Cell type-specific manipulation of salt tolerance genes in wheat and barley

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Declaration

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“I love you to pieces

I dedicate to you, this thesis”

-Jorge Cham

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Conference posters

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List of Abbreviations

ABA	abscisic acid
ACPFG	Australian Centre for Plant Functional Genomics
At	*Arabidopsis thaliana*
AtAVP1	VP1 from *Arabidopsis thaliana*
ATP	adenosine triphosphate
AUD	Australian dollar
BLAST	Basic local alignment search tool
bp	base pairs
BSA	bovine serum albumin
CBL4	calcineurin B-like 4 protein
ccdB	cytotoxic coupled cell division
cDNA	complementary DNA
CIPK24	CBL-interacting protein kinase 24
CPA	cation/ proton antiporters
Cps	counts per second
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetraacetate
EST	expressed sequence tags
FACS	fluorescence-activated cell sorting
GAPdh	glyceraldehydes- 3- phosphate dehydrogenase
GAS	groundwater-associated salinity
GFP	Green fluorescent protein
GSS	genome survey sequence
GUS	β-glucuronidase
H^+*-ATPase*	proton translocating ATPase
H^+*-PPase*	proton translocating pyrophosphatase
HA	haemagglutinin

HKT	high- affinity K^+ transporter
Hv	*Hordeum vulgare*
HvHVP1	VP1 from *Hordeum vulgare*
Hyg	Hygromycin
KAc	Potassium acetate
LB	Luria Bertani
MCS	multiple cloning site
MgAc	Magnesium acetate
MPSS	Massively parallel signature sequence
NHX1	Na^+/H^+ exchanger
Nos	Nopaline synthase
Os	*Oryza sativa*
OsOVP1	VP1 from *Oryza sativa*
PEG	polyethylene glycol
PPi	pyrophosphate
PTGS	post-transcriptional gene silencing
qPCR	quantitative PCR
RNA	ribonucleic acid
RNAi	RNA interference
RO	reverse osmosis
PCR	reverse transcriptase PCR
S.E.M	standard error of the mean
SDS	sodium dodecyl sulphate
SKOR	stelar K^+ outward rectifying channel
SOS	salt overly sensitive
SSC	salt and sodium citrate solution
Ta	*Triticum aestivum*
TaCycl	gene encoding cyclophillin in *T.aestivum*
TaEFa	gene encoding elongation factor a in *T.aestivum*

TE	tris EDTA
TPM	transcripts per million
uidA	β-glucuronidase
USDA	U.S. Department of Agriculture
UTR	untranslated region
v/v	volume per volume
VP	vacuolar H^+ pyrophosphatase
w/v	weight per volume
WEA	Wheat Exports Australia

Abstract

More than 67% of Australian cropping land is at risk of becoming saline and agriculture is increasingly utilising salt effected land (Rengasamy, 2002). Salinity has a significant impact on crop yield, and the identification and manipulation of genes that help to ameliorate yield penalties resulting from salinity can enhance agricultural production.

Bread wheat, a hexaploid with AABBDD genome, has been long considered more salt tolerant than the tetraploid durum wheat with an AABB genome. The D genome, originally from *Aegilops tauschii,* contains a locus important for maintaining high K^+/Na^+, *Kna1,* on chromosome 4, which contains the *HKT1;5* gene encoding a Na^+ specific transporter, *TaHKT1;5-D.* The transcript of this gene was knocked down through RNAi. Plants containing the RNAi construct were found to accumulate higher levels of Na^+ in the 4th leaf regardless of whether they were grown under control or mild salt stress conditions (75mM). This result supports previous findings that orthologues of *HKT1;5* in other plants influence Na^+ translocation from root to shoot (Ren *et al.*, 2005; Davenport *et al.*, 2007). The impact of *TaHKT1;5* on salt tolerance was studied by subjecting transgenic plants to control or salt stress (75mM) conditions. Changes in phenotype were measured through non-destructive plant imaging (LemnaTec® Scanalyzer), but no phenotypic variation was observed as a result of the salt stress that was applied, suggesting the stress may have been too mild.

In parallel with the knockdown approach, the *HvHKT1;5* gene, an orthologue of the bread wheat Na^+ transporter (*TaHKT1;5-D*), and a barley inorganic proton pyrophosphatase, *HvHVP1*, were overexpressed in barley through use of promoters thought to control cell type-specific expression. Promoters were identified through an MPSS database search for genes with low to moderate transcript levels and specificity for root-cortex or root-stele. The promoters controlling these genes were then isolated to drive *HvHKT1;5* in root cortex and stele and *HvHVP1* in root cortex. Four promoters were found to be promising: two stelar-specific and two cortex-specific and were placed upstream of *HvHKT1;5* and *HvHVP1.* These constructs were then transformed into barley (cv. Golden Promise). Transgenic plants were grown in 100mM salt stress with two independent lines for each promoter:gene construct.

Independent lines which included a stelar-specific promoter controlling *HvHKT1;5* transcription showed reduced Na^+ accumulation and increased K^+ accumulation in 4th leaf xylem sap. Transgene mRNA was detected in both shoots and roots of the plant.

In conclusion, while lowering levels of *HKT1;5* transcript in wheat were not found to impact whole plant salinity tolerance, it did increase Na^+ accumulation in the shoot. This was supported by the results in barley where overexpression of *HvHKT1;5* resulted in lower Na^+ levels and a concomitant increase in K^+ levels in the shoot. Further study on whether this result impacts barley salt tolerance is currently underway.

1 General introduction

1.1 Soil salinity and its impacts on Agriculture with a focus on Australia

High soil salinity and in particular, sodium-salt induced salinity, is a prevalent form of mineral toxicity found in Australia and worldwide. Land naturally contains salts and minerals in the soil resulting from various geological and aeolian processes (Rengasamy, 2006). In Australian soil, sodium chloride, sodium carbonate and sodium bicarbonate are the predominant salts and a high representation of sodium in soil gives rise to soil sodicity which reduces soil porosity and increases soil strength, restricting soil-water and soil-air interactions (Rengasamy, 2006). Australia has a considerably high proportion of soil affected by salinity. While it is thought that the establishment of agriculture is to blame- and to a certain extent it is, Australia was already naturally high in salinity (Sexton and Corporation, 2003). The drainage of the rivers and creeks in Australia is mainly inland rather than out to sea and as a result much of the salt from the rain and carryover from the water flows accumulates on land (Beresford *et al.*, 2001; Sexton and Corporation, 2003). Another factor which compounds the problem is the aeolian deposit of up to 200 kg of salt per hectare in areas proximal to the coast and up to 20 kg of salt per hectare inland annually (Sexton and Corporation, 2003).

Australian crops are grown in dryland conditions on more than 12 million of the 20 million hectares of arable land (Rengasamy, 2002). Soil sodicity in dryland areas is a major determinant of the soil structure and a lot of water is retained at the surface subjecting much of it to evaporation; sodic soils in Australia, due to the presence of sodium carbonates, also have a high pH (Beresford *et al.*, 2001; Rengasamy, 2002). Dryland farming is carried out in areas that receive approximately between 250 and 600mm of rainfall annually and the salinity can measure up to 16 dS/m (Rengasamy, 2002; Rengasamy, 2006; Rengasamy, 2010). This level of salinity is four times the basal measurement of a saline soil which is 4 dS/m and can greatly affect crop survival (Munns and Sharp, 1993; Rengasamy, 2010).

Groundwater-associated salinity or GAS which has long been thought to be a common form of salinity is caused by groundwater bringing up salts in the process of rising to the surface when draining into surface water bodies or through transpiration. The salt being brought up to

the surface is either stored in the deeper layers of vadose or in the groundwater itself if it is present at shallow soil horizons. Native species with deep-roots generally keep the groundwater table at a stable level and prevent it from rising to the topsoil. When native vegetation is replaced with shallow rooted agricultural crops, the increased rate of evaporation and resulting capillary action of the groundwater increases and causes the water table to rise and consequently bring up the associated salts to the root zone. This type of salinity is thought to affect around 16% of arable land in Australia. However, recent studies (Rengasamy, 2002; Rengasamy, 2006) have reported that arable lands have the potential to also be affected by a type of non-groundwater associated salinity called transient salinity.

In soils where water permeability is low as a result of high concentrations of sodium, moisture accumulates in the subsoil and forms a temporary 'perched watertable'. Due to the solubility of salts in water, they accumulate in this saturated zone and when the moisture evaporates, the salts are concentrated in that zone. There is also salt naturally present in rainwater which accumulates in the saturated zone. This salinity, called transient salinity, combined with GAS has the potential to contribute to losses in the agricultural industry in the vicinity of AUD $ 1.3 billion dollars (Rengasamy, 2002; Rengasamy, 2006). Figure 1.1 illustrates the prevalence of soil salinity in Australian Soil. Salinity reduces yield in plants in two ways; through osmotic stress and salt-specific stress.

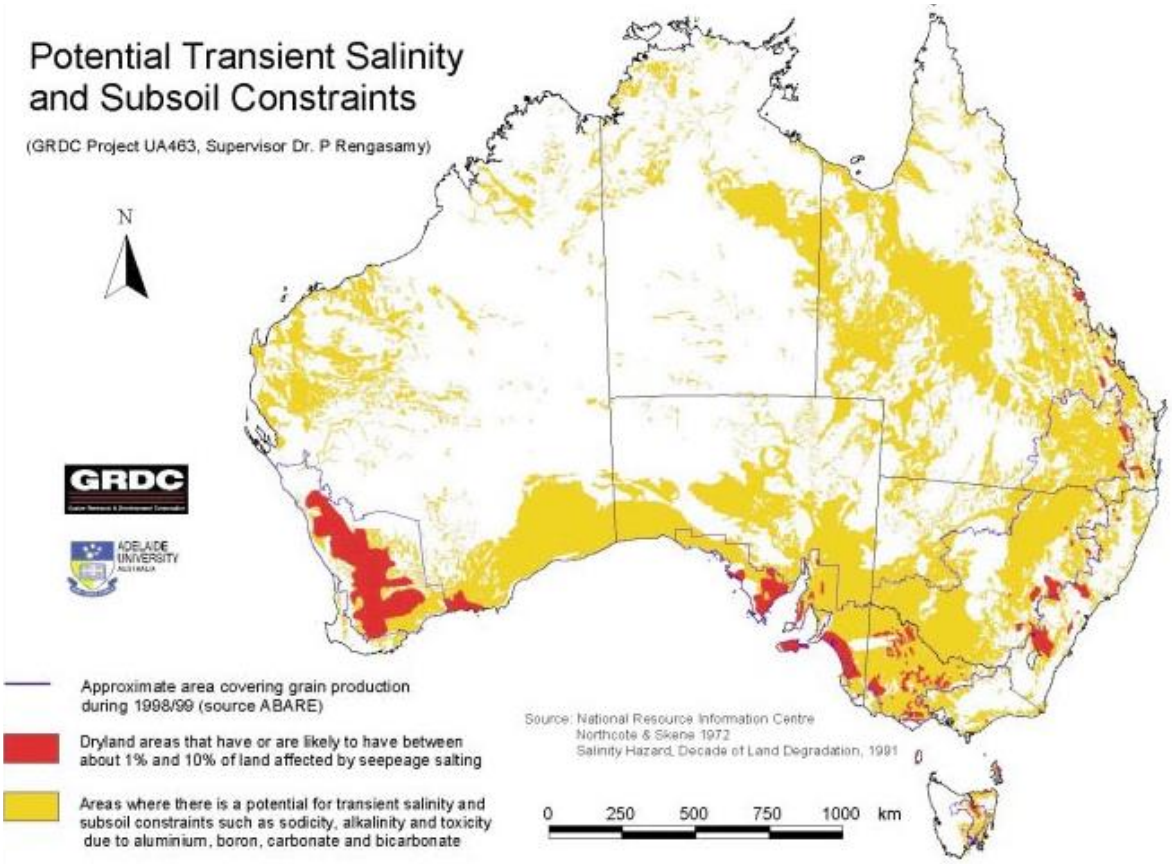


Figure 1.1: A map showing the prevalence of soil salinity in Australian Soil with groundwater associated salinity (GAS). *Areas affected by GAS are shown in red and areas with the potential for developing transient salinity are shown in yellow. Areas in which grain production occurs are outlined in black. Taken from (Rengasamy, 2002).*

1.2 Osmotic stress in plants

Water movement up the plant is restricted when there is a high amount of solute outside the plant (Munns and Termaat, 1986). We know this because osmotic adjustment is seen in glycophytic crop plants which increase their organic osmolytes within their system to maintain turgor and thus maintain normal growth rate (Turner *et al.*, 1986). This initial loss in turgor, although brief, is followed by reduction in shoot growth rate. This type of response is not only seen in plants exposed to sodium chloride (NaCl) but also plants growing in solutions containing other osmotica such as polyethylene glycol (PEG) and mannitol (Munns and Termaat, 1986; Yeo *et al.*, 1991; Sumer *et al.*, 2004). The reduction in leaf growth and thus area reduces the photosynthetic output of the plant. This occurs in conjunction with increased stomatal closure due to soil-water relations experienced by the roots (Davies *et al.*, 2005). A reduction in photosynthetic output in the plant reduces yield. Furthermore, it has been shown that hormonal signals such as abscisic acid and jasmonic acid, involved in both biotic and abiotic stress signalling, are also involved in cellular responses to saline environments (Munns, 2002). This reiterates the non-specific solute effect on plant growth (Munns, 2002). Some plants have osmotic stress tolerance and are able to maintain green leaves and growth rate in spite of the reduced water availability (Rajendran *et al.*, 2009). Upon long-term salinity stress, however, the plants progress past the osmotic stress phase and experience sodium salt-specific stress or ionic stress.

1.3 Ionic stress in plants

Salt-specific stress can be identified once senescence of older leaves increases following the reduced shoot growth resulting from an initial osmotic stress. Salt-induced damage can be a result of high salt levels in the cell that can interfere with enzymatic functions or decrease cellular integrity by accumulating in plant cell walls. Potassium (K^+), which is important for many enzymatic processes in plants treated with high levels of NaCl, decline in the xylem sap. This might be responsible for the stunted growth of shoots often observed in plants (although the converse has also been observed in some plants) after treatment with salt (Tester and Davenport, 2003; Munns, 2007).

Another change observed as a result of sodium (Na^+) accumulation in shoots is an increase in carbohydrate stores correlating with a decrease in carbohydrate metabolism implying that a long-term metabolic change could be the cause of stunted shoot growth. This has also been observed in water-stressed plants (Delane *et al.*, 1982; Byrt *et al.*, 2007; Munns, 2007). A reduction in photosynthesis has been observed in NaCl-treated plants. The relationship between NaCl-treated plants and reduced leaf growth is probably an indirect one. For example, NaCl-treated plants have increased leaf cell death and hence a proportional decrease in photosynthetic output can result. This may be due to the diverted supply of energy to the sequestering of excess salt in the vacuoles of the plant cells and as a result, photosynthetic rates decline. The declining photosynthetic processes might be responsible, in turn, for a reduction in shoot growth. Reduction in shoot growth combined with a concomitant reduction in photosynthetic activity affects overall plant productivity (Munns and Termaat, 1986).

The degree to which plants are affected by salt stress varies between different species. Some plants, called halophytes, are able to grow in higher salt conditions than glycophytes (salt-sensitive plants). Although enzymes from both plant types are equally sensitive to salt, halophytes are able to effectively 'exclude' Na^+ from the xylem and hence limit shoot Na^+ accumulation or sequester away salt into their vacuoles with the end result being the restriction of Na^+ interference from important plant metabolic processes (Munns and Tester, 2008).

1.4 How do plants cope with high salt levels?

Plants cope with salt-stress in a number of different ways. For most plants, including cereal crops, Na^+ is one of the most toxic ions. The degree of Na^+-specific effects on a plant depends on its ability to either 'exclude' the ion from the plant, or to 'tolerate' the Na^+ that does enter. In particular, the ability to exclude Na^+ from the shoot is important as it is the primary zone of Na^+ induced damage. Na^+- specific effects manifest differently varies between and within different plant species (Munns, 2002). This difference in salinity tolerance between plants is due to the variation in genetic profiles. It is these differences which can be exploited in the identification of key genes which may be responsible for phenotypic differences.

1.4.1 **Na^+ exclusion**

The cytoplasm of the plant cell is where Na^+ has its principal toxic effects, thus managing salt levels and preventing Na^+ from accumulating in the cytoplasm are important survival mechanisms in plants (Tester and Davenport, 2003). This is achieved by initially preventing the entry of salt into roots. Some halophytes, for example, have a Casparian band that acts as a physical barrier to Na^+ that is much wider than non-halophytes (Poljakoff-Mayber, 1975; Tester and Davenport, 2003). Na^+ exclusion from the xylem is also an important trait for limiting Na^+ accumulation in the shoot (Davenport *et al.*, 2007; Møller *et al.*, 2009; Munns *et al.*, 2012). Na^+ transporters play an important role in limiting shoot Na^+ accumulation by unloading excess Na^+ from the shoot xylem thereby decreasing the amount of Na^+ that is carried from the roots to the shoots in the transpiration stream (Davenport *et al.*, 2007).

Recirculation of the Na^+ from the shoot back to the root through the phloem is another strategy of decreasing Na^+ accumulation (Berthomieu *et al.*, 2003; Sunarpi *et al.*, 2005).There is very limited evidence, however, to suggest that phloem recirculation alone is sufficient to reduce Na^+ in shoot (Flowers and Yeo, 1986; Tester and Davenport, 2003; Sunarpi *et al.*, 2005). Studies have also outlined the importance of maintaining a high K^+/Na^+ ratio in plant cells. It has been suggested that a high K^+ concentration maintained in the cytosol increases the rate of Na^+ efflux from the cell by depolarising the cell membrane and increasing expression of non-selective cation channels which can facilitate the removal of cytosolic Na^+ (Obata *et al.*, 2007).

1.4.2 **Na^+ tolerance**

Limiting Na^+ translocation to the shoot is an effective strategy of excluding Na^+ from interfering with plant metabolic processes. When the Na^+ overwhelms the exclusion strategies of the plant and the rate of cellular Na^+ accumulation exceeds the rate of its expulsion, plants need to manage the Na^+ that eventually enters the cells. This can be achieved through vacuolar sequestration of Na^+ ions and limiting its presence in the cytoplasm where it can interfere with important metabolic processes (Figure 1.2). There are Na^+/H^+ exchangers found in the tonoplast membrane like *NHX1* and *NHX2* which facilitate the intravacuolar transport through the use of an H^+ gradient set up by proton pumps like vacuolar proton translocating

pyrophosphatase (*H^+-PPase*) and proton translocating ATPase (*H^+-ATPase*). The genes important in all these processes will be covered in the following sections of this chapter.

Figure 1.2 outlines the different transport strategies employed by plants to manage Na^+ that enters the plant.

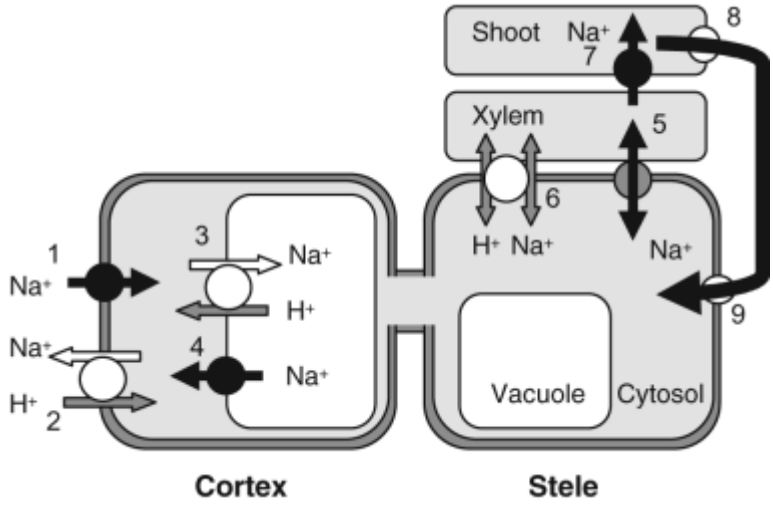


Figure 1.2: A schematic view of the different pathways of Na^+ fluxes and possible strategies employed by plants to limit net Na^+ accumulation in the shoot. *Under high salt conditions, Na^+ enters the root through non-selective cation channels (1). Transporters such as Na^+/H^+ antiporters can be used to pump the excess Na^+ back into the soil (2), Vacuolar compartmentalisation of Na^+ through tonoplast Na^+/H^+ antiporters can help increase salt-tolerance (3). Excess Na^+ can also passively reach the cytosol through non-selective channels (4) and this can be pumped out by mechanisms depicted in (2). The xylem part of the stele is loaded with Na^+ and this most likely occurs through passive mechanisms and this flow can be bi-directional (6). Unloading of xylem Na^+ into the shoot, is possible through passive mechanisms like non-selective cation channels (7) which can then potentially be recirculated back to stele through the phloem (8). Taken from Davenport et al. (2007)*

1.5 Genes important in controlling root to shoot translocation of Na^+

1.5.1 The exclusion mechanism

1.5.1.1 The high-affinity K^+ transporter (HKT) *gene family*

Na^+ predominates in the majority of the saline soils in the world (Rengasamy, 2006) and as it competes with K^+ for binding sites in enzymes, it is important to study the genes involved in Na^+ and K^+ homeostasis. Of particular interest to this study is the high affinity K^+ transporter (*HKT*) gene family. The *HKT* family is involved in high affinity Na^+ transport, high affinity K^+ transport and Na^+/K^+ symport (Platten *et al.*, 2006; Apse and Blumwald, 2007). The *HKT* gene family can be divided into two subfamilies, based on whether a serine (subfamily 1) or glycine (subfamily 2) is present at the first pore loop of the predicted protein (Platten *et al.*, 2006). All of the *HKT* members in subfamily 1 contain a serine residue as opposed to a glycine residue as found in subfamily 2, in the first pore loop of the predicted protein (Platten *et al.*, 2006). This serine or glycine residue substitution is most likely responsible for the high and low specificity for Na^+, respectively (Platten *et al.*, 2006; Cotsaftis *et al.*, 2012). Members of *HKT* subfamily 1 have been found in both eudicots and monocots but, to date, no subfamily 2 members have been found in eudicots; monocot species are the only ones that appear to have *HKT* subfamily 2 gene members (Platten *et al.*, 2006).

1.5.1.2 AtHKT1; 1 *and its role in salinity tolerance*

The single HKT member found in *Arabidopsis thaliana*, *AtHKT1;1* (Platten *et al.*, 2006) is involved in xylem unloading of Na^+ into the root (Davenport *et al.*, 2007). In studies involving *hkt1;1* mutants, it has been shown that not only does it affect unloading of Na^+ from the xylem, (Sunarpi *et al.*, 2005) but it also affects the rate of Na^+ uptake into vacuoles in root cells (Davenport *et al.*, 2007). The function of *AtHKT1;1* as a transporter that mediates the unloading of Na^+ from the xylem is further supported by evidence that the protein is localised to the plasma membrane of xylem parenchyma cells (Ren *et al.*, 2005; Sunarpi *et al.*, 2005). It has been shown by promoter-*GUS* fusion studies that there also appears to be some localisation in the phloem. It is thought, however, that the *HKT1;1* gene plays a relatively minor role in the phloem (Berthomieu *et al.*, 2003; Davenport *et al.*, 2007). The orthologue of *AtHKT1;1* in cereals is *HKT1;5* and this gene also functions to unload xylem Na^+ into the roots (Byrt *et al.*, 2007; Gao *et al.*, 2007). *HKT1;5*-like genes have also been identified in

wheat, rice and barley and might perform similar functions to *AtHKT1;1* (Byrt *et al.*, 2007). Figure 1.2 shows possible strategies which can be employed by a plant to limit Na^+ accumulation in the shoot, including the employment of transporters such as *HKT1;5*.

1.5.1.3 **HKT1;5** *in barley and wheat and its role in salinity tolerance*

Nine *HKT* gene members in bread wheat and two in barley, the plants of interest to this study, have so far been reported (Huang *et al.*, 2008). Of the *HKT* genes studied in wheat, as in other cereals, *HKT1;5* has been the most characterised. Recently a study by Munns *et al.* (2012) has demonstrated its importance in a durum wheat line, line 149. Durum wheat does not appear to contain a native *HKT1;5* member that is active and line 149 described in Munns *et al.* (2000) and Munns *et al.* (2012) had a *Triticum monococcum HKT1;5* member (*TmHKT1;5-A*) introgressed into the genome. The durum wheat line, 149, was found to have 25% increased grain yield compared with near isogenic lines as a result of this gene being present (Munns *et al.*, 2012). This member also bears high similarity to the D genome member, *TaHKT1;5-D*, found in bread wheat thought to have originated from *Aegilops tauschii* (Byrt *et al.*, 2007) which will be characterised in this study. Both genes are reported to work by limiting Na^+ translocation to the shoot through xylem unloading in the root as in the orthologues in rice and *A. thaliana* (Ren *et al.*, 2005; Byrt *et al.*, 2007; Davenport *et al.*, 2007; Munns *et al.*, 2012).

Transgenic studies involving the knockout of *AtHKT1;1* in *A. thaliana* resulted in plants with increased salt sensitivity (Mäser *et al.*, 2002; Rus *et al.*, 2004) but interestingly constitutive expression of *AtHKT1;1* under the control of a 35S promoter also rendered a similar result (Møller *et al.*, 2009). The latter finding alluded to the importance of spatial localisation of gene expression which was elucidated by Møller *et al.* (2009) who showed that cell type-specific overexpression of *AtHKT1;1* in a similar pattern to its native function does enhance salinity tolerance in *A. thaliana*. *HKT1;5*, therefore, is an ideal gene to be manipulated to enhance salt tolerance of crop plants. Studies have already shown that overexpression of *AtHKT1;1* under tissue-specific promoters can increase salt tolerance of the transgenic plants (Møller *et al.*, 2009; Plett *et al.*, 2010). Stelar- and pericycle-specific expression of *AtHKT1;1* resulted in reduced Na^+ translocation to the shoot as a result of increased Na^+ unloading from

the transpiration stream. Plett *et al.* (2010) uncovered an interesting mode of action of *AtHKT1;1* overexpressed in root cortex and epidermis of *A. thaliana* and rice. These plants also had low shoot Na^+ as reported in (Møller *et al.*, 2009). It was suggested that this reduction in shoot Na^+ was caused by the transgenes pleiotropically increasing endogenous *AtHKT1;1/OsHKT1;5* levels and therefore increasing Na^+ unloading back into the root. The mechanisms by which this occurred was suggested to be through RNA-mediated signalling but this is yet to be confirmed.

A successful demonstration of increasing salt tolerance of a cereal through cell type-specific overexpression of *AtHKT1;1* has been documented and achieved by Plett *et al.* (2010).

1.5.1.4 Salt overly sensitive (SOS) pathway

The salt overly sensitive (SOS) pathway is of particular interest to this project as the removal of Na^+ , to the external medium, from the root tissues that has been unloaded by *HKT1;5* is most likely through a Na^+/H^+ antiporter that is located on the plasma membrane. The SOS1 protein, is an Na^+/H^+ antiporter and is part of a larger superfamily of cation proton antiporters (CPA) which is subdivided into three families (Brett *et al.*, 2005). The *SOS1* gene is part of the CPA1 family that has its origins in prokaryotes (Brett *et al.*, 2005). It is the 7th member of the *NHX* family of genes which is comprised of eight genes. *NHX 1→4* are found in the vacuole, *5* and *6* in the endosomal sections and *7* and *8* found in the plasma membrane. *NHX8* has been proposed to transport Li^+/H^+ (An *et al.*, 2007).

The *SOS1* gene is induced by salt stress and is expressed in the vasculature throughout the plant and in the epidermis of the root tip (Shi *et al.*, 2000). This gene induction is regulated by a complex formed by *SOS3*, also known as calcineurin B-like 4 protein (CBL4) and SOS2, also known as CBL-interacting protein kinase (CIPK24). The *CBL4* member in *A. thaliana*, *AtCBL4*, has been found to transduce Ca^{2+} signals induced by NaCl stress (Shi *et al.*, 2000). Upon sensing the Ca^{2+} signal induced by salt stress, SOS3 then activates and recruits SOS2 (Halfter *et al.*, 2000) which, in turn, activates SOS1 function through phosphorylation (Qiu *et*

al., 2002). The SOS1 protein is mainly expressed in the roots and it is possible that it works in conjunction with HKT1;5 to shunt Na^+ from the roots of the plant.

With both *SOS1* and *AtHKT1;1/HKT1;5* being expressed in the vasculature, polar localisation of both *HKT1;5* and *SOS1* on opposite sides of the cell can envisage a cooperative model in which these genes might operate. *HKT1;5* can be expressed on the proximal side which unloads Na^+ from the transpiration stream. This 'unloaded' Na^+ can be moved to outer root layers by SOS1 which might be localised on the distal side of the cell facing the root epidermal layers. This speculation is borne out of the findings in radial boron transport in *A. thaliana* reported by Takano *et al.* (2010) where preferential localisation of a boron influx and an efflux proteins to the distal and proximal regions of the root cell, respectively, was reported. This cooperative model cannot be applied to cell layers beyond the vasculature as that is the one region that both SOS1 and AtHKT1;1/HKT1;5 are co-located. While it is known that Na^+ is eventually extruded to the external medium, with SOS1 presumably playing a major role, it is not entirely clear how this occurs. The findings by Rus *et al.* (2001) who reported a root growth inhibition in *sos1* plants that is then subsequently ameliorated in *sos1*, *hkt1;1* double knockout plants lends support to this theory. The reason why a knockout of *HKT1;1* in *sos1* mutants ameliorated the growth reduction that was observed in the single sos1 mutant lines was probably a result of Na^+ hyperaccumulation. The hyperaccumulation of Na^+ in roots would have been a result of Na^+ removal from the transpiration stream by HKT1;1 but no expulsion to the external medium as a result of a defective SOS pathway (Rus *et al.*, 2001; Davenport *et al.*, 2007).

This forms the basis for increasing the Na^+ exclusion in barley through overexpression of *HvHKT1;5* in specific root cell-types. It is hypothesised that by increasing the rate of unloading of Na^+ from the transpiration stream will enable the shunting of Na^+ ions to the external medium by Na^+/H^+ antiporters like SOS1 and thereby limit Na^+ translocation to the shoot.

1.5.2 **Tissue tolerance mechanism**

1.5.2.1 Vacuolar H^+ translocating pyrophosphatase (H^+-PPase)

The vacuolar H^+ translocating pyrophosphatase (H^+-PPase)or VP is found in all 3 domains- archaea, bacteria and eukaryota although is not necessarily found in all organisms in these domains (Drozdowicz and Rea, 2001).

The vacuolar *H^+-PPase* is of high importance to plants where it is expressed in the tonoplast membrane and operates to clear the pyrophosphate (PPi) generated by plant metabolism (Ferjani *et al.,* 2011). Along with the vacuolar H^+-ATPase which uses ATP to pump H^+ across the tonoplast, the vacuolar H^+-PPase also functions in the acidification of the vacuole.

There are two types of inorganic pyrophosphatases, type 1 and 2. Type 1 pyrophosphatases are dependent on K^+ and are reversibly inhibited by Ca^{2+}. Type 2 pyrophosphatases are K^+ insensitive but sensitive to Ca^{2+} (Drozdowicz and Rea, 2001).

The pyrophosphatase of importance to this study is a type 1 H^+-PPase. The relevance of H^+-PPase to salt tolerance, or drought tolerance for that matter, is related to its ability to generate a H^+ gradient in vacuoles that can be exploited by Na^+/H^+ antiporters like NHX1 to sequester Na^+ or K^+ in the vacuole (Blumwald, 1987; Gaxiola *et al.*, 2001a). Sequestration of Na^+ in the vacuole benefits the plant two fold- the limiting of Na^+ presence in the cytoplasm thereby preventing it from interfering with important metabolic processes and the sequestered Na^+ also decreases the cell's overall water potential and acts as a cheap osmoticum. This is supported by studies in plants that have overexpressed the vacuolar H^+-PPase and have reported an increase in solute accumulation in plant tissues accompanied with an increase in salt and drought tolerance (Gaxiola *et al.*, 2001b; Brini *et al.*, 2007; Bao *et al.*, 2009; Pasapula *et al.*, 2011; Zhang *et al.*, 2011).

H^+-PPase is also crucial to plant growth (Li *et al.*, 2005; Ferjani *et al.*, 2011). Plants defective in *AVP1* (an *A. thaliana* member of H^+-PPase) show severe growth defects and do not

develop properly. Concomitantly, plants overexpressing *AVP1* were found to have more vigorous growth compared to wildtype plants and this was initially attributed to the protein's ability to acidify the vacuole and causing acid leakage which resulted in auxin-related growth (Li *et al.*, 2005). This suggestion has recently been challenged by Ferjani *et al.* (2011) who have shown that PPi removal is a more critical role of H^+-PPase as far as normal plant growth is concerned. Ferjani *et al.* (2011) also used *DR5:GUS* lines that were defective in *AVP1*. *DR5* is an auxin responsive element and activates any genes downstream like *uidA* (GUS) as in the case of this study which can then be visualised (Ulmasov *et al.*, 1997). GUS patterns were compared between plants with a defective *AVP1* and wildtype plants and no differences in auxin signalling was observed as was claimed in Li *et al.* (2005). It has been proposed that PPi removal is actually important for gluconeogenesis based on the findings that plants with defective *AVP1* were lacking in sucrose by 50% compared to wildtype and were subsequently rescued by supplementing the growth media with sucrose (Ferjani *et al.*, 2011).

Additionally, *avp1* mutants in Ferjani *et al.* (2011) had the same growth defects reported in Li *et al.* (2005) ameliorated through transformation with a yeast pyrophosphatase lacking the proton pumping activity. This highlights the importance of the H^+PPase as a pyrophosphatase more than an H^+ pump though slight alkalisation of the vacuole was reported in the *avp1* mutants when compared with wildtype.

Thus, pyrophosphatase does contribute to vacuolar acidification and facilitates sequestration of Na^+ (or K^+). Findings by Gaxiola *et al.* (2001a), D'yakova *et al.* (2006), Gao *et al.* (2006), Bao *et al.* (2009) and Zhang *et al.* (2011), just to name a few, clearly demonstrate increased solute accumulation in plant tissues as a result of the overexpression of the vacuolar *H^+-PPase*.

Plant H^+-PPases of vacuolar, Golgi and plasma membranes share approximately 85% similarity in terms of their amino acid sequences (Sakakibara *et al.*, 1996). This indicates a high level of sequence conservation amongst the proteins across plant species.

There are two types of vacuolar pyrophosphatases in barley; the *HVP1* and *HVP10*. Both function to facilitate the sequestration of Na^+ in the vacuole and are differentiated functionally by their spatial expression patterns (Fukuda *et al.*, 2004; Shavrukov *et al.*, 2013). While both genes are found in both shoots and roots, *HVP1* appears to be preferentially expressed in the shoots and *HVP10* in the roots of barley (Fukuda *et al.*, 2004; Shavrukov *et al.*, 2013). *HVP1* expression is also induced in response to osmotic stress although not to the same extent as salt stress whereas *HVP10* gene expression seems to respond to salt stress only (Fukuda *et al.*, 2004; Shavrukov *et al.*, 2013). It has been conjectured by Shavrukov *et al.* (2013) that, as a result of the reciprocal expression patterns, these two genes might play reciprocal roles in the plant. *HVP10*, a gene candidate in the locus *HvNax3* identified as co-segregating with reduced Na^+ in the shoot of barley plants has been suggested as facilitating Na^+ accumulation in the roots thereby reducing Na^+ translocation to the shoot.

The *HVP* gene of interest to this study is the *HVP1*. HVP1 enzyme activity was also increased (Fukuda *et al.*, 2004) as was seen with HvNHX1 (Fukuda *et al.*, 1998) in response to salt stress. It is therefore possible that HvHVP1 and HvNHX1 work in a coordinated fashion and an increase in HVP1 activity, through its overexpression, can be hypothesised to increase salt tolerance as reported in other plant species. This gene will be overexpressed in barley root cortex tissues. An increase in pyrophosphatase would better facilitate the endogenous HvNHX1 activity in this study. An increase in vacuolar sequestration of Na^+ as a result of *HvHVP1* overexpression in plant roots would mean translocation of Na^+ to shoot would be limited thereby maintaining shoot growth as supported by the previously discussed findings of Shavrukov *et al.* (2013) on *HVP10*.

1.5.2.2 Na^+/H^+ antiporter (*NHX1*)

The H^+ gradient established by the pyrophosphatase is crucial for the Na^+/H^+ transport activities of NHX1 which works in a coordinated fashion with the H^+-PPase.

As mentioned previously, the *NHX1* gene is part of a larger superfamily of cation proton antiporters (CPA) which is subdivided into three families (Brett *et al.*, 2005). *NHX1* is part of

the CPA1 subfamily that has its origins in prokaryotes (Brett *et al.*, 2005). There have been eight *NHX* members identified in *A. thaliana*. *NHX 1→4* are found in the vacuole, *5* and *6* in the endosomal sections and *7* and *8* found in the plasma membrane. *NHX7* is also known as *SOS1,* another important salt tolerance determinant (Brett *et al.*, 2005) which will briefly be covered later in this section and *NHX8* has been proposed to transport Li^+/H^+ (An *et al.*, 2007).

The *NHX* members of importance to this project are *1* and *2* that have been shown to be functionally redundant in *A. thaliana* (Bassil *et al.*, 2011; Barragán *et al.*, 2012). Support for these NHX transporters having a critical role in plant K^+ nutrition has recently emerged as double knockout mutants of *NHX1* and *2* in *A. thaliana* result in plants with poor growth and low K^+ and water content (Bassil *et al.*, 2011; Barragán *et al.*, 2012). Under salt stress conditions, however, where *AtNHX1* has been overexpressed Na^+ transport into vacuoles has been reported (Apse *et al.*, 1999).

NHX1 functions by exploiting a H^+ gradient set up within the vacuole by a H^+ pump (H-PPase or H^+-ATPase) (Callow *et al.*, 1997). NHX1 then transports K^+ (or Na^+ under salt stress) into the vacuole (Apse *et al.*, 1999; Bassil *et al.*, 2011; Barragán *et al.*, 2012; Bassil *et al.*, 2012). The importance of NHX1 (or NHX2 for that matter) in a plant's Na^+ tolerance is borne out of its ability to sequester Na^+ into the vacuole where it can function as a cheap osmoticum and also be kept away from the cytoplasm where it can interfere with key metabolic processes.

The *NHX* member in barley, *HvNHX1,* that has been studied by Fukuda *et al.* (2004) was shown to rescue an *nhx1* yeast mutant. Osmotic stress in barley plants also induced expression of *NHX1* indicating that the gene is possibly involved in the maintenance of turgor pressure (Fukuda *et al.*, 2004) as suggested for *AtNHX1* (Barragán *et al.*, 2012).

The NHX transporters are of particular interest to this project as one of the aims of the project is to enhance salt tolerance of barley plants by overexpression of *HvHVP1*, a barley vacuolar H^+-PPase. The overexpression of this gene should result in acidification of the vacuole; this can then be exploited by NHX proteins that can sequester excess Na^+ from the cytoplasm into the vacuole.

1.6 The role of different cell types in a plant's salinity tolerance

A plant's ability to manage Na^+ levels internally is a sum of different cell type-specific processes. Tissue types which act as barriers to solute entry are a clear example of cell specialisation. Suberisation of the exodermis in rice and cotton to form a Casparian strip, in response to external salt, is an example of how cells can act as a deterrent to Na^+ entry into plants (Reinhardt and Rost, 1995; Cai *et al.*, 2011). In fact, in the study by Cai *et al.* (2011), it was found that the formation of a Casparian strip in the exodermis was more essential to a plant's salinity tolerance than that in the endodermis. This highlights the requirement of specialised cell types for controlling Na^+ entry into a plant. Additionally, some genes for which their function in salt tolerance is dependent on their expression in specific cell-types have already been mentioned in this chapter (*HKT1;1, SOS1, HVP10*). The importance of the spatial control has been exemplified by the findings in Møller *et al.* (2009) whereby constitutive expression of *HKT1;1* in *A.thaliana* increased shoot Na^+ accumulation. The study by Plett *et al.* (2010) supported this finding with a caveat that the same cell types might not be appropriate for all organisms. Plett *et al.* (2010) reported that expression of *AtHKT1;1* in stelar cells of rice actually increased shoot Na^+ accumulation and that expression of *AtHKT1;1* in cortex cells was better suited for increasing Na^+ tolerance in rice. This discrepancy was suggested to be a result of rice having different cell types and, by extension, different processes important in regulating Na^+ influx to *A. thaliana* (Plett *et al.*, 2010). Also, cell type-specific expression of *AtAVP1* in the root cortex and epidermis of *A. thaliana* reduced shoot Na^+ accumulation (EL-Hussieny, 2006) demonstrating that manipulation of a gene which might be more constitutively expressed in its native form can result in reduced shoot Na^+. The result from EL-Hussieny (2006) is further supported by the study by Shavrukov *et al.* (2013) who suggest that Na^+ is reduced in the barley shoot as a result of increased *HVP10* in plant roots in response to salt stress. This highlights the importance of

cell types crucial for managing Na^+ levels within a plant and forms the basis for the expression of salt tolerance genes in a cell type-specific manner.

1.7 Increasing salt tolerance of plants

While attempts are being made in conventional breeding to exploit wild relatives' genetic resources and have been successful at improving salinity tolerance (Munns *et al.*, 2012), there is generally little natural variation amongst major crop varieties that can be utilised for gene transfer into commercial varieties (Colmer *et al.*, 2005; Pardo, 2010). Moreover, inherent in plant breeding programs may be complications with linkage drag that have to be bred out of a population (Flowers, 2004). This compounded with the unpredictability of conventional breeding as a result of independent assortment and homologous recombination make introgression of salt tolerant genes into commercial crops a long and cumbersome process (Pardo, 2010).

The identification of genes important for plant salt tolerance as described in the previous sections in this chapter avails an opportunity for exploiting these genes through biotechnology to enhance crop salt tolerance. With the increasing availability of sequenced genomes thanks to next generation sequencing methods, identification of crucial genes has been accelerated and is ever increasing. Mutant studies with the aid of sequenced genomes have also enabled more effective characterisation of gene function. Many studies have outlined the importance of spatial control of gene expression as a result of their function (Rus *et al.*, 2001; Sunarpi *et al.*, 2005; Davenport *et al.*, 2007; Møller *et al.*, 2009; Plett *et al.*, 2010).

This study will build on these studies with an aim to overexpress salt tolerance genes in a cell type-specific manner in barley. The genes of interest to this study are the barley orthologue of the relatively well-characterised *AtHKT1;1* from *A. thaliana*, *HvHKT1;5* and *HvHVP1*, the barley orthologue of *AtAVP1* from *A.thaliana*.

1.7.1 **Strategy for increasing salt tolerance employed by this study**

The overall aim of this research study is to increase salt tolerance in barley plants through enhancing the Na^+ exclusion mechanism. It is expected that by limiting Na^+ translocation to the shoot and thereby reducing shoot Na^+ accumulation, plants will maintain normal/adequate growth under salt stress conditions.

1.7.1.1 Aim- Enhance Na+ exclusion through the unloading mechanism through cell type-specific overexpression of **HvHKT1;5**

The expression of *HvHKT1;5* will be restricted to the root-stele based on studies that have shown that native expression of the orthologues in *A. thaliana* and wheat are in stele and this is beneficial to the plant's whole salinity tolerance (Sunarpi *et al.*, 2005; Davenport *et al.*, 2007; Møller *et al.*, 2009; Munns *et al.*, 2012). *HvHKT1;5* will also be expressed in root-cortex as Plett *et al.* (2010) found that expression of the *A. thaliana AtHKT1;1* in the root-cortex of rice pleiotropically increased the endogenous *OsHKT1;5* levels and, thereby, increased Na^+ exclusion in the shoot. Additionally, based on the increased Na+ accumulation that was observed in rice plants overexpressing *AtHKT1;1* in stelar cells (Plett *et al.*, 2010), it would be prudent to include a subset of plants overexpressing *HvHKT1;5* in a cortex-specific manner. Cell type-specific overexpression will be achieved through identification and isolation of putative stelar and cortex-specific promoters.

1.7.1.2 Aim- Study the importance of **HKT1;5** *for salt tolerance in bread wheat through employing a gene knockdown approach through RNAi*

The overexpression study of *HvHKT1;5* to increase salt tolerance through enhancing the Na^+ exclusion capacity of barley will be complemented by studying the effects of lower gene expression through RNAi. This will be performed in bread wheat plants which contain an important Na^+ exclusion locus, *Kna1*, containing a *HKT1;5* member; *TaHKT1;5-D*. The plants will contain an RNAi construct targeting the *TaHKT1;5-D* member. Changes in salinity tolerance of the RNAi containing wheat plants will be studied in comparison to control null segregant lines by assessing physiological indicators (changes in shoot Na^+/K^+) and growth changes (biomass accumulation and growth rate).

