Improving salt stress resistance in cereals

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Abstract

Salinity is a complex environmental stress that affects growth and production of agriculturally important crops world-wide. Tolerance to salt stress is variable for different plants and involves integration of various physiological and biochemical mechanisms. This thesis investigates the role in salt tolerance of various transporter genes in important agricultural cereal crops, i.e. rice and barley. Transgenic lines overexpressing *OsTPKa*, *OsTPKb* and *OsAKT1* were generated using *Agrobacterium* mediated rice transformation, while for *OsAKT1* a loss of function mutant was also isolated. Transgenic and mutant plants were characterized to study the role of these genes in rice. For barley, transgenic lines overexpressing *HvHKT2;1* were obtained and studied for its role in barley salt tolerance.

Transgenic rice plants overexpressing *TPKa* did not show any growth phenotype under salt stress conditions, however, the performance of *TPKa* overexpressors was better at different K^+ stress conditions compared to control lines. In contrast, transgenic lines overexpressing *TPKb* showed improved growth under all K^+ and Na^+ stress conditions, suggesting that TPK channels plays a crucial role in K^+ nutrition and in maintaining a higher K^+/Na^+ ratio under different K^+ and Na^+ stress conditions. Characterization of rice *AKT1* mutants and overexpressors showed the involvement of the AKT1 channel in Na^+ uptake at low $[K^+]_{ext}$ or high $[Na^+]_{ext}$ concentrations and both overexpression and loss of function resulted in reduced growth under these conditions. On the other hand, data from experiments with barley HKT2;1 overexpressing lines showed improved growth under salt stress conditions possibly via Na^+ exclusion or accumulation of excessive Na^+ in the shoots.

Overall, the findings point to two important aspects of salt tolerance: firstly, the contribution of *TPKa* and *TPKb* to K^+ homeostasis, particularly that of *TPKb* in maintaining ion homeostasis during different K^+ and Na^+ stress conditions. Secondly, a role of AKT1 and HKT2;1 in Na^+ uptake at the root soil boundary is inferred. These findings reconfirm the idea that maintaining a high K^+/Na^+ ratio is crucial for salt tolerance in both rice and barley. In barley, *HvHKT2;1* overexpressors showed improved salt tolerance via Na^+ redistribution from shoot to root and accumulation of Na^+ in older leaves. The transgenic lines overexpressing *TPKs* and *HKT2;1* and the information gained from this study could be used in future breeding programs or to generate multiple

overexpressors to study the additive or synergistic effects of traits that will add to the present knowledge of ion transport in rice and barley.

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List of abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
Arabidopsis	*Arabidopsis thaliana*
At	*Arabidopsis thaliana*
ATP	Adenosine tri phosphate
AVP	*Arabidopsis thaliana* vacuolar pyrophosphatase
bp	base pair
Ca^{2+}	calcium (ion)
cAMP	cyclic adenosylmonophosphate-ribose
CaMV	Cauliflower mosaic virus
CBL	Calcineurin B-like (protein)
CCC,	Cation Chloride Co-transporter
cDNA	complementary DNA
CHX,	Cation/H^+ exchanger
Cl^-	Chloride ion
CLC,	Voltage gated Cl^- channel
CNGC,	Cyclic Nucleotide Gated Channel;
DNA	Deoxyribonucleic acid
dNTP	2' deoxylnucleotide 5'-triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid
GFP	Green Fluorescent Protein
GLR,	Glutamate like receptor;
H^+	proton (hydrogen ion)
H^+-ATPase	Vacuolar proton-pumping adenosine triphosphatase
H^+-PPase	Vacuolar proton-pumping pyrophosphatase
HATS	High-affinity transport system
HKT,	High affinity K^+ transporter
Hv	*Hordeum vulgare L.*
$[ion]_{cyt}$	Cytosolic ion concentration
$[ion]_{ext}$	External ion concentration
$[ion]_{vac}$	Vacuolar ion concentration
K^+	potassium (ion)
KCO	K^+ outward rectifying channel
KHX	K^+/H^+ exchanger
KIR	Shaker type K^+ inward rectifier
KOR	Shaker type K^+ outward rectifier
KUP/HAK	K^+ uptake permease
LATS	Low-affinity transport system

LB	Luria Bertani media
LCT1	Low affinity cation
Mg^{2+}	Magnesium (ion)
min	minutes
mRNA	messenger RNA
MS	Murashige and Skoog
MS salt	Murashige-Skoog salt
Na^{+}	sodium ion
NHX	Na^{+}/H^{+} exchanger;
NSCC	Non-Selective Cation Channel
N-terminus	Amino-terminus
ORF	Open Reading Frame
Os	*Oryza sativa*
PCR	Polymerase-chain-reaction
qRT-PCR	quantitative Reverse Transcriptase PCR
RNA	Ribonucleic acid
RT	Reverse Transcriptase
T-DNA	Transferred DNA
TPK	Two-pore K^{+} channel
Tris	tris (hydroxymethyl) aminomethane
v/v	volume by volume
VICC	Voltage independent cation channel
w/v	weight by volume
YPD	Yeast Peptone Dextrose medium

Genetic annotation

Uppercase	Referring to the protein e.g. AKT
Uppercase	Italicized referring to the gene e.g. *AKT*
Lowercase	Italicized referring to a mutant form of the gene e.g. *akt*

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Afaq A. Mian

AUTHOR'S DECLARATION

Unless otherwise acknowledged, I declare that the work presented here is original

Chapter 1

1 General Introduction

One of the most critical problems currently affecting mankind is the increasing shortage of water and land to support global food requirements. The high demand for agricultural based products must therefore be met primarily by increasing productivity from cultivated land as well as the use of marginal land for agricultural production (O'Leary, 1995). Such endeavours are often frustrated by environmental stresses that have a large negative impact on agronomy. For example, over 800 million hectares of land are affected by high levels of salinity worldwide (FAO, 2008), an area that accounts for more than 6% of the total land area all over the world (Table 1-1). In general, there is a strong negative correlation between agricultural production worldwide and soil salinity. However, agricultural productivity in salt affected environments can be potentially raised by growing crops with high tolerance to salt stress.

Table 1-1: Regional distribution of salt-affected soils, in million hectares.

Regions	Total area	Saline soils		Sodic soils	
	Mha	Mha	%	Mha	%
Africa	1,899	39	2.0	34	1.8
Asia, the Pacific and Australia	3,107	195	6.3	249	8.0
Europe	2,011	7	0.3	73	3.6
Latin America	2,039	61	3.0	51	2.5
Near East	1,802	92	5.1	14	0.8
North America	1,924	5	0.2	15	0.8
Total	12,781	397	3.1%	434	3.4%

FAO Land and Plant Nutrition Management Service

1.1 What is soil salinity?

Salinity can be defined as the excess of ions of soluble salts such as sodium (Na^+), chloride (Cl^-), calcium (Ca^{2+}), sulphate (SO_4^{2-}), and bicarbonate (HCO_3^-) in soil that affect plant

growth and development (Lewis, 1984). Soil salinity is measured by its electrical conductivity. The SI unit of electrical conductivity (EC) is dS/m. When the electrical conductivity of the soil exceeds 4 dS/m, that soil is called saline (Salinity Laboratory Staff, 1954). Saline soils are categorized into saline, sodic and saline sodic soils depending on EC, soil pH, exchangeable sodium percentage and soil physical condition. Table 1-2 summarizes all the three categories.

Table 1-2: Classification of salt affected soils.

Classification	**Electrical conductivity (dS/m)**	**Soil pH**	**Exchangeable Sodium Percentage**	**Soil Physical Condition**
Saline	> 4.0	<8.5	<15	Normal
Sodic	< 4.0	>8.5	>15	Poor
Saline-Sodic	> 4.0	<8.5	>15	Normal

> = greater than, < = less than

1.2 Types of salinity

Salinity can be categorized into primary and secondary salinity on the basis of sources of salinity. The accumulation of salts on the surface of soil over long periods of time through natural processes is termed primary salinity. For example, the deposition of oceanic salts carried by wind and water, or the release of salts through weathering of rocks. Secondary salinisation is due to human activities. The most common causes are land clearing and the use of salt-rich irrigation water.

1.3 Plants vary greatly in terms of salt tolerance

Plants vary in their ability to tolerate salinity. Generally, *Arabidopsis* and rice are quite sensitive. Corn, grain sorghum, soybeans are moderately tolerant while wheat, oats, sunflower and alfalfa are tolerant. Barley and tall wheatgrass are very tolerant and can grow on soils with exchangeable sodium percentages (ESPs) of 50 and higher. Table 1-3 illustrates the rating of various crops in terms of salinity tolerance.

Table 1-3: Rating of various crops in terms of salinity tolerance.

Sensitive	Moderately Tolerant	Tolerant	Very Tolerant
Arabidopsis	Corn	Wheat	Barley
Rice	Grain Sorghum	Oats	Tall wheatgrass
	Soyabean	Sunflower	
		Alfalfa	

Plants can be divided into glycophytes and halophytes on the basis of their abilities to grow on different salt concentrations (Flowers et al., 1977). Halophytes can grow on high concentrations of salts. For example, *Atriplex vesicaria*, a species common to salt marshes, can grow and complete its life cycle at concentrations of 700 mmol L^{-1} NaCl.

There are many facets to the remarkable tolerance of halophytes, including effective balancing of salt accumulation to lower the tissue osmotic potential and salt compartmentation to avoid toxicity. In addition, several halophytes have specialized cells such as salt glands in leaves and stems to extrude surplus ions and thus avoid toxic levels of accumulation (Zhu, 2007). In contrast, the majority of terrestrial plants, including most agriculturally important crops, is glycophytic. Glycophytes typically restrict salt translocation to shoot tissue and are therefore sometimes classified as 'excluders'. However, glycophytes appear to share most tolerance mechanisms that have been detected in halophytes but the levels and regulation of these systems are not well adapted to moderate and high levels of salt, resulting in a greater degree of sensitivity. Similarly, there may be important ecophysiological variation between di- and mono-cotyledonous plants: for example, in *Arabidopsis* shoot Na^+ accumulation and salt tolerance do not show a close correlation whereas this is the case for most tested cereals (Moller and Tester, 2007).

1.4 Effect of salt stress on plants

There are two main components to salinity stress in plants; an initial osmotic stress and a subsequent accumulation of toxic ions which negatively affects cellular metabolism (Munns et al., 2006) and in addition can lead to secondary stresses such as nutritional imbalance and oxidative stress (Alscher et al., 1997). The Na^+ cation is predominantly associated with the deleterious effect of salinity, and therefore, most research has focussed

on this mineral. However, plant adaptation to salt stress also requires appropriate regulation of Cl^- homeostasis (Munns and Tester, 2008). Indeed, for species such as soybean, citrus and grapevine where Na^+ is predominantly retained in the roots and stems, Cl^- is considered more toxic because this ion is accumulated at high levels in shoot tissues, negatively impacting on essential processes such as photosynthesis.

The osmotic component of salinity is caused by excess inorganic ions such as Na^+ and Cl^- in the environment that decrease the osmotic potential of the soil solution and hence water uptake by the plant root. Uptake of abundantly available Na^+ and Cl^- therefore offers a comparatively cheap way to lower the tissue osmotic potential. To avoid the risk of ion toxicity associated with this strategy, Na^+ and Cl^- are generally compartmentalized in the vacuole and/or less sensitive tissues. In parallel, adjustment of the cytoplasmic compartment is achieved via production of compatible osmolytes such as proline, mannitol, sorbitol and glycine betaine. The latter also act as antioxidants and thus detoxify reactive oxygen species (ROS). However, when plants are growing in high salt concentrations, an adequate sequestration of ions in the vacuole can become a limiting factor, especially in the case of glycophytes. In this scenario, plants can accumulate excessive amounts of Na^+ in the cytosol which negatively affects many aspects of cellular physiology. For example, the physicochemical similarities between Na^+ and K^+ lead to competition at transport and catalytic sites that normally bind the essential cation K^+ and maintaining a high cytosolic K^+/Na^+ ratio is believed to improve salt tolerance (Maathuis and Amtmann, 1999; Zhu, 2001).

Oxidative stress is another aspect of salinity stress which is in fact a consequence of salinity-induced osmotic and/or ionic stress (Hernandez et al., 2001). The salt induced production of ROS such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^\bullet$) is counteracted by different detoxifying enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.11) and glutathione reductase (GR, EC 1.6.4.2). Indeed, transgenic rice overexpressing a yeast Mn superoxide dismutase was shown to have improved salinity tolerance (Tanaka et al., 1999).

1.5 Salt stress signalling in plants

Plants need to sense the osmotic and ionic components of salt stress. This is important to maintain correct functioning of the roots and shoots in response to water deficit and the presence of high Na^+ concentrations. Plants show rapid responses after the addition of

NaCl to the external medium (Knight et al., 1997) but the details are not very well understood. Increases in extracellular Na^+ are presumably sensed at the plasma membrane although no sensors have been identified as yet. A rapid increase in cytosolic free Ca^{2+} suggests that the increase in extracellular NaCl facilitates the Ca^{2+} flux into the cytosol across plasma membrane and tonoplast (Tracy et al., 2008). The rise in cytosolic Ca^{2+} may be relayed by Ca^{2+} sensors such as SOS3, a calcineurin B-like protein. Although the affinity of SOS3 for Ca^{2+} is not known, increases in cytosolic Ca^{2+} likely facilitate the dimerization of SOS3 and its subsequent interaction with SOS2, a calcineurin B-like interacting protein kinase (CIPK24). The SOS3/SOS2 complex is targeted to the plasma membrane through myristoylation and subsequently phosphorylates the Na^+/H^+ antiporter, SOS1 (Zhu, 2002). SOS1 has also been suggested to act as a Na^+ sensor (Shi et al., 2002).

Several putative osmosensors have been identified in plants. The HKT-type Na^+/K^+ co-transporters of *Eucalyptus camaldulensis* have been shown to increase ion uptake when expressed in *Xenopus laevis* oocytes exposed to hypo-osmotic conditions (Liu et al., 2001) while the hybrid two-component histidine kinase AtHK1 activates a MAP kinase cascade in *Arabidopsis* in response to hyperosmotic conditions (Urao et al., 1999).

Cyclic nucleotides such as cAMP and cGMP have also been implicated in salt stress signalling. Electrophysiological data suggest that cyclic nucleotides may reduce Na^+ entry into the cell by down regulating voltage-independent cation channels in *Arabidopsis* (Maathuis and Sanders, 2001) and a rapid increase in cytosolic cGMP was observed when *Arabidopsis* plants were exposed to salt and osmotic stress (Donaldson et al., 2004).

1.6 Strategies for coping with high salinity

The large number of studies on plant salt tolerance has established that salt tolerance is a multigenic, complex trait which involves many physiological and biochemical mechanisms that vary between species with diverging resistance. However, a common theme of tolerance is the adequate control of salt uptake at the root level, regulation of influx into cells, control over long distance transport, and the compartmentation at the cellular and tissue level (Blumwald, 2000; Flowers and Colmer, 2008). These processes are mediated by membrane transporters and manipulating the activity of this class of proteins has therefore enormous potential to affect plant performance in saline conditions (Maathuis, 2007).

