on to multiply seed numbers for subsequent experiments the fresh weight of the whole plant was recoded and the 4th leaf harvested for ion analysis. Approximately 5 cm of green leaf blade material from each plant was collected, frozen in liquid N_2 and stored at -80°C until required for DNA and RNA analysis. Plant material was then dried for 2 days at 70°C in an oven (Contherm Scientific Ltd, Wellington, New Zealand) and weighed to obtain dry weight measurements.

Table 3.1: Components and final concentrations in 80 L hydroponic systems of the standard ACPFG growth solution (modified from Genc, et al. 2007)

Component	Chemical Formula	Final Concentration (mM)
Ammonium nitrate	NH_4NO_3	0.2
Potassium nitrate	KNO_3	5
Calcium nitrate tetrahydrate	$Ca(NO_3)_2{\cdot}4H_2O$	2
Magnesium sulfate heptahydrate	$MgSO_4{\cdot}7H_2O$	2
Potassium dihydrogen orthophosphate	KH_2PO_4	0.1
Disodium trisilicate	$Na_2Si_3O_7$	0.5
Sodium iron EDTA	NaFe(III) EDTA	0.05
Manganese(II) chloride tetrahydrate	$MnCl_2{\cdot}4H_2O$	0.005
Zinc sulphate heptahydrate	$ZnSO_4{\cdot}7H_2O$	0.1
Copper sulphate pentahydrate	$CuSO_4{\cdot}5H_2O$	0.0005
Sodium molybdate dihydrate	$Na_2MoO_4{\cdot}2H_2O$	0.0001

3.2.3 DNA extraction and genotyping analysis

DNA was extracted from leaf material as described in Section 2.2.4.

In each plant the presence or absence of the *TaTVP1* gene (AY296911) was used as a control to check the success of the extractions. *TaTVP1* was amplified using PCR with the primers and setting variables described in Table 3.2. The presence or absence in each plant of the transgene *AtCIPK16* (At2g25090) was identified using two primers sets specific to *AtCIPK16,* one set (*qPCR AtCIPK16*) which bind in a unique region and amplify a short 229 bp fragment and another set (*Full AtCIPK16*) which amplifies the full length of the coding sequence (Table 3.2). The PCR conditions used to amplify these genes were as follows: an initial denaturation at 94°C for 2 mins, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at the appropriate temperature for the primers (Table 3.2) for 30 s and extension at 68°C for the appropriate length of time (Table 3.2), followed by a final extension

at 68°C for 5 mins. All PCRs were performed with either OneTaq® or CrimsonTaq® (New England Biolabs) in 25 µL reaction volumes. Each reaction contained 1 µL of extracted gDNA, 5 µL 5× OneTaq standard reaction buffer/ 5× CrimsonTaq (with Mg) reaction buffer, 0.5 µL 10 mM dNTPs, 0.625 U OneTaq/ CrimsonTaq DNA polymerase, 5 µL 10 mM forward primer and 5 µL 10 mM reverse primer for the appropriate gene. PCR products were visualised using gel electrophoresis with a 1.5 % agarose gel containing 5 µL/100 mL SYBRsafe® stain (Invitrogen).

3.2.4 RNA extraction and gene expression analysis

Total RNA was extracted from representative samples from each *AtCIPK16* transgenic lines and the null line using a Direct-zol RNA purification kit (Zymo Research, Irvine, USA) with modifications from the manufacturer's specifications. Frozen leaf material was ground in 2 mL tubes using 3 small stainless steel ball bearings and a vortex, before ball bearings were removed. 500 µL of TRIzol-like reagent (Invitrogen) was added to the samples before they were mixed at room temperature for 10 mins on an orbital shaker. Samples were then centrifuged at 12,000 *g* for 5 mins and supernatant transferred to a new 2 mL tube containing 500 µL of 100 % (v/v) ethanol and mixed using a vortex. All of the supernatant was transferred to a Zymo-Spin IIC column and centrifuged for 1 min at 12,000 *g* to bind RNA to the spin column and the flow-through discarded. An in-column DNase I digestion was performed to remove any contaminating DNA which involved first washing the isolated RNA by adding 400 µL of Zymo RNA Wash Buffer to spin column and centrifuging for 1 min at 12,000 *g* with the flow-through discarded. To each column 5 µL of DNase I (1 U/µL) and 75 µL of Zymo DNA Digestion Buffer was added. Samples were left to incubate at room temperature for 15 mins, centrifuged at 12,000 *g* for 30 s and the flow-through discarded. To wash the RNA, 400 µL of Zymo RNA PreWash Buffer was added then samples were centrifuged for 1 min at 12,000 *g* and flow-through discarded, these steps were then repeated. To purify the RNA, 700 µL of Zymo RNA Wash Buffer was added to samples, which were then centrifuged for 1 min at 12,000 *g*, the flow-through was discarded, and the samples centrifuged again at 12,000 *g* for 2 mins. The spin columns were transferred to a final 1.5 mL collection tube and left open to air dry for 5 – 10 min to allow any excess ethanol in wash buffers to evaporate. RNA was resuspended in 30 µL of DNase/RNase-Free water and left to incubate for 10 min. Samples were then centrifuged for 1 min at 12,000 *g*, the elution put back into spin column and samples centrifuged again for 2 min at 12,000 *g*. The spin columns were discarded and the RNA stored on ice (for immediate use) or at -80°C. The quality of the extracted RNA was checked using spectrophotometry (see Section 2.2.4) and gel electrophoresis. 5 µL of RNA was visualised using gel electrophoresis with a 1. % agarose gel containing 5 µL/100 mL SYBRsafe® stain (Invitrogen) to

check for the presence of clear 18S and 28S RNA bands as well as a band containing small RNA molecules such as tRNAs.

cDNA was synthesised using a SuperScript® III First-Strand Synthesis System kit (Cat. No. 18080-051, Invitrogen) using the protocol in the manufacturer's instructions. First, 1 µL of oligo $(dT)_{20}$ (50 µM), 1 µL of dNTP mix (10 µM), 0.5 µg of total RNA and sterile water to a volume of 10 µL was pipette mixed in 100 µL tubes. Samples were incubated at 65°C for 5 min to remove secondary RNA structures and immediately placed on ice for at least 1 min. Samples were briefly centrifuged, then to each tube the following was added: 2 µL of 10× RT Buffer, 4 µL of $MgCl_2$ (25 mM), 2 µL of DTT (0.1 M), 1 µL of RNaseOUT™ Recombinant RNase Inhibitor and 200 U of Superscript® III Reverse Transcriptase. The solutions were gently pipette mixed, centrifuged and incubated at 50°C for 50 min. To terminate the reaction, samples were heated to 85°C for 5 mins, then briefly centrifuged and chilled on ice. To remove any RNA, 2 U of *E. coli* RNase H was added and samples incubated at 37°C for 20 mins. cDNA was then stored at -20°C.

To test the quality of the cDNA synthesis, a control PCR was run on all samples to check to expression of the house-keeping gene *TaGAPDH* (EF592180) using primers described in Table 3.2.
Due to issues attempting to detect expression of the transgene *AtCIPK16* (At2g25090), several sets of primer pairs binding to different regions of the transgene were designed (Table 3.2). The PCR conditions used to amplify these genes/ gene fragments were as follows: an initial denaturation at 94°C for 2 mins, followed by 37 cycles of denaturation at 94°C for 30 s, annealing at the appropriate temperature for the primers (Table 3.2) for 30 s and extension at 68°C for the appropriate length of time (Table 3.2), followed by a final extension at 68°C for 5 mins. All PCRs were performed with OneTaq® (New England Biolabs) in 25 µL reaction volumes. Each reaction contained 1 µL of extracted gDNA, 5 µL 5× OneTaq standard/ GC reaction buffer, 0.5 µL 10 mM dNTPs, 0.625 U OneTaq, 5 µL 10 mM forward primer and 5 µL 10 mM reverse primer for the appropriate gene. PCR products were visualised using gel electrophoresis with a 1.5 % agarose gel containing 5 µL/100 mL SYBRsafe® stain (Invitrogen).

3.2.5 Ion analysis of leaf and root tissue

Ion analysis was conducted on both 4th leaf and root samples as described in Chapter 2 section 2.2.6.

Table 3.2: Details of gene specific primers and PCR conditions used for the amplification of gDNA and/or cDNA from leaf tissue samples of null segregant and three independent *AtCIPK16* transgenic wheat lines.

Gene	Primer set	Forward primer sequence (5' - 3')	Reverse primer sequence (5' - 3')	Annealing	Extension	Expected band size (bp)
TaTVP1		GACGACGACCCTGGAAGCAAGGAAG	ATAGAAGCAACAACAAGAGCAGCG	58°C	30 s	578 - gDNA
TaGAPDH		TTCAACATCATTCCAAGCAGCA	CGTAACCCAAAATGCCCTTG	52°C	30 s	230 - cDNA
AtCIPK16	Full Length	ATGGAAGAATCAAACCGTAGTAGTACTGTC	TCATGAAACATTATTTATTTTGTTATCATTTGTG	55°C	2 mins	2069 - gDNA 1410 - cDNA
AtCIPK16	Exon 1	GAACAGAGATCTCCACCGG	TGTACAATGTCATGACGT	55°C	40 s	606 - cDNA
AtCIPK16	Middle	TTTTCCACCGCGATATTAAACC	TTCTTTCTCGTTTAGGTCTTTCTTCTTTTTCTTT	53°C	40 s	536 - cDNA
AtCIPK16	qPCR	ACTCTCAAGATTGCTTGTGCCG	TGATGTGATGAATTGGAAGGCG	53°C	30 s	229 – gDNA 229 - cDNA
AtCIPK16	Exon 2	TTACACCATCAGTAGCCTTTTCGATAG	ACCTTGCCATGACCATACAA	53°C	30 s	491 - cDNA

3.3 Results

3.3.1 Gene presence and expression analysis of *AtCIPK16* transgenic lines

The presence of the native *TaVP1* gene was confirmed in gDNA samples from plants in all CIPK16-1, CIPK16-2 and CIPK16-3 lines as well as the null line and null segregants, establishing that gDNA extracted was of good quality (Fig. 3.1A i). The presence of the 200 bp unique region of the AtCIPK16 transgene was confirmed in transgenic plants from the transgenic lines using the *AtCIPK16* qPCR primer set (Table 3.2) and found to be absent in the null line and null segregants (Fig. 3.1A ii). The full length of the transgene was absent from all nulls, but was present in only some of the plants previously identified as transgenic (Fig. 3.1A iii).

RT-PCR confirmed the expression of the native *TaGAPDH* housekeeping gene in representative samples from null segregant, CIPK16-1, CIPK16-2 and CIPK16-3 lines, illustrating that cDNA was successfully synthesised (Fig. 3.1B i). In the null segregants no expression of *AtCIPK16* was detected for any primer set (Fig. 3.1B). For most plants previously identified as genotypically transgenic, no expression of *AtCIPK16* was detected using any of the primer sets which bound to different regions of the *AtCIPK16* mRNA; although expression was detected for a few samples particularly using primer set *AtCIPK16 Exon 2* (Fig. 3.1B iii, iv and vi).

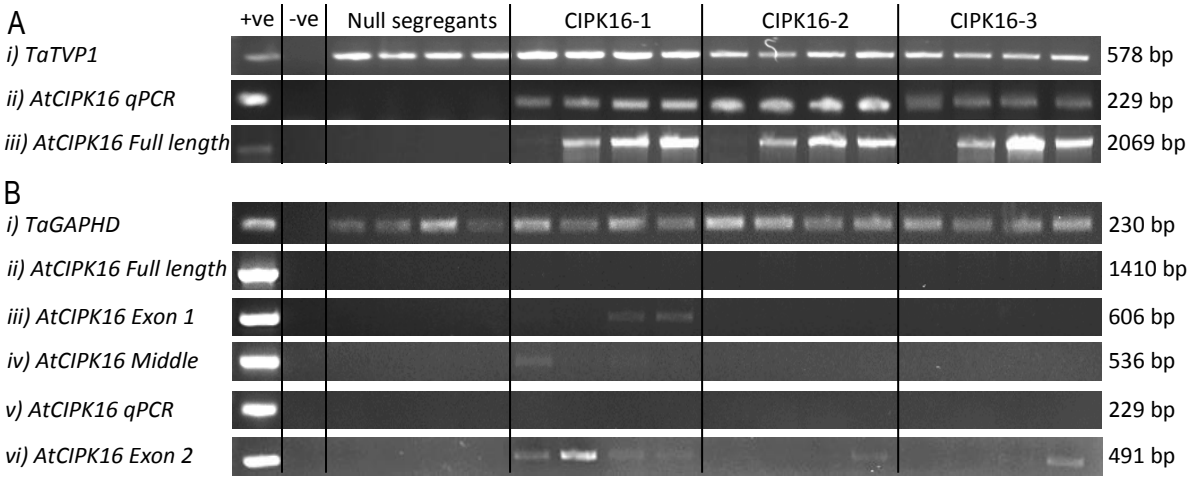


Figure 3.1: Electrophoresis gel showing representative results of genotyping and expression for null segregants and three transgenic *AtCIPK16* wheat lines. Genotyping by PCR amplification on gDNA (A) shows the presence of the native *TaTVP1* gene (A i), the qPCR region of the *AtCIPK16* transgene (A ii) and the full length of the transgene (A iii). RT-PCR shows the expression in the corresponding cDNA samples (B) of the native *TaGAPDH* gene (B i) is shown, as well as the expression of the *AtCIPK16* transgene using primer sets: Full length (B ii), Exon 1 (B iii), Middle (B iv), qPCR (B v) and Exon 2 (B vi). Each gel includes a positive control (+ve), a water negative control (-ve) and four replicates from each line.

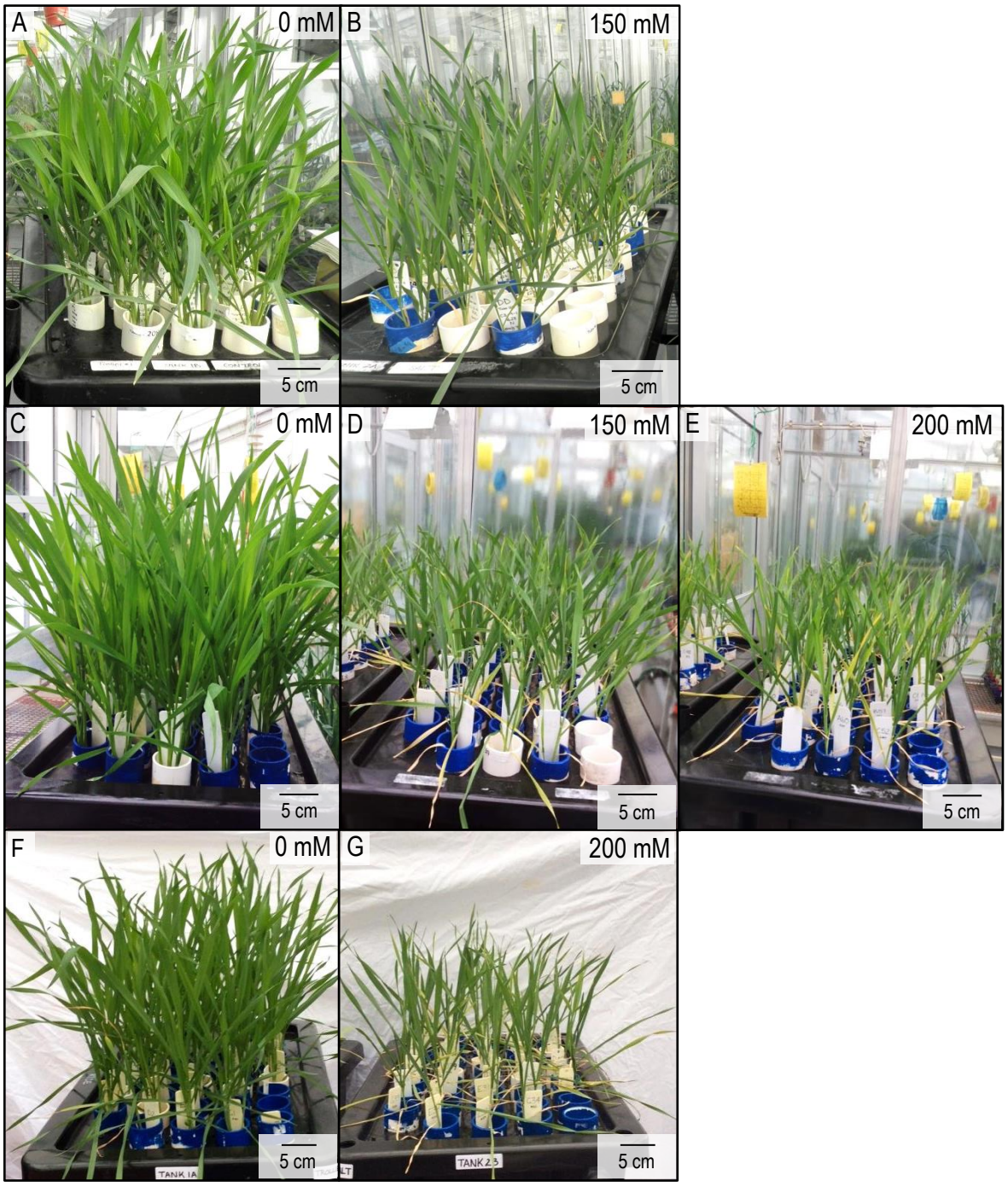


Figure 3.2: Photographs of null segregant and three transgenic *AtCIPK16* wheat lines at 24 days grown in 80 L flood-drain hydroponic systems under different salt treatments. Growth at 24 days of null segregant and three independent *AtCIPK16* transgenic wheat (cv. Gladius) lines grown in hydroponic experiment #1 (autumn 2014) in A) 0 mM and B) 150 mM NaCl; hydroponic experiment #2 (late spring 2014) in C) 0 mM, D) 150 and E) 200 mM NaCl; and hydroponic experiment #3 (winter 2015) in F) 0 mM and G) 200 mM NaCl.

3.3.2 Transgenic *AtCIPK16* lines have varied biomass production

Considerable variation was observed in biomass and tiller number production between experiments, consistent with seasonal differences (Fig. 3.2). Plants grown in hydroponics #1, which was conducted in autumn 2014, produced the most biomass and tillers compared to the other experiments; with plants in hydroponics #2 (late spring 2014) growing on average 20 % less than those in hydroponics #1 (Fig. 3.3). Plants grown in hydroponics #3 (winter 2015) produced nearly half (43 %) the biomass seen in hydroponics #1 (Fig. 3.3). Between the three hydroponic experiments, the sibling lines from the same independent transformation events showed consistent trends in biomass and tiller number production particularly between lines CIPK16-1·1 and CIPK16-1·2 (Fig. 3.3 and Table 3.3). CIPK16-1 lines had significantly decreased whole plant biomass and tiller number under control (0 mM NaCl) conditions compared to nulls and other transgenic lines, and little to difference from nulls under 150 and 200 mM salt treatments; a trend also seen in shoot and root biomass (Sup. Fig. 3.1). On the other hand CIPK16-2 lines showed small increases in biomass under all salt conditions. CIPK16-3 lines had considerable variation between siblings, as CIPK16-3·1 had significantly reduced growth under 0 mM NaCl and some decrease in biomass under 150 mM NaCl. CIPK16-3·2 performed differently from its sibling line showing either no or a slightly decrease in biomass under either salt treatment. Results for the CIPK16-3 line are inconclusive due to the lack of transgenic plants identified in these transgenic lines. Root and shoot biomass measurements show the same seasonal and line trends as whole plant biomass (Sup. Fig. 3.1). Due to having a lower biomass under 0 mM NaCl conditions, CIPK16-1 lines had apparent increased salt tolerance (retention of biomass under salt conditions) compared to nulls and other transgenics. The increased biomass production of CIPK16-2·2 under all salt conditions also resulted in increased salt tolerance compared to null plants (Fig. 3.4). Differences between each transgenic sibling line and null line are summarised in Table 3.3.

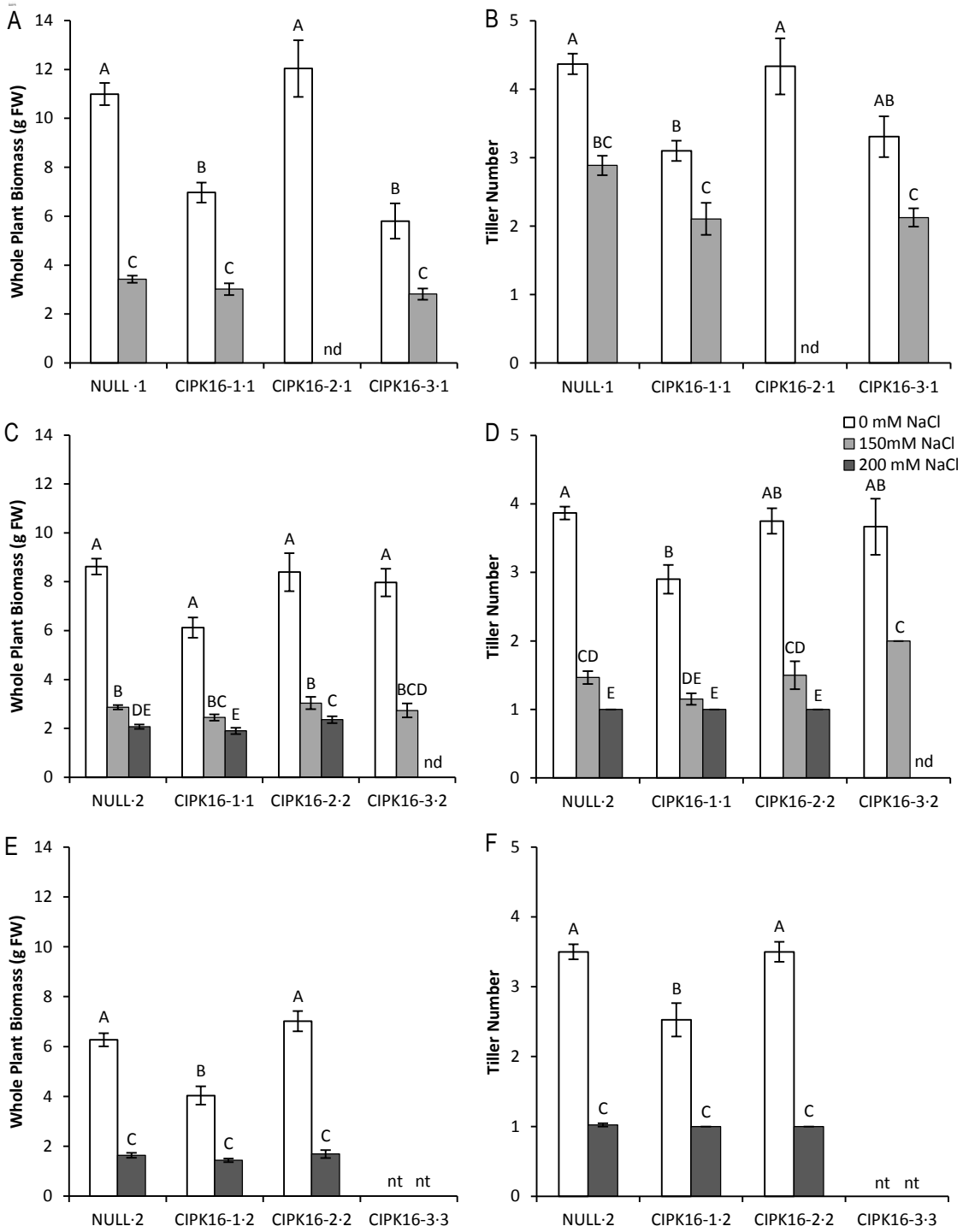


Figure 3.3: Whole plant biomass measurements and tiller number of null segregant and three transgenic *AtCIPK16* wheat lines grown in hydroponic experiments. Whole plant biomass (FW) of null segregant and three independent *AtCIPK16* transgenic wheat (cv. Gladius) lines grown in hydroponics in A) experiment #1 (autumn 2014), C) experiment #2 (late spring 2014) and E) experiment #3 (winter 2015). Tiller number of null segregant and three independent *AtCIPK16* transgenic wheat (cv. Gladius) lines grown in hydroponics in B) experiment #1 (autumn 2014), D) experiment #2 (late spring 2014) and F) experiment #3 (winter 2015) Plants were grown under 0 mM NaCl (white bars), 150 mM NaCl (light grey bars) and 200 mM NaCl (dark grey bars) treatments. Values are mean ± s.e.m (n = 3 – 47). nd = not determined due to no transgenics identified in one of the treatments. nt = line is not transgenic based on genotyping. Significant differences (one-way ANOVA, Tukey-Kramer, $P \leq 0.05$) indicated by letters.