1.7.1.3 *Aim- Enhance Na+ exclusion through the tissue tolerance mechanism through cell type-specific overexpression of* HvHVP1

Overexpression of *HvHVP1* which helps facilitate the vacuolar sequestration of Na^+ by cation/proton antiporters like HvNHX1 in the root cortex will enable storage in vacuoles in root cells. Increased storage of Na^+ in root cells will, in turn, reduce Na^+ transport to the shoots thereby effectively 'excluding' Na^+ from the shoot. Plus, given the growth enabling effects of H^+*-PPase* overexpression (Li *et al.*, 2005; Ferjani *et al.*, 2011), it is possible that root growth could be increased and a more extensive root network, in turn, could help increase water uptake. Enhanced pyrophosphatase activity in the root cells has been demonstrated to reduce shoot Na^+ accumulation where overexpression of *AtAVP1* in A. thaliana root cortex and epidermal cells resulted in significantly lower Na^+ levels in the shoot (EL-Hussieny, 2006). This is further supported by the new findings of Shavrukov *et al.* (2013) who suggested that *HVP10* that is preferentially expressed in root cells helped maintain low shoot Na^+ by facilitating root vacuolar sequestration of Na^+. Cell type-specific overexpression will be achieved through identification and isolation of putative cortex-specific promoters.

1.8 Thesis outline

Chapter 2 will detail the identification of promoters which control gene expression in root-cortex and root-stele. These promoters will be isolated to drive expression of *HvHKT1;5* and *HvHVP1* in root cortex/stele and root-cortex, respectively in barley plants. The spatial control of the putative promoters will be studied through reporter gene assays using green fluorescent protein (*GFP)* and *uidA* (β-glucuronidase or GUS).

Chapter 3 will detail the phenotypes of transformed barley plants containing constructs of the putative promoters identified in chapter 3 driving the expression of *HvHKT1;5* and *HvHVP1*.

Chapter 4 will detail the phenotypic studies of bread wheat plants containing an RNAi construct targeting the wheat *HKT1;5* member, *TaHKT1;5-D*. Correlation between lowered gene expression levels, Na^+ accumulation in the shoots and changes in biomass accumulation

will be detailed. The aim of this study was to ascertain the importance of *TaHKT1;5-D* as a salt tolerance determinant. It will also address the question of whether *TaHKT1;5-D* is the gene in the large unresolved locus, *Kna1*, responsible for the lowering of shoot Na^+ levels observed in plants containing this locus.

Finally, chapter 5 will summarise the results of this research and discuss the importance of the various salt tolerance strategies employed by cereals and discuss future prospects of the data generated in this study.

2 Identification and isolation of putative root cortex- and stelar-specific promoters from maize and rice

2.1 Introduction

The salt tolerance of plants has been enhanced by overexpressing key genes involved in whole plant salt tolerance (Apse *et al.*, 1999; Gaxiola *et al.*, 2001a; Shi *et al.*, 2003; Møller *et al.*, 2009; Plett *et al.*, 2010). Many of these studies have expressed salt tolerance genes constitutively and ubiquitously (Apse *et al.*, 1999; Gaxiola *et al.*, 2001a; Shi *et al.*, 2003). However, some studies have shown that when genetically manipulating processes involving Na^+ transport, constitutive overexpression is not the ideal means to achieve increased salinity tolerance, and in some cases it has been shown to increase salt sensitivity (Rus *et al.*, 2004; Møller *et al.*, 2009; Plett *et al.*, 2010). As a solution, the overexpression of genes in specific cell-types is an emerging strategy to enhance whole plant salinity tolerance (Møller *et al.*, 2009; Plett *et al.*, 2010). The control of gene expression in specific cell-types is commonly determined by the promoter used to drive the genes of interest. Thus, promoter discovery is an important initial step in the development of plants expressing transgenes in a tissue-specific manner.

Recent developments in gene and enhancer trapping have enabled identification of various tissue-specific promoters in *A. thaliana* and rice for use in the genetic modification of plants (James, 1998; Johnson *et al.*, 2005). Building on this, the use of a promoter that controls not only spatial or temporal specific expression, but also particular levels of expression (low, medium, high) would be highly desirable.

A key aim of this project was to identify promoters that have activity only in the root-stelar and cortex tissues to drive specific expression of the salt tolerance genes, *HvHKT1;5* and *HvHVP1,* in barley. While the candidate promoters were selected for tissue-specificity, care was also taken to select for low to medium levels of expression; this was done to ensure that ectopic expression of *HvHKT1;5* and *HvHVP1* were not so high as to result in any growth/metabolic penalties which can result with certain genes (Tarczynski *et al.*, 1992; Karakas *et al.*, 1997).

Identification of root-stelar and cortex promoters was achieved through mining a massively parallel signature sequencing (MPSS) database generated by DuPont Pioneer which contained expression libraries from root-cortex and root-stele of B73 maize. The MPSS method involves processing of mRNAs in a sample to generate 16-20 bp reads referred to as signature tags. These signature tags are detected in a sample at levels of transcripts per million (TPM) (Brenner *et al.*, 2000). An MPSS candidate approach was adopted for this project as the database generated by DuPont was the only database known at the time to contain specific gene expression information pertaining to root-cortex and root-stele in cereals. Following identification of a maize MPSS candidate, orthologues were identified in rice and promoters upstream of the orthologues in rice were isolated for use in this project. Barley promoters would have been preferred but identification of orthologous tissue-specific promoters in barley was not possible due to the incomplete nature of the genome sequence at the time. As patterns of regulation may vary between barley and rice or maize, promoter- reporter fusions were generated in barley in order to study the activity of the promoters.

In this chapter, the process of promoter identification, development of transformation constructs, and assays of plants containing constructs with promoter and reporter gene fusions will be described in detail.

2.2 Materials and methods

Initially, gene candidates that had specificity to root-cortex or root-stele were identified through searching maize MPSS data. Candidates which were found to be specific for either root-cortex or root-stele and that also had low-to-moderate levels of transcript abundance were selected for subsequent analysis.

2.2.1 MPSS database searches

The DuPont-Pioneer MPSS database contained various gene expression libraries from different maize tissues isolated from plants grown in different treatments. An initial search for candidates involved mining a root-cortex and root-stelar expression library generated from 10 day old B73 maize seedlings (kindly performed by Dr. Andrew J. Harvey, Dept of Genetics

and Bioengineering, Yeditepe University, Turkey). Both the cortex and stelar-specific libraries contained two subsets, each of which had been grown under control conditions or treated for 4 hours with 5 mM nitrate. Cortex-specific and stelar-specific candidates were identified initially on the basis that expression patterns were mutually exclusive, i.e. cortex-specific candidates were not present in the stelar-specific library and vice versa. Candidates were also selected on the basis of their transcript levels of expression being low to moderate as established at 100-1000 TPM (Dr. Andrew J. Harvey, Dept of Genetics and Bioengineering, Yeditepe University, Turkey pers. comm. 2007). MPSS candidates were rejected if there were differences in transcript level between the two different nitrate conditions (0 mM and 5 mM nitrate). This was to ensure that the promoters which would ultimately be isolated upstream of these genes or their orthologues were not affected by nitrate availability, and that downstream gene expression was consistent. Likewise, a search against drought-treated and well-watered root libraries was performed. The drought treatment library and the nitrate treatment libraries were the only two libraries that had transcript data pertaining specifically to maize roots; consequently, only these libraries were used for comparison with root-stele and root-cortex libraries.

The candidates which were retrieved from the root cortex and stelar libraries were searched against all other MPSS maize tissue libraries. Apart from ensuring tissue-specificity, this step was to ensure that there was no transcript measured in critical developmental tissues such as the embryo, endosperm or seed, or in any reproductive tissues. Elimination of MPSS candidates found in the developing/reproductive tissues was to prevent isolation of corresponding promoters that would also induce expression of downstream genes in those tissues. Ectopic gene expression in those tissues could have deleterious effects and/or reduce the yield of the plant.

2.2.1.1 MPSS candidate naming system

After selection on the basis of transcript levels (100-1000 TPM), MPSS candidates were placed in a spreadsheet and given numbers in the order of appearance - C1 →CX for cortex, and for stele, S1 →SX. This nomenclature will be used when referring to the MPSS candidates and their corresponding EST sequences.

2.2.2 **EST sequence retrieval and primer design for semi-quantitative PCR**

Signature tags, selected from the MPSS database, were used to retrieve maize expressed sequence tags (EST), or maize gene sequences from the website PlantGDB.org (Dong *et al.*, 2004) using the basic local alignment search tool (BLAST). Respective EST sequences were then used to design primers for testing their tissue specificity through PCR. Primers were designed by inputting EST sequences into Primer3 (Rozen and Skaletsky, 1999) to generate candidate primers which were analysed using NetPrimer (Premier Biosoft International). Default parameters of both online primer design software platforms were employed to generate primer sequences. Where an EST could not be found in public databases, the DuPont EST database was searched and the corresponding gene identified for design of primers. In these cases, a PlantGDB.org accession number for the genome survey sequence (GSS) contig corresponding to the gene will be provided in place of the EST.

2.2.3 **Tissue isolation**

In order to test the MPSS data, maize tissues at various relevant growth stages were isolated. These tissues included root-stele, root-cortex, root tip, shoots, tassel, silk and ear (Prof. Scott Tingey, DuPont Pioneer, pers.comm.) and were used to confirm the MPSS data. mRNA from these tissues was used in semi- quantitative PCR experiments aimed at confirming the transcript patterns observed in the MPSS database. Once harvested, all tissues were snap-frozen in liquid nitrogen for RNA extraction.

2.2.3.1 Root-cortex and -stelar tissue isolation

B73 maize seeds, kindly supplied by Dr Trevor Garnett (Australian Centre for Plant Functional Genomics (ACPFG), The University of Adelaide, Australia), were aerated in reverse osmosis (RO) water for 2 hours to prevent growth of mould, laid out to germinate on wet filter paper in Petri dishes and left until the cotyledon was approximately 5 mm in length (5-7 days). Seedlings were then transferred to an aerated hydroponics system which consisted of a 3L rectangular container covered with black tape. A tray was placed in the tub and overlayed with a 50 mm x 50 mm plastic grid to support the seedlings (Figure 2.1).

Seedlings were grown in RO water containing 1mM $CaSO_4$ in two batches and grown in a growth room for 10 days (Figure 2.1) at 24°C with day length varying between 10-14 hrs. One batch was a control treatment and the other treated with 100 μM abscissic acid (ABA, Sigma-Aldrich) added 3 hours prior to tissue collection. The ABA treatment was performed to simulate water stress and to confirm with PCR that there were no changes in gene transcript patterns in response to a water stress (as was seen in the comparisons with drought-treated and well-watered root MPSS libraries, Section 2.2.1). A 3 hour pre-treatment with 100 μM ABA was deemed sufficient to simulate water stress (Dr Matthew Gilliham, Wine and Horticulture Unit at the School of Agriculture, Food and Wine, The University of Adelaide, pers. comm. 2007). The addition of 100 μM ABA to simulate water stress was based on studies which measured water stress in plants and its association with K^+ homeostasis in cells. A stelar K^+ outward rectifying channel (SKOR), in particular, was found to be down regulated in water stress and similar down regulation was seen after treatment with 10 μM ABA (Gaymard *et al.*, 1998; Lacombe *et al.*, 2000; Roberts and Snowman, 2000). A higher concentration was selected to ensure SKOR downregulation and, by extension, causing the plants to experience high water stress.

Figure 2.1 Ten day old B73 maize seedlings in two aerated growth treatments. *Left tub contains 100 µM ABA treatment and right tub is control treatment.*

Isolation of cortex and stele was performed as described in Leonard *et al.* (1975). Shoots and root tips (root apical meristems) were cut off the 10 day old seedlings and harvested separately. Two independent experimental replicates of control and ABA treated root-cortex, root-stele, and shoots were obtained.

2.2.3.2 Maize reproductive tissue isolation

Tassel, silk and ear tissues were harvested from maize Buckler lines kindly supplied by Dr. Trevor Garnett (ACPFG, The University of Adelaide, Australia). Following discussion with Prof. Scott Tingey (DuPont Pioneer, pers.comms. 2007), early and late stages of tassel (pre-pollination (end of stage VT) and post-pollination (beginning of R1)), silk (pre- and post-pollination, R1) and ear (milky-R3 and dough consistency-R4) were selected to sample for presence of ESTs corresponding to the MPSS candidate tags in crucial reproductive tissues. These tissues were harvested using http://www.agronext.iastate.edu/corn/ as a reference for the various growth stages.

2.2.4 **RNA extraction of maize tissue**

RNA was extracted from root-cortex, root-stele, shoot, root tip, tassel (pre- and post-pollination), silk (pre- and post-pollination) and ear (early-milk and late-dough) to replicate the relevant tissue libraries in the MPSS database.

RNA was extracted from approximately 100 mg of frozen tissue which was ground using a mortar and pestle. Ground tissue was suspended in1ml of Trizol-like reagent (38% phenol at pH 4.3, 1M guanidine thiocyanate, 1M ammonium thiocyanate). Samples were mixed on a rotor suspension mixer (model # RSM6 Ratek Instruments Pty. Ltd., Victoria, Australia) for 5 min and centrifuged at 14,000 rpm at 4°C for 15 min (Eppendorf Model 5810R, Hamburg, Germany). Chloroform (200 µL) was added to the supernatant which was shaken vigorously for 15 sec and incubated at room temperature for 5 min. The chloroform-supernatant mix was spun at 14,000 rpm for 20 min at 4°C. The aqueous phase was separated into a new tube to which 500 µL of 100% isopropanol was added and the mixture was incubated at room temperature for 10 min and spun at 14,000 rpm for 15 min at 4°C. The resultant pellet was retained and washed with 1 ml of 70% ethanol by spinning at 14,000 rpm for 5 mins at 4°C.

The pellet was left to air dry and was resuspended in RNase free water (Applied Biosystems/Ambion).

2.2.5 **Maize PCR to validate MPSS data**

PCR was performed on various maize tissues to validate the MPSS transcript data. This was done by studying the presence/absence of gene transcript in the different tissues. RNA was quantified using a spectrophotometer (Nanodrop ND-100, Thermo Fisher Scientific, Waltham, MA). Ten micrograms of RNA was treated with 5 µL of DNase I (Applied Biosystems/Ambion) in a total volume of 50 µL and incubated at 37°C for 30 min. The reaction was stopped by the addition of 5 µL of DNase Inactivation Reagent and incubated at 24°C for 5 min before being spun down at 13,200 rpm from which the supernatant containing the RNA was collected. RNA (0.4 µg) was used for synthesis of cDNA in a 20 µL volume using SuperscriptIII Reverse Transcriptase (Invitrogen, Carlsbad CA) (Table 1) according to the manufacturer's protocol. The cDNA was used for PCR in a Tetrad (PCR conditions outlined in Table 1) (MJ Research MPTC-225, Waltham, MA) for amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPdh) for 30 cycles to check the integrity of the cDNA and also to check the consistency of template loading between the various samples. This was done by adding 1 µL of the template (cDNA) to a total PCR volume of 25 µL. One microlitre of the same cDNA was used as a template to check the tissue specificity of the EST sequences identified in section 1.3.2. PCR was performed using 0.1 µL Platinum Taq DNA Polymerase (Invitrogen), 2.5 µL 10X PCR buffer, 2 µL dNTP (5mM), 0.75 µL 50mM $MgCl_2$, 0.2 µM each of forward and reverse primer, 5% dimethyl sulfoxide (DMSO) (v/v) and made up to 25 µL with high-pure water (18.2 MΩ•cm). The respective annealing temperatures and cycle numbers were varied for each gene target and these are outlined in Table 2.1. Each PCR set contained cDNA from tissue samples, a positive control for which the template was B73 genomic DNA, and a negative control which contained no template DNA.

Table 2.1 MPSS candidates, primer sequences used for amplifying maize EST sequences and PCR conditions employed for PCR of maize mRNA

MPSS candidate name	Forward primer for maize PCR	Reverse primer for maize PCR	Annealing temperature (°C	Extension time (s)	Number of cycles	Product size
Root cortex candidates						
C5	CAACGGTGGTTGAGTTCAGA	CCACATCGAGTCCCATTTCT	52	30	30	154 bp
C6	CATATCCAGGTCGCGGTAGT	ACATCCAGTACGGCTCCAAC	54	30	30	152 bp
C34	ATTATTCGGCGACAGACAGG	AGAAGCCAAGAGGGTGATCC	52	30	25	208 bp
C62	AACTACCCGCTGATGCAGAA	GGCACTCCCTGATCCTGTT	52	30	35	211 bp
C226	TCAGCTTGTGACGAATGACC	CTGTTGAACTTGGCGATGAA	50	30	25	206 bp
C257	CAACCTCAACCTCACCAAGG	ATGAGCGACATGCAGAACAC	52	30	35	171 bp
C270	CCATGCAAGAGTTTTGCTCA	TCCTCCCACGGCTAAATATG	50	30	25	250 bp
C280	CAACCTCAACCTCACCAAGG	ATGAGCGACATGCAGAACAC	52	30	35	171 bp
Root stelar candidates						
S9	TTCCGAAAATAAAGCCATGC	GACATAAGGCTTCCGTTCCA	48	30	35	170 bp
S147	GCTAGGTGTTGGTGGGTGTT	TAGCTGATCGGTACGGTGGT	54	30	35	214 bp
Control gene						
Zea mays GAPdh	GACAGCAGGTCGAGCATCTTC	GTCGACGACGCGGTTGCTGTA	55	30	30	114bp

2.2.6 **Identification of EST/gene orthologues in rice and isolation of promoters**

Following PCR on maize RNA to check for tissue-specificity, candidates that were specific to either root-cortex or stele were searched against RAP-db (Ohyanagi *et al.*, 2006) for rice orthologues. The retrieved rice sequences were then searched against a microarray database that was generated from root-cortex and root-stele cells isolated using fluorescence-activated cell sorting (FACS) (Dr Alexander Johnson, ACPFG, unpublished data). The search against the database was performed mainly to ensure that the spatial gene transcript patterns seen in maize was correlated with that observed in rice, that is, a cortex-specific gene from maize for which an orthologue was present in rice was also found in the root-cortex of rice, but not in the root-stele and vice versa. This library, however, did not have gene transcript data for other tissues and so a search on a rice MPSS database was performed through http://mpss.udel.edu/rice (Nobuta *et al.*, 2007). This MPSS database contained gene transcript information from a variety of tissues and treatments (Table 2.2). Where rice orthologues were identified and found to have the expected transcript pattern according to the criteria outlined above, the 2kb region immediately upstream of that gene would be isolated as putative promoter. If no rice orthologue was found or if transcript patterns were not congruent with those in the maize data, the 2kb region directly upstream of the gene from maize was isolated as a promoter.

Table 2.2 Tissues and treatments used to generate rice MPSS data

Tissue	Treatment
Young root	Roots: 14 days
Mature root A	Roots: 60 days
Mature root B	Roots: 60 days
Mature root combined	Mature root A + B
Young leaf	Leaves: 14 days
Mature leaf A	Leaves: 60 days
Mature leaf B	Leaves: 60 days
Mature leaf C	Leaves: 60 days
Mature leaf D	Leaves: 60 days
Mature leaf combined	Mature leaf A + B + C + D
Etiolated seedling	Dark grown seedlings: 10 days
Germinating seed	12 h day/night cycle: 3 days
Stem	Stems: 60 days
Meristem	Meristematic tissues: 60 days
Mature pollen	Mature pollen
Mature stigma + ovary	Mature stigma and ovary
Immature pannicle	Immature panicle: 90 days
Callus	Callus: 35 days
Abiotic stress libraries	
Root + salt	Roots: 250 mM salt for 24 h
Leaf + salt	Leaves: 250 mM salt for 24 h
Root + drought	Roots: drought for 5 days
Leaf + drought	Leaves: drought for 5 days
Root + cold	Roots: 4°C for 24 h
Leaf + cold	Leaves: 4°C for 24 h

2.2.7 Generation of promoter- *uidA/GFP* fusion lines

2.2.7.1 Isolation of putative cell type-specific promoters

The 2kb region immediately upstream of either the maize or rice gene, corresponding to the MPSS searches, was amplified by PCR with 0.3 µL of Expand High Fidelity PCR taq (Roche), 2.5 µL of buffer with $MgCl_2$, 0.2µM each of forward and reverse primers, 2 µL dNTPs (5mM), 1 µL of genomic DNA template made up to a total volume of 25 µL with high-pure water (18.2 MΩ•cm). The PCR cycling conditions are outlined in Table 2.3. Another promoter found upstream of *TaHKT1;5-D* in bread wheat thought to control root-stelar gene expression was kindly provided by Dr Caitlin S. Byrt (ARC Centre of Excellence in Plant Cell Walls, The University of Adelaide, Australia). This gene is thought to be expressed predominantly in the xylem parenchyma cells of the root (Byrt *et al.*, 2007) and so was used as a candidate root stelar-specific promoter. The putative promoter sequences are presented in Appendix 2.1.

Table 2.3 Origin of promoter sequences and respective primer sequences used in PCR to amplify promoters

Promoter name	Origin	Forward primer	Reverse primer	Annealing temperature (°C)	Extension time	Number of cycles
C34	Rice (Nipponbare)	GCCAACTGAAACGCCAC	GTATTTATCATGCATATGGCG	51	2 min 30 secs	35
C257	Maize (B73)	GAGCATCTCCAACTAAAAAAG	TATATTGCTGCTAGCGCTAT	50	2 min 30 secs	35
S147	Rice (Nipponbare)	CTGATGATCGACCACTATTAG	GCTTGTAAACTACGCAAGAG	51	2 min 30 secs	35
Ta	Bread wheat (Chinese Spring)	TTGCAGATGTTCGCATACAC	TTCTACTGTAAGTTGTGTAGAG	50	2 min 30 secs	35

2.2.7.2 Generating entry vectors

The PCR products were cloned into the pCR8/GW/TOPO TA vector (Invitrogen). Two microlitres of the PCR product, 1 µL of salt solution (Invitrogen), 1 µL of TOPO vector and high-pure water (18.2 MΩ•cm) to bring the volume to 6 µL were mixed together and incubated at 24°C for 5 min. Two microlitres of the reaction was added to a vial of Mach 1 *Escherichia coli* cells (Invitrogen) which was incubated on ice for 30 min. The cells were transformed by heat shock at 42°C for 30 sec before being placed on ice again for 2 min. A volume of 250 µl of Luria Bertani (LB) liquid culture was added to the cells and incubated shaking at 225 rpm at 37°C for one hour. The transformed cells were grown on LB agar plates containing 100µg/ml Spectinomycin. Colonies were picked and grown in 2 ml LB liquid culture containing 100 µg/ml Spectinomycin shaking at 150 rpm at 37°C for 16 hours (overnight).

A volume of 2 ml of the overnight culture was centrifuged (Eppendorf) at 13,200 rpm for 2 min. The supernatant was discarded and the bacterial pellet was resuspended in 100 µL of 25mM Tris-HCl buffer (pH 8) containing 10mM ethylenediaminetetraacetic acid (EDTA) and 15% (v/v) of 50 mM glucose to which 200 µL of 0.2 M NaOH solution containing 1% (v/v) SDS was added. The solution was slowly inverted to ensure complete breaking of the cells and 150 µL of 3M potassium acetate buffer (pH 5.2) was added to neutralise the pH solution. The tubes were incubated at -20°C for 10 min. The solution was centrifuged at 13,200 rpm for 15 min. The supernatant was retained; 1 ml of 100% ethanol added to it, and it was placed at -20°C for 5 min. The solution was centrifuged at 13,200 rpm for 10 min. The supernatant was discarded and the pellet was washed by adding 200 µL of 70% ethanol and centrifuged for 2 min. The supernatant was removed and the DNA pellet was left to dry after which it was resuspended in Tris EDTA (TE) buffer containing RNase A (Sigma-Aldrich).

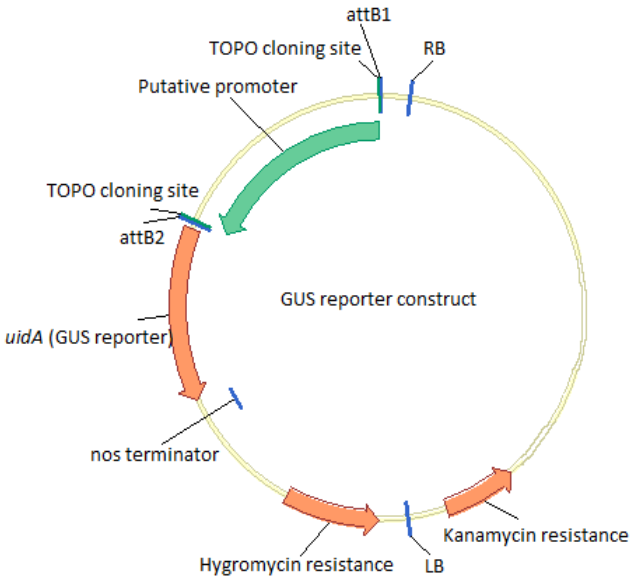
Correct orientation of the gene of interest was determined through restriction digest analysis of the recombinant plasmids. Promoter inserts were sequenced using promoter specific primers to ensure the correct insert was present in the entry vectors before they were recombined into plant transformation vectors (Table 3). (Appendix 2.1).

2.2.7.3 Generating destination vectors

Entry vectors containing candidate promoters in the correct orientation were recombined with the gateway destination vectors pMDC164 (Curtis and Grossniklaus, 2003) containing *uidA* (Figure 2a) and pMDC107 (Curtis and Grossniklaus, 2003) containing *GFP* (Figure 2b). The promoters in pCR8/GW/TOPO TA were transferred to pMDC107 and pMDC164 through a gateway reaction using LR clonase II enzyme mix (Invitrogen) which contained 150 ng each of the entry and destination vectors, 2 μL of buffer enzyme mix made up to 10 μL with TE buffer (10mM Tris-HCl, 1mM EDTA adjusted to pH of 8). The reaction mix was incubated at 24-25°C for 16 hrs. The orientation of the promoter in pMDC107 and pMDC164 was checked using restriction digests (Table 2.4).

The promoter reporter constructs were used to transform the AGL-1 strain of *Agrobacterium tumefacians* and then transformed into barley embryos (Tingay *et al.*, 1997; Matthews *et al.*, 2001) by Konny Beck-Oldach (ACPFG) and Rohan Singh (ARC Centre of Excellence in Plant Cell Walls, The University of Adelaide, Australia).

(a)



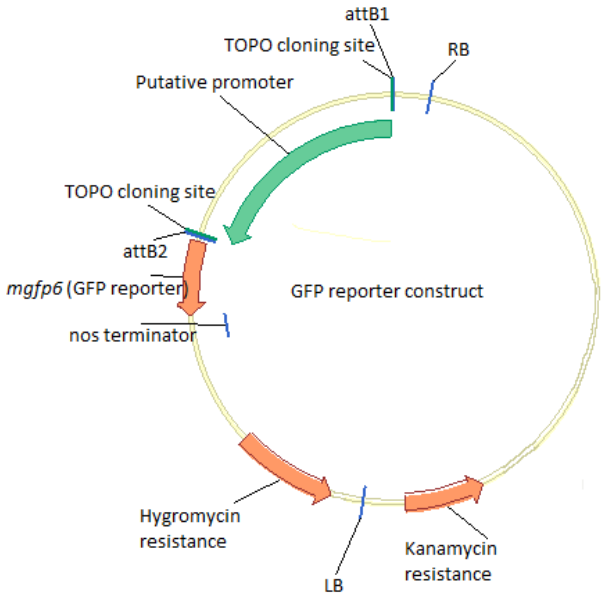


Figure 2.2 Vector maps of reporter constructs used in plant transformation containing promoters and downstream reporters *mgfp6* or *uidA* (a) Promoter- *uidA* (b) Promoter- *mgfp6*. *The recombination sites (TOPO cloning site), into which the insert is transferred from the entry vector, are labelled attB1 and attB2; the hygromycin and kanamycin resistance genes enable in* planta *and microbial selection, respectively.*

2.2.8 **Assaying for reporter gene activity**

T_1 transgenic seeds were treated with 30% Domestos containing bleach (Unilever Australasia) for 10 min and rinsed 10 times with RO water and 5 times with high-pure water (18.2 MΩ•cm). Seedlings were germinated on Petri dishes and then allowed to grow in sunlight for 10 days before being harvested for a GFP or GUS assay.

2.2.8.1 Green fluorescent protein (GFP) detection

Transgenic barley lines containing *GFP* under the control of the various promoters were first studied under a Leica MZ FLIII fluorescent stereo microscope with a GFP2 filter (480/40 nm, barrier filter LP510 nm). Images were captured with a DC300F digital camera and analysed with the associated software, IM50 version 1.20 (Leica Microscopie Systems, Heerbrugg, Switzerland). Confocal microscopy was performed on a Zeiss Axioskop 2 plus LSM5 PASCAL argon laser confocal microscope (Carl Zeiss, Jana Germany). GFP signal was detected at an excitation wavelength of 488 nm and emission range of 505-530 nm using PASCAL version 3.2 SP2 software (Carl Zeiss).

Seedlings aged between 7-15 days were suspended in high-pure water (18.2 MΩ•cm) and segmented manually for leaf, sheath, crown and root. The tissues were mounted in high-pure water (18.2 MΩ•cm) on glass slides with a glass cover slip and the prepared slides were then placed on the microscope platform and imaged.

2.2.8.2 Beta-glucuronidase (GUS) assay

Transgenic barley lines containing *uidA* under the control of the various promoters were studied by performing a GUS stain assay. The GUS staining solution was based on a 50 mM sodium phosphate buffer (pH 7) which contained 10 mM EDTA (w/v), 0.1% Triton X-100 (v/v), 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, 1mg/ml 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc; Gold Biotechnology Inc.) and 106 μg/ml chloramphenicol.

Stain was applied to segmented seedlings in Petri dishes and care was taken to ensure that all the plant tissues were fully submerged in solution. The solution was then vacuum infiltrated into the tissues at 25-27 Hg (Napco 5831-220 vacuum oven, Thermo Fisher Scientific, Waltham, MA linked to Edwards Vacuum Pump E2M5, Edwards, Singapore) for 20-30 min and incubated at 37°C overnight in the dark. The seedlings were then destained through the addition of 95% ethanol for 10 minutes. The ethanol was discarded and replaced with 70% ethanol to remove all chlorophyll from the tissues.

2.3 Results

2.3.1 **MPSS signature tags**

After performing searches to obtain cortex and stelar specific signature tags, those that had ideal transcript levels of 100-1000 TPM were retained. A total of 293 cortex candidates and 287 stelar candidates were found to have the desired transcript levels. These initial candidates were numbered in order of appearance, that is, C1-C293 for cortex candidates, and S1-S287 for stelar candidates.

Of these candidates, those that were confirmed to be expressed only in root-cortex or in root-stele through ensuring no expression was found in MPSS expression libraries from other tissues were C5, C6, C34, C62, C226, C257, C270, C280, S9 and S147 (Table 2.4).

The tags were then used to retrieve maize EST/gene sequences (Table 2.5). The EST sequences were targets for PCR to check for presence in various maize tissues (Figure 2.3).

Table 2.4 Cell type-specific maize MPSS candidates with signature tag and corresponding transcript levels

Cortex candidate name	**MPSS signature tag**	**Transcript level (TPM)**
C5	GATCCATTTCGTTCTTT	101
C6	GATCCCACTGACTACTT	102
C34	GATCGAGCACATTTTCA	108
C62	GATCGCCATGAACCAGC	116
C226	GATCGAACGAAAACAAG	270
C257	GATCCATCATCCATGTT	387
C270	GATCTGCTCGACGGGCG	611
C280	GATCACCTATACCTCTT	805
Stelar candidate name	**MPSS signature tag**	**Transcript level (TPM)**
S9	GATCGCGTGTTGTAACG	103
S147	GATCGGGTTGAATAATT	1062

2.3.2 **Maize EST sequences corresponding to the candidate MPSS signature tags**

Maize B73 EST sequences that were retrieved through mining Plant GDB databases and their respective accession numbers are presented in Table 2.6. Some EST sequences were not publicly available and having been sourced from the DuPont Pioneer proprietary EST database corresponding to the signature tags, these sequences cannot be provided here. Instead, a genome survey sequence (GSS) ID has been provided.

Table 2.5 Matching sequence ID and corresponding rice probe set ID for maize MPSS candidates

MPSS candidate name	Maize Plant GDB sequence ID	Corresponding rice probe set ID
C34	18173673; 18179728; 18179452; 16927013; 32909590; 37375838; 33101955; 18180906; 18178012; 33101215; 37375027; 37378733; 33100895; 45567310; 33101125; 33102464; 33102349; 33101393; 28984190 (EST)	LOC_Os12g36240.1
C257	29412128; 20135913 (EST)	N/A
S147	ZmGSStuc11-12-04.3487.1 (GSS contig)	LOC_Os04g52720
C62	37382343	N/A
C226	14204210; 14203965; 37374021; 37381148; 32830625; 32929984; 32928136; 32929710; 32929317; 32850578; 32828291	N/A
C270	45568463; 17932269; 18173615; 5762037	N/A
C280	62116471	N/A
S9	ZmGSStuc11-12-04.29373.1	N/A

2.3.3 **Validation of spatial patterns of mRNA in MPSS data with PCR on maize tissue series**

Most of the cortex candidates that were tested for tissue specificity using PCR had transcript mostly confined to the root cortex. Candidates C6, C62, C226 and C280 had transcript present in various other tissues aside from root (Figures 2.3 b, d, e and h). The transcript for C6 and C62 were present in all tissues tested (Figures 2.3 b and d), whereas transcript for C226 was confined mainly to the cortex but with some transcript in shoots and young tassel (Figure 2.3 e). C280 appeared to be transcribed in all tissues tested but at low levels except in the untreated root tip where mRNA levels appeared to be higher (Figure 2.3h). C5 had relatively low transcript levels confined mainly to the root cortex with little transcript apparent in one of the replicates of shoot tissue which had been treated with ABA (shoot 100μM ABA2) and in the untreated root tip (Figure 2.3a). C34 and C257 had transcript levels in the root cortical tissues that were higher than in the other tissues tested, with some low transcript in the root tips (Figures 2.3c and f). C270 had the same transcription pattern as C34 and C257 but with more mRNA in the root tips than in the root cortex (Figure 2.3g).

Only two stelar candidates from the MPSS search were tested for tissue specificity using PCR and, as they had transcript that was present mainly in the root-stele, S147 was established to be the most suitable candidate as a stelar-specific promoter (Figure 2.3j). Of the replicates of a root-cortex set which had been treated with 100 μM ABA (cortex 100 μM ABA1), there was mRNA in one but not in the other replicate (Figure 2.3j). Some transcript could also be observed in the untreated root tip, mature tassel, mature silk and young ear (milk stage). S9 had ubiquitous transcript patterns with stronger signals in the stele compared with other tissues (Figure 2.3i).

For MPSS candidates that were tested using PCR, a positive and negative control was used in all cases (including GAPdh). The negative control gave a null result in all but four of the experiments (C34, C226, C270 and S147) and the positive control containing the genomic DNA of B73 failed to amplify a product (results not shown).

Figure 2.3a Agarose gel showing transcript patterns of cortex-specific candidate, C5, in root-cortical, root-stelar, shoot tissues and root tips under control and 3 hr treatment with 100 µM ABA and young/mature tassel, young/mature silk and ear at milk and dough stage.

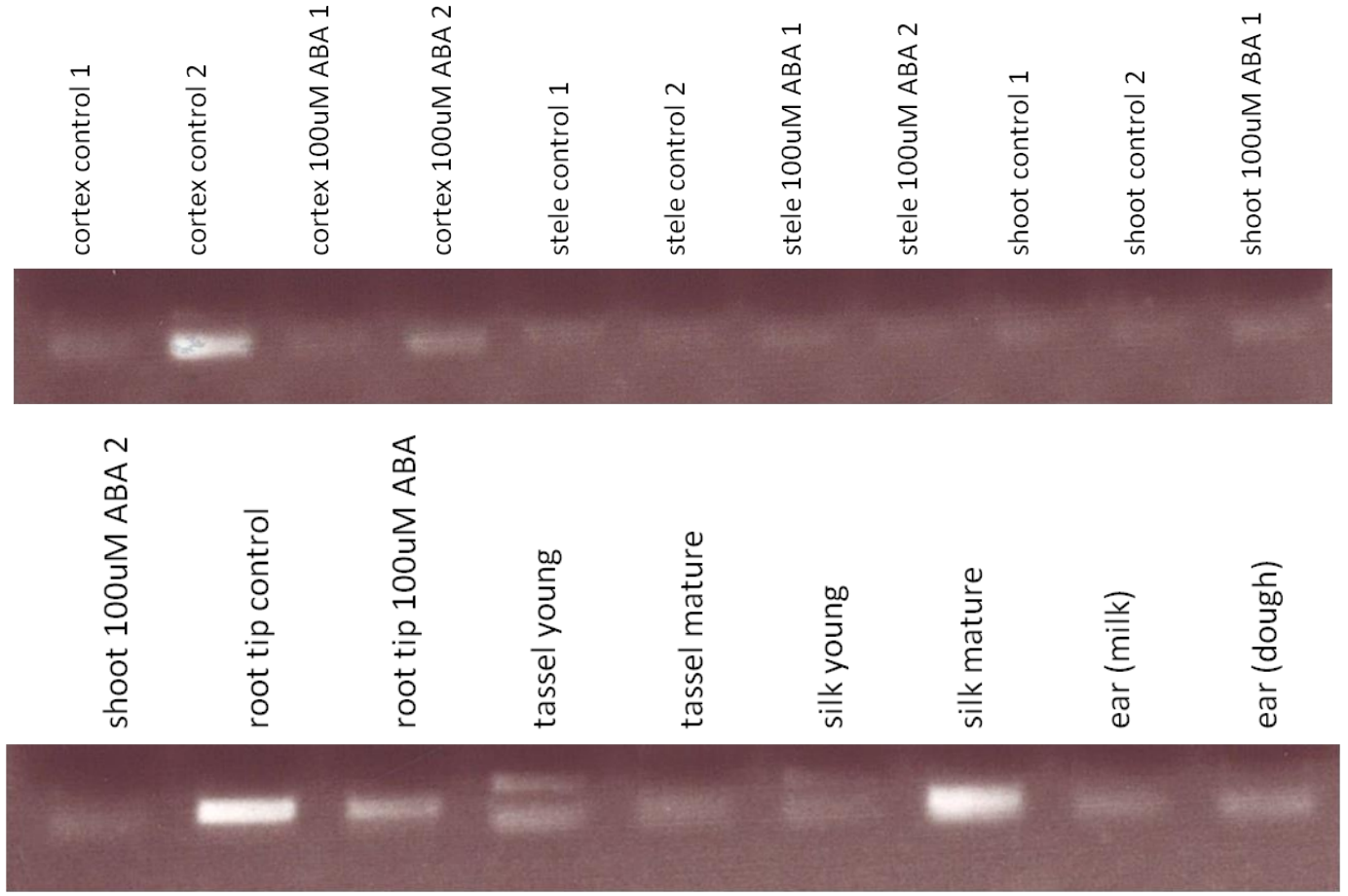


Figure 2.3bAgarose gel showing transcript patterns of cortex-specific candidate, C6, in root-cortical, root-stelar, shoot tissues and root tips under control and 3 hr treatment with 100 µM ABA and young/mature tassel, young/mature silk and ear at milk and dough stage.

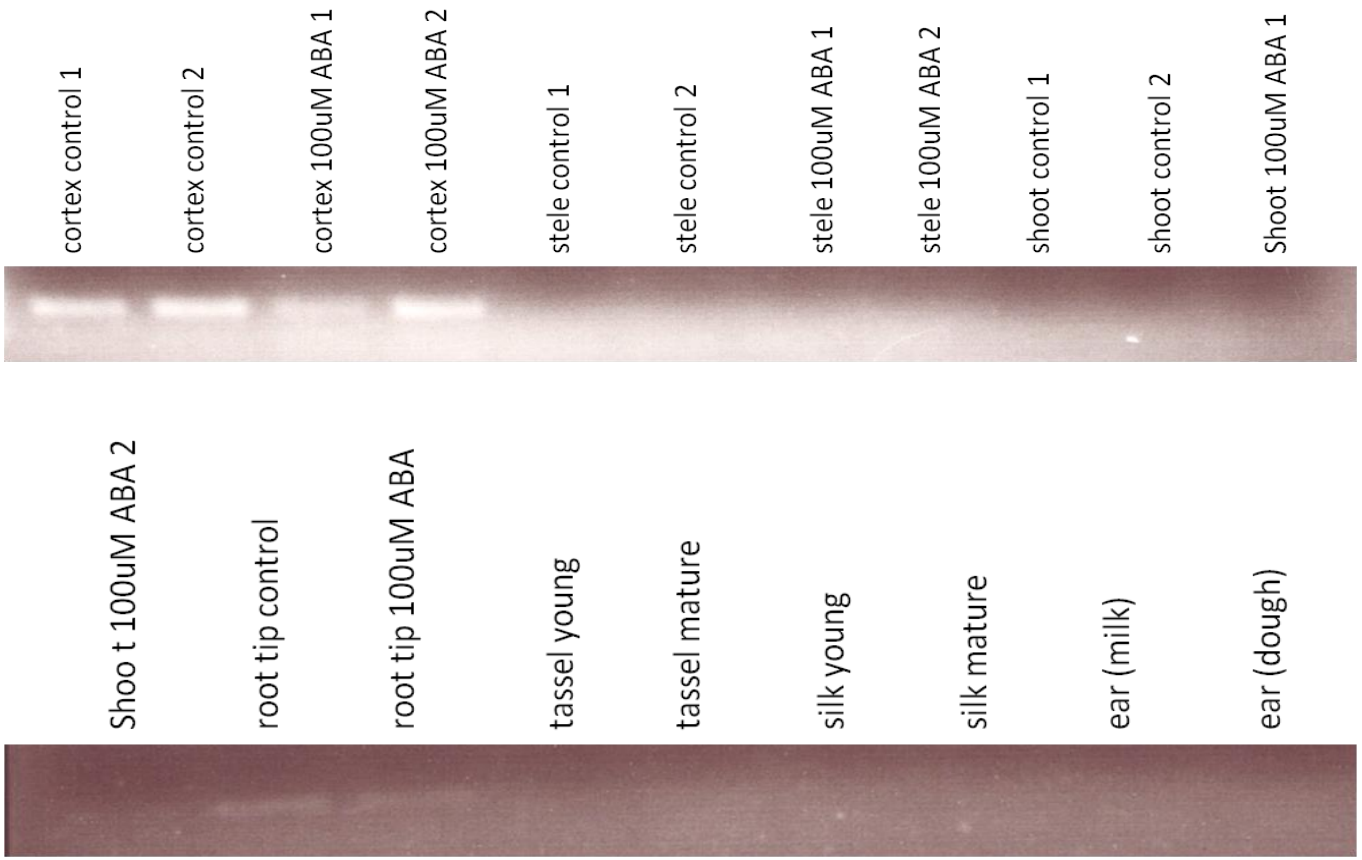


Figure 2.3c Agarose gel showing transcript patterns of cortex-specific candidate, C34, in root-cortical, root-stelar, shoot tissues and root tips under control and 3 hr treatment with 100 µM ABA and young/mature tassel, young/mature silk and ear at milk and dough stage.

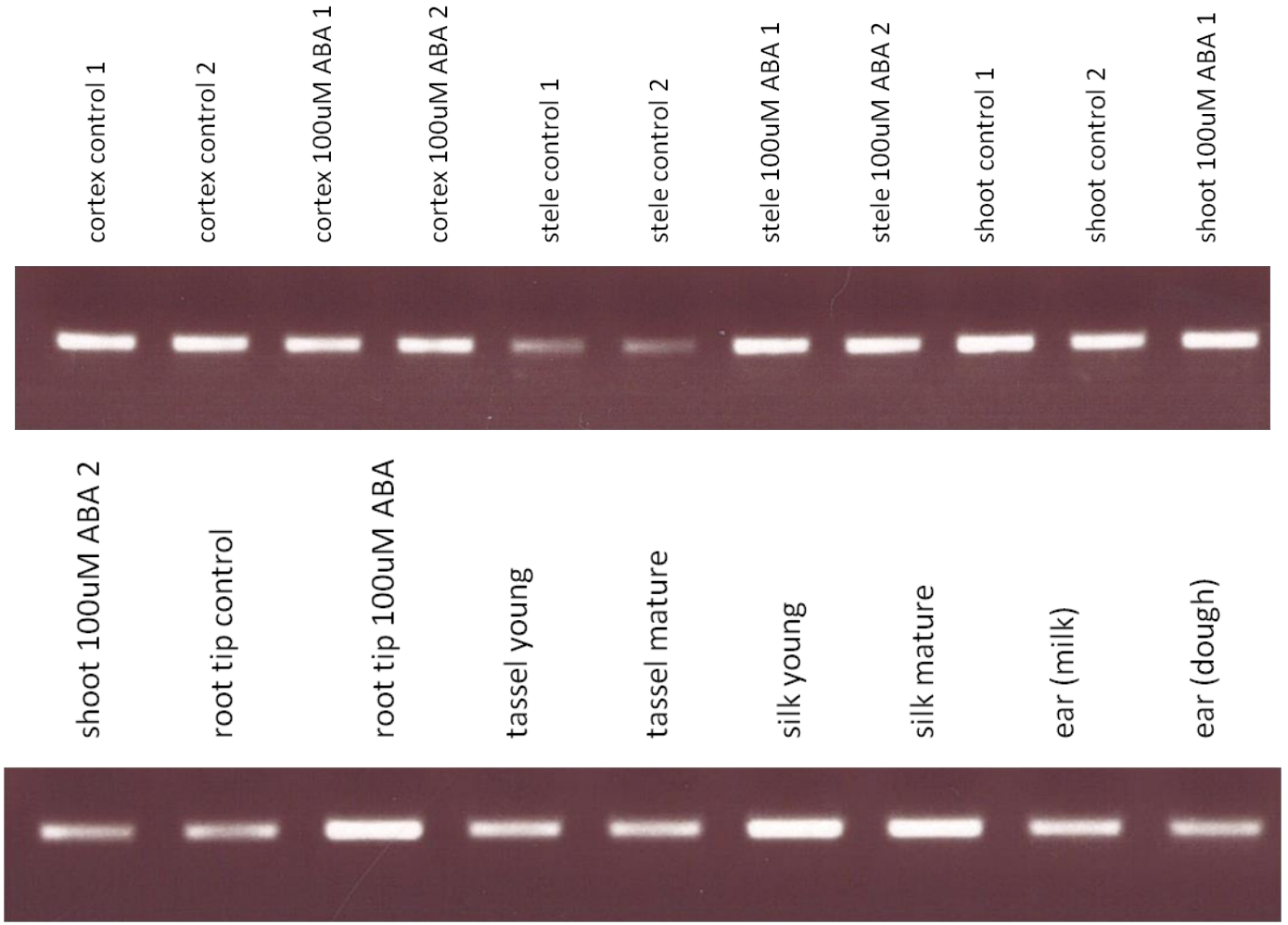


Figure 2.3d Agarose gel showing transcript patterns of cortex-specific candidate, C62, in root-cortical, root-stelar, shoot tissues and root tips under control and 3 hr treatment with 100 µM ABA and young/mature tassel, young/mature silk and ear at milk and dough stage.