1.7 Role of transporter proteins in uptake and distribution of Na^+, K^+ and Cl^-

The combination of molecular, biochemical and physiological analyses has facilitated the identification and characterization of some important Na^+, K^+ and Cl^- transporter genes and proteins. These transporters play crucial roles in uptake, efflux, translocation and compartmentation of Na^+, K^+ and Cl^- ions. With the genomes of various plants having been sequenced, the total complement of potential proteins involved in Na^+, K^+ and Cl^- transport can be surmised. Figure 1-1 gives an overview of the main classes of monovalent ion transporters that totals several hundreds of isoforms, often derived from large gene families.

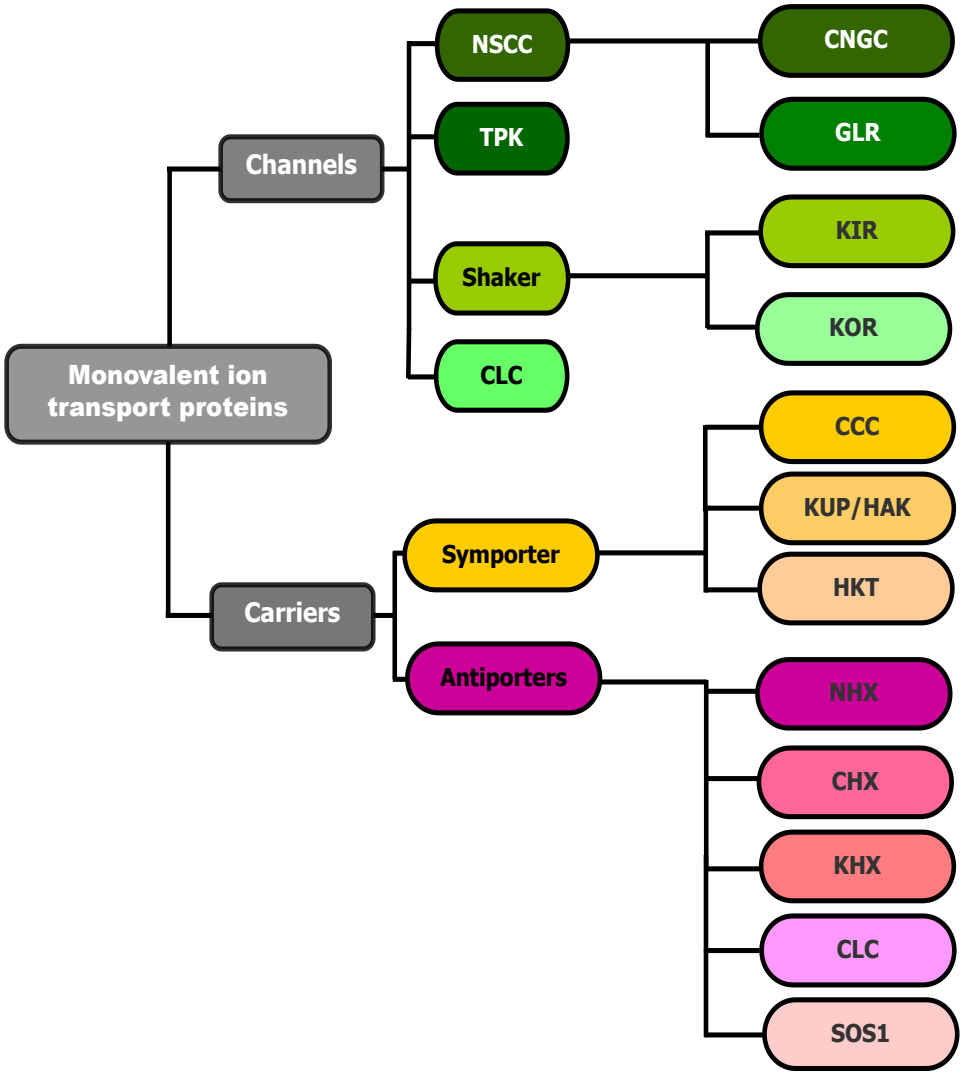


Figure 1-1:Overview of main gene families involved in Na^+, K^+ and Cl^- homeostasis in rice during salt stress. Abbreviations: CCC, cation chloride co-transporter; CHX, cation/H^+ exchanger; CLC, voltage gated Cl^- channel; CNGC, cyclic nucleotide gated channel; GLR, glutamate like receptor; HKT, high affinity K^+ transporter; KHX, K^+/H^+ exchanger; KIR, Shaker type K^+ inward rectifier; KOR, Shaker type K^+ outward rectifier; KUP/HAK, K^+ uptake permease; NHX, Na^+/H^+ exchanger; NSCC, non-selective cation channel; TPK, two-pore K^+ channel.

1.8 Salt uptake from soil

One of the main strategies to cope with salt stress is a tight control of Na^+ and Cl^- influx at the root:soil boundary. Whereas halophytes largely balance the uptake of inorganic ions with growth and osmotic requirements (Flowers et al., 1977), unidirectional Na^+ influx greatly exceeds net uptake in most glycophytes (Kronzucker et al., 2003). Thus, limiting

influx in the latter category would potentially alleviate stress but requires detailed insights into the molecular mechanisms that underlie root Na^+ and Cl^- uptake.

It is well established that Na^+ enters into root cells passively (Cheeseman, 1982). Whether plants have specific transport systems for low affinity Na^+ uptake from the soil remains an open question (Xiong and Zhu, 2002) and the exact mechanisms responsible for root Na^+ and Cl^- uptake are only partially clear and likely include transporters from several gene families and transport classes.

1.8.1 The role of non-selective cation channels in Na^+ uptake

On the basis of similarity between Ca^{2+} inhibition of radioactive Na^+ influx and Na^+ current through non-selective cation channels (NSCCs) it was hypothesised that the latter played a significant role in root Na^+ uptake (Amtmann et al., 1999; Tester and Davenport, 2003; Maathuis, 2007). However, the exact proportion and conductance of this pathway may vary substantially. For example, Kader and Lindberg, (2005) employed the Na^+ reporter dye SBFI to directly monitor cellular Na^+ levels and concluded that NSCCs mediate a significantly greater proportion of overall Na^+ uptake in cells of salt sensitive rice cultivars.

Primary sequences indicate the presence of two major plant NSCC families (Figure 1-1): the glutamate like receptors (GLRs) and cyclic nucleotide gated channels (CNGCs), both encoded by large gene families. In *Arabidopsis*, *CNGC3* contributes to short term Na^+ uptake in plant roots (Figure 1-2) and its presence has a moderate impact on plant salt tolerance (Gobert et al., 2006). *AtCNGC10* was also reported to be involved in sodium uptake and long distance transport (Guo et al., 2008). In addition, transcriptomics studies showed significant regulation of five *CNGC* isoforms in *Arabidopsis* in response to salinity stress (https://www.genevestigator.ethz.ch/; Maathuis, 2006) suggesting further members of this family may be involved.

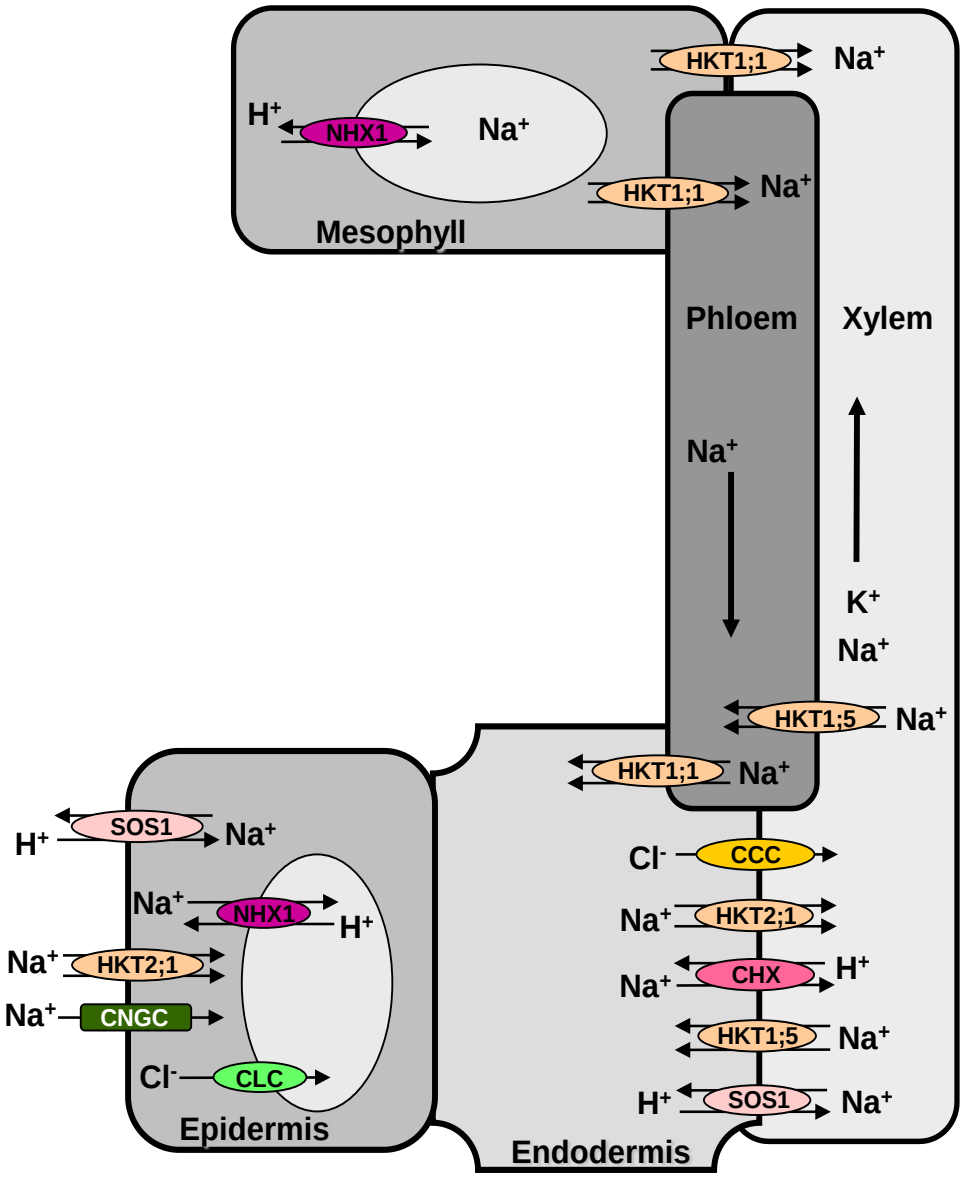


Figure 1-2: Generalised functions and localisation of Na^+ and Cl^- transporter proteins. Na^+ uptake at the soil-root boundary is thought to occur predominantly via non-selective cation channels like CNGCs and carriers from the HKT family. *HKT2;1* mediates high affinity uptake of Na^+ in rice but may also participate in Na^+ xylem loading. *HKT1;5*, located in rice xylem parenchyma cells, reduces the xylem Na^+ concentration and thus reduces shoot Na^+ load. Na^+ efflux into the vacuole and apoplast occurs via antiport systems such as *NHX1* at the tonoplast and *SOS1* at the plasma membrane, mechanisms that are conserved across many species. *SOS1* may also mediate xylem loading of Na^+ along with other antiporters such as CHXs. Chloride uptake and transports are not well understood. Chloride channels (CLCs) may be involved in compartmentation of Cl^- into the vacuole and chloride cation co-transporters (CCCs) may mediate xylem loading of Cl^- in the plant. The mechanism and identity of Cl^- uptake are not known.

1.8.2 Potassium channels may contribute to Na^+ uptake

Plants contain two general classes of K^+ selective ion channels, shaker type channels and TPKs. Shaker type K^+ channels such as KAT1 and AKT1 form the predominant inward K^+ conductance observed in plant plasma membranes. Such channels generally have a high K^+:Na^+ selectivity and were generally regarded not to play a significant role in Na^+ uptake (e.g. (Schachtman et al., 1991; Amtmann and Sanders, 1999). However, more recent work suggests that the picture is more complex and there may be ecophysiological variation in this respect: Wang et al., (2007) used a pharmacological approach to characterise Na^+ uptake in the halophyte *Suaeda maritima* and concluded that the low affinity Na^+ uptake pathway in this species resembles an AKT1 channel. Similarly, Kader and Lindberg, (2005) provide evidence that K^+ channels mediate substantial Na^+ influx in a salt sensitive rice cultivar but not in a tolerant one. In both cases the conclusions were derived from applying channel blockers and inhibitors which can be notoriously non-specific but these finding do suggest that K^+ channels are potential pathways for root Na^+ influx.

1.8.3 Carrier-type transporters that mediate Na^+ uptake

High affinity potassium transporters (HKTs) are carrier-type proteins that mediate Na^+ and K^+ transport (Haro et al., 2005). The first HKT was cloned from wheat and thought to function mainly as high affinity K^+ uptake system (Schachtman and Schroeder, 1994) but subsequently it was found that HKTs function primarily as Na^+:K^+ symporters or Na^+ uniporters (Haro et al., 2005).

There is ample evidence that HKTs contribute to Na^+ uptake from the soil (Figure 1-2): In wheat, antisense expression of wheat HKT1 caused significantly less Na^+ uptake in transgenic plants and enhanced growth under high salinity (Laurie et al., 2002). In *Arabidopsis*, only one HKT isoform is reported, i.e. *AtHKT1*, which is expressed mainly in roots and is reported to show high levels of Na^+ uptake activity in both yeast and *Xenopus oocytes* (Uozumi et al., 2000). In *Arabidopsis*, *AtHKT1* was believed to function as a potential Na^+ uptake pathway (Rus et al., 2001) but later work shows that *AtHKT1* is probably mostly involved in Na^+ reabsorption from the shoot (Berthomieu et al., 2003; Sunarpi et al., 2005).

In contrast to *Arabidopsis*, rice contains nine HKT isoforms (Garciadeblas et al., 2003): *OsHKT2;1* (previously *HKT1*) has been extensively studied and shown to be a high affinity Na^+ transporter that is of particular importance during low K^+ conditions (Garciadeblas et al., 2003; Horie et al., 2007). Expression of *OsHKT2;1* is localised to the root epidermis, cortical cells and vascular tissues of both roots and leaves (Golldack et al., 2002; Garciadeblas et al., 2003; Horie et al., 2007) and expression patterns in roots were found to be different in salt tolerant and sensitive varieties in response to NaCl stress (Golldack et al., 2002). Loss of function mutants in *OsHKT2;1* show reduced growth in low K^+ conditions and accumulated less Na^+ (Horie et al., 2007). Thus, it appears that *OsHKT2;1* augments monovalent cation uptake by providing high affinity Na^+ uptake in K^+ deficient conditions. However, *OsHKT2;1* relevance in Na^+ uptake during salinity stress may be limited since it has a micromolar affinity for Na^+ and its activity is rapidly downregulated at higher ambient concentrations of Na^+.