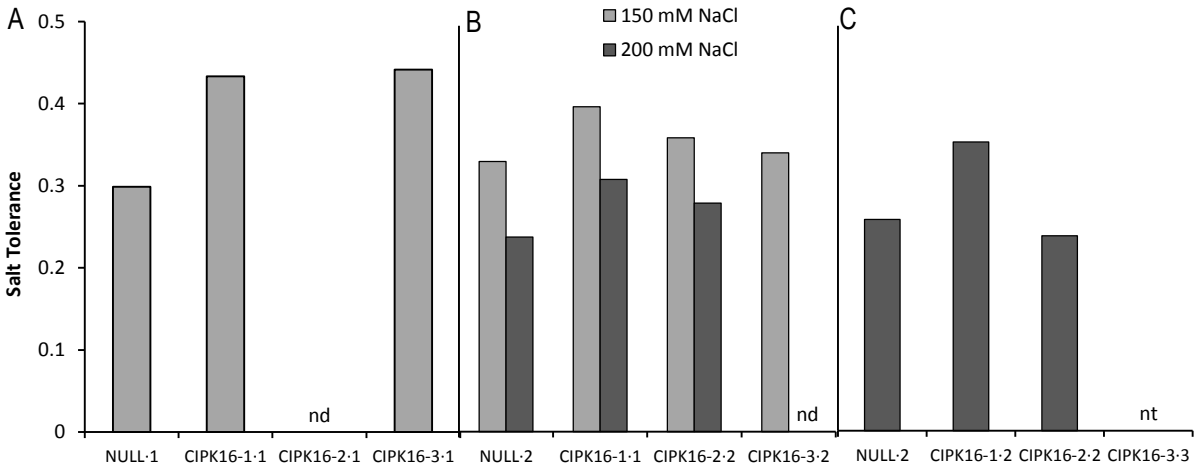


Figure 3.4: Relative salt tolerance of null segregant and three transgenic *AtCIPK16* wheat lines grown under hydroponic experiments. Salt tolerance as defined by the retention of average biomass under saline conditions compared to control conditions of null segregant and three independent *AtCIPK16* transgenic wheat (cv. Gladius) lines grown in hydroponics in (A) experiment #1 (autumn 2014); (B) experiment #2 (late spring 2014); and (C) experiment #3 (winter 2015).Salt tolerance under 150 mM NaCl (light grey bars) and 200 mM NaCl (dark grey bars) treatments. nd = not determined due to no transgenics identified in one of the treatments. nt = line is not transgenic based on genotyping.

3.3.3 Transgenic *AtCIPK16* lines have varying responses in leaf ion accumulation

Similar to biomass, considerable seasonal trends were seen in leaf Na^+ and Cl^- concentration under 150 mM and 200 mM salt conditions (Fig. 3.5). Plants grown in hydroponics #1, which had the highest biomass production, had the lowest concentrations of Na^+ and Cl^- (40 – 50 % less), compared to plants in hydroponics #2 at 150 mM NaCl. While plants in hydroponics #3, which produced the least biomass overall, accumulated the highest concentration of ions (10 – 35 % more), compared to hydroponics #2 plants grown at 200 mM NaCl (Fig. 3.5). In general under all salt treatments (0 mM, 150 mM and 200 mM) null and transgenic lines accumulated more Cl^- in leaf tissue than Na^+, with up to 40 % more Cl^- seen in the leaf tissue of some lines under 150 mM or 200 mM salt stress. Similar trends were seen between leaf Na^+ and Cl^- concentrations, with lines which accumulated more leaf Na^+ (e.g. CIPK16-1·2) also accumulating more Cl^- and those lines which showed decreased Na^+ also showed decreased Cl^- content (Fig. 3.5 and Table 3.3).

Unlike biomass measurements, some sibling lines of the same transgenic CIPK16 lines did not show consistent trends for Na^+ and Cl^- concentration. CIPK16-1·1 had increased Na^+ and Cl^- content in leaf tissue under 0 mM, 150 mM (significantly) and 200 mM salt treatments compared to null segregants (Fig. 3.5). Sibling line CIPK16-1·2 had increased Na^+ and Cl^- under 0 mM NaCl conditions and a slight decrease in Na^+ and Cl^- concentration under 200 mM salt treatment, which suggested possible Na^+ and Cl^- exclusion in this sibling line (Fig. 3.5 and Table 3.3). CIPK16-3 lines both had increased Na^+

concentration under 150 mM NaCl conditions compared to null segregants (Fig. 3.5) , although only CIPK16-3·1 had slightly increased Na^+ under control conditions as well (Table 3.3). Unlike other lines CIPK16-3 lines had differing Na^+ and Cl^- responses, as CIPK16-3·1 had increased Cl^- concentration under both salt treatment, while CIPK16-3·2 had decreased Cl^- concentration under 200 mM salt treatment. Results for the CIPK16-3 were once again inconclusive as sibling lines showed considerable variation in leaf Na^+ and Cl^- concentration. Unlike lines from the other two independent transgenic events, CIPK16-2 sibling lines shared a fairly consistent phenotype with little variability. CIPK16-2 lines had no difference in leaf Na^+ and Cl^- content at 0 mM and CIPK16-2·2 also had no difference in ion content compared to nulls at 150 mM salt treatment; while under 200 mM salt treatment line CIPK16-2·2 showed exclusion of Na^+ and Cl^- (Fig. 3.5 and Table 3.3). Differences between each transgenic sibling line and the null line are outlined in Table 3.3.

Unlike leaf Na^+ and Cl^- content, leaf K^+ showed no seasonal variations in concentration, although all null and transgenic lines had significant decreases in leaf K^+ concentration under 150 mM NaCl or 200 mM NaCl compared to control conditions (Fig. 3.6). Under 0 mM NaCl conditions none of the transgenic lines, except CIPK16-2·1 which had a 20 % increase, had any differences in K^+ concentration from the null segregants, unlike trends seen for leaf Na^+ and Cl^- (Table 3.3). Under 150 mM or 200 mM salt treatments a few lines had minimal increases in leaf K^+, except for CIPK16-1·1 which again accumulated increased amounts of ions (Fig. 3.6). Differences between the null line and each transgenic sibling line are summarised in Table 3.3.

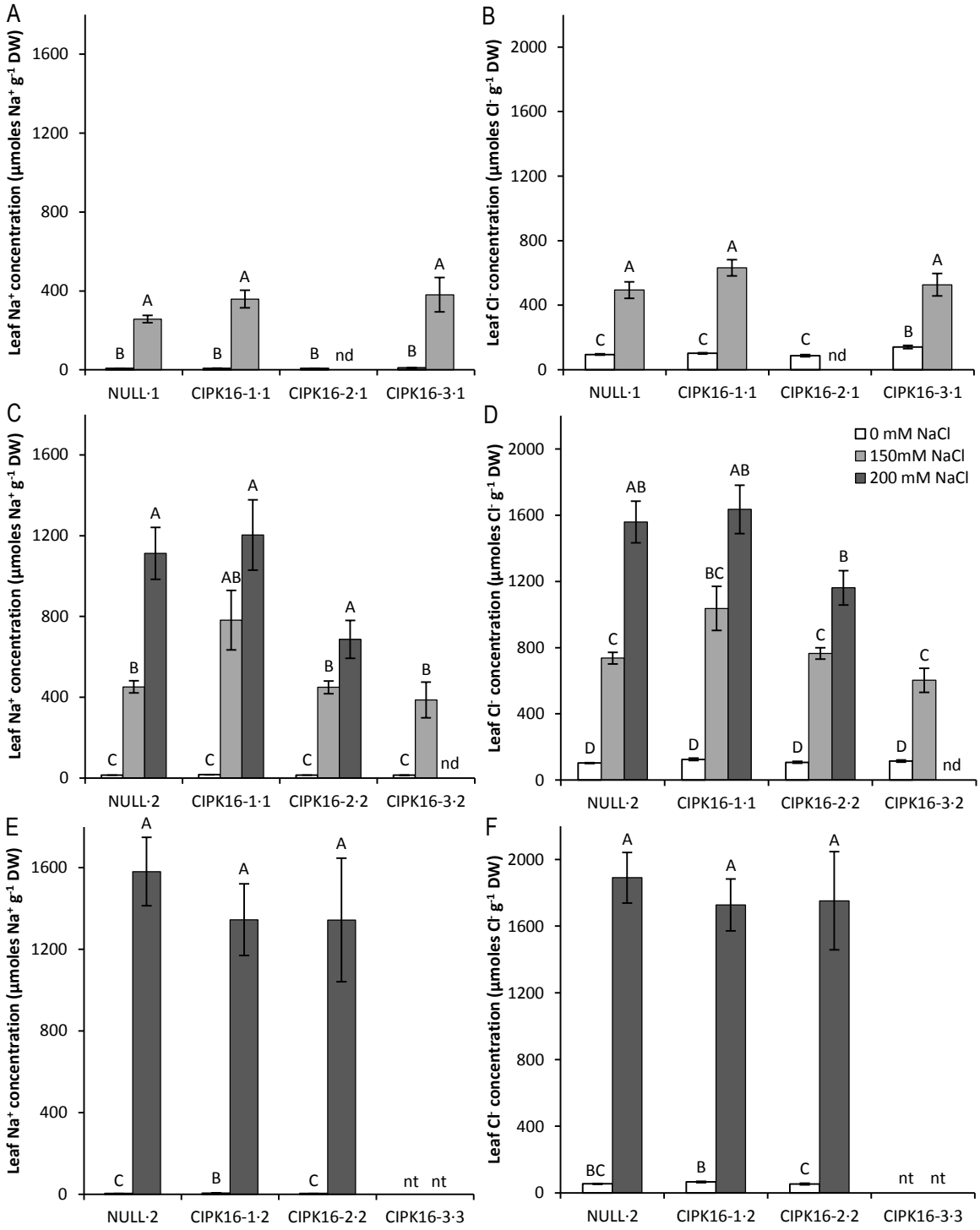


Figure 3.5: Leaf Na^+ and Cl^- concentration of null segregant and three transgenic *AtCIPK16* wheat lines grown in hydroponic experiments. Leaf Na^+ concentration (µmoles Na^+ g^{-1} DW) of null segregant and three independent *AtCIPK16* transgenic wheat (cv. Gladius) lines grown in hydroponics in A) experiment #1 (autumn 2014), C) experiment #2 (late spring 2014) and E) experiment #3 (winter 2015). Leaf Cl^- concentration (µmoles Cl^- g^{-1} DW) of null segregant and three independent *AtCIPK16* transgenic wheat (cv. Gladius) lines grown in hydroponics in B) experiment #1 (autumn 2014), D) experiment #2 (late spring 2014) and F) experiment #3 (winter 2015). Plants were grown under 0 mM NaCl (white bars), 150 mM NaCl (light grey bars) and 200 mM NaCl (dark grey bars) treatments. Values are mean ± s.e.m (n = 3 – 47). nd = not determined due to no transgenics identified in one of the treatments. nt = line is not transgenic based on genotyping. Significant differences (one-way ANOVA, Tukey-Kramer, $P \leq 0.05$) indicated by letters.

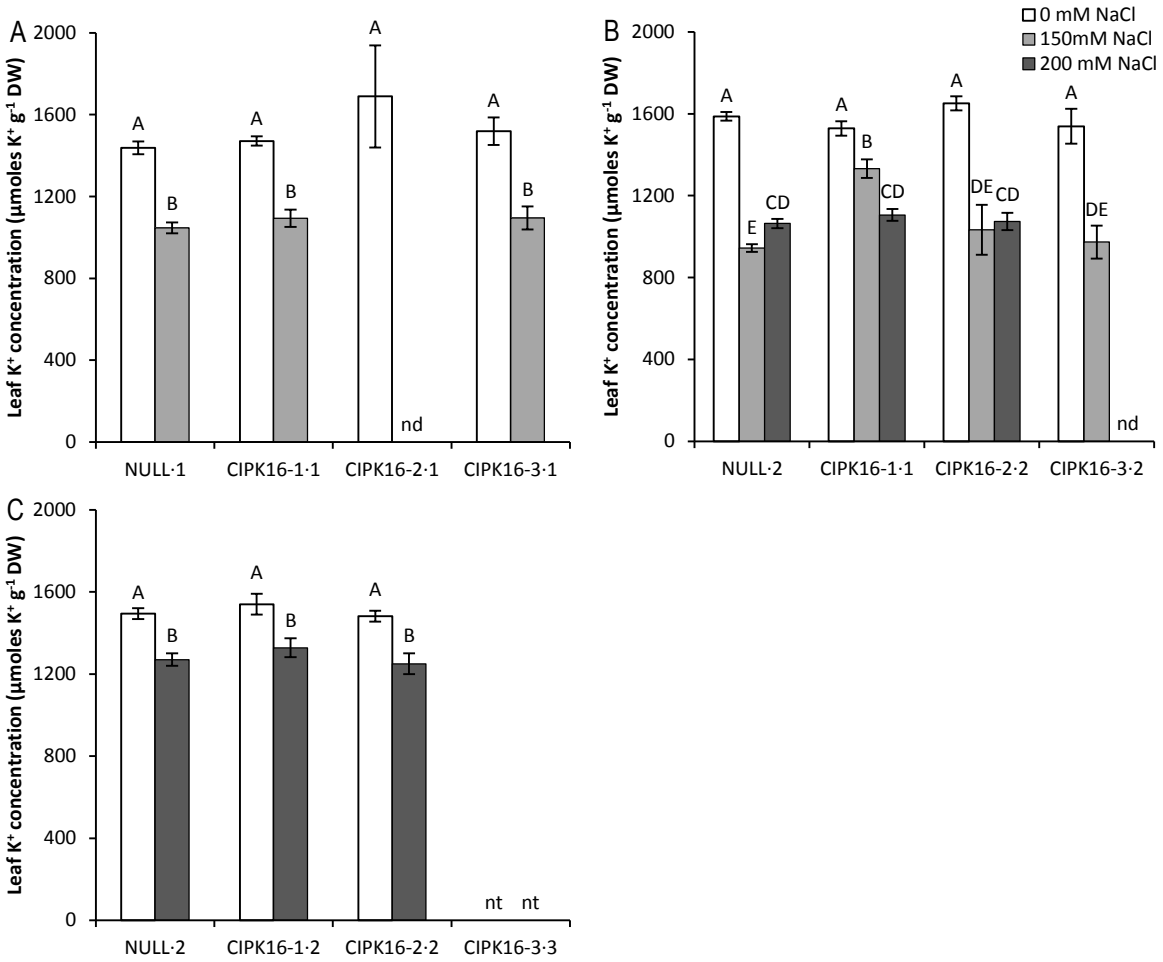


Figure 3.6: Leaf K^+ concentration of null segregant and three transgenic *AtCIPK16* wheat lines grown in hydroponic experiments. Leaf K^+ concentration (µmoles K^+ g^{-1} DW) of null segregant and three independent *AtCIPK16* transgenic wheat (cv. Gladius) lines grown in hydroponics in A) experiment #1 (autumn 2014), B) experiment #2 (late spring 2014) and C) experiment #3 (winter 2015). Plants were grown under 0 mM NaCl (white bars), 150 mM NaCl (light grey bars) and 200 mM NaCl (dark grey bars) treatments. Values are mean ± s.e.m (n = 3 – 47). nd = not determined due to no transgenics identified in this treatment. nt = line is not transgenic. Significant differences (one-way ANOVA, Tukey-Kramer, $P \leq 0.05$) indicated by letters.

3.3.4 Transgenic *AtCIPK16* lines show varied root ion accumulation trends

Seasonal variations between hydroponics #2 and hydroponics #3 were seen in root Na^+ and Cl^- concentrations, as lines in hydroponics #3 accumulated 8 – 20 % more Na^+ and Cl^- than those lines in hydroponics #2 (Fig. 3.7). All transgenic and null lines showed similar trends for both root Na^+ and Cl^- concentrations under all salt treatments (Fig. 3.7). Interestingly, unlike in leaf tissue, plants accumulated less Cl^- in the root tissue than Na^+ under 150 mM and 200 mM salt treatments; with the absolute values in the root reversed for Na^+ and Cl^-, as roots accumulated more Na^+ than leaves and less Cl^- than leaf tissue under salt stress (Fig. 3.5 & 3.7). CIPK16-1 lines showed some variation in the concentrations of Na^+ and Cl^-. CIPK16-1·1 showed increased concentrations of Na^+ and Cl^- under all salt treatments while CIPK16-1·2 only showed slight increases in Na^+ and Cl^- concentrations under 0 mM and only Cl^- at 200 mM NaCl (Table 3.4). CIPK16-2·2 had varying responses to salt treatments with small increases in Na^+ and Cl^- under 150 mM NaCl but less than 10 % decreases in ions under 200 mM salt treatment, with a similar trend seen for line CIPK16-3·2 (Table 3.4). Like leaf K^+ content, few differences were seen between lines for root K^+ concentration, particularly CIPK16-1 lines which had decreased K^+ under 0 mM. In the same trend as leaf tissue, roots under 150 mM or 200 mM NaCl treatment saw decreased K^+ content although this drop in K^+ concentration was much more significant with concentration reduced by over 50 % under salt treatment for all lines (Fig. 3.7E & F). Therefore despite roots having contained slightly more K^+ under 0 mM NaCl, under 150 mM or 200 mM NaCl treatments root tissue had limited K^+ content. Differences between the null line and each transgenic sibling line for which roots and shoot were harvested are summarised in Table 3.4.

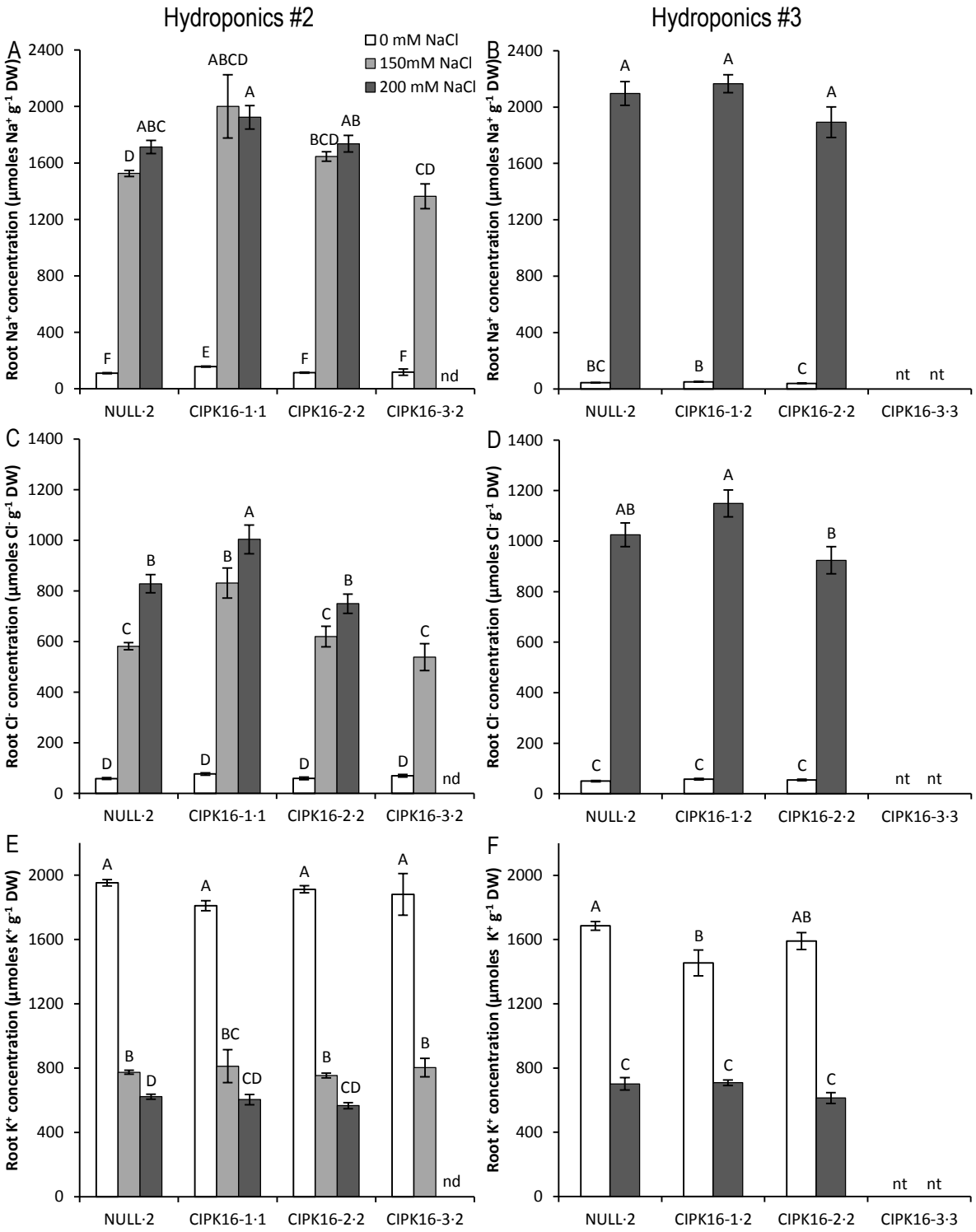


Figure 3.7: Root Na^+, Cl^- and K^+ concentration of null segregant and three transgenic *AtCIPK16* wheat lines grown in hydroponic experiments. Root Na^+ concentration (µmoles Na^+ g^{-1} DW) of null segregant and three independent *AtCIPK16* transgenic wheat (cv. Gladius) lines grown in hydroponics in A) experiment #2 (late spring 2014) and B) experiment #3 (winter 2015). Root Cl^- concentration (µmoles Cl^- g^{-1} DW) of null segregant and three independent *AtCIPK16* transgenic wheat (cv. Gladius) lines grown in hydroponics in C) experiment #2 (late spring 2014) and D) experiment #3 (winter 2015). Root K^+ concentration (µmoles K^+ g^{-1} DW) of null segregant and three independent *AtCIPK16* transgenic wheat (cv. Gladius) lines grown in hydroponics in E) experiment #2 (late spring 2014) and F) experiment #3 (winter 2015). Plants were grown under 0 mM NaCl (white bars), 150 mM NaCl (light grey bars) and 200 mM NaCl (dark grey bars) treatments. Values are mean ± s.e.m (n = 3 – 47). nd = not determined due to no transgenics identified in one of the treatments. nt = line is not transgenic based on genotyping. Significant differences (one-way ANOVA, Tukey-Kramer, $P \leq 0.05$) indicated by letters.

Table 3.3: Comparison of mean results for biomass and leaf ion concentrations for each sibling transgenic line grown in all three hydroponic experiments to the respective null segregants in the same experiment. * = significant difference from null segregant line.

Line	Experiment # & NaCl treatment		Biomass	Leaf Na^+ concentration	Leaf Cl^- concentration	Leaf K^+ concentration
CIPK16-1·1	1 & 2	0 mM	30 – 40 % decrease *	7 – 15 % increase	7 – 17 % increase	No difference
		150 mM	20 % decrease	45 % increase	20 – 25 % increase	5 – 30 % increase *
		200 mM	No difference	10 % increase	5 % increase	No difference
CIPK16-1·2	3	0 mM	30 % decrease *	35 % increase *	20 % increase	No difference
		200 mM	No difference	14 % decrease	8 % decrease	No difference
CIPK16-2·1	1	0 mM	10 % increase	No difference	No difference	20 % increase
		150 mM	Not determined			
CIPK16-2·2	2 & 3	0 mM	0 – 10 % increase	No difference	No difference	No difference
		150 mM	30 % increase	No difference	No difference	9 % increase
		200 mM	0 – 20 % increase	14 – 35 % decrease	5 – 30 % decrease	No difference
CIPK16-3·1	1	0 mM	45 % decrease *	20 % increase	30 % increase *	No difference
		150 mM	17 % decrease	30 % increase	6 % increase	No difference
CIPK16-3·2	2	0 mM	7 % decrease	No difference	10 % increase	No difference
		150 mM	No difference	14 % decrease	18 % decrease	No difference
		200 mM	Not determined			
CIPK16-3·3	3	0 mM	Line not transgenic			
		200 mM	Line not transgenic			

Table 3.4: Comparison of mean results for root ion concentrations for each sibling transgenic line grown in all three hydroponic experiments to the respective null segregants in the same experiment. * = significant difference from null segregant line.