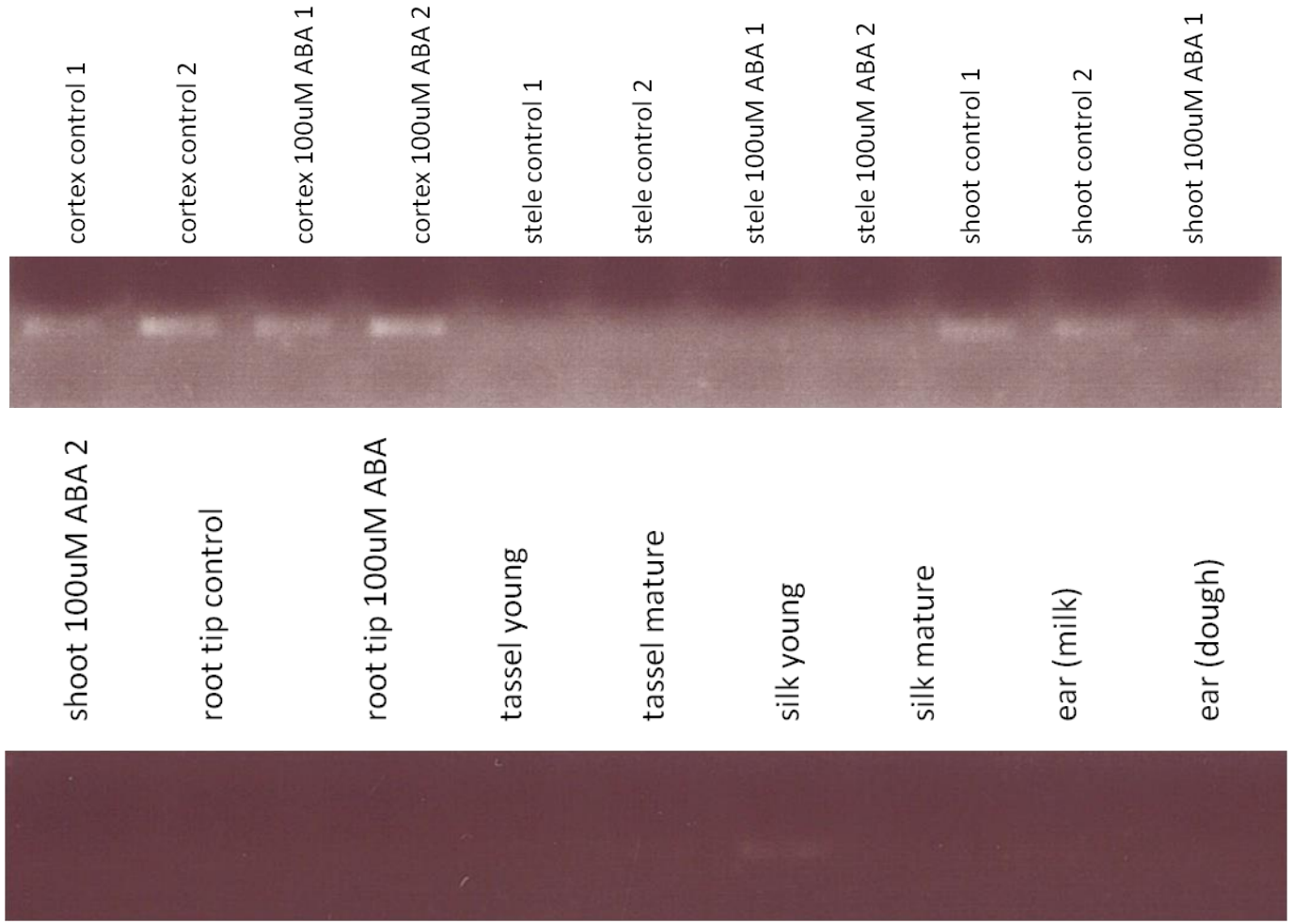


Figure 2.3e Agarose gel showing transcript patterns of cortex-specific candidate, C226, in root-cortical, root-stelar, shoot tissues and root tips under control and 3 hr treatment with 100 µM ABA and young/mature tassel, young/mature silk and ear at milk and dough stage.

Figure 2.3f Agarose gel showing transcript patterns of cortex-specific candidate, C257, in root-cortical, root-stelar, shoot tissues and root tips under control and 3hr treatment with 100 µM ABA and young/mature tassel, young/mature silk and ear at milk and dough stage.

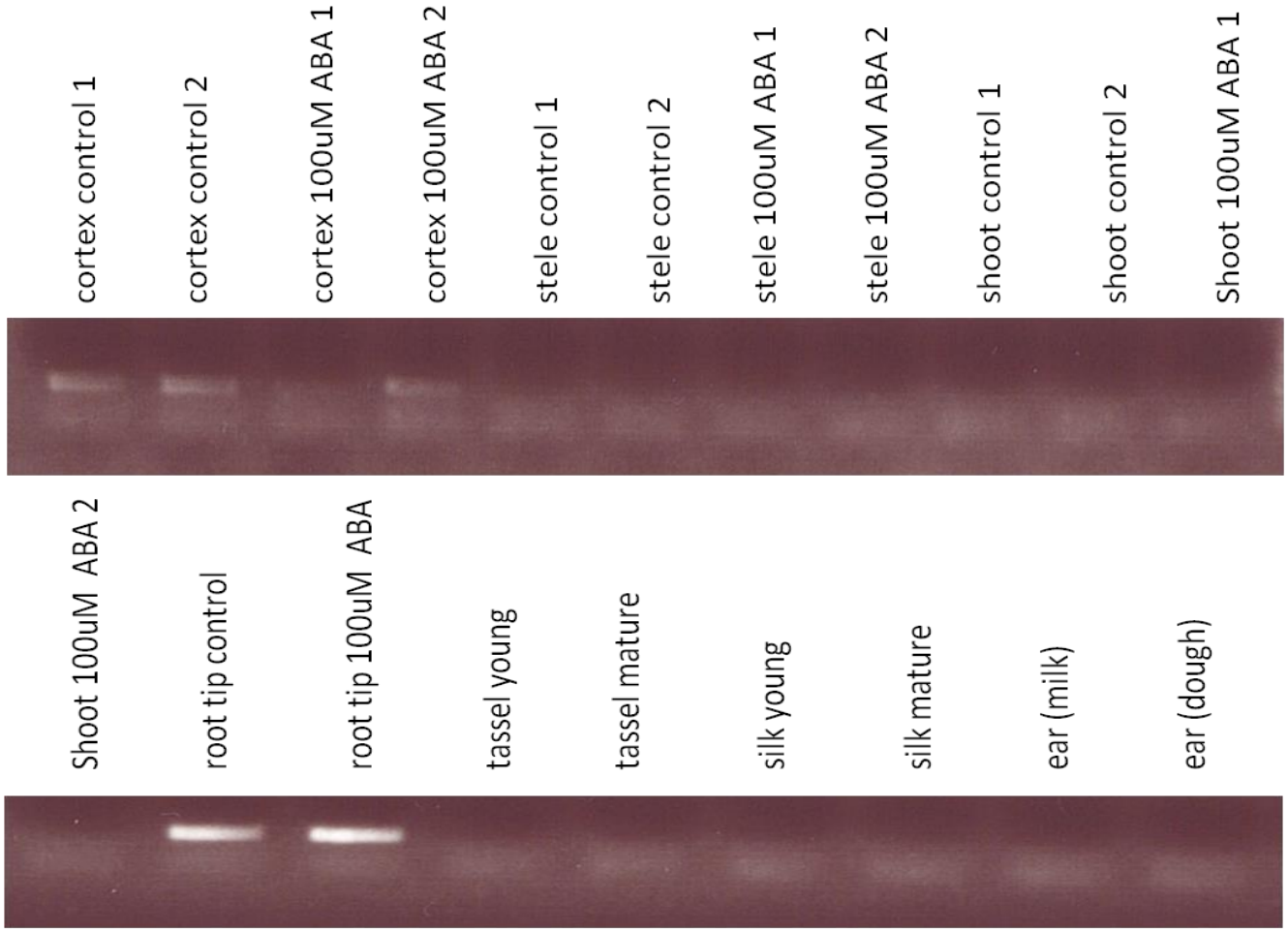


Figure 2.3g Agarose gel showing transcript patterns of cortex-specific candidate, C270, in root-cortical, root-stelar, shoot tissues and root tips under control and 3 hr treatment with 100 µM ABA and young/mature tassel, young/mature silk and ear at milk and dough stage.

Figure 2.3h Agarose gel showing transcript patterns of cortex-specific candidate, C280, in root-cortical, root-stelar, shoot tissues and root tips under control and 3 hr treatment with 100 µM ABA and young/mature tassel, young/mature silk and ear at milk and dough stage.

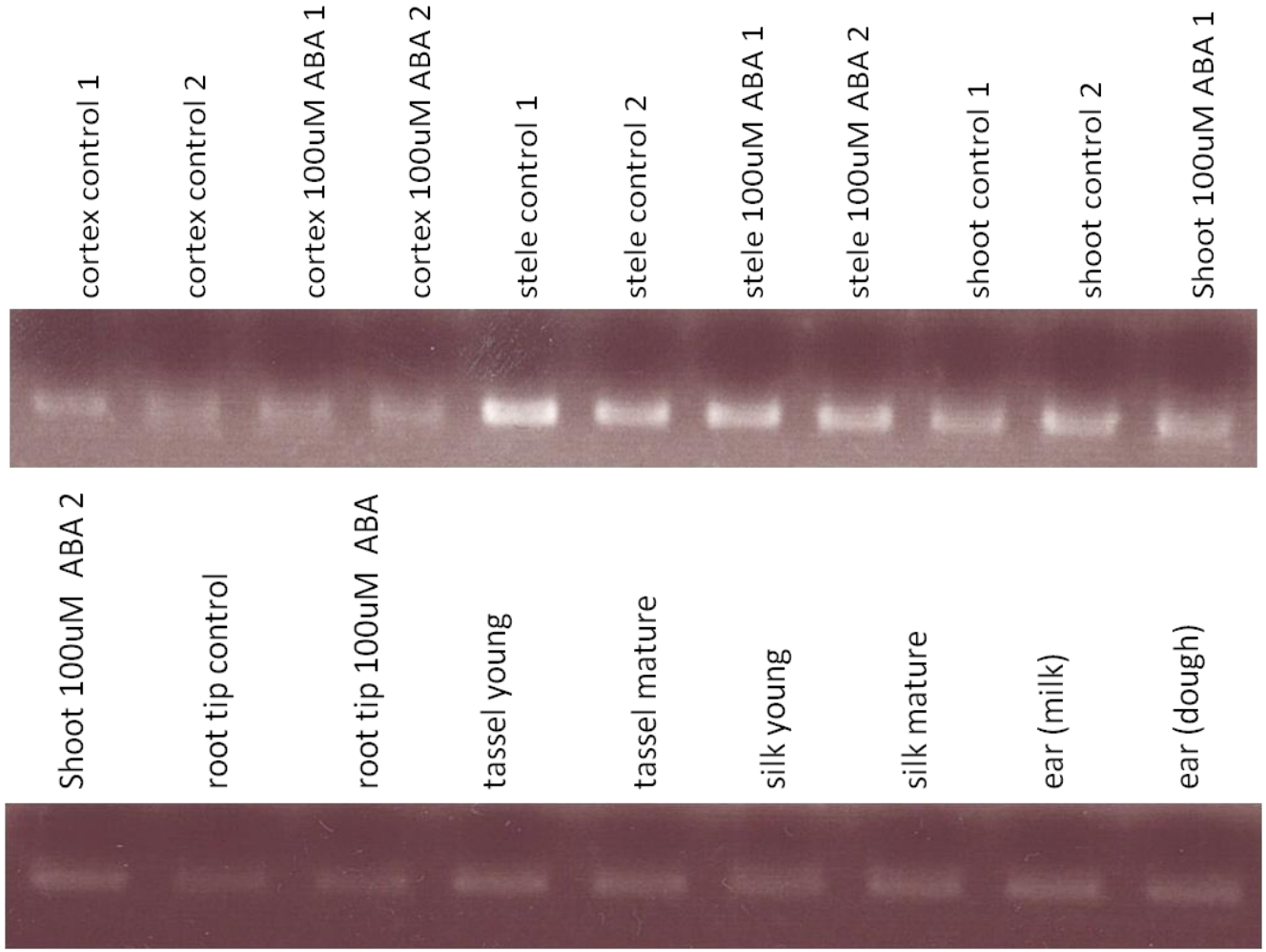


Figure 2.3i Agarose gel showing transcript patterns of stelar-specific candidate, S9, in root-cortical, root-stelar, shoot tissues and root tips under control and 3 hr treatment with 100 µM ABA and young/mature tassel, young/mature silk and ear at milk and dough stage.

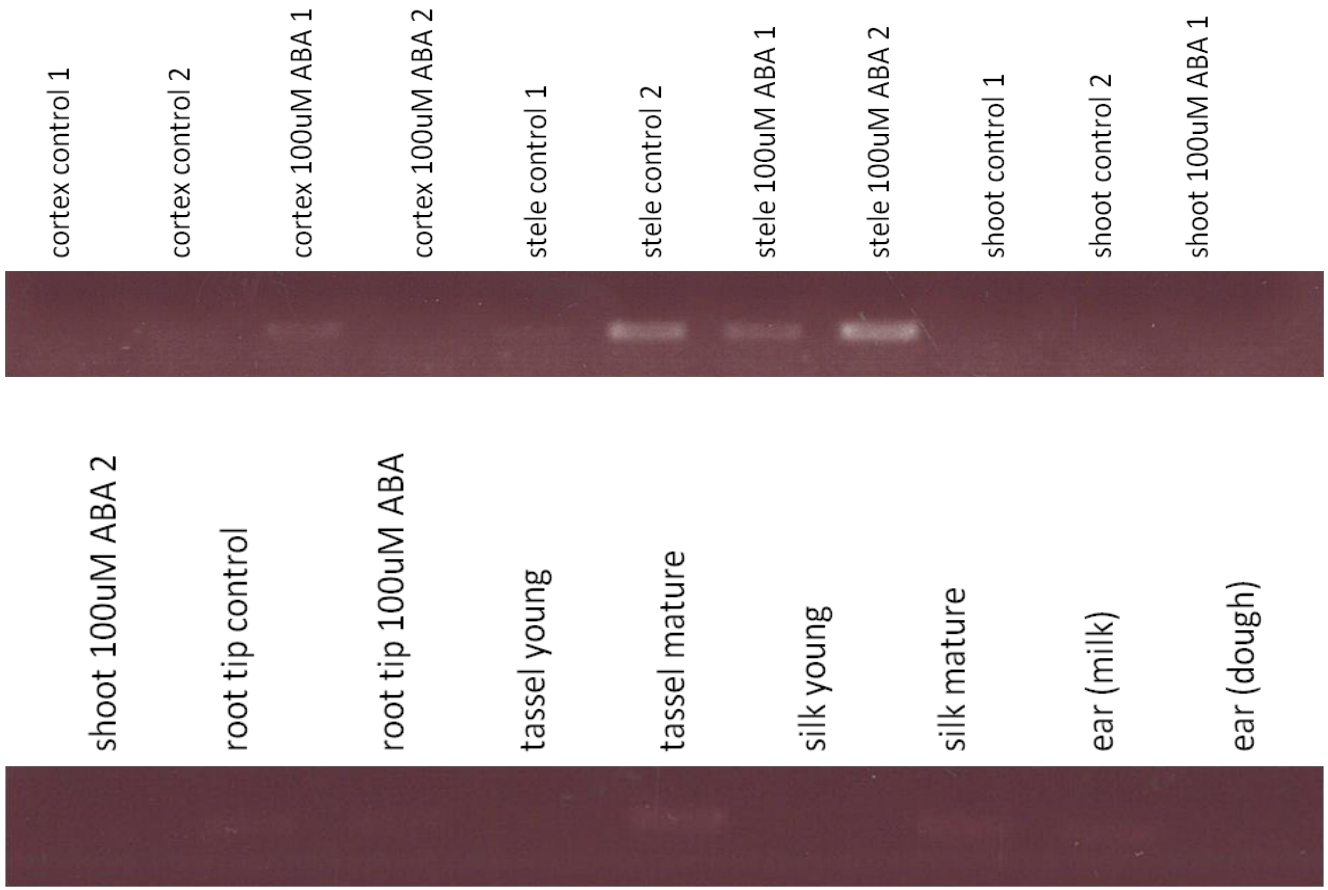


Figure 2.3j: Agarose gel showing transcript patterns of stelar-specific candidate, S147, in root-cortical, root-stelar, shoot tissues and root tips under control and 3 hr treatment with 100 µM ABA and young/mature tassel, young/mature silk and ear at milk and dough stage.

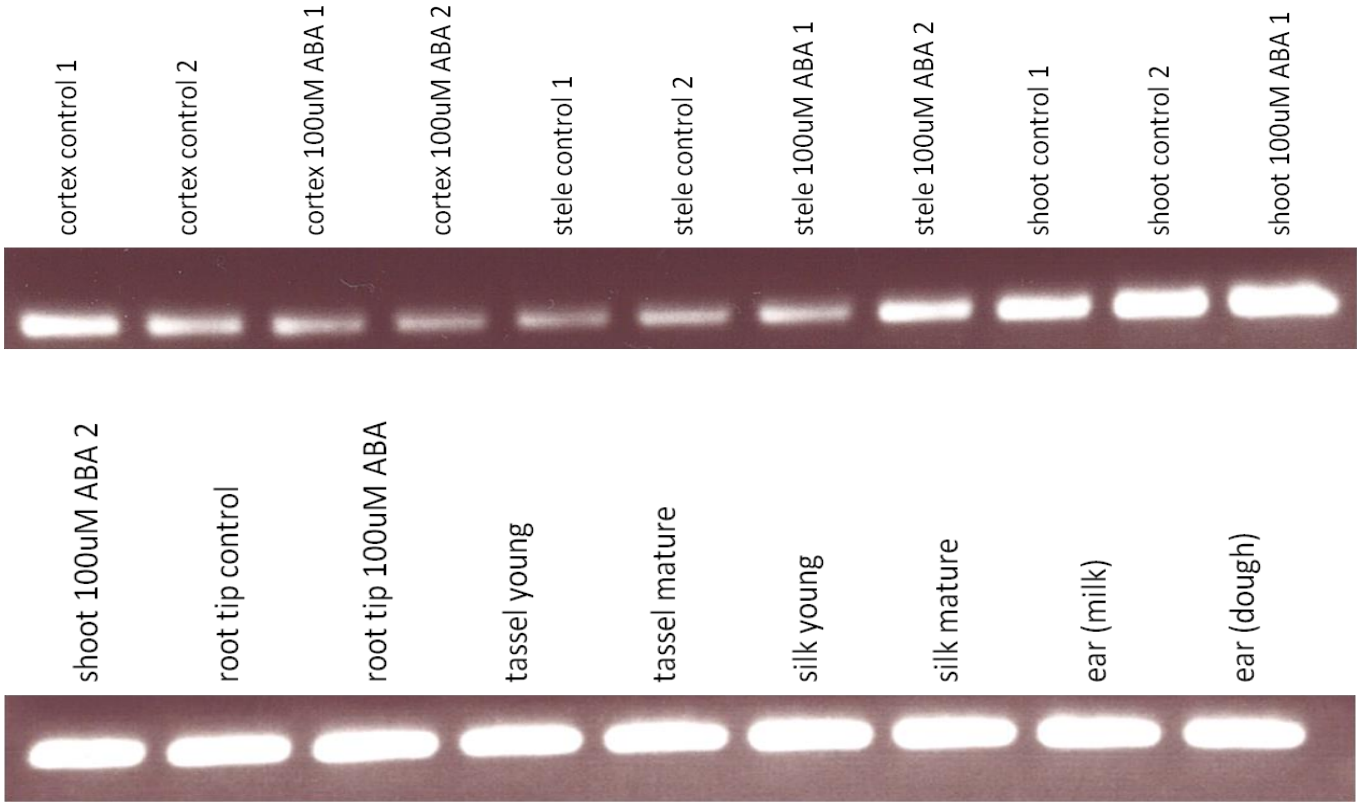


Figure 2.3k Agarose gel showing transcript patterns of maize GAPdh as a loading control, in root-cortical, root-stelar, shoot tissues and root tips under control and 3 hr treatment with 100 µM ABA and young/mature tassel, young/mature silk and ear at milk and dough stage.

2.3.3.1 Rice orthologues

The sequences of maize gene candidates where PCR validated MPSS transcript patterns were used to identify rice orthologues (Table 2.5). These rice orthologues were then further selected based on their presence/absence in microarray data from root cortex/stelar cells isolated using FACS. Following this selection, the candidates were compared with a rice root MPSS database. All candidates were found to be expressed below background level of 25 TPM.

2.3.3.2 GFP assay

Plants containing the promoter reporter constructs in pMDC107 were analysed under both stereo and confocal microscope for detection of GFP but no reporter signal was observed in any of the plants examined (results not shown).

2.3.3.3 GUS assay

Staining results were inconsistent across the samples examined (results not shown). Most attempts at GUS staining were negative and where GUS activity was detected, it was not possible to replicate the result in subsequent attempts making it hard to distinguish any possible staining due to promoter activity from background GUS staining. This rendered the reporter activity non-conclusive.

2.4 Discussion

The aim of the work outlined in this chapter was to identify promoters that would not only control expression in particular cell types (the root stele and root cortex) but would also drive low to moderate levels of transcription. This was important as the promoter used to drive the genes of interest (see Chapter 4) should not divert so much energy for the expression of the ectopic gene that it might impede plant growth and development (Tarczynski *et al.*, 1992; Karakas *et al.*, 1997). Also, the overexpression of transporters might be toxic to the cells in which they are highly expressed and thus might lead to cell death (Tarczynski *et al.*, 1992).

The use of the MPSS database was an effective strategy for the identification of promoters by first identifying genes that were expressed in specific cell types at specific levels (low to moderate). The DuPont MPSS database was the only known plant MPSS database that contained gene transcript data for the cortex and stele of roots. As this database which was generated using B73 maize was not well annotated at the time the database search was performed, orthologues needed to be identified in rice which had a better annotated genome. The upstream promoter of the rice orthologue was then used as a candidate promoter to be transformed into barley.

2.4.1 **Confirmation of MPSS transcript patterns**

For the purposes of identifying cell type-specificity, a semi qRT-PCR approach was used where only the presence or absence of the MPSS candidates in maize was assessed. Due to financial constraints, qRT-PCR was not used to assess the gene transcript levels.

The non-amplification of the positive control which contained B73 genomic DNA as a template in the semi qRT-PCR experiments might have been a result of inhibitory factors resulting from the DNA extraction process. Multiple attempts were made to amplify a product using the positive control but due to time constraints this was eventually abandoned. As the semi qRT-PCR results were somewhat consistent (discussed below) with the MPSS expression database and as the loading control was consistent, identification of appropriate candidates was still able to be carried out despite the non-amplification of the positive control.

The results from maize PCR of stelar and cortical candidates mostly correlated with their respective transcript patterns on the MPSS database, with some low levels seen in other tissue types. The candidates which were selected for further work were C34, C257, and S147 (Figures 3c, 3f, 3g and 3j). The remaining MPSS candidates were not taken further as the transcript patterns did not concur with those in the MPSS data. This was because while transcript was present in the relevant tissues, i.e. cortex-specific candidates identified in the MPSS search were present in the cortex, transcript was also found in other tissues; likewise for the stelar candidate, S9, which was taken no further. The inconsistency of the data (PCR

vs. MPSS) of the same candidates from different maize varieties would imply that the promoters controlling these candidates, if introduced into a different organism like barley, could control spatial patterns in an unexpected manner (Zakharov *et al.*, 2004). This has been proposed to be because non-coding regions are not usually subject to the same level of selective pressure as coding regions are and are thus less likely to be conserved between species (Guo and Moose, 2003). It is important to reiterate at this point that cell type-specificity is important as plants overexpressing *AtHKT1;1* under CaMV35S promoter had increased shoot Na^+ in the plants resulting in increased salt sensitivity (Møller *et al.*, 2009). This was in contrast to having stelar-specific expression (Møller *et al.*, 2009).

While having the same transcript patterns as C34, C270 had less pronounced transcript in the root cortex than in the root tips - a developing tissue type of the plant - and consequently was discarded. In order to avoid any disruptions in plant growth, development and ultimately, grain production, promoters controlling expression in any developmental tissues and/or reproductive tissues were not used.

The cortex candidates which were selected for further work were weakly transcribed in the root tips. This inconsistency with observations from the MPSS database is most likely to be a result of tissue contamination. Separation of the root tips from the more mature root was performed by hand and, when isolated, it is distinctly possible that the root tips had some region of the primary root attached, which could be responsible for the observed transcript pattern.

Some stelar-specific transcript (of S147) was observed in the reproductive tissues (tassel, silk and ear, Figure 3j) but this was not of particular concern as the PCR bands in these tissues were faint indicating mRNA levels were low. Also, given that the reproductive tissues derived were from a different maize variety (Buckler) from other tissues, subtle changes in gene activity between the different varieties might be present due to genetic differences in the cis-regulatory regions (Guo and Moose, 2003; Zakharov *et al.*, 2004).

This has implications for the expression patterns of orthologous genes in rice, from which the upstream promoter would ultimately be isolated i.e. to what extent, will promoter control of gene orthologues in rice be different to those found in maize? This uncertainty was mitigated through comparison with microarray data from cells isolated using FACS (Section 2.2.6) of root cortex and stelar cells of rice (Nipponbare) which confirmed that some cortex or stelar-specific expression patterns are maintained. The additional step of comparison with the rice MPSS data (covered in section 2.2.6) (Nobuta *et al.*, 2007) was not useful in discerning expression patterns of candidates as transcript abundance registered below the background level of 25 TPM (Dr Andrew J. Harvey, Dept of Genetics and Bioengineering, Yeditepe University, Turkey, pers.comm.). This lack of measurable transcript was probably due to a dilution effect, the number of cortical and stelar transcripts being a small fraction of the transcripts for the whole root and so perhaps remaining undetected. The problem of the dilution effect was probably mitigated in the candidates which were found in the microarray data from FACS cells as the mRNA was collected from only cortex or only stele of the root. Consequently, it was assumed that if cortex-specific candidates were found only in the cortex and not in the stele, and the converse in the microarray dataset from FACS cortex and stelar cells, as was seen in the MPSS database and PCR of maize, that this was sufficient data with which to proceed. The maintenance of spatial activity of gene orthologues in maize and rice, i.e. if they were cortex-specific in both maize and rice was an indicator of similar gene regulation in rice. Whether these rice genes that correlated with cortex or stelar-specificity of the orthologues in maize had these candidates expressed elsewhere in the plant was not established as there was no microarray data for other tissues from rice that was generated from tissues isolated through FACS.

For the maize candidates that had rice orthologues that did not exhibit similar expression patterns in the microarray generated from FACS cells, such as C257, the corresponding promoter was isolated from B73 maize (Appendix 2.1).

2.4.2 **Rice promoters**

Rice promoters were preferred over promoters isolated from other cereals as the genome sequence for rice is complete and well annotated and so delineation of coding from non-

coding regions is more straightforward. (Appendix 2.1). A promoter isolated upstream of the *TaHKT1;5-D* (Byrt *et al.*, 2007) gene found on chromosome 4D of bread wheat was provided by Dr C.S. Byrt; this was a stelar-specific candidate to be used upstream of *HvHKT1;5* and transformed into barley (Appendix 2.1).

The spatial control of the promoters isolated from rice and maize and transformed into barley were tested by studying the expression patterns of the reporter genes *GFP* and *uidA*. The GFP reporter system was intended for non-destructive visualisation of promoter activity. However, no unambiguous fluorescent signal was detected in any of the seedlings. This might be due to an anatomical limitation. Barley roots have fairly thick epidermal layers and root hairs which develop early in the seedlings making it difficult for the laser to penetrate to the inner tissues. Sectioning of the tissues to help mitigate this anatomical limitation was also performed but with no conclusive result. This difficulty was compounded by the fact that the expected signal was low as a result of low promoter activity. The GFP in the construct pMDC107 also had no targeting signal which meant that it would be diffused throughout the cytoplasm. The promoters were identified for their low- moderate activity which might have been too low for detection of GFP which will be diffused through the cytosol as it does not contain a targeting signal. In the future, this difficulty can be tackled by using GFP with an endoplasmic reticulum (ER)-targeting signal, meaning that the signal can be concentrated in one location and thus potentially provide greater contrast and so be more readily discerned. Another possibility for increasing the GFP signal would be through exploiting an enhancer system similar to that developed by (Haseloff, 1999). By placing the enhancer cassette like GAL4 downstream of the weak promoter, the GFP signal can be amplified.

GUS reporter lines were developed to circumvent the problem of detecting low promoter activity. Despite multiple attempts at assaying for GUS activity using negative and positive controls, the staining results were inconclusive, as they could not be replicated and were inconsistent between assays. Again, this might have been a result of low promoter activity combined with thick epidermal layers in the barley roots. Detection of reporter gene expression through an RT-PCR approach could have been attempted but due to time

constraints this was not carried out. Enhanced GUS expression through use of an enhancer cassette as described by Haseloff (1999) would reduce ambiguity in the reporter assay.

As promoter activity could not be assessed successfully through reporter gene expression, the regions of control of the promoters will need to be assessed by studying transgenic barley plants containing salt tolerant genes. These plants will contain constructs with promoters driving *HvHKT1;5* and *HvHVP1* (as discussed in this chapter) and will be studied for phenotypic changes by measuring leaf sodium and potassium. Transgene activity will also be studied through PCR of *HvHKT1;5* and *HvHVP1* in transgenic plants and this will be indicative of promoter activity as well.

2.5 Conclusion

With promoter engineering gaining increasing significance due to its application in the generation of plants with targeted genetic manipulation, the process of identifying potential candidates through first searching MPSS data is a viable strategy. Here an attempt was made to identify promoter candidates that control low-moderate expression of genes, as it would be particularly useful if the gene being controlled by the promoter is toxic to the plant at high levels or diverts energy away from plant growth and development.

While the experimental results generated through PCR were generally consistent with the *in silico* MPSS data, the promoters isolated upstream which control these gene candidates still need to be tested for specificity. This is especially important here as the gene candidates identified in maize were used to find orthologues in rice. The region upstream of this gene was used as promoters to be transformed into barley. Gene regulation could vary between the different species. Placing a reporter gene downstream of a promoter and then transforming this construct into a plant can help confirm spatial and temporal patterns of the downstream gene of the promoter although this was not successful in this study. It is suggested that the reporter constructs used for this project had expression levels which could not be detected and so promoter control could not be visualised through GFP or GUS staining. A promoter that controls gene expression levels in the 1000 TPM range is apparently not sufficiently active for

standard reporter gene assays but as outlined above, there are means of amplifying the reporter gene signal which can be used in the future (Haseloff, 1999).

The promoters identified in this study were simultaneously used to control transgene expression of *HvHKT1;5* and *HvHVP1* in barley to study the effects of cell type-specific expression of these two genes in Na^+ accumulation in the shoots. Transcript levels of the transgene will also be studied to confirm promoter activity and also correlate any changes in shoot Na^+ or K^+.

3 Cell type-specific overexpression of *HvHKT1;5* and *HvHVP1* in barley as a strategy to increase its salt tolerance

3.1 Introduction

Of the commercially grown cereal crops barley is the most salt tolerant (Colmer *et al.*, 2005). Barley is an efficient accumulator of shoot Na^+ and studies have revealed that there is little correlation between the presence of Na^+ in the barley shoot and salt tolerance (Greenway and Munns, 1980; Gorham *et al.*, 1990; Chen *et al.*, 2005; Shabala *et al.*, 2010). The line Golden Promise is salt tolerant and also has a high regeneration rate following gene transformation using *Agrobacterium tumefacians* (Finnie *et al.*, 2004; Dahleen *et al.*, 2007). Golden Promise was used in this study to investigate the effects of two known salt tolerance genes on overall phenotype. A known shoot salt accumulator was used to study the role of genes in shoot salt exclusion on the basis that any effect of the genes in limiting root-to-shoot sodium translocation would be particularly pronounced given the inherent tendency of the Golden Promise line to allow root-to-shoot Na^+ translocation. Further, it was anticipated that any Na^+ exclusion observed in the transformed plants would be due primarily to the presence of the transgenes.

HKT1;5, found in monocots and its orthologue in *A. thaliana*, *AtHKT1;1* have been characterised as having Na^+- specific transport capabilities. *AtHKT1;1*/*HKT1;5* is found expressed mainly in the stelar cells of the root in order to unload Na^+ from the xylem sap back into the cells immediately around the xylem vessels (Mäser *et al.*, 2002; Berthomieu *et al.*, 2003; Ren *et al.*, 2005; Byrt *et al.*, 2007; Davenport *et al.*, 2007; Møller *et al.*, 2009; James *et al.*, 2011; Munns *et al.*, 2012). There are also reports of shoot expression of *AtHKT1;1*/*HKT1;5* (Mäser *et al.*, 2002; Berthomieu *et al.*, 2003; Ren *et al.*, 2005; Sunarpi *et al.*, 2005) which invoke a phloem recirculation theory whereby excess Na^+ in shoot is loaded into the phloem which is then recirculated to the roots (Mäser *et al.*, 2002; Berthomieu *et al.*, 2003; Sunarpi *et al.*, 2005). However, evidence does not support a role for *HKT* activity in the shoot in controlling Na^+ accumulation in the shoot (Rus *et al.*, 2006; Byrt *et al.*, 2007; Davenport *et al.*, 2007; James *et al.*, 2011; Munns *et al.*, 2012).

The Na^+ transporter in barley encoded by *HvHKT1;5* has not been functionally characterised, although it shares approximately 88% amino acid similarity with bread and durum wheat orthologues (Appendix 3.1). The assumption made here is that the barley Na^+ transporter functions in the same way as the wheat orthologues, that is, by reducing root-to-shoot translocation of Na^+ by unloading Na^+ from the root xylem parenchyma into the cells surrounding the xylem vessels of the root. By transforming barley plants with cortex and stelar-specific promoters driving *HvHKT1;5*, the aim of this study was to establish whether *HvHKT1;5* can play a role in barley salt tolerance through the Na^+ exclusion mechanism. Transforming barley plants with *HvHKT1;5* under the control of cell type-specific promoters would also help establish whether increased exclusion of Na^+ could be engineered, as had been done in *A. thaliana*.

A. thaliana plants overexpressing the *HKT1;5* orthologue, *AtHKT1;1* using the 35S promoter were found to hyperaccumulation Na^+ in the shoot (Møller *et al.*, 2009). Although the root Na^+ concentration was not measured, it was suggested by Møller *et al.* (2009) that this shoot hyperaccumulation of Na^+ was a result of increased unidirectional influx. Thus, cell type-specificity of *AtHKT1;1* or *HKT1;5*, for that matter, appears to be crucial to limiting Na^+ accumulation in the shoot. It is expected that, by expressing *HvHKT1;5* under the control stelar- or cortex-specific promoters, that similar results to the study by Møller *et al.* (2009) and Plett *et al.* (2010) will be observed, with reduced Na^+ accumulation in the shoot.

The inorganic vacuolar H^+ pyrophosphatase (*H^+-PPase*), *HvHVP1*, in barley has been found to be upregulated in roots during salt stress and, to a lesser extent, osmotic stress (Fukuda *et al.*, 2004). Overexpression studies of orthologues of *HvHVP1* in *A. thaliana* and alfalfa demonstrate that the Na^+ content in leaves is increased suggesting that the pyrophosphatase works in conjunction with Na^+/H^+ antiporters that sequester Na^+ into the vacuole (Guo *et al.*, 2006; Brini *et al.*, 2007; Bao *et al.*, 2009). By establishing a proton gradient within the vacuole, the H^+-PPase, facilitates the inward Na^+ transport by Na^+/H^+ antiporters into the vacuole (Venema *et al.*, 2002; Barragán *et al.*, 2012). This sequestration of Na^+ into the

vacuole limits rises in the Na^+ content in the cytoplasm upon salt stress, thereby enabling normal cytoplasmic processes to continue.

Additionally, aside from the increased salt and drought tolerance in plants overexpressing the H^+-PPase biomass accumulation appears to be greater in the transgenic plants compared with null segregant/wildtype controls. A study in rice in which *OVP1* was constitutively overexpressed revealed that the rice plants accumulated more biomass and grew more vigorously. The mechanisms underpinning the observed increase in growth, however, remain unclear. A study by Li *et al.* (2005) suggested that increased expression of the pyrophosphatase results in acid leakage into the extracellular space which promotes auxin-induced growth. A more recent study, however, has established that the increase in growth observed in plants overexpressing the pyrophosphatase is caused not by the movement of H^+ but instead by the liberation of phosphates from pyrophosphate molecules. High concentrations of pyrophosphate in the cytoplasm inhibits plant growth and its breakdown makes available phosphates which can then be reused in the plant metabolic pathways (Ferjani *et al.*, 2011).

Native expression of *HvHVP1* has reported to be in both the shoot and root, but is increased only in the roots when the plant is under salt stress (Fukuda *et al.*, 2004). This indicates its primary role in the root, in the sequestration of Na^+. This is the basis for the overexpression of *HvHVP1* under a putative cortex-specific promoter. Cortex cells were selected as ideal cell-types as they have relatively large cell volumes and can act as a pre-stelar barrage for the influx of Na^+ in roots. It can also sequester any Na^+ being unloaded from the xylem tissues by native *HvHKT1;5*.

The aim of this study was to increase the unloading of Na^+ by *HvHKT1;5* from the xylem transpiration stream and to facilitate an increase in sequestration of Na^+ in the cortical vacuoles through *HvHVP1*, the overall effect being an anticipated lowering of Na^+ accumulation in the shoot.

3.2 Materials and methods

3.2.1 Isolation of *HvHKT1;5* and *HvHVP1*

3.2.1.1 Primer design

Sequences for the genes of interest were downloaded from Genbank, *HvHKT1;5* and *HvHVP1* having Genbank accession numbers DQ912169.1 and AB032829.1, respectively. The sequences were used to design gene-specific primers (Table 3.1) using the default parameters of an online primer design software platform (Netprimer, Premier Biosoft International). Aside from gene specific primers, primers were also designed to contain restriction enzyme recognition sites in both forward and reverse primers (Table 3.1). The restriction sites were selected in order that they were not present in the genes and were present once only in the destination vector, pTOOL36 (modified pMDC32 which does not contain a 2 x 35S promoter). The *HvHKT1;5* forward and reverse primers contained a Pac I and a Dra I recognition site at the 5' and 3', respectively; the *HvHVP1* forward and reverse primers contained a Pac I and a Sac I site at the 5' and 3' site, respectively. An additional reverse primer - containing the same restriction sites but with a haemagglutinin (HA) epitope tag from the gene of interest with the stop codon removed - was designed for each gene (Table 3.1). The HA tag was incorporated into the gene sequence to enable immunolocalisation of the ectopically expressed gene products in the transformed plants.

Table 3.1: *HvHKT1;5* and *HvHVP1* PCR primers and cycling conditions

Primer name	Forward primer	Reverse primer	Annealing temperature (°C)	Extension time (min)	Cycle number	Details
HvHKT1;5 FS/RE	5'-ATGGGTTCTTTGCATGTCTC-3'	5'-CTACACTATCCTCCATGCC-3'	50	2	40	Gene specific primers to amplify *HvHKT1;5* includes stop codon for GOI; length- 1,533 bp
HvHKT15 PacI F/HA Dra I R	5'-TTAATTAAATGGGTTCTTTGCATGTCTC-3'	5'-TTTAAAGCCCGCATAGTCAGGAACATCGTATGGGTACACTATCCTCCATGCC-3'	50	2	35	Gene specific primers with Pac I restriction site in the forward primer and a HA tag starting in place of the normal stop codon; reverse primer with Dra I restriction site
HvHVP1.F3/R3	5'-ATGGTGGCGGCGGCGAT-3'	5'-CTACAGAATCTTGAAGAGGATTCCTCCATA-3'	54	2.24	35	Gene specific primers to amplify *HvHVP1* includes stop codon for GOI; length- 2,398 bp
HvHVP1 PacI F/HA Sac I R	5'-TTAATTAAATGGTGGCGGCGGCGAT-3'	5'-GAGCTCGCCCGCATAGTCAGGAACATCGTATGGGTACAGAATCTTGAAGAGGATTCCTCC-3'	54	3	4	Gene specific primers with Pac I restriction site in the forward primer and a HA tag starting in place of the normal stop codon; reverse primer with Sac I restriction site. First 4 cycles with lower annealing temperature to allow annealing of reverse primer, followed by 31 cycles with annealing temperature of 58 °C
			58	3	31	

3.2.1.2 Growing **Hordeum vulgare** *(cv. Golden Promise) for RNA extraction*

Barley seeds of the variety Golden Promise were treated with 30% Domestos (Unilever Australasia) for 10 mins and rinsed 10 times with RO water and 5 times with high-pure water (18.2 MΩ•cm). Seedlings were germinated on Petri dishes at 24°C in sunlight and allowed to grow for 10 days before being transplanted for growth in hydroponics.

Plants were grown in a 12L tub covered with a lid containing drilled holes made to accommodate 50 ml BD Falcon Tubes (BD Sciences, NJ, USA) containing 10.5 L of aerated, modified Hoagland's Solution (Figure 3.1). The solution contained final concentrations of 0.2 mM NH_4NO_3, 5 mM KNO_3, 2 mM $Ca(NO_3)_2.H_2O$, 2 mM $MgSO_4.7H_2O$, 0.1 mM KH_2PO_4, 0.5 mM Na_2SiO_3, 0.05 mM NaFe(III)EDTA, 50 µM H_3BO_3, 5 µM $MnCl_2.4H_2O$, 10 µM $ZnSO_4.7H_20$, 0.5 µM $CuSO_4.5H_2O$, and 0.1 µM $Na_2MoO_4.2H_2O$ in RO water with the pH adjusted to 6.5.

The plants were grown for 3 weeks before having the shoots and roots harvested separately and snap frozen in liquid nitrogen for RNA extraction (Section 1.2.1.3).

Figure 3.1: Growth of barley plants in hydroponics.

The seedlings were placed in 1.5ml microcentrifuge tubes with the bottom cut off to allow root growth. These tubes were then placed in 50 ml Falcon tubes also with the bottom cut off and with a hole in the lid to accommodate the 1.5 ml tube. The 50 ml Falcon tubes containing the seedlings were placed on a lid which was placed in 12L rectangular tubes containing 10.5 L of modified Hoagland's solution.

3.2.1.3 RNA extracted from shoot and root

RNA was extracted from approximately 100 mg of frozen tissue as described in Chapter 2 section 2.2.4.

3.2.1.4 Generating cDNA

cDNA was synthesised as described in Chapter 2 section 2.2.5.

3.2.1.5 PCR conditions

The cDNA was used for PCR (MJ Research MPTC-225 Tetrad) with primers HKT FS/RE for amplification of *HvHKT1;5* (cycling conditions described in Table 3.1). One microlitre of the product from this PCR was then used for another PCR to conjugate a haemagglutinin (*HA*) epitope tag to the 3' end of the gene for subsequent immunolocalisation experiments. The gene-specific primers, HvHKT15 PacI F/HA Dra IR, also had restriction enzyme sites adapted onto the ends for insertion into the backbone of the destination vector, pTOOL36 (explained later in section 3.2.2.2), downstream of the multiple cloning site (MCS).

The same process was employed for *HvHVP1* which also had a *HA* epitope tagged to the 3' end of the gene for immunolocalisation purposes. As was done with *HvHKT1;5*, the forward and reverse primers for the nested PCR, HvHVP1 PacI/HA Sac IR also had restriction sites added in order to allow ligation into the backbone of the destination vector, pTOOL36.

3.2.2 **Generating constructs**

3.2.2.1 Entry vectors

The PCR products were cloned into the plasmid vector pCR8/GW/TOPO TA (Invitrogen) and then transformed into Mach 1 *Escherichia coli* strain cells (Invitrogen) using the heat shock method, both steps according to the manufacturer's instructions. This was performed as per the details in Chapter 2 section 2.2.7.2.