OsHKT1;1 (previously HKT4) has also been studied in heterologous expression systems and found to be a low affinity Na^+ transporter. Transcript abundance of *OsHKT1;1* was found to be relatively insensitive when [Na^+] was raised from 1 to 40 mM but its transport characteristics and high expression levels in roots suggest it may form part of the low affinity Na^+ uptake pathway in rice (Garciadeblas et al., 2003).

Transcriptomics studies also show altered transcript levels of *OsHKT2;3* and *OsHKT2;4* in roots, when plants were exposed to salt, suggesting involvement of further HKTs in Na^+ uptake (Garciadeblas et al., 2003).

In addition to HKTs, other carriers have been implicated in Na^+ uptake. Some members of the high affinity K^+ uptake transporter family HAK/KUP/KT may transport Na^+ with low affinity in the presence of high soil Na^+:K^+ ratios (Pardo and Quintero, 2002). Furthermore, yeast expression studies revealed that the normal function of HAK/KUP/KTs, high affinity K^+ uptake, is competitively inhibited by Na^+, pointing to a shared transport pathway of the two monovalent cations (Santa-Maria et al., 1997; Fu and Luan, 1998).

Several studies have shown substantial transcriptional regulation of HAK/KUP/KT isoforms by salt stress (e.g. Chao et al., 2005; Walia et al., 2005; Walia et al., 2007). For example, Su et al., (2002) observed that the expression of HAKs in *Mesembryanthemum crystallinum* was upregulated during salt stress and K^+ starved conditions. However,

whether this result and those for other HAKs relates to a potential role in Na^+ uptake or augmentation of K^+ uptake during salinity stress remains to be established.

The low affinity cation transporter, *LCT1* from wheat, functions as a non-selective cation carrier conducting K^+, Rb^+, Na^+, and Ca^{2+} transport in yeast (Schachtman et al., 1997; Clemens et al., 1998). Expression of *LCT1* in yeast increased salt sensitivity and Na^+ contents of the cells (Amtmann et al., 2001) suggesting that it might form a Na^+ uptake pathway in plant roots. However, the membrane localization and physiological roles of *LCT1* in plants has yet to be explored.

1.8.4 Transporters involved in Cl^- uptake

Cl^- is a major solute in plant vacuoles, particularly during salt stress, and is involved in both turgor and osmoregulation (White and Broadley, 2001). In contrast to Na^+, Cl^- uptake in most conditions must be energised but although there is a substantial amount of information regarding K^+ and Na^+ transport in plants, yet very little is clear about the molecular mechanisms behind the substantial Cl^- influx that results from salinisation (Flowers and Colmer, 2008). Plants contain CLC-type anion channels which are believed to participate in turgor regulation, stomatal movement and anionic nutrient transport such as NO_3^- (Hechenberger et al., 1996). Although the transcript abundance of several CLCs is affected by salinity (e.g. Diedhiou and Golldack, 2006) they are unlikely to contribute to root Cl^- uptake: firstly, plant CLCs have only been detected at endomembranes which appears to exclude a role in Cl^- uptake, and secondly the thermodynamics of Cl^- uptake rule out passive channel type mechanisms (Figure 1-2).

A second class of potential Cl^- transporters is formed by the cation chloride cotransporters (CCCs) encoding one gene in *Arabidopsis* and two genes in rice. *AtCCC*, expressed in root and shoot tissues, probably functions as a $2Cl^-:K^+:Na^+$ cotransporter. Loss-of-function of *AtCCC* in *Arabidopsis* led to a changed root:shoot Cl^- ratio but also to a large increase in net Cl^- uptake arguing against a role of *AtCCC* in the uptake of this ion (Colmenero-Flores et al., 2007). Thus the exact mechanism of Cl^- uptake and the involved proteins are two important questions that have yet to be answered.

1.8.5 Na^+ efflux mechanisms at the plasma membrane

In *Arabidopsis*, the plasma membrane Na^+/H^+ antiporter *AtSOS1* (Figure 1-2) was identified from a forward genetics mutant screen (Zhu, 2000). *SOS1* transcription is augmented after salt stress and transport assays showed it capable of exchanging H^+ coupled Na^+ and K^+ antiport activity (Shi et al., 2002). Although it may participate in other processes, the hypersensitivity displayed by loss of function mutants in *SOS1* clearly showed its paramount importance in salt tolerance (Shi et al., 2003). Further work revealed that regulation of *SOS1* activity by *SOS3* and *SOS2* also functions in rice and may be conserved in other plant species (Martinez-Atienza et al., 2007).

In roots, *SOS1* is primarily found in root tips and around the vasculature but is largely absent in other root tissue. Thus, it is unlikely that *SOS1* is responsible for a large part of Na^+ efflux into the apoplast and the likelihood of additional efflux systems in epidermal and cortical cells is great.

1.8.6 Na^+ compartmentation and the role of antiporters in vacuoles

Na^+ compartmentation in the vacuole occurs in all tissues and is an important mechanism for osmotic adjustment and Na^+ detoxification in the cytosol. Tonoplast H^+:Na^+ antiport activity has been reported in many species such as sugar beet (Blumwald and Poole, 1987), *Atriplex nummularia* (Hassidim et al., 1990), *Plantago maritima* (Staal et al., 1991) and *Mesembryanthemum crystallinum* (Barkla et al., 1995).

The tonoplast NHX1 antiporter was initially thought to be a primary tonoplast Na^+ antiporter and has been characterised in multiple species including *Arabidopsis* (Apse et al., 1999), wheat (Xue et al., 2004), tomato (Zhang and Blumwald, 2001), soybean (Li et al., 2006b) and rice (Fukuda et al., 2004). However, NHX1 was also shown to be involved in K^+ uptake into the vacuole under normal growth conditions, suggesting the dual affinity of NHX1 for Na^+ and K^+ (Yamaguchi et al., 2005). Leidi et al., (2010) also reported the involvement of AtNHX1 in the subcellular partitioning of K^+, affecting plant K^+ nutrition and improved salt tolerance. Overexpression of *NHX1* in several species endowed a greater level of salt tolerance. For example, transgenic tomato plants overexpressing *AtNHX1* produced fruits in the presence of salt concentrations where wild type plants could not survive (Zhang and Blumwald, 2001). Similarly improved salt tolerance was observed in rice after overexpression of *OsNHX1* (Chen et al., 1999; Fukuda et al., 2004). However,

the transgenic rice plants showed less vigorous growth in control conditions (Chen et al., 1999).

Other members of the NHX family are also capable of moving Na^+: Yokoi et al., (2002) reported that *AtNHX2* and *AtNHX5* could be important salt tolerance determinants, and observed that *AtNHX2* has a major function in vacuolar Na^+ sequestration. In *Ipomoea nit*, the vacuolar antiporter *InNHX2* was identified which is expressed in leaves, stems and roots and its transcription is significantly induced by salt stress (Ohnishi et al., 2005). Heterologous expression of *InNHX2* in yeast revealed that In*NHX2* catalyzes both Na^+ and K^+ transport into the vacuoles and also promotes vacuolar alkalization (Ohnishi et al., 2005).

1.8.7 Role of Cl^- channels in vacuolar Cl^- compartmentation

In addition to Na^+, Cl^- compartmentation is also important for salt tolerance, as elevated levels of Cl^- in the cytosol may be harmful, particularly in the case of citrus crops (Xu et al., 2000). Since the vacuole is moderately positive with reference to the cytoplasm, part of the vacuolar Cl^- sequestration could proceed through ion channels and several voltage-gated anion channels of the CLC family have been detected in the tonoplast of various species. In *Arabidopsis, CLCa* was recently shown to function primarily as a H^+ coupled antiporter to drive vacuolar nitrate accumulation (De Angeli et al., 2007) whereas *CLCc* may also be involved in NO_{3-} homeostasis rather than vacuolar Cl^- sequestration. However, CLC transcription has been found to respond to salinity: in rice, *OsCLCa* was significantly upregulated in salt sensitive cultivars in response to salinity stress and *OsCLCc*, which is expressed both in leaves and roots showed transcript reduction in the chloride accumulating salt sensitive IR29 while transient induction occurred in the chloride excluding variety Pokkali (Diedhiou, 2006). Diedhiou and Golldack, (2006) showed a coordinated regulation of anion and cation homeostasis in salt-treated rice and suggested a function for *OsCLCc* in osmotic adjustment at high salinity. A similar coregulation was recorded in soybean for *NHX1* and *CLC1* (Li et al., 2006a). Nakamura et al., (2006) showed that the same CLC channels partially complimented the yeast *gef1* mutant which lacks the yeast CLC channel. In conjunction, these findings suggest that CLC type anion channels are important in mediating Cl^- sequestration in the vacuole (Figure 1-2).

1.8.8 Long distance transport of Na^+

Most glycophytes can be classified as salt excluders, i.e. species that prevent large accumulation of salt in photosynthesising tissues (Weicht and Macdonald, 1992). Such species show a relatively high K^+:Na^+ selectivity where salt translocation is concerned, possibly via reabsorption of salts in the basal parts of the root vasculature (Lessani and Marschner, 1978). In addition, retranslocation of Na^+ from shoot to root has also been described and such mechanisms would also contribute to low shoot salt loads (Pitman, 1977).

In contrast, many halophytes are salt includers and store high levels of Na^+ and Cl^- in stems and leaves. Many salt includers are succulent because of the accumulation of salt in the large vacuoles of the mesophyll cells. Long distance transport of salt therefore provides another key step in the control of overall salt distribution within plant tissues.

Several proteins have been implicated in root shoot translocation of Na^+: the plasma membrane antiporter SOS1 is expressed in root parenchyma and in *Arabidopsis* impacts on Na^+ loading into the xylem sap during moderate salt stress (Shi et al., 2002). However, its exact function may depend on the severity of the salinity stress and include removal of Na^+ from the xylem stream when salt stress is excessive.

In *Arabidopsis,* loss-of-function mutations in the *HKT1* gene lead to overaccumulation of Na^+ in shoots and rendered the plant Na^+ hypersensitive (Maser et al., 2002b; Berthomieu et al., 2003). RNA *in situ* hybridizations showed that *HKT1* is expressed mainly in leaf phloem tissues and mediates Na^+ loading into the phloem vessels. In addition, *HKT1* may be involved in Na^+ unloading from the phloem sap in roots (Berthomieu et al., 2003) thus providing a mechanism for Na^+ retranslocation from shoot to root.

In other species too, HKT isoforms have been implicated in long distance Na^+ movement: in rice, *OsHKT1:5* (previously *HKT8*) is a plasma membrane Na^+ transporter expressed in xylem parenchyma cells that retrieves Na^+ from the xylem sap (Ren et al., 2005). The activity of *OsHKT1;5* results in a lower Na^+ load in shoot tissue and therefore a considerably higher K^+:Na^+ ratio in leaf tissues. *OsHKT1;5* transcript abundance is responsive to salt treatment (Walia et al., 2005) and interestingly, shows allelic variation between cultivars that differ in salt tolerance (Ren et al., 2005).

In wheat, *NAX1* and *NAX2* were characterized as being involved in Na^+ exclusion in this species (Munns et al., 2003). Both NAX genes have been identified as Na^+ transporters belonging to the HKT gene family (Huang et al., 2006; Byrt et al., 2007). Like *OsHKT1;5*, *NAX1* and *NAX2* appear to remove Na^+ from the xylem whereas NAX1 is also responsible for restricting Na^+ at the leaf base.

Members of the H^+:monovalent cation exchanger family (CHX) are also likely to contribute to Na^+ translocation. *AtCHX21* is mainly expressed in the root endodermis and loss of function in this gene reduced levels of Na^+ in the xylem sap without affecting phloem Na^+ concentrations (Hall et al., 2006). The *Atchx21* mutant showed a complex phenotype with reduced root growth in the presence of 50 mM NaCl (Hall et al., 2006). In rice, salt induced expression of *OsCHX11* in roots was cultivar dependent and higher in a tolerant cultivar (Senadheera et al., 2009). The differential expression correlated with a higher K^+:Na^+ ratio in the tolerant cultivar suggesting that *CHX11* may be involved in long distance transport of Na^+ and/or K^+.

1.9 Background and Rationale for thesis

Salt stress affects plant growth and development by causing various detrimental effects. The most important of them are primary stresses such as ionic, osmotic and nutrient stresses. These primary stresses interfere with a plant's ability to absorb water and nutrients, and accumulation of high levels of salts (mainly Na^+ and Cl^-) can cause ion toxicity. Primary stress can lead to secondary stresses such as oxidative stress, making salt tolerance a very complex trait. Studies at the molecular level have revealed that salt tolerance is controlled by interactions between several physiologically and biochemically coordinated processes (Kawasaki et al., 2001; Seki et al., 2002). It is well established that uptake, efflux, translocation and compartmentation of toxic ions (mainly Na^+ and Cl^-) provide important bases for salinity tolerance in plants, and hence, a potential avenue to improve crops. However, a lack of understanding regarding the molecular entities and complex interactions of the responsible membrane transport proteins has hindered progress in this respect and hence other physiological traits should also be considered for salt tolerance of the plants.

It is well established that Na^+ competes with K^+ for uptake sites at the plasma membrane, and major binding sites in the cytoplasm such as those involved in enzymatic reactions, protein synthesis, and ribosome function. Salt stress therefore often induces K^+ deficiency

in the plants (Shabala and Cuin, 2008). Maintenance of high intracellular K^+/Na^+ ratio is considered crucial for cell metabolism and a key component of salinity tolerance in plants (Maathuis and Amtmann, 1999; Hasegawa et al., 2000; Shabala, 2000; Tester and Davenport, 2003; Chen et al., 2007). Therefore, excessive cytosolic Na^+ accumulation should be prevented along with retention of optimal concentrations of K^+ in the cytosol for proper functioning of the plants under salt stress conditions. K^+ transporters are believed to play important roles in salt stress signalling and tolerance by controlling intracellular K^+ homeostasis (Chen et al., 2007). For example, vacuolar two pore K^+ channels were suggested to function as a major pathway for K^+ release into the cytosol during stomatal closure and play important role in K^+ homeostasis (Ward and Schroeder, 1994; Gobert et al., 2007). Hirsch et al., (1998) reported that *Arabidopsis* AKT1, a shaker type channel, can contribute to root K^+ accumulation from soil, and play important roles in salt tolerance. Similarly, K^+/H^+ antiporters are suggested to be key players in vacuolar K^+ accumulation, essential for maintaining cell turgor (Leigh and Jones, 1996; Song et al., 2004). In *Arabidopsis*, rice and wheat, HKT transporters were shown to protect plant leaves from overaccumulating Na^+ to toxic levels (Maser et al., 2002, Ren et al., 2005, Sunarpi et al., 2005). These results suggest that K^+ channels play multiple roles in K^+ homeostasis which can be of prime importance in salt tolerance of the plants.