Line	Experiment # & NaCl treatment		Root Na^+ concentration	Root Cl^- concentration	Root K^+ concentration
CIPK16-1·1	2	0 mM	30 % increase *	24 % increase	7 % decrease
		150 mM	24 % increase	30 % increase *	5 % increase
		200 mM	11 % increase	17 % increase *	No difference
CIPK16-1·2	3	0 mM	11 % increase	13 % increase	14 % decrease *
		200 mM	No difference	11 % increase	No difference
CIPK16-2·2	2 & 3	0 mM	0 – 12 % decrease	0 – 7 % increase	No difference
		150 mM	7 % increase	6 % increase	No difference
		200 mM	0 – 9 % decrease	10 % decrease	9 – 13 % decrease
CIPK16-3·2	2	0 mM	7 % increase	16 % increase	No difference
		150 mM	10 % decrease	7 % decrease	No difference
		200 mM	Not determined		
CIPK16-3·3	3	0 mM	Line not transgenic		
		200 mM	Line not transgenic		

3.4 Discussion

3.4.1 Response of Gladius wheat to NaCl treatment

Bread wheat is considered a moderately salt tolerant crop species due to its ability to exclude Na^+ particularly from shoot tissue under normal growth conditions (Munns & Tester 2008). Under salt stress conditions (150 mM or 200 mM NaCl) all lines suffered significant reductions in tiller number, whole plant, shoot and root biomass production, with more severe reductions seen under 200 mM stress (Fig. 3.3), consistent with growth reductions seen in the literature (Genc, et al. 2007; Rahnama, et al. 2010; Rahnama, et al. 2011). Consistent with the typical behaviour of bread wheat, plants accumulated more Na^+ in leaf tissue as the salt stress conditions increased (Fig. 3.5) (Rahnama, et al. 2010; Rahnama, et al. 2011) accompanied by the same pattern of increase in leaf Cl^- accumulation. Under all growth conditions plants accumulated more Cl^- in the leaf tissue than Na^+ suggesting that bread wheat is unable to regulate the transport of Cl^- up to shoot tissue as well as its regulation of Na^+. This is also supported by the root ion data which illustrates that under salt stress root tissue accumulates more Na^+ than the leaf, suggesting possible retrieval of Na^+ from the transpiration stream and storage in root tissue (Munns & Tester 2008; Roy, et al. 2014). Cl^- on the other hand shows the opposite phenotype, with more Cl^- accumulating in leaf tissue than root tissue under salt stress conditions, once again supporting the hypothesis that wheat is unable to control the transport of Cl^- into shoot tissue as well as other ions such as Na^+. Interestingly, in the root tissue there is little difference in the Na^+ and Cl^- concentrations at 150 mM and 200 mM, which is quite different to leaf tissue, where large differences in ion accumulation are seen between these two salt concentrations. This suggests that at high salt concentrations wheat is stressed beyond the capacity of the root tissue to remove and store Na^+ and some Cl^- from the xylem and thus reduce the amount of Na^+ reaching the shoot tissue. Another critical factor in plant salt tolerance is the ability to maintain a high Na^+/K^+ ratio to enable correct cellular metabolic functioning, it is therefore expected that bread wheat accumulates more K^+ than Na^+ in shoot tissue (Munns & Tester 2008). Under 0 mM both leaf and root tissue maintained similar amounts of K^+ well above Na^+ levels. In leaf tissue at 150 mM NaCl, K^+ content was above Na^+ content, although when plants were subjected to 200 mM NaCl the ratio switched to plants accumulating more Na^+ than K^+, despite only a small decrease in the overall leaf K^+ concentration between 0mM and 200 mM NaCl stress (Fig. 3.3). Under salt stress the root K^+ concentration drops significantly, likely due to increased movement of K^+ to the leaf tissue to help maintain higher levels of K^+ in these critical tissues. These changes in leaf and root K^+ and Na^+ concentrations are echoed in literature such as Rahnama et al. 2010 and Rahnama et al. 2011. Overall null segregant and transgenic Gladius wheat plants demonstrated a range of normal

phenotypes for salt stress wheat, although seasonal variations due to the timing of different hydroponic experiments has made the interpretation of results difficult.

Large differences between hydroponic experiments were observed particularly in biomass production as well as leaf Na^+ and Cl^- concentrations, likely due to variations in sunlight availability and temperature. Hydroponic #1 was conducted in autumn 2014, hydroponics #2 in late spring 2014 and hydroponics #3 in winter 2015, which all had different average minimum/maximum temperatures and daily hours of sunlight throughout the respective growing seasons: 12°C/23°C and 7.2 hrs of sunshine, 15°C/27°C and 9.8 hrs of sunlight and 7°C/17°C and 4.7 hrs of sunlight, respectively (Weather Station 023090 Kent Town, Adelaide SA and 023034 Adelaide Airport, SA; http://www.bom.gov.au/climate/).

Plants from all lines produced increased biomass in hydroponic #1 compared to the other experiments like due to the ideal growth conditions plants experienced. Plants grown in this experiment experienced warm temperatures but not high enough to subject plants to temperature stresses with plenty of sunlight to promote photosynthesis (Porter & Gawith 1999). Plants were therefore able to grow, involving the uptake of nutrient solution (including Na^+ and Cl^-), but due to the optimal conditions were able to dilute the incoming Na^+ and Cl^- ions with the production of water filled new tissue (Munns, et al. 1995; Colmer, et al. 1995), thus resulting in the low ion concentrations seen (Fig. 3.2A).

Despite also having plenty of sunshine to promote photosynthesis, growth conditions during hydroponic experiment #2 were less optimal, due to a slight increase in average minimum/maximum temperatures throughout the experiment as well as several days over 30°C. The higher temperatures prompted plants to increase transpiration as a method of evaporative cooling (Gates 1964; Paulsen 1994) which also increased Na^+ and Cl^- uptake with the higher water demand. The increased energy use from higher transpiration and slight temperature stress would have also decreased the energy available for plant growth, resulting in the smaller plants seen in this experiment (Fig. 3.2C).

Experiment #3 produced the least amount of biomass out of the three experiments due to the experiment being conducted in winter, with little sunlight and much cooler temperatures. With not as much biomass being produced, plants were unable to dilute incoming ions in new tissue and therefore accumulated large amounts of Na^+ and Cl^-. It is commonly acknowledged that planting season affects wheat growth, although there is little published data supporting this. The differences in biomass and

leaf ion concentrations seen in this project due to growth seasons are similar to trends seen in Genc et al. 2007.

3.4.2 One transgenic line, CIPK16-2·2, demonstrates a Na^+ and Cl^- exclusion phenotype

The constitutive expression of *AtCIPK16* in both Arabidopsis and barley has previously resulted in clear increases in biomass production and Na^+ exclusion under salt stress, in all three independent transgenic lines for each species (Roy, et al. 2013). In this project not only was considerable phenotypic variation observed between the three independent transgenic lines, CIPK16-1, CIPK16-2 and CIPK16-3, but disparity within each transformation event between sibling lines was also seen. The two sibling lines CIPK16-1·1 and CIPK16-1·2 showed reasonably consistent results between all salt treatments in all hydroponic experiments. Under control (0 mM NaCl) conditions plants from this transformation event produced significantly less (30 – 40 %) root, shoot and therefore whole plant biomass, due not only to fewer tillers but smaller roots, shoots and leaves. Under 150 mM salt treatment this decrease in biomass compared to null segregants was not as severe, while under 200 mM plants from the CIPK16-1 sibling lines performed the same as nulls. Consistent with not producing much biomass these plants also had increased Na^+ and Cl^- concentrations in both the leaf and root tissue under all salt conditions, likely due to their inability to dilute the ions in new tissue growth. Although unlike its sibling line CIPK16-1·2 showed a possible shoot Na^+ and Cl^- exclusion phenotype under 200 mM salt stress, although due to large variation within the data set these decreases were not significant. In these plants root Na^+ was not affected although root Cl^- concentration did increase, which may suggest that in this sibling Na^+ and Cl^- was being effectively excluded not only from the shoot but Na^+ from the roots as well, although this did not have any effect on biomass production. *AtCIPK16* in CIPK16-1 wheat does caused reduced plant biomass especially under normal growth conditions, although due to variation between siblings lines it is inconclusive whether or not Na^+ and Cl^- are accumulated or excluded from this line under salt stress.

The most variable of the transgenic lines was CIPK16-3, which despite evaluating three sibling lines, produced very few transgenic plants, with the analysis of sibling line CIPK16-3·3 not possible due to all plants being null segregants. Sibling line CIPK16-3·1 behaved in a manner similar to CIPK16-1·1, producing reduced biomass especially under 0 mM salt treatment, as well as accumulating more Na^+ and Cl^- in leaf tissue at both 0 mM and 150 mM NaCl. CIPK16-3·2 on the other hand produced very similar biomass to null segregants and showed a possible leaf and root Na^+ and Cl^- exclusion phenotype under 150 mM salt stress, unlike CIPK16-1·2 which only showed likely exclusion in shoot tissue. Data at 200 mM is not available for CIPK16-3·2 due to a lack of transgenic plants. Due to the

lack of transgenic CIPK16-3 plants and the considerable variation in salt tolerance and Na^+ exclusion capacity between siblings, it is inconclusive whether or not *AtCIPK16* improves the salt tolerance through exclusion in CIPK16-3 wheat.

Unlike other lines CIPK16-2 siblings performed the same under measured salt conditions with both sibling lines CIPK16-2·1 and CIPK16-2·2 showing slightly increased biomass production and leaf K^+ content with no differences in leaf Na^+ and Cl^- concentration from null segregants. A lack of transgenic plants in the CIPK16-2·1 sibling line under 150 mM NaCl means that further comparisons between siblings cannot be made. Under high salt stress (150 mM and 200 mM NaCl) CIPK16-2·2 continued to show improved growth over null segregants, although the increase in biomass varied between experiments. Under 200 mM salt stress CIPK16-2·2 showed substantial leaf Na^+ and Cl^- exclusion accompanied by a smaller decrease in ion concentration in root tissue, despite no differences being noted in leaf ion concentrations at 150 mM. These results suggest that at 0 – 150 mM NaCl *AtCIPK16* has no real effect on CIPK16-2 transgenic wheat other than increased biomass under all conditions, but when plants are grown under high salt (200 mM) CIPK16-2·2 has increased biomass production accompanied by leaf ion exclusion and some root ion exclusion. It should be noted that for scientific purposes understanding the mechanisms behind the salt tolerant phenotype of CIPK16-2·2 is important. Therefore at least three independent transgenic events showing the same phenotype need to be obtained before results can be published. However in a commercial setting, where large bioscience companies patent individual transformation events as well as genes (Hairmansis 2014), this single transformation event would be of value as a breeding resource for increasing biomass production and Na^+ exclusion under salt stress in elite cultivar production.

The results from this project do not reflect the main trends seen in a preliminary experiment conducted with the T_1 generation of these transgenic lines under 200 mM NaCl stress in the same supported hydroponic experimental system (Roy, et al. unpublished). Results from this preliminary experiment demonstrated that under salt stress all three transgenic lines expressing *AtCIPK16* had increased relative biomass accompanied by Na^+ exclusion, with up to a 45 % decrease in leaf Na^+ concentration (Roy, et al. unpublished). Data collected from three separate hydroponic experiments in this project instead show one line CIPK16-2·2 which displays the same phenotype, two lines CIPK16-1·2 and CIPK16-3·2 showed signs of some of the desired phenotype, while all the other transgenic lines show differing phenotypes from the preliminary experiment or the phenotype was not able to be established due to genotype issues.

A significant issue for all the CIPK16 lines was the lack of good quality seed material. This often hindered the capacity for growth of the same sibling line in multiple hydroponic experiments, particularly for sibling CIPK16-3 lines. Disparity between original seed quality could explain some of the variation seen within individual plants of the same transgenic line, which has impacted analysis of the data by producing very large error bars in some cases. Seed quality issues also impacted on the amount of transgenic material available to phenotype, as many seeds did not germinate and few died after being transplanted into the hydroponic systems. Genotyping analysis then revealed that for certain sibling lines most plants grown where null segregants, which meant that analysis of data was once again hindered due to the low number of individual replicated (plants) for certain transgenic lines.

While transgenic plants were confirmed to possess the at least some of the *AtCIPK16* transgene (most possessed the full *AtCIPK16* gene) in the gDNA, the transgene gene was not expressed in most of the plants of these lines. In a few plants, expression of small parts of the transgene was identified but expression of the full length of *AtCIPK16* transgene could not be identified in any plants. The lack of phenotype in all of the CIPK16 lines was therefore likely due to them not expressing the transgene resulting in no protein being produced and so the lines behaving similarly to the null segregants. Phenotype differences between the three independent transgenic lines may also be due to effects from the insertion site of the transgene which may have knocked out/disrupted a native gene or promoter. Overall the lack of full *AtCIPK16* expression complicated the analysis of the data as it is possible that most of the genomically identified transgenic plants actually behaved similar to nulls to due to a lack of *AtCIPK16* expression.

3.4.3 Disruption of transgene expression: hypothesised reason for lack of phenotype

Unlike for transgenic plants in the Roy, et al. (unpublished) study, stable and reliable expression of the *AtCIPK16* transgene was not confirmed in any of the experiments performed in this project. Expression of the full length of the *AtCIPK16* transgene was never identified, although occasionally using primer pairs which amplified smaller fragments (Exon 1, Middle and Exon 2; see Table 3.2) a cDNA product could be amplified which indicated that mRNA for at least some of the transgene was present in a few plants. The issues with identifying *AtCIPK16* expression may have been due to technical difficulties in the expression analysis process, such as RNA degradation and fragmentation which would make the amplification of longer sections of specific mRNAs, i.e. *AtCIPK16,* difficult or the presence of secondary structures in the original mRNA which inhibit the RT-PCR process (Malboeuf, et al. 2001; Bustin & Nolan 2004). Other explanations for the lack of transgene expression

involve possible underlying genetic and epigenetic problems associated with these *AtCIPK16* wheat lines, i.e. incomplete insertion/s of the transgene, incorrect initiation of *AtCIPK16* transcription or epigenetic silencing.

The process of transformation is currently quite imprecise in terms of transgene insertion with many issues arising from the process which can affect transgene expression and thus affect plant phenotype. While transformation efficiency is generally quite low, it is quite common for multiple inset lines to be generated containing the promoter+transgene inserted in several different places within the genome or even sequentially at the same locus (De Block 1993; Kohli, et al. 1998; Pawlowski & Somers 1998; Jackson, et al. 2001; Wright, et al. 2001; Zhou, et al. 2003). The lines used in this project were originally transformed using biolistics, a method know to create fragmentation of the insertion vector and result in the insertion of multiple copies of the truncated transgene on top of any full length copies inserted (De Block 1993; Pawlowski & Somers 1998; Wright, et al. 2001). While it is known that between one and four copies of the full *AtCIPK16* transgene was inserted into the three independently transformed CIPK16 transgenic lines, it is possible that multiple truncated copies of *AtCIPK16* fragments could also having been inserted at each transformation event. It is also unknown where in the genome the transgene/s inserted, although through further investigation it is possible to visualise the insertion of the transgene through fluorescence in situ hybridization (FISH) (Pedersen, et al. 1997; Jackson, et al. 2001). Insertion of the transgene may have disrupted a native gene, promoter, enhancer or repressor element, or misaligned the spacing between such elements such that the insertion of the transgene not the transgene itself causes a change in the expression of other native genes and therefore the plant phenotype. With it likely that each of the original CIPK16 transgenic lines contained multiple copies or fragments of the *AtCIPK16* transgene, the natural process of chromosome segregation during meiosis may explain some of the differences in phenotype. The preliminary experiment was conducted using T_1 plants while the experiments in this project were conducted on subsequent generation T_3 plants. Being a subsequent generation, lines have undergone segregation and therefore likely lost copies of the transgene or gene fragment (Srivastava, et al. 1996). The lack of plants containing the transgene in the gDNA in the CIPK16-3 sibling lines may be explained by the loss of the transgene present in the T_1 generation by segregation. Segregation may also explain why by the T_3 generation sibling lines from the same transgenic event show different phenotypes due to different numbers of transgene/fragment inserts and their locations in the genome. Techniques such as Southern analysis or quantitative-PCR could be used to identify *AtCIPK16* insert number in these lines (Ingham, et al. 2001; Li, et al. 2004), with the use of multiple probes, binding to different sections of the transgene, hopefully allowing for the

identification of truncated transgene fragments as well as full length copies. Visualisation of the transgene inserts and fragments via techniques such as FISH (Pedersen, et al. 1997; Jackson, et al. 2001) could also be valuable in understanding not only the positon of the inserts but changes in the insert locations through generations. The screening of multiple lines and generations could help to reveal which inserts/fragments have been inherited through the generations and which have been lost through segregation. The changes in insert copy number and location data over generations could be used in conjunction with phenotypic data to identify which of the original inserts in the T_1 generation had the most positive phenotypic effect and are therefore valuable inserts.

In the three hydroponic experiments a considerable number genomic null segregants were identified likely due to issues discussed above. Yet most of the genomically transgenic plants identified seemed to behave similar to the null segregants, due to an additional problem of the lack of *AtCIPK16* expression, as seen by the lack of full length transgene transcript and only the occasional partial transcript identification. Changes in the epigenome of the transgenic lines may explain the differences in expression and phenotype between the plants, lines and generations of *AtCIPK16* expressing wheat. While the transgene insertion site and positional effects can disrupt native genes it can also affect transgene expression. Certain locations in the genome are naturally silenced by chromatin modifications and DNA methylation resulting in no expression of any genes in these regions, therefore transgene insertion into one of these regions would automatically result in silencing and a lack of expression of the transgene (Finnegan & McElroy 1994; Matzke & Matzke 1998; Kooter, et al. 1999). Literature has shown that in some cases the presence of more than one copy of a gene per genome, whether the introduced gene is completely novel or another copy of a native gene, can result in silencing of all gene copies in a process known as homology-dependent gene silencing (Finnegan & McElroy 1994; Flavell 1994; Kohli, et al. 1999; Kooter, et al. 1999). This may explain why in some CIPK16 lines and plants, which possibly contain more than one transgene insert, gene expression is completely lost after the T_1 generation, consistent with the expression analysis in this project and the change in phenotype from the preliminary experiment. The most likely explanation of the considerable overall variation seen on many levels, between plants, sibling lines, transformation events and preliminary versus current experiments, is transgene silencing (TGS). TGS is the gradual silencing of inserted transgenes by DNA methylation often of cytosine residues which results in a stable and heritable changes in the methylation pattern and ultimately chromatin structure, and blocks gene expression (Kooter, et al. 1999). This epigenetic change occurs on an individual plant basis although some changes can be inherited, and often progresses with subsequent generations leading to total loss of expression after several generations (Kilby, et al. 1992; Srivastava, et al. 1996; Demeke, et al.

1999; Kohli, et al. 1999; Kooter, et al. 1999; Anand, et al. 2003). It should be noted that in some plants partial *AtCIPK16* transcripts were amplified, although no full transcripts were identified, which would not occur if TGS was occurring in these plants. It is therefore possible that these lines were not able to produce the full *AtCIPK16* transcript which either resulted in no protein or truncated non-functional peptides being produced. The lack of expression and therefore salt tolerance or Na^+ exclusion phenotype seen in just about all transgenic lines in this experiment is therefore likely due to a lack of full *AtCIPK16* transcript (therefore protein) and/or TGS which started in the T_1 plants and has become more severe in subsequent generations with only a few individual plants still showing some expression of the *AtCIPK16* transgene or gene fragments.

3.5 Conclusions & Future directions

With the considerable variation in the data on many levels from growing seasons to epigenomes it is difficult to interpret the results obtained in the project to conclude whether the presence or expression of *AtCIPK16* in wheat has any positive effects on wheat salt tolerance via increased Na^+ exclusion capacity.

Considerable work needs to be conducted in the future to determine whether the constitutive expression of *AtCIPK16* can improve the salt tolerance via increased Na^+ exclusion in bread wheat (cv. Gladius). To do so T_2 or T_3 material from the wide range transgenic lines available needs to be reanalysed and the correct lines selected to perform the same characterisation as performed in this project. Selected lines need to contain a single copy of the *Ubi:AtCIPK16* insert, ideally inserted through *Agrobacterium*-mediated transformation, into region of the genome known not to be naturally silenced. Expression and DNA methylation of the transgene in plants from subsequent generations of three independent transformation events should also be tested. Once this information is known about the selected lines further characterisation in glasshouse and field conditions can take place to examine the effects of *AtCIPK16* expression in wheat.

Although no direct AtCIPK16 ortholog exists in wheat (Amarasinghe, et al. in press), wheat does possess close relative to AtCIPK16, CIPK5 and CIPK25, and with future characterisation may harbour a CIPK with similar targets or expression profiles to AtCIPK16. These native wheat CIPKs would be excellent targets for gene editing using CRISPR/Cas9. Characterisation of wheat lines with edited expression profiles of closely related CIPKs may further the understanding of the effects of AtCIPK16 expression in bread wheat.

Chapter 4: Determination of whether the presence/absence of TATA-box in the AtCIPK16 promoter is responsible for the AtCIPK16 expression differences observed between Arabidopsis ecotypes

4.1 Introduction

Gene expression is complex interconnected process regulated at multiple levels within the cell. Gene promoters play an important role in influencing the level of gene transcription though the presence of *cis*-, *trans*- and basal motifs at key locations throughout the promoter (Komarnytsky & Borisjuk 2003). Key to gene transcription is the core promoter, the region of the promoter up to 100 bp 5' of the transcription start site (TSS) where the RNA polymerase and associated pre-initiation complex (PIC) binds (Aso, et al. 1994; Singh 1998; Molina & Grotewold 2005; Juven-Gershon & Kadonaga 2010; Kumari & Ware 2013). In certain promoters a key element of the core promoter is the TATA-box, a motif where TATA-box binding factors (TBPs) are able to bind, recruiting other proteins such as transcription initiation transcription factors aiding the formation of the PIC, which allow for the precise initiation of transcription at the TSS by RNA polymerase II (Aso, et al. 1994; Reindl & Schöffl 1998; Singh 1998; Juven-Gershon & Kadonaga 2010). TATA-boxes are not present all gene promoters, instead are more predominant in the promoters of stress-responsive genes and are least predominant in the promoters of 'housekeeping' genes (Molina & Grotewold 2005; Bernard, et al. 2010; Zuo & Li 2011; Kumari & Ware 2013). Genes which contain TATA-boxes also have shorter 5' UTRs (Molina & Grotewold 2005; Kumari & Ware 2013). It is estimated that only 29 % of Arabidopsis, 19 % of rice, 17.7 % of monocot and 17.6 % of dicot genes have promoters containing TATA-boxes (Molina & Grotewold 2005; Civán & Svec 2009; Kumari & Ware 2013).

TATA boxes are typically located between -36 and -28 bp before the TSS, optimally around -32 bp (Sawant, et al. 1999; Molina & Grotewold 2005; Zuo & Li 2011). It is known that promoters can contain

multiple TATA-box elements which all play an important role in gene regulation, with the addition of single or multiple TATA-boxes shown to enhance expression (Grace, et al. 2004). Despite being now defined by a consensus sequence, TATAWAW (W = A or T), many sequence variances for plant TATA-boxes exist (Joshi 1987; Vankan & Filipowicz 1989; Heard, et al. 1993; Zhu, et al. 1995; Sawant, et al. 1999; Shahmuradov, et al. 2003; Molina & Grotewold 2005; Kiran, et al. 2006; Juven-Gershon & Kadonaga 2010; Zuo & Li 2011). Literature shows that depending on the gene or gene family, mutations in the TATA-box can either have no effect, reduced expression or lead to no gene expression (Vankan & Filipowicz 1989; Heard, et al. 1993; Zhu, et al. 1995; Grace, et al. 2004; Kiran, et al. 2006). The position of the mutation within the TATA-box also has an effect on subsequent gene expression, with complete deletion of the TATA-box, either by replacement with another DNA sequence or true deletion, resulting in little to no expression (An, et al. 1986; Ha & An 1989; Vankan & Filipowicz 1989; Heard, et al. 1993; Zhu, et al. 1995; Kiran, et al. 2006). Sensitivity to changes in the TATA-box is thought to be related to the ability of TBPs to recognise and bind to these mutations (Heard, et al. 1993). Spacing of the TATA box relative to other promoter elements is also know to affect gene expression (Zhu, et al. 1995; Grace, et al. 2004). Overall despite playing an important role in transcription initiation of highly expressed stress responsive genes, little is still known about the role of TATA-boxes in individual gene promoters.

While examining the sequence for a putative salt tolerance gene *AtCIPK16* in two Arabidopsis ecotypes, Bay-0 and Shahdara, it was discovered that the only major sequence difference between the two alleles was in the promoter region (Roy, et al. 2013). The Bay-0 allele had a 10 bp deletion in the promoter -31 bp before the TSS which added a TATA-box (TATATAA) not present in the Shahdara sequence (Roy, et al. 2013). Reporter promoter constructs indicated that the Bay-0 allele had the highest expression especially under salt, while the Shahdara allele had basal levels of expression (Roy, et al. 2013). While the different reporter constructs trialed in Roy, et al. (2013) showed that differences in *AtCIPK16* expression were likely due to promoter allele differences it was not shown directly that the TATA-box was directly responsible for the phenotype differences. It was therefore hypothesised that by performing site directed mutagenesis on the gene's promoter the role of the TATA-box in *AtCIPK16* expression could be tested by removing the TATA-box from the Bay-0 allele and creating a TATA-box in the Shahdara allele. This chapter outlines the work conducted to begin testing the previously outlined hypothesis.

4.2 Materials and Methods

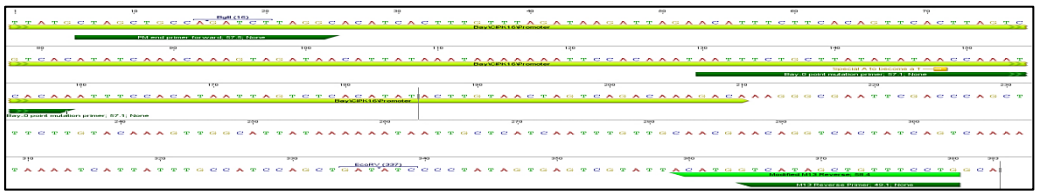
4.2.1 Analysis of promoter regions to identify mutation sites

Bay-0 and Shadara *AtCIPK16* promoter sequences from Roy, et al. (2013) were analysed using Geneious 6.0 (http://www.geneious.com, Kearse et al. 2012) to identify the possibility to modify the promoters to give the presence/ absence of a TATA box (TATAA). Once a suitable site was found primers were designed using Geneious 6.0 (http://www.geneious.com, Kearse et al. 2012) and Primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/, Ye et al. 2012) (see Table 4.1) to perform site directed mutagenesis by introducing point mutations into the desired location. For each promoter sequence, one primer was designed to introduce the point mutation, as the set was designed to contain the desired point mutation in the centre of the primer.