3.2.2.2 Destination vectors

pCR8- *HvHKT1;5* was digested with the endonucleases Pac I and Dra I and pCR8- *HvHVP1* with Pac I and Sac I (NEB Enzymes). One unit of each restriction endonuclease was added to 2 µl of 10X SDB buffer (containing 330 mM Tris, pH7.8, 650 mM KAc, 100 mM MgAc, 40 mM Sperimidine and 50 mM Dithiothreitol). The digests were incubated at 37°C for 16 hrs and were run on a gel and the gene fragment gel-purified (QIAquick Gel Extraction kit, Qiagen) according to the manufacturer's instructions. Simultaneously, the destination vector pTOOL36 (modified pMDC32) (Figure 3.2) was digested with Pac I and Ale I to be ligated with *HvHKT1;5*, and Pac I and Sac I to be ligated with *HvHVP1*, and gel-purification was carried out as with entry vectors as mentioned above. As it had multiple Dra I recognition sites, the destination vector backbone was digested with Ale I instead of Dra I. Conversely an Ale I recognition site was present in *HvHKT1;5* and so could not be conjugated to the primer sequence used to amplify *HvHKT1;5*. Both Ale I and Dra I generated blunt ends which meant that the resulting ends from the vector and the gene could be conjugated.

A volume of 90 fmol of the insert and 30 fmol of the pTOOL36 backbone were mixed with 4 µl of 5 X first strand buffer, 1 µl of T4 ligase and high-pure water was used to make up a total volume of 20 µl. The reaction was incubated at room temperature for 5 min before being incubated for 16 hrs at 4℃.

The ligations had to be transformed into the DB3.1 strain of *E. coli* cells as the destination vector contained a cytotoxic coupled cell division B (ccdB) gene (Hiraga *et al.,* 1986) in a Gateway recombination cassette. The DB3.1 strain is resistant to the gene product of the ccdB selection gene that is present in the destination vector.

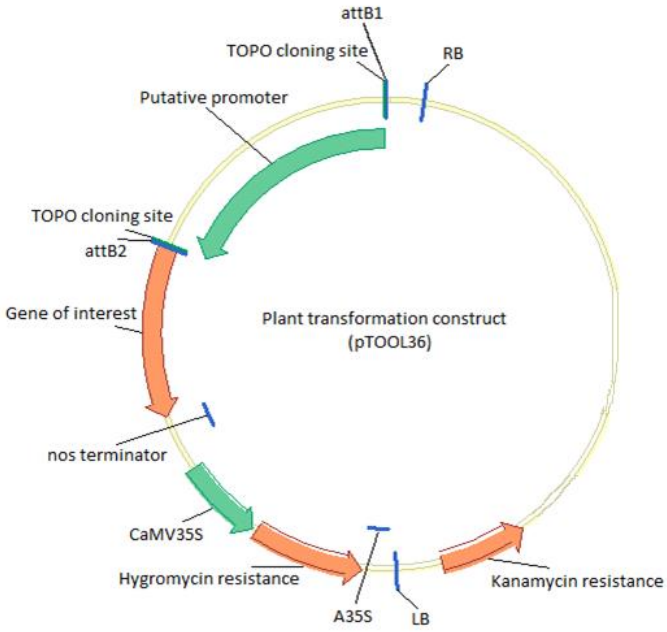


Figure 3.2: Destination vector pTOOL36 containing promoter upstream of the gene of interest; other features include Nos terminator; CaMV35S driving hygromycin resistance gene; A35S driving kanamycin resistance gene; left border and right border.

3.2.2.3 Promoter insertion into destination vectors

Once *HvHKT1;5* and *HvHVP1* were integrated downstream of the gateway site of pTOOL36, the putative promoters C34, C257, S147 and Ta (Chapter 2) in pCR8 (Invitrogen) were inserted upstream of the genes through an LR clonase reaction (detailed in Chapter 2 Section 2.2.7.3).

The plant transformation vectors containing the genes of interest downstream of the putative promoters were digested with different restriction enzymes to ensure correct orientation before being transformed into barley embryos. Restriction digests contained 0.2 µl of the restriction enzyme, with 2 µl of the relevant 10X reaction buffer, 4 µl of DNA and high-pure water made up to a total volume of 20 µl. All reactions were incubated for 16 hrs at 37℃.

3.2.3 **Transgenic plants**

The promoter reporter constructs were used to transform the AGL-1 strain of *Agrobacterium tumefacians* and then transformed into barley embryos, as described in Tingay *et al.* (1997) and Matthews *et al.* (2001), by Konny Beck-Oldach (ACPFG) and Rohan Singh (ARC Centre of Excellence in Plant Cell Walls, The University of Adelaide, Australia).

3.2.4 **Assaying transgenic plants**

3.2.4.1 Identifying single insert lines

A fully developed leaf blade was collected from transgenic plants and put into 1 ml tubes placed in a 96-well rack; each tube contained 3 small steel ball bearings and was placed in a -80℃ freezer for 16 hrs. The sample tubes without their lids were placed in a Christ Alpha 1-2 LD freeze-drier set at -50℃ and a vacuum pressure of 0.11 mBar for 16 hrs. The freeze-dried tissue was ground in a grinder (Retsch mill, Type MM 300, Düsseldorf, Germany) for 2.5 min at 25 rotations per second. A volume of 600 µL of extraction buffer (0.1M Tris-HCl at pH 7.5, 0.05M EDTA pH 7.5, 1.25% SDS made up in high-pure water) was added to the ground tissue, shaken thoroughly and incubated at 65℃ for 30 min. Samples were cooled at 4℃ for 15 min. Three hundred microlitres of 6M ammonium acetate was added to the samples which were incubated at 4℃ for 15 min. The plates containing the samples were centrifuged (Sigma

4-15 centrifuge, Germany) at 4,000 rpm for 15 min. Volumes of 600 µL of the supernatant were transferred to new collection tubes to which 360 µl of isopropanol was added and tubes were incubated at room temperature for 5 min to precipitate DNA. The samples were centrifuged at 4,000 rpm for 15 min so that the DNA formed a pellet, the resulting supernatant being discarded. The pellet was washed by adding 400 µl of 70% ethanol and centrifuged at 4000 rpm for 15 min. The supernatant was discarded and the pellet dried after which it was resuspended in 50 µl of high-pure water (18.2 MΩ•cm).

The DNA concentration of the samples was quantified using a spectrophotometer (Nanodrop ND-100, Thermo Fisher Scientific, Waltham MA). A mass of 7-8 µg of DNA was digested with 20 units of Hind III (New England Bioloabs) in a total volume of 10 µl with a final concentration of 1 X SDB buffer (33 mM Tris, pH 7.8, 65 mM potassium acetate, 10 mM magnesium acetate, 4 mM spermidine, 5 mM dithiothreitol). The reaction was incubated at 37℃ for 16 hrs to ensure complete digestion of DNA. The digested samples were loaded on a 0.5 mm thick agarose gel and electrophoresed at 33V for 16 hrs. The gel was stained with 100 ml of ethidium bromide staining solution (final concentration of 1 µg/ml ethidium bromide made up in high-pure water) for 20 min. The gel was rinsed with high-pure water and visualised on a UV transilluminator to confirm complete digestion of DNA samples.

The DNA from the agarose was transferred to a nylon membrane (Hybond N+, Amersham GE Healthcare Australia, NSW) by the Southern transfer method (Southern, 1975) using 0.4 M NaOH. The transfer was allowed to run for 16 hrs, the 0.4 M NaOH being topped up regularly.

Following transfer of the DNA to the nylon membrane, the membrane was washed with 2 X SSC (0.3 M NaCl, 0.03M Tri-sodium citrate made up in high-pure water) and then dried with paper towel. The DNA was cross-linked to the nylon membrane through brief (5 s) exposure to UV light and then incubated in 30 ml of prehybridisation solution which contained 5 X SSC, 0.25 mg/ml salmon sperm DNA, 10% Denhardt's III solution (100% v/v Denhardt's III

solution- 2% w/v BSA, 2% w/v Ficoll 400, 2% w/v PVP) made up in high-pure water in a hybridisation bottle. The hybridisation bottles, containing the membranes which were unravelled so that the DNA-containing side was exposed to the pre-hybridisation solution, were placed into a rotary hybridisation oven (Ratek Instruments, NSW, Australia) at 65℃ for 16 hrs. The pre-hybridisation solution was then replaced with 5 ml hybridisation solution which contained 0.075 X HSB (5X HSB-3 M NaCl, 100 mM Pipes, 25 mM Na_2EDTA at pH 6.8 adjusted with NaOH), 1.5ml of 100% v/v Denhardt's III solution, 0.375% w/v Dextran Sulphate and 12.5 µg/µl salmon sperm DNA made up in high-pure water). The hybridisation bottles containing the membranes and hybridisation solution were placed back into the hybridisation oven at 65℃ for 5 min after which the radioactive DNA probe was added.

The DNA probe was an 815 bp fragment from the gene hygromycin (kindly provided by Jodie Kretschmer, Australian Centre for Plant Functional Genomics, Adelaide, Australia) which was amplified by PCR using primers Hyg1 (5'-GTCGATCGACAGATCCGGTC-3') and Hyg2 (5'-GGGAGTTTAGCGAGAGCCTG-3') for 35 cycles using Platinum Taq DNA Polymerase (Invitrogen, Carlsbad CA) as described in Chapter 2, Section 2.2.5. The annealing temperature for the amplification of hygromycin probe was 55℃ and the extension time was 1min. The PCR product was run on an agarose gel and subsequently purified using QIAquick Gel Extraction Kit (Qiagen, VIC Australia) and quantified using a spectrophotometer (Nanodrop ND-100, Thermo Fisher Scientific, Waltham MA). Approximately 50 ng of probe DNA, 3 µl of 9-mer random primer mix (0.1 ng/mL) and high-pure water made up to 7.5 µl was mixed and boiled for 5 min and then rapidly chilled by placing on ice for 5 min. A volume of 12.5 µl of oligo buffer (containing 60 µM for dATP, dTTP and dGTP, 150 mM Tris-HCl pH 7.6, 150 mM NaCl, 30 mM $MgCl_2$ and 300 µg/ml BSA) was added to the mix containing the probe. To the mix containing the oligo buffer and probe DNA, 4 µl of radioactively labelled (α-^{32}P) dCTP and 1 µl of Klenow reagent (New England Biolabs) was added and this was incubated at 37℃ for 45 min. The reaction was run through a Sephadex G-100 column (Sigma-Aldrich) constantly topped up with 1X TE buffer. A Geiger counter (Mini-instruments, Mini-Monitor gm meter Series 900) was placed adjacent to two collection tubes. The flow through was collected in the first tube until the counts per second (cps) reached 500 cps, after which the flow through was collected in a second tube. Collection in

the second tube was stopped after the radiation counts fell below 500 cps. This second collection tube contained the labelled probe to which 500 µl of salmon sperm carrier DNA (5 mg/ml) was added, boiled 5 min and then chilled on ice for 5 min. The probe mixture was then added carefully to the centre hybridisation bottle containing the hybridisation solution and membrane and then incubated for 16 hrs at 65℃ in a hybridisation oven.

Following hybridisation, the membranes were washed by first replacing the hybridisation buffer in the hybridisation bottle with 40ml 2XSSC, 0.1% SDS and incubating in the hybridisation oven for 20 min at 65℃. The membranes were then removed from the hybridisation bottle and placed in a rectangular sealed container. Three successive membrane washes were performed in the container with the second wash containing 1XSSC, 0.1% SDS, the third wash containing 0.5XSSC, 0.1% SDS and the final wash containing 0.2XSSC, 0.1% SDS; all washes were incubated at 65℃ for 20 min in a shaking water bath (Ratek Instruments, SWB20, Victoria, Australia). The membranes were blotted dry, covered with a plastic sheet and placed in a pre-cooled (-20℃) X-ray film cassette with an X-ray film (Fuji Super HR-T30) in a dark room. The cassette containing the X-ray film and the radioactively labelled membrane were then stored at -80℃ for two weeks. The X-ray film was developed in an X-ray developer (AGFA Curix X-ray film developer, Mortsel Belgium) in a dark room.

3.2.4.2 Salt stress study

Following Southern hybridisation of the transgenic barley DNA, single transgene insert lines were identified for further study. Two independent, single insert lines from each construct and 8 seeds (biological replicates) from each line were used for salt assays along with a set of null segregants generated from a separate study (kindly provided by Hweiting Tan, PhD student, ARC Centre of Excellence in Plant Cell Walls, University of Adelaide, Australia). Null segregants were chosen as more appropriate controls than wild-type as they were generated through the same process of transformation under similar conditions and stored for a similar length of time in a similar manner to the seeds that were generated in the current project. Null segregants from another study were used as the number of available seeds from this study were low. Seeds were germinated on Petri dishes and then transferred to two hydroponics

solution treatments, a control and a saline hydroponics solution. After 10 days of growth the 4th leaf was harvested and the Na^+ and K^+ content measured in order to investigate the effect of the transgene on leaf sodium accumulation.

3.2.4.2.1 Growth of transgenic barley plants

Barley seeds were soaked in 30% Domestos (Unilever Australasia) for 3 min and rinsed with RO water and finally with high-pure water (18.2 MΩ•cm) to remove any traces of bleach. The seeds were placed in Petri dishes containing 110 mm diameter Whatman filter paper and 10 ml of high-pure water. The seeds were placed on the filter paper with the embryo side facing up and stored at 4℃ for a week to allow imbibition of water into the seed and then were allowed to germinate at room temperature. Once the cotyledon measured 10-20 mm and the first leaf had emerged from the coleoptiles, the plants were transplanted into 1.5 ml Eppendorf tubes without lids which were inserted into a hole in the lid of a 50 ml Falcon tube. The Falcon tube was placed into a cavity made by cutting out a hole in the lid of an 11 L tub as depicted in Figure 3.1. The tub contained 10.5 L ACPFG hydroponics solution (Section 3.2.1.2) with an aeration stone to provide oxygen for the roots. The solution was replaced every 2 to 3 days.

After the 3rd leaf had emerged, 100 mM NaCl with 3 mM $CaCl_2$ was added to one of the treatments. The salt application was made in increments of 25 mM NaCl, 0.75 mM $CaCl_2$ daily for 4 days. The time at which the 4th leaf emerged was recorded and, following 10 days of growth, was harvested. A week after 4th leaf was harvested, leaves, some sheath and roots were harvested from the plants for RNA extraction to confirm that the transgene was being transcribed. Some sheath and root tissue was left for transplantation into soil- modified U.C.Davis mix to mature and set seed.

3.2.4.2.2 Genotyping barley individuals in salt stress study

Sections of the leaf tip of 1-2 mm were cut with a sharp blade and stored in 20 µl of dilution buffer (Phire Plant Direct PCR Kit). The blade was wiped clean with 70% ethanol after collection of each sample to avoid contamination of subsequent samples. An 815 bp region in the hygromycin gene was amplified using primers Hyg 1- 5'- GTCGATCGACAGATCCGGTC-3' and Hyg 2- 5'- GGGAGTTTAGCGAGAGCCTG-3'. The PCR mix contained 0.5 µl of the dilution buffer that contained the leaf sample, 10 µl of 2 X Phire Plant PCR buffer, 0.5 µM of Hyg 1 and 2 primers, 0.4 µl of Phire Hot Start DNA polymerase and high-pure water to make up a total volume of 20 µl. The PCR consisted of an initial denaturation step at 98℃ for 5 min followed by 40 cycles of 98℃ for 5 s, 66℃ for 5 s and 72℃ for 20 s and then a final extension step of 72℃ for 1 min.

3.2.4.2.3 Measuring leaf sodium

The 10 day old 4th leaf had its fresh weight recorded immediately after harvest and was then dried at 65℃ in a drying oven (Contherm Designer 150 litre Model 8150, Petone, New Zealand) for 16 hrs and the dry weight recorded. The dried leaf samples were digested in 20 ml of 1% nitric acid for 4 hrs at 85℃ in a heat block (Environmental express hot block digester, Charleston SC). The Na^+ and K^+ content were measured using a flame photometer (Sherwood Flame Photometer Model 420, Cambridge, UK).

3.2.4.2.4 Management of data

The average leaf Na^+ or K^+ concentration for each independent transgenic barley line and standard error values were calculated using Microsoft Excel. All null segregants from each experiment were grouped together.

3.2.4.3 Confirming the transcription of the transgene

RNA was extracted from shoot and root tissue of barley plants (Chapter 2, section 2.2.4), and was quantified using a spectrophotometer (Nanodrop ND-100, Thermo Fisher Scientific, Waltham MA). Five micrograms of RNA was treated with 3 µl of DNase I (Applied Biosystems/Ambion) in a total volume of 30 µl as described in Chapter 2, section 2.2.5. The reaction was deactivated with the addition of 3 µl of DNase inactivation reagent to the reaction and incubation at 24℃ for 10 min. A volume of 5 µL of DNase I-treated RNA was used for cDNA synthesis as described in Chapter 2, section 2.2.4 in a total volume of 20 µl.

One microlitre of cDNA was used in a 25 µl PCR with Platinum Taq (Invitrogen) (Chapter 2 section 2.2.5) of glyceraldehyde-3-phosphate dehydrogenase (GAPdh) for 30 cycles to check both the integrity of the cDNA and the template loading consistency between the various samples.

Two microlitres of cDNA was used for a 25 µl PCR with Platinum Taq to detect the presence of transgene mRNA. Primers used for this reaction were a forward primer from the middle of the transgene, and a reverse primer for the Nos terminating sequence. Ectopic *HvHKT1;5* transcript was detected with a forward primer HvHKT15 SF2- 5' CGGCTACGACCACCTC 3' and reverse NosT primer 5'CATCGCAAGACCGGCAACAGGATTC 3' which generated a 749 bp product. Ectopic *HvHVP1* transcript was detected with a forward primer HvHVP1 SF3- 5' ACGACCGTTGATGTCCTGA 3' and reverse NosT primer- 5' CATCGCAAGACCGGCAACAGGATTC 3' which generated a 685 bp product. PCR for experiment batches 1 (lines 308-2, 308-5, 310-3 and 310-8) and 2 (lines 312-4, 312-5, 314-6, 314-7) involved the PCR conditions, as instructed by the manufacturer, with 30 cycles of 94℃ for 30 s, 55℃ for 30 s, 72℃ for 1 min and a final incubation step at 72℃ for 10 min.

PCR for batch 3 (lines 316-10, 316-20, 318-10, 318-11) involved similar conditions to those used for batches 1 and 2, but with 28 rather than 30 PCR cycles.

The cDNA samples generated from the null segregant lines had both HvHKT15 SF2 and HvHVP1 SF3 added as forward primers with NosT as the reverse.

3.3 Results

3.3.1 **Identification of single insert line**

Only those lines which displayed single bands for hybridising with the Hygromycin probe in the Southern blot were selected as single insert lines and used for further analysis (Table 3.2).

Table 3.2: Independent transformants containing a single insert of the transgene. *The transgenic line number corresponds to the construct and the individuals noted in the table are single insert lines.*

Line number	Individual	Construct name	Description
308	2,5,9,10,11,13,14,19,21,22,23	MK13	Cortex promoter C34 upstream of *HvHKT1;5*
309	1,2,4,14,18,19	MK14	Cortex promoter C34 upstream of *HvHKT1;5* with *HA* epitope tag
310	3,8,9,12,13,20	MK15	Cortex promoter C34 upstream of *HvHVP1*
311	1,2,3,8,9,10,14,15,17,18,20,22	MK16	Cortex promoter C34 upstream of *HvHVP1* with *HA* epitope tag
312	4,5,8,10,13,17,19,20	MK17	Cortex promoter C257 upstream of *HvHKT1;5*
313	1,14,16,17	MK18	Cortex promoter C257 upstream of *HvHKT1;5* with *HA* epitope tag
314	6,7,11,12,15,16,22	MK19	Cortex promoter C257 upstream of *HvHVP1*
315	1,2,7,8,9,10,11	MK20	Cortex promoter C257 upstream of *HvHVP1* with *HA* epitope tag
316	4,10,15,20	MK25	Stelar promoter S147 upstream of *HvHKT1;5*
317	1,3,6,10,12,13,15,17,19	MK26	Stelar promoter S147 upstream of *HvHKT1;5* with *HA* epitope tag
318	10,11,19	MK29	Stelar promoter Ta upstream of *HvHKT1;5*
319	4,17	MK30	Stelar promoter Ta upstream of *HvHKT1;5* with *HA* epitope tag

3.3.2 **Accumulation of Na^+ and K^+ in 4th leaf of transgenic barley**

Plants containing the transgene insert were grouped into their respective lines and those that were null segregants were grouped with other null plants.

Three batches of control and salt treatments were carried out, each with a group of null segregants (G297-17-10 null segregant line provided by Ashley Tan and null segregants from lines generated in this project) and two independent lines from a single construct.

3.3.2.1 Control treatments

Control treatments of the transgenic plants were carried out in order to assess the Na^+ and K^+ profile of the 4th leaf sap under non-stressed conditions. The results from these treatments would be compared with the results from salt-stressed plants in order to assess whether the rate of Na^+ or K^+ accumulation differs with differing growth environments.

The 4th leaf of plants grown under control conditions generally had low Na^+ and high K^+ levels. The accumulation of Na^+ and K^+ was also highly variable within each line and the resultant data failed normality tests. Other non-parametric methods were inapplicable to the measurements. Consequently, the significance of differences between the means of the different lines could not be determined. However, a trend has been inferred from comparing the means of the Na^+ and K^+ content in the 4th leaf of the plants from the different lines.

3.3.2.1.1 Transgenic plants containing HvHKT1;5 *or* HvHVP1 *under the control of C34 accumulate less Na^+ than the null segregant group under control conditions. Accumulation of K^+ tends to be constant in these lines*

Independent lines used in this experiment were 308-2 and 308-5 which contained *HvHKT1;5* driven under C34 (cortex-specific promoter) and also 310-3 and 310-8 which contained *HvHVP1* downstream of the same promoter. As these lines were T1 and thus segregating for

the transgene there were no individuals that survived containing the transgene for line 308-5. Surviving individuals of 308-5 were null for transgene and so were included in the null segregant group.

Under control conditions, 310-8 accumulated the least Na^+ in 4th leaf with a 27% reduction compared with the null segregants although the Na^+ levels were very low. Lines 308-2 and 310-3 also accumulated less Na^+ in 4th leaf than the null segregants with a 9% and 3% reduction, respectively (Table 3.3).

Under control conditions, line 308-2 containing the transgene accumulated 9% less Na^+ than the null segregant group, but was no different from line 310-3 in terms of accumulation of Na^+. Line 310-8 accumulated the least 4th leaf sap sodium compared with the null segregants, 308-2 or 310-3. The potassium levels in 4th leaf sap were found to be essentially the same for null segregants, 308-2, 310-3 and 310-8 (Figure 3.3b). The K/Na ratio was also not remarkable in that there was not much variability between the various lines (Figure 3.3c).

Table 3.3: Percentage differences of Na^+, K^+ and K^+/Na^+ for transgenic lines grown under control or salt-stress conditions compared with null segregants

Control treatment	Percentage difference from respective null group in [Na^+] in 4th leaf sap (%)	Percentage difference from respective null group in [K^+] in 4th leaf sap (%)	Percentage difference from respective null group in K^+/Na^+ in 4th leaf sap (%)
1st experiment			
308-2 (n=6)	-9	-18	-10
310-3 (n=4)	-3	-2	+1
310-8 (n=4)	-27	-13	+14
2nd experiment			
312-4 (n=7)	+1	-2	-2
312-5 (n=5)	+1	5	+4
314-6 (n=7)	+5	5	-1
3rd experiment			
316-10 (n=5)	-4	-3	+0.2
316-20 (n=6)	-8	-3	+5
318-10 (n=5)	-5	-1	+4
318-11 (n=5)	-11	-7	+6
Salt stress treatment (100 mM NaCl)	Percentage difference from respective null group in [Na^+] in 4th leaf sap (%)	Percentage difference from respective null group in [K^+] in 4th leaf sap (%)	Percentage difference from respective null group in K^+/Na^+ in 4th leaf sap (%)
1st experiment			
308-2 (n=2)	-8	+1	+7
308-5 (n=2)	-3	-4	-3
310-3 (n=7)	+5	-5	-10
310-8 (n=2)	+4	-12	-18
2nd experiment			
312-4 (n=4)	+14	-18	-28
312-5 (n=2)	+3	+3	-0.4
314-6 (n=6)	+11	-15	-24
314-7 (n=2)	+6	-9	-16
3rd experiment			
316-10 (n=6)	-15	-9	+8
316-20 (n=4)	-8	+5	+14
318-10 (n=5)	-2	-7	-4
318-11 (n=8)	-0.02	-11	-11

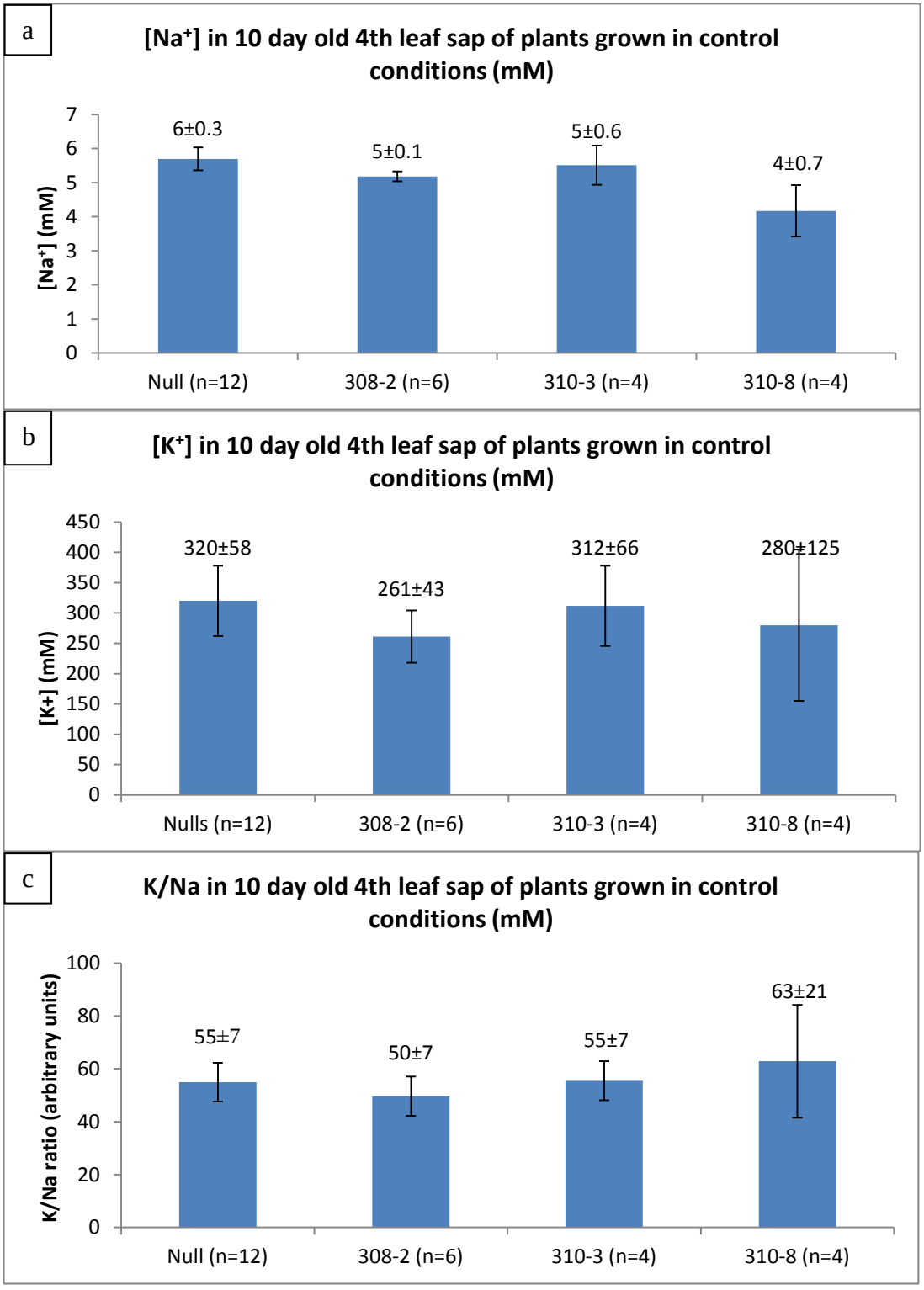


Figure 3.3: Concentrations of Na^+ (a), K^+ (b) in 4th leaf sap and K^+/Na^+ ratio (c). Raw values are indicated above the respective bars. *Number of individuals per line is indicated adjacent to the lines. Line 308-2 contains a putative cortex-specific promoter C34) upstream of HvHKT1;5; the other independent line (308-5) had no surviving individuals containing the transgene. Lines 310-3 and 310-8 contain HvHVP1 downstream of a putative cortex-specific promoter, C34.*

3.3.2.1.2 Transgenic plants containing HvHKT1;5 *or* HvHVP1 *under control of C257 show no changes in Na^+ accumulation compared with the null segregant group and in fact appear to increase Na^+ content under control conditions; K^+ levels appear to be generally higher in the transgenic plants.*

The independent lines used in this batch were 312-4 and 312-5 which contained *HvHKT1;5* driven under C257 (cortex-specific promoter), and 314-6 and 314-7 which contained *HvHVP1* driven under the same promoter. Surviving individuals of line 314-7 did not have the transgene and thus were grouped with null segregants from other lines. There were no remarkable differences in terms of Na^+ accumulation between the null segregants, 312-4 and 312-5 (Figure 3.4a). Line 314-6 had 5% higher Na^+ in the 4th leaf sap than the null segregants whilst not differing from 312-4 or 312-5 in terms of Na^+ accumulation (Table 3.3). Interestingly, 314-6 also accumulated higher K^+ in the 4th leaf sap compared with the null segregants, as did 312-5; 312-4, however, actually appeared to accumulate the least 4th leaf K^+ compared with the other lines (Figure 3.4b). The K/Na ratios did not differ greatly between the lines (Figure 3.4c).

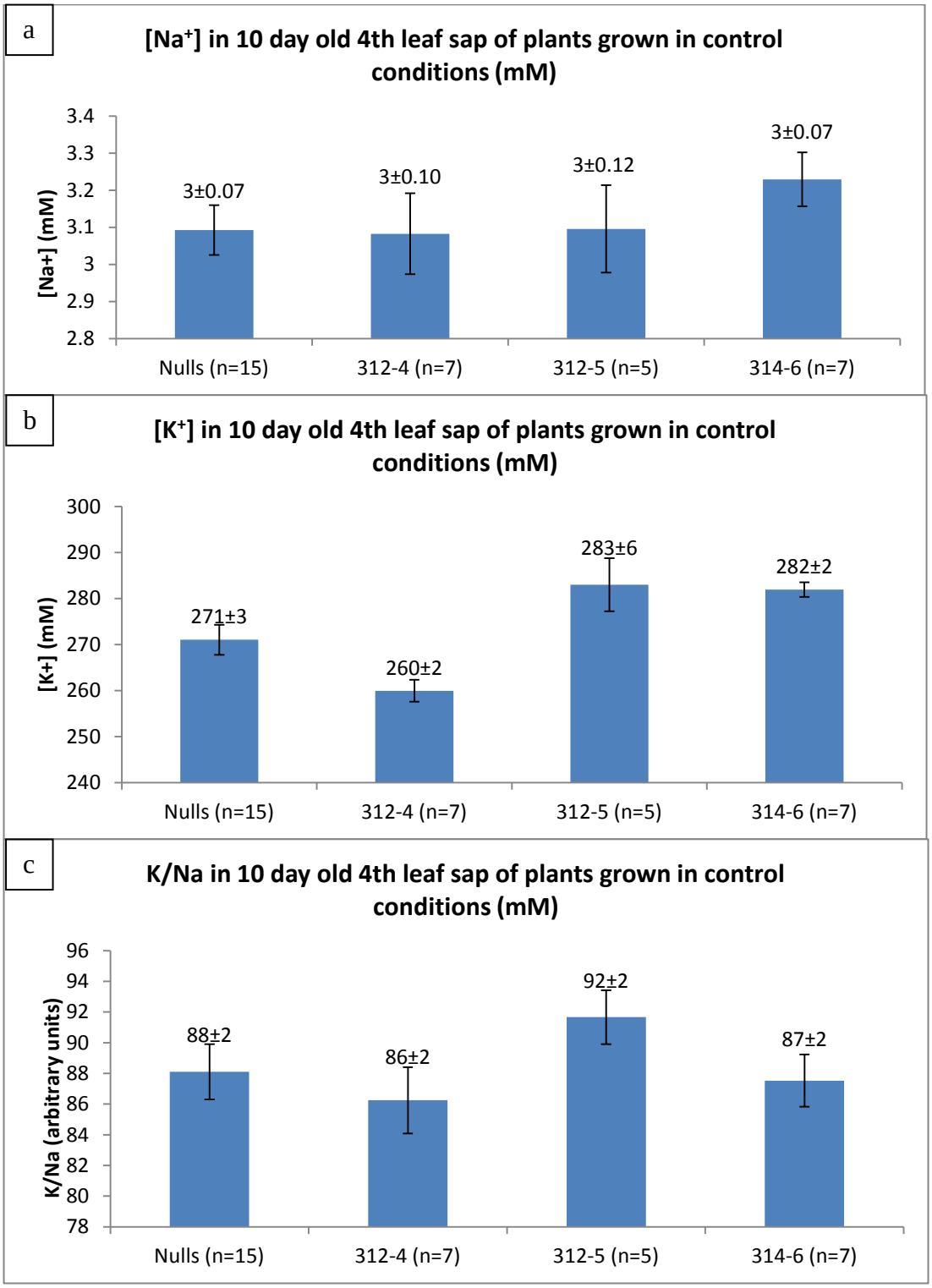


Figure 3.4 Concentrations of Na^+ (a), K^+ (b) in 4th leaf sap and K^+/Na^+ ratio(c). Raw values are indicated above the respective bars. *Number of individuals per line is indicated next to the lines. Lines 312-4 and 312-5 contain a putative cortex-specific promoter, C257, upstream of HvHKT1;5; line 314-6 contains HvHVP1 downstream of a putative cortex-specific promoter, C257.*

3.3.2.1.3 *Transgenic plants containing* HvHKT1;5 *under control of S147 and Ta accumulate less Na^+ and K^+ compared with null segregant group; K^+/Na^+ ratios, however, did not vary much between the different lines.*

The independent lines used in this experiment were 316-10 and 316-20, which contained *HvHKT1;5* under the control of S147; and 318-10 and 318-11 which contained *HvHKT1;5* under the control of Ta. All lines- 316-10, 316-20, 318-10 and 318-11 have lower Na^+ accumulation in the 4^{th} leaf sap compared with the null segregants (Figure 3.5a), 318-11 having the most dramatic reduction of 11% (Table 3.3). Line 318-11 also had the lowest K^+ accumulation in the 4^{th} leaf sap compared with other lines and 7% less than the null segregants (Table 3.3), whereas line 316-10 had slightly lower K^+ in the 4^{th} leaf sap compared with the null segregants but was not different from lines 316-20, 318-10 and 318-11 (Figure 3.5b). The K/Na ratios in this study did not vary between the lines and the variability within the lines was quite high (Figure 3.5c).

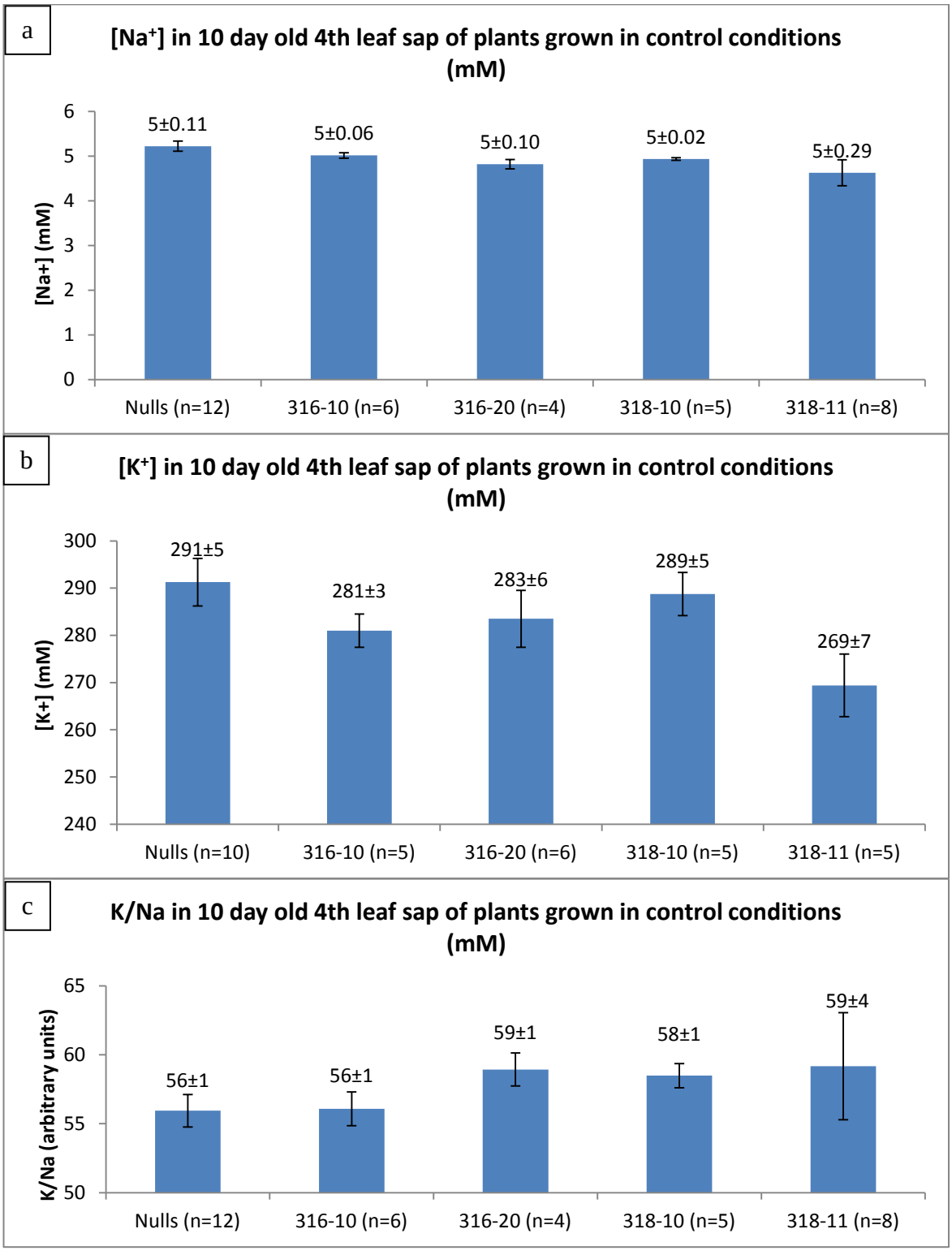


Figure 3.5 Concentrations of Na^+ (a), K^+ (b) in 4th leaf sap and K^+/Na^+ ratio(c). Raw values are indicated above the respective bars. *Number of individuals per line is indicated next to the lines. Lines 316-10 and 316-20 contain a putative stelar-specific promoter, S147, upstream of HvHKT1;5; lines 318-10 and 318-11 contain HvHKT1;5 downstream of a putative stelar-specific promoter, Ta.*

3.3.2.2 Salt stress treatment

The salt stress treatment was the key assay of the effect of high external Na^+ on the plants in terms of the exclusion of Na^+ and acquisition of K^+ in the 4th leaf.

3.3.2.2.1 Transgenic plants containing HvHKT1;5 *or* HvHVP1 *under control of C34 tend to accumulate less Na^+ in the 4th leaf than do null segregant group under salt stress, but K^+/Na^+ ratio is generally lower than for null segregant group*

The independent lines used in this experiment were 308-2, 308-5, which contained *HvHKT1;5* driven under C34 (cortex-specific promoter), - and 310-3 and 310-8 which contained *HvHVP1* downstream of the same promoter. As the T1 generation was segregating for the transgenic construct, many individuals were null for the transgene or lacked transgene activity (no transcript) and so were grouped with other null segregants.

With a reduction of 8%, line 308-2 had the lowest Na^+ accumulation compared with the null segregants; at 3%, its sibling line 308-5 had a less dramatic reduction in 4th leaf Na^+ compared with the null segregants (Table 3.3). Both sibling lines 310-3 and 310-8 had a 4% increase in 4th leaf Na^+ compared with the null segregants (Figure 3.6a). There were clear inverse trends observed for K^+ accumulation in all the lines: with the exception of 308-5, where Na^+ levels in the 4th leaf sap decreased, K^+ generally increased (Figure 3.6b).

While there was not a remarkable increase in 4th leaf [K^+] in 308-2 compared with the null segregants the K^+/Na^+ ratio increased by 7% as a result of the reduced Na^+ accumulation (Figure 3.6c). Line 310-8 had an almost 20% reduction in K^+/Na^+ compared with the null segregant group as a result of an approximate 10% reduction in K^+ accumulation in the 4th leaf sap compared with the null segregants. The reduction in K^+/Na^+ observed in line 310-3 was not as dramatic as that for 310-8, but 310-3 did have almost a 10% reduction in its K^+/Na^+ ratio compared with the null segregants, while having a reduction in [K^+] compared with the null segregants similar to that for 308-5.

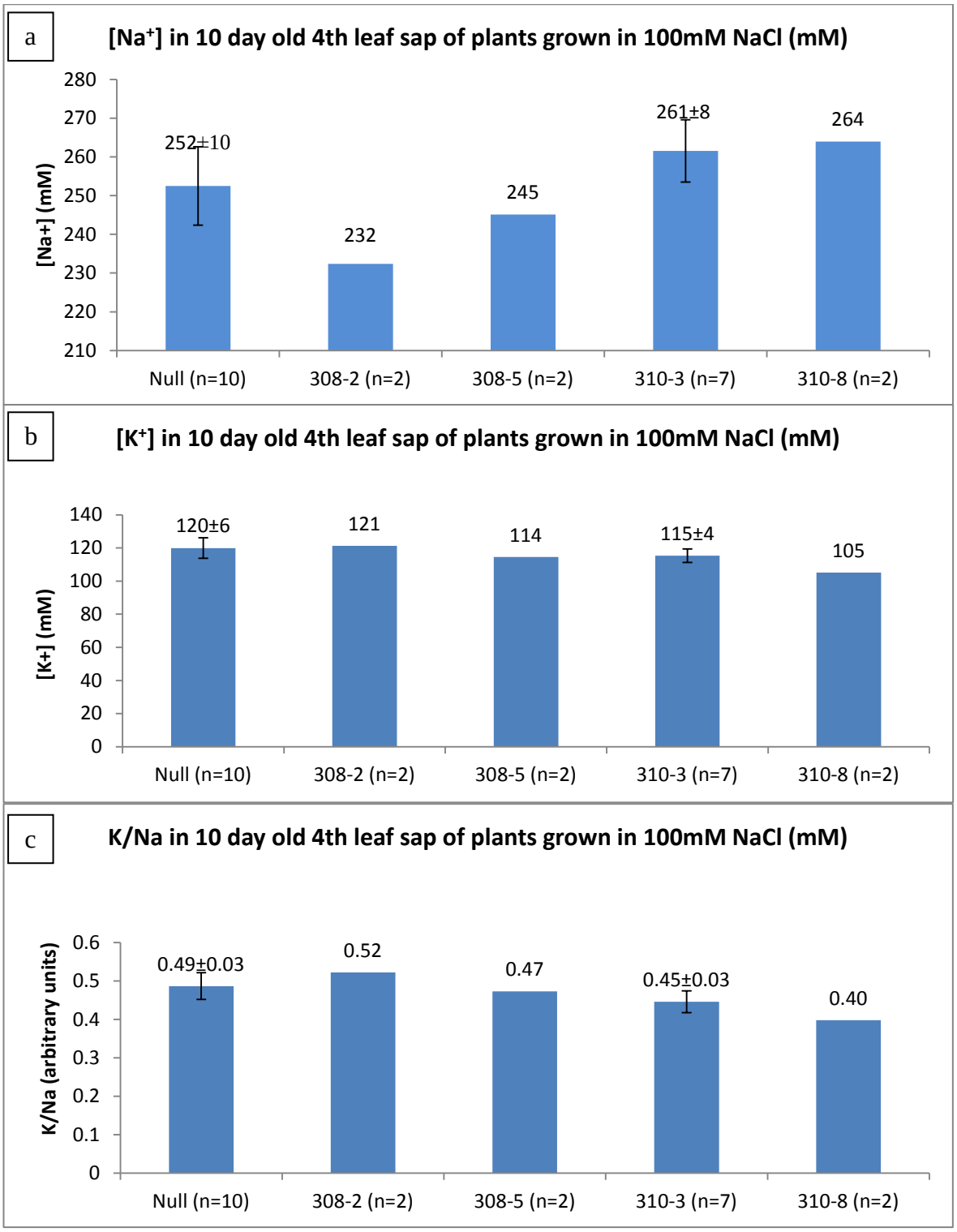


Figure 3.6 Concentrations of Na^+ (a), K^+ (b) in 4th leaf sap and K^+/Na^+ ratio (c) of plants grown in 100 mM NaCl + 3 mM $CaCl_2$. Raw values are indicated above the respective bars. *Number of individuals per line is indicated next to the lines. Lines 308-2 and 308-5 contain a putative cortex-specific promoter, C34, upstream of HvHKT1;5; lines 310-3 and 310-8 contain HvHVP1 downstream of a putative cortex-specific promoter, C34.*

3.3.2.2.2 Transgenic plants containing HvHKT1;5 *or* HvHVP1 *under control of C257 tend to accumulate more Na^+ in 4th leaf than null segregant group. K^+/Na^+ ratio, by extension, is also lower in transgenic plants than in null segregant group*

The independent lines used in this batch were 312-4 and 312-5 which contained *HvHKT1;5* driven under C257 (cortex-specific promoter) and 314-6 and 314-7 which contained *HvHVP1* driven under the same promoter.