Despite many years of intensive research and a reasonable understanding of ion transport regulation in *Arabidopsis* and rice, the role of many genes and the basic mechanism by which halophytes maintain favourable ion gradients during salt stress is not clear. Therefore, this research work focuses on both a glycophytic (rice) and a halophytic (barley) species to understand how salt sensitive and salt tolerant crops cope with salt stress.

Rice is one of the most important staple food crops in the world and more than half of the world's population consumes rice as their primary source of food. Rice has the smallest genome of all the cereals (430 million nucleotides) and serves as a model system for one of the two main groups of flowering plants, the monocotyledons, in the same way as *Arabidopsis thaliana* is the model for dicotyledons. Rice has low salt tolerance and its production and planting area are greatly affected by soil salinity (Akbar and Ponnamperuma, 1980). Despite many years of intensive research and a reasonable understanding of ion transport regulation in rice, the role of many rice genes in salt tolerance remains unknown.

Barley is another important agricultural crop widely cultivated throughout the world. In a 2007 ranking of cereal crops, barley was declared fourth both in terms of quantity produced (136 million tons) and in area of cultivation (566,000 km²). Although barley and rice belong to the same family of poaceae, yet the two cereal crops, resembling each other in appearance, differ greatly in salt tolerance. Barley is tolerant to salt stress, compared to rice and although the genome of barley is around 10 times larger than that of rice, the sequence homology is quite high between the two species (Gale and Devos, 1998). Therefore, this research work focuses on both a glycophytic (rice) and a halophytic (barley) species to understand how salt sensitive and salt tolerant crops cope with salt stress.

1.10 Aims of the project

The majority of agriculturally important crops is glycophytic, and therefore seriously threatened by soil-salinity. The main aims of the project were to identify various transporter genes in glycophytic model crop (rice) and a halophytic crop barley: to study the exact roles of these genes for better understanding of the mechanisms that can contribute in salt tolerance of cereals; to understand better how tolerant crop species gradually adapt to salt stress to enable further development of additional salt tolerance traits. The objectives of this work were as follows:

1. To study the exact physiological role of rice *TPKa* and *TPKb* genes and to know the relationship between salt-tolerance and transporter gene function using overexpression approach.
2. To study how loss-of-function mutations and overexpression in rice *AKT1* affect plant growth and development under various Na^+ and K^+ stress conditions.
3. To study the role of *HvHKT2;1* in plant salt tolerance of halophytic barley under various K^+ and Na^+ stress conditions.

Chapter 2

2 Characterisation of Rice Vacuolar Two Pore K^+ channels

2.1 Introduction

The importance of vacuoles in plant cells is evident from the fact that vacuoles comprise more than half the volume of fresh weight of herbaceous vascular plants, the non woody tissue of woody plants, some algae and bryophytes (Raven, 1987). In plants, vacuoles play vital roles in many crucial physiological processes such as turgor provision, storage of minerals and nutrients, and cellular signalling. The majority of these processes has been associated with the large central lytic vacuole (LV) which can occupy as much as 90% of the cellular volume. In addition to lytic vacuoles, storage of nutrients can occur in protein storage vacuoles (PSVs) found in abundance in tissues such as seeds, where large quantities of proteins and minerals are stored for the developing embryo (Isayenkov et al., 2010). These PSVs have also been demonstrated in vegetative cells, where they may function in the accumulation of vegetative storage proteins in response to developmental or environmental cues (Jauh et al., 1998).

For proper functioning of the vacuole, V-ATPase and V-PPase provide the proton motive force (PMF) for the transport of sugars, amino acids and minerals (K^+, Na^+, Ca^{2+} and NO_3^-) across the tonoplast (Isayenkov et al., 2010). Vacuolar Ion channels with K^+ and Na^+ permeability have been well studied for their importance in salt tolerance. Compartmentation of Na^+ and K^+ in vacuoles is one of the main strategies for salt tolerance of various plant species to prevent the deleterious effects of Na^+ in the cytosol while maintining osmotic adjustment for water uptake into cells (Leigh and Wyn-Jones, 1984; Walker et al., 1996; Blumwald, 2000). Several studies suggest that vacuolar antiporters at the tonoplast mediate the transport of Na^+ into the vacuole, and improve plant salt tolerance (Apse et al., 1999; Gaxiola et al., 1999; Francisco et al., 2000). For example, *Arabidopsis* vacuolar antiporter, AtNHX1 was believed to be involved in Na^+ compartmentation and improve salt tolerance (Apse et al., 1999; Gaxiola et al., 1999). However, the exact mechanism by which these transporters improve salt tolerance is not clear (Pardo et al., 2006). Many studies suggest that vacuolar antiporters catalyze both

Na^+/H^+ and K^+/H^+ exchanges with similar affinity (Apse et al., 2003; Yamaguchi et al., 2005). Rodriguez-Rosales et al., (2008) found even greater K^+ content compared to Na^+ content in tissues of transgenic plants. K^+ is an essential macronutrient often referred to as "the regulator" for its involvement in regulation of more than 60 enzymes required for plant growth and development (Mohammad and Naseem, 2006). K^+ helps plants to tolerate stresses such as extreme temperatures, drought and pests (Cakmak, 2005). K^+ is also involved in protein synthesis, photosynthesis, stomatal movement and water-relations (turgor regulation and osmotic adjustment) in plants (Marschner, 1995). Plants take up K^+ from the soil and plant cells accumulate K^+ in concentrations ranging from 50 – 150 mM (Leigh and Wyn-Jones, 1984). However, the cytoplasmic K^+ concentration is controlled at around 100 mM (Walker et al., 1996). Three types of K^+ channels are described at the tonoplast namely slow-activating vacuolar (SV) channels, the fast vacuolar (FV) channels and the vacuolar K (VK) channels. SV channel was shown to be encoded by TPC1 gene (Peiter et al., 2005), and play a role in cation homeostasis and Ca^{2+} signalling (Ranf et al., 2008). The role of FV channel in plants is largely unknown, however, it has been postulated that FV and VK channels play important role in guard cell turgor regulation and K^+ homeostasis (Voelker et al., 2010). The VK channels have been proposed to be highly selective for K^+ ions (Allen and Sanders, 1996).

TPKs represent the VK channels (Gobert et al., 2007; Latz et al., 2007), found in guard cells and also in other cell types derived from root and shoot tissues (Gobert et al., 2007). In *Arabidopsis*, 5 TPK isoforms have been reported, of which four isoforms are highly homologous (TPK1, 2, 3 and 5) and localized to the tonoplast (Voelker et al., 2006). However, TPK4 is expressed at the plasma membrane (Becker et al., 2004). TPK families have also been found in genomes of other species such as rice, tobacco and *Physcomitrella* (Latz et al., 2007) as shown in table 2-1.

AtTPK1 has been functionally characterized using overexpression, loss of function and heterologous expression approaches: it is ubiquitously expressed and has a role in cellular K^+ homeostasis and K^+ release during stomatal functioning (Gobert et al., 2007). *AtTPK1* was found to be highly expressed in embryonic tissue, and overexpressing *AtTPK1* accelerated seed germination (Czempinski et al., 2002; Gobert et al., 2007). However, the exact physiological functions of other members of the TPK family are not clear.

Table 2-1: List of TPK channels in *Arabidopsis*, rice and tobacco

Gene	Expression	Localization	Function	Reference
AtTPK1	Ubiquitous	Tonoplast	K^+ homeostasis, K^+ release during, stomatal functioning, seed germination	(Gobert et al., 2007 ; Czempinski et al., 2002)
AtTPK2	Pollen	Tonoplast	Unknown	(Voelker et al., 2006)
AtTPK3	Ubiquitous	Tonoplast	Unknown	(Voelker et al., 2006)
AtTPK4	Pollen	Plasma membrane	K^+ homeostasis	(Becker et al., 2004)
AtTPK5	Ubiquitous	Tonoplast	Unknown	(Voelker et al., 2006)
OsTPKa	Ubiquitous	Tonoplast	Unknown	(Isayenkov et al. 2010)
OsTPKb	Seeds	Protein storage vacuole	Unknown	(Isayenkov et al. 2010)
NtTPK1		Tonoplast	Unknown	(Hamamoto et al., 2008)

TPKs have a four transmembrane two pore structure, each pore containing a GYGD K^+ selectivity motif, and one or two predicted C-terminal EF hands as shown in figure 2-1. AtTPK1 is regulated by intracellular Ca^{2+} (Gobert et al., 2007). TPK1 activity is also influenced by cytoplasmic pH, phosphorylation and 14-3-3 proteins (Gobert et al., 2007; Latz et al., 2007). The activity of NtTPK1 in tobacco is also sensitive to spermidine and spermine (Hamamoto et al., 2008).

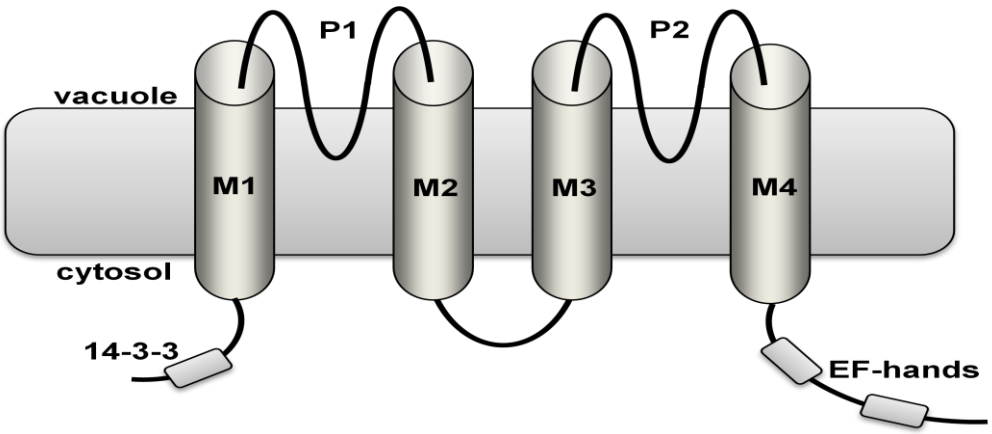


Figure 2-1:Proposed topology of the two pore K^+ channel, showing four transmembrane domains (M1 – M4), the two pore regions (P1 – P2), the predicted 14-3-3 binding site in the amino terminus, and two carboxyterminal Ca^{2+} binding EF-hands.

Salt tolerance has been extensively studied, but still the exact mechanism of salt tolerance remains largely unknown because of the complex nature of salinity stress. Salinity stress is always associated with interaction between different ions i.e. Na^+, Ca^{2+} and K^+. Salt tolerance requires not only the adaptation to Na^+ toxicity, but also the acquisition of K^+ as an essential nutrient with its uptake strongly affected by elevated levels of Na^+. A high cytosolic K^+/Na^+ ratio is important for maintaining cellular homeostasis and proper functioning of the cell during salt stress. K^+ ion channels of the two pore K^+ (TPK) family have extensively studied in *Arabidopsis* and tobacco, and are proposed to play an important role in K^+ homeostasis and seed germination. However, the role of various TPKs during salt stress is not clear, particularly in cereal crops.

In rice, two TPK isoforms, *OsTPKa* and *OsTPKb* have been cloned and shown to be highly homologous to each other and to *AtTPK1*. However, *OsTPKa* is localized to the tonoplast of the central lytic vacuole, whereas *OsTPKb* is localized to protein storage vacuoles suggesting particular roles of different TPKs (Isayenkov et al., Unpublished). It is hypothesized that the overexpression of the *OsTPKa* from rice would facilitate the K^+ homeostasis during salt stress condition and *OsTPKb* overexpression would help in increasing the rate of seed germination under salinity stress. To investigate the hypothesis that K^+ ion channels of the two pore K^+ (TPK) family facilitate K^+ homeostasis and seed germination during salt stress, *OsTPKa* and *OsTPKb* were introduced into wild type rice, expressed under control of the CaMV 35S promoter. The expression of TPKs in transgenic

lines was analyzed using RT-PCR. Growth and ion contents of the transgenic *OsTPKa* and *OsTPKb* overexpresssors was compared with non transgenic control plants. This chapter will describe the data from a study that compared transgenic TPKs overexpressing lines compared to non-transgenic lines and the results will be discussed in relation to other studies.

2.2 Materials and Methods

2.2.1 Chemicals and consumables

Laboratory chemicals used throughout the work were purchased from: Bio-Rad (UK), Merck (UK), MWG-Biotech (UK) and Sigma (UK). The laboratory consumables were purchased from Eppendorf (UK).

2.2.2 Enzymes and reaction kits

Restriction enzymes from New England Biolabs (Schwalbach) were used for DNA digestion. DNA Taq polymerase from New England Biolabs (Schwalbach) was used for genotyping of recombinant bacteria and transgenic plants by PCR. The following kits were used:

QIAprep spin Miniprep kit	Qiagen UK
QIAquick gel extraction kit	Qiagen UK
QIAquick PCR purification kit	Qiagen UK
RNase easy KIT	Qiagen UK

2.2.3 Media stock solutions and buffers

All buffers, growth media and solutions used in this study were prepared according to standard protocols, as described at the end of the corresponding method description. Antibiotics and other heat-sensitive additives were prepared as stock solutions, filter-sterilized and added to the growth media after cooling to 50°C.

2.2.4 Plasmid vectors

Modifiedbinary vectors pGreen and pSoup were used for rice transformation (Vain et al., 2004) as shown in figure 2-2. The pG0179 is a pGreen based vector which contains the cauliflower mosaic virus 35S promoter-driven gene encoding resistance to hygromycin for

selection of the transformants. pGreen is a Ti binary vector which can replicate in *Escherichia coli* but is unable to replicate in *Agrobacterium* without the presence of another binary plasmid, pSoup, in the same strain. pSoup contains the tetracycline resistance gene for selection and has an oriV region which has a replication function in trans for pGreen-based vectors in *Agrobacterium*.