4.2.2 Introducing point mutations by PCR mutagenesis.

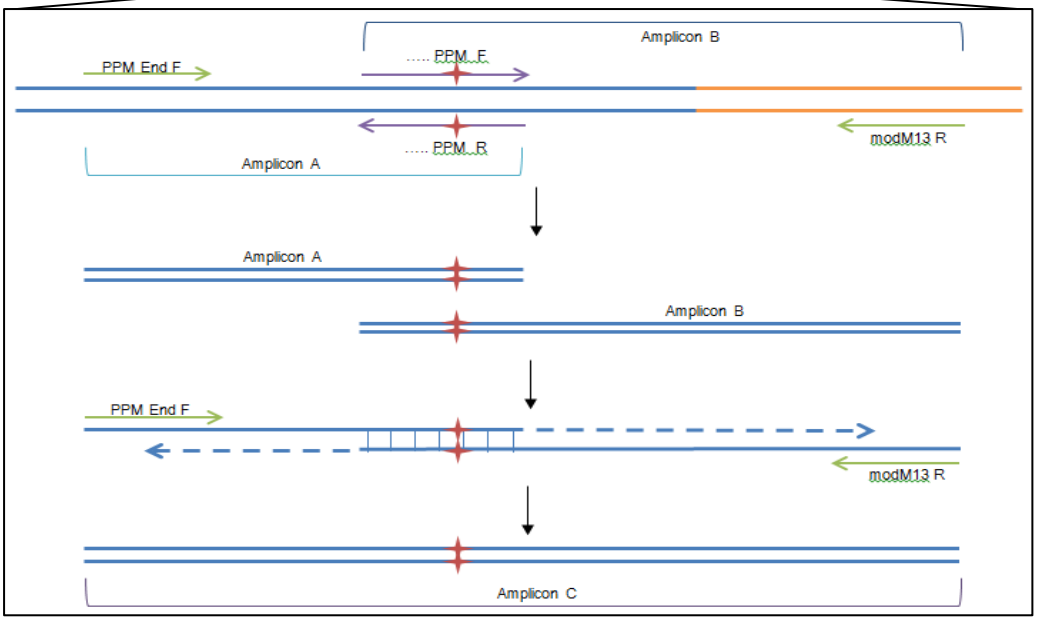
To begin introducing the point mutations into the Bay-0 and Shahdara promoters amplicons A and B (Fig. 4.1) were first created using primers designed to incorporate the desired point mutation (see Table 4.1). Template DNA, the full *AtCIPK16* promoters from both Bay-0 and Shahdara in pCR8 (kindly supplied by Dr. Stuart Roy), was diluted a concentration of 10 – 20 ng/µL. All PCRs to amplify amplicons A and B from either allele were performed with Phusion® High-Fidelity DNA polymerase (New England Biolabs), to ensure no addition mutations were introduced, in 50 µL reaction volumes. Each reaction contained 3 µL of diluted plasmid for the appropriate allele, 10 µL 5× Phusion HF reaction buffer, 1 µL 10 mM dNTPs, 1 U Phusion HF DNA polymerase, 2.5 µL 10 mM forward primer and 2.5 µL 10 mM reverse primer for the appropriate reaction. The PCR conditions used to amplify amplicons A and B were as follows: an initial denaturation at 98°C for 30 s, followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 63°C for 30 s and extension at 72°C for 15 s, followed by a final extension at 72°C for 5 mins. 10 µL of PCR products were visualised using gel electrophoresis with a 1.5 % agarose gel containing 5 µL/100 mL SYBRsafe® stain (Invitrogen). Bands of the correct size were excised from the agarose gel under UV light and purified using the DNA extraction from agarose gels protocol of a NucleoSpin® Gel and PCR Clean-Up Kit (Macherey-Nagel). Spectrophotometry was used to check quality and quantity of amplicons A and B purified (see Section 2.2.4).

4.2.1 Sequence analysis and primer design

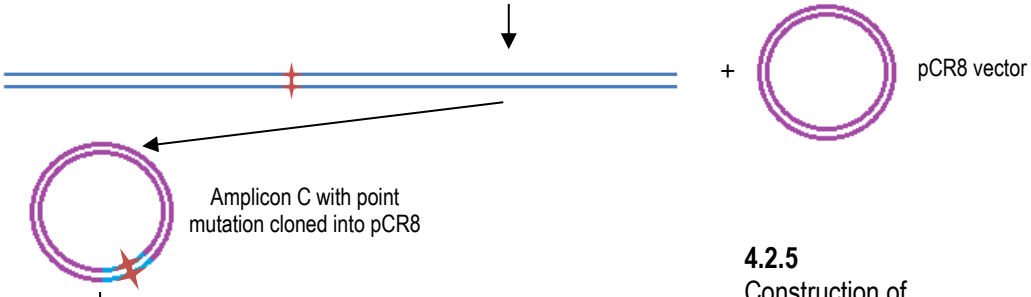


4.2.2 PCR mutagenesis

Full *AtCIPK16* promoter in pCR8 vector



4.2.3 Cloning of Amplicon C into pCR8



4.2.3 & 4.2.4 Digest and ligation of plasmids

BglII and *EcoRV* digest

4.2.5 Construction of expression vector and transformation

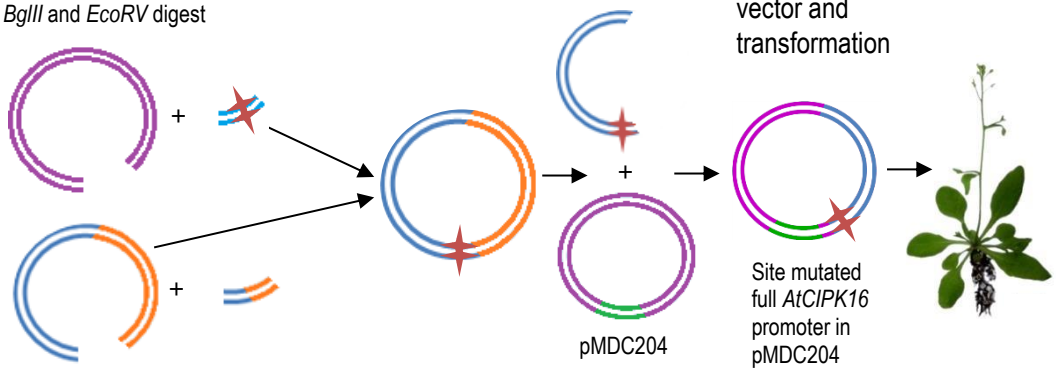


Figure 4.1: Flow diagram outlining the methods undertaken to perform site directed mutagenesis by PCR on a reporter construct plasmid. 4.2.1 Identification of suitable mutation site and designing of primers to add or remove a TATA box; 4.2.2 PCR mutagenesis to introduce desired point mutations; 4.2.3 Cloning of promoter amplicon containing point mutation into pCR8; 4.2.4 Digestions and ligations of amplicon C – pCR8 plasmids and original promoter plasmids; 4.2.5 Construction of final reporter construct plasmids and transformation into Arabidopsis. Plasmid sections outlined in orange = part of the original pCR8 plasmid, blue = *AtCIPK16* promoter, purple = final entry or destination vector and green = GFP. Red stars = point mutations. Green arrows = primers. Bold numbers indicate Methods sections describing the process.

Synthesis of amplicon C (Fig. 4.1) was conducted using amplicons A and B which introduced the point mutations to both strands of the same DNA oligo. Once again PCRs from both alleles were performed with Phusion® HF DNA polymerase (New England Biolabs), to prevent any new mutation introduction, in 50 µL reaction volumes. In the first stage of synthesis each reaction contained 10 ng of amplicon A for the appropriate allele, 10 ng of amplicon B for the same allele, 10 µL 5× Phusion HF reaction buffer, 1 µL 10 mM dNTPs and 1 U Phusion HF DNA polymerase. The PCR conditions used to for the first stage of synthesis were as follows: an initial denaturation at 98°C for 30 s, followed by 5 cycles of denaturation at 98°C for 10 s, annealing at 63°C for 30 s and extension at 72°C for 15 s. This allowed the complementary overlap at the 3' end of each amplicon where the point mutation was located to bind and act like primers leading to the extension of each amplicon (A and B) forming amplicon C (Fig. 4.1). After the 5 rounds of PCR, 2.5 µL 10 mM forward primer and 2.5 µL 10 mM reverse primer for the appropriate amplicon were added (see Table 4.1) to each reaction. Samples then continued cycling under the following conditions: an initial denaturation at 98°C for 30 s, followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 63°C for 30 s and extension at 72°C for 15 s, followed by a final extension at 72°C for 5 mins. Amplicon C for each allele was visualised using gel electrophoresis with a 1.5 % agarose gel containing 5 µL/100 mL SYBRsafe® stain (Invitrogen). PCR product (amplicon C) was purified using the PCR clean-up protocol of a NucleoSpin® Gel and PCR Clean-Up Kit (Macherey-Nagel). Spectrophotometry was used to check quality and quantity of amplicon C purified (see Section 2.2.4).

To ensure the single point mutation had been introduced into amplicons A, B and C for both alleles, combinations of the purified amplicons and corresponding primers were submitted to the Australian Genome Research Facility (AGRF) (Waite Campus, Urrbrae, SA) for purified DNA Sanger sequencing. DNA sequencing analysis and alignment were performed using Geneious 6.0 (http://www.geneious.com, Kearse et al. 2012).

4.2.3 Restriction digest and DNA ligation reactions

Double restriction digests were performed to facilitate: 1) the reformation of the full *AtCIPK16* promoters containing the point mutations, 2) cloning and 3) the identification of plasmids containing inserts and the orientation. Restriction enzymes used for all digests were *BglII* (recognition site A▼GATCT) and *EcoRV* (recognition site GAT▼ATG) (New England Biolabs). Double restriction digests were performed in 25 µL reactions containing 2.5 µL of 10× NEBuffer 3.1, 10 U of *BglII* restriction enzyme, 10 U of *EcoRV* restriction enzyme and 500 – 1000 ng of template DNA. Reactions were incubated at the recommended temperature for optimum activity of 37°C for 1 – 4 hr, and then at 80°C for 20 min to heat inactivate *EcoRV*. Digests were visualised using gel electrophoresis on a 1.5 % agarose gel containing 5 µL/100 mL SYBRsafe® stain (Invitrogen). If necessary bands of the correct size were excised from the agarose gel under UV light and purified using the DNA extraction from agarose gels protocol of a NucleoSpin® Gel and PCR Clean-Up Kit (Macherey-Nagel). Spectrophotometry was used to check quality and quantity of digested DNA purified (see Section 2.2.4).

DNA ligation was used to insert digested amplicon C back into the original full length *AtCIPK16* promoters in pCR8 vectors. DNA ligations were performed using T4 DNA Ligase (New England Biolabs) and set up on a 1:3 molecular ratio of vector DNA and insert DNA. Each 20 µL reaction contained 2 µL of 10× T4 reaction buffer, 1 µL of T4 DNA ligase and 1:3 molecular ratio of plasmid backbone (50 ng) to amplicon C. The reaction was incubated at 16°C overnight then terminated by incubation at 65°C for 10 min, before being transformed into *E.coli* for multiplication (Section 4.2.4)

4.2.4 Generation of amplicon C – pCR8 Gateway® vectors

PCR products generated using Phusion HF Taq polymerase do not contain a poly A overhang at the 3' end of each strand that is required for Gateway® cloning. Elongase® Enzyme Mix (Thermo-Fisher Scientific) was used to add a poly A overhang to amplicon Cs for both alleles. Each reaction contained 2 µL of amplicon C, 1 µL of 10 mM dNTPs and 7 µL of MilliQ H_2O pipette mixed together. To this cocktail, 2.5 µL of 5× Buffer A, 2.5 µL of 5× Buffer B, 1 µL of Elongase enzyme mix and 9 µL of MilliQ H_2O, pre-mixed together, was added. Samples were incubated at 68°C for 10 mins.

Purified, digested, poly A tailed amplicon C, containing the desired point mutation for each allele, were cloned into the Gateway® entry vector pCR®8/GW/TOPO® (Invitrogen) using TOPO® cloning reactions preformed following the manufacturers protocol. Each reaction contained 2 – 3 µL of amplicon C, 1

µL of pCR8 TOPO® vector, 1 µL of accompanying salt solution and MilliQ H_2O to a final volume of 6 µL. Samples were incubated at room temperature for 30 mins, then all the reaction mix was transformed into Turbo high-efficiency competent *E. coli* (New England Biolabs) following a modified version of the manufacturer's high-efficiency transformation heat shock protocol. In brief, all the reaction mix from the cloning procedure was mixed gently with 50 µL of Turbo competent cells on ice and left to incubate on ice for 20 mins. The cells were heat shocked to allow entry of the plasmid into the cells by incubating at 42°C for 45 s then were transferred to ice for 3 mins. 300 µL of Super Optimal Broth, containing 20 mM glucose, (SOC medium) was gently pipette mixed into cells and the cells incubated at 37°C for 1 hr with shaking. A 300 µL bacterial culture was spread onto Luria Betani (LB) agar plates containing spectomycin (100 µL per 100 mL of agar) for positive selection of transformed colonies. The plates were incubated at 37°C overnight. Positive colonies which grew on the agar and were therefore resistant to spectomycin were selected and cultured in liquid LB media with spectomycin (100 µL per 100 mL of media) at 37°C overnight. Plasmids were recovered and purified from cells using a ZR Plasmid Miniprep – Classic Kit (Zymo Research) following the manufacturers protocol. Plasmids were restriction enzyme digested (Section 2.2.3) and sequenced (Section 2.2.2) to check for presence and orientation of insertion as well as existence of the desired point mutations.

4.2.5 Further steps needed to transform final destination vectors into Arabidopsis

Due to technical difficulties and time constraints in the Master's program this project was not completed. The following outlines in the steps that would have been taken to finish this project. Once the full *AtCIPK16* promoter with point mutation in pCR8 vector for each allele was completed the full promoter would have been transferred to the expression vector pMDC204 (Curtis & Grossniklaus 2003) using LR clonase reactions (Invitrogen). pMDC204 contains an insertion site for promoters, such as the modified *AtCIPK16* promoters, which drive expression of the GFP (mGFP6HDEL) reporter gene (Curtis & Grossniklaus 2003). Product from the LR reactions would have then been used to transform *E.coli* competent cells to increase the plasmid concentration as described in Section 4.2.4. The pMDC204 transformation vector would have then been transformed into *Agrobacterium tumefaciens* ready for plant transformation. Arabidopsis ecotype Col-0 would have been transformed using the floral dip method (Clough & Bent 1998; Weigel & Glazebrook 2002) using *Agrobacterium* with either the mutated Bay-0 promoter in pMDC204 or the mutated Shahdara promoter in pCMD204. T_1 seeds would have been germinated and grown on agar plates under hygromycin selection to determine primary transformants. These transformants would then have been transferred to control

and salt stress plates for experimental analysis, then after 7 days of growth any GFP fluorescence would have been observed using confocal microscopy (Roy, et al. 2013). DNA and RNA would have been extracted from each of the plants to confirm the presence and activity of the reporter construct. Finally positive transformants would have been transferred to soil and grown to produce T_2 seed, which would have been used for more rigorous testing.

4.3 Results

4.3.1 Analysis of *AtCIPK16* promoters to introduce point mutations and design primers

Analysis of the *AtCIPK16* promoter for both the Bay-0 and Shahdara alleles revealed that it was possible to use site directed mutagenesis to remove/introduce a TATA-box into the promoter. The mutation of the Bay-0 TATA-box, TATAA to TATAT was hypothesised to result in decreased promoter activity due to the effective removal of the TATA-box. The mutation of the corresponding Shahdara sequence, TATAT to TATAA was hypothesised to increase promoter activity due to the creation of a TATA-box in the sequence. The region of the promoter and pCR8 for both Bay-0 and Shahdara alleles used to generate the desired mutations is illustrated in Fig. 4.2.

For PCR mutagenesis several sets of primers needed to be designed, including one set common to both alleles and all three amplicons A, B and C (see Fig. 4.1), as well as one set overlapping the mutation site and containing the desire point mutation for each allele. Due to the need to incorporate two unique restriction site, ensure the PCR product was between 100 – 500 bp in length and the location of the mutation site relative to the end of the promoter, the length between the two common primers needed to be over 350 bp which meant that part of the pCR8 vector was included in the PCR mutation area. All primers were designed with the following constraints in mind: approximately the same melting temperature above 55°C, a GC content of about 50 % and minimal secondary structures. The primers which were designed and the specifics of the amplicons created using these primers are described in Table 4.1. The binding location of the primers in the promoter and pCR8 regions of Bay-0 and Shahdara are shown in Fig. 4.2

Table 4.1: Description of primers designed for site directed mutagenesis of the *AtCIPK16* promoter by PCR and details of the amplicons created. (A) Primers described are those used for the generation of amplicons A, B and C for both Bay-0 and Shahdara alleles. Bay-0/Shahdara PPM Forward/Reverse primers include the desired point mutations (in bold) which will result in the addition or removal of a TATA box from the desired promoter. (B) Primers used to create each amplicon and amplicon length.

A

Primer Name	Primer Sequence (5' to 3')	Primer T_m	Amplicons created…
PPM End Forward	CTAGCTGCCAGATCTTAGGC	57.5°C	A and C
Modified M13 Reverse	CAGGAAACAGCTATGACCATGT	58.4°C	B and C
Bay-0 PPM Forward	AATTTCTGCTTATATATA**T**CCAAATCACAA	57.1°C	B
Bay-0 PPM Reverse	TTGTGATTTGG**A**TATATATAAGCAGAAATT	57.1°C	A
Shahdara PPM Forward	ATTTCTGCTTATATATA**A**AGATATGTAACCAAAT	56.7°C	B
Shahdara PPM Reverse	ATTTGGTTACATATCT**T**TATATATAAGCAGAAAT	56.7°C	A

B

Amplicon Name	Primers Used	Amplicon Length
Bay-0 Amplicon A	PPM End Forwards Bay-0 PPM Reverse	154 bp
Bay-0 Amplicon B	Bay-0 PPM Forward Modified M13 Reverse	251 bp
Bay-0 Amplicon C	PPM End Forward Modified M13 Reverse	375 bp
Shahdara Amplicon A	PPM End Forwards Shahdara PPM Reverse	159 bp
Shahdara Amplicon B	Shahdara PPM Forward Modified M13 Reverse	260 bp
Shahdara Amplicon C	PPM End Forward Modified M13 Reverse	385 bp

Figure 4.2: Sequence of the region of the *AtCIPK16* promoter in the pCR8 vector and the primers involved in the site directed mutagenesis. (A) Sequence of the Bay-0 promoter shows the location of the primers used to mutate the existing TATA-box by changing the A to a T (highlighted in yellow). (B) Sequence of the Shahdara promoter shows the location of the primers used to mutate the existing sequence to form a TATA-box by changing the T to an A (highlighted in yellow). PPM End Forward and Modified M13 Reverse primers are common primers used in the site directed point mutation process. Bay-0/Shahdara PPM Forward/Reverse primers overlap the mutation site and contain the desired point mutation. *AtCIPK16* promoter is highlighted in pink, pCR8 vector in red and primers in greens. *BglII* and *EcoRV* restriction sites are shown.

4.3.2. Successful creation of amplicons A, B and C containing the desired point mutation for both alleles

Using PCR point mutagenesis Bay-0 amplicons A and B as well as Shahdara amplicons A and B containing the desired point mutations were successfully generated using the original promoter – pCR8 constructs as template DNA (Fig. 4.3A). Sequencing was used to confirm the presence of the point mutation in Bay-0 (Fig. 4.3B) and Shahdara (Fig. 4.3C) promoters. Amplicons A and B were used as templates to effectively synthesis amplicon C which introduced the desired point mutation into both strands on the DNA oligo, for both alleles (Fig. 4.4).

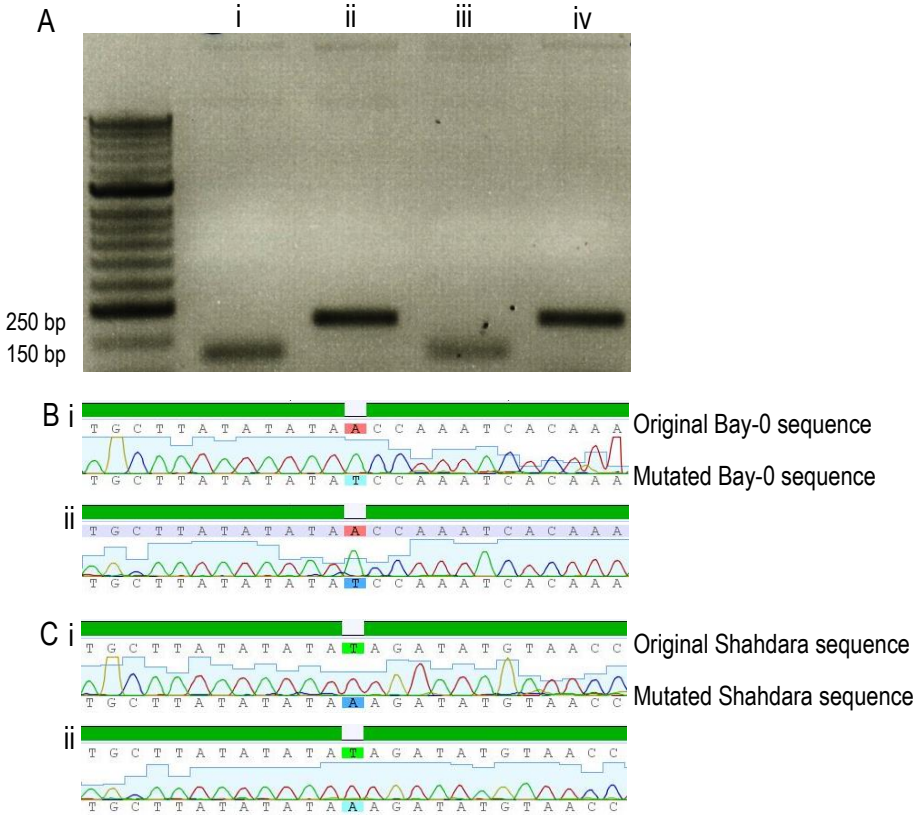


Figure 4.3: Electrophoresis gel and chromatograph with sequence alignment of amplicons A and B from both Shahdara and Bay-0 alleles containing the desired point mutations. (A) Electrophoresis gel showing amplicon A from Bay-0 (i), amplicon B from Bay-0 (ii), amplicon A from Shahdara (iii) and amplicon B from Shahdara (iv) synthesised during PCR point mutagenesis. First well = 100 bp ladder. (B & C) Sequencing results show the section of each amplicon around the desired point mutations. Each sequencing results shows the consensus sequence in green, the reference Col-0 sequence at the top, then the chromatograph and sequencing for the amplicon. (B) Both Bay-0 amplicons show the desired mutation A to T (highlighted) for both amplicon A (B i) and amplicon B (B ii). (C) Both Shahdara amplicons show the desired mutation T to A (highlighted) for both amplicon A (C i) and amplicon B (C ii).

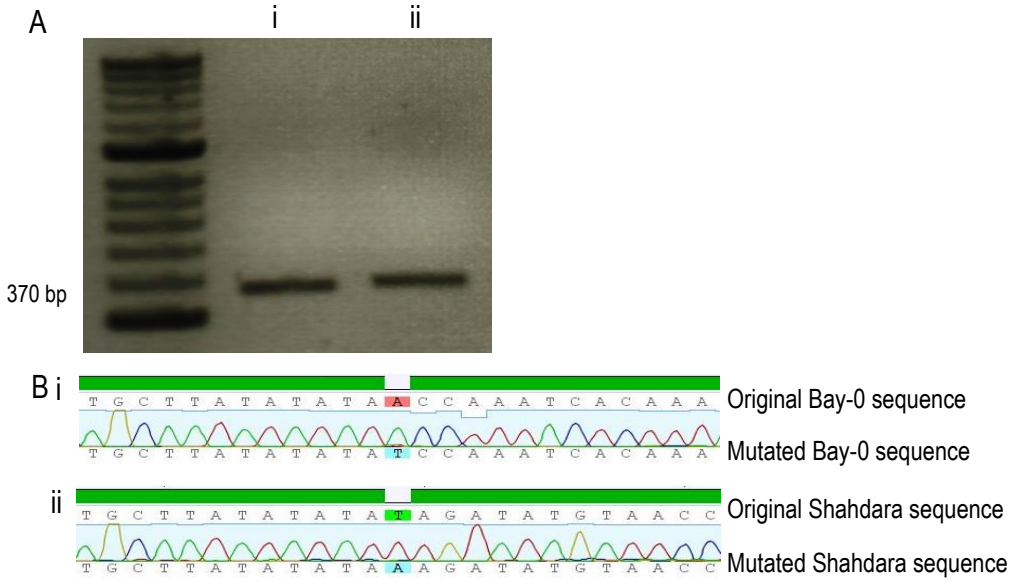


Figure 4.4: Electrophoresis gel and chromatograph with sequence alignment of amplicon C from both Shahdara and Bay-0 alleles containing the desired point mutations. (A) Electrophoresis gel shows the production of (i) amplicon C from Bay-0 and (ii) amplicon C from Shahdara synthesised during PCR point mutagenesis. First well = 100 bp ladder. (B) Sequencing results confirm the desired point mutation with in each promoter. Each sequencing results shows the consensus sequence in green, the reference Col-0 sequence at the top, then the chromatograph and sequencing for the amplicon. Both the Bay-0 amplicon C (Bi) shows the desired mutation A to T (highlighted) and Shahdara amplicon C (Bii) shows the desired mutation T to A (highlighted).