All lines in this experiment accumulated higher Na^+ in the 4th leaf compared with the null segregants, albeit, to varying levels (Figure 3.7a).

Lines 312-4 and 314-6 accumulated most 4th leaf Na^+ with a more than 10% increase compared with the null segregants. Lines 312-5 and 314-7 accumulated considerably less Na^+ than their sibling lines compared with the null segregants (Table 3.3). Concomitantly, there was generally a decrease in 4th leaf K^+ in all lines, 312-4 and 314-6 accumulating almost 20% less K^+ than the null segregants (Figure 3.7b). Line 312-5 had slightly increased K^+ compared with the null segregants but this increase was not large nor did it increase the K^+/Na^+ ratio with respect to the null segregants (Figure 3.7c). Line 312-4 had the lowest K^+/Na^+ ratio with an almost 30% reduction when compared with the null segregants, closely followed by 314-6 which had an almost 25% lower K^+/Na^+ compared with the null segregants. The reduction in K^+/Na^+ for line 312-5 was negligible, while line 314-7 had an almost 20% lower K^+/Na^+ when compared with the null segregants (Figure 3.7c).

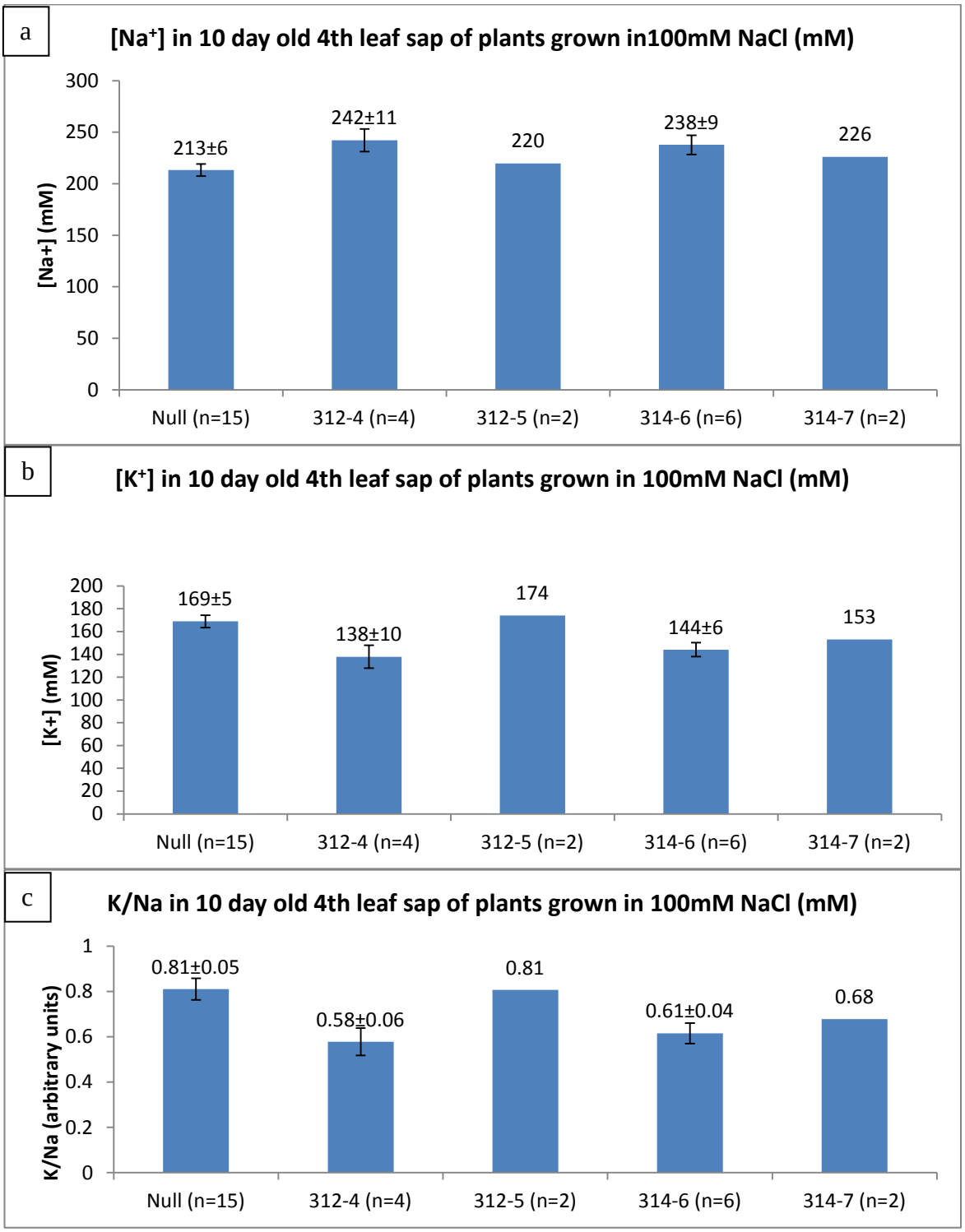


Figure 3.7 Concentrations of Na^+ (a), K^+ (b) in 4th leaf sap and K^+/Na^+ ratio (c) of plants grown in 100 mM NaCl + 3 mM $CaCl_2$. Raw values are indicated above the respective bars. *Number of individuals per line is indicated next to the lines. Lines 312-4 and 312-5 contain a putative cortex-specific promoter, C257, upstream of HvHKT1;5; lines 314-6 and 314-7 contain HvHVP1 downstream of a putative cortex-specific promoter, C257.*

3.3.2.2.3 Transgenic plants containing HvHKT1;5 *under control of S147 and Ta accumulate less Na^+ than null segregant group; lines containing* HvHKT1;5 *under control of S147 also have higher K^+/Na^+ ratio than null segregants.*

The independent lines used in this experiment were 316-10 and 316-20 which contained *HvHKT1;5* under the control of S147 and 318-10 and 318-11 which contained *HvHKT1;5* under the control of Ta. Compared with the null segregant group, both lines 316-10 and 316-20 had lower Na^+ content in the 4th leaf (Figure 3.8a), 316-10 having a dramatic reduction of 17% less Na^+ in the 4th leaf (Table 3.3). Lines 318-10 and 318-11 had similar levels of Na^+ accumulated in the 4th leaf sap compared with the null segregant group and by extension had higher Na^+ levels in the 4th leaf compared with 316-10 and 316-20 (Figure 3.8a).

Aside from line 316-10 which had lower Na^+ accumulation than line 318-11, all the other transgenic lines accumulated similar amounts of Na in the 4th leaf sap (Figure 3.8a).

The concentrations of K^+ were slightly lower in 316-10 but higher in 316-20 compared with the null segregants. Lines 318-10 and 318-11 both had lower K^+ levels in 4th leaf than the null segregants (Figure 3.8b).

The resultant K^+/Na^+ ratio was higher than the null segregant group for both lines 316-10 and 316-20 (Figure 3.8c), these lines having a 9% and 15% increase, respectively (Table 3.3). Lines 318-10 and 318-11 both had lower K^+/Na^+ levels in the 4th leaf proportional to the lower K^+ accumulation in the leaf sap when compared with the null segregants.

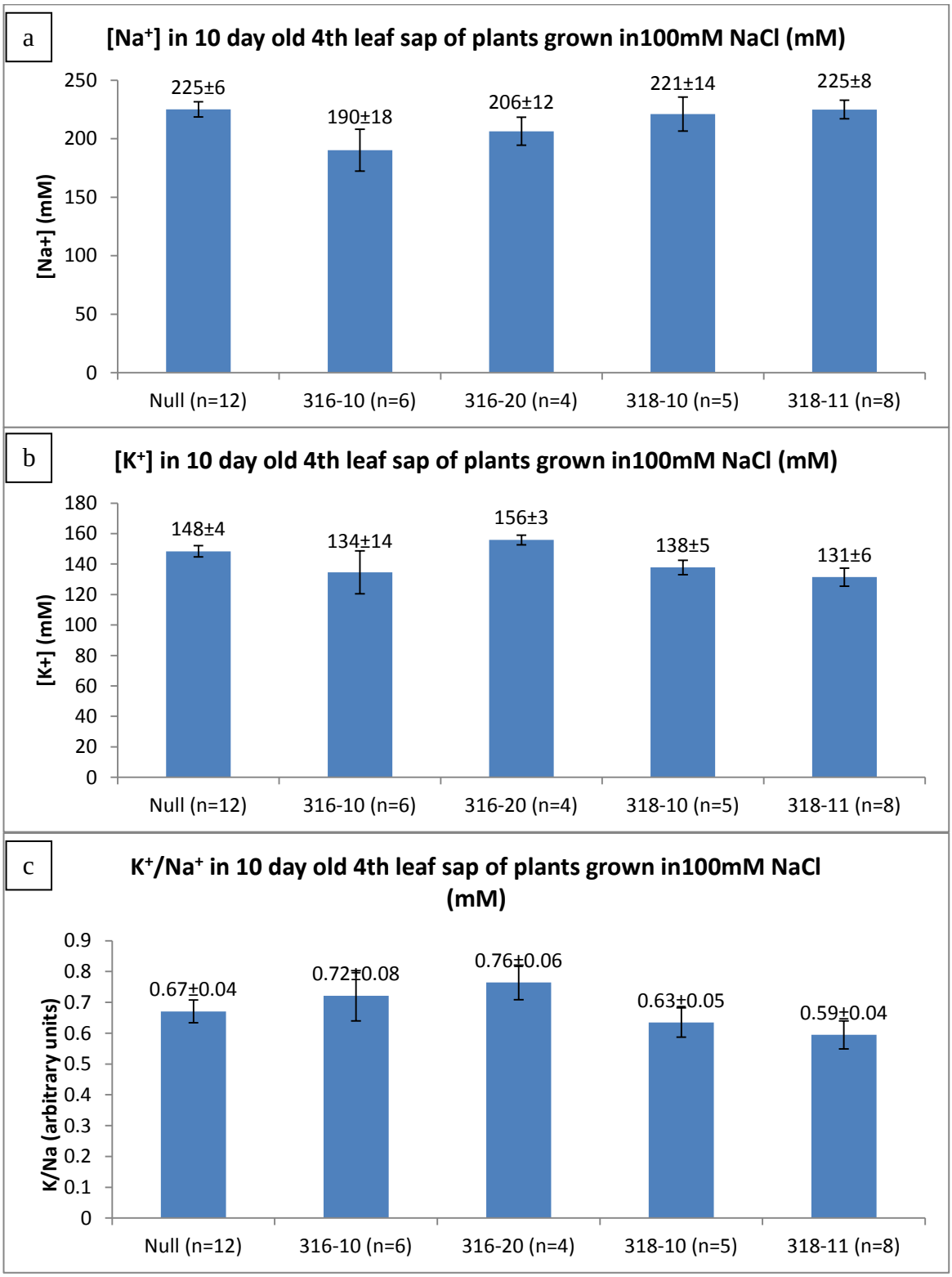


Figure 3.8: Concentrations of Na^+ (a), K^+ (b) in 4th leaf sap and K^+/Na^+ ratio (c) of plants grown in 100 mM NaCl + 3 mM $CaCl_2$. *Raw values are indicated above the respective bars. Number of individuals per line are indicated next to the lines. Lines 316-10 and 316-20 contain a putative stelar-specific promoter, S147, upstream of HvHKT1;5; lines 318-10 and 318-11 contain HvHKT1;5 downstream of a putative stelar-specific promoter, Ta.*

3.3.3 **Transgene mRNA**

Shoot and root RNA was extracted from the individuals in the salt stress experiments and synthesised into cDNA. This cDNA acted as a template for a PCR to confirm the presence of transgene mRNA. The PCR used a gene-specific forward primer and nosT reverse primer and a positive result implied that the transgene was expressed.

3.3.3.1 Salt stress experiment 1: transgene mRNA

The results of the PCR to detect the presence of the transgene for salt stress experiment 1 are presented in Figure 3.9 but have been summarised in Table 3.4 for easy perusal, as some of the results were faint. The loading control is not consistent throughout the samples but this was not seen as a problem as the purpose of this study was to detect presence/absence of transgene mRNA. The individuals from the lines 308-2 and 308-2 which were overexpressing *HvHKT1;5* under the putative cortex-specific promoter, C34 had variable spatial transgene activity (Table 3.4). Of a total of 16 individuals across the two independent lines, only 10 survived out of which 3 were null for the transgenic construct and one which lacked transgene transcript. Two individuals in 308-2 and 308-5, respectively, had transgene activity in both shoot and root (Table 3.4). Line 308-2 had one individual which had transgene activity only in the shoot and not root. Line 308-5 had one individual which had transgene activity only in the root and another just in the shoot (Table 3.4). The individuals from the line 310-8 and 310-8 which were overexpressing *HvHVP1* under the putative cortex-specific promoter, C34, mostly had transgene activity in both shoot and root (Table 3.4). Line 310-3 predominantly had transgene activity throughout the plant but 1 individual had activity only in the roots and not in the shoot (Table 3.4). Another individual appeared to have only root-specific activity but on closer inspection of the GAPdh loading control PCR, the sample containing cDNA of the mRNA from shoots did not amplify indicating possibly degraded cDNA. Due to lack of template, the RT-CPR could not be repeated. The other sibling line, 310-8, also had 1 individual which appeared to be root-specific but was found to have no GAPdh result for the shoot sample indicating, again, that the mRNA might have been degraded. One individual of line 310-8 had shoot-specific activity and the two other individuals with activity throughout the plant (Table 3.4).

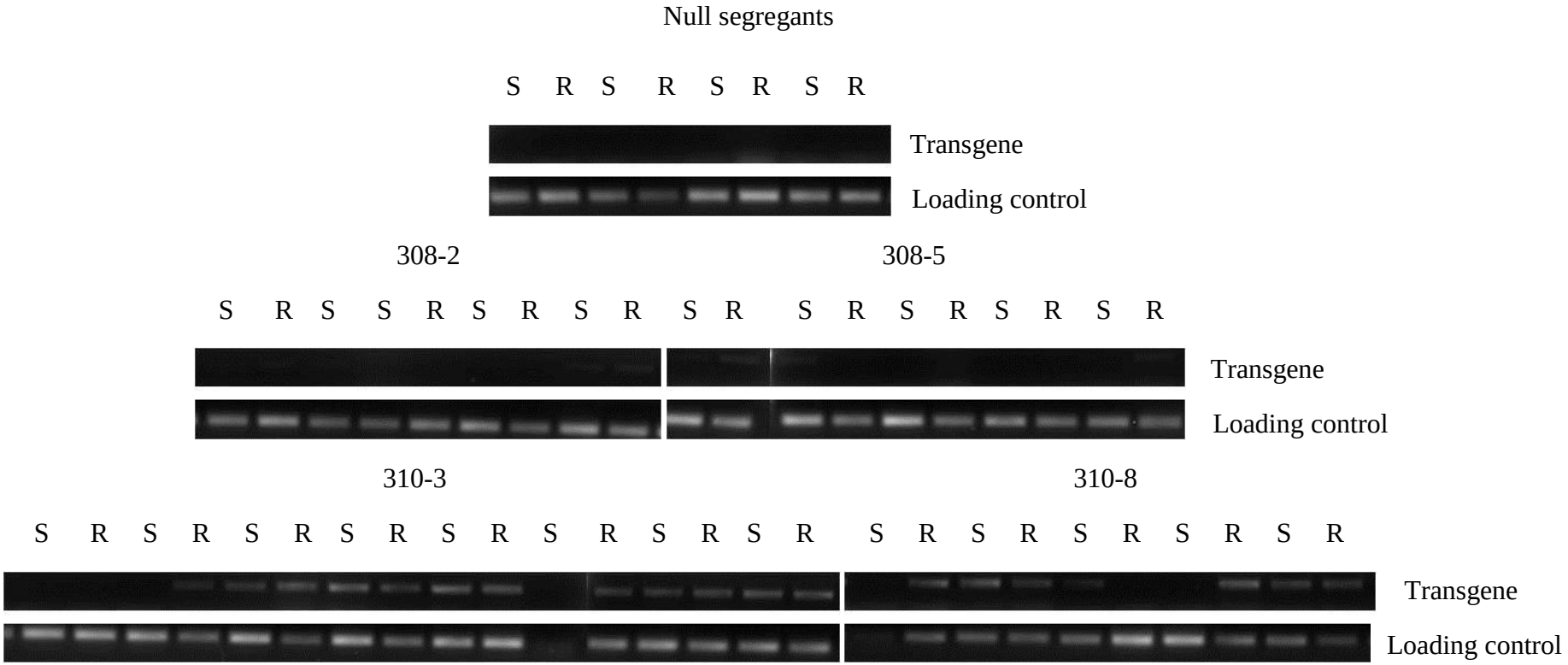


Figure 3.9: Photograph of agarose gel showing presence or absence of transgene mRNA in shoot (S) and root (R) in the respective lines of salt stress study 1. *Top row is the PCR for the transgene and bottom row is the PCR for GAPdh loading control as indicated. Lines from salt stress experiment 1 including null segregants, 308-2 and 308-5 containing ectopic HvHKT1;5 under the control of putative cortex-specific promoter C34 and 310-3 and 310-8 containing ectopic HvHVP1 also under the same promoter, C34.*

Table 3.4 Summary table of transgene data for lines 308-2, 308-5, 310-3 and 310-8. The table indicates the number of individuals that survived the treatment, the number of individuals that contained the transgenic construct, the number of individuals that had transgene expression, the number of individuals that had shoot-only transgene activity and root-only transgene activity. The differentiation was made as some plants contained activity in both the shoot and root, while others only had root or shoot-specific activity.

	308-2	308-5	310-3	310-8
Surviving individuals	5	5	8	5
Individuals positive for transgene	4	3	7	5
Individuals with transgene activity	3	3	7	5
Individuals with shoot-specific transgene activity	1	1	0	1
Individuals with root-specific transgene activity	0	1	1	1

3.3.3.2 Salt stress experiment 2: transgene mRNA

The results of the PCR to detect the presence of the transgene for salt stress experiment 2 are presented in Figure 3.10 but have been summarised in Table 3.5 for easy perusal as some of the results were faint. The loading control is not consistent throughout the samples but this was not seen as a problem as the purpose of this study was to detect presence/absence of transgene mRNA. Lines 312-4 and 312-5 which had more than half of the surviving individuals positive for the transgene appeared to have a substantial number of individuals with no transgene expression. Line 312-4 had 4 out of 7 individuals positive for the transgene but only 3 which produced the transcript. Line 312-5 had 5 out of 7 individuals positive for the transgene out of which only 2 had the transgenic transcript. Lines 314-6 and 314-7 appeared to not have the same gene silencing effect as seen in lines 312-4 and 312-5. The majority of individuals of 312-4 had root-specific activity and no individuals with shoot-specific activity. The majority of individuals for both 312-5 and 314-7 appeared to have shoot-specific activity. With the exception of one individual which appeared to have root-specific activity, 314-6 had activity in both shoot and root (Table 3.5).

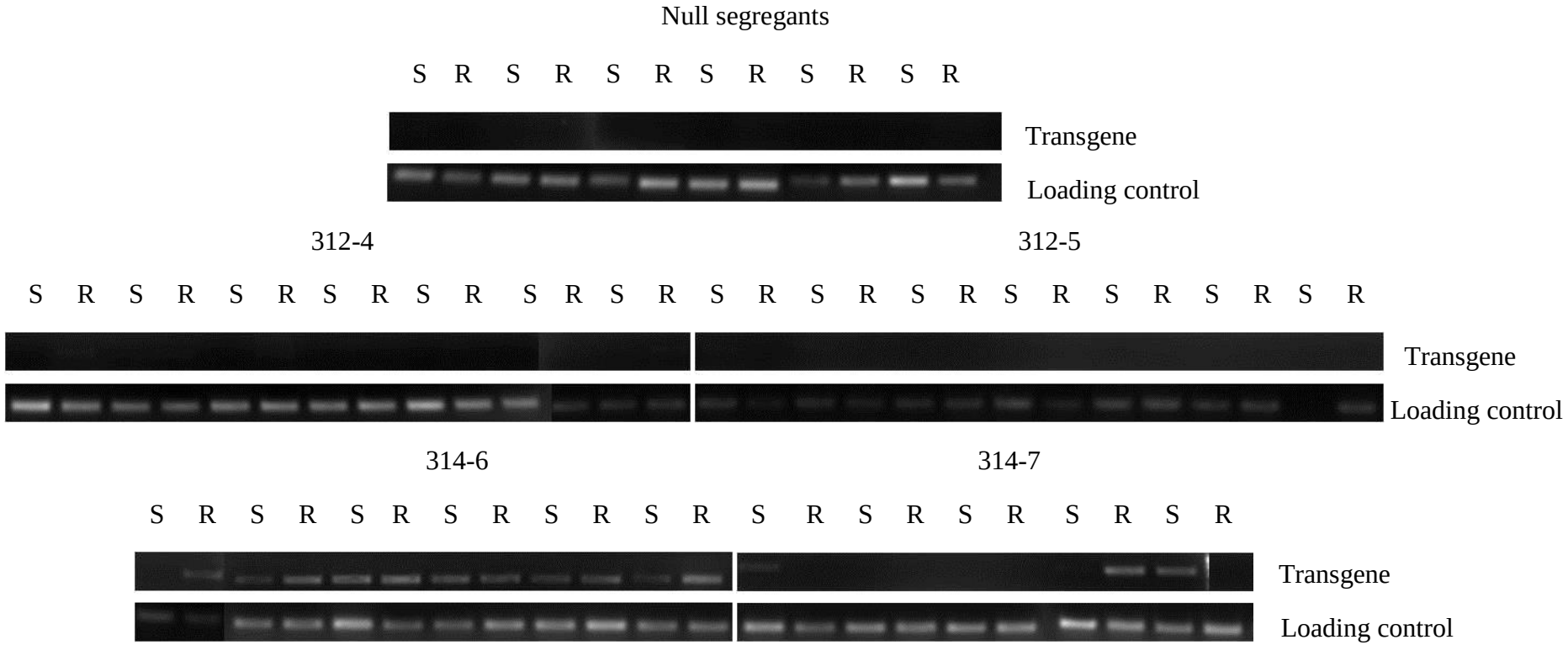


Figure 3.10: Photograph of agarose gel showing presence or absence of transgene mRNA in shoot (S) and root (R) in the respective lines of salt stress study 2. *Top row is the PCR for the transgene and bottom row is the PCR for GAPdh loading control as indicated. Lines from salt stress experiment 2 including null segregants, 312-4 and 312-5 containing ectopic HvHKT1;5 under the control of putative cortex-specific promoter C257 and 314-6 and 314-7 containing ectopic HvHVP1 also under the same promoter, C257***.**

Table 3.5: Summary table of transgene data for lines 312-4, 312-5, 314-6 and 314-7. The table indicates the number of individuals that survived the treatment, the number of individuals that contained the transgenic construct, the number of individuals that had transgene expression, the number of individuals that had shoot-only transgene activity and root-only transgene activity. The differentiation was made as some plants contained activity in both the shoot and root, while others only had root or shoot-specific activity.

	312-4	312-5	314-6	314-7
Surviving individuals	7	7	6	5
Individuals positive for transgene	4	5	5	3
Individuals with transgene activity	3	2	6	3
Individuals with shoot-specific transgene activity	0	2	0	2
Individuals with root-specific transgene activity	2	0	1	1

3.3.3.3 Salt stress experiment 3: transgene mRNA

The results of the PCR to detect the presence of the transgene for salt stress experiment 3 are presented in Figure 3.11 but have been summarised in Table 3.6 for easy perusal. The PCR data indicates that transgene mRNA is present both in the shoots and roots of the transgenic plants; the only anomaly is the lack of a PCR band for the root sample of one of the individuals of line 316-10 despite being present in the shoot of the individual (Table 3.6). From the PCR performed to test the loading control a faint result was also obtained for this sample. As expected, those lines that were identified as null segregants have no transgenic mRNA (Table 3.6).

The results obtained here indicate that the phenotypic changes occurring in the transgenic plants are most likely to be due to the transgene being transcribed and expressed.

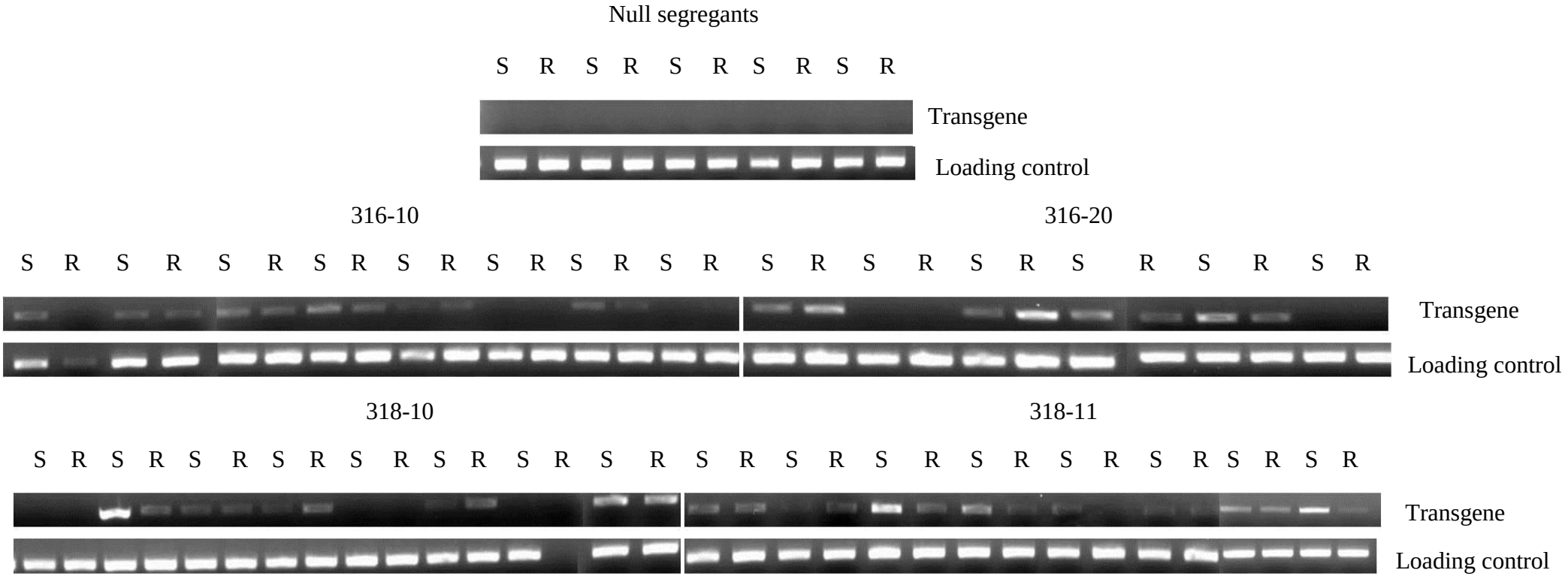


Figure 3.11: Photograph of agarose gel showing presence or absence of transgene mRNA in shoot (S) and root (R) in the respective lines of salt stress study 3. *Top row is the PCR for the transgene and bottom row is the PCR for GAPdh loading control as indicated. Lines from salt stress experiment 3 including null segregants, 316-10 and 316-20 containing ectopic HvHKT1;5 under the control of putative stelar-specific promoter S147 and 318-10 and 318-11 containing ectopic HvHKT1;5 under the control of putative stelar-specific promoter Ta.*

Table 3.6: Summary table of transgene data for lines 316-10, 316-20, 318-10 and 318-11. The table indicates the number of individuals that survived the treatment, the number of individuals that contained the transgenic construct, the number of individuals that had transgene expression, the number of individuals that had shoot-only transgene activity and root-only transgene activity. The differentiation was made as some plants contained activity in both the shoot and root, while others only had root or shoot-specific activity.

	316-10	316-20	318-10	318-11
Surviving individuals	8	8	8	8
Individuals positive for transgene	7	6	8	8
Individuals with transgene activity	6	6	5	8
Individuals with shoot-specific transgene activity	1	0	0	0
Individuals with root-specific transgene activity	0	0	0	0

3.4 Discussion

Shoot Na^+ concentrations in plants grown in control conditions were very low and the K^+ levels high in all the plants. The Na^+ and K^+ data were also highly variable, possibly as a result of the inherent genetic backgrounds of the plants. As such, only those results from the salt stress treatment will be discussed in detail in this section as there is less variability between the independent lines containing the same construct, used for the study.

3.4.1 Plants containing stelar-specific S147 promoter upstream of *HvHKT1;5* accumulate less Na^+ than null segregants

The greatest reduction in shoot Na^+ accumulation was seen in plants expressing the *HvHKT1;5* gene under control of the putative S147 promoter, identified in this project for its specific control of gene expression in the root stele, grown under salt stress. A 17% reduction in Na^+ accumulation compared with the null segregants was observed in line 316-10 and a less dramatic reduction of 10% was seen in line 316-20. While there was a slight reduction in K^+ uptake in line 316-10 under salt stress, line 316-20 accumulated more K^+ in the 4^{th} leaf compared with the null segregants. In both lines, the resultant K^+/Na^+ ratio, which is often considered to be an important salt tolerance determinant, was higher than for the null segregant group with a 9% and 15% increase for 316-10 and 316-20, respectively.

A basic PCR was performed on cDNA from these plants to investigate where transgene expression was being controlled. Although not anticipated, S147 controlled transgene expression was not restricted to the roots but was also present in shoots. This result was unexpected as in previous studies involving expression of *HvHKT1;5* orthologues under the control of the 35S promoter, where expression of the gene is ubiquitous, plants become more salt sensitive than the controls (Møller *et al.*, 2009). Moller *et al*. (2009) showed that expression of *AtHKT1;1* in the stelar cells of *A. thaliana* plant roots increased the ability of the plant to exclude shoot sodium. Plett *et al.* (2010) showed that expression of *AtHKT1;1* in rice root cortex and in the root epidermal and root cortex tissues of *A. thaliana* also increased Na^+ exclusion in the shoot. Interestingly, stelar specific expression of *AtHKT1;1* in rice roots actually increased Na^+ accumulation in the shoots (Plett *et al.*, 2010). These findings

emphasise the importance of the gene expression of *HKT1;5* being restricted to particular cell types. The findings in this study where transgenic *HvHKT1;5* transcript was observed in both shoots and roots do not necessarily contradict previous studies which demonstrate that cell-type specificity is important. As the reporter gene assays for *GFP* and *uidA* did not yield any conclusive data, it can only be conjectured as to why the apparent lack of spatial specificity of *HvHKT1;5* did not have deleterious effects on the plants.

While it is generally agreed that native *HKT1;5* and expression of its orthologues is thought to occur in the xylem (Sunarpi *et al.*, 2005; Byrt *et al.*, 2007; Davenport *et al.*, 2007), the study by Plett *et al*. (2010) has demonstrated that it need not be restricted to those cell types alone and that the spatial control of *AtHKT1;1/HKT1;5* might be more robust than initially thought. A possibility is that the *HvHKT1;5* transgene was expressed predominantly in the 'right' or in a part of the root hat negate any deleterious effects that might have been caused by transgene expression in a less ideal region of the plant like the shoots.

Gene transcript regulation could also ensure that the appropriate cell-types express the gene and enable removal of Na^+ by ensuring high levels in the crucial cell-types where expression is beneficial and low levels in the cell-types where *HvHKT1;5* expression could be detrimental. The study by Plett *et al.* (2010) alluded to a positive feedback loop where the cortex-specific *AtHKT1;1* gene transcript was also enhancing the transcription of the endogenous counterpart. This raises the issue as to why endogenous gene regulation did not suppress the salt sensitivity in the 35S::*AtHKT1;1* plants in the study by Møller *et al.* (2009) as was reported by Plett *et al.* (2010). It could be a case of dosage - as the putative promoters in this study are assumed to have low activity, it may be that the gene transcript levels are low enough to be managed by the regulatory mechanisms of the plant, some of which have been suggested by Plett *et al.* (2010). The transgene expression in the appropriate tissue type combined with a possible increase in the endogenous *HvHKT1;5* could be counteracting any deleterious effects that expression in other tissue types might have brought about. It is only possible to speculate what might be taking place in the transformed plants with respect to *HvHKT1;5*, as not much is known about the regulatory aspects of the gene and its

orthologues. The process involved in gene activation, transcript regulation and finally, gene product translation and function remains elusive for *HvHKT1;5* and its orthologues.

3.4.2 Why isn't accumulation of shoot Na^+ reduced in plants transformed with *HvHKT1;5* under the control of Ta, putative stelar-specific promoter isolated from upstream of wheat *TaHKT1;5-D*?

It was anticipated that the promoter Ta, isolated upstream of the bread wheat *TaHKT1;5-D*, would control gene expression in the root stele as has been suggested by Byrt *et al.* (2007) and that this would reduce Na^+ translocation from the root to the shoot. This was not the case with the ectopic expression of *HvHKT1;5* detected in both roots and shoots of the transgenic plants. Shoot Na^+ was also not reduced in the transgenic barley plants generated in this study.

The PCR with mRNA isolated from the shoots and roots of both the lines of 318 (318-10 and 318-11) indicated that transgene expression was present in both roots and shoots of plants and this result was further confounding. Based on a radioactive Na^+ flux experiment by Davenport *et al.* (2007) in *A. thaliana* and immunolocalisation experiments by (Sunarpi *et al.*, 2005) on native *HKT1;1* protein in *A. thaliana*, it has been suggested that *HKT1;5* is found mainly in the root xylem parenchyma. It has also been suggested that the same expression patterns are to be expected in bread wheat from which the promoter was isolated and transformed into the 318 plant lines (James *et al.*, 2006; Byrt *et al.*, 2007; Munns *et al.*, 2012). As barley and wheat are relatively closely related, it was expected that these expression patterns would be seen when the wheat promoter, Ta, was transformed into barley. While transcript appears to be produced, there is no apparent change in phenotype. Again, perhaps the regulatory factors in the tissue types in which the gene is transcribed are preventing the translation of the gene product or are preventing the gene product from being functional. A suite of potentially different trans-regulatory factors in barley compared with that in wheat could activate transgene expression in an unexpected fashion as gene regulatory regions tend to have higher rates of mutations than coding regions (Guo and Moose, 2003).

3.4.3 Cortex-specific promoters driving transgenic *HvHKT1;5* and *HvHVP1* produce no changes in shoot Na^+ accumulation

Plants which were transformed with either *HvHKT1;5* or *HvHVP1* under cortex-specific promoters C34 or C257 did not show the same level of reduction in shoot Na^+ as lines 316-10 and 316-20.

3.4.3.1 Putative cortex-specific promoters driving HvHKT1;5 *do not appear to reduce shoot Na^+*

With the exception of line 308-2, which contained the C34 promoter driving expression of *HvHKT1;5*, other plant lines - 310, 312 and 314- did not have a reduction in shoot Na^+ accumulation and, in fact, there were elevated levels of Na^+, particularly in line 314 which had significantly higher levels compared with the null segregants.

The *A. thaliana* orthologue of *HvHKT1;5*, *AtHKT1;1*, has been shown to be important in its unloading of Na^+ from the transpiration stream back into the stelar cells. Thus, the spatial-specificity of *AtHKT1;1* gene expression is critical. Moller *et al* (2009) showed that Na^+ exclusion was optimal with *AtHKT1;1* expression limited to the root-stele. Plett *et al* (2010), in an attempt to identify a 'wrong' cell-type in which *AtHKT1;1* could be expressed, limited its expression to the 'outer layers of the root' in *A. thaliana*, that is, the epidermis and the cortex, and found that Na^+ accumulation in the shoot actually decreased. The same was seen in rice when *AtHKT1;1* was expressed in root-cortex cells and the converse when the gene was expressed in the stelar cells. The use of a cortex promoter to drive *HvHKT1;5* in barley plants was based on this finding. However, as the spatial control of the cortex promoters in barley has not been confirmed, it is impossible to discern the reason for the lack of change in accumulation of shoot Na^+.

It was also interesting to observe that there were a number of individuals which had a putative cortex-specific promoter driving the *HvHKT1*;5 which appeared to contain the transgene but lacked any corresponding transcript. Whether this is a consequence of similar gene regulatory

mechanisms as suggested by Plett *et al.* (2010) or those individuals that had no transgene activity were in fact false-positive for transgene presence needs to be further investigated. Perhaps future genotyping methods should involve extraction of DNA from plants and then amplification of transgene target using conventional Taq polymerases. It is possible that the PHIRE system used in this study to genotype the individuals was too sensitive and was susceptible to generating false positives, though known null segregants were found to be consistently negative so this is unclear.

Finally, it is not possible based on the data collected in this study to determine whether this apparent lack of reduction of shoot Na^+ actually relates directly to the overall salt tolerance of the plant. This will be discussed further in a subsequent section of this chapter.

3.4.3.2 Putative cortex-specific promoters driving HvHVP1 do not appear to reduce shoot Na^+

Na^+ exclusion is not the only important strategy employed by plants to maintain non-toxic levels of Na^+ in the cells. Sequestration of Na^+ into vacuoles, thereby limiting its presence in the cytosol, has also been cited as an important salt tolerance mechanism. Studies overexpressing vacuolar pyrophosphatases have shown that overall plant biomass is less affected in plants overexpressing *H^+-PPase* than controls under salt stress conditions (Gaxiola *et al.*, 2001a; Guo *et al.*, 2006; Brini *et al.*, 2007; Pasapula *et al.*, 2011; Zhang *et al.*, 2011). The proton pyrophosphatase encoded by *HvHVP1* has been found to be highly expressed under salt-stress conditions and is a major factor of driving sequestration of Na^+ in the vacuole (Fukuda *et al.*, 2004). Many studies that have overexpressed *H^+-PPase* from plants including *A. thaliana*, bread wheat and salt cress in different plants have shown that the salt tolerance of the plant increases with increased Na^+ accumulation in the shoot (Gaxiola *et al.*, 2001a; Guo *et al.*, 2006; Brini *et al.*, 2007; Bao *et al.*, 2009). It is possible that the plants that are expressing the pyrophosphatase are better sequestering the Na^+ ions in the shoot and are therefore more salt tolerant. The plants overexpressing *HvHVP1* under the cortex-specific promoters do not appear to have reduced shoot Na^+ content. What cannot be ruled out,

however, are any pleiotropic effects that might cause an increase in endogenous *HvHKT1;5* and *HvHVP1* as reported in the study by Plett *et al.* (2010).

3.4.3.3 Limitations of performing experiments with T_1 plant lines

Physiological mechanisms aside, a problem encountered while studying these lines was a lack of sufficient replicate numbers (n>3) for each line due to transgene segregation. As the plants were first generation transformants, a number of the individuals were null for the transgene and thus had to be grouped with the other null segregants. Some lines had no positive transformants or just one which were not used for data analysis as meaningful comparisons between the lines could not be made. The high degree of variability within each line for Na^+ and K^+ could be attributed to the small number of biological replicates.

The T_1 material also had to be used conservatively to ensure collection of T_2 seed. The use of T_2 seed would be beneficial as lines homologous for the transgene could be identified and selected for use. This would also increase the number of biological replicates available for study and would allow investigation of other salt tolerance parameters as destructive analyses would be possible.

3.4.4 **Na^+ exclusion and salt tolerance**

Na^+ exclusion is an important strategy employed by plants to limit Na^+- induced damage to the shoot. Na^+ exclusion is an important aspect of salt tolerance in barley (Chen *et al.*, 2007b) as in wheat but barley is able to tolerate more shoot Na^+ than wheat (Munns and James, 2003; Colmer *et al.*, 2005). This means that tissue tolerance of Na^+ in barley is also an important feature of barley salt tolerance. The recent study which increased barley salt tolerance through the constitutive overexpression of *HvHKT2;1* and reported that shoot Na^+ levels actually increased provides evidence of this (Mian *et al.*, 2011).

Thus, while there was not a reduced amount of shoot Na^+ for most of the lines in this study, this does not necessarily mean that the lines were not more salt tolerant. Due to factors such as sparseness of plant material it was not possible to measure other salt tolerance indicators. As well as shoot Na^+ and root Na^+ accumulation, it would be ideal to test percentage of green compared with necrotic regions of the shoot, the biomass of the plant and, ultimately, the grain yield.

3.4.5 **Transgenic gene activity**

Apart from assessment of phenotypic changes, a good understanding of gene regulation and location of gene activity is necessary. While the promoter- reporter assays were unsuccessful, immunolocalisation of the HA epitope fused downstream of both *HvHVP1* and *HvHKT1;5* can be performed to study where the gene product is located and whether or not the transgenic protein is being expressed. Assessing the location of gene activity by studying the presence/absence of gene product in the various cell-types of the transgenic plants and comparing this with the resulting phenotype might help elucidate the roles of the different tissue types in Na^+ exclusion and hence the appropriate cell-types in which expression of *HvHKT1;5* or *HvHVP1* can be beneficial or detrimental to the plant. This could not be performed in this study but is currently underway to ascertain location of transgene activity.

3.5 Conclusion

While reporter gene assays (detailed in Chapter 3) could not conclusively indicate regions of putative promoter control, semi-quantitative PCR of the genes *HvHKT1*;5 and *HvHVP1* helped identify in which organs the promoters were controlling transgene activity. Although *HvHKT1;5* is expected to be primarily expressed in root-stele, activity in the shoot does not appear to necessarily have deleterious effects as evidenced by the 2 sibling lines of 316. While both independent lines 316-10 and 316-20 were successful in reducing leaf Na^+, other lines which were generated in this study cannot be ruled out as having increased salt tolerance. Those lines which had increased leaf Na^+ or those that contained *HvHVP1* could enhance salinity tolerance of the plant by boosting vacuolar sequestration of Na^+. Further studies investigating changes in biomass would be ideal to determine the effects of these

transgenes in whole plant salinity tolerance. Immunolocalisation of the transgenes fused with HA epitope tag will help confirm spatial control of the putative promoters identified in this research and the permissive regions where *HvHKT1*;5 and *HvHVP1* can be expressed in the plants to increase salt tolerance. Additionally, an understanding of native gene activity of *HvHKT1;5* in barley could help elucidate its biological function within the plant and whether the transgenic lines 316-10 and 316-20 overexpressing *HvHKT1;5* have similar expression patterns. Functional characterisation of *HvHKT1;5* could also help clarify its biological role in barley and also whether it is involved in whole plant salt tolerance. Thus, knockout/knockdown studies to complement the overexpression work covered in this chapter would be very informative in terms of the role *HvHKT1;5* plays in barley.

The following chapter will partly address the above knockout/knockdown suggestion, but with respect to the *HvHKT1;5* orthologue in bread wheat, *TaHKT1;5-D*. RNAi knockdown lines were generated to study the importance of *TaHKT1;5-D* in bread wheat salt tolerance. This gene is of particular interest as it was located in the well known *Kna1* locus reported more than two decades ago by Gorham *et al.* (1987) that was found to be important in bread wheat shoot Na^+ exclusion. The importance and relevance of *TaHKT1;5-D* to the *Kna1* locus was studied by looking at the effects of lowered *TaHKT1;5-D* levels on plant phenotype.

4 Role of *TaHKT1;5-D* as a salt tolerance determinant in bread wheat

4.1 Introduction

During the year 2011-2012 Australia produced 4% of the world's wheat and contributed to 16% of total world wheat exports; of the wheat grown in Australia that year, approximately 83% was exported (WEA, 2012; USDA, 2013). Wheat exports alone earned the Australian economy more than $6 billion AUD in 2011 and these exports are highly regarded globally for the low moisture content of the grain and the resultant white flour (WEA, 2012). While anomalous weather conditions have supported an unusually high wheat yield for the year 2011/12 (WEA, 2012), Australian agriculture often faces losses in yield as a result of harsh environmental conditions (Rengasamy, 2002; Rengasamy *et al.*, 2003; Rengasamy, 2006). A study by Rengasamy (2002) depicted the areas in Australia that are affected by salinity; this same area is also the region in which much of the Australian grain such as wheat and barley is produced (Chapter 1, Figure 1.1). The projected losses to the Australian agricultural sector due to salinity have been estimated to be more than $1 billion per year (Rengasamy, 2002; Rengasamy, 2006). With the need for food production to increase by 2050, the ability of cereal crops like wheat to withstand limiting growth conditions including drought or subsoil constraints such as salinity must be enhanced.

Two major species of the cultivated wheats are the hexaploid bread wheat *Triticum aestivum* (AABBDD) and the tetraploid durum wheat *Triticum turgidum* ssp. durum (AABB). Durum wheat is more salt sensitive than bread wheat due to its tendency to accumulate high levels of Na^+ in the shoot and maintain poor K^+/Na^+ ratios (Munns *et al.*, 2006). The ability of bread wheat to better exclude Na^+ and thus maintain higher K^+/Na^+ ratios than durum wheat has long been attributed to the presence of the D genome which originated from *Aegilops tauschii* (Gorham *et al.*, 1987; Feuillet *et al.*, 2008). It has been proposed that the D genome contains a locus, *Kna1*, important for lowering shoot Na^+ and increasing shoot K^+, that houses the *HKT1;5* gene on the long arm of chromosome 4 (Gorham *et al.*, 1987; Byrt *et al.*, 2007). This *HKT1;5* gene in bread wheat, *TaHKT1;5-D*, has been found to function in the same way as its orthologues in *A. thaliana* (*AtHKT1;1*) and rice (*OsHKT1;5*) by allowing the inward flow of Na^+ ions into the cytosol (Uozumi *et al.*, 2000; Ren *et al.*, 2005; Byrt, 2008). Physiological

studies of gene transport function have demonstrated that the gene product of all the *HKT1;5*s and their orthologues specifically transport Na^+ (Ren *et al.*, 2005; Byrt, 2008; Munns *et al.*, 2012).