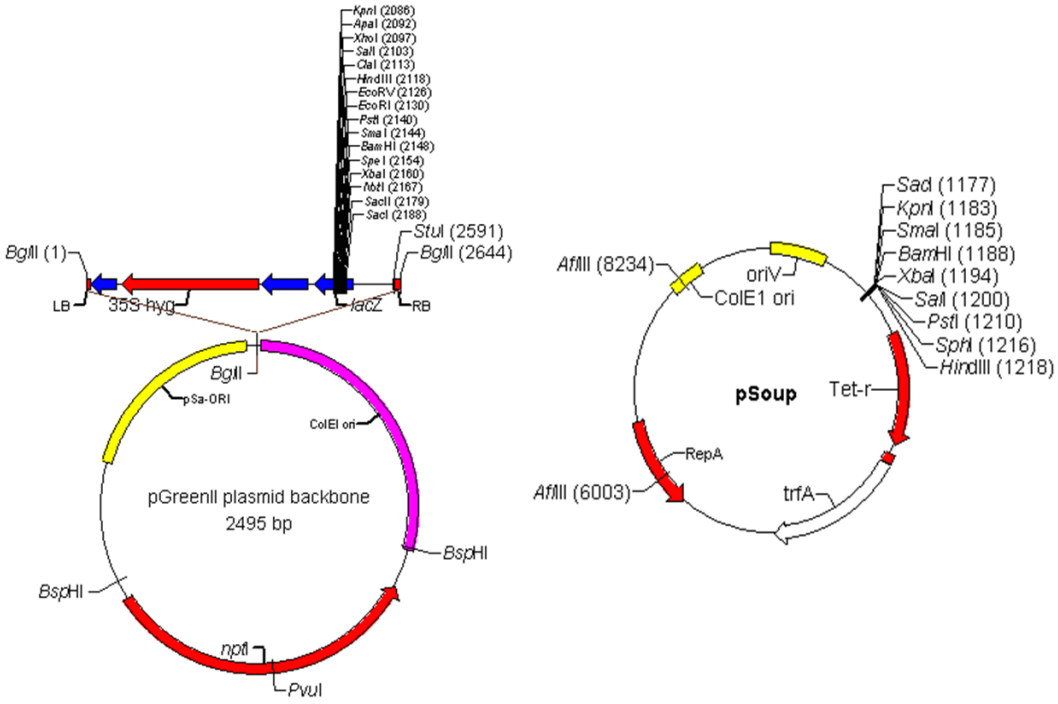


Figure 2-2: pGreen/pSoup-based vectors used for rice transformation.

2.2.5 Oligonucleotide primers

All primers used in this study were manufactured and HPLC purified by MWG-Biotech. Stock solutions of 100 pmol were prepared in autoclaved and filter sterilized water and stored at -20°C.

2.2.6 *Escherichia coli* strain

E.coli strain DH5α (Ausubel et al., 1994) was used as recipient of foreign DNA for the propagation and isolation of plasmid DNA throughout this study.

2.2.7 *Agrobacterium tumefaciens* strain

Agrobacterium strain AGL1 was used for *Agrobacterium*-mediated gene transfer.

2.2.8 Plants

For better understanding the molecular mechanism of salt tolerance in rice, mature seeds of rice plants (*Oryza sativa* L. Japonica cv Nipponbare), obtained from the International Rice Research Institute (IRRI; Laos Banos, Philippines) were used in this study.

2.2.9 Biological methods

2.2.9.1 Growth and maintenance of *Escherichia coli*

E. coli cells were grown at 37°C in LB medium (see below) in a shaker incubator at 225 rpm, or on agar-solidified 1.5 % (w/v) LB plates. For long-term storage, 500 mL of an overnight liquid culture was mixed with an equal volume of 40 % (v/v) glycerol and stored at - 80°C.

LB medium

Peptone	1 % (w/v)
Yeast extract	0.5 % (w/v)
NaCl	0.5% (w/v)
pH	7.4

2.2.9.2 Growth and maintenance of *Agrobacterium tumefaciens*

Agrobacterium tumefaciens cells were grown in YEB medium (see below) after adding 100 µg/ml rifampicin and 50 µg/ml kanamycin at 28 °C in a shaker incubator at 200 rpm. For selection of recombinant bacteria 25 µg/ml tetracycline was added to the growth medium. For long-term storage 500 µL of an overnight liquid culture was mixed with an equal volume of glycerol stock medium (GSM) and stored at – 80 °C.

YEB medium

Nutrient broth or beef extract	0.5% (w/v)
Yeast extract	0.1% (w/v)
Peptone	0.5% (w/v)
Sucrose	0.5% (w/v)

pH 7.4

2 mM $MgSO_4$, 50 µg/ml kanamycin, 25 µg/ml rifampicin were added after autoclaving and cooling.

GSM

Glycerol	50 % (v/v)
$MgSO_4$	100 mM
Tris-HCl	25 mM
pH	7.4

2.2.9.3 Preparation of competent *E. coli* cells for heat-shock transformation

E. coli strain DH5α competent cells were prepared for heat shock transformation. A single bacterial colony was inoculated in 5 ml of LB broth and cultured at 37°C overnight. Afterwards, 0.5 ml of fresh overnight culture was transferred into 100 ml of LB broth. The cells were cultured at 37°C for 3 hours until the OD600 nm reached 0.4-0.5 and then transferred to an ice-cold tube. After placing on ice for 10 min, the cells were recovered by centrifugation (2,000 g / 4 °C/10 min). Cells were washed 3 times by spinning at 4000 rpm for 5 minutes at 4 ^{o}C and the pellet was re-suspended in 50 ml of ice cold 10% glycerol. Finally the cells were re-suspended in 1 ml of 10 % glycerol. Aliquots of 100 µl of the suspension were dispensed into pre-chilled eppendorf tubes, frozen immediately in liquid nitrogen and stored at -80°C.

2.2.9.4 Transformation of *E. coli* by heat-shock

As soon as the competent cells were thawed, plasmid DNA or ligation products were diluted 1:5 in sterile dH_2O and mixed gently with the competent cells, then stored on ice for 30 min. The cells were incubated for 90 seconds at 42°C and placed on ice for 2 min. A volume of 800 µl of SOC medium was added to the eppendorf tubes and transformed cells were incubated at 37°C for 45 min. Around 200 µl of cells were plated onto a LB-agar plate supplemented with appropriate antibiotics and incubated at 37°C overnight.

SOC medium

Bacto tryptone	1% (w/v)
Yeast extract	0.5% (w/v)
NaCl	10 mM
KCl	2.5 mM
Autoclaved and added;	
Sterile $MgCl_2$	1 M, 10 ml
Sterile $MgSO_4$	1 M, 10 ml
Sterile glucose	1 M, 20 ml

Filled up to 1 L water and filter sterilized.

2.2.9.5 Preparation of competent *A. tumefaciens* cells for electroporation

A single colony of *A. tumefaciens* grown on a YEB-agar containing 100 µg/ml rifampicin (Rif) and 25 µg/ml kanamycin (Km) (YEB-Rif-Km) was inoculated in 5 ml of YEB-Rif-Km medium in a 100 ml Erlenmeyer flask and incubated at 28°C for two days with shaking (250 rpm). One ml of the culture was transferred into 100 ml of YEB-Rif-Km medium and cultivated at 28°C for 15-20 hours with shaking (250 rpm) until the OD600 reached 1-1.5. The cells were chilled on ice for 15 min and spun down by centrifugation (4,000 g / 4 °C / 5 min). The culture medium was decanted and the cells were washed and centrifuged three times with 10 ml of dH_2O and resuspended in 500 µl of sterile 10% (v/v) glycerol. 45 µl-aliquots of the suspension were dispensed into pre-chilled eppendorf tubes, frozen immediately in liquid nitrogen and stored at -80°C.

2.2.9.6 Transformation of *A. tumefaciens* by electroporation

The electroporation apparatus was set to 2.5 KV. The pulse controller was set to 200 ohms. One µl plasmid DNA was added to tubes containing 40 µl fresh or thawed cells on ice, mixed by swirling with a pipette tip. The DNA and cells were transferred to a pre-chilled electroporation cuvette (0.2 cm electrode gap) using a narrow pipette tip and placed into the small chamber in the electroporator. The electroporation apparatus was energized and pulses were delivered until a short beep was heard. The cuvette was removed from the sample chamber and immediately 1 ml SOC medium was added to the cells. The cells were transferred to a sterile polypropylene culture tube using a Pasteur pipette and were placed in a shaker for 4 hours at 28 ^{o}C. The aliquots of the electroporation mixture were plated on LB agar plates supplemented with the appropriate antibiotics and were incubated at 28°C.

2.2.9.7 Isolation of plasmid DNA

To prepare DNA for nucleotide sequence analysis, Qiagen plasmid "DNA Mini prep kit" was used according to manufacturer's instructions.

2.2.9.8 Restriction enzyme digestion and precipitation of DNA

Restriction enzyme digestions were carried in the buffer supplied with the enzyme and in accordance with the supplier's recommendations. 1 µg DNA was mixed with 1 unit of the appropriate enzyme and incubated for 1-3 hours at the enzyme's optimal temperature. For double digestions, combination of the two enzymes at the most suitable buffer was carried out. Reactions were stopped by freezing at -20°C until required for further processing. For precipitation of plasmid DNA, DNA solution was mixed with 0.1 volumes of 0.3 M NaAc (pH 5.2) and 2.5 volumes of absolute ethanol. After storing this mix at -80°C for 30 min, DNA was pelleted via centrifugation (10 min/ 13000 g/ 4 °C). Subsequently, the DNA pellet was washed with 70% ethanol, air dried at room temperature and finally suspended in 10 mM Tris-HCl (pH 8.5).

2.2.9.9 Agarose gel electrophoresis

Gel electrophoresis of double-stranded DNA fragments was performed as described by Sambrook et al., (1996) in 0.8-1.5% agarose gels supplemented with Sybr Safe (0.1µg/ml). As markers, a defined amount of 2-Log DNA ladder (New England Biolabs) was included for determination of fragment size and estimation of concentration. Bands were visualized using a UV transilluminator at 302nm. For the documentation of the bands, the video

documentation system of the Herolab Company together with the Software "Easy Quant" was employed. In preparative electrophoresis, the desired fragment was excised with a sterile scalpel directly on the transilluminator and the DNA was extracted from the agarose gel using the Qiaex gel extraction kit (Qiagen, UK).

2.2.9.10 Determination of DNA concentrations

The concentration of isolated and purified DNA was determined by spectrophotometric measurement by measuring the absorbance of the solution at 260 nm, the wavelength at which both DNA and RNA absorb maximally, 50µg/ml DNA gives an absorbance of 1 at 260 nm (Sambrook et al., 1996).

2.2.9.11 Ligation of DNA fragments

DNA ligations were carried out in a total volume of 20µl. The insert:vector molar ratio was adjusted to 3:1 for sticky ends in 1 x T4 ligase buffer (New England Biolabs) and 0.1U of T4 DNA ligase was added. The reactions were incubated overnight at 16°C. Ligated DNA was precipitated, washed with 70% (v/v) ethanol at room temperature, resuspended in 10µl of sterile ultrapure water and stored at -20°C until required for transformation.

2.2.9.12 PCR amplification

Polymerase chain reaction (PCR) offers a fast and convenient method of amplifying a specific DNA segment. With this technique, a denaturated DNA sample is incubated with Taq DNA polymerase, dNTPs and two oligonucleotide primers whose sequences flank the DNA segment of interest so that they direct the DNA polymerase to synthesize new complementary strands. Multiple cycles of the process allows amplification of the genes of interest (Saiki et al., 1988). The reactions were performed in 0.2 ml PCR reaction tubes using DNA thermal cycler. PCR reactions were carried out in a total volume of 50 µl PCR, products were resolved on a 1-1.2% (w/v) agarose gel to check the amplification and integrity of the amplified product.

2.2.10 *Agrobacterium* mediated rice transformation

Mature seeds of japonica rice cultivar Nipponbare were transformed using *Agrobacterium* strain AGL1 based on a procedure described by Vain et al., (2004) with some modifications.

2.2.10.1 Media composition for rice transformation

The chemical composition of media that were used for *Agrobacterium* mediated rice transformation is described in Table 2-2.

2.2.10.2 Seed Sterilization

Dehusked seeds were sterilized with 70 % ethanol for 1 minute prior to washing in sterile water. These seeds were further sterilized with 2.5% sodium hypochlorite containing 1 drop of Tween-20 per 50 ml for 15 min and then washed five times in sterile water. This step was carried out once without Tween-20.

2.2.10.3 Callus induction

The sterilized seeds were inoculated on N6D medium (Table 2-2) and cultured under continuous light at 30 °C for 3 weeks. Loose embryogenic translucent globules, around 1 mm in size, were separated by rolling the callus grown from the seeds on gelling agent and were cultured for an additional 10 days on fresh N6D medium to produce embryogenic nodular units (ENU), that were used as targets for transformation (Figure 2-3).

2.2.10.4 *Agrobacterium* mediated transformation of calli

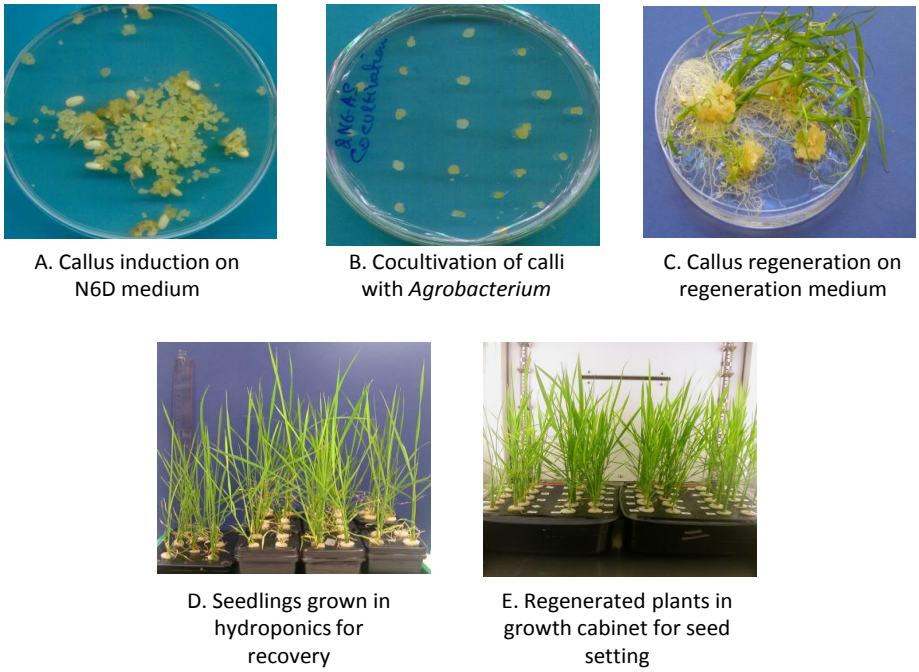
Agrobacterium strains were grown for 2 days at 28 °C on AB medium supplemented with 200 mM acetosyringone and suitable antibiotics. Bacterial cells were scooped up from the plate, re-suspended in 20 ml of AAM liquid medium (Table 2-2) and shaken for 1 h at 28 °C. Culture plates containing ENUs were flooded with bacterial suspension OD = 1.0 (600 nm) for 5 min. Liquid culture was removed, and each ENU was picked up and blotted onto sterile filter paper before being placed onto co-cultivation medium for 2 days in the dark at 25 °C. After co-culturing, ENUs were put onto selection medium (N6D medium containing 150 mg l^{-1} timentin plus appropriate antibiotics) in the dark at 28 °C (Figure 2-3). After a 2-week long period, each callus (grown from an individual ENU) was split into two to five pieces. Pieces of callus were cultured for 3 additional weeks onto fresh N6D-based selection medium.

Table 2-2: Media composition used for transformation of rice.