4.3.3 Creation of pCR8 vector with full *AtCIPK16* promoter with point mutation

While it was possible to generate amplicon C (Fig. 4.4), restriction digestion of amplicon C to allow for insertion back into the original pCR8 promoter vector failed (Fig. 4.5). A new approach was then trialled to first clone amplicon C into a new pCR8, then use restriction enzymes to cut out amplicon C from pCR8 for insertion into a digested pCR8 vector which contained the allelic specific promoter.

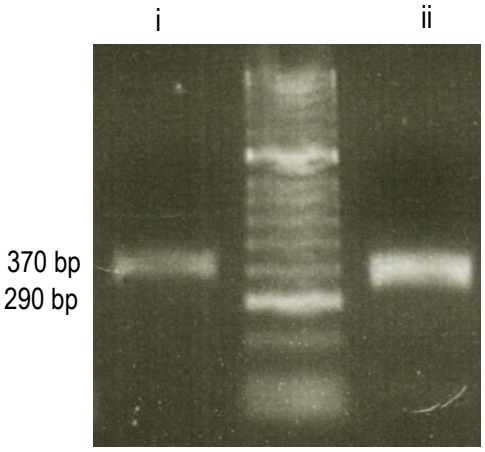


Figure 4.5: Electrophoresis gel of failed double restriction enzyme digest of Bay-0 and Shahdara amplicon Cs. Electrophoresis gel shows amplicon C from Bay-0 (i) and Shahdara (ii) at the original size of approximately 370 bp, after digestion with BglII and EcoRV which if successful would have produced bands at 290 bp. Middle lane = 100 bp ladder

After the addition of a poly A overhang to amplicon Cs, amplicon Cs contained the point mutations were successfully cloned into an empty pCR8. Plasmids from positively transformed colonies were retrieved and purified, then checked using double restriction enzyme digestions and sequencing. These methods demonstrated that amplicon C for Bay-0 and Shahdara were successfully cloned into pCR8 (digest Fig. 4.6A and sequencing Fig. 4.6B).

To attempt to create a vector containing the full *AtCIPK16* promoter with the desired point mutation, double restriction enzyme digestion of both the original promoter in pCR8 vectors (Bay-0 and Shahdara) and the amplicon C in pCR8 vectors (Bay-0 and Shahdara was necessary. Digestion of any plasmid listed above was not successful with only a small fraction of the plasmid added digested, and recovery of the desired fragments not possible in the case of amplicon Cs (Fig. 4.7). Technical difficulties also prevent the distinction of cut and uncut original promoter in pCR8 vectors which also contributed to the failure of subsequent ligation steps. Due to time constraints the project was left at this stage having not yet been able to successfully create the full *AtCIPK16* promoters containing the point mutations.

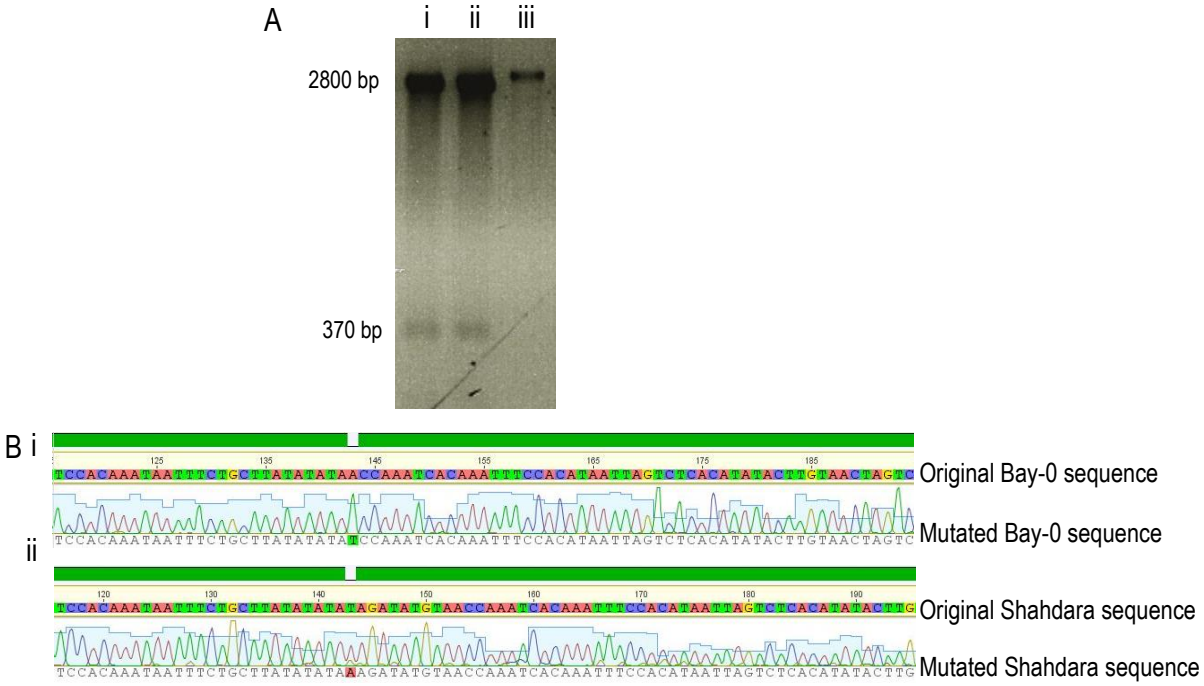


Figure 4.6: Electrophoresis gel and chromatograph with sequence alignment of amplicon C in pCR8 vector for both Shahdara and Bay-0 alleles containing the desired point mutations. (A) Electrophoresis gel shows (i) amplicon C from Bay-0 cloned into pCR8 and (ii) amplicon C from Shahdara cloned into pCR8, double digested with *BglII* and *EcoRV* restriction enzymes. Plasmid backbone is at 2800 bp and amplicon C runs at 370 bp. (iii) shows a pCR8 plasmid with no amplicon C insert. (B) Sequencing results show the section of each plasmid around the desired point mutations. Each sequencing results shows the consensus sequence in green, the reference Col-0 sequence at the top, then the chromatograph and sequencing for the plasmid. Both the Bay-0 amplicon C in pCR8 (B i) and the Shahdara amplicon C in pCR8 (B ii) shows the desired mutation (highlighted).

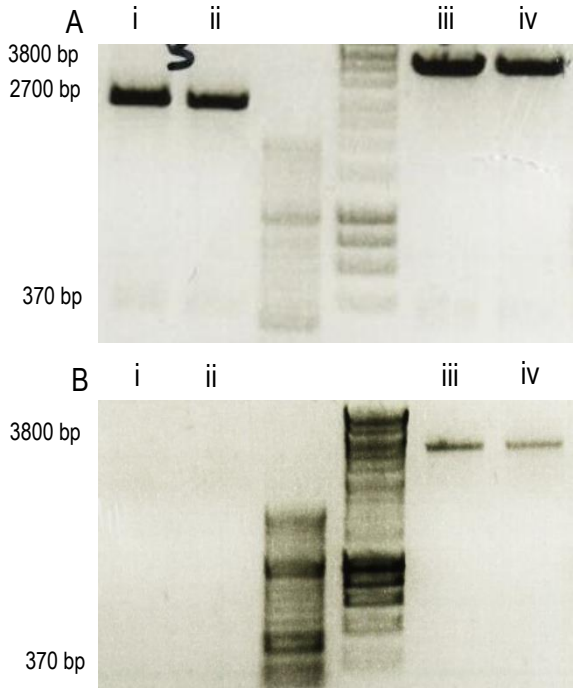


Figure 4.7: Electrophoresis gels of double restriction enzyme digests and results of gel purification of bands excised from the gel of amplicon Cs in pCR8 vectors and original promoters in pCR8. (A) Electrophoresis gel shows amplicon C from (i) Bay-0 and (ii) Shahdara cloned into a new pCR8 entry vector, (iii) the original Bay-0 and (iv) Shahdara promoters which have undergone a double digest with *BglII* and *EcoRV* restriction enzymes. (B) Second electrophoresis gel shows amplicon C from Bay-0 cloned into pCR8 (i), amplicon C from Shahdara cloned into pCR8 (ii), original Bay-0 promoter in pCR8 (iii) and original Shahdara promoter in pCR8 (iv) after gel purification of excised fragments (amplicon C and original plasmid backbone). Plasmid backbone is at 2800 bp or 3800 bp and amplicon C runs at 370 bp.

4.4 Discussion

4.4.1 Difficulties in plasmid construction

The protocol used in the project to introduce the desired point mutations via overlap-driven PCR mutagenesis was based on previously developed methods (Ho, et al. 1989; Tomic, et al. 1990; Heckman & Pease 2007). Overlap PCR mutagenesis has previous been used to demonstrate: the importance of certain amino acid residues, the effects of mutation in the read-through domain on virus expression and gene mutation effects on herbicide resistance (Kobayashi, et al. 1993; Ott, et al. 1996; Brault, et al. 2000; Qiu 2015). In this project overlap PCR was also used successfully to introduce 1 bp point mutations into specific nucleotide sequences. In the case of this experiment point mutations were successfully introduced into sections of the *AtCIPK16* promoter for both the Bay-0 and Shahdara alleles. The Bay-0 allele which original contained a TATA-box encoded by TATAA was successfully mutated to the new sequence TATAT which theoretically removed the TATA-box (Fig. 4.3 & 4.4). The Shahdara allele which original did not contain a TATA-box but instead had the sequence TATAT was successfully mutated to theoretically contain a TATA-box encoded by the sequence TATAA (Fig. 4.3 & 4.4).

Technical difficulties were encountered in attempting to introduce the part of the promoter sequence containing the point mutation (amplicon C) back into the original pCR8 vectors containing the full AtCIPK16 promoters. Double restriction digestion of amplicon C failed to result in cleavage at either of the restriction sites (Fig. 4.5), likely due to the fact that the digestion was only meant to remove in total 80 bp from the amplicon. To circumvent this problem and ensure that the restriction sites were adequately spaced away from the end of any oligo, amplicon Cs were successfully transformed in pCR8 and the amplicon C – pCR8 plasmids recovered (Fig. 4.6). Cloning amplicon C in pCR8 did result is some digestion and formation of the correct digested amplicon C fragment needed for vector construction (Fig. 4.7A). As illustrated in Fig. 4.7, despite several attempts at troubleshooting either by using new stocks of each restriction enzyme, altering the enzyme: plasmid ratio, increasing the digestion time or repeating the reaction, very little digestion of the amplicon C – pCR8 plasmid

occurred. Time permitting several options could be explored in the future: the conditions of the digestion buffer, experimenting with different enzyme: plasmid ratios, the residual salt concentrations in the purified DNA samples or the use of sequential single digests. Both *EcoRV* and *BglII* were chosen due to both enzymes being insensitive to methylation, therefore methylation of the restriction sites should not have been an issue affecting digestion. The lack of digested amplicon C (300 bp fragment) and the natural loss of DNA during gel purification meant that no or very little digested amplicon C was recovered (Fig. 4.7B). For the construction of the vector it was also necessary to digest the original full promoter – pCR8 plasmids with the same restriction enzymes to remove the same amplicon C DNA fragment which doesn't contain the point mutation. Like the digestion of the amplicon C – pCR8 plasmids, the digestion reactions with the original plasmids also had very limited success with only a few of the plasmids digested (Fig. 4.7A). Unfortunately due to so few of the plasmids being digested and the cut plasmids only being 300 bp shorter it was impossible to distinguish between uncut and cut plasmid. Thus despite recovery of the uncut/cut plasmid being possible, it seems most of the plasmid recovered was uncut plasmid. Ligation reactions between almost no digested amplicon C and likely no cut plasmid backbone therefore failed. Due to time limitations further troubleshooting of the restriction enzyme digestions and thus ligation reactions were not able to be carried out and so testing of the hypothesis was not completed.

4.5 Future work

More work is required to complete the construction of mutated *PromoterAtCIPK16::GFP* pMDC204 vectors necessary to test the hypothesis of whether the presence or absence of the TATA-box in the *AtCIPK16* promoter is the cause of the differential gene expression. The double restriction digest reaction still needs to be properly optimised to increase the yield of digested product, or new restriction enzymes need to be investigated. Once digestion of the amplicon C – pCR8 and promoter – pCR8 vectors is complete, the ligation reaction between amplicon C and the promoter backbone can occur, which will be transformed into *E.coli* competent cells for multiplication. Once sequencing has confirmed the sequence and orientation of the full *AtCIPK16* promoters including the desired point mutations, promoters will be transferred to pMDC204 via an LR clonase reaction to ultimately drive GFP expression. Mutated Bay-0 and Shahdara *PromoterAtCIPK16::GFP* pMDC204 vectors would then be transformed into Arabidopsis (Col-0) via the *Agrobacterium* floral dip method and transformants used to identify differences between the promoter in GFP fluorescence under salt stress (see Section 4.2.5) (Roy, et al. 2013). Variations in GFP fluorescence will be compared

between the original Bay-0 promoter, original Shahdara promoter, mutated Bay-0 promoter and mutated Shahdara promoter. If differences in fluorescence, in the expected pattern, for both alleles was seen between original and mutated promoters this would be confirmation that the presence/absence of the TATA-box (defined by TATAA) was responsible for the expression differences. The switch in fluorescence pattern would also validate the hypothesis that the TATA-box caused the enhanced *AtCIPK16* expression seen in the Bay-0 ecotype and explains the lower shoot Na^+ and enhanced growth phenotype of Bay-0. If no differences in GFP fluorescence were observed between the original and mutated promoters, this would suggest that it was not the TATA-box formation but some other factor associated with the 10 bp deletion that caused the expression differences between ecotypes. The other possible observation would be a change in fluorescence pattern somewhere in between which would suggest that the presence/absence of the TATA-box did contribute to the expression differences but that the actual physical 10 bp deletion in the Bay-0 allele was also necessary. While no differences in the amino acid sequences of the codding regions of two *AtCIPK16* alleles were reported by Roy, et al. (2013) there were some other small differences between Bay-0 and Shahdara promoters further 5' of the TSS. If no significant changes in GFP fluorescence were seen these small differences in the promoters would need to be further investigated to understand their possible effect of *AtCIPK16* expression. The expected results from this project help to contribute to the understanding of different promoter elements, particularly the TATA-box motif, giving information on key nucleotides in the coding sequences, as well as the effects of the removal/creation by point mutation of an element on gene expression.

Chapter 5: General Discussion

5.1 Review of thesis aims

Arabidopsis thaliana Calcineurin B-like Interacting Protein Kinase 16 (*AtCIPK16*) has been identified as a likely candidate gene involved in Na^+ exclusion (Roy, et al. 2013). Previous work demonstrated that transgenic barley (cv. Golden Promise) expressing *35S:AtCIPK16* had larger biomass and improved Na^+ exclusion under saline conditions compared to null segregants in a glasshouse-based experiment (Roy, et al. 2013). A preliminary field experiment conducted during 2012 with these transgenic barley lines showed similar results under saline field conditions (Roy, et al. unpublished), although these results were yet to be verified over several years of field trials. Preliminary work had also shown that transgenic wheat (cv. Gladius) expressing *Ubi:AtCIPK16* produced increased biomass and accumulated less Na^+ in leaf tissue under 200 mM NaCl treatment compared to null segregants in glasshouse-based experiments (Roy, el al. unpublished). However these results had yet to be confirmed in larger scale experiments with subsequent generations, and thus further characterisation of these wheat lines had yet to occur. To understand how *AtCIPK16* transgene expression may influence the salt tolerance of barley and wheat further characterisation of these lines in both field and glasshouse-based experiments were needed.

These aims of this Masters project were:

1. To further characterise the effects of *35S:AtCIPK16* expression on field grown transgenic barley (cv. Golden Promise) in 2013 and 2014 **(Chapter 2)**
2. To evaluate the salt tolerance and Na^+ exclusion capacity of transgenic *Ubi:AtCIPK16* wheat (cv. Gladius) in glasshouse-based experiments **(Chapter 3)**
3. To demonstrate, via editing of the AtCIPK16 promoter, that the presence/absence of a TATA box causes differences in gene expression between Arabidopsis ecotypes **(Chapter 4)**

5.2 Summary of main findings

In Chapter 2, the Na^+ exclusion capacity and yield of transgenic *AtCIPK16* barley was evaluated in low and high salinity field sites at Kunjin, Western Australia in 2013 and 2014. Considerable variation was seen between years due to environmental factors, particularly rainfall. In 2014, transgenic lines G298-2-17 and G298-10-15 showed increased shoot Na^+ and Cl^- exclusion, with decreased growth and yield, compared to wildtype and null segregants in the high salt sites. In 2012 (a dry year), these two transgenic lines also demonstrated shoot Na^+ and Cl^- exclusion, though in this year the ion exclusion correlated with increased yield. These findings suggest that transgenic *AtCIPK16* barley may be able to exclude both Na^+ and Cl^-, although whether the exclusion has a positive or negative effect on yield depends on the annual rainfall.

In Chapter 3, the salt tolerance and Na^+ exclusion capacity of transgenic *AtCIPK16* wheat was evaluated in three glasshouse-based hydroponic experiments. Interpretation of the data was hindered by several factors including the large number of null segregants in some transgenic lines, the lack of apparent transgene expression in most plants, the huge variation between experiments due to the growth season and the variability between siblings of the same transgenic line. One sibling line CIPK16-2·2 had increased shoot and root Na^+ and Cl^- exclusion which corresponded to slightly higher biomass production. These findings suggest that considerable work needs to be done to clean up the genetic background of these transgenic lines before any conclusions on the effects of AtCIPK16 in wheat can be drawn.

In Chapter 4, the promoters of Bay-0 and Shahdara *AtCIPK16* alleles were mutated to enable investigation into whether to presence or absence of the TATA box drove the expression differences of the two alleles. The desired point mutations were successfully introduced into small sections of both alleles via overlap PCR mutagenesis; removing the Bay-0 TATA box (TATAA) to TATAT and creating a TATA box in Shahdara TATAT to TATAA. Due to technical difficulties and time constraints this part of the project was not completed.

5.3 Implications of thesis findings

5.3.1 Benefits of *AtCIPK16* expression in barley and wheat may depend on environment

This project suggested that improving the salt tolerance of barley via the expression of *35S:AtCIPK16* was only feasible depending on the rainfall environment and only under high salt field conditions (Chapter 2). Na^+ and Cl^- exclusion was observed in high salt for two transgenic lines, in both 2012 and 2014, although the effect of the exclusion on yield greatly differed between years. Ion exclusion in 2012, (a quite dry year) resulted in increased grain yield, while in 2014 (an above average rainfall year) ion exclusion resulted in severe decreases in grain yield per plant and per plot. This suggests that the effect of AtCIPK16 in promoting shoot ion exclusion can either interfere or enhance barley's natural growth behaviours on saline soils depending on the rainfall environment. Barley, like all plants, does exclude excess Na^+ and Cl^- in attempts to maintain optimal growth conditions under stress (Munns, et al. 1999), but ion exclusion is maintained in balance with the use of these ions as ionic osmoticum, used to promote normal plant growth (Blumwald, et al. 2000; Adem, et al. 2015). The increased ion exclusion brought about by *AtCIPK16* expression in these transgenic plants would therefore have disrupted this balance. In dry years (i.e. 2012) this would benefit barley by increasing the exclusion of Na^+, as plants are very limited in the amount of water that can be extracted from dry saline soils (Rengasamy 2006) and therefore cannot dilute or store as much Na^+ in these conditions. On the other hand in above average rainfall years (i.e. 2014) increased ion exclusion would be detrimental to the growth and therefore yield of barley due to the decreased internal store of growth promoting osmoticum.

A common phenotype for all transgenic, wildtype and null segregant lines was seen in low salt conditions for all traits measured in all three years. This suggests that AtCIPK16 has no effect on barley growth, ion exclusion or yield at lower salt conditions. For this project results were only seen at high salt levels with increases in yield only seen in dry conditions. This suggests that *AtCIPK16* transgenic barley would only be relevant to areas with highly saline soils and low rainfall; although the growth response of *35S:AtCIPK16* barley to other factors such as soil texture, water holding capacity, pH and disease may also limit the regions of Australia where *AtCIPK16* barley would be beneficial over current commercial cultivars. Currently *AtCIPK16* has only been transformed into Golden Promise barley, a cultivar not commercially grown in Australia, to therefore be relevant to grain growers *AtCIPK16* would need to be bred into elite Australian cultivar breeding stocks. Cultivars in Australia are generally bred for the wide range of soil and climatic environments found across Australia and therefore do not incorporate any genes, such as *AtCIPK16,* which may inhibit yield

under any environmental conditions. Including *AtCIPK16* in breeding material would need to be accompanied by a shift towards breeding crop cultivars that are specific to certain environmental areas and conditions, which may not be a practical business model for many breeding companies. Low rainfall, highly saline areas are often used for purposes other than cropping due to the low yield returns and profits, therefore breeding specifically for environments such as these may not ultimately be practical even if yield returns are increased. One way of mitigating the negative effects of *AtCIPK16* expression on barley yields under other environmental conditions would be through the use of a different promoter to control transgene expression. The use of a specific stress inducible promoter, e.g. high salt or drought, would enable *AtCIPK16* to be incorporated into general breeding lines and still have a positive effect when grown under certain growth conditions.

5.3.2 Role of CIPK16 in salt tolerance

Findings from this project showed that in certain transgenic lines *AtCIPK16* was driving increased exclusion of not only Na^+ but also Cl^- from the shoot in both barley and wheat, as well as root tissue in wheat (Chapter 2 & 3). The increased Na^+ exclusion was consistent with work previously published on the overexpression of *AtCIPK16* in Arabidopsis, as well as preliminary experiments out carried on previous generations of the same *AtCIPK16* barley and wheat lines used in this project (Roy, et al. 2013; Roy, et al. unpublished). As Cl^- concentration had not been measured in previous experiments it is difficult to know if the accompanying exclusion of Cl^- seen in this project was a truly novel result or just undetected in previous work. These results raise questions about the salt tolerance mechanisms and pathways that AtCIPK16 may regulate and function in. Although the CBLs which bind to AtCIPK16 in Arabidopsis are known (Huang 2015) the targets of AtCIPK16 regulation have yet to be identified. It has been hypothesised that AtCIPK16 localises to the nucleus where it may behave like a transcription factor for at least one gene, possibly a Na^+ transporter (Roy, et al. 2013; Huang 2015). The exclusion of both Na^+ and Cl^- seen in this project suggested that if AtCIPK16 was acting as a regulator of genes involved in Na^+ or Cl^- transport AtCIPK16 would have been regulating several targets due to the known separation of these two transport pathways (Teakle & Tyerman 2010; Tavakkoli, et al. 2011). Although not supported by the results of this project, it has also been hypothesised that AtCIPK16 may have a role in regulating K^+ levels, through a possible interaction with AKT1 (Lee, et al. 2007), and the exclusion of Na^+ and Cl^- may be a side effect of increased K^+ transport under saline conditions. Soil salinity reduces the uptake of K^+ into the plant and it has been suggested that plants which are able to maintain high levels of K^+ uptake under salt stress have increased salt tolerance (Maathuis & Amtmann 1999; Shabala & Cuin 2008). It has therefore been

hypothesised, but not yet tested, that overexpression of genes encoding K^+ transporters or their regulators could improve salt tolerance. As the K^+ levels in the transgenic barley or wheat lines with Na^+ exclusion did not differ from those with Na^+ accumulation it appears that AtCIPK16 does not impact salt tolerance through the regulation of K^+ transport pathways, rather through the regulation of Na^+ and Cl^- transport.