The study by Byrt (2008) revealed that a deletion comprising about 14% of the distal part of chromosome 4D resulted in increased shoot Na^+ accumulation. This deleted region, aside from being part of *Kna1*, also contained the *HKT1;5* gene. This deleted region, however, is large and could possibly contain salt tolerance genes other than *TaHKT1;5-D* that are not yet characterised that could also play an important role in the plant's ability to exclude salt. As bread wheat is a hexaploid containing two copies of three genomes (AABBDD) it is laborious to knockout a single gene. A more effective way of studying the importance of *TaHKT1;5-D* in salt exclusion in bread wheat is to silence the gene using RNAi (Travella *et al.*, 2006; Byrt, 2008). Byrt (2008) generated two RNAi sequences, one of 600 bp and the other of 559 bp in length, designed to silence *TaHKT1;5-D*. These sequences were transformed into *T. aestivum* cv. Bobwhite plants. T_2 seeds collected from the transformed wheat were kindly provided by Dr Caitlin Byrt (ARC Centre of Excellence in Plant Cell Walls, University of Adelaide). In this Chapter, details will be given of the studies performed on these plants, and of the relevance of *TaHKT1;5-D* to the *Kna1* locus, shoot Na^+ exclusion and to overall plant salt tolerance.

4.2 Materials and methods

4.2.1 Assaying the effect of the RNAi on T_2 lines of *T. aestivum* cv. Bobwhite

T_2 seed from transgenic Bobwhite plants containing the RNAi constructs RNAi 1 and RNAi 2 (Appendix 4.1) was kindly provided for this study by Dr Caitlin Byrt (ARC Centre of Excellence in Plant Cell Walls, University of Adelaide). Lines were selected on the basis of preliminary observations of high Na^+ accumulation in the 3rd leaf of T_1 plants. Two to three sibling lines from each transformation event was used for further study including one null segregant line as a control (Table 4.1). Any null segregants for the RNAi were also grouped with the respective null segregant control (Table 4.1). In order to simplify identifiers the

collective of sibling plants used to study the two constructs will be referred to as RNAi 1 or RNAi 2, and the corresponding null segregants will be RNAi 1 null or RNAi 2 null.

Transgenic Bobwhite plants along with null segregants as controls were grown in 50 mM NaCl and 0.825 mM $CaCl_2$. After 10 days of leaf growth from the point of emergence, the 4^{th} leaves of the individuals were sampled and the Na^+ and K^+ content in the leaf sap was measured.

Table 4.1: Sibling lines used in experiments with T_2 and T_3 generation. The column containing the individual line designators contains the nomenclature used by Dr Byrt and for clarity has been labelled as either RNAi 1 or RNAi 2.

Lines used in the study with T_2 generation	
Group of sibling lines	Individual lines
RNAi 1 nulls	1S02-10 and any null segregants of 1S02-7 and 1S02-8
RNAi 1	1S02-7, 1S02-8
RNAi 2 nulls	2S11-4
RNAi 2	2S11-1, 2S11-2, 2S11-5
Lines used in the study with T_3 generation	
Group of sibling lines	Individual lines
RNAi 1 nulls	1S02-10 and any null segregants of 1S02-7 and 1S02-8
RNAi 1	1S02-7, 1S02-8
RNAi 2 nulls	2S11-4
RNAi 2	2S11-1, 2S11-2

4.2.1.1 Growth of transgenic T. aestivum *cv. Bobwhite plants*

Seeds were sterilised in 30% Domestos containing bleach (Unilever Australasia) for three minutes, then rinsed with RO water and, finally, with high-pure water (18.2 MΩ•cm) to remove any traces of bleach. The seeds were placed in Petri dishes containing Whatman paper and 5 ml of high-pure water (18.2 MΩ•cm) and incubated at 4°C in the dark for 7 days. The Petri dishes were left at room temperature and the seeds were allowed to germinate in sunlight. Once the coleoptiles had grown to 5.0 mm in length, the seedlings were transferred into a hydroponics solution containing a modified Hoagland's mix as described in (Chapter 3 section 3.2.1.2). When the 2nd leaf of the plants was half the length of the first leaf (Zadok's stage 1.1), a salt stress was applied at increments of 12.5 mM NaCl and 0.206 mM supplementary $CaCl_2$ to reach a final concentration of 50 mM NaCl and 0.825 mM $CaCl_2$ over 2 days.

4.2.1.2 Leaf and root sampling of plants

The 4th leaves of the plants grown under salt stress were allowed to develop for 10 days following emergence and were then sampled for Na^+ and K^+ analysis. The fresh weight of the leaf material was recorded immediately after harvest then dried at 65°C in a drying oven (Contherm Designer 150 litre Model 8150, Petone, New Zealand) for 16 hrs, after which the dry weight was measured and recorded.

Following a recovery period of one week after 4th leaf sampling, root samples were taken from each individual and these were snap frozen in liquid nitrogen and stored at -80°C for RNA extraction. In order to genotype the individuals as being positive or null for the transgenic construct, leaf samples were also taken for extraction of genomic DNA.

4.2.1.3 Genotyping transgenic T. aestivum *cv. Bobwhite plants*

4.2.1.3.1 DNA extraction

Leaf material was collected from plants for DNA extraction as described in Chapter 3, Section 3.2.4.1.

4.2.1.3.2 PCR determination of plants as positive or null for construct

Primer sequences had already been tested by Dr Caitlin Byrt who kindly shared the sequence data.

Genomic DNA was used for PCR (MJ Research MPTC-225 Tetrad) with primers FD4 (5'GCTTGGCCATCTTCATCGCCGTG-3') and
RD1 (5'GGCCACAGCTGTACCCGGTGCTG 3'). Plants containing a transgenic construct would generate two PCR products - one 147bp and another 322 bp in length, whereas any plants without a construct would generate only a 322 bp product. As the RNAi sequence traverses part of the coding region of *TaHKT1;5-D*, the 322 bp product would be a result of the genomic DNA containing an intron in the coding region as opposed to the 147 bp product generated from the RNAi insert which lacked this intron.

The PCR was performed using 0.1 µL Platinum Taq DNA Polymerase (Invitrogen, Carlsbad CA), 2.5 µL 10X PCR buffer, 2 µL dNTP (5mM), 0.75 µL 50mM $MgCl_2$, 0.2 µM each of forward and reverse primer, 5% DMSO (v/v) and made up to 25 µL with high-pure water (18.2 MΩ•cm). The PCR cycle involved an incubation step at 94°C for 5 min followed by 30 cycles of 94°C for 1 min; 60°C for 30 s; and 72°C for 1 min (Byrt, 2008).

4.2.1.4 RNA extraction of root tissue

RNA was extracted using the methods described in Chapter 2, Section 2.2.4.

4.2.1.5 Generating cDNA

RNA was quantified using a spectrophotometer (Nanodrop ND-100, Thermo Fisher Scientific, Waltham MA). Ten micrograms of RNA was treated with DNase I (Applied Biosystems/Ambion, Austin, TX) in a total volume of 20 ul and 0.4 µg RNA was used for synthesis of cDNA in a 20 µl volume using SuperscriptIII Reverse Transcriptase (Invitrogen, Carlsbad CA). The cDNA was synthesised as described in Chapter 2 section 2.2.5.

4.2.1.6 Quantitative PCR Primer design

As the RNAi insert spanned 600bp and 559bp of the 3' end of the coding region including the untranslated region (Appendix 4.2), the primers targeted a portion of the gene that did not overlap with the RNAi sequence. These primers would only detect changes in endogenous *TaHKT1;5* levels and not any RNAi transcripts. The primers could not, however, differentiate between the *TaHKT1;5* on the D genome and the homeologues on the B genome. The forward primer used for the qPCR was 5'-CTGCGGCTTCGTCCCGA-3' and the reverse, 5'-CGCTAGCACGAACGCCG-3' with the target sequence being 253 bp in length (Appendix 4.3).

Transcript analysis of *TaNHX1* and *TaSOS1* from bread wheat was also performed and the primer sequences were kindly provided by Dr. Deepa Jha (The University of Adelaide). The forward primer for *TaNHX1* was 5'- GCCTGGTTCACCCATAGAGA-3' and the reverse 5'-CACCGAAAGAATCCCAAGAG-3'. The forward primer for *TaSOS1* was 5'-AGAAGCCGATCTGCAAAGAA-3' and the reverse 5'- TGCTGCCATACATGCTGACT-3'.

4.2.1.7 Quantitative PCR

Quantitative PCR (qPCR) was kindly performed by Dr Neil Shirley (ARC Centre of Excellence in Plant Cell Walls, Adelaide, Australia). The endogenous *TaHKT1;5, TaNHX1 and TaSOS1* transcripts were normalised with 3 control genes- *TaGAPdh*, *TaActin, TaCycl* and *TaEFa*. Each line had a minimum of three biological replicates. The qPCR was

performed as described in Burton *et al.* (2004). The lines which were genotyped in order to establish whether they were positive or null for the constructs were grouped with their respective lines and an average expression level was calculated along with standard error.

4.2.1.8 Leaf Na^+ and K^+ measurements

Leaves were sampled and dried as detailed in Chapter 3 section 3.2.4.2.3. The dried leaf samples were digested in 20 ml of 1% nitric acid for 4 hrs at 85°C in a heat block (Environmental express hot block digester). The Na^+ and K^+ contents were measured using a flame photometer (Sherwood Flame Photometer Model 420).

The values generated from this analysis were expressed as concentrations of Na^+ and K^+ in tissue sap.

The lines which were genotyped as positive or null for the constructs were grouped with their respective lines and an average Na^+ or K^+ concentration was calculated along with the standard error of the mean.

4.2.2 *TaHKT1;5-D*, *TaSOS1*, *TaNHX1* transcript analysis of wildtype *T.aestivum* cv. Bobwhite plants

Six wildtype Bobwhite plants were grown in a control treatment (ACPFG solution) and six in a salt stress treatment (as in section 4.2.1.1). Root tissues were harvested for RNA extraction to study the transcript patterns of *TaHKT1;5-D*, *TaSOS1*, *TaNHX1* (as in section 4.2.1.6). cDNA was prepared (as in section 4.2.1.5) and used for qPCR (as described in section 4.2.17) using the same primers as for the RNAi plants. This study was performed to assess the expression patterns of these genes in wild-type plants; whether there was an induction in *TaHKT1;5-D* expression and whether this was correlated with changes in *TaSOS1* and *TaNHX1* levels in response to salt stress.

4.2.3 High-throughput phenotyping of T_3 plants containing RNAi transgene in The Plant Accelerator

Forty biological replicates of transgenic lines containing RNAi 1 and 20 biological replicates of transgenic lines containing RNAi 2 were each used for a control and salt stress treatment (see Table 4.1). A larger number of replicates were chosen for RNAi 1 in order to compensate for the number of plants which would segregate as null for the transgene. The salt stress imposed on the plants was 75 mM NaCl. After the length of the coleoptiles reached 5-10 mm, the plants were transplanted into coco-peat soil and watered to 40% total pot weight. Ten days after emergence, the fourth leaf was sampled from the plants for measurement of Na^+ and K^+ levels in the leaf sap, and the plants were grown to maturity to allow seed collection. Biomass of the plants was measured non-destructively through a commercial image capture system, LemnaTec 'Scanalyzer 3D' (Wurselen, Germany). Three images were captured of each plant from 3 different angles consisting of a top view and two side views, each rotated at 90 angles. Number of pixels were taken to be the projected shoot area and were used to infer biomass changes over time. The use of this technique to non-destructively measure biomass accumulation for the assessment of salinity tolerance has been detailed in Rajendran *et al.* (2009) and Berger *et al.* (2012).

4.2.3.1 Preparation of soil for growth of TaHKT1;5-D *knockdown lines*

For the pots used in the control treatment 1.6 kg of coco peat soil was added. Those pots that were to contain the salt stress treatment were filled to 800 g of soil to which was added 320 ml of RO water containing 150 mM NaCl + 5mM $CaCl_2$ and then topped up with another 800g of coco peat soil. When the pots were watered to 640 ml (40% of total pot weight) the resulting salt stress would be 75 mM NaCl+ 2.5 mM $CaCl_2$. NaCl and $CaCl_2$ were added to the bottom half of the pot so that the plants did not immediately experience salt stress upon transplantation.

4.2.3.2 Growth of T.aestivum TaHKT1;5-D *knockdown lines*

Seeds were set to germinate in Petri dishes as described in section 4.2.1.1. After the coleoptiles were 5-10 mm in length, the plants were transplanted into the prepared pots. These

pots were placed on the conveyor system in The Plant Accelerator (The University of Adelaide, Australia) to be imaged and watered once every 2 days. Imaging of the plants commenced from the first day of watering and finished after the plants bolted. The plants were then transferred to a glasshouse to mature and set seed. Seeds were collected, counted and the 100-seed weight was measured to assess changes in grain yield between the treatments and the lines.

4.2.3.3 Genotyping T_3 RNAi plants grown in the APPF Plant Accelerator

Plants grown in The Plant Accelerator (The University of Adelaide, Australia) were genotyped using Phire Plant Direct PCR Kit (Finnzymes/Thermo Scientific). Sections of the leaf tip of 1-2 mm were cut with a sharp blade and stored in 20 µL of dilution buffer (Phire Plant Direct PCR Kit, Finnzymes/Thermo Scientific). The blade was wiped clean with 70% ethanol after collection of each sample to avoid contamination of subsequent samples. The primers - FD4 and RD1- were used in PCR. The PCR mix contained 0.5 µL of the dilution buffer that contained the leaf sample, 10 µL of 2 X Phire Plant PCR buffer, 0.5 µM of FD4 and RD1 primers, 0.4 µL of Phire Hot Start DNA polymerase and high-pure water (18.2 MΩ•cm) to make up a total volume of 20 µL. The PCR consisted of an initial denaturation step at 98°C for 5 min followed by 40 cycles of 98°C for 5 s and 72°C for 20 s, and a final extension step of 72°C for 1 min.

4.2.3.4 Studying changes in phenotype as a result of the RNAi construct in **T. aestivum** *grown in soil*

4.2.3.4.1 Fourth leaf Na^+ and K^+

The 4th leaf was sampled from all the plants to study the rate of Na^+ and K^+ accumulation in the leaf sap. The samples were prepared and measured as described in Chapter 4, Section 4.2.1.8.

4.2.3.4.2 Non-destructive imaging of plant biomass changes over time

Plants were imaged using the LemnaTec imaging system which calculated the percentage of living (green) tissue compared with dying/necrotic tissue (yellow/brown) over time, as described in Rajendran *et al.* (2009). Data output was in pixels which were compared between the different lines and treatments to infer the effect of the RNAi construct on the overall growth of the plants.

The values were then fitted to a 4-parameter sigmoid curve with the formula

$$y = y_0 + \frac{a}{1 + e^{-(\frac{x-x_0}{b})}}$$

and 95% confidence intervals were calculated using Sigmaplot (Systat Software, San Jose, CA). The numerical output was graphed in Microsoft Excel and the growth rate ($\frac{dW}{dx}$), where W represents pixels (plant biomass) and relative growth rate ($\frac{growth\ rate}{W}$), calculated as described in Hunt (1978).

4.3 Results

4.3.1 Native gene repression in RNAi knockdown *T.aestivum* lines

T_2 transgenic plants containing the RNAi constructs showed a reduction in *TaHKT1*;5-D mRNA levels compared with their respective null segregant controls (Figure 4.1a). Mean *TaHKT1*;5-D mRNA levels in RNAi 1 were nearly half those in RNAi 1 null segregants. The reduction in *TaHKT1*;5-D transcript levels seen in RNAi 2 was significantly lower ($p<0.01$), less than half compared with the RNAi 2 nulls. Plants containing the RNAi 1 construct were segregating and thus the number of individuals positive for RNAi 1 was considerably fewer compared with the non-segregating RNAi 2. This affected the ability to attribute any significance to the differences between the RNAi 1 null and RNAi 1 lines.

While no significant differences in *TaSOS1* transcript levels were observed between either RNAi line and its respective control, RNAi 1 nulls and RNAi 1 had significantly higher ($p<0.05$) levels of *TaSOS1* transcript than RNAi 2 nulls and RNAi 2 (Figure 4.1b). The same trend was observed for TaNHX1 (Figure 4.1c) possibly reflecting the difference in the genetics of the donor material used for transformation with the RNAi constructs.

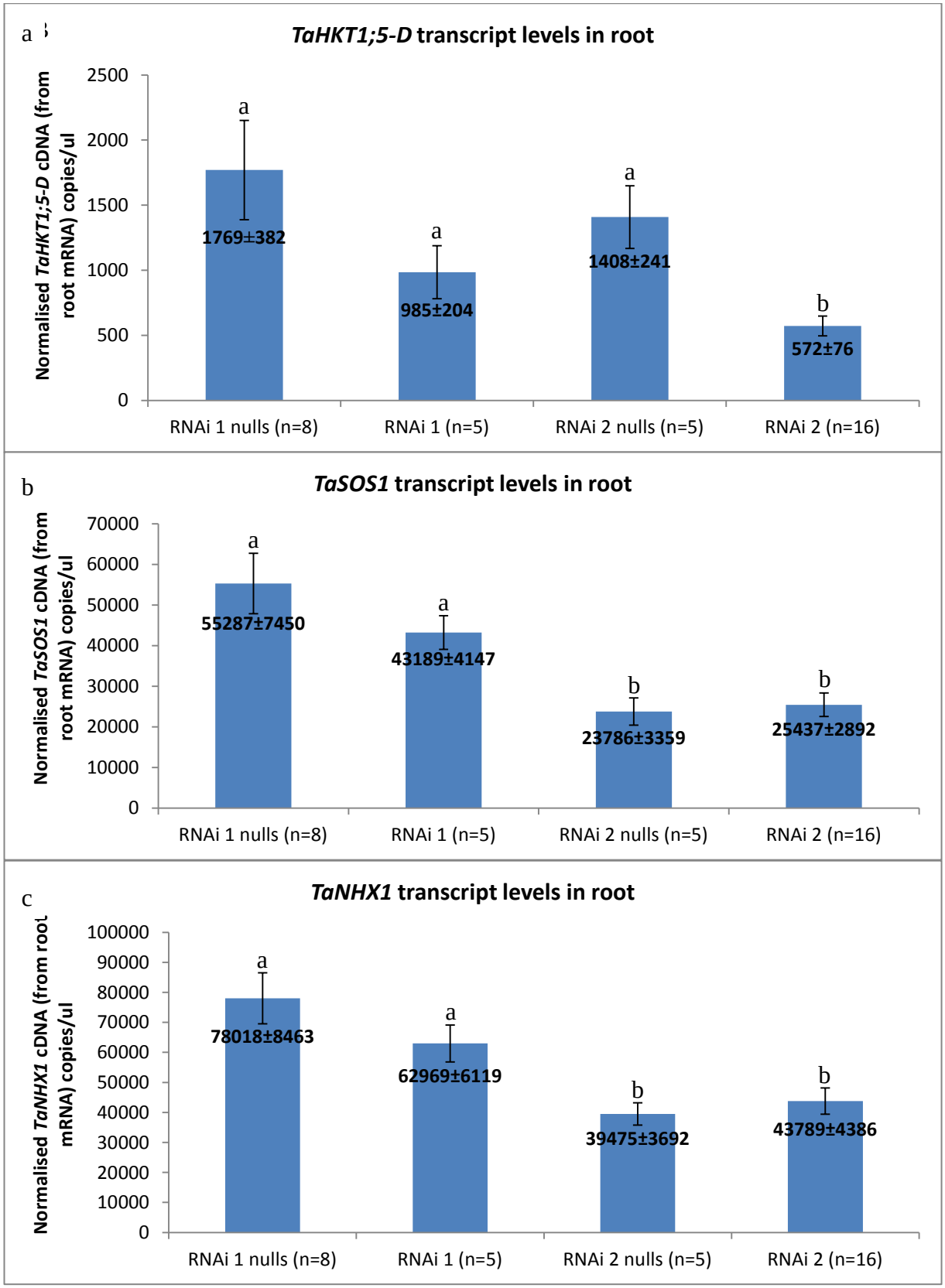


Figure 4.1: Graphs showing native gene transcript patterns in RNAi lines and respective null segregants. *The number of biological replicates is indicated next to the different plant lines. Significant differences at p<0.05. Raw mean values with S.E.M. presented within the closed bars of each plant line. A. Endogenous levels of root TaHKT1;5-D transcript in RNAi lines and their respective null segregants. B. Endogenous levels of root TaSOS1 transcript in RNAi lines and their respective null segregants. C. Endogenous levels of root TaNHX1 transcript in RNAi lines and their respective null segregants.*

4.3.2 Na^+ and K^+ accumulation in 4th leaf sap in RNAi knockdown *T. aestivum* lines

The accumulation of Na^+ in the leaves was correlated with the levels of *TaHKT1;5-D* mRNA (Figure 4.2a). Where there were low levels of *TaHKT1;5-D* transcript, an elevated Na^+ content was measured in the 4th leaf sap. The Na^+ content in the leaves of the plants containing the RNAi constructs was in the order of the NaCl treatment applied to the hydroponics solution, that is, 50 mM (Figure 4.2a). The Na^+ content in the 4th leaf sap of RNAi 1 plants was significantly higher ($p<0.01$) by almost 1.5 fold compared with their null counterpart. RNAi 2, which had a greater level of *TaHKT1;5-D* repression compared with its null segregant than did RNAi 1, also had a higher Na^+ content in the 4th leaf sap with a Na^+ concentration of more than double that of the RNAi 2 nulls ($p<0.01$).

The levels of K^+ in the lines were not as consistent as for Na^+ (Figure 4.2b). There were significant levels of variability ($p<0.01$) between the null segregant lines, the RNAi 1 nulls having lower K^+ in the 4th leaf sap compared with RNAi 2 nulls. RNAi 2 also had significantly lower levels of K^+ ($p<0.01$) in the 4th leaf sap compared with its null counterpart. RNAi 1, however, does not appear to have lower levels of K^+ compared with its null segregant line and in fact appears to have a higher K^+ content. Whether or not this is a consequence of there being fewer biological replicates for RNAi 1 could not be resolved in this study.

The resulting K^+/Na^+ ratios (Figure 4.2c) were correlative with the high Na^+ content in the 4th leaf sap, i.e. where there was high Na^+ in the leaf, a concomitant decrease in K^+/Na^+ ratio was observed. The plants containing the RNAi has significantly lower K^+/Na^+ ratios compared with their respective null segregants ($p<0.05$). RNAi 2, which had a more dramatic reduction in *TaHKT1;5-D* mRNA compared with its null segregant, showed a more dramatic reduction in K^+/Na^+ compared with RNAi 1 and its null segregant.

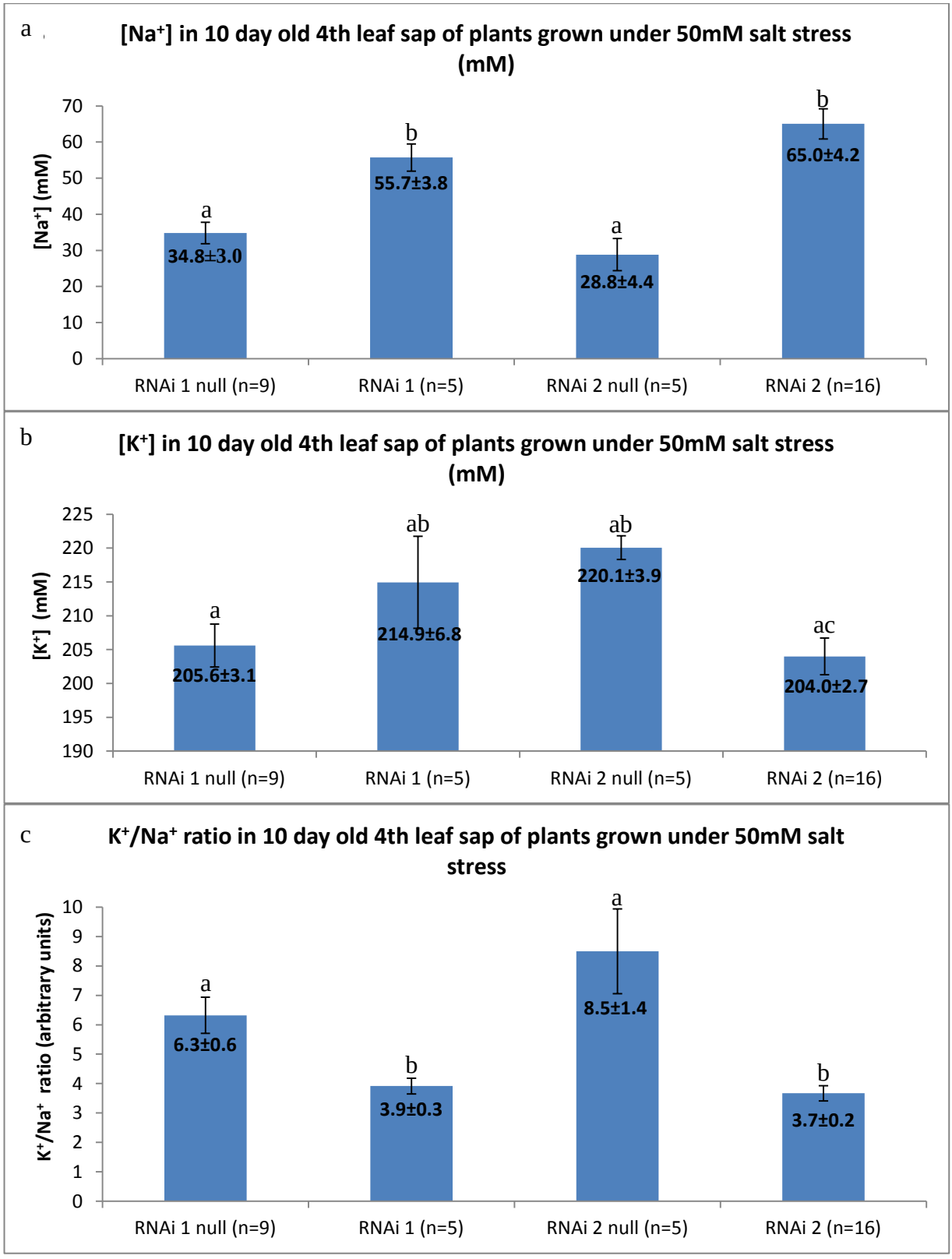


Figure 4.2: Graphs showing Na^+ and K^+ concentrations in the 4th leaf sap of RNAi lines and respective null segregants grown under 50 mM salt stress. *The number of biological replicates is indicated next to the different plant lines. Significant differences at $p<0.05$. Raw mean values with S.E.M. presented within the closed bars of each plant line. (a) Na^+ concentrations in 10 day old 4th leaf sap in mM in RNAi lines and their respective null segregants; (b) K^+ concentrations in 10 day old 4th leaf sap in mM in RNAi lines and their respective null segregants; (c) K^+/Na^+ ratios in 10 day old 4th leaf sap in RNAi lines and their respective null segregants*

4.3.3 Transcript patterns of *TaHKT1;5-D*, *TaSOS1* and *TaNHX1* in wild type *T. aestivum* Bobwhite plants show little difference between control and salt stress treatment

There appeared to be no differences in the transcript levels of *TaHKT1;5-D* (Figure 4.3a) or *TaSOS1* (Figure 4.3b) and a slight induction in the transcription of *TaNHX1* (Figure 4.3c) in the wildtype Bobwhite plants grown in the salt treatment compared with those in control treatment; however, this difference is not statistically significant.

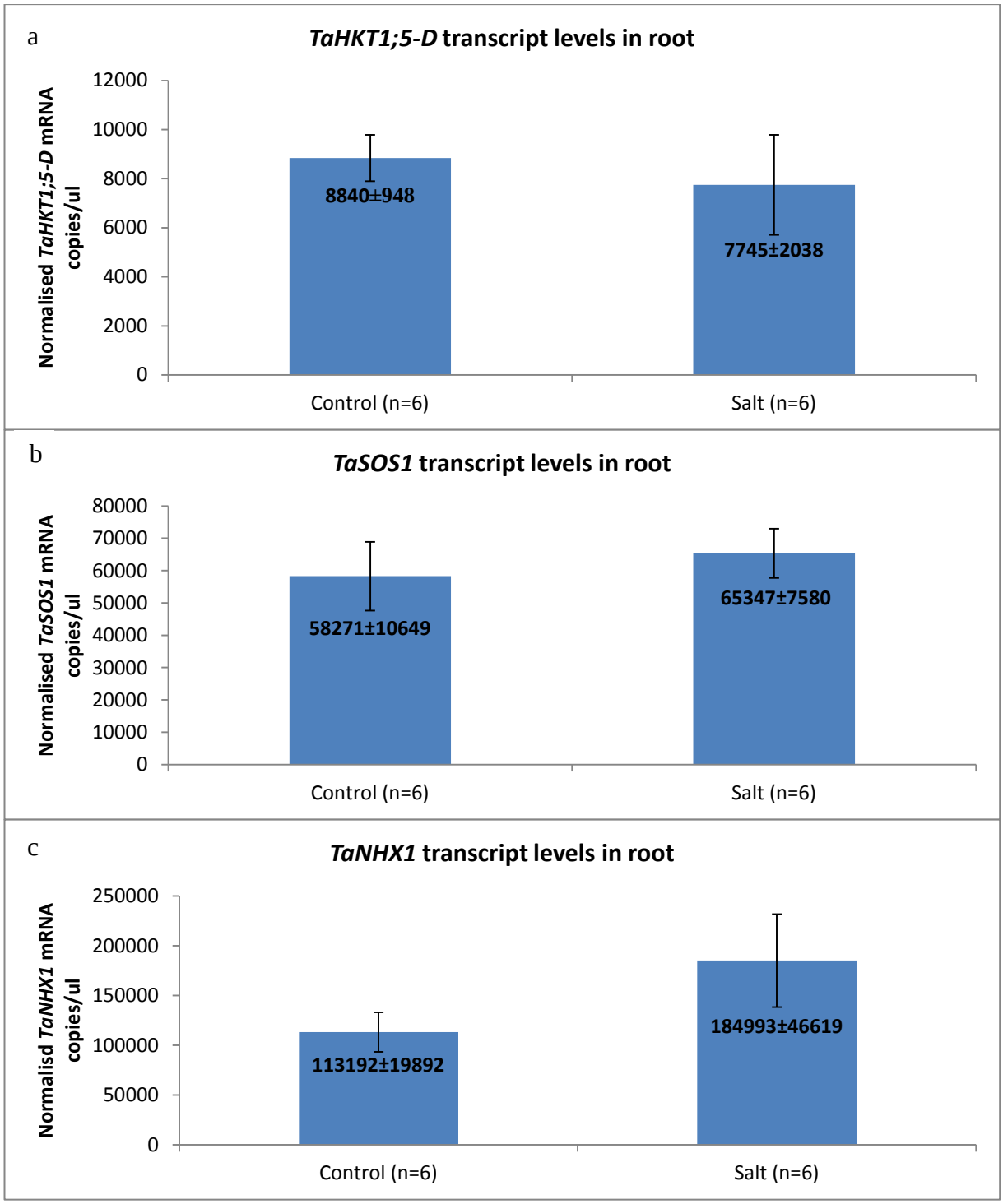


Figure 4.3: Gene transcript patterns in wildtype *T.aestivum* cv. Bobwhite plants in control and 50 mM salt stress conditions. *Number of biological replicates is indicated next to the different plant lines. Raw mean values with S.E.M presented within the closed bars of each plant line. (a) TaHKT1;5-D mRNA levels in roots of wildtype bread wheat plants grown under control and salt stress (50mM) conditions; (b) TaSOS1 mRNA levels in roots of wildtype bread wheat plants grown under control and salt stress (50mM) conditions; (c) TaNHX1 mRNA levels in roots of wildtype bread wheat plants grown under control and salt stress (50mM) conditions*

4.3.4 **Salt tolerance of *T.aestivum* lines containing RNAi construct**

While low levels of *TaHKT1;5-D* mRNA were correlated with increased accumulation of Na^+ in the shoot, this is not necessarily an indication of changes in whole plant salinity tolerance. Whether or not *TaHKT1;5-D* actually influences whole plant salinity tolerance was studied by measuring biomass changes over time in RNAi and null segregant lines under control and salt stress conditions. The changes in biomass were inferred using a non-destructive imaging system employed at The Plant Accelerator (The University of Adelaide, Australia) using the LemnaTec 3D scanalyser. Analysis of the Na^+ and K^+ content in the fourth leaf sap was also performed.

The Na^+ data for both control and salt treatments showed a significantly increased accumulation of Na^+ in the 4^{th} leaf sap in the RNAi lines compared with their respective null segregants ($p<0.01$) (Figures 4.4a-4.4d). Similar trends, in terms of Na^+ accumulation, were seen in the RNAi lines compared with their null counterparts for both the control and salt conditions. The only difference in the accumulation of Na^+ seen in plants under control and salt conditions was that under salt stress. RNAi 1 accumulates significantly higher levels of Na^+ compared with its null counterpart than does RNAi 2 ($p<0.01$) (Figures 4.4b, 4.4d). An approximately 5 mM increase in Na^+ in all plant lines is also seen in the salt stress treatment compared with the levels observed in the control treatment., Despite the differences between the RNAi lines and their null segregants, the accumulation of Na^+ was still low, the highest level being a concentration of 31 mM Na^+ in the 4^{th} leaf sap as measured in the RNAi 1 line grown in the salt treatment (Figure 4.4b).

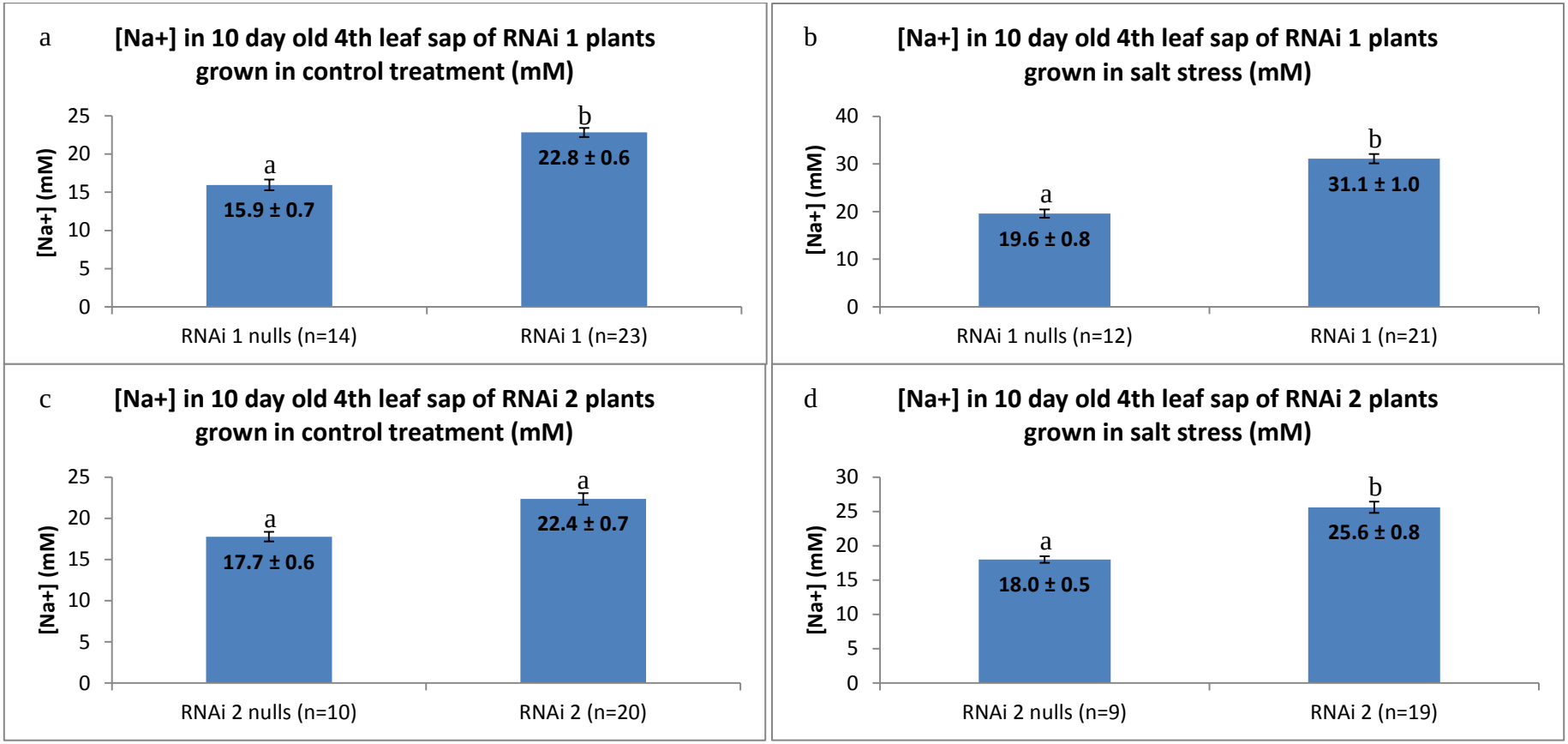


Figure 4.4: Graphs showing Na^+ concentrations in the 4th leaf sap of RNAi lines and respective null segregants grown under control and 75 mM salt stress conditions. *The number of biological replicates is indicated next to the different plant lines. Significant differences at $p<0.05$. Raw mean values with S.E.M. are presented within the closed bars of each plant line. (a) Na^+ concentrations in 10 day old 4^{th} leaf sap in mM in RNAi 1 and its respective null segregant line grown under control conditions; (b) Na^+ concentrations in 10 day old 4^{th} leaf sap in mM in RNAi 1 and its respective null segregants grown under 75mM salt stress; (b) Na^+ concentrations in 10 day old 4^{th} leaf sap in mM in RNAi 2 and its respective null segregant line grown under control conditions; (d) Na^+ concentrations in 10 day old 4^{th} leaf sap in mM in RNAi 2 and its respective null segregants grown under 75mM salt stress.*

The levels of K^+ did not differ greatly between the different lines or between the two treatments, control or salt stress (Figures 4.5a, b, c, d), there being only a slight reduction in the level of K^+ in all plants in the salt treatment (Figures 4.5b and 4.5d) when compared with the control treatment (Figures 4.5a and 4.5c).

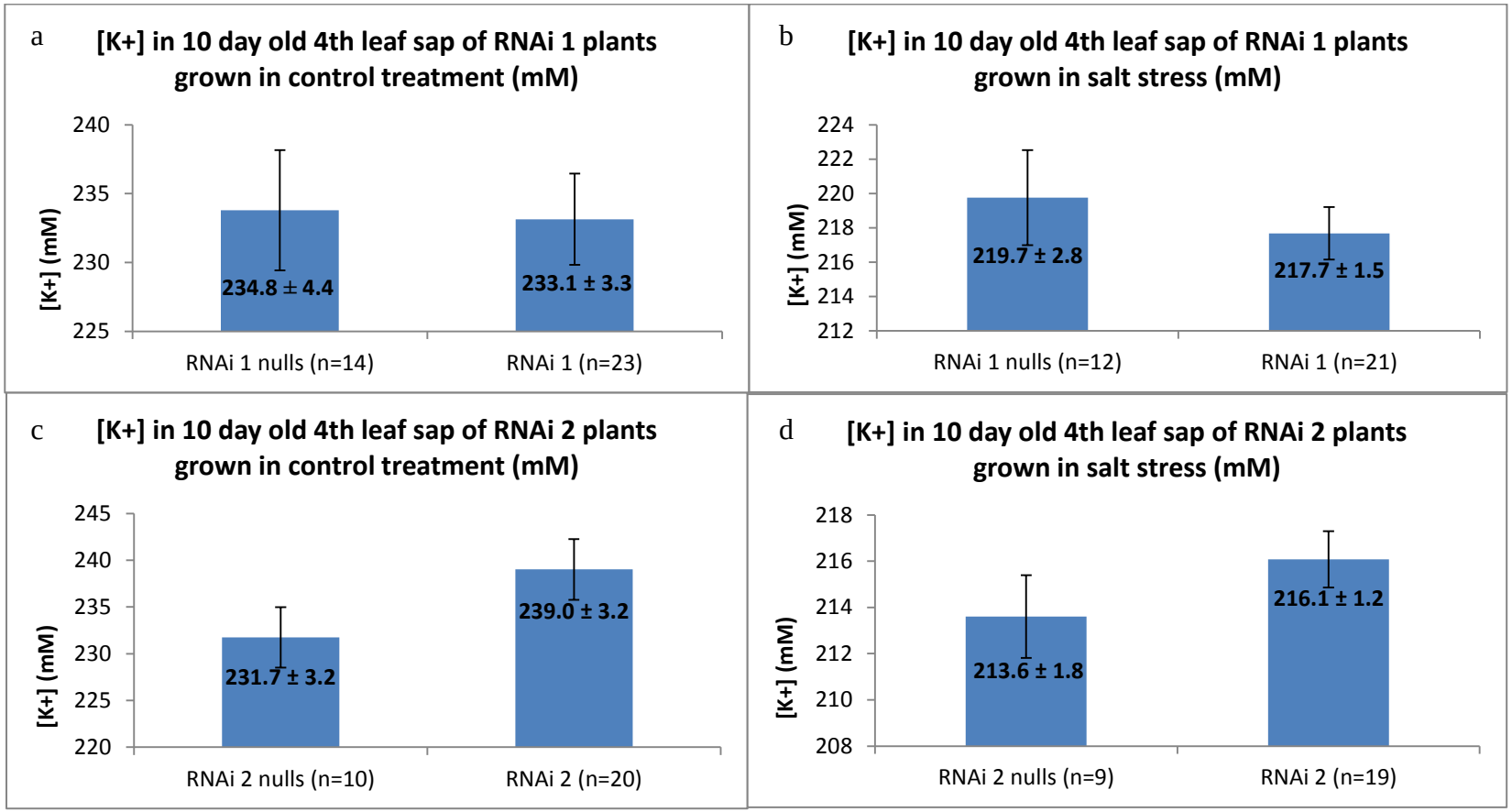


Figure 4.5: Graphs showing K^+ concentrations in the 4th leaf sap of RNAi lines and respective null segregants grown under control and 75 mM salt stress conditions. *The number of biological replicates is indicated next to the different plant lines. Significant differences at $p<0.05$. Raw mean values with S.E.M. are presented within the closed bars of each plant line. (a) K^+ concentrations in 10 day old 4th leaf sap in mM in RNAi 1 and its respective null segregant grown under control conditions; (b) K^+ concentrations in 10 day old 4th leaf sap in mM in RNAi 1 and its respective null segregants grown under 75 mM salt stress; (c) K^+ concentrations in 10 day old 4th leaf sap in mM in RNAi 2 and its respective null segregant grown under control conditions; (d) K^+ concentrations in 10 day old 4th leaf sap in mM in RNAi 2 and its respective null segregants grown under 75 mM salt stress.*

The K^+/Na^+ ratios in the plants follow similar trends as the Na^+ data in both control and salt stress treatments. In the control treatment, RNAi 1 has significantly lower K^+/Na^+ than the RNAi 1 null (Figure 4.6a) ($p<0.01$) while RNAi 2 has lower K^+/Na^+ than its null counterpart (Figure 4.6c) but this difference is not significant as a result of RNAi 2 nulls having a much lower K^+/Na^+ ratio compared with the RNAi 1 nulls.

The differences in K^+/Na^+ ratios in the RNAi plants and their respective null segregants in the salt treatment were more pronounced. Both RNAi 1 and RNAi 2 had a significantly lower level of K^+/Na^+ than their respective controls ($p<0.01$) (Figures 46b and 4.6d). In line with the lower Na^+ seen in RNAi 2 compared with RNAi 1 (Figures 4.4b and 4.4d), RNAi 2 also had a significantly higher K^+/Na^+ ratio than RNAi 1.