Composition	N6D (mg/l)	2N6-AS (mg/l)	AAM (mg/l)	RE-III (mg/l)	HF (mg/l)	AB (mg/l)
Macronutrient Components						
KNO_3	2,830	2,830		1,900	1,900	
NH_4Cl						
NH_4NO_3				1650	1650	
$(NH_4)_2SO_4$	463	463				
$MgSO_4.7H_20$	185	185	250	370	370	296
$CaCl_2.2H_20$	166	166	150	440	440	10
$NaH_2PO_4.2H_20$			150			1300
K_2HPO_4						3000
KH_2PO_4	400	400		170	170	
KCl			3,000			150
Micronutrient components						
Na_2EDTA	37.3	37.3		37.3	37.3	
$FeSO_4.7H_20$	27.8	27.8		27.8	27.8	2.5
Fe-EDTA			40			
$MnSO_4.\ 4H_20$	4.4	4.4	10	22.3	22.3	
$ZnSO_4.\ 7H_20$	1.5	1.5	2.0	8.6	8.6	
$CuSO_4.5H_20$			0.025	0.025	0.025	
$CoCl_2.7H_20$			0.025	0.025	0.025	
KI	0.8	0.8	0.75	0.83	0.83	
H_3BO_3	1.6	1.6	3.0	6.2	6.2	
$Na_2MoO_4.2H_2O$			0.25	0.25	0.25	
Organic components						
Casamino Acid	300	300	500	2,000		
Glycine	2.0	2.0	7.5	2.0	2.0	
L-Arganine			176.7			
L-Proline	2,878					
L-Glutamine			900			
L-Aspartic Acid			300			
myo-Inositol	100	100	100	100	100	
Nicotinic Acid	0.5	0.5	1.0	0.5	0.5	
Pyridoxine HCl	0.5	0.5	1.0	0.5	0.5	
Thiamine HCl	1.0	1.0	10	0.1	0.1	
Phytohormones						
2,4-D	2.0	2.0				
NAA				0.02		
Kinetin				2.0		
Acetosyringone		10~20	10~20			
Carbon Source						
Sucrose	30,000	30,000	68,500	30,000	30,000	
Sorbitol				30,000		
Glucose		10,000	36,000			5,000
pH	5.8	5.2	5.2	5.8	5.8	7.2

2.2.10.5 Regeneration of resistant calli

The resistant calli were transferred to pre-regeneration medium REIII (Table 2-2) for 9 days in the dark at 28 °C. Calli showing clear differential growth (Figure 2-3) were then transferred to regeneration medium HF (Table 2-2) for 2–3 weeks in the light at 28 °C.



A. Callus induction on N6D medium

B. Cocultivation of calli with *Agrobacterium*

C. Callus regeneration on regeneration medium

D. Seedlings grown in hydroponics for recovery

E. Regenerated plants in growth cabinet for seed setting

Figure 2-3:Different stages of rice transformation using *OsTPKa* and *OsTPKb* constructs: A. Callus induction on N6D medium, B. Cocultivation of calli with *Agrobacterium*, C. Callus regeneration on regeneration medium, D. Seedlings grown in hydroponics for recovery and E. Regenerated plants grown in growth cabinet for seed setting.

2.2.10.6 Histochemical GUS assay

GUS activity assays were carried out according to Kosugi et al., (1990), using 5-bromo-4-chloro-3-indolylglucuronide (X-gluc) as a substrate. Tissues were cleared after staining by soaking in 100% methanol. GUS expression was measured one week after transferring on selection medium, by counting the GUS-positive spots appearing as blue zones (1mm or more in diameter) on a given callus after the staining procedure.

2.2.11 Growth and selection of regenerated seedlings

Only one plant was regenerated from each original ENU to guarantee that each plant represents an independent transformation event. Plants were developed on HF medium (Table 2-2) for 2–3 weeks at 28 °C in the light. Plants were transferred to a controlled environment room for growth to maturity as shown in Figure 2-3.

2.2.11.1 Growth medium and growth conditions

Rice seeds were germinated in sterile conditions, at 28ºC, 100% relative humidity and kept in darkness for five days. Seedlings produced from the seeds were then transferred to a hydroponic culture system consisting of boxes containing 8 liters of aerated nutrient solution and holding up to 30 plants each. Nutrient medium consisting of macronutrients (1.25 mM KNO_3, 0.5 mM $Ca(NO_3)_2.4H_20$, 0.5 mM $MgSO_4.7H_20$, 42.5 µM Fe-EDTA and 0.625 mM KH_2PO_4) and micronutrients (0.16 µM $CuSO_4.5H_20$, 0.38 µM $ZnSO_4.5\ H_20$, 1.8 µM $MnSO_4.H\ H_20$, 45 µM H_3BO_3, 0.015 µM $(NH_4)_2Mo_7O_{24}.4H_20$ and 0.01 µM $CoCl_2$) as described by (Miyamoto et al., 2001), was used as a control medium. The hydroponic containers were placed in a growth chamber set to a light/dark cycle of 16/8 h daily (PAR 300 µmol/m^2 per second), 25/20ºC day/night temperature, and 60/40% relative humidity. Nutrient solution was changed every seven days. Plants were subjected to various Na^+ and K^+ stress conditions after 3 weeks of germination. Non-stressed plants were grown in parallel and harvested at the same time and served as a control. For growth, ion content and xylem sap experiments, Na^+ and K^+ treatments were made by addition of stocks of NaCl (5 M) and KCl (2 M) to the control medium. However, for 0 K^+ treatments, all potassium salts in the control medium were replaced with equimolar quantities of the corresponding sodium salts.

2.2.11.2 DNA extraction from rice plants

DNA was extracted from rice plants according to the CTAB method with some modification. Plant material up to 100 mg was ground to fine powder in liquid nitrogen. Ground tissues were quickly mixed with pre-warmed 450 µl of CTAB buffer and incubated at 65 °C for 50 min. After vortexing the mixture, 300 µl of chloroform:isoamylalcohol solution (24:1) was added. The mixture was vigorously shaken and centrifuged for 5 min in a microfuge. The top aqueous layer was transferred to clean sterilized eppendorf tubes and DNA was precipitated by adding 2 volumes of 96 % ethanol and 4 % 3 M NaAc (pH 5.2). The mixture was vortexed and left at room temperature for 30 minutes to precipitate the DNA. The mixture was then centrifuged for 10 min at 13000 rpm to obtain the DNA pellet. Finally, the pellet was rinsed in 70 % ethanol, dried for 10 min and resuspended in 100 µl TE buffer.

CTAB extraction buffer: 2 % CTAB, 1.4 M NaCl, 100 mM Tris-HCL (pH 8) and 20 mM Na-EDTA.

2.2.11.3 Total RNA isolation from rice tissues

Total RNA was extracted from leaf tissues of plants, using an RNase easy KIT (Qiagen, UK), according to the manufacturer's instructions.

2.2.11.4 cDNA synthesis and RT-PCR

The method of RT-PCR is a powerful technique used to quantify the expression of genes. Being simple and fast it was used as an alternative to other gene quantification methods like Northern blotting. cDNA is a DNA copy synthesized from mRNA. The enzyme used is the reverse transcriptase RNA-dependent DNA polymerase isolated from a retrovirus (AMV or MMLV). The amplification is achieved through the poly (A) tail found at the 3' end of most eukaryotic mRNAs to which a short complementary synthetic oligonucleotide (oligo dT primer) is hybridized. Together with all 4 deoxynucleotide triphosphates, magnesium ions and at neutral pH, the reverse transcriptase synthesises a complementary DNA on the mRNA template. The cDNA synthesis procedure consists of adding a total of 2 µg RNA to 1 µl oligo dT (500 µg/ml) primer in a total volume of 12 µl. After briefly mixing and centrifugation (2 sec) RNA was denatured by heating 10 min at 70°C and quickly chilled on ice for 2 min. After brief centrifugation, 6 µl of 5x First Strand Buffer (Sigma UK) was added for maintaining a favourable pH, 2 µl of 0.1 M DTT for stabilising the strand and 1 µl dNTP for the synthesis. The mixture was briefly centrifuged and then incubated at 42°C for 2 min before adding 1 µl of SuperScript RT II. The enzyme (Invitrogen UK) was added and mixed with the solution by gently pipeting five times up and down and the mixture then incubated at 42°C for the synthesis. The reaction was stopped after 1 hour and the enzyme was inactivated by incubating at 70°C for 15 min. The obtained cDNA was kept on ice for 2 min, centrifuged briefly and stored at -20°C for further study.

2.2.11.5 PCR analysis of transgenic rice plants

To analyze the integration of the transformed gene in transgenic rice plants, genomic DNA was isolated and PCR methods were used. Leaf tissues were used for extraction of the DNA. PCR was performed for 30 cycles (30 sec. at 94°C, 30 sec. at 60°C, 60 sec. at 72°C), using the hygromycin phosphotransferase (HPT) gene specific primers and genomic DNA from the transformants as template. The nucleotide sequence of the sense primer HygNfor was 5P'-ATTTGTGTACGCCCGACAGT-3' and the nucleotide sequence of the antisense

primer HygNrev was 5P'-GGATATGTCCTGCGGGTAAA-3'. The PCR products were resolved by electrophoresis in a 1.2 % agarose gel.

2.2.12 Expression analyses of *OsTPKa* and *OsTPKb* transcript level by semi-quantitative RT-PCR

Total RNA was extracted from leaf tissues of three week old control and *OsTPKa and OsTPKb* overexpressor lines, using an RNase easy KIT (Qiagen, UK), following the manufacturer's instructions. A total of 1 μg RNA was used to synthesize first strand cDNA using the transcriptor high-fidelity cDNA synthesis KIT (Roche, Mannheim, Germany). PCR was applied to 1 μl of cDNA with gene-specific primers against *OsTPKa* and *OsTPKb*. The house-keeping gene, α-tubulin was used as the control. PCR consisted of 35 cycles of 45 s at 53 °C, 1 min 30 s at 72 °C and 30 s at 95 °C. The primers used for semi-quantitative RT-PCR analyses are listed in table 2-3.

Table 2-3: Primers used to detect gene expression in control and *OsTPKa* and *OsTPKb* overexpressing lines using semiquantitative RT-PCR.

Gene ID	Primer Sequence	Product Size
αhistone-F	5'-CGAGAAGCGAAGAGGAGATG-3'	465
αhistone-R	5'-TCAACAAGTTGACCACGTCAC -3'	465
TPKa_cDNA-F	5'-TTCAAGGCCCCCCTGAAAAG -3'	552
TPKa_cDNA-R	5'-TTGGTCTCACCACCCTTCAGA -3'	552
TPKb_cDNA-F	5'-CGCCCACGGACAAGGACAAC -3'	596
TPKb_cDNA-R	5'-CGTCCCTGACGCCACCGCCGC -3'	596

2.2.13 Growth analyses on *OsTPKa* and *OsTPKb* overexpressors and wild type control lines

Rice plants overexpressing *OsTPKa, OsTPKb* and control plants were grown for 2 weeks on terra green in the green house. After two weeks, the seedlings were transferred to hydroponic growth medium. At the three week stage, three plants each from *OsTPKa* and *OsTPKb* overexpresssors, and control plants were exposed to different Na^+ and K^+ stress conditions i.e. control, 0 K^+, 50 mM KCl and 50 mM NaCl (see growth media and conditions) for one week. Experiments were repeated three times and relative growth rate (RGR) was calculated using the equation described by Poorter and Garnier, (1996).

2.2.14 Na^+ and K^+ content analyses on *OsTPKa* and *OsTPKb* overexpressors and wild type control lines

Na^+ and K^+ content of leaves, stems, and roots was measured using flame photometry. Rice plants overexpressing *OsTPKa, OsTPKb* and wild type control plants were grown for 2 weeks on terra green in the green house and then transferred to hydroponic control medium for one week. At the three week stage, three plants each from *OsTPKa* and *OsTPKb* overexpressors and control plants were exposed to different Na^+ and K^+ stress conditions i.e. control, 0 K^+, 50 mM KCl and 50 mM NaCl (see growth media and conditions) for one week. The plants were then separated into roots and shoots and roots were washed with 20 mM $CaCl_2$ solution for 10 minutes. Fresh weights of the samples from roots and shoots were noted after blotting with tissue papers. Samples were then dried at 80^{o}C for 3 days. Dried samples were treated with 5 ml of 20 mM $CaCl_2$ for 24 hours and Na^+ and K^+ content of the samples were recorded using a flame photometer (Sherwood flame photometer-410, Cambridge, UK). The experiments were replicated three times.

2.2.15 Xylem sap analyses on *OsTPKa* and *OsTPKb* overexpressors and wild type control lines

Three week old *OsTPKa* and *OsTPKb* overexpressing lines and control plants were treated with different Na^+ and K^+ regimes (control, 50 mM KCl, 50 mM NaCl) for one week before collecting the xylem sap. Plants were transferred to a pressure chamber (Digital plant water potential apparatus, EL540-300), and the shoot was excised about 20 mm above the root/shoot junction. Pressure exceeding the osmotic pressure of the external solution was applied to the chamber. Xylem sap was collected for 30 minutes. Aliquots of 0.3 ml xylem sap were collected from 3 plants in parallel and immediately put on ice. Na^+ and K^+ content of the xylem sap was measured with a flame photometer (Sherwood flame photometer-410 Cambridge, UK).

2.2.16 Statistical analysis

All the data were obtained from experiments carried out across a minimum of three biological replications. The experimental data were subjected to unpaired two-tailed t-tests to identify significance at the $P < 0.05$ level.

2.3 Results

2.3.1 Establishing a hydroponic rice culturing system

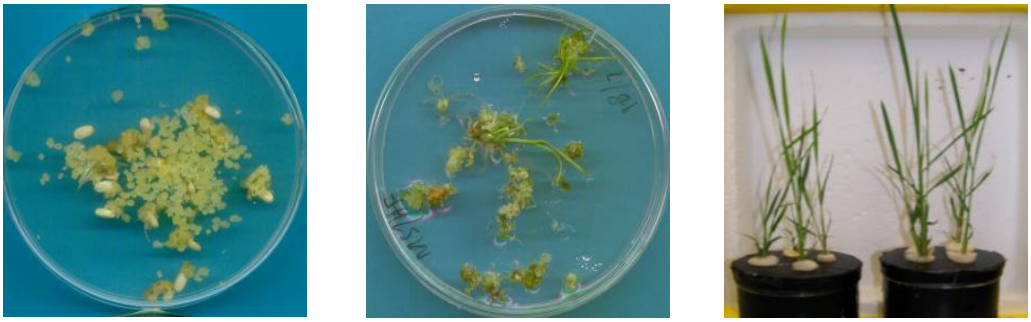
A rice hydroponic culture system was established for the first time in the Biology Department. This was done to optimize the conditions, i.e. medium composition, day and night length, temperature and humidity for rice growth, and to be able to grow the regenerated calli to seedling and seed setting stage. Rice seeds were germinated in sterile conditions and seedlings were transferred to hydroponic culture system. Nutrient medium for rice growth in hydroponic culture system was prepared as described in material and methods. The hydroponic containers were placed in a growth chamber set to a light/dark cycle of 16/8 h daily (PAR 300 µmol/m^2 per second), 25/20ºC day/night temperature and 60/40% relative humidity. Nutrient solution was changed every seven days. Plants were successfully grown to the seed production stage in the hydroponic culture system and seeds were obtained as shown in figure 2-4.