5.3.3 Is exclusion the best mechanism to pursue in these crops?

The results of this project also questions whether Na^+ exclusion is the best salt tolerance mechanism to pursue in barley and wheat research. Previous work has shown that increasing the Na^+ exclusion capacity of wheat is a viable option for improving wheat salt tolerance. The expression of *Triticum monococcum Nax1* (*TmHKT1;4*) and/or *Nax2* (*TmHKT1;5*) in durum or bread wheat, along with the native wheat Na^+ transporting HKTs, resulted in improved biomass and increased shoot Na^+ exclusion (James, et al. 2011; Munns, et al. 2012). The results from the work on these Na^+ transporters suggest that increased Na^+ exclusion whether, by overexpressing transporter genes or their regulators such as *AtCIPK16*, would be beneficial to the growth and yield of bread wheat. Although other literature suggests that while Na^+ exclusion may be a more functional mechanism in bread wheat than other crops, for certain cultivars other salt tolerance mechanisms may be more important in determining overall salt tolerance, i.e. the most salt tolerance bread wheat cultivars may not be those with the highest rates of Na^+ exclusion (Genc, et al. 2007; Genc, et al. 2010). Literature also suggests that increasing the Na^+ exclusion capacity of barley may not be the best mechanism for its salt tolerance. This has been illustrated by the fact that increased shoot Na^+ accumulation, via the overexpression of *HvHKT1;2*, led to the improved growth and therefore salt tolerance of these transgenic lines under saline hydroponic conditions (Mian, et al. 2011). In this project *AtCIPK16* expressing barley had increased Na^+ and Cl^- exclusion in average to above average rainfall years, although the exclusion of ions caused severe decreases in biomass and yield production. These results match the theory that increased Na^+ exclusion negatively impacts barley growth and yield, although it seems that a caveat to this is that in field conditions rainfall and therefore soil water availability does impact on the effect of ion exclusion in barley. While Na^+ exclusion is an important mechanism for shoot ion dependent stress, more important tolerance mechanisms in wheat and barley may be associated with shoot ion independent (non-ionic) stress (Munns & Tester 2008; Roy, et al. 2014). The effects of non-ionic salt stress – reductions in cell expansion, photosynthesis etc. – are seen relatively quickly (Munns, et al. 1995; Munns & Tester 2008) and can impact longer on plant growth and yield than ionic stress, especially at lower salt concentrations. This project and current literature (Munns, et al. 2012) has

illustrated that increasing the Na^+ exclusion capacity of wheat (and barley under certain conditions) does not result in increased growth and thus yield unless transgenic plants are grown under high to very high salt stress conditions. This suggests that under low to moderate salinity levels, the salinity levels likely to be seen in more cropping areas, Na^+ exclusion is not the key tolerance mechanism, but rather non-ionic tolerance mechanisms involved in osmotic tolerance are. Given these principles, for long-term wide reaching salinity tolerance, the development of crop cultivars with improved osmotic tolerance mechanisms may be the future for salinity research.

5.4 Future Research

5.4.1 GM field trials of transgenic *AtCIPK16* barley in Australia

This project continued on with previously established saline GM field trials of transgenic barley (Schilling 2014) with data presented from three years of field trials. However, the analysis of the data from these trials was hindered by several factors which were variable between all three years. The timing and amount of rainfall was considerably variable not only within a year but also between years, with periods of low and high rainfall which caused drought (2012 growth season) and waterlogging (July 2013) which all limited plant growth. For example, the average rainfall for July in Corrigin, WA (near the Kunjin field site) is 60 mm, however in 2013 three weeks after sowing the field site received 60 mm of rain in one day, which resulted in waterlogging of the high salt site. Another issue was the often extreme variation in soil salinity between individual plots within the low and high salt trial areas which unfortunately resulted in many plots which were planted in one salt area actually having the opposite salinity when measured. This was particularly an issue in 2014, where as well as the salinity of individual plots not matching the location, it was possible that within a plot the salinity varied. Despite the plots originally being sown at a desired density, rainfall and soil salinity as well as other possible complicating soil factors, variations in plot density occurred. In 2014 the variations in plant establishment and therefore plot density were more pronounced with considerable patchiness seen not only in high salt plots like that seen in previous years but also individual low salt plots. While most of the data presented was from individual randomly sampled plants and therefore not affected by the plot density issues, the final grain yield per plot measurements were dependent on the number of plants per plot and thus were more difficult to analysis. Due to issues harvesting interstate in field conditions, gene expression was unable to be determined for these samples, so it is unknown whether *AtCIPK16* is expressed in all the transgenic barley plants sampled in this project.

The significant differences in results between above and below average rainfall years seen for the transgenic *AtCIPK16* expressing barley, as well as the described difficulties in some of the analysis of the data collected, strongly suggest that further field trials are necessary. Field trials designed to clarify whether the positive effects of *AtCIPK16* on barley salt tolerance and yield under dry conditions and the negative effects under average or well-watered conditions are stable and replicable need to be carried out. This could either involve multiple field locations in different rainfall environments with varying soil types, the use of different levels of irrigation and/or the use of rainout shelters/ polytunnels. Unfortunately due to considerable expense and regulation GM field trials on this scale would likely not be feasible. The small scale (84×: 2 m × 1 m plots) trials conducted in this project cost $35,000 p.a. with an additional $15,000 p.a. of postharvest monitoring for two years afterwards, compared to a larger non-GM (1,000×: 7 m × 3 m plots) trial which costs $22,000 p.a. (Roy, personal communication). The use of rainout shelters/polytunnels or irrigation would also increase costs due to the infrastructure needed. For maximum efficiency in field trial assessment of these lines and relevance to breeders, larger plot sizes than those used in this project would be needed which would also be additional costs. To allow for the desired field assessment of these *AtCIPK16* wheat and barley lines in different soil types, rainfall environments and salinity levels, approximately 15 different GM field trial site would need to be grown in each cereal cropping state: Western Australia, South Australia, Victoria and New South Wales. With the considerable expense and GM regulatory issues regarding trial site location, site size, monitoring and the transport of GM material, it is unlikely that field trials of this size and scale could be conducted especially considering the current political and social views on GM crops.

To help address some of the factors affecting the analysis of the data such as varying salinity, several measures could be used. These measures could include: cultivation of both low and high salt trial sites to promote soil and therefore salinity uniformity, the use of raised beds to reduce the incidence of waterlogging and saline groundwater rises throughout the growing season (Bakker, et al. 2005; Bakker, et al. 2010) or the irrigation of non-saline fields with saline water to control the salinity level (Pulvento, et al. 2012). Although there may be issues with these approaches, particularly raised beds, as implementing these measures would result in the destruction of any natural subsoil structure and is likely to be unrepresentative of the majority of Australian cropping conditions. In this project, all field grown plants were hand sampled for biomass measurements and thus the number of plants that were sampled per plot (n = 3 – 6) was limited, which in some cases hindered the analysis of data due to the restricted number of transgenic plants in certain lines. Due to the limitations of hand sampling and processing in this project, soil and plants were only sampled at one time-point in the growing season

to restrict the number of samples to be processed. The lack of sampling throughout the growing season limited the data available on any changes in soil salinity throughout the season and any changes in the effect of ion exclusion throughout different growth stages. The understanding of the effects of *AtCIPK16* expression in barley may be better understood in future field trials with sampling throughout the growing season at key developmental stages as well as an increase in the number of individually sampled plants per plot. To also improve phenotyping in future field trials, high-throughput field phenotyping options such as aerial drones or ground-based vehicles fitted with high-resolution and/or infrared thermal cameras could be employed (Comar, et al. 2012; Andrade-Sanchez, et al. 2014; Araus & Cairns 2014; Sankaran, et al. 2015; Zaman-Allah, et al. 2015).

5.4.2 Further characterisation of transgenic *AtCIPK16* wheat lines

In this project, only one transgenic sibling *AtCIPK16* wheat line showed the desired Na^+ exclusion phenotype coupled with increased biomass production, although this phenotype was only seen at the highest salt treatment tested (200 mM NaCl). Unfortunately several complicating factors made it difficult to reach any conclusion about the influence of *AtCIPK16* expression on the salt tolerance of wheat. Seed number and quality issues meant that for each of the three independent transgenic lines and the null segregant line several sibling lines were grown over the three experiments. The use of several siblings for each transgenic line, as well as the genotyping results, revealed that the genetic makeup of these transgenic lines is complicated. Genotyping revealed non-Mendelian segregation ratios for the T_3 generation of the transgenic lines. Although the original full length copy number of these lines are known (n = 1 – 4), the number of transgene fragments is still currently unknown. Segregation over the past generations has resulted in the loss of some copies of the *AtCIPK16* transgene which may explain why in one transgenic line very few transgenic individuals were identified. The lack of transgenic plants in one independent transgenic line made comparisons between lines and salt treatments very difficult, particularly in the second full experiment where one sibling line consisted of all null segregants. Another complication was the apparent lack of transgene expression in the transgenic plants tested with no correlation to salt treatment or independent transgenic line. The profile of the minimal *AtCIPK16* expression suggests that epigenetic transgene silencing by DNA methylation is occurring in these transgenic plants. DNA methylation silencing can differ between plants from the same transgenic line and can accumulate over generations (Kilby, et al. 1992; Demeke, et al. 1999; Anand, et al. 2003) which would explain the expression results seen in the transgenic *AtCIPK16* wheat lines used in this project. Another possible explanation for the lack of full *AtCIPK16* transcript amplification, separate or in conjunction with TGS, is the possible fragmentation

of the one or multiple copies of the transgene, or other errors introduced during the transformation process could have resulted in truncated transcripts being produced or the inhibition of transcription entirely.

To allow conclusions about the effects of *AtCIPK16* expression on the salt tolerance of bread wheat to be drawn, considerable work on understanding the genetic background of these transgenic lines needs to be done. To continue the characterisation of these transgenic lines, sibling lines which possess only a single copy of the full transgene need to be identified through either quantitative real-time PCR or Southern analysis (Ingham, et al. 2001; Li, et al. 2004; Shou, et al. 2004). Lines also need to be checked for *AtCIPK16* transgene expression, production of the full length of the *AtCIPK16* transcript and methylation induced silencing which could be detected by high-resolution melt analysis (Malentacchi, et al. 2009; Rodríguez López, et al. 2010). If lines without *AtCIPK16* silencing could not be identified then it may be possible to reverse the DNA methylation, although this would result in reversing the silencing of more than just the *AtCIPK16* transgene (Chen & Pikaard 1997; Lee & Chen 2001). Re-characterisation of the three *AtCIPK16* transgenic lines with known genetic and epigenetic backgrounds could then occur, via repetition of the second full scale hydroponic experiment, as previous results were mainly seen at 200 mM NaCl.

5.4.3 What is the AtCIPK16 network pathway in wheat and barley?

The CBL-CIPK network is believed to be conserved across the plant kingdom, with CBLs and CIPKs having been identified throughout the evolutionary tree from algae and mosses to angiosperms (Kolukisaoglu, et al. 2004; Xiang, et al. 2007; Batistič & Kudla 2009; Weinl & Kudla 2009; Chen, et al. 2011b; Ye, et al. 2013; Kleist, et al. 2014; Zhang, et al. 2014; Mohanta, et al. 2015; Sun, et al. 2015; Wang, et al. 2015a; Hu, et al. 2015b; Meena, et al. 2015b). The conserved nature of CBL-CIPK interactions in plant species has also been demonstrated by the ability of CBLs and CIPKs from different species to interact and form functional complexes, despite the often limited number of CBLs a specific CIPK can bind to (Kim, et al. 2003b; Hwang, et al. 2005; Martínez-Atienza, et al. 2007; Tripathi, et al. 2009; Yoon, et al. 2009; Hu, et al. 2012; Wang, et al. 2012; Chen, et al. 2012; Deng, et al. 2013a; Abdula, et al. 2015). For several of the original Arabidopsis CIPKs, such as CIPK24 (SOS2) functional orthologs in other species have now been identified and characterised (Tang, et al. 2010; Hu, et al. 2012; Huertas, et al. 2012; Zhang, et al. 2014; Zhou, et al. 2014; Li, et al. 2014a; Hu, et al. 2015a; Liu, et al. 2015; Sun, et al. 2015). Unlike *AtCIPK24*, it is very unlikely that a functional ortholog of *AtCIPK16* will be identified in barley or bread wheat due *AtCIPK16* forming a clade unique to the

Brassicales (Amarasinghe in press). There is therefore no native *AtCIPK16* ortholog in barley or wheat which could be used help understand the interactions AtCIPK16 form in these plants. While no actual AtCIPK16 ortholog may be identifiable another CIPK may be identified in barley/wheat which is a functional replacement in these species. This CIPK would need to possess the same functional domains (particularly a nuclear localisation sequence), have a similar expression profile (upregulated under salt stress in stellar tissue) and regulate pathways involved in Na^+ exclusion (possibly Na^+ retrieval from the xylem).

While in a commercial setting the development of lines with improved salt tolerance and yield would be enough, it is also important to understand on a molecular scale how *AtCIPK16* is affecting the salt tolerance of transgenic barley and wheat lines. Previous work has demonstrated which AtCBLs can bind and activate AtCIPK16 but the targets of AtCIPK16 are not yet known, with targets suggested to either be membrane bound transporters, nuclear localized proteins or the chromatin itself (Roy, et al. 2013; Huang 2015). Future work should aim to identify the targets of AtCIPK16 in Arabidopsis, allowing for a better understanding of AtCIPK16's involvement in salt tolerance. Once the targets are known in Arabidopsis, their identification in barley and wheat may be possible. With no native *CIPK16* ortholog in barley or wheat it is possible that the introduction of the transgene into these species has created novel regulation networks. Although as the Na^+ exclusion phenotype was preserved, some similar elements to the native Arabidopsis must be present in barley and wheat. Important future work would include identifying which barley or wheat CBLs bind and activate AtCIPK16, and where these CBL*x*-AtCIPK16 complexes localise in the cell, e.g. the nucleus. Knowing the targets of AtCIPK16 in these transgenic lines would also aid in the understanding of the involvement of AtCIPK16 in salt tolerance. Since *AtCIPK16* is a transgene in these barley and wheat lines it is possible that AtCIPK16 is being activated in different locations or phosphorylating different targets than in Arabidopsis which may explain some of the different phenotypic results seen in this project. For example, AtCIPK16 may be regulating the similar targets in Arabidopsis, barley and wheat for Na^+ exclusion, but when acting as a transgene in barley/wheat AtCIPK16 may regulate a target which negatively affects plant growth or yield. Key to future understanding the phenotypes seen in the *AtCIPK16* expressing barley and wheat lines used in this project will be molecular work on the gene networks formed by *AtCIPK16* in these plants.

5.4.4 *AtCIPK16* expression: which promoter to use?

Manipulation of CIPK16 expression is limited to genetic modification pathways due to the lack of native AtCIPK16 orthologs in bread wheat or barley. All the lines used in this project expressed *AtCIPK16* under the control of a constitutive promoter, *Cauliflower mosaic virus 35S* (*35S*) for barley lines and maize *ubiquitin 1* (*Ubi*) for wheat lines. Constitutive promoters are typically used to confer high levels of ectopic gene expression in transgenic plants (Dutt, et al. 2014). Previous work has revealed issues with the two constitutive promoters used in this project, such as varied expression levels between species, tissue types and developmental stages (Battraw & Hall 1990; Christensen, et al. 1992; Cornejo, et al. 1993; Rooke, et al. 2000; Pret'ová, et al. 2001; Sunilkumar, et al. 2002). It is therefore possible that the lack of expression seen in the *Ubi:AtCIPK16* wheat lines may also be the result of issues with the constitutive promoter, as well as transgene silencing. The constitutive expression of stress-responsive transcription factors, which *AtCIPK16* may well be, has previously been demonstrated to often result in the desired stress phenotype coupled with defects in growth and development (Kasuga, et al. 1999; Kasuga, et al. 2004; Morran, et al. 2011; Kovalchuk, et al. 2013), such as those seen in the field grown *35S:AtCIPK16* barley lines under above average rainfall conditions. Literature showed that expression of these transcription factors under the influence of stress-inducible promoters allowed for the desired the phenotype without growth retardation (Kasuga, et al. 1999; Kasuga, et al. 2004; Morran, et al. 2011; Kovalchuk, et al. 2013). This work raises the question of where other *AtCIPK16* expressing barley and wheat lines should be developed, with expression driven by different promoters, such as a cell-type specific or a stress inducible promoter. The cell-type specific promoter should be stellar specific, as this is where native *AtCIPK16* expression occurs, which could restrict any negative effects of ectopic expression. Other options would be a salt-stress inducible promoter, which would prevent any negative effects of *AtCIPK16* expression at no or low salt concentrations; or the use of a drought-inducible promoter, which would mean *AtCIPK16* would only have an effect under low rainfall conditions where expression of the gene seems to be beneficial. Since the Bay-0 allele of the native *AtCIPK16* promoter has high gene expression only under salt stress and in only one cell-type, future work may also involve investigating the creation of *AtCIPK16* expressing barley/wheat lines driven by the Bay-0 *AtCIPK16* promoter.

This project attempted to demonstrate that addition or removal of a TATA box by site-directed PCR mutagenesis could result in changes in promoter activity and thus gene expression. Future work should aim to finish producing the mutated *AtCIPK16* promoter – GFP reporter constructs for both the Bay-0 and Shahdara alleles. If testing of the constructs in Arabidopsis revealed that the single point mutations had the desired effects on GFP fluorescence – Bay-0: TATAA to TATAT to remove the

TATA box, Shahdara: TATAT to TATAA to produce a TATA box – then this proof of concept could have implications on the future development of new promoters. The outcomes of these experiments could demonstrate that increased gene expression need not use constitutive or foreign promoters but rather edited versions of the gene of interest's native promoter. Native gene promoters could be edited to include motifs such as TATA boxes or stress responsive elements, instead of using constitutive or stress-inducible promoters. The development of end product non-GM technologies such as Transcription Activator-Like Effector Nucleases (TALEN) (Li, et al. 2012; Zhang, et al. 2013b; Wang, et al. 2014; Liang, et al. 2014) or Clustered Regularly Interspersed Short Palindromic Repeats (CRISPR)/CRISPR-associated (Cas) (Brooks, et al. 2014; Wang, et al. 2014; Liang, et al. 2014; Feng, et al. 2014; Ma, et al. 2015; Wang, et al. 2015b) could be used to achieve such modifications. As development of these technologies are still occurring, particularly the CRISPR/Cas systems, as of yet no attempts to mutate gene promoters has occurred although general genome editing has been successful. With no native *AtCIPK16* homolog in barley or wheat, other candidates for gene editing to improve salt tolerance, such as HKTs, would have to be identified.

5.5 Concluding Remarks

In this project transgenic barley expressing *35S:AtCIPK16* showed increased Na^+ and Cl^- exclusion in two transgenic lines grown in high salt field trials. Although in years with above average rainfall ion exclusion led to decreased shoot biomass and yield, while in dry years ion exclusion caused increased growth and yield production. The findings of this project also show that one sibling transgenic line of the *Ubi:AtCIPK16* wheat had Na^+ and Cl^- exclusion from both root and shoot tissue coupled with increased biomass production. Despite this result, complications in the genetic and epigenetic background of the transgenic wheat lines meant that no conclusions on the effects of *AtCIPK16* in wheat could be drawn. This project demonstrated that site-directed PCR mutagenesis could be used to introduce specific point mutations into the promoters of *AtCIPK16* alleles, although this section of the project was not completed. Overall, this project suggests that *AtCIPK16* expression may be able to increase the salt tolerance of cereals under certain growth conditions.

Chapter 6: Appendices

Appendix 1

Plant Growth Regul
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ORIGINAL PAPER

The role of the CBL–CIPK calcium signalling network in regulating ion transport in response to abiotic stress

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Abstract Plants are sessile organisms and have multiple tolerance mechanisms which allow them to adapt to the environmental stresses to which they may be exposed. Key to a plant's tolerance of abiotic stresses is the ability to rapidly detect stress and activate the appropriate stress response mechanism. The calcineurin B-like (CBL) and CBL-interacting protein kinase (CIPK) signalling pathway is a flexible Ca^{2+} signalling network which allows a plant to fine tune its response to stress, via both pre- and post-translational mechanisms. Genes encoding CBLs and CIPKs have now been identified in a variety of plant species. Plants have been found to have large gene families of *CBL*s and *CIPK*s, each encoding proteins with specific upstream and downstream targets, thus providing the flexibility required to allow a plant to adapt to a variety of stresses. Characterisation of *CBL* and *CIPK* mutants have shown them to be important for a plant to survive cold, drought, heat, salinity and low nutrient stresses. Many CBLs and CIPKs have been shown to be involved in the transport of ions through a plant, either limiting the supply of toxic ions to certain tissues or maximising the uptake of beneficial nutrients from the soil. This review will provide an update into the current knowledge of CBL and CIPK interactions and their role in ion transport during abiotic stress.

Keywords Calcium signalling · Calcineurin B-like protein · CBL-interacting protein kinase · Abiotic stress

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Introduction

Plants are able to survive multiple abiotic and biotic stresses throughout their lifecycles, which requires them to have mechanisms to detect and react to each individual stress. Exposure to abiotic stresses, such as cold/frost, drought/water stress, heat and salinity as well as nutrient deficiencies, results in decreased germination rates, seedling survival, biomass production, leaf expansion, photosynthesis (carbon assimilation) and overall growth (Munns et al. 1995; Jacobs and Pearson 1999; Zeng and Shannon 2000; Zhao et al. 2001; Alexieva et al. 2001; Sairam et al. 2002; Gill et al. 2003; Reddy et al. 2004; Zhou et al. 2014). As the occurrences of these stresses are often unpredictable, plants have developed mechanisms for the early detection of abiotic stresses, so that they can initiate the appropriate tolerance mechanism(s) before damage occurs. Vital to a plant's survival under abiotic stress is not only the sensing of the stress, but also the ability to signal the occurrence of the stress throughout the plant.

Ca^{2+} has been implicated in signalling the occurrence of stresses and subsequently enabling the activation of tolerance mechanisms (Sanders et al. 1999, 2002; Bose et al. 2011; Choi et al. 2014). Calcineurin B-like protein (CBL)–CBL-interacting protein kinase (CIPK) complexes are one of several calcium signalling mechanisms which decode intra-cellular calcium signals and regulate the transcriptional and translational response of a plant cell to the stress

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(Luan et al. 2002; Batistič and Kudla 2012). As well as possessing a N-terminal kinase domain, CIPKs also have a C-terminal regulatory domain consisting of a CBL binding NAF/FISL domain and a protein–phosphate interaction (PPI) domain which are involved in CIPK–PPC2 (protein phosphatase 2C-type) interactions (Albrecht et al. 2001; Ohta et al. 2003; Batistič and Kudla 2004; Ye et al. 2013a; Lyzenga et al. 2013). Upon of the occurrence of a stress, plants produce a stress specific Ca^{2+} signature (Rudd and Franklin-Tong 2001; Sanders et al. 2002; Dodd et al. 2010) resulting in Ca^{2+} to binding to four EF hands domains in the CBL protein. This enables CBLs to bind to the NAF domain of CIPKs thereby activating the kinase (Kolukisaoglu et al. 2004). The N-terminal of the CBL, then directs the CBL–CIPK complex to a specific cellular target region resulting in the activated CIPK phosphorylating the appropriate target proteins (Quan et al. 2007; Cheong et al. 2007; Batistič et al. 2008, 2010; Held et al. 2011; Drerup et al. 2013).

CBLs and CIPKs have been identified in a range of terrestrial plants from mosses, ferns and gymnosperms to monocots and dicots (Kolukisaoglu et al. 2004; Xiang et al. 2007; Zhang et al. 2008; Weinl and Kudla 2009; Batistič et al. 2010; Ye et al. 2013a; de la Torre et al. 2013; Zhang et al. 2014; Kleist et al. 2014). In all species they have been found to regulate a plant's response to drought, heat, cold, salt and low nutrient availability (Table 1) (Xiang et al. 2007; Wang et al. 2007, 2012; Chen et al. 2011a, 2012; Li et al. 2012a; Cuéllar et al. 2013). This review will cover the known roles of CBLs and CIPKs in the direct regulation of ion transport.

Table 1 CBLs and CIPKs demonstrated to involved in the regulation of plant responses to various abiotic stresses

Ion	CBLs	CIPKs
Sodium	*AmCBL1*	*HbCIPK2*
	AtCBL1	*AtCIPK3*
	BnCBL1	*OsCIPK3*
	GmCBL1	*AtCIPK6*
	AtCBL4 (SOS3)	*BnCIPK6*
	ZmCBL4	*GhCIPK6*
	PtSOS3	*MdCIPK6L*
	SlSOS3	*TaCIPK14*
	AtCBL5	*OsCIPK15*
	PeCBL6	*AtCIPK16*
	OsCBL8	*ZmCIPK16*
	AtCBL9	*ZmCIPK21*
	ThCBL9	*AtCIPK24 (SOS2)*
	AtCBL10	*HaSOS2*
	PeCBL10	*PtSOS2*
	PtCBL10A & B	*MdSOS2*
		SlSOS2
		PeCIPK26
		TaCIPK29
Potassium	*AtCBL1*	*AtCIPK1*
	GmCBL1	*AtCIPK3*
	OsCBL1	*VvCIPK3*
	PeCBL1	*VvCIPK4*
	VvCBL1	*AtCIPK6*
	AtCBL2	*GhCIPK6*
	VvCBL2	*MdCIPK6L*
	AtCBL3	*AtCIPK9*
	AtCBL4	*OsCIPK12*
	AtCBL5	*AtCIPK16*
	AtCBL9	*AtCIPK23*
	AtCBL10	*OsCIPK23*
	PeCBL10	
Phosphorous	*BnCBL1*	*BnCIPK6*
Nitrogen	*AtCBL9*	*AtCIPK8*
		AtCIPK23

Sodium

The salt overly sensitive (SOS) pathway is an example of a CBL–CIPK signalling pathway which was first identified in Arabidopsis (Wu et al. 1996; Liu and Zhu 1997; Zhu et al. 1998; Halfter et al. 2000; Quintero et al. 2002). Comprised of SOS3 (AtCBL4), SOS2 (AtCIPK24) and the plasma membrane Na^+/H^+ antiporter, SOS1, the pathway plays a vital role in Na^+ tolerance through the efflux capacity of SOS1, which pumps Na^+ from the plant cell (Fig. 1) (Qiu et al. 2002). The formation of a SOS2–SOS3 complex in the roots in response to salt stress allows SOS2 to phosphorylate and activate the transport properties of SOS1 (Qiu et al. 2002; Quintero et al. 2002) (Figs. 1, 2). Plants which lack the ability to activate SOS1 (such as *sos3* or *sos3* mutants) accumulate more Na^+ through a reduced efflux capacity, resulting in decreased growth under saline conditions (Liu and Zhu 1997; Zhu et al. 1998; Elphick et al. 2001; Guo et al. 2004; Quan et al. 2007; Ye et al. 2013b).