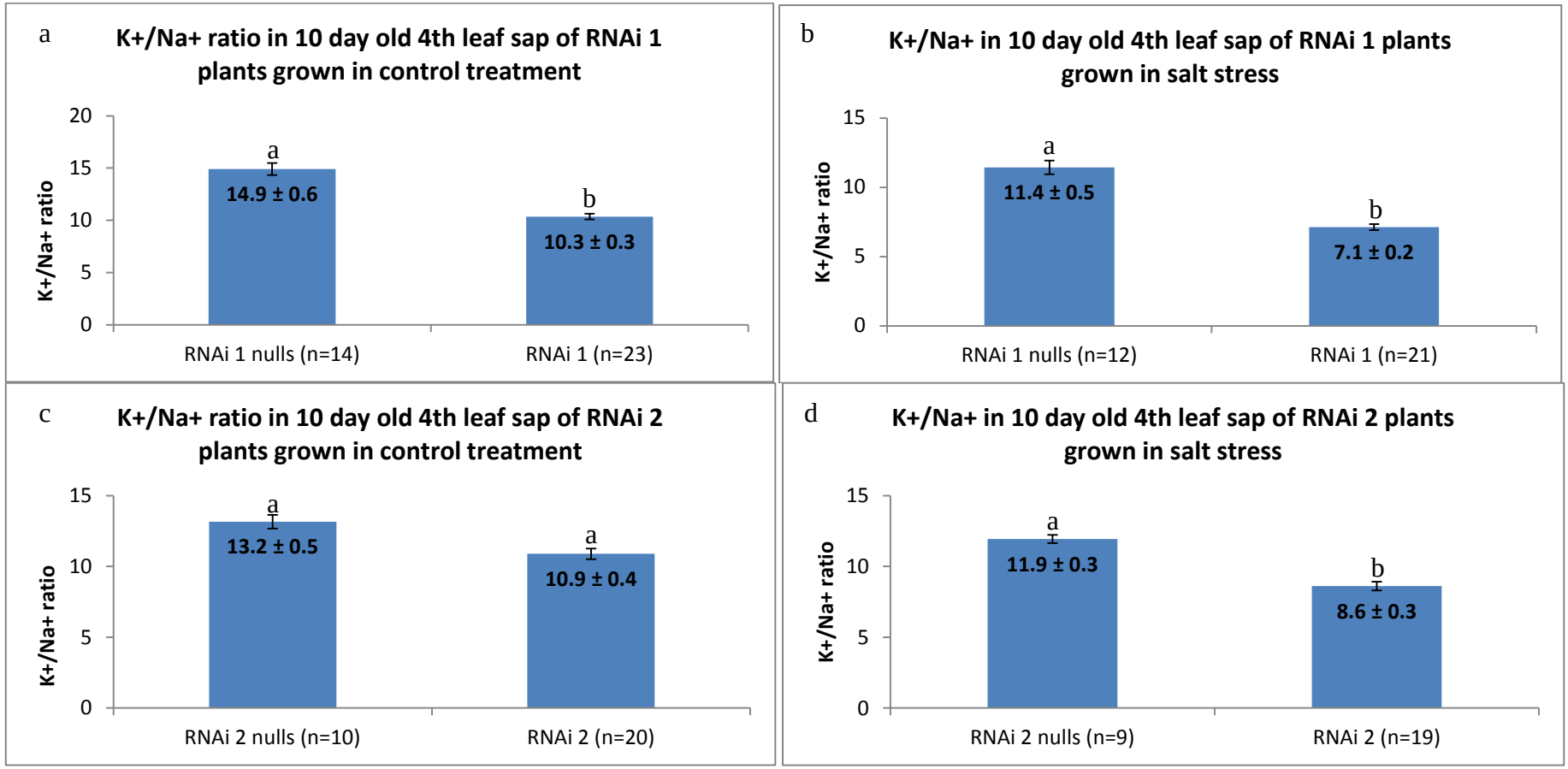


Figure 4.6: Graphs showing K^+/Na^+ ratio in the 4th leaf sap of RNAi lines and respective null segregants grown under control and 75 mM salt stress conditions. *The number of biological replicates is indicated next to the different plant lines. Significant differences at $p<0.05$. Raw mean values with S.E.M. are presented within the closed bars of each plant line. (a) K^+/Na^+ concentrations in 10 day old 4th leaf sap in mM in RNAi 1 and its respective null segregant grown under control conditions; (b) K^+/Na^+ concentrations in 10 day old 4th leaf sap in mM in RNAi 1 and its respective null segregants grown under 75mM salt stress; (c) K^+/Na^+ concentrations in 10 day old 4th leaf sap in mM in RNAi 2 and its respective null segregant grown under control conditions; (d) K^+/Na^+ concentrations in 10 day old 4th leaf sap in mM in RNAi 2 and its respective null segregants grown under 75 mM salt stress.*

4.3.5 **Yield component analysis**

There were no significant differences in the 100 seed weight between the RNAi containing wheat lines and their respective controls (Figures 4.7a-d). There appears to be a slight decrease in the 100 seed weight in the RNAi 1 line compared with its null counterpart grown under control conditions (Figure 4.7a) but this difference is not maintained in the salt stress treatment (Figure 4.7b). RNAi 2 has slightly reduced 100 seed weight compared to its control in the salt stress treatment but this difference is not remarkable (Figure 4.7d). There were no observable differences between RNAi 2 and its null counterpart grown under control conditions (Figure 4.7c).

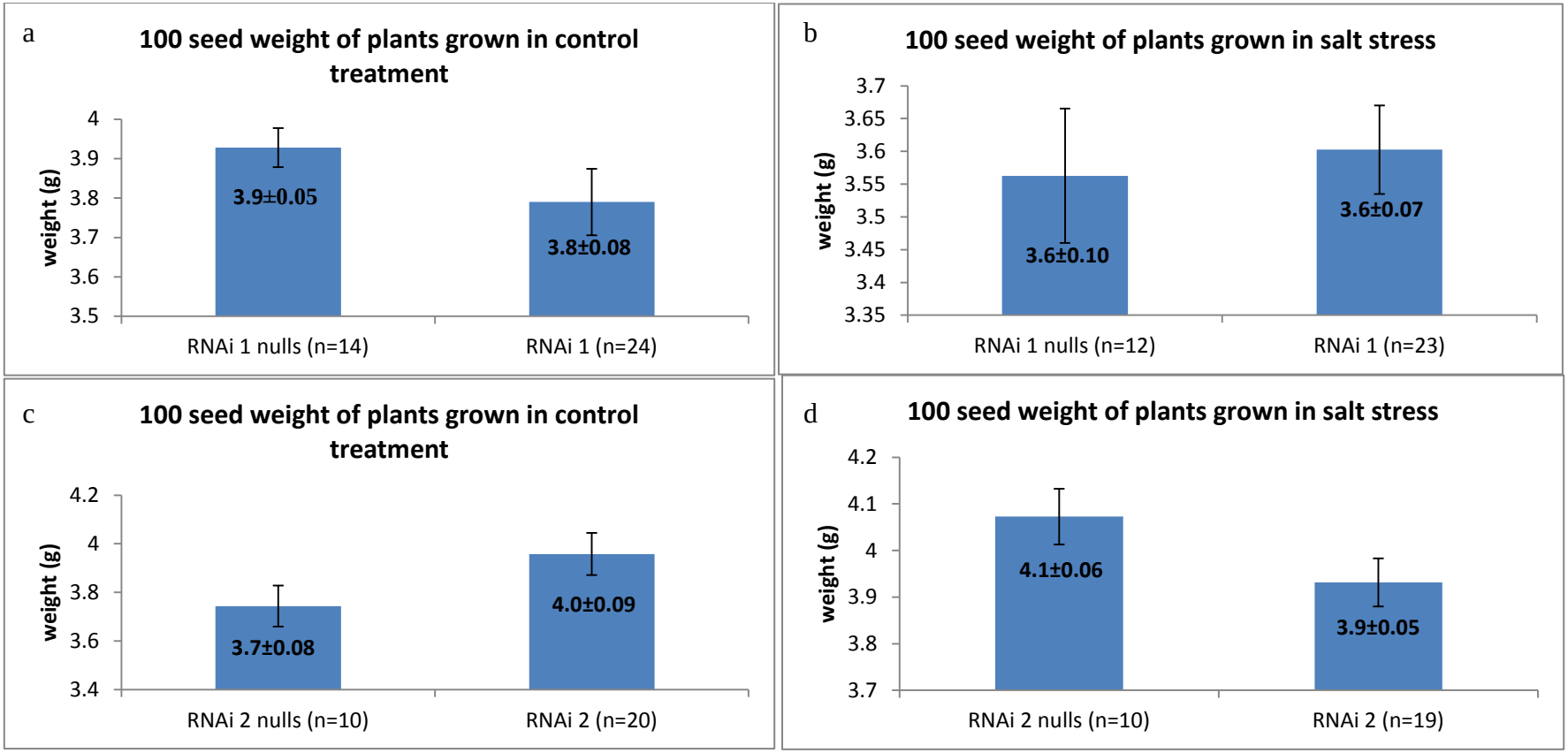


Figure 4.7: Graphs showing 100 seed weight (gm) of RNAi 1 and RNAi 2 compared with their respective controls grown in control treatment and salt stress. *The number of biological replicates is indicated next to the different plant lines. Raw mean values with S.E.M. are presented within the closed bars of each plant line. (a) 100 seed weight of RNAi 1 and its respective null segregant grown under control conditions; (b) 100 seed weight of RNAi 1 and its respective null segregants grown under 75mM salt stress; (c) 100 seed weight of RNAi 2 and its respective null segregant grown under control conditions; (d) 100 seed weight of RNAi 2 and its respective null segregants grown under 75 mM salt stress.*

4.3.6 **Changes in plant biomass**

Changes in plant biomass were quantified in terms of pixels over time. The growth data fitted a 4-parameter sigmoid curve the best for which 95% confidence intervals were also plotted.

4.3.6.1 Biomass over time and relative growth rate of RNAi plants grown in control conditions do not show much difference compared to their respective null segregants

Under control conditions, RNAi 1 nulls appeared to accumulate more biomass over time compared with RNAi 1 until the completion of the experiment (grain filling stage) (Figure 4.8a). The relative growth rate, however, shows that there is no difference in the rate of biomass accumulation between RNAi 1 and RNAi 1 nulls (Figure 4.8b). The RNAi 2 nulls and RNAi 2 do not appear to differ in biomass over time (Figure 4.9a), but RNAi 2 appears initially to have a greater relative growth rate although this eventually matches that of the RNAi 2 nulls (Figure 4.9b).

4.3.6.2 Biomass over time and relative growth rate of RNAi plants grown in salt stress conditions do not show much difference compared to their respective null segregants

Biomass accumulation over time for the RNAi 1 nulls and RNAi 1 were more similar in the salt treatment (Figure 4.8c). The differences in the relative growth rate between RNAi 1 and RNAi 1 nulls are negligible, if they exist at all (Figure 4.8d). RNAi 1 does appear to have a slower relative growth rate than the RNAi 1 nulls.

RNAi 2 nulls and RNAi 2 share similar biomass levels initially but at the completion of the experiment, RNAi 2 has accumulated less than the RNAi 2 nulls (Figure 4.9c). This is reflected in their relative growth rate, RNAi 2 consistently having a lower relative growth rate when compared with the RNAi 2 nulls (Figure 4.9d).

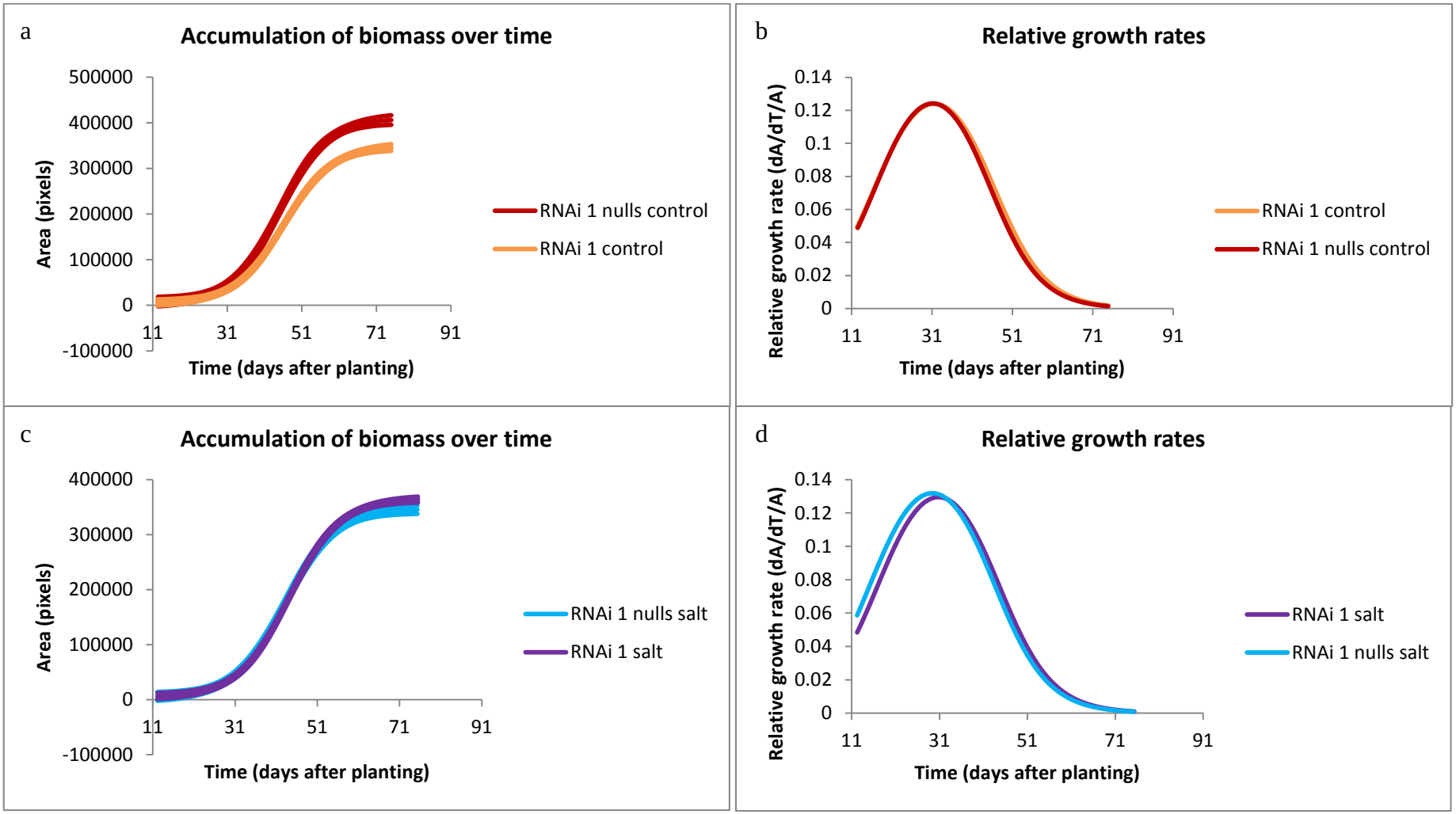


Figure 4.8: Plots showing changes in biomass over time of RNAi 1 and respective null segregants in control and salt stress (75 mM NaCl) treatment. *(a) Change in biomass over time in RNAi 1 null segregants (n=10) and RNAi 1 (n=18) with 95% confidence intervals in control treatment. Biomass represented in pixels. (b) Relative growth rate of RNAi 1 null segregants (n=10) and RNAi 1 (n=18) in control treatment. (c) Change in biomass over time in RNAi 1 null segregants (n=10) and RNAi 1 (n=17) with 95% confidence intervals in salt stress. (d) Relative growth rate of RNAi 1 null segregants (n=10) and RNAi 1 (n=17) in salt stress.*

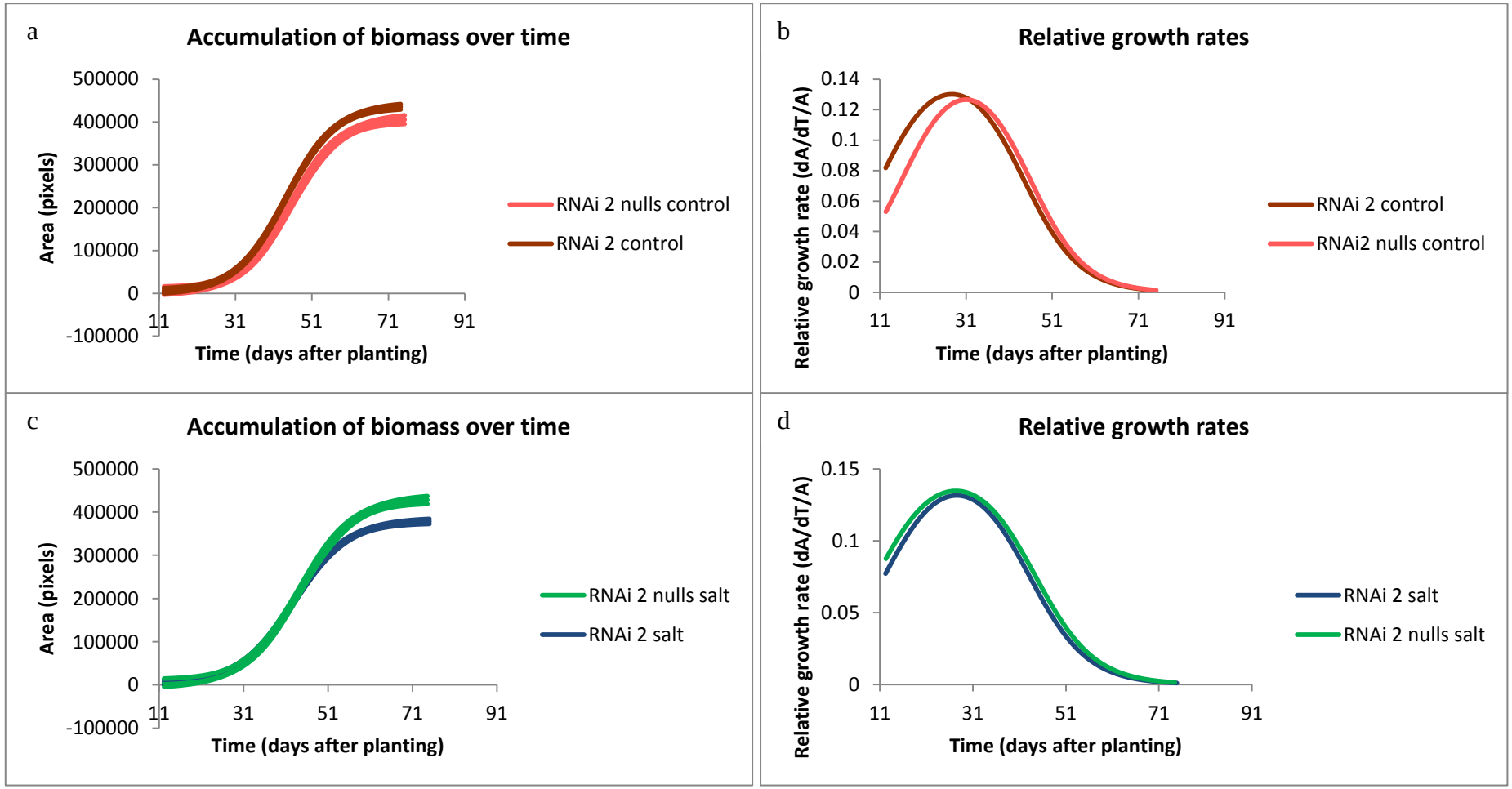


Figure 4.9: Plots showing changes in biomass over time of RNAi 2 and respective null segregants in control and salt stress (75 mM NaCl) treatment. *(a). Change in biomass over time in RNAi 2 nulls (n=8) and RNAi 2 (n=17) with 95% confidence intervals in control treatment. Biomass represented in pixels. (b) Relative growth rate of RNAi 2 nulls (n=8) and RNAi 2 (n=17) in control treatment. (c) Change in biomass over time in RNAi 2 nulls (n=7) and RNAi 2 (n=17) with 95% confidence intervals in salt stress. (d) Relative growth rate of RNAi 2 nulls (n=7) and RNAi 2 (n=17) in salt stress.*

4.4 Discussion

4.4.1 Effect of the RNAi on endogenous *TaHKT1;5-D* expression

The RNAi lines do show lower levels of endogenous *TaHKT1;5-D* transcript compared with the null segregants (figure 4.1a). The transcript levels are inherently low in the null segregants compared to wildtype and fall to about half in the lines with the RNAi 1 construct. Observing the mRNA levels in the null segregants, it appears that *TaHKT1;5-D* is not highly transcribed in the null segregant lines as the level that is detected is slightly above the background level of 500 copies/µl (Dr. Neil Shirley, pers. comms., ARC Centre of Excellence in Plant Cell Walls, University of Adelaide). The wildtype lines however, on average, appear to have approximately 5 times more *TaHKT1;5-D* expression (Figure 4.3a) compared to the RNAi null segregants (Figure 4.1a), though the S.E.M. amongst the wildtype plants also appears to be greater. The RNAi 2 null segregant line also consistently appears to have dramatically lower levels of *SOS1* and *NHX1* levels and RNAi 1 null segregant line appears to have lower levels of *NHX1* levels compared with the wildtype plants (Figures 4.1b-c). A difference in genetic background of the RNAi null segregant lines and/or a result of the transformation process on the RNAi null segregants could be the cause of the discrepancy in *TaHKT1;5-D* levels observed in the RNAi null segregants and wildtype. The possibility of the transgene being inserted into a regulatory region which pertains to either *SOS1* or *NHX1* thereby causing the reduced levels observed in the null segregant lines cannot be ruled out either, although this would be very unlikely.

TaHKT1;5-D also appears not to be induced by salt stress (Figure 4.3a) which was expected as a similar result was observed with the rice *HKT1;5* in a study by Ren *et al.* (2005) where no increase in endogenous gene expression is observed in salt stress when compared with control. Thus, *TaHKT1;5-D* is probably not a facultative gene despite having a specific role in the plant as a Na^+ transporter. While *TaHKT1;5-D* levels were reduced in both the RNAi lines, less consistent transcript patterns were observed for *TaSOS1* and *TaNHX1* (Figure 4.1b-c). *TaSOS1* and *TaNHX1* follow similar trends for transcript levels in the different lines (Figures 4.1b-c and 4.3b-c). This was as anticipated as *TaSOS1* and *TaNXH1* are both Na^+/H^+ antiporters and it has been suggested that as well as SOS1 the SOS pathway also regulates NHX1 in *A. thaliana* (Qiu *et al.*, 2004). It is possible that *TaSOS1* and *TaNHX1* function in a similar manner. These two gene pathways, however, appear to be decoupled from that of

TaHKT1;5-D (Figures 4.1a-c and 4.3a-c) as found by Rus *et al.* (2001) in the gene orthologues in *A. thaliana*.

4.4.2 **Issue of RNAi specificity**

Other gene members of the *HKT* gene family were not studied. Such studies are important in order to determine the effects of the RNAi construct in genes which might harbour regions that are similar to the target gene. While RNAi sequences are ultimately processed into ~25 mer sequences which are highly specific to the target gene (Hamilton and Baulcombe, 1999), sequence similarity with other genes has been demonstrated to increase the number of gene targets for the RNAi (Van Houdt *et al.*, 2003). In this study, the RNAi constructs were generated such that there was slight overlap with the 3'UTR region to ensure specificity of the targeting of the RNAi. As the RNAi sequences used in this study were quite large, both being between 500- 600 bp in length, there are many possible ~25 mer PTGS sequences that could be generated. Although much care was taken to ensure gene specificity for silencing, there are also homeologues of *TaHKT1;5-D* on the B genome which could be affected by the RNAi (Byrt, 2008). Likewise, as *TaHKT1;5-D* is part of a larger *HKT* gene family, it could share short sequences that may be homologous to the other 8 gene members of the family. This sequence similarity combined with the large number of possible PTGS that could be generated could result in other *HKTs* being targeted (Van Houdt *et al.*, 2003).

4.4.3 **Dosage effect of RNAi**

In this study, it was observed especially for data generated through experiments on T_2 RNAi lines that RNAi 2 consistently had lower levels of *TaHKT1;5-D* (Figure 4.1a) and therefore higher Na^+ (Figure 4.2a) and lower K^+ (Figure 4.2b) in the shoot than RNAi 1. This might be explained by RNAi insert copy number or amount of RNAi transgene expression. It has been shown that high levels of RNAi expression can cause greater levels of gene repression (Travella *et al.*, 2006) which might be the case seen in this study where RNAi 2 consistently appears to have less *TaHKT1;5-D* expression, high Na^+ and lower K^+. Na^+ (Figure 4.4a-d) and K^+ differences (Figure 4.5a-d) seen between RNAi 1 and 2 in T_2 plants grown in hydroponics are somewhat mitigated in the T_3 generation possibly as a result of being grown in soil. There was not sufficient DNA collected from the wheat plants to carry out a Southern

blot to assess insert number for each of the lines but establishing this may help explain the discrepancy observed throughout the study.

Another indication of RNAi insert copy number being greater in RNAi 2 than RNAi 1 was the lack of null segregants found in any of the RNAi 2 lines; whereas, RNAi 1 lines were segregating in both T_2 and T_3 generation (Table 4.1). This would also imply that a higher number of RNAi inserts are present in RNAi 2 than RNAi 1.

The transgene integration site also needs to be determined to ensure that it has not been inserted into a region that might control or regulate expression of *TaHKT1;5-D* although the hypothesis regarding the insert copy number seems more likely because of what has been observed.

4.4.4 Role of *TaHKT1;5-D* in K^+/Na^+ discrimination

In line with studies that show reduction/lack of *HKT1;5* transcript level, a decrease in *TaHKT1;5-D* gene transcript levels has been found to increase Na^+ accumulation in the shoot of bread wheat plants containing the RNAi construct (Gorham *et al.*, 1987; Mäser *et al.*, 2002).

The study by Gorham *et al.* (1987) also showed that associated with a reduction in shoot Na^+ was an increase in shoot K^+ accumulation and, therefore, higher shoot K^+/Na^+. While K^+/Na^+ ratios (Figures 4.2c and 4.6a-d) decreased in lines with reduced *TaHKT1;5-D* transcript (Figure 4.1a), the concentrations of shoot K^+ (Figures 4.2b and 4.5a-d) in the lines were not consistent unlike what was observed in the study by Gorham *et al.* (1987). In the T_2 generation where plants were grown in hydroponics, RNAi 2 had a lower concentration of K^+ in 4th leaf sap compared with its null segregant line but RNAi 1 appeared to have a higher K^+ concentration compared with the RNAi 1 null segregant line (figure 4.2b). It is not clear whether this is a result of there being insufficient biological replicates with RNAi 1 having only five compared with sixteen replicates for RNAi 2. There was also a less dramatic reduction in *TaHKT1;5-D* mRNA levels for RNAi 1 than RNAi 2 compared with the null counterparts (Figure 4.1a); RNAi 1 also had a less dramatic increase in Na^+ with respect to its null line than RNAi 2 (Figure 4.2a). This reduced Na^+ accumulation might have had less

impact on K^+ entry into the shoot. Higher salt stress such as 100 mM NaCl might have rendered results more in line with what was expected, that is, a high concentration of Na^+ and a low concentration of K^+ in leaf tissue from all the lines.

4.4.5 Is *TaHKT1;5-D* a salt tolerance determinant?

While *TaHKT1*;5-D in bread wheat appears to control the Na^+ and K^+ content in leaves its role as a salt tolerance determinant, as seen in other systems, remains to be confirmed. In order to study the role of *TaHKT1*;5-D in whole plant salt tolerance in wheat, plants containing the RNAi constructs (RNAi 1 and RNAi 2) and their respective null segregants were assessed for changes in biomass using non-destructive methods. Fourth leaf Na^+ analyses in these plants demonstrated that the imposed salt stress of 75 mM NaCl was not sufficient, despite the RNAi lines accumulating significantly higher Na^+ than their respective null segregants (Figures 4.4a-d). The plants containing the RNAi construct and their null segregant controls of the study performed in soil accumulated leaf sap Na^+ in the order of 30 mM. It has been suggested that the plant cytosol can tolerate 30 mM Na^+ without being stressed (Munns and Tester, 2008). While the measured shoot Na^+ in bread wheat in this study was not from the cytosol, it is possible that the leaf sap measurements are indicative of similar levels of intracellular Na^+. This would mean that Na^+ levels in the shoot were not so high to cause any major growth impediments. Such an inference was borne out by observation: there was very little, if any, change in relative growth rate between the lines containing the RNAi and their null counterparts. The lack of any difference seen in 4^{th} leaf K^+ levels between the RNAi lines and their respective controls could also be explained by the lack of sufficient salt stress. The low levels of Na^+ in the 4^{th} leaf sap were most likely not sufficient to displace K^+ from the shoot.

There were also no differences observed in the 100 seed weights of the different lines compared with the controls with the exception of RNAi 2 which had a slight reduction compared with its null counterpart, although this was not significant. Again, a higher salt stress might cause a more dramatic reduction in the yield of the RNAi plants compared with their respective null segregants.

It is possible that differences in plant behaviour when grown in hydroponics rather than soil could result in the different Na^+ levels observed between the two growth conditions. Where plants in hydroponics were treated with 50 mM NaCl, the plants accumulated concentrations of Na^+ in the order of the concentration applied to the growth solution. In soil, however, the plants were more efficient at excluding external Na^+. This observation may have been due to the high water retention capabilities of coco peat soil combined with the presence of other cations, such as Ca^{2+}, which were effective in displacing Na^+ from the soil.

Based on the observations from this study, it can be surmised that the imposed salinity stress was insufficient and that higher levels of salt stress will need to imposed in subsequent studies on the effect of *TaHKT1;5-D* on plant biomass.

Studies involving the importance of *HKT1;5* or *HKT1;1* have shown that the gene does have an impact on whole plant salinity tolerance (Berthomieu *et al.*, 2003; Rus *et al.*, 2004; Sunarpi *et al.*, 2005; Davenport *et al.*, 2007; Møller *et al.*, 2009; Plett *et al.*, 2010; James *et al.*, 2011; Munns *et al.*, 2012). While there is support for the *Kna1* locus playing an important role shoot Na^+ exclusion, its importance in salt tolerance has not been established (Gorham *et al.*, 1987; Byrt *et al.*, 2007). The results presented in this chapter provide support for the importance of *TaHKT1;5-D* as being an important contributor to *Kna1's* role in Na^+ exclusion but the role of the gene in plant salt tolerance could not be resolved in this study.

4.5 Conclusion

Lowering *TaHKT1*;5-*D* transcript levels resulted in increased Na^+ accumulation in the 4^{th} leaf sap of bread wheat plants both under control and salt-stressed conditions. This confirms the importance of *TaHKT1;5-D* to the *Kna1* locus' role in lowering shoot Na^+ levels. While the transcript levels of *TaHKT1*;5-*D* were inversely proportional to Na^+ content in the 4^{th} leaves, the impact of the gene on whole plant salinity tolerance was not resolved in this study. Plant biomass and growth rate appear not to be affected by the lack of *TaHKT1*;5-*D* expression. This is most likely to be due to the low levels of salt which were applied to the plant resulting in a salt stress treatment that was insufficient to elicit a response from the plant.

The results detailed in this chapter where reduced *HKT1;5* transcript in bread wheat increases Na^+ accumulation in the shoot, supports the results from Chapter 3 where overexpressing the barley orthologue decreases Na^+ accumulation in the shoot. The question of Na^+ exclusion and its effect on salinity tolerance, however, was not able to be resolved in this study. This, however, does not mean that the results are incongruous with the current literature that clearly states that Na^+ exclusion is an important salt tolerance trait for glycophytes.

The following chapter will synthesise all the results presented in this study and will discuss the implications of the study with regard to the current literature.

5 General discussion of the results

5.1 Genetically engineering Na^+ exclusion in barley plants

5.1.1 Conservation of promoter control between different species- lessons learned

Promoter identification was a critical component in this study. The identification of promoters which controlled either root cortex or stelar-specific gene expression at relatively low levels was best achieved for this project through studying gene expression patterns in a maize MPSS database. The identification of the rice orthologues of genes identified in maize and the subsequent isolation of the immediate 2kb upstream non-coding region as promoter to be transformed into barley was undertaken assuming that *cis* and *trans* gene regulatory factors between the three species would be conserved. There have been studies which have shown promoter activity to be conserved between different plant species (Kaplinsky *et al.*, 2002; Guo and Moose, 2003). Also, given the high level of synteny between the grasses, it is highly conceivable that not only gene regions are orthologous but also some of the regions that control the expression of these genes as spatial and temporal control of gene activity is important for gene function (Devos and Gale, 2000). As promoter regions are non- coding, however, the rate of sequence mutation is often greater than that in the coding regions and, thus, may experience non-concerted evolution (Guo and Moose, 2003). This means that the isolation of rice promoters of maize orthologues used to control downstream gene expression maybe unpredictable in barley (Zakharov *et al.*, 2004).

This is not to say that the method used in this study is not an effective predictive strategy for the identification of cell type-specific promoters from one organism and its function in another. It is hard to predict, however, which regulatory regions will be conserved between plant species as different genes have different rates of mutations; for instance, a disease resistance gene and, by extension its promoter, will have a higher rate of mutation than one that is involved in important metabolic processes and by extension so will the regulatory regions (Leister *et al.*, 1998). Attempts at identifying spatial and temporal control of gene regulatory regions through using gene enhancer traps have helped identify important gene enhancer regions which have been subsequently exploited to drive genes of interest (Haseloff,

1999; Johnson *et al.*, 2005; Møller *et al.*, 2009; Plett *et al.*, 2010). Further expansion on such resources to other important cereals can help the identification of regulatory regions which can be selected on the basis of their temporal and spatial specificity. In this study, however, while the promoter activities were not maintained from maize to rice to barley as transgene expression was not limited to the root alone, the constructs containing the putative promoter fused to the genes of interest did render favourable results.

5.1.2 Unintended consequences- promoter control in barley was different to what was expected but still generated a result where Na^+ exclusion was improved

5.1.2.1 Na^+ exclusion through HvHKT1;5 *overexpression*

PCR studies on transgene expression indicated that transgene expression was present in the roots as well as the shoots. According to previous studies, cell type-specificity of *AtHKT1;1* expression (the *A. thaliana* orthologue of the cereal *HKT1;5*) is paramount for its role in whole plant salt tolerance (Møller *et al.*, 2009). It is widely accepted that *AtHKT1;1* and other *HKT1;5*s are expressed primarily in the roots (Berthomieu *et al.*, 2003; Sunarpi *et al.*, 2005; Byrt *et al.*, 2007; James *et al.*, 2011; Munns *et al.*, 2012). As for whether *HKT1;5* expression is found in the shoots, the jury is still out, with studies in rice and *A. thaliana* reporting that it is expressed in shoots, albeit, at lower levels than in roots (Berthomieu *et al.*, 2003; Ren *et al.*, 2005; Sunarpi *et al.*, 2005) and studies in wheat reporting no shoot expression (Byrt *et al.*, 2007; James *et al.*, 2011; Munns *et al.*, 2012). It appears, in the case of this study, that shoot expression of *HvHKT1;5* was not detrimental, suggesting the flexibility of *HKT1;5*-permissive organs. As HKT1;5 is implicated in xylem unloading of Na^+ to limit shoot Na^+ accumulation, it is possible that sufficient Na^+ could have been unloaded by the roots leaving little to be translocated to the shoot (Byrt *et al.*, 2007; Davenport *et al.*, 2007; Møller *et al.*, 2009; Plett *et al.*, 2010; James *et al.*, 2011; Munns *et al.*, 2012) .

5.1.2.2 Possible enhancement of tissue Na^+ tolerance mechanism through HvHVP1 *overexpression*

HvHVP1 has been shown to be important in cation sequestration in vacuoles and when overexpressed constitutively in plants has given rise to increased Na^+ accumulation when

grown under salt stress (Park *et al.*, 2005; D'yakova *et al.*, 2006; Gao *et al.*, 2006; Guo *et al.*, 2006; Brini *et al.*, 2007; Bao *et al.*, 2009; Pasapula *et al.*, 2011; Zhang *et al.*, 2011). This is because it facilitates the inward flow of cations into vacuoles by cation/H^+ antiporters like NHX1 through the maintenance of a large proton gradient (Fukuda *et al.*, 1998; Gaxiola *et al.*, 1999; Fukuda *et al.*, 2004; Fukuda and Tanaka, 2006; Brini *et al.*, 2007). In this study, it was intended for the *HvHVP1* ectopic expression to be limited to the cortical cells in the root to increase the amount of Na^+ storage in root cells and thereby limit its transport to the shoot. Expression of *HvHVP1* in all the overexpressing lines was found in both shoots and roots as opposed to the root-cortex only expression which was expected when isolating the putative promoters in this study. As *HvHVP1* is likely to be important in enhancing tissue tolerance by increasing intracellular sequestration of Na^+, its expression in the shoots would potentially increase shoot Na^+ accumulation. The plants overexpressing *HvHVP1* do tend to accumulate more Na^+ than controls, especially in lines 310-3 and 314-6. Thus, the strategy of increasing Na^+ exclusion in shoot through overexpression of *HvHVP1* was not successful. It is, however, entirely possible that salt tolerance has still been improved but not necessarily by exclusion. The increase in Na^+ accumulation seen in the transgenic barley lines could be a result of the enhanced vacuolar sequestration of Na^+ compared with null segregants. There is a slight drop, however, in leaf K^+ accumulation which could be a result of Na^+ displacing K^+ in the vacuoles in order to maintain charge and osmotic balance. An increase in solute accumulation in the shoots accompanied with normal shoot growth compared with null segregants or wildtype plants would be a strong indicator of increased salinity tolerance.

5.1.3 **Spatial location of transgene activity- strategies to resolve the black box**

Immunolocalisation of the transgenic protein would help establish the putative promoters' regions of control and where the transgenes were being expressed. Transgenic barley plants containing a haemagglutinin epitope tag fused to the C-terminus of both *HvHKT1;5* and *HvHVP1* had been generated as part of this study. Characterisation of these lines through *in situ* immunolocalisation would be a better alternative to reporter assays as it will demonstrate the areas in which the transgene product is active. This will also help elucidate regions of the plant in which *HvHKT1;5* can be expressed and help gain a better idea of how the gene can be manipulated to better enhance Na^+ exclusion and thus tolerance in plants.

5.2 How important is shoot Na^+ exclusion to whole plant salinity tolerance?

5.2.1 In barley

Although effects of transgene expression in barley had been assessed through studying shoot Na^+ accumulation, the genes' role in Na^+ tolerance could not be resolved. As the plants were T_1 generation, all of the material had to be grown through to the next generation and be allowed to set seed. As a result, biomass studies could not be performed. Studying changes in biomass accumulation between the transgenic plants compared with null segregant controls would have helped establish the effects of the transgenes on the plant's ability to tolerate salt.

Also, a reduced Na^+ profile in the shoot does not necessarily lead to salt tolerance. Xylem loading of Na^+ appears to be less restricted compared with wheat or other cereals (Shabala *et al.*, 2010) and barley does tend to accumulate more shoot Na^+ than wheat and still maintain growth (Munns and James, 2003; Colmer *et al.*, 2005). This made barley a good model to study the exclusion mechanism contributed by the constructs generated in this project. As barley is naturally a Na^+ accumulator, any Na^+ reduction observed in the shoot of barley would be a result of transgene activity. Whether these changes in Na^+ accumulation are relevant to whole plant salt tolerance is yet to be established in this study and biomass studies need to be performed to address this.

5.2.2 In bread wheat

It was not possible to determine the importance of *HKT1;5* as a salt tolerance determinant in this study. What could be concluded, however, was its role in shoot Na^+ accumulation. The Na^+ data with respect to *HKT1;5* expression, in this study, concurred with what has been previously reported in the literature about *HKT1;5* playing a positive role in reducing Na^+ accumulation in the shoot (Byrt *et al.*, 2007; Davenport *et al.*, 2007; Møller *et al.*, 2009; Plett *et al.*, 2010; James *et al.*, 2011; Munns *et al.*, 2012).

Lowering *TaHKT1;5-D* (*HKT1;5* member found in bread wheat) transcript levels through RNAi in bread wheat resulted in significantly higher Na^+ levels in the shoot in the transgenic

lines compared with null segregant controls. There was little correlation between *TaHKT1;5-D* levels and K^+ accumulation in the shoot, which reinforced the role of *HKT1;5* as a Na^+-specific transporter. This gene member is found in an important locus, *Kna1*, first reported by Gorham *et al.* (1987). This locus, found on the long arm of chromosome 4D, is large and unresolved and has been known to be important in maintaining high K^+/Na^+ ratio observed in bread wheat (Gorham *et al.*, 1987). The findings of this study correlate in part with that reported in Gorham *et al.* (1987) in that shoot Na^+ accumulation is significantly affected by *TaHKT1;5-D* but not shoot K^+ accumulation. Plants were grown under glasshouse conditions and were subject to mild salt stress to assess changes in biomass accumulation and whether lower *TaHKT1;5-D* expression had any bearing on general plant growth. The importance of *TaHKT1;5-D* in salt tolerance also could not be resolved in this study most likely as a result of insufficient salt stress being imposed on the plants. Increased Na^+ stress could help provide a clearer picture on the impacts of lowered *TaHKT1;5-D* on biomass accumulation.

5.3 Future directions

While transforming plants with single salt tolerance genes might be effective in lowering Na^+ accumulation and thus increasing salt tolerance, pyramiding salt tolerance genes might be more effective. For example, as *NHX1* has been shown to operate in a coordinated manner with the vacuolar *H^+-PPase*, overexpression of both the pyrophosphatase and the Na^+/H^+ antiporter could result in enhanced salinity tolerance. In addition, further pyramiding the genes involved in tissue tolerance with those that are important for Na^+ exclusion could result in an ideal combination of genes that can work in a cooperative manner to achieve a salt tolerant plant. Expression of the *HKT1;5* in the root stele combined with the expression of *H^+-PPase* and *NHX1* in the outer layers of the root like the cortex and the epidermis could could assist the unloading of Na^+ in the roots. This approach is currently being trialled in a new project where with the crossing of 316-10 and 310-3 lines is being used to generate plants that have both the ectopic exclusion and tolerance mechanism.

Given the more rapid mutation rates of promoter regions in plants compared with their genes, thorough characterisation of the promoters in both the source plant and also in the plant to which it will be transferred would be prudent. Ideally, promoters of the same plant would be

used to drive ectopic expression of the genes of interest, i.e., barley promoters being used in barley to ectopically control gene expression. This would mitigate issues with regard to the predictability of promoter control as found in this study. Characterisation of promoters is key to the improvement of crop abiotic stress tolerance through targeted gene expression.

Discovery and functional characterisation of genes important in salt tolerance have enabled the manipulation of these genes through use of biotechnology in crops. Much proof-of-concept work with regard to overexpression of important salt tolerance genes has been conducted in model plants under laboratory conditions (Karakas *et al.*, 1997; Sheveleva *et al.*, 1997; Apse *et al.*, 1999; Gaxiola *et al.*, 1999; Shi *et al.*, 2003; D'yakova *et al.*, 2006; Gao *et al.*, 2006; Guo *et al.*, 2006; Brini *et al.*, 2007; Chen *et al.*, 2007a; Duan *et al.*, 2007; Bao *et al.*, 2009; Møller *et al.*, 2009; Plett *et al.*, 2010). The results of these studies, however, have little relevance if the same benefits in commercially important plants under field conditions are not realised. Thus, further characterisation of the barley lines generated in this study is important and part of the work needs to be carried out under field conditions to see whether the same advantageous phenotypes are upheld in the field as they have been observed in the glasshouse.

6 References

An, R., Chen, Q.-J., Chai, M.-F., Lu, P.-L., Su, Z., Qin, Z.-X., Chen, J. & Wang, X.-C. 2007. *AtNHX8, a member of the monovalent cation: proton antiporter-1 family in Arabidopsis thaliana, encodes a putative Li/H antiporter*.

Apse, M. P., Aharon, G. S., Snedden, W. A. & Blumwald, E. 1999. Salt tolerance conferred by overexpression of a vacuolar Na+/H+ antiport in Arabidopsis. *Science,* 285**,** 1256 - 1258.

Apse, M. P. & Blumwald, E. 2007. Na+ transport in plants. *FEBS Letters,* 581**,** 2247-2254.

Bao, A.-K., Wang, S.-M., Wu, G.-Q., Xi, J.-J., Zhang, J.-L. & Wang, C.-M. 2009. Overexpression of the Arabidopsis H+-PPase enhanced resistance to salt and drought stress in transgenic alfalfa (Medicago sativa L.). *Plant Science,* 176**,** 232-240.

Barragán, V., Leidi, E. O., Andrés, Z., Rubio, L., De Luca, A., Fernández, J. A., Cubero, B. & Pardo, J. M. 2012. Ion Exchangers NHX1 and NHX2 Mediate Active Potassium Uptake into Vacuoles to Regulate Cell Turgor and Stomatal Function in Arabidopsis. *The Plant Cell Online,* 24**,** 1127-1142.

Bassil, E., Coku, A. & Blumwald, E. 2012. Cellular ion homeostasis: emerging roles of intracellular NHX Na/H antiporters in plant growth and development. *Journal of Experimental Botany,* 63**,** 5727-5740.

Bassil, E., Tajima, H., Liang, Y.-C., Ohto, M.-A., Ushijima, K., Nakano, R., Esumi, T., Coku, A., Belmonte, M. & Blumwald, E. 2011. The Arabidopsis Na+/H+ Antiporters NHX1 and NHX2 Control Vacuolar pH and K+ Homeostasis to Regulate Growth, Flower Development, and Reproduction. *The Plant Cell Online,* 23**,** 3482-3497.

Beresford, Q., Bekle, H., Phillips, H. A. & Mulcock, J. 2001. *The Salinity Crisis: Landscapes, Communities and Politics,* Crawley, University of Western Australia Press.

Berger, B., Regt, B. & Tester, M. 2012. High-Throughput Phenotyping of Plant Shoots. *In:* Normanly, J. (ed.) *High-Throughput Phenotyping in Plants.* Humana Press.

Berthomieu, P., Conejero, G., Nublat, A., Brackenbury, W. J., Lambert, C., Savio, C., Uozumi, N., Oiki, S., Yamada, K., Cellier, F., Gosti, F., Simonneau, T., Essah, P. A., Tester, M., Very, A. A., Sentenac, H. & Casse, F. 2003. Functional analysis of AtHKT1 in Arabidopsis shows that Na+ recirculation by the phloem is crucial for salt tolerance. *Embo Journal,* 22**,** 2004-2014.

Blumwald, E. 1987. Tonoplast vesicles as a tool in the study of ion transport at the plant vacuole. *Physiologia Plantarum,* 69**,** 731-734.