Figure 2-4: Rice plants in growth cabinet for seed production.

2.3.2 Tissue culturing of Rice (*Oryza sativa* L. Japonica cv Nipponbare)

Another critical step in rice transformation was the establishment of tissue culture condition in plates. It was important to check the procedure were working well and to see the different stages of rice growth on culture plates. Therefore, culture media were prepared (Table 2-2) as described by Vain *et al.,* (2004). Rice seeds were first sterilized and the sterilized seeds were then put on N6D medium for 3 weeks under continuous light at 32 ^{0}C to produce embryogenic nodular units (ENU). The ENUs were then transferred to regeneration medium (REIII, Table 2-2) for 9 days in the dark at 28 ^{0}C. Calli showing clear differential growth were then transferred to regeneration medium (REIII) for 2–3 weeks in

the light at 28 ^{0}C. One seedling was regenerated from each original ENU. The regenerated plants were grown to maturity in a controlled environment as described in Material and Methods. Tissue culturing conditions were optimized and plants were grown to maturity from the callus as shown in figure 2-5.



A. Rice seeds grown on N6D medium

B. Callus on Regeneration medium

C. Regenerated plants in hydroponic medium

Figure 2-5: Different stages of tissue culturing of rice, on callus induction medium (A), regeneration medium (B) and hydroponic culture pots (C).

2.3.3 Histochemical GUS assay of transformed calli

GUS expression was measured (as described in material and methods) one week after growing on selection medium. Small patches of blue colouration on the surface of the transformed calli, could be observed using a light microscope (Figure 2-6).

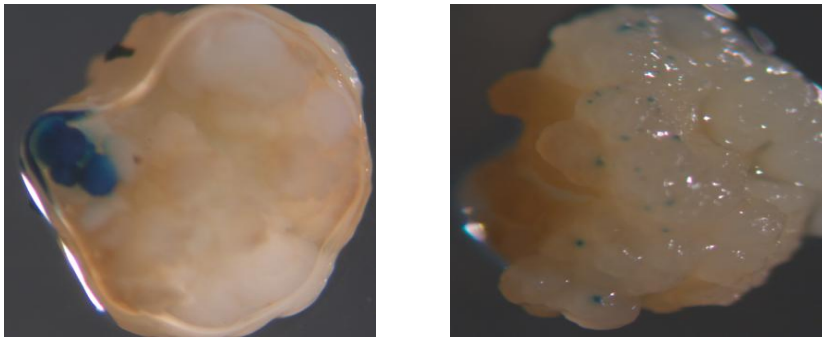


Figure 2-6: Transgenic calli showing GUS expression (blue spots).

2.3.4 Construction of vectors containing Hygromycin resistance genes

Once the protocol for rice transformation was established and the calli with GUS expression were generated. The constructs for overexpression of *OsTPKa* and *OsTPKb* genes were made by Dr. Stanislav Isayenkov at The University of York, using a binary

vector system pGreen/pSoup as described in the material and methods **(2.2.4).** Rice calli obtained from the seeds were transformed with the 35S:*TPKa* and 35S:*TPKb* gene and transgenic plants were regenerated. Regenerated seedlings (T1) were grown to the next generation (T2) for selection of homozygous lines.

2.3.5 Characterisation of rice plants overexpressing *OsTPKa* and *OsTPKb* genes

For selection of homozygous lines for *OsTPKa* and *OsTPKb*, a germination test was carried out on ½ MS media agar plates (Murashige and Skoog, 1962) supplemented with 50 mg l^{-1} hygromycin (Figure 2-7) as described in the Methods section. Plants producing well developed roots on hygromycin plates were selected and grown for seed production. A total of 12 transgenic lines was analysed for *OsTPKa*. Among these, two transgenic lines overexpressing *OsTPKa* (*OsTPKa* 133 and *OsTPKa* 212) were found to be homozygous, eight lines were heterozygous and one line was found homozygous with regard to hygromycin sensitivity. For *OsTPKb*, 9 lines were screened, out of which 2 lines (*OsTPKb* 13 and *OsTPKb* 155) were found homozygous overexpressing while 1 line was found to be homozygous hygromycin sensitive (*OsTPKb*55). The sensitive line, termed as (WT), was used as control line for all experimentation.

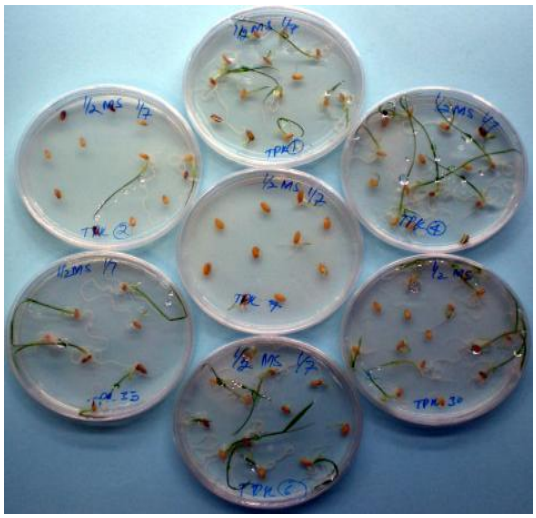


Figure 2-7: Screening of rice seeds overexpressing *OsTPKa* and *OsTPKb* gene on ½ MS hygromycin selection media.

The presence of the *OsTPKa* and *OsTPKb* constructs in the selected *Oryza sativa* lines used in this study were confirmed by PCR analysis of genomic DNA extracted from these plants using hygromycin gene specific primers (2.2.11.5) as shown in in Figure 2-8.

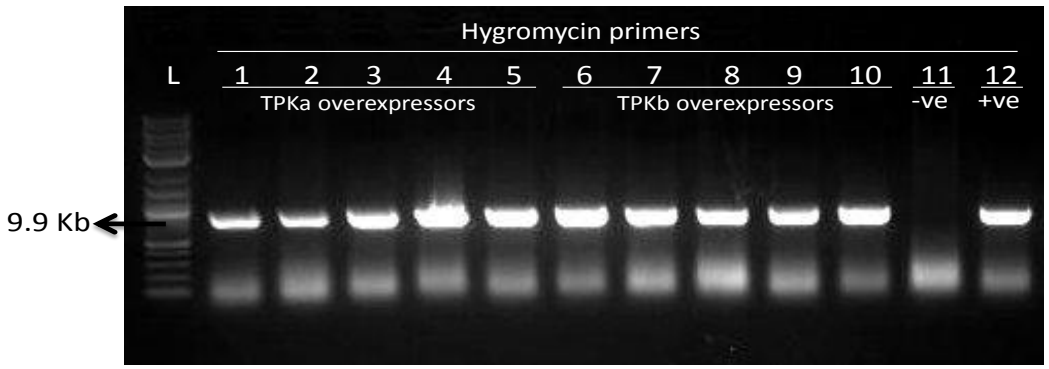


Figure 2-8: PCR analysis of *OsTPKa* and *OsTPKb* overexpressors for selection of homozygous lines and confirmation of germination tests using hygromycin gene specific primers. Lane L contains the ladder. Lanes 1 – 5 contain DNA of different plants from *OsTPKa* overexpressing lines and Lanes 6 – 10 contain DNA from *OsTPKb* overexpressing lines that showed hygromycin sensitivity during the germination tests. –ve and +ve are the controls. Arrow indicates band size of the product (990 bp).

2.3.6 Expression levels of *OsTPKa* and *OsTPKb* overexpressing lines compared to wildtype plants using reverse transcriptase PCR

Semiquantitative RT-PCR analyses were carried out to investigate the transcript levels in the homozygous *OsTPKa* and *OsTPKb* overexpressing lines and control plants. cDNA samples from control plants and homozygous overexpressing *OsTPKa* and *OsTPKb* lines were obtained as described in the methods section, and were semi-quantitatively assayed using RT-PCR. The amplicon of the rice histone gene was used as a control to normalise the data. RT-PCR analyses showed increased expression in one of the two homozygous lines from each *OsTPKa* and *OsTPKb* compared to wild type plants as shown in figure 2-9. However, the second line from both *OsTPKa* (*OsTPKa* 212, Figure 2-9) and *OsTPKb* (*OsTPKb* 155, Figure 2-9) overexpresssors showed very low or no increase in transcript level.

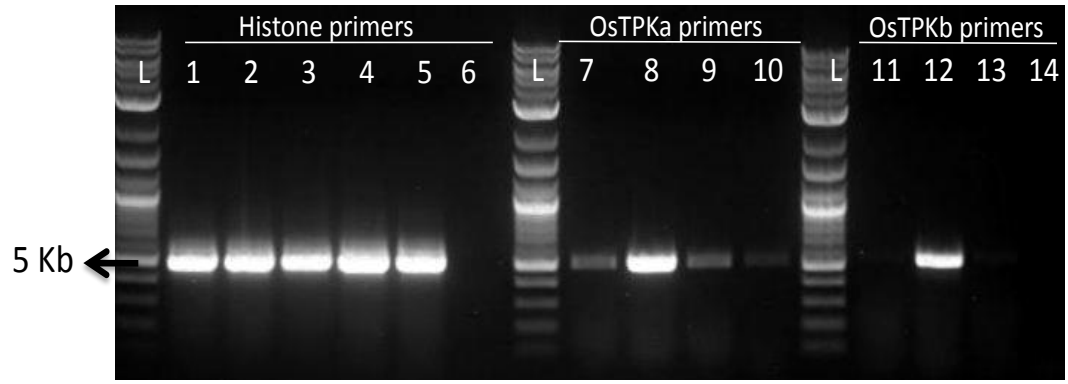


Figure 2-9: Expression analysis of *OsTPKa* and *OsTPKb* overexpressors by RT-PCR. The *OsTPKa* 133 (lane 8) and *OsTPKb* 13 (lane 12) showed increased expression compared to wild type control line *OsTPKb 55* (Lane 1, 7, 11).

2.3.7 Growth analyses of the *OsTPKa* and *OsTPKb* overexressors compared with wild type at different stress conditions

To investigate the effect of *OsTPKa* and *OsTPKb* overexpression on the growth of the plants, relative growth rates of *OsTPKa and OsTPKb* overexpressors and wild type control plants were recorded at control, 0 K^+, 50 mM K^+ and 50 mM NaCl conditions as described in the methods section. Wild type control plants showed increased relative growth rates (RGR) compared with rice *TPKa* (TPKa1) and *TPKb* (TPKb1) overexpresssors under control conditions (Figure 2-10), while the non-overexpressing lines for both *OsTPKa* (TPKa2) and *OsTPKb* (TPKb2) behaved like wild type control line (*OsTPKb 55*).

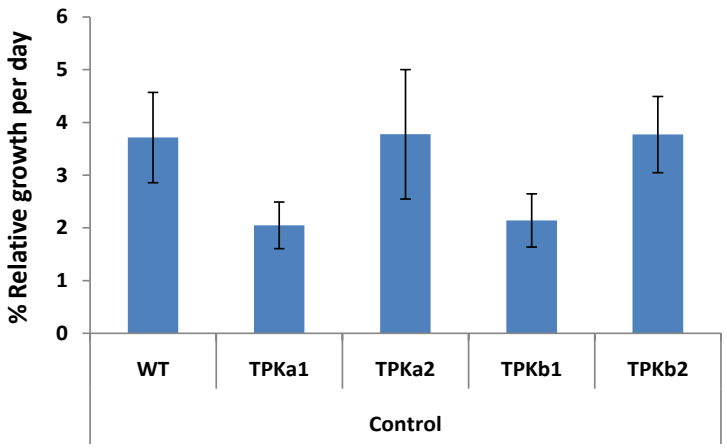


Figure 2-10: Growth of rice *TPK* overexpressors (TPKa1, TPKa2 and TPKb1, TPKb2) and wild type control plants (WT) at control condition. Twentyone-day-old rice seedlings were exposed to control condition and RGR was measured after 7 days of treatment. Values are the mean ± S.D. (n = 3).

However, when the data were normalized at control condition, both *OsTPKa* and *OsTPKb* overexpressing lines showed significantly improved relative growth compared to wild type and non-overexpressing lines at 0 K^+ and 50 mM KCl conditions (Figure 2-11). In contrast, at 50 mM NaCl the *OsTPKb* (OsTPKb1) showed more relative growth compared to control and non overexpressing lines (TPKa2 and TPKb2), however, the *OsTPKa* overexpressing line struggled along with wild type control plants to grow at 50 mM NaCl concentration. The non overexpressing *OsTPKa* line showed improved growth compared to wild type control plants at 50 mM NaCl. These results suggest that *OsTPKa* and *OsTPKb* activity mainly affects K^+ homeostasis while *OsTPKb* also influences salt tolerance at high NaCl (50 mM NaCl) conditions (Figure 2-11).

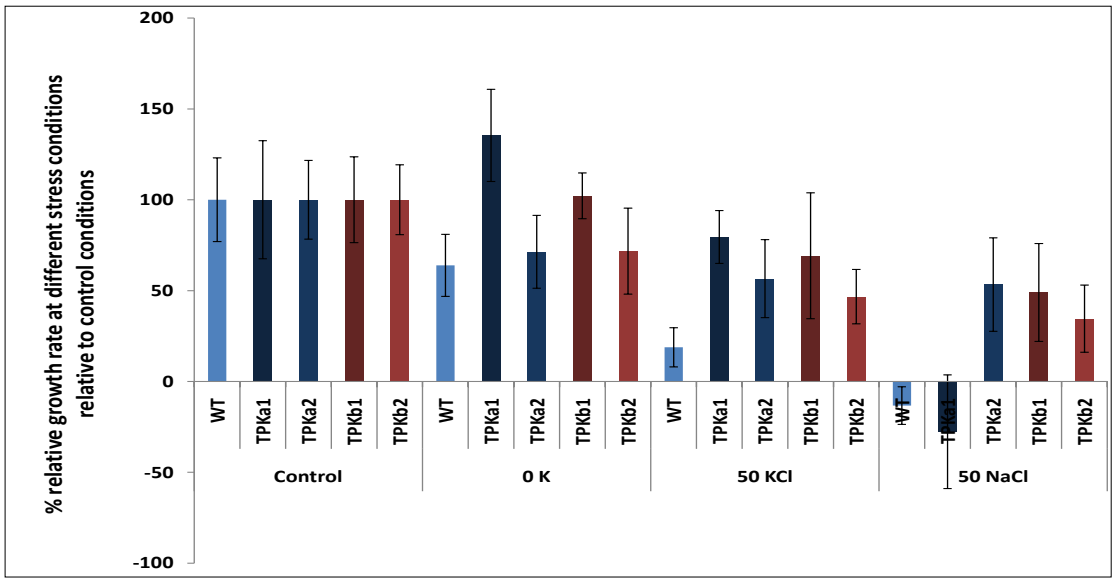


Figure 2-11: Growth of rice lines in different growth conditions. Rice *TPK* overexpressors (TPKa1, a2 and TPKb1, b2) and wild type control plants (WT) after exposure to different Na^+ and K^+ treatment. Twentyone-day-old rice seedlings were exposed to control conditions, 0 K^+, 50 mM KCl and 50 mM NaCl in hydroponic medium. RGR was measured after 7 day of treatment. Values are the mean ± S.D. (n = 3).