AtSOS2 has also been hypothesised to be involved in Na^+ sequestration by regulating a tonoplast Na^+/H^+ antiporter via its interaction with AtCBL10 (Figs. 1, 2) (Qiu et al. 2004; Kim et al. 2007). This interaction between AtCBL10 and AtCIPK24 appears to occurs in aerial tissues and *cbl10* mutants show defects in seedling shoot and mature stem growth, as well as increased wilting under saline conditions (Quan et al. 2007; Kim et al. 2007). The tomato (*Solanum lycopersicum*) SOS2 protein (SlSOS2) is also hypothesised to regulate the activity of tonoplast $Na^+/$

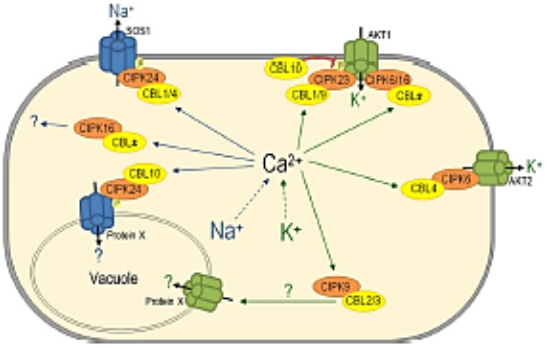

Fig. 1 Model of calcineurin B-like protein (CBL)–CBL-interacting protein kinase (CIPK) interactions and functions in regulating sodium (Na^+) and potassium (K^+) homeostasis. See main text for details. P represents those CBL–CIPK interactions which result in target phosphorylation. SOS1, salt overly sensitive 1; AKT1, Arabidopsis K^+ transporter 1; AKT2, Arabidopsis K^+ transporter 2. CBLx and Protein X indicate where an interaction is known to take place but where the identity of the protein the CIPK is interacting with is still unknown

H^+ antiporters. Overexpression of *SlSOS2* in difference tomato cultivars leads to an increase in the activity of K^+/H^+ transporters and H^+-ATPases, suggesting other possible tonoplast and endosomal targets for SOS2 regulation (Huertas et al. 2012).

In addition to AtCBL4 and AtCBL10, AtCBL1 can form a complex with AtSOS2 at the plasma membrane, prompting speculation that it may phosphorylate plasma membrane bound proteins (Kolukisaoglu et al. 2004; Kim et al. 2007). Interestingly, while AtCBL1, AtCBL4 and AtCBL10 are able to recruit AtSOS2 to either the plasma membrane or tonoplast (Fig. 2), AtSOS2 is also able to associate with membranes independent of a CBL interaction, although the mechanism behind this is not yet understood (Quan et al. 2007; Kim et al. 2007).

Members of the SOS pathway have now been identified in a many plant species: *Aeluropus lagopoides* (Jannesar et al. 2014), apple (*Malus domestica*) (Hu et al. 2012), barley (*Hordeum vulgare*) (Rivandi et al. 2011), canola (*Brassica napus*) (Chakraborty et al. 2012), maize (*Zea mays*) (Wang et al. 2007; Chen et al. 2014), mustard greens (*Brassica juncea*) (Kushwaha et al. 2011), poplar (*Populus sp.*) (Wu et al. 2007; Tang et al. 2010, 2014; Li et al. 2012a; Zhang et al. 2013; Zhou et al. 2014; Lv et al. 2014), rice (*Oryza sativa*) (Martínez-Atienza et al. 2007; Xiao et al. 2009; Chen et al. 2011b), soybean (*Glycine max*) (Li et al. 2012c), sunflower (*Helianthus annuus*) (Saadia et al. 2013) and tomato (*Solanum lycopersicum*) (Olías et al. 2009; Huertas et al. 2012). Overexpression of individual SOS components from different species has been shown to increase salt tolerance through both increased Na^+ efflux and sequestration (Cheong et al. 2003; Shi et al. 2003; Wang et al. 2007; Yang et al. 2009; Chen et al. 2011a, 2012; Li et al. 2012a, 2012c, 2013; Hu et al. 2012; Huertas et al. 2012; Zhou et al. 2014; Tang et al. 2014). Co-expression of all three main components of the SOS pathway (*AtSOS3*, *AtSOS2* and *AtSOS1*) has also been shown to increase growth under saline conditions in sweet potato and tall fescue (Gao et al. 2012; Ma et al. 2014), signifying that the manipulation of *SOS* gene expression levels may be a way to improve plant salt tolerance.

Functional conservation between species is also common for SOS pathway components, for example *MdSOS2* (*Malus domestica*) is able to compliment *AtCIPK24* in Arabidopsis *cipk24* mutants with MdSOS2 able to interact with AtCBL4/SOS3 (Martínez-Atienza et al. 2007; Wang et al. 2007; Hu et al. 2012; Huertas et al. 2012; Tang et al. 2014; Lv et al. 2014). Two other CIPKs have been identified as able to partly or completely compliment AtCIPK24. ZmCIPK16 is able to partly replace the function of AtCIPK24 in Arabidopsis *sos2* knockouts at germination and early development, although plants still display some reduced growth compared to wildtype plants, perhaps due to ZmCIPK16 being involved in only SOS1 regulation and not the other mechanisms which AtCIPK24 may be involved in (Zhao et al. 2009). *HbCIPK2* (*Hordeum brevisubulatum*) expression in an Arabidopsis *sos2* mutant background

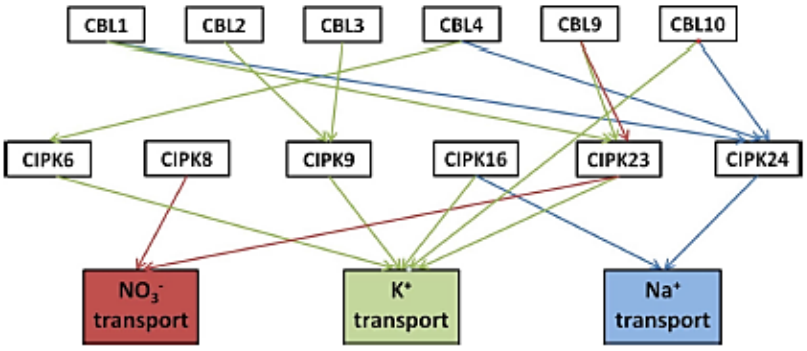

Fig. 2 Interactions observed between different *Arabidopsis thaliana* CBLs and CIPKs and the ion transport mechanism they are involved in

results in even better tolerance to salinity than is seen in wildtype Arabidopsis, suggesting that HbCIPK2 is more efficient in regulating plant salt tolerance than AtCIPK24 or has an additional roles in salinity tolerance (Li et al. 2012b). It has been speculated that HbCIPK2 may be involved in the regulation H^+-pumps, leading to the maintenance of the H^+ gradient under salt which would enable reduced efflux of important ions such as K^+ (Li et al. 2012b).

The over-expression of other *CBLs* or *CIPKs* have also resulted in enhanced salt tolerance in a variety of different plant species, through increased germination, seedling survival and biomass production, although the mechanisms they function in are as yet unknown or not fully understood (Xiang et al. 2007; Sun et al. 2008; Gu et al. 2008; Tripathi et al. 2009; Cheong et al. 2010; Li et al. 2012a, 2013; Wang et al. 2012; Chen et al. 2012, 2013, 2014; He et al. 2013; Deng et al. 2013a, b; Roy et al. 2013). Whilst the mechanisms behind these improvements have yet to be clarified, AtCBL9, AtCIPK16 (Fig. 1), TaCIPK14, TaCIPK29 (*Triticum aestivum*) and ZmCIPK21(*Zea mays*) have been shown to have roles in Na^+ transport, ROS metabolism, K^+ homeostasis and or ABA sensitivity in salt stressed plants (Pandey et al. 2004, 2008; Deng et al. 2013a, b; Roy et al. 2013; Chen et al. 2014). Table 1 summarises all of the CBLs and CIPKs implicated in the regulation of salt tolerance.

Of the numerous CBL and CIPKs that have been identified as being involved in salt stress only a few have been implicated as negative regulators of salt tolerance. Unlike AtCBL1, PeCBL1 (*Populus euphratica*) was found to negatively influence Na^+ efflux from the cell under saline conditions, although the mechanisms behind this are also unclear (Zhang et al. 2013). Over-expression of *OsCIPK3* (*Oryza sativa*), results in increased sensitivity to salt, perhaps by interfering with the synthesis of compatible solutes or ion homeostasis (Rao et al. 2011).

Potassium

Potassium nutrition involves controlling the uptake and translocation of K^+ through the plant. AKT1 is a low-affinity inwardly-rectifying K^+ channel involved in the cellular uptake of K^+ (Schroeder et al. 1994; Lagarde et al. 1996; Xu et al. 2006; Li et al. 2006; Cheong et al. 2007; Nieves-Cordones et al. 2012). The activity and flux of K^+ through AKT1 is regulated by a multitude of proteins including CBLs/CIPKs (Li et al. 2006; Lee et al. 2007; Liu et al. 2013, reviewed in Alemán et al. 2011). AtCIPK23 interacting with AtCBL1 or AtCBL9 is able to phosphorylate AKT1 resulting in an inward movement of K^+ into cells (Fig. 1) (Xu et al. 2006; Li et al. 2006; Lee et al. 2007; Cheong et al. 2007). Knockout *akt1* lines exhibit increased sensitivity to K^+ deficiencies and have reduced growth and chlorotic leaves (Dennison et al. 2001; Xu et al. 2006) which are very similar to symptoms observed in Arabidopsis *cipk23* and *cbl1/cbl9* knockout lines (Xu et al. 2006; Cheong et al. 2007).

AKT1 is highly expressed in root tissue, although low levels have been observed in leaves, particularly in stomatal guard cells and hydathodes (Lagarde et al. 1996; Lacombe et al. 2000; Szyroki et al. 2001; Pilot et al. 2003). *AtCIPK23*, *AtCBL1* and *AtCBL9* are also expressed throughout root cells and in aerial tissues such as the guard cells and vasculature (Cheong et al. 2007). These overlapping expression profiles indicate that as well as regulating the root uptake of K^+; AtCBL1, AtCBL9 and AtCIPK23 may be involved in the regulation of AKT1 activity in aerial tissue, for guard cell turgor regulation, K^+ redistribution and membrane repolarisation (Fig. 2) (Schroeder et al. 1994; Szyroki et al. 2001; Dennison et al. 2001; Pilot et al. 2003; Xu et al. 2006; Cheong et al. 2007; Nieves-Cordones et al. 2012). AtCBL1, AtCBL9 and AtCIPK23 have also been shown to regulate the activity of anion transporters SLAC1 and SLAH3 in the guard cells (Maierhofer et al. 2014), suggesting multiple roles for these CBLs and CIPK in the same cell type. Knockout mutation analysis indicates that AtCBL1, AtCBL9 and AtCIPK23 are involved in drought tolerance by regulating stomatal water movements, increasing guard cell sensitivity to ABA and regulating K^+ uptake by the guard cells (Guo et al. 2002; Albrecht et al. 2003; Xu et al. 2006; Cheong et al. 2007). AtCBL1 is likely a positive regulator of drought stress with knockout mutants showing accelerated water loss (Cheong et al. 2003; Albrecht et al. 2003; D'Angelo et al. 2006) while Arabidopsis *cbl9* knockout mutants showed decreased growth under drought but with no changes in stomatal regulation (Pandey et al. 2004).

Although overexpression of *AKT1* in Arabidopsis doesn't result in any improvement in growth when plants are grown with low K^+ availability, overexpression of *AtCBL1*, *PeCBL1*, *AtCBL9* or *AtCIPK23* in Arabidopsis does result in enhanced tolerance to low K^+ abundance (Lagarde et al. 1996; Pilot et al. 2003; Xu et al. 2006; Cheong et al. 2007; Zhang et al. 2013). Transgenic approaches to improve tolerance to low K^+ availability in commercial crops has also been investigated, with *AtCIPK23* overexpressed in potato and *AtCBL9*, *AtCIPK23* and *AKT1* co-expressed in sugarcane, resulting in increased growth, survival and K^+ uptake under low K^+ conditions (Wang et al. 2010; Li et al. 2014b). The enhanced tolerance in both transgenic species was hypothesised to be due to an increased ability to uptake K^+ at lower concentrations (Xu et al. 2006), with the expression changes possibly contributing to other processes allowing increased tolerance to low K^+ environments.

The rice homologs OsCIPK23 and OsCBL1 have been demonstrated to interact and activate the AKT homolog OsAKT1 (Li et al. 2014a) and studies into the grapevine (*Vitis Vinifera*) AKT1 homologs, VvKT1.1and VvKT1.2, have also shown their regulation by CBL and CIPKs during berry filling (Cuéllar et al. 2010, 2013). Both the VvCBL1–VvCIPK4 and the VvCBL2–VvCIPK3 complexes are able to activate and regulate the activity of VvKT1.2 influencing whole plant K^+ transport (Cuéllar et al. 2013). The CBL–CIPK network also appears to be involved in the negative regulation of AKT1 activity, with AtCBL10 thought to compete with AtCIPK23 for direct binding to AKT1, therefore stopping AtCIPK23 from binding and activating AKT1 (Ren et al. 2013). This negative regulation is supported by the observation that overexpression of *AtCBL10* causes significantly reduced inward K^+ currents resulting in decreased plant health especially under low K^+ conditions and demonstrating that the excess AtCBL10 acts an inhibitor for AKT1–AtCIPK23 binding (Ren et al. 2013). AtCIPK6 and AtCIPK16 are other CIPKs that have been found to be involved in the regulation of AKT1, although their ability to interact with AKT1 *in planta* has not yet been fully investigated (Lee et al. 2007).

AKT2 is another K^+ transporter found to be involved in moving K^+ across the plasma membrane and is involved in stomatal regulation and phloem loading (Szyroki et al. 2001; Deeken et al. 2002; Gajdanowicz et al. 2011; Sandmann et al. 2011; Held et al. 2011). However, post-translational modifications are necessary to switch it from being involved with K^+ influx to K^+ efflux (Chérel et al. 2002; Sandmann et al. 2011). A de/phosphorylation network is thought to regulate the functional switch from influx to efflux, with AtPP2CA dephosphorylation found to repress the ability of AKT2 to move K^+ out of the cell (Chérel et al. 2002; Sandmann et al. 2011). While AtCIPK6 has been shown not to phosphorylate AKT2, interaction of the AtCBL4–CIPK6 complex with AKT2 is necessary to activate AKT2's K^+ efflux pathways (Figs. 1, 2) (Held et al. 2011). The single knockout mutants *akt2, cipk6* and *cbl4* all show similar growth phenotypes, reductions in growth and delayed bolting, likely due to inhibited sugar and K^+ movement throughout the plant to growing tissues, especially under conditions of reduced carbon fixing (Deeken et al. 2002; Gajdanowicz et al. 2011; Sandmann et al. 2011; Held et al. 2011).

AtCBL2, AtCBL3 and AtCIPK9 are also implicated in the distribution and translocation of K^+ and can be found in root epidermal and vascular cells (Figs. 1, 2) (Pandey et al. 2007; Liu et al. 2013). Again there appears to be functional redundancy within the system, as only *cbl2cbl3* mutants have abnormal embryo development, stunted growth and necrosis, which are typical symptoms of nutrient deficiencies, under normal growth conditions (Tang et al. 2012; Eckert et al. 2014). The growth defect observed in *cbl2cbl3* mutants are likely linked with reduced levels of vacuolar-ATPase activity, which affects ion compartmentalisation and redistribution, including K^+ (Tang et al. 2012). The single knockout mutant *cbl3*, shows reduced sensitivity to low K^+ conditions, which may be linked to the regulation of V-ATPase (Liu et al. 2013). Conversely, single overexpression lines of *AtCBL2* or *AtCBL3* both show increased low K^+ sensitivity, exhibiting earlier development of necrosis and lower K^+ content, particularly in root tissue when grown under low K^+ conditions (Liu et al. 2013). These symptoms are very similar to those seen in knockout *cipk9* when grown on K^+ deficient media (Liu et al. 2013). AtCIPK9 interacts with both AtCBL2 and AtCBL3 at the tonoplast (Liu et al. 2013) with knockout *cipk9* lines having increased tolerance to low K^+, while overexpression of *AtCIPK9* results in early chlorosis, reduced chlorophyll content and overall lower K^+ content (Liu et al. 2013). As *AtCBL2*, *AtCBL3* and *AtCIPK9* are all expressed in root epidermal cells, and alterations in expression levels of these genes affects plant K^+ content, it is likely that they may play a role in the regulation of K^+ uptake. Although the downstream K^+ transporter regulated by the kinase has yet to be identified it is speculated that the target of the CBL2/3-CIPK9 complex may be vacuolar ATPases (Fig. 1). All three genes are also highly expressed in root vascular tissue, suggesting a role in the regulation of K^+ distribution and translocation throughout the plant (Liu et al. 2013). This hypothesis is supported by an increased root/shoot K^+ ratio in the low K^+ tolerant *cbl3* and *cipk9* knockdown mutants, suggesting more K^+ is translocated to the shoot tissue, while in *AtCBL3* and *AtCIPK9* overexpressing plants less K^+ is translocated to the shoots (Liu et al. 2013).

Phosphorus

CBLs and CIPKs have been implicated in a plant's response to low inorganic phosphorus (P_i) (Table 1). *BnCBL1* and *BnCIPK6* are upregulated in *Brassica napus* under P_i deficient conditions and have been shown to interact in both yeast two-hybrid screens and split-YFP studies in plant cells (Chen et al. 2012). Therefore *BnCBL1* and *BnCIPK6* may regulate processes involved in the plant's response to P_i deficiencies, although the mechanism and pathway is yet unknown. Overexpression of either *BnCBL1* or *BnCIPK6* in Arabidopsis enhances growth and biomass production under low P_i conditions, particularly the establishment and growth of lateral roots (Chen et al. 2012). *BnCIPK6* is also able to fully compliment Arabidopsis *cipk6* mutants grown on low P_i, suggesting that *AtCIPK6* is also involved in responses to low P_i conditions (Chen et al. 2012).

Nitrate

Plants uptake nitrate through two systems: the high-affinity system and the low affinity system (Forde 2002; Krouk et al. 2010). The low-affinity system is responsible for most of the primary nitrate uptake and response under sufficient conditions, while the high-affinity system is induced under nitrate deficits (Forde 2002; Krouk et al. 2010). NRT1.1 (CHL1) is involved in both high-affinity and low-affinity nitrate transport (Huang et al. 1996; Liu et al. 1999), with CIPK-mediated phosphorylation of NRT1.1 shown to change NRT1.1 from a low-affinity to high-affinity nitrate transporter (Ho et al. 2009). During nitrate sufficient conditions NRT1.1, in its unphosphorylated state, facilitates low-affinity nitrate transport (Ho et al. 2009). However under nitrate deficient conditions, the AtCBL19–AtCIPK23 complex is responsible for phosphorylation of NRT1.1, increasing its high-affinity transport capacity allowing for improved nitrate uptake (Fig. 2) (Ho et al. 2009).

As well as possessing the ability to uptake nitrate, NRT1.1 has also been shown to affect the expression of nitrate-inducible genes, suggesting its involvement in nitrate sensing as well as nitrate uptake (Hu et al. 2009). A role in primary nitrate sensing has been suggested for AtCIPK8 as the gene's expression level is strongly induced by nitrate, even in *ntr1.1* plants lacking nitrate uptake (*NRT1.1* knockout mutants) (Hu et al. 2009). Although the mechanism is unclear, AtCIPK8 is likely involved in a mechanism which senses high nitrate concentrations and thus activates the low-affinity nitrate response (Hu et al. 2009). In addition to showing a reduced primary nitrate response, *cipk8* mutants also had reduced expression of several nitrate response genes, including *AtNRT1.2* and *AtNRT2.1*, suggesting that it may play a role in nitrate-dependent transcriptional regulation (Hu et al. 2009). This may be due to AtCIPK8 regulating a currently unknown mechanism which in turn impacts on transcription or mRNA stability, or AtCIPK8 may directly interact with transcription factors, transcriptional machinery or impact on mRNA stability. As well as regulating nitrate-dependent gene expression, AtCIPK8 has also been implicated in the regulation of nitrate dependent stimulation of root growth and the transport of other anions, such as boron or malate/fumarate (Hu et al. 2009).

Manipulation of CBLs and CIPKs to improve ion transport during abiotic stress

Many abiotic tolerance mechanisms, such as drought tolerance, heat tolerance, salt tolerance and tolerance to low nutrient deficiencies, rely at least partially on the control of ions through a plant. Manipulation of the expression of specific *CBL*s and *CIPK*s is an attractive strategy to improve the stress tolerance of plants, perhaps more so than the manipulation of the transporters they regulate, as a single CBL or CIPK may regulate several tolerance mechanisms. Care still needs to be taken, however, that the genes encoding CBLs and CIPKs are tightly controlled as what may work under one stress may be detrimental under another. The overexpression of *AtCBL1* for example would be beneficial for developing plants with increased drought, low potassium or salt tolerance, but may negatively affect the cold tolerance of the same plants (Cheong et al. 2003). Knockdowns of *AtCIPK23* would encourage stomatal closures which would result in reduced water loss especially under drought conditions, but would negatively affect plants under heat stress, a problem since both stresses often occur simultaneously (Cheong et al. 2007; Nieves-Cordones et al. 2012). The use of stress and tissue specific promoters to drive the expression of transgenes of *CBL*s and *CIPK*s will therefore be important, as is a greater understanding of the downstream targets of different CIPKs under different stresses.

To date, the majority of research in the CBL and CIPK field has primarily focused on identifying the interactions between CBLs and CIPKs, the location of their interaction and the phenotypic analysis of CBL or CIPK mutants exposed to different abiotic stresses. Only a small number downstream targets of CIPKs have been identified, for example SOS1 (Shi et al. 2000; Qiu et al. 2002), CHL1 (Hu et al. 2009; Ho et al. 2009), AKT1 (Xu et al. 2006; Lee et al. 2007; Cheong et al. 2007) and AKT2 (Held et al. 2011). Identification of the downstream targets is essential to gain a complete understanding to the CIPK and CBL signalling network, the processes they control and how that affects plant phenotype. Now that (for some species) much is known about which CBLs interact with what CIPKs and where this interaction occurs within the tissue and cell, it is now time to expand the scope of research to the proteins being regulated.

Conclusions

The diverse CBL–CIPK calcium signalling pathway enables the control over the movement ions throughout a plant under a range of stress conditions. The CBL–CIPK pathways have been shown to be instrumental in the moemnnt of sodium, potassium, nitrate and phosphorous through out a plant. While manipulation of these pathways through the insertion of *CBL* and *CIPK* transgenes initially appears to be an attractive way of engineering plants with improved stress tolerance, care must be taken in how these transgenes are controlled and in the selection CBLs and CIPKs which are not detrimental to other biological processes.