Brenner, S., Johnson, M., Bridgham, J., Golda, G., Lloyd, D. H., Johnson, D., Luo, S., Mccurdy, S., Foy, M., Ewan, M., Roth, R., George, D., Eletr, S., Albrecht, G., Vermaas, E., Williams, S. R., Moon, K., Burcham, T., Pallas, M., Dubridge, R. B., Kirchner, J., Fearon, K., Mao, J.-I. & Corcoran, K.

2000. Gene expression analysis by massively parallel signature sequencing (MPSS) on microbead arrays. *Nat Biotech,* 18**,** 630-634.

Brett, C. L., Donowitz, M. & Rao, R. 2005. Evolutionary origins of eukaryotic sodium/proton exchangers. *American Journal of Physiology - Cell Physiology,* 288**,** C223-C239.

Brini, F., Hanin, M., Mezghani, I., Berkowitz, G. A. & Masmoudi, K. 2007. Overexpression of wheat Na+/H+ antiporter TNHX1 and H+-pyrophosphatase TVP1 improve salt- and drought-stress tolerance in Arabidopsis thaliana plants. *Journal of Experimental Botany,* 58**,** 301-308.

Burton, R. A., Shirley, N. J., King, B. J., Harvey, A. J. & Fincher, G. B. 2004. The CesA Gene Family of Barley. Quantitative Analysis of Transcripts Reveals Two Groups of Co-Expressed Genes. *Plant Physiology,* 134**,** 224-236.

Byrt, C. S. 2008. *Genes for sodium exclusion in wheat.* PhD (Science), The University of Adelaide.

Byrt, C. S., Platten, J. D., Spielmeyer, W., James, R. A., Lagudah, E. S., Dennis, E. S., Tester, M. & Munns, R. 2007. HKT1;5-Like Cation Transporters Linked to Na+ Exclusion Loci in Wheat, Nax2 and Kna1. *Plant Physiol.,* 143**,** 1918-1928.

Cai, X., Chen, T., Zhou, Q., Xu, L., Qu, L., Hua, X. & Lin, J. 2011. Development of Casparian strip in rice cultivars. *Plant Signaling & Behavior,* 6**,** 59-65.

Callow, J. A., Leigh, R. A. & Sanders, D. 1997. *The Plant Vacuole: The Plant Vacuole*, Elsevier Science.

Chen, H., An, R., Tang, J. H., Cui, X. H., Hao, F. S., Chen, J. & Wang, X. C. 2007a. Over-expression of a vacuolar Na+/H+ antiporter gene improves salt tolerance in an upland rice. *Molecular Breeding,* 19**,** 215-225.

Chen, Z., Newman, I., Zhou, M., Mendham, N., Zhang, G. & Shabala, S. 2005. Screening plants for salt tolerance by measuring K+ flux: a case study for barley. *Plant, Cell & Environment,* 28**,** 1230-1246.

Chen, Z., Pottosin, I. I., Cuin, T. A., Fuglsang, A. T., Tester, M., Jha, D., Zepeda-Jazo, I., Zhou, M., Palmgren, M. G., Newman, I. A. & Shabala, S. 2007b. Root Plasma Membrane Transporters Controlling K+/Na+ Homeostasis in Salt-Stressed Barley. *Plant Physiol.,* 145**,** 1714-1725.

Colmer, T. D., Munns, R. & Flowers, T. J. 2005. Improving salt tolerance of wheat and barley: future prospects. *Australian Journal of Experimental Agriculture,* 45**,** 1425-1443.

Cotsaftis, O., Plett, D., Shirley, N., Tester, M. & Hrmova, M. 2012. A Two-Staged Model of Na+ Exclusion in Rice Explained by 3D Modeling of HKT Transporters and Alternative Splicing. *PLoS ONE,* 7**,** e39865.

Curtis, M. D. & Grossniklaus, U. 2003. A Gateway Cloning Vector Set for High-Throughput Functional Analysis of Genes in Planta. *Plant Physiology,* 133**,** 462-469.

D'yakova, E. V., Rakitin, A. L., Kamionskaya, A. M., Baikov, A. A., Lahti, R., Ravin, N. V. & Skryabin, K. G. 2006. A study of the effect of expression of the gene encoding the membrane H+-

pyrophosphatase of Rhodospirillum rubrum on salt resistance of transgenic tobacco plants. *Doklady Biological Sciences,* 409**,** 346-348.

Dahleen, L. S., Manoharan, M. & Lakshmanan, P. 2007. Recent advances in barley transformation. *In Vitro Cellular and Developmental Biology - Plant,* 43**,** 493-506.

Davenport, R. J., Munoz-Mayor, A., Jha, D., Essah, P. A., Rus, A. N. A. & Tester, M. 2007. The Na+ transporter AtHKT1;1 controls retrieval of Na+ from the xylem in Arabidopsis. *Plant, Cell & Environment,* 30**,** 497-507.

Davies, W., Kudoyarova, G. & Hartung, W. 2005. Long-distance ABA Signaling and Its Relation to Other Signaling Pathways in the Detection of Soil Drying and the Mediation of the Plants Response to Drought. *Journal of Plant Growth Regulation,* 24**,** 285-295.

Delane, R., Greenway, H., Munns, R. & Gibbs, J. 1982. Ion Concentration and Carbohydrate Status of the Elongating Leaf Tissue 4Hordeum vulgare Growing at High External NaCl: I. RELATIONSHIP BETWEEN SOLUTE CONCENTRATION AND GROWTH. *J. Exp. Bot.,* 33**,** 557-a-573.

Devos, K. M. & Gale, M. D. 2000. Genome Relationships: The Grass Model in Current Research. *The Plant Cell Online,* 12**,** 637-646.

Dong, Q., Schlueter, S. D. & Brendel, V. 2004. PlantGDB, plant genome database and analysis tools. *Nucleic Acids Research,* 32**,** D354-D359.

Drozdowicz, Y. M. & Rea, P. A. 2001. Vacuolar H+ pyrophosphatases: from the evolutionary backwaters into the mainstream. *Trends in Plant Science,* 6**,** 206-211.

Duan, X. G., Yang, A. F., Gao, F., Zhang, S. L. & Zhang, J. R. 2007. Heterologous expression of vacuolar H+-PPase enhances the electrochemical gradient across the vacuolar membrane and improves tobacco cell salt tolerance. *Protoplasma,* 232**,** 87-95.

El-Hussieny, G. S. 2006. *Control of shoot elemental accumulation by cell type-specific alteration of gene function in roots.* PhD, University of Cambridge.

Ferjani, A., Segami, S., Horiguchi, G., Muto, Y., Maeshima, M. & Tsukaya, H. 2011. Keep an Eye on PPi: The Vacuolar-Type H+-Pyrophosphatase Regulates Postgerminative Development in Arabidopsis. *The Plant Cell Online*.

Feuillet, C., Langridge, P. & Waugh, R. 2008. Cereal breeding takes a walk on the wild side. *Trends in Genetics,* 24**,** 24-32.

Finnie, C., Steenholdt, T., Roda Noguera, O., Knudsen, S., Larsen, J., Brinch-Pedersen, H., Bach Holm, P., Olsen, O. & Svensson, B. 2004. Environmental and transgene expression effects on the barley seed proteome. *Phytochemistry,* 65**,** 1619-1627.

Flowers, T. J. 2004. Improving crop salt tolerance. *J. Exp. Bot.,* 55**,** 307-319.

Flowers, T. J. & Yeo, A. R. 1986. Ion Relations of Plants Under Drought and Salinity. *Functional Plant Biology,* 13**,** 75-91.

Fukuda, A., Chiba, K., Maeda, M., Nakamura, A., Maeshima, M. & Tanaka, Y. 2004. Effect of salt and osmotic stresses on the expression of genes for the vacuolar H+-pyrophosphatase, H+-ATPase subunit A, and Na+/H+ antiporter from barley. *J. Exp. Bot.,* 55**,** 585-594.

Fukuda, A. & Tanaka, Y. 2006. Effects of ABA, auxin, and gibberellin on the expression of genes for vacuolar H+-inorganic pyrophosphatase, H+-ATPase subunit A, and Na+/H+ antiporter in barley. *Plant Physiology and Biochemistry,* 44**,** 351-358.

Fukuda, A., Yazaki, Y., Ishikawa, T., Koike, S. & Tanaka, Y. 1998. Na+/H+ Antiporter in Tonoplast Vesicles from Rice Roots. *Plant and Cell Physiology,* 39**,** 196-201.

Gao, F., Gao, Q., Duan, X., Yue, G., Yang, A. & Zhang, J. 2006. Cloning of an H+-PPase gene from Thellungiella halophila and its heterologous expression to improve tobacco salt tolerance. *Journal of Experimental Botany,* 57**,** 3259-3270.

Gao, J.-P., Chao, D.-Y. & Lin, H.-X. 2007. Understanding Abiotic Stress Tolerance Mechanisms: Recent Studies on Stress Response in Rice. *Journal of Integrative Plant Biology,* 49**,** 742-750.

Gaxiola, R. A., Li, J., Undurraga, S., Dang, L. M., Allen, G. J., Alper, S. L. & Fink, G. R. 2001a. Drought- and salt-tolerant plants result from overexpression of the AVP1 H+-pump. *Proc Natl Acad Sci USA,* 98**,** 11444 - 11449.

Gaxiola, R. A., Li, J. S., Undurraga, S., Dang, L. M., Allen, G. J., Alper, S. L. & Fink, G. R. 2001b. Drought- and salt-tolerant plants result from overexpression of the AVP1 H+-pump. *Proceedings of the National Academy of Sciences of the United States of America,* 98**,** 11444-11449.

Gaxiola, R. A., Rao, R., Sherman, A., Grisafi, P., Alper, S. L. & Fink, G. R. 1999. The Arabidopsis thaliana proton transporters, AtNhx1 and Avp1, can function in cation detoxification in yeast. *PNAS,* 96**,** 1480-1485.

Gaymard, F., Pilot, G., Lacombe, B., Bouchez, D., Bruneau, D., Boucherez, J., Michaux-Ferrière, N., Thibaud, J.-B. & Sentenac, H. 1998. Identification and Disruption of a Plant Shaker-like Outward Channel Involved in K+ Release into the Xylem Sap. *Cell,* 94**,** 647-655.

Gorham, J., Bristol, A., Young, E. M., Jonesh, R. G. W. & Kashour, G. 1990. Salt Tolerance in the Triticeae: K/Na Discrimination in Barley. *J. Exp. Bot.,* 41**,** 1095-1101.

Gorham, J., Hardy, C., Wyn Jones, R. G., Joppa, L. R. & Law, C. N. 1987. Chromosomal location of a K/Na discrimination character in the D genome of wheat. *Theoretical and Applied Genetics,* 74**,** 584-588.

Greenway, H. & Munns, R. 1980. Mechanisms of Salt Tolerance in Nonhalophytes. *Annual Review of Plant Physiology,* 31**,** 149-190.

Guo, H. & Moose, S. P. 2003. Conserved Noncoding Sequences among Cultivated Cereal Genomes Identify Candidate Regulatory Sequence Elements and Patterns of Promoter Evolution. *The Plant Cell Online,* 15**,** 1143-1158.

Guo, S. L., Yin, H. B., Zhang, X., Zhao, F. Y., Li, P. H., Chen, S. H., Zhao, Y. X. & Zhang, H. 2006. Molecular cloning and characterization of a vacuolar H+-pyrophosphatase gene, SsVP, from the halophyte Suaeda salsa and its overexpression increases salt and drought tolerance of Arabidopsis. *Plant Molecular Biology,* 60**,** 41-50.

Halfter, U., Ishitani, M. & Zhu, J.-K. 2000. The Arabidopsis SOS2 protein kinase physically interacts with and is activated by the calcium-binding protein SOS3. *Proceedings of the National Academy of Sciences,* 97**,** 3735-3740.

Hamilton, A. J. & Baulcombe, D. C. 1999. A Species of Small Antisense RNA in Posttranscriptional Gene Silencing in Plants. *Science,* 286**,** 950-952.

Haseloff, J. 1999. Met. Cell Biol.

Hiraga, S., Jaffé, A., Ogura, T., Mori, H. & Takahashi, H. 1986. F plasmid ccd mechanism in Escherichia coli. *Journal of Bacteriology,* 166**,** 100-104.

Huang, S., Spielmeyer, W., Lagudah, E. S. & Munns, R. 2008. Comparative mapping of HKT genes in wheat, barley, and rice, key determinants of Na+ transport, and salt tolerance. *J. Exp. Bot.,* 59**,** 927-937.

Hunt, R. 1978. *Plant Growth Analysis,* London, Edward Arnold.

James, H. 1998. Chapter 9: GFP Variants for Multispectral Imaging of Living Cells. *In:* Kevin, F. S. & Steve, A. K. (eds.) *Methods in Cell Biology.* Academic Press.

James, R. A., Blake, C., Byrt, C. S. & Munns, R. 2011. Major genes for Na+ exclusion, Nax1 and Nax2 (wheat HKT1;4 and HKT1;5), decrease Na+ accumulation in bread wheat leaves under saline and waterlogged conditions. *Journal of Experimental Botany,* 62**,** 2939-2947.

James, R. A., Davenport, R. J. & Munns, R. 2006. Physiological Characterization of Two Genes for Na+ Exclusion in Durum Wheat, Nax1 and Nax2. *Plant Physiology,* 142**,** 1537-1547.

Johnson, A. a. T., Hibberd, J. M., Gay, C., Essah, P. A., Haseloff, J., Tester, M. & Guiderdoni, E. 2005. Spatial control of transgene expression in rice (Oryza sativa L.) using the GAL4 enhancer trapping system. *The Plant Journal,* 41**,** 779-789.

Kaplinsky, N. J., Braun, D. M., Penterman, J., Goff, S. A. & Freeling, M. 2002. Utility and distribution of conserved noncoding sequences in the grasses. *Proceedings of the National Academy of Sciences,* 99**,** 6147-6151.

Karakas, B., Ozias-Akins, P., Stushnoff, C., Suefferheld, M. & Rieger, M. 1997. Salinity and drought tolerance of mannitol-accumulating transgenic tobacco. *Plant, Cell & Environment,* 20**,** 609-616.

Lacombe, B., Pilot, G., Michard, E., Gaymard, F., Sentenac, H. & Thibaud, J.-B. 2000. A Shaker-Like K^+ Channel with Weak Rectification Is Expressed in Both Source and Sink Phloem Tissues of Arabidopsis. *The Plant Cell,* 12**,** 837-851.

Leister, D., Kurth, J., Laurie, D. A., Yano, M., Sasaki, T., Devos, K., Graner, A. & Schulze-Lefert, P. 1998. Rapid reorganization of resistance gene homologues in cereal genomes. *Proceedings of the National Academy of Sciences,* 95**,** 370-375.

Leonard, R. T., Nagahashi, G. & Thomson, W. W. 1975. Effect of Lanthanum on Ion Absorption in Corn Roots. *Plant Physiology,* 55**,** 542-546.

Li, J. S., Yang, H. B., Peer, W. A., Richter, G., Blakeslee, J., Bandyopadhyay, A., Titapiwantakun, B., Undurraga, S., Khodakovskaya, M., Richards, E. L., Krizek, B., Murphy, A. S., Gilroy, S. & Gaxiola, R. 2005. Arabidopsis H+-PPase AVP1 regulates auxin-mediated organ development. *Science,* 310**,** 121-125.

Mäser, P., Eckelman, B., Vaidyanathan, R., Horie, T., Fairbairn, D. J., Kubo, M., Yamagami, M., Yamaguchi, K., Nishimura, M., Uozumi, N., Robertson, W., Sussman, M. R. & Schroeder, J. I. 2002. Altered shoot/root Na+ distribution and bifurcating salt sensitivity in Arabidopsis by genetic disruption of the Na+ transporter AtHKT1. *FEBS Letters,* 531**,** 157-161.

Matthews, P. R., Wang, M. B., Waterhouse, P. M., Thornton, S., Fieg, S. J., Gubler, F. & Jacobsen, J. V. 2001. Marker gene elimination from transgenic barley, using co-transformation with adjacent `twin T-DNAs' on a standard Agrobacterium transformation vector. *Molecular Breeding,* 7**,** 195-202.

Mian, A., Oomen, R. J. F. J., Isayenkov, S., Sentenac, H., Maathuis, F. J. M. & Véry, A.-A. 2011. Over-expression of an Na+- and K+-permeable HKT transporter in barley improves salt tolerance. *Plant Journal,* 68**,** 468-479.

Møller, I. S., Gilliham, M., Jha, D., Mayo, G. M., Roy, S. J., Coates, J. C., Haseloff, J. & Tester, M. 2009. Shoot Na+ Exclusion and Increased Salinity Tolerance Engineered by Cell Type–Specific Alteration of Na+ Transport in Arabidopsis. *The Plant Cell Online,* 21**,** 2163-2178.

Munns, R. 2002. Comparative physiology of salt and water stress. *Plant, Cell & Environment,* 25**,** 239-250.

Munns, R. 2007. Utilizing genetic resources to enhance productivity of salt-prone land. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources,* 2.

Munns, R., Hare, R. A., James, R. A. & Rebetzke, G. J. 2000. Genetic variation for improving the salt tolerance of durum wheat. *Australian Journal of Agricultural Research,* 51**,** 69-74.

Munns, R. & James, R. 2003. Screening methods for salinity tolerance: a case study with tetraploid wheat. *Plant and Soil,* 253**,** 201-218.

Munns, R., James, R. A. & Läuchli, A. 2006. Approaches to increasing the salt tolerance of wheat and other cereals. *Journal of Experimental Botany,* 57**,** 1025-1043.

Munns, R., James, R. A., Xu, B., Athman, A., Conn, S. J., Jordans, C., Byrt, C. S., Hare, R. A., Tyerman, S. D., Tester, M., Plett, D. & Gilliham, M. 2012. Wheat grain yield on saline soils is improved by an ancestral Na+ transporter gene. *Nature Biotechnology,* 30**,** 360-364.

Munns, R. & Sharp, R. E. 1993. Involvement of Abscisic Acid in Controlling Plant Growth in Soil of Low Water Potential. *Functional Plant Biology,* 20**,** 425-437.

Munns, R. & Termaat, A. 1986. Whole-Plant Responses to Salinity. *Functional Plant Biology,* 13**,** 143-160.

Munns, R. & Tester, M. 2008. Mechanisms of Salinity Tolerance. *Annual Review of Plant Biology,* 59**,** 651-681.

Nobuta, K., Venu, R. C., Lu, C., Belo, A., Vemaraju, K., Kulkarni, K., Wang, W., Pillay, M., Green, P. J., Wang, G.-L. & Meyers, B. C. 2007. An expression atlas of rice mRNAs and small RNAs. *Nat Biotech,* 25**,** 473-477.

Obata, T., Kitamoto, H. K., Nakamura, A., Fukuda, A. & Tanaka, Y. 2007. Rice Shaker Potassium Channel OsKAT1 Confers Tolerance to Salinity Stress on Yeast and Rice Cells. *Plant Physiol.,* 144**,** 1978-1985.

Ohyanagi, H., Tanaka, T., Sakai, H., Shigemoto, Y., Yamaguchi, K., Habara, T., Fujii, Y., Antonio, B. A., Nagamura, Y., Imanishi, T., Ikeo, K., Itoh, T., Gojobori, T. & Sasaki, T. 2006. The Rice Annotation Project Database (RAP-DB): hub for Oryza sativa ssp. japonica genome information. *Nucleic Acids Research,* 34**,** D741-D744.

Pardo, J. M. 2010. Biotechnology of water and salinity stress tolerance. *Current Opinion in Biotechnology,* 21**,** 185-196.

Park, S., Li, J., Pittman, J. K., Berkowitz, G. A., Yang, H., Undurraga, S., Morris, J., Hirschi, K. D. & Gaxiola, R. A. 2005. Up-regulation of a H+-pyrophosphatase (H+-PPase) as a strategy to engineer drought-resistant crop plants. *Proceedings of the National Academy of Sciences of the United States of America,* 102**,** 18830-18835.

Pasapula, V., Shen, G., Kuppu, S., Paez-Valencia, J., Mendoza, M., Hou, P., Chen, J., Qiu, X., Zhu, L., Zhang, X., Auld, D., Blumwald, E., Zhang, H., Gaxiola, R. & Payton, P. 2011. Expression of an Arabidopsis vacuolar H+-pyrophosphatase gene (AVP1) in cotton improves drought- and salt tolerance and increases fibre yield in the field conditions. *Plant Biotechnology Journal,* 9**,** 88-99.

Platten, J. D., Cotsaftis, O., Berthomieu, P., Bohnert, H., J. Davenport, R., J. Fairbairn, D., Horie, T., A. Leigh, R., Lin, H.-X., Luan, S., Mäser, P., Pantoja, O., Rodríguez-Navarro, A., P. Schachtman, D., I. Schroeder, J., Sentenac, H., Uozumi, N., Véry, A.-A., Zhu, J.-K., S. Dennis, E. & Tester, M.

2006. Nomenclature for HKT transporters, key determinants of plant salinity tolerance. *Trends in Plant Science,* 11**,** 372-374.

Plett, D., Safwat, G., Gilliham, M., Skrumsager Møller, I., Roy, S., Shirley, N., Jacobs, A., Johnson, A. & Tester, M. 2010. Improved Salinity Tolerance of Rice Through Cell Type-Specific Expression of <italic>AtHKT1;1</italic>. *PLoS ONE,* 5**,** e12571.

Poljakoff-Mayber, A. 1975. Morphological and Anatomical Changes in Plants as a Response to Salinity Stress. *In:* Poljakoff-Mayber, A. & Gale, J. (eds.) *Plants in Saline Environments.* Springer Berlin Heidelberg.

Qiu, Q.-S., Guo, Y., Dietrich, M. A., Schumaker, K. S. & Zhu, J.-K. 2002. Regulation of SOS1, a plasma membrane Na+/H+ exchanger in Arabidopsisthaliana, by SOS2 and SOS3. *Proceedings of the National Academy of Sciences of the United States of America,* 99**,** 8436-8441.

Qiu, Q.-S., Guo, Y., Quintero, F. J., Pardo, J. M., Schumaker, K. S. & Zhu, J.-K. 2004. Regulation of Vacuolar Na+/H+ Exchange in Arabidopsis thaliana by the Salt-Overly-Sensitive (SOS) Pathway. *J. Biol. Chem.,* 279**,** 207-215.

Rajendran, K., Tester, M. & Roy, S. J. 2009. Quantifying the three main components of salinity tolerance in cereals. *Plant, Cell & Environment,* 32**,** 237-249.

Reinhardt, D. H. & Rost, T. L. 1995. Salinity accelerates endodermal development and induces an exodermis in cotton seedling roots. *Environmental and Experimental Botany,* 35**,** 563-574.

Ren, Z.-H., Gao, J.-P., Li, L.-G., Cai, X.-L., Huang, W., Chao, D.-Y., Zhu, M.-Z., Wang, Z.-Y., Luan, S. & Lin, H.-X. 2005. A rice quantitative trait locus for salt tolerance encodes a sodium transporter. *Nat Genet,* 37**,** 1141-1146.

Rengasamy, P. 2002. Transient salinity and subsoil constraints to dryland farming in Australian sodic soils: an overview. *Australian Journal of Experimental Agriculture,* 42**,** 351-361.

Rengasamy, P. 2006. World salinization with emphasis on Australia. *J. Exp. Bot.,* 57**,** 1017-1023.

Rengasamy, P. 2010. Soil processes affecting crop production in salt-affected soils. *Functional Plant Biology,* 37**,** 613-620.

Rengasamy, P., Chittleborough, D. & Helyar, K. 2003. Root-zone constraints and plant-based solutions for dryland salinity. *Plant and Soil,* 257**,** 249-260.

Roberts, S. K. & Snowman, B. N. 2000. The effects of ABA on channel-mediated K+ transport across higher plant roots. *Journal of Experimental Botany,* 51**,** 1585-1594.

Rozen, S. & Skaletsky, H. 1999. Primer3 on the WWW for General Users and for Biologist Programmers.

Rus, A., Baxter, I., Muthukumar, B., Gustin, J., Lahner, B., Yakubova, E. & Salt, D. E. 2006. Natural Variants of At<italic>HKT1</italic> Enhance Na⁺ Accumulation in Two Wild Populations of <italic>Arabidopsis</italic>. *PLoS Genet,* 2**,** e210.

Rus, A., Lee, B.-H., Munoz-Mayor, A., Sharkhuu, A., Miura, K., Zhu, J.-K., Bressan, R. A. & Hasegawa, P. M. 2004. AtHKT1 Facilitates Na+ Homeostasis and K+ Nutrition in Planta. *Plant Physiol.,* 136**,** 2500-2511.

Rus, A., Yokoi, S., Sharkhuu, A., Reddy, M., Lee, B. H., Matsumoto, T. K., Koiwa, H., Zhu, J. K., Bressan, R. A. & Hasegawa, P. M. 2001. AtHKT1 is a salt tolerance determinant that controls Na+ entry into plant roots. *Proc Natl Acad Sci USA,* 98**,** 14150 - 14155.

Sakakibara, Y., Kobayashi, H. & Kasamo, K. 1996. Isolation and characterization of cDNAs encoding vacuolar H+-pyrophosphatase isoforms from rice (Oryza sativa L.). *Plant Molecular Biology,* 31**,** 1029-1038.

Sexton, M. & Corporation, A. B. 2003. *Silent Flood: Australia's Salinity Crisis*, ABC Books.

Shabala, S., Shabala, S., Cuin, T. A., Pang, J., Percey, W., Chen, Z., Conn, S., Eing, C. & Wegner, L. H. 2010. Xylem ionic relations and salinity tolerance in barley. *The Plant Journal,* 61**,** 839-853.

Shavrukov, Y., Bovill, J., Afzal, I., Hayes, J., Roy, S., Tester, M. & Collins, N. 2013. HVP10 encoding V-PPase is a prime candidate for the barley HvNax3 sodium exclusion gene: evidence from fine mapping and expression analysis. *Planta***,** 1-12.

Sheveleva, E., Chmara, W., Bohnert, H. J. & Jensen, R. G. 1997. Increased Salt and Drought Tolerance by D-Ononitol Production in Transgenic Nicotiana tabacum L. *Plant Physiology,* 115**,** 1211-1219.

Shi, H., Ishitani, M., Kim, C. & Zhu, J.-K. 2000. The Arabidopsis thaliana salt tolerance gene SOS1 encodes a putative Na+/H+ antiporter. *Proceedings of the National Academy of Sciences of the United States of America,* 97**,** 6896-6901.

Shi, H., Lee, B.-H., Wu, S.-J. & Zhu, J.-K. 2003. Overexpression of a plasma membrane Na+/H+ antiporter gene improves salt tolerance in Arabidopsis thaliana. *Nat Biotech,* 21**,** 81-85.

Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology,* 98**,** 503-517.

Sumer, A., Zorb, C., Feng, Y. & Schubert, S. 2004. Evidence of sodium toxicity for the vegetative growth of maize (Zea mays L.) during the first phase of salt stress. *Journal of Applied Botany,* 78**,** 135-139.

Sunarpi, Horie, T., Motoda, J., Kubo, M., Yang, H., Yoda, K., Horie, R., Chan, W.-Y., Leung, H.-Y., Hattori, K., Konomi, M., Osumi, M., Yamagami, M., Schroeder, J. I. & Uozumi, N. 2005. Enhanced salt tolerance mediated by AtHKT1 transporter-induced Na+ unloading from xylem vessels to xylem parenchyma cells. *The Plant Journal,* 44**,** 928-938.

Takano, J., Tanaka, M., Toyoda, A., Miwa, K., Kasai, K., Fuji, K., Onouchi, H., Naito, S. & Fujiwara, T. 2010. Polar localization and degradation of Arabidopsis boron transporters through distinct trafficking pathways. *Proceedings of the National Academy of Sciences*.

Tarczynski, M. C., Jensen, R. G. & Bohnert, H. J. 1992. Expression of a bacterial mtlD gene in transgenic tobacco leads to production and accumulation of mannitol. *Proceedings of the National Academy of Sciences,* 89**,** 2600-2604.

Tester, M. & Davenport, R. 2003. Na+ Tolerance and Na+ Transport in Higher Plants. *Ann Bot,* 91**,** 503-527.

Tingay, S., Mcelroy, D., Kalla, R., Fieg, S., Wang, M., Thornton, S. & Brettell, R. 1997. Agrobacterium tumefaciens-mediated barley transformation. *The Plant Journal,* 11**,** 1369-1376.

Travella, S., Klimm, T. E. & Keller, B. 2006. RNA Interference-Based Gene Silencing as an Efficient Tool for Functional Genomics in Hexaploid Bread Wheat. *Plant Physiology,* 142**,** 6-20.

Turner, N. C., Passioura, J. B., Physiology, I. a. O. P. & Physiologists, A. S. O. P. 1986. *Plant Growth, Drought and Salinity*, CSIRO.

Ulmasov, T., Murfett, J., Hagen, G. & Guilfoyle, T. J. 1997. Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. *The Plant Cell Online,* 9**,** 1963-71.

Uozumi, N., Kim, E. J., Rubio, F., Yamaguchi, T., Muto, S., Tsuboi, A., Bakker, E. P., Nakamura, T. & Schroeder, J. I. 2000. The Arabidopsis HKT1 gene homolog mediates inward Na+ currents in Xenopus laevis oocytes and Na+ uptake in Saccharomyces cerevisiae. *Plant Physiol,* 122**,** 1249 - 1260.

Usda 2013. World Agricultural Supply and Demand Estimates. *In:* Agriculture, U. S. D. O. (ed.). Washington, D.C.: World Agricultural Outlook Board.

Van Houdt, H., Bleys, A. & Depicker, A. 2003. RNA Target Sequences Promote Spreading of RNA Silencing. *Plant Physiology,* 131**,** 245-253.

Venema, K., Quintero, F. J., Pardo, J. M. & Donaire, J. P. 2002. The Arabidopsis Na+/H+ exchanger AtNHX1 catalyzes low affinity Na+ and K+ transport in reconstituted liposomes. *J Biol Chem,* 277**,** 2413 - 2418.

Wea 2012. Report for growers 2011/12. *In:* Australia, W. E. (ed.) Final ed. Deakin, Australia: Commonwealth of Australia.

Yeo, A. R., Lee, Λ.-S., Izard, P., Boursier, P. J. & Flowers, T. J. 1991. Short- and Long-Term Effects of Salinity on Leaf Growth in Rice (Oryza sativa L.). *Journal of Experimental Botany,* 42**,** 881-889.

Zakharov, A., Giersberg, M., Hosein, F., Melzer, M., Müntz, K. & Saalbach, I. 2004. Seed-specific promoters direct gene expression in non-seed tissue. *Journal of Experimental Botany,* 55**,** 1463-1471.

Zhang, H., Shen, G., Kuppu, S., Gaxiola, R. & Payton, P. 2011. Creating drought- and salt-tolerant cotton by overexpressing a vacuolar pyrophosphatase gene. *Plant Signaling & Behavior,* 6**,** 861-863.

Appendix

Appendix 2.1: Table containing putative promoter sequences for C34, C257, S147 and Ta.

C34
gccaactgaaacgccacgtcatgctgaacgttaatggggaggaaggtgcttttacccatgtcaattccttctttttaccatgcatccccccgttcgttttcc catgattttagatacttttttttttgtccccgatcgaatcacagatcatttttcattactgcagttgcaaggatgtggcaaaaaatatcatctagaccagttggtt gatttgtcacctacatttattcagatctaataagaatataagtcaacaagttggtttttaaattcgatgtgtatatatctttaagccgacgatatgtatttcagta atgacctttacccggtgaagctttttttttcttaattggtatatacaggataatagtatgttttaaactctctatcaagaaagagcagctaagctggatgggct ctccaatttatgttaatttaataattccctcgataacaagataaatgatctccagtcatttcttgaccattgttctactttacctatgactttataaaatcctgtag caacgtgctgagtatcacccaataaacataagaaaacacaccatgcatgttctagtgagcatcttgatgctggtaggataactagcgagctaaataaa gctaagggtcggatgcgacatgggtgctagcgaatagagcttttctgccttgttagggtgcgtttggaatcagctgttgcatgtccgagcagtatatga gcgcgtgattaattaagtattttctttttttttcaaaaatagataatataatttttttaagcaactttcgtatataaacttttttttaaaaaacacgccgtttagtagttta aaaaacgtgcgcgcgaaatacaacggagagggattggaaacacaggattccaaacacagcctgaatcgtcattgatacaaggaaagatgggagg aggaacaagagggagaggagcaatagtttgggggatgatcccctccattaatttctcattctggatctgtgctacttcctccgtttcataatataagtcat tctagcatttatctatctagattcattaatatcaatatgaatgtggaaaataccagaatgacttacattgtgaaacgggggagagtagctaggaatggata aataaataatgttatcccaaacagaaaaagttacgtacggccgggtatgtgcttcacgtgaaatgggaaatatatacaatcaatgacgactaatttggc agcaaacatgcatgcatgggccagttaattaattatgctaaatggatagtttgattcgatgagcagccggacgactgaggctttctttttgttgggaaaaa aattaggtttgattgtcacatcagatatacggatatacatttgaagtattaaacgtagtttaaaaacaaaataaattatagattctactaaaaaactgcgag ataaatttattaagcctaattaattcgttattagcaaatgcttactgtagcaccacattgtcaaatcatggtgcaatttggtttaaaaattttgtctcgtaattta cacgtaatatgtgtaattggttttttcctacattaaatacttcatgcgtatgtctaaacatttgatgtgacagtgtgaaatttttttttttaaatagggcctgagag caaaacacatgctgcatgaatagtagtcgctcgctcgtgtatcatacttaaagaaagtttaactaggaaaaaaaaacctacttcatatataaaatcacaa gacctttttatccaaaaattgaaactttcaactaaaagttcaaaaaaattatactcttggctcaaactttcaatataaaaacccaaaacttctaatccaaaaac tgaaacttcccaaaaaaatcaaaaactttgtgctcatggctctcaatctttcgatacgaagttacagaatcatggtagggtagctagctaaataaaggga cggatgcgatatgggtgctagcgaatagagcctttctaccttgctgaatcgtcattgataattaggggaggaggaacaagagggaaaggagcaata gtttggggatgatcccctccattgaataatttctcattctggatccgtgctagggatggatcaataatgttatcccaaacagaaacagctagtgttaggta cgggtatgtgcttcacgtgaaatgggaaatatatacaatcaacgatgactaatttggtagtaagcatacatgcatgggccaattaattaattatgctaaat ggatatagtttgattcgatcagcagctgaacgactgatcgagagcaaaacacatgcataatgcataatgcattaatttatatatatgaatagtagtcgctc gctcgtgtatcacgcataaattatatatcaatgatcgatcacatgcaacaccggcaaattaaattgacccatcatcgatccatctagctagctatgcctat atatatacgccatatgcatgataaatac
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agccctcgcgcgcttggtgtactctagtttagtacaccaatccgcatgcattctttttgtttgtttgtttgtttgttttgattgacaaatatatgcggcag
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cgtgtcatcgtccagttagatgtcaactcccatctgaatctcctcctccatggcggctttcctataaatacgtcgcctttccatgttggatgtcattccattc
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S147

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gatagctaaaaatagttatattttaggataaaggcagtaccttaaaacaagtggtctaaacaatccaatacaaggatgcatttttgaagcgttgagcgaa
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Ta
Ttgcagatgttcgcatacactcaaccataagaatgcatgcacacacacactcctactaaatgcacatcgccgaaaggcctgaaatgaatgcaagaa aatgcgaccaccagtgtcaagtctagaacttgaaccctggtgggttatttccatcacaagcaacctaaccatttgagttaccctcagctcgctatgcca acattaattaacaatagcaaacttgtttcactatatttatcataatataatttctagatatatagtcaaaataatttcaaatatttatgaatgaagggagcacca tgctatggtaatatagatgcattactttggaggagctagttgtaggtagctctaaacatgtattttcatagtttctaatttttggcatgtattttctatcttctatgt gtatatctttttcgggattctgtatgtatatgtgtatatgtacttttcgttgcacttagtacaacacaagtcaggtggttgccctgagctccttctcttcatgatg ccacgctcacaccctacgatacatatccaacggagcggggcatcgcacccggtgggcaccaactgactcttgttcgttaccggtgatacggacgtg gaacttatcactcacccgcaaaaaaaaaagttatcactcgattccattgtttcttccacaagtctgctctcttgtaggagtacctaattttcgtcatatgatat gcctcgcaaaaaagatatgcctcccacgagctcccattgtgcgctagcttttgcgattagattcagtaattaagacactataatgtcgttgcagggagta aagcaacatcaacggacaaatttttacagacctcacgggatgggctgtcgtagcagatctatttggaaaaagaaattagagattttctttgtagtccgtc cgtttgtctagcatttttgcgtccacccccctttttgggtataataatccattagtctctgattgcctccaacaaaacagaccaagaagtctctacacaact tacagtagaa

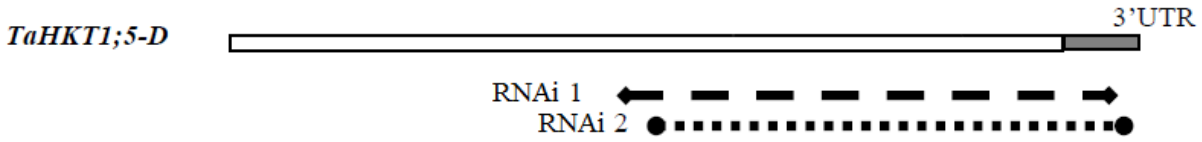
Appendix 3.1: Amino acid sequence alignment of TaHKT1;5-D and HvHKT1;5 identical sequences are highlighted in yellow and conservative sequence differences are highlighted in green.

```
                    1                                                  50
TaHKT1;5-D      (1) MGSLHVS-SSATQHSKLERAYQLLVFHVHPFWLQLLYFVSISFFGLVILK
HvHKT1;5        (1) MGSLHVSGSTTTQHSRVQRAYQLLFFHVHPFWPQLLYFVSISFFGFVMLR
Consensus       (1) MGSLHVS SS TQHSKL RAYQLL FHVHPFW QLLYFVSISFFG VILK

                     51                                                 100
TaHKT1;5-D     (50) ALPMKTSTVPRPMDLDLIFTSVSATTVSSMVAVEMESFSNPQLLLLTLLM
HvHKT1;5       (51) ALPMKTN---MPTDLDLIFTSVSATTVSSMQAVEMESFSNPQLLLLTLLM
Consensus      (51) ALPMKT     P DLDLIFTSVSATTVSSM AVEMESFSNPQLLLLTLLM

                    101                                                150
TaHKT1;5-D    (100) LLGGEVFTSMLGLHFTYVKSKKKEAQAPHDHDDGDKGKPAPSSSLELAVT
HvHKT1;5       (98) LLGGEVFTSMLGMYFTYVKSKKKEAQAP--HDDGAKVKPAPSSLELTAAS
Consensus     (101) LLGGEVFTSMLGLHFTYVKSKKKEAQAP  HDDG K KPAPSS    A S

                    151                                                200
TaHKT1;5-D    (150) TGMDD---VDRVEQGFKDQPRYDRAFLTRLLLFIVLGYHVVVHLAGYSLM
HvHKT1;5      (146) ICMDDGTAQDRMEQGFKDQPRYGRAFLTRLLLFIVVGYHAVVHPAGYSLM
Consensus     (151   MDD    DRMEQGFKDQPRY RAFLTRLLLFIVLGYH VVH AGYSLM

                    201                                                250
TaHKT1;5-D    (197) LVYLSVVSGARAVLTGKGISLHTFSVFTVVSTFANCGFVPNNEGMIAFRS
HvHKT1;5      (196) LVYLSVVSGARTVLAGKGISMHTFSVFTIVSTFANCGFMPNNEGMASFRS
Consensus     (201) LVYLSVVSGAR VL GKGISLHTFSVFTIVSTFANCGFMPNNEGM AFRS

                    251                                                300
TaHKT1;5-D    (247) FPGLLLLVMPHVLLGNTLFPVFLRLAIWALRRVTRRPELGELRSIGYDHL
HvHKT1;5      (246) FPGLLLLVMPHVLLGNTLFPVFLRLAIWALQRFTKRPELGELRSIGYDHL
Consensus     (251) FPGLLLLVMPHVLLGNTLFPVFLRLAIWAL R TKRPELGELRSIGYDHL

                    301                                                350
TaHKT1;5-D    (297) LTSRHTWFLAFTVAAFVLAQLSLFCAMEWGSNGLRGLTAVQKLVAGLFMS
HvHKT1;5      (296) LTSRHTRFLAFTVAVFVLAQLSLFCAMEWGSDGLRGLTAAQKLVAALFMS
Consensus     (301) LTSRHT FLAFTVA FVLAQLSLFCAMEWGS GLRGLTA QKLVAALFMS

                    351                                                400
TaHKT1;5-D    (347) VNSRHTGEMVVDLSTVSSALVVLYVVMMYLPPYTTFLPVEDDSDQQVGAD
HvHKT1;5      (346) VNSRHAGEMVVDLSTVSSAVVVYMVMMYLPPYTTFLPVEDS-NQQVGTD
Consensus     (351) VNSRH GEMVVDLSTVSSALVVLYMVMMYLPPYTTFLPVED   QQVG D

                    401                                                450
TaHKT1;5-D    (397) QRDQKRITSMWRKLLMSPLSCLAIFIAVVCITERRQISDDPLNFNVLNIT
HvHKT1;5      (395) QKR----TSIWHKLLMSPLSCIAIFVVVCITERRQISDDPLNFNVLSIA
Consensus     (401) QK     TSIW KLLMSPLSCIAIFI VVCITERRQISDDPLNFNVL I

                    451                                                500
TaHKT1;5-D    (447) VEVISAYGNVGFSTGYSCGRQVTPDGGCRDTWVGFSGKWSWQGKLALIAV
HvHKT1;5      (441) VEVISAYGNVGFSTGYSCGRQVTPDGSCRDAWVGFSGKWSREGKLALIAV
Consensus     (451) VEVISAYGNVGFSTGYSCGRQVTPDG CRD WVGFSGKWS  GKLALIAV

                    501             520
TaHKT1;5-D    (497) MFYGRLKKFSMHGGEAWRIV
HvHKT1;5      (491) MFYGRLKKFSMHGGQAWRIV
Consensus     (501) MFYGRLKKFSMHGG AWRIV
```

Appendix 4.1: Sequences for RNAi 1 and RNAi 2

RNAi 1
Ccgtgtcgtcggccctcgtggtgctctatgtggtcatgatgtacctaccaccttacactacatttctaccagtggaagacgacagtgaccaacagtgg gagcagatcagcgcgaccagaaaaggataacaagcatgtggcggaagctgctcatgtcgccgctctcgtgcttggccatcttcatcgccgtggtgt gcatcacggagcggcggcagatctccatgacccccctcaacttcaacgtcctcaacatcaccgtcgaggttatcagtgcgtacggaaacgtggggtt cagcaccgggacagctgtggccggcaggtgacgcccgacggcggctgcagggacacgtgggttggcttctctgggaagtggagttggcaagg gaagctggctctcattgctgtcatgttctacggcaggctcaagaattcagcatgcatggtggcgaggcatggaggatagtataacctagtagcagact gcatatttctcaatgatctctcttcagacagagactagctacatctcgctctagcctaaaaccatctgaacatatttccattatgccgagtacctcaatcac tgcatgcatat
RNAi 2
ctcgtggtgctctatgtggtcatgatgtacctaccaccttacactacatttctaccagtggaagacgacagtgaccaacagtgggagcagatcagcgc gaccagaaaaggataacaagcatgtggcggaagctgctcatgtcgccgctctcgtgcttggccatcttcatcgccgtggtgtgcatcacggagcgg cggcagatctccatgacccccctcaacttcaacgtcctcaacatcaccgtcgaggttatcagtgcgtacggaaacgtggggttcagcaccgggacag ctgtggccggcaggtgacgcccgacggcggctgcagggacacgtgggttggcttctctgggaagtggagttggcaagggaagctggctctcatt gctgtcatgttctacggcaggctcaagaattcagcatgcatggtggcgaggcatggaggatagtataacctagtagcagactgcatatttctcaatgat ctctcttcagacagagactagctacatctcgctctagcctaaaaccatctgaacatatttccattatgccga

Appendix 4.2: Regions RNAi 1 and RNAi 2 corresponding to *TaHKT1;5-D*. Taken from Byrt (2008).



Appendix 4.3: *TaSOS1*, *TaNHX1* and *TaHKT1;5-D* target regions for qPCR

***TaSOS1* qPCR fragment**
Agaagccgatctgcaaagaagtgcatcattgctgtcttcaaccctcggaccatcacgaacacagagcaaagagcatgtcggtttgctcaggtggcc ggagagtttccggagatccagcgggcctgggaatgcaagcctagctgaaatcagaggtcagcctggtagcttctctgctagagccttgcaagtcag catgtatggcagca
***TaNHX1* qPCR fragment**
Gcctggttcacccatagagaggagcgtccatgggcctggcttgttgggcactgtgacggaggcagaagaccgtagttaagtcgaagcccagaag gtgcaagtgtatttcttgtaaatgctcagatatcactcagttttgctcttgggattctttcggtg
***TaHKT1;5-D* qPCR fragment**
ctgcggcttcgtcccgaacaacgaagggatgatcgccttccggtccttcccgggcctcctgctcctagtcatgccgcacgtcctcctcggcaacaca ctcttccccgtcttcctcaggctggccatctgggctctccggagagtcaccaggaggcccgagctcggtgagctgaggagcatcggctacgacca cctgctgacgagccggcacacgtggttcttggctttcaccgtggcggcgttcgtgctagcg