2.3.8 Na^+ and K^+ content of rice *TPK* overexpressors and control plants

Na^+ and K^+ content of *OsTPKa* and *OsTPKb* overexpressors and control plants were recorded using a flame photometer as described in the methods section. *OsTPKa* overexpressors showed increased Na^+ content in the roots and shoots compared to wild type control plants under control conditions. However, *OsTPKb* overexpressors showed no significant differences in the roots but significantly lower Na^+ content was recorded for *OsTPKb* overexpressors in the shoot under control condition. At 0 K^+ and 50 mM KCl

conditions, no significant differences were observed for Na^+ content in the roots and in 50mMKCl, both *OsTPKa* and *OsTPKb* overexpressors showed lower Na^+ content in the shoots compared to wild type control plants. At high salt stress (50 mM NaCl condition) transgenic plants overexpressing *OsTPKa* and *OsTPKb* showed higher Na^+ content in roots but lower Na^+ contents in shoots compared to wild type control plants.

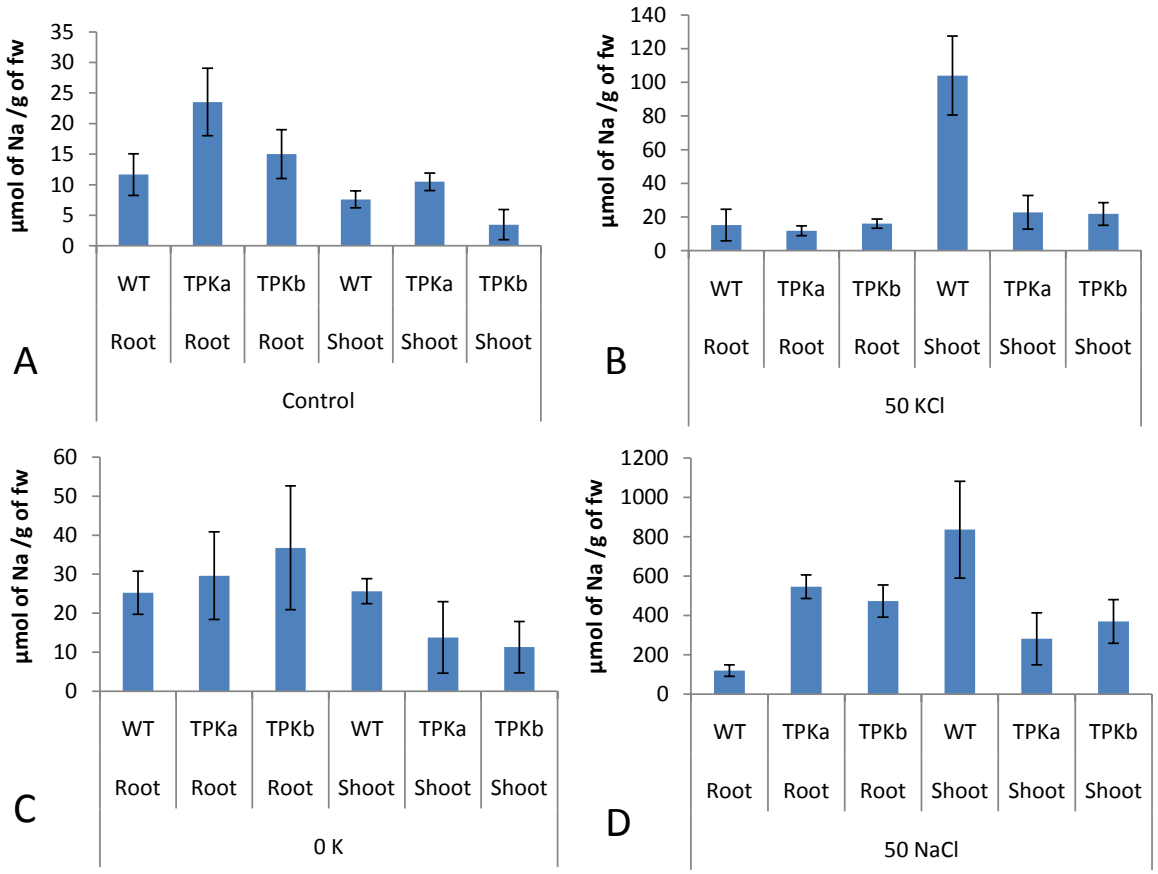


Figure 2-12:Long-term accumulation of Na^+. Fifteen-day-old plants were exposed to different Na^+ and K^+ stress treatments. At the end of 7 days of treatment, Na^+ content in roots and shoots of rice plants overexpressing OsTPKa and OsTPKb, and control plants was determined at control (A), 50 mM K (B), 50 mM NaCl (C), 0 K^+ and (D) 50 mM NaCl. Values are the mean ± S.D. (n = 3).

OsTPKa and *OsTPKb* overexpressors showed higher K^+ content in roots but no significant differences were observed for K^+ content in the shoots in control and 0 K^+ conditions. At 50 mM KCl, transgenic plants overexpressing *OsTPKa* and *OsTPKb* showed no significant differences in the root K^+ content while in the shoots wild type control plants showed higher K^+ content than the rice *TPK* overexpressors. These results further strengthen the idea that rice TPKs play an important role in K^+ nutrition and salt stress, possibly via

maintaining a high K^+/Na^+ ratio at root and shoot level, which is crucial for plant growth and development.

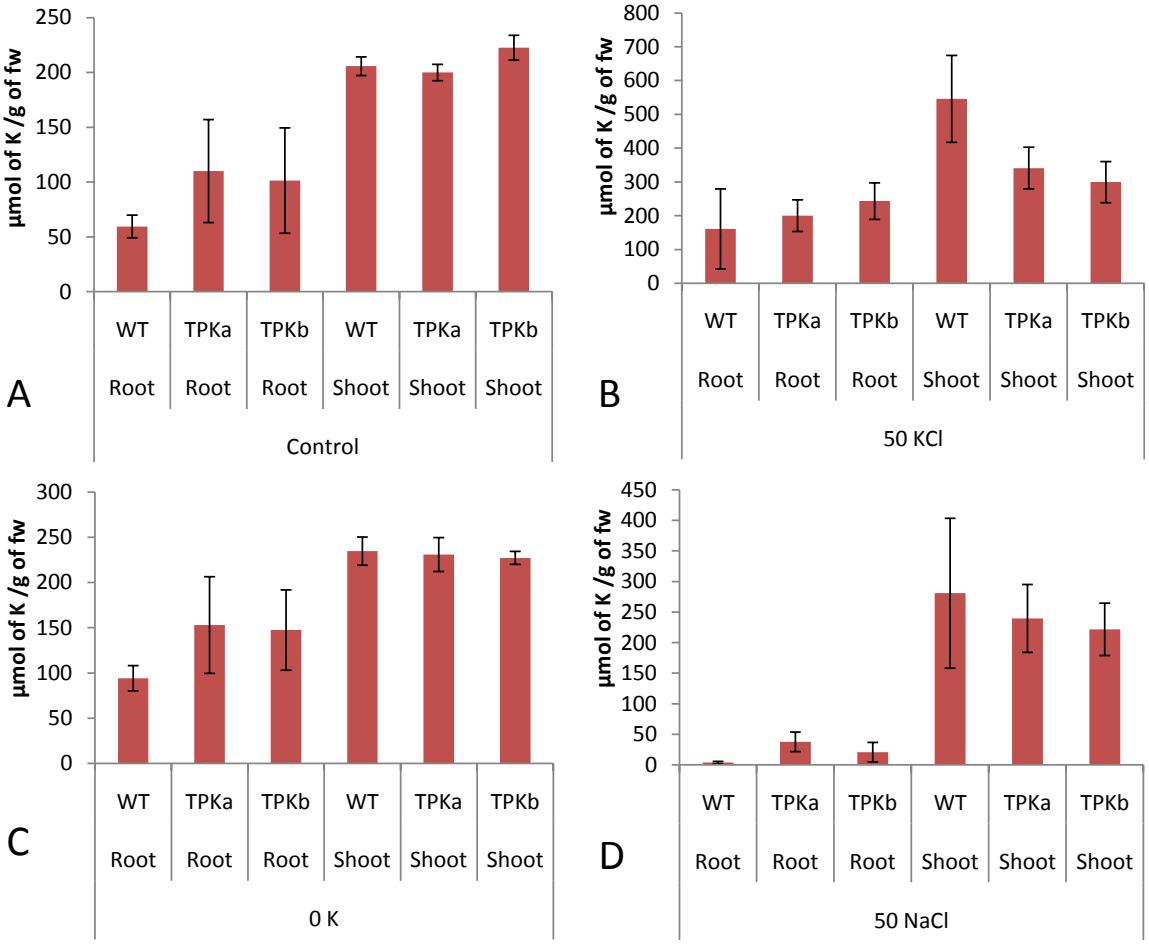


Figure 2-13:Long-term accumulation of Na^+. Fifteen-day-old plants were exposed to different Na^+ and K^+ stress treatments. At the end of 7 days of treatment, Na^+ content in roots and shoots of rice plants overexpressing *OsTPKa* and *OsTPKb*, and control plants and control plants was determined at control (A), 50 mM K (B), 50 mM NaCl (C), 0 K^+ and (D) 50 mM NaCl. Values are the mean ± S.D. (n = 3).

2.3.9 Xylem sap analysis of rice *TPK* overexpressors and control plants

To study the role of *OsTPKa* and *OsTPKb* in Na^+ and K^+ distribution between root and shoot, Na^+ and K^+ content was measured in the xylem sap using a flame photometer as described in the material and methods section. For this, xylem sap was collected from the cut shoot base of TPK overexpressors and control plants, exposed to control, 50 mM K^+ and 50 mM NaCl conditions. *TPK* overexpresssors showed lower Na^+ content at control and 50 mM NaCl conditions but at 50 mM KCl the overexpresssors showed increased Na content compared to wild type control plants. Where K^+ is concerned rice TPK

overexpresssors showed lower K^+ content at control, 50 mM KCl and 50 mM NaCl conditions compared to wild type control plants (Figure 2-13). These results further strengthen the idea of *TPKb* involvement in maintaining higher K^+ content in root tissues.

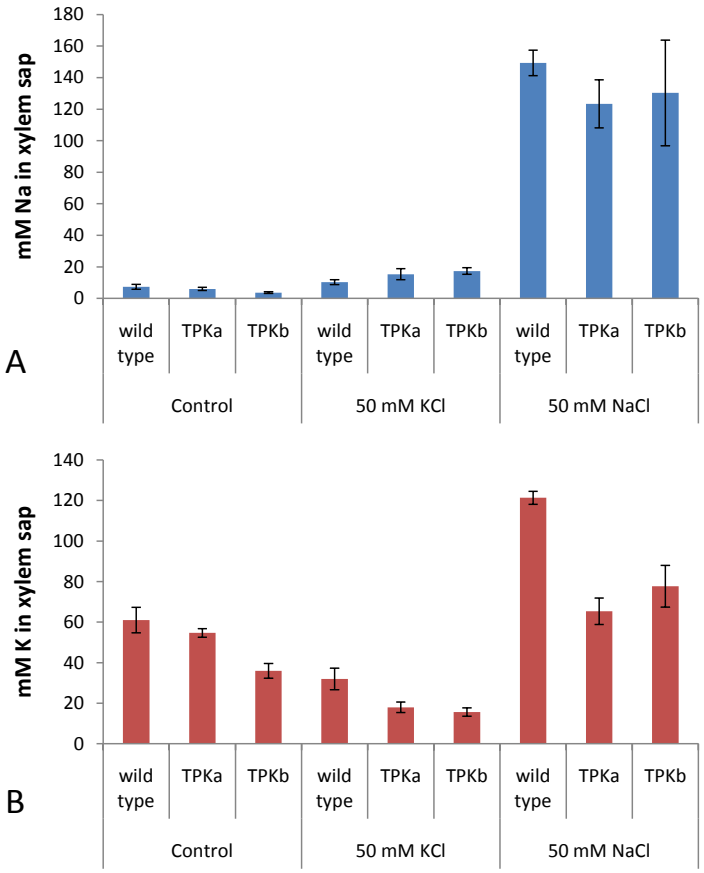


Figure 2-14:Na^+ content (A) and K^+ content (B) in xylem sap of rice *TPKa* and *TPKb* overexpresssors (TPKa and TPKb) and wild type control (wild type) plants. Fifteen-day-old plants were exposed to different Na^+ and K^+ stress treatment by adding NaCl and KCl to the hydroponic control medium to final concentrations of 50 mM KCl and 50 mM NaCl. At the end of 7 days treatment, xylem sap was collected and Na^+ and K^+ content was measured. Values are the mean ± S.D. (n = 3).

2.4 Discussion

Vacuoles are multifunctional organelles playing fundamental roles in important plant developmental processes. They act as reservoirs for various ions and metabolites and are involved in detoxification and cellular homeostasis. They are also crucial for cellular responses to environmental and biotic stress factors. Minerals such as K^+ are stored in the vacuoles and play important roles in maintaining turgor and act as buffer for

cytosolic homeostasis. Several K^+ transporters have been shown to transport K^+ in plants and across vacuoles including the tandem pore K^+ (TPK) channels.

TPKs are two-pore K^+ channels localized to the tonoplast or plasma membrane. In *Arabidopsis*, *AtTPK1* is located at the tolonoplast, was shown to be involved in stomatal function and K^+ nutrition (Gobert et al., 2007) and *AtTPK4*, localized at the plasma membrane, participated in the transport of K^+ in the pollen tube. Rice also has multiple TPK genes but the physiological role of these genes is not known. To obtain insights about the specific roles of *OsTPKa* and *OsTPKb* in rice, a gain-of-function approach was used and potential phenotypic changes resulting from increases in gene expression were recorded in parameters such as relative growth rate, Na^+ and K^+ tissue content, and xylem sap composition.

The growth analyses showed reduced relative growth rates (RGR) for rice *TPKa* and *TPKb* overexpressors compared to wildtype in control conditions, suggesting a non-specific growth effect in the transgenic lines (Figure 2-10). This might be due to the production of stress related proteins under normal conditions by the transgenic lines, resulting in the dwarf phenotypes of overexpresssors at control condition as earlier proposed by Kurusu et al., (2004). However, when the data were normalized at control conditions to study the particular effect of transgenes, the transgenic lines overexpressing *OsTPKa* and *OsTPKb* showed increased growth rates compared to wild type control plants under low K^+ (0 K^+) and high K^+ (50 mM KCl) conditions (Figure 2-11). Similar results were reported for