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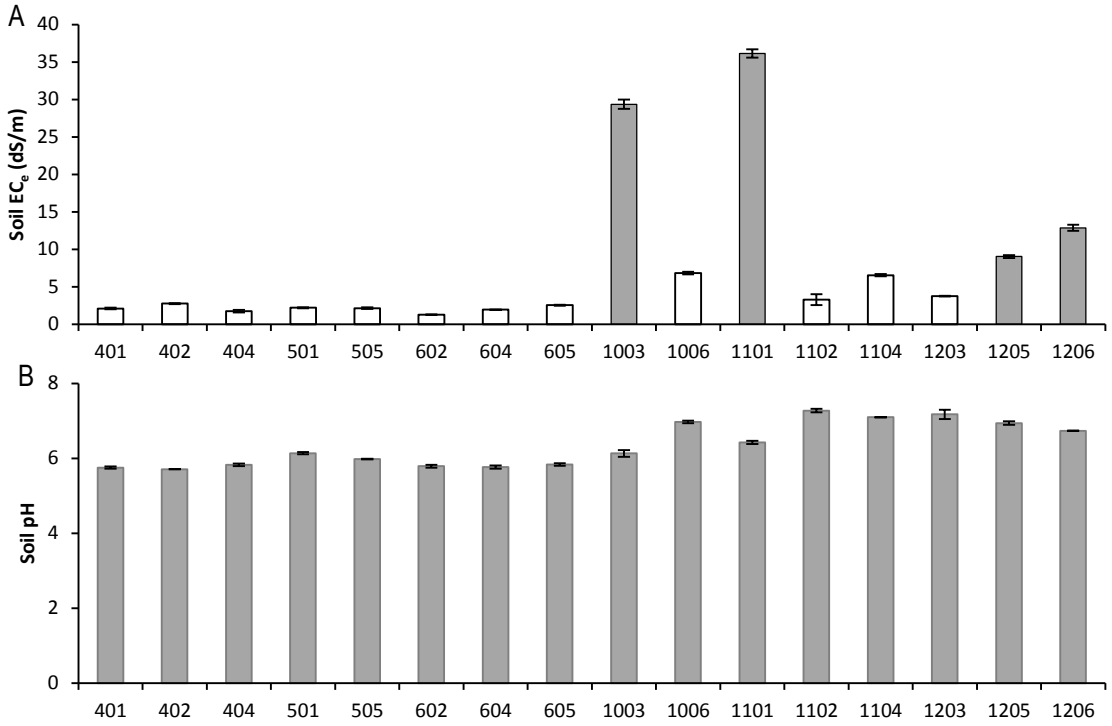
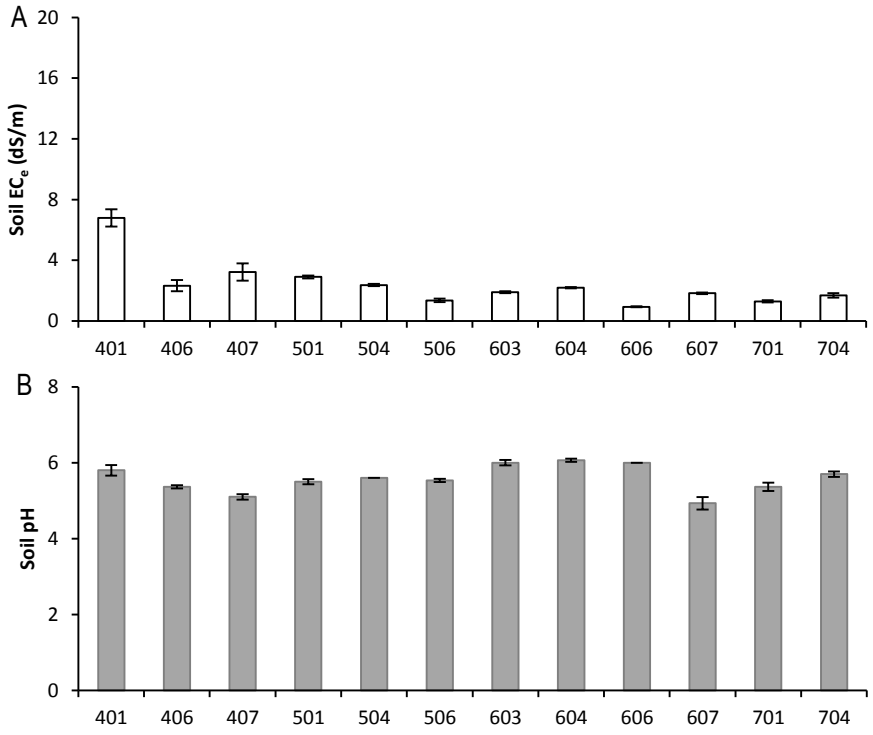
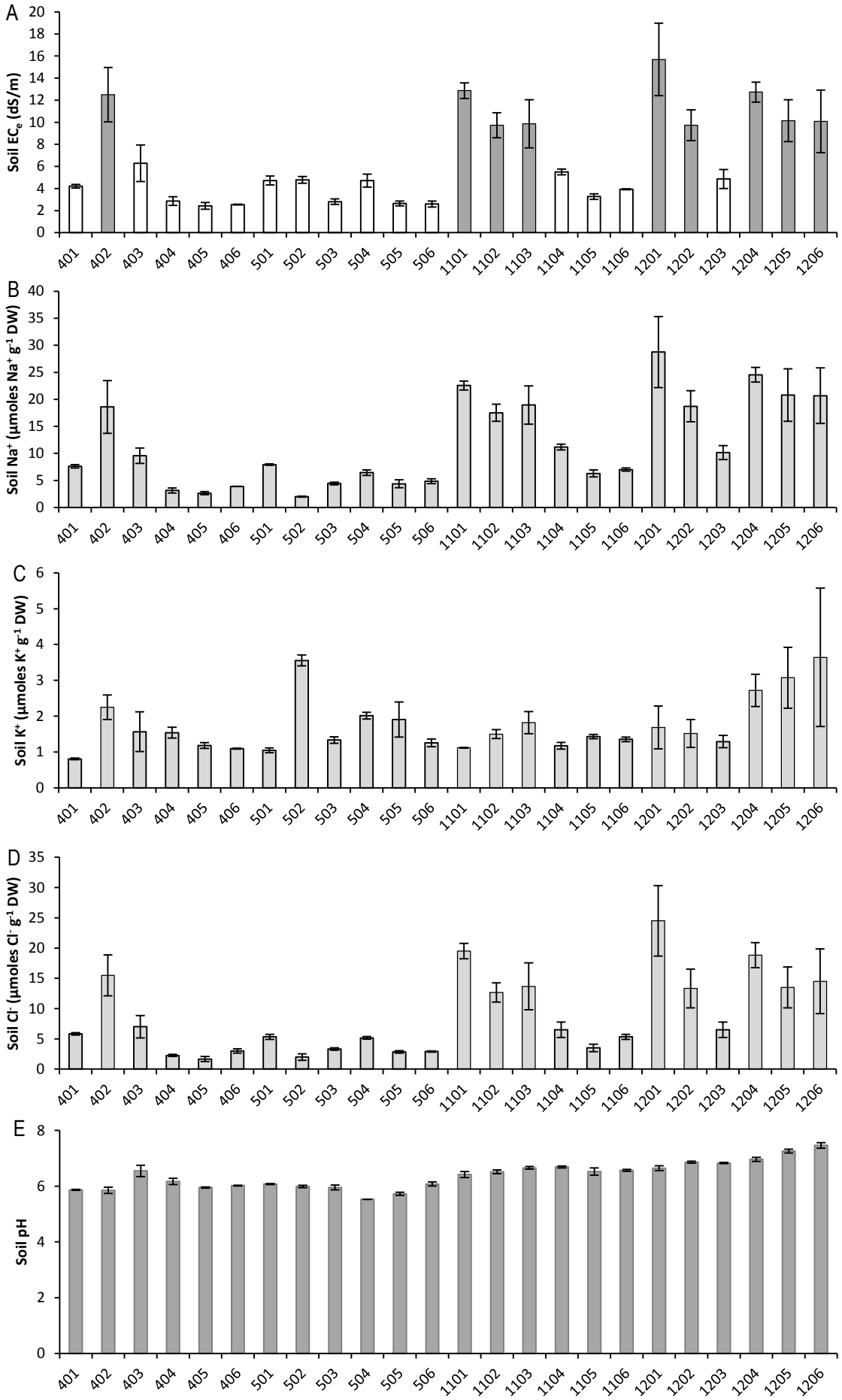
Appendix 2



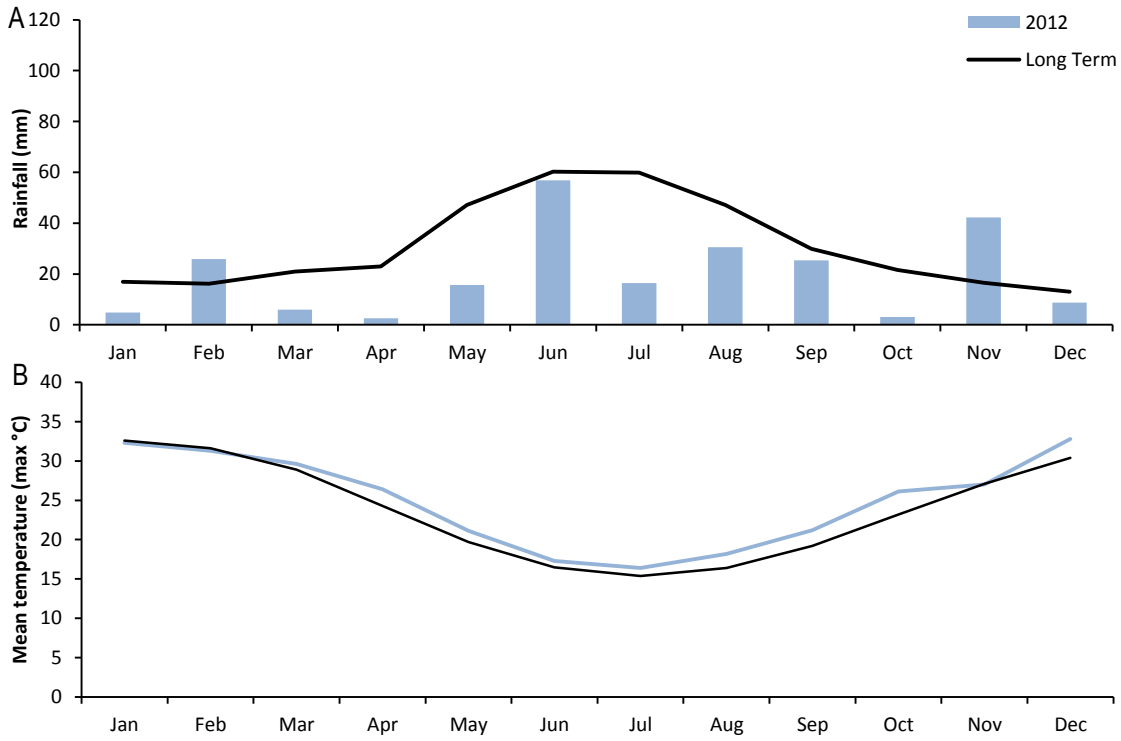
Supplementary Figure 2.1: The electrical conductivity (EC_e) (dS/m) (A) and pH (B) of soil collected from individual plots in the low (401 – 605) and high (1003 – 1206) salt trial areas at Kunjin, Western Australia in September 2012 using a soil core (0 -10 cm). Plots with EC_e values below 8 dS/m (white bars) where analysed separately to plots with EC_e values above 8 dS/m (grey bars). Values presented as the mean ± s.e.m (n = 3).



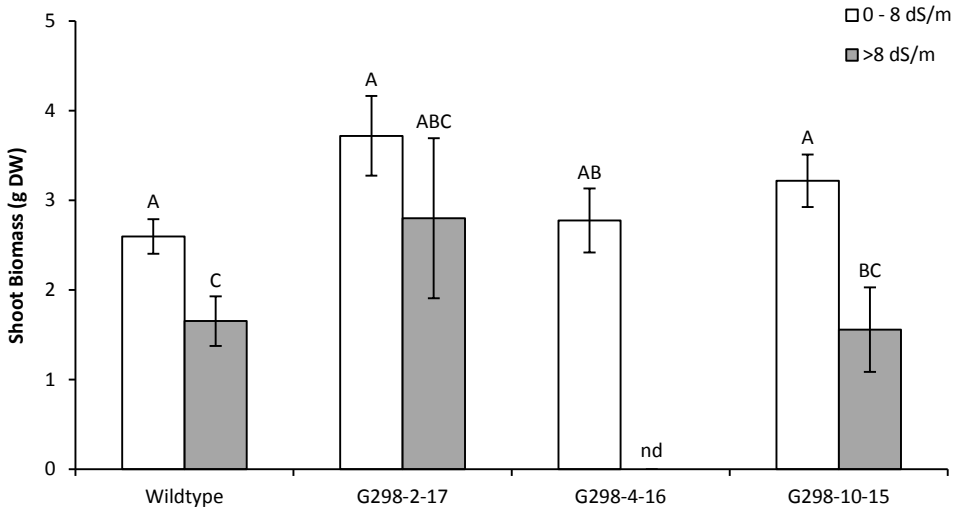
Supplementary Figure 2.2: The electrical conductivity (EC_e) (dS/m) (A) and pH (B) of soil collected from individual plots in the low (401 – 605) salt only trial area at Kunjin, Western Australia in September 2013 using a soil core (0 -10 cm). Values presented as the mean ± s.e.m (n = 3).


A
Soil EC_e (dS/m)
B
Soil Na^+ (µmoles Na^+ g^{-1} DW)
C
Soil K^+ (µmoles K^+ g^{-1} DW)
D
Soil Cl^- (µmoles Cl^- g^{-1} DW)
E
Soil pH
401 402 403 404 405 406 501 502 503 504 505 506 1101 1102 1103 1104 1105 1106 1201 1202 1203 1204 1205 1206

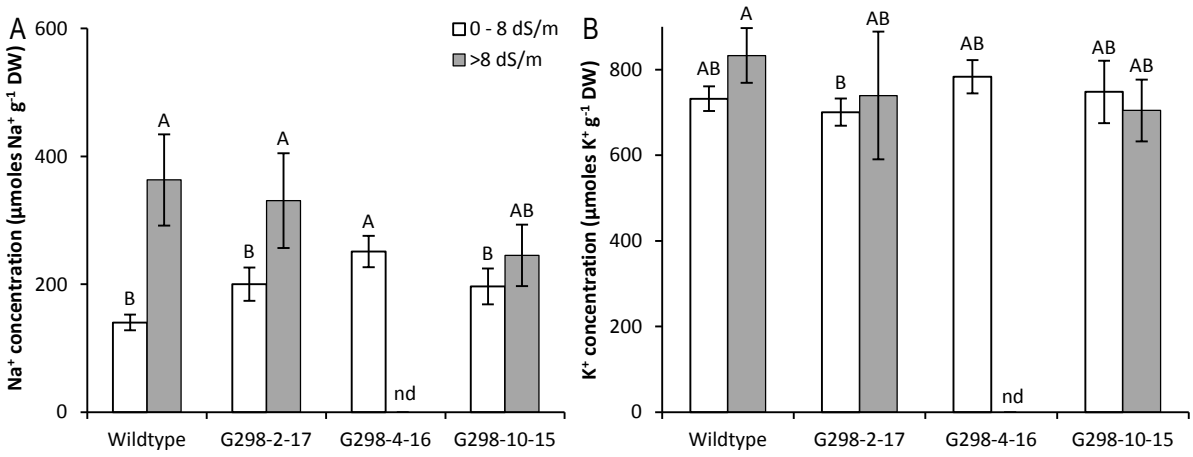
Supplementary Figure 2.3: The electrical conductivity (EC_e) (dS/m) (A), Na^+ concentration (µmoles Na^+ g^{-1} DW) (B), K^+ concentration (µmoles K^+ g^{-1} DW) (C), Cl^- concentration (µmoles Cl^- g^{-1} DW) (D) and pH (E) of soil collected from individual plots in the low (401 – 506) and high (1101 – 1206) salt trial areas at Kunjin, Western Australia in September 2014 using a soil core (0 -10 cm). Plots with EC_e values below 8 dS/m (white bars) where analysed separately to plots with EC_e values above 8 dS/m (grey bars). Values presented as the mean ± s.e.m (n = 3).



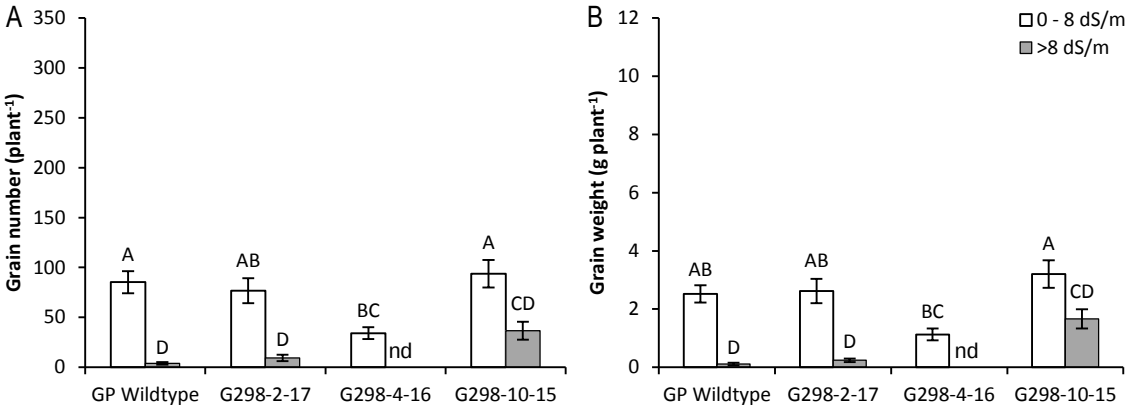
Supplementary Figure 2.4: Average rainfall (mm) (A) and maximum temperature (°C) (B) at Corrigin, Western Australia for 2012 (blue). Black represents the long term average over the last 65 years. Rainfall for each month is the total monthly rainfall. Temperature for each month is the mean maximum daily temperature. Data were obtained from weather station 010536 (http://www.bom.gov.au/climate/).



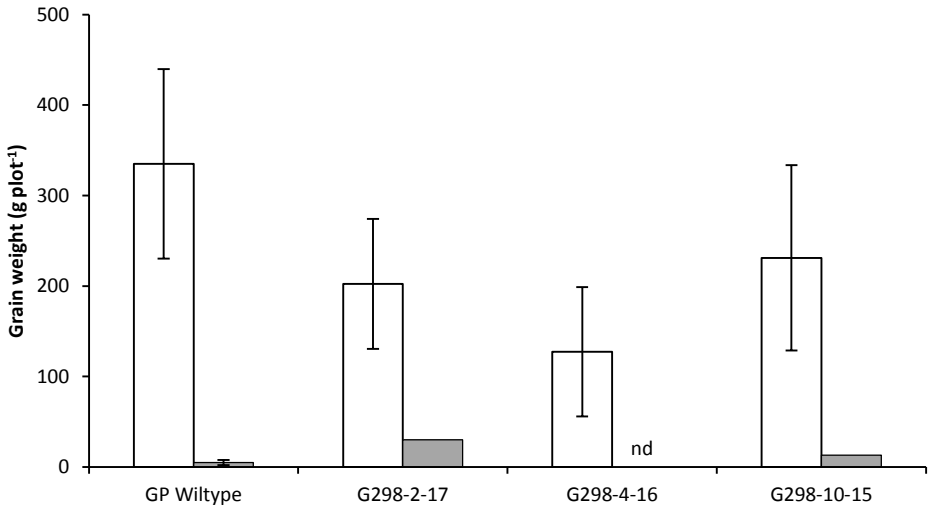
Supplementary Figure 2.5: Shoot biomass per plant of wildtype (cv. Golden Promise) and three independent transgenic *AtCIPK16* expressing barley grown in 2012 at Kunjin, Western Australia in low salt (EC_e 0 – 8 dS/m) (white bars) and in high salt (EC_e >8 dS/m) (grey bars). Values are mean ± s.e.m (n = 6 – 23). Significant differences (one-way ANOVA, Tukey-Kramer, $P \leq 0.05$) indicated by letters. nd = not determined.



Supplementary Figure 2.6: Na^+ (A) and K^+ (B) concentration (µmoles ion per g DW) of wildtype (cv. Golden Promise) and three independent transgenic *AtCIPK16* expressing barley grown in 2012 at Kunjin, Western Australia in low salt (EC_e 0 – 8 dS/m) (white bars) and in high salt (EC_e >8 dS/m) (grey bars). Values are mean ± s.e.m (n = 6 - 23). Significant differences (one-way ANOVA, Tukey-Kramer, $P \leq 0.05$) indicated by letters. nd = not determined.

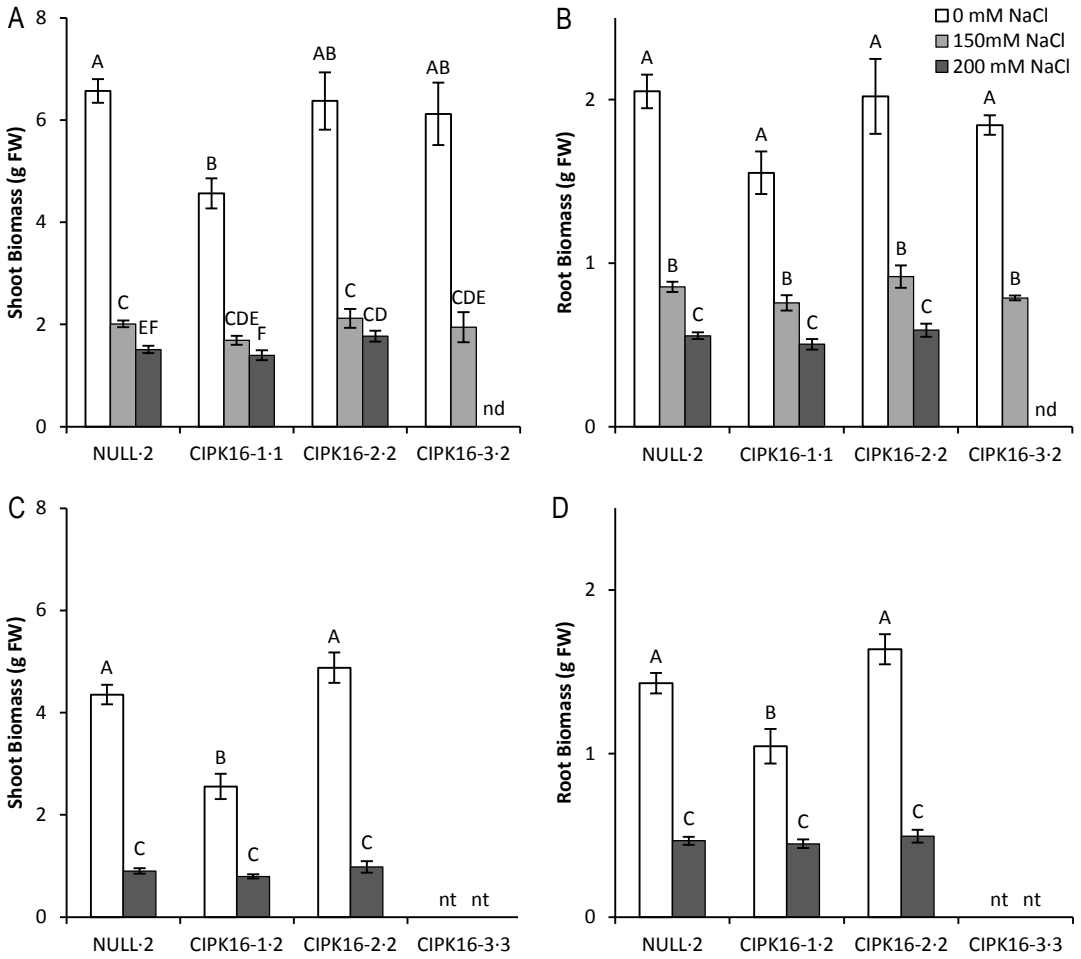


Supplementary Figure 2.7: Grain yield per plants parameters of wildtype and transgenic *AtCIPK16* expressing barley grown at Kunjin, Western Australia. Grain number (per plant) (A) and grain weight (per plant) (B) of wildtype (cv. Golden Promise) and three independent *AtCIPK16* expressing transgenic barley lines grown in 2012 in low salt (EC_e 0 – 8 dS/m) (white bars) and in high salt (EC_e >8 dS/m) (grey bars). Values are mean ± s.e.m (n = 6 – 23). Significant differences (one-way ANOVA, Tukey-Kramer, $P \leq 0.05$) indicated by letters. nd = not determined.



Supplementary Figure 2.8: Grain yield per plot for wildtype (cv. Golden Promise) and three transgenic *AtCIPK16* expressing barley lines grown in 2012 at Kunjin, Western Australia in low salt (EC_e 0 – 8 dS/m) (white bars) and in high salt (EC_e >8 dS/m) (grey bars). Values are mean ± s.e.m (n = 1 – 4). nd = not determined.

A



Supplementary Figure 3.1: Shoot and root biomass of null segregant and three transgenic *AtCIPK16* wheat lines grown in hydroponic experiments. Shoot biomass (FW) of null segregant and three independent *AtCIPK16* transgenic wheat (cv. Gladius) lines grown in hydroponics in A) experiment #2 (late spring 2014) and C) experiment #3 (winter 2015). Root biomass (FW) of null segregant and three independent *AtCIPK16* transgenic wheat (cv. Gladius) lines grown in hydroponics in B) experiment #2 (late spring 2014) and D) experiment #3 (winter 2015). Plants were grown under 0 mM NaCl (white bars), 150 mM NaCl (light grey bars) and 200 mM NaCl (dark grey bars) treatments. Values are mean ± s.e.m (n = 3 – 47). nd = not determined due to no transgenics identified in one of the treatments. nt = line is not transgenic based on genotyping. Significant differences (one-way ANOVA, Tukey-Kramer, $P \leq 0.05$) indicated by letters.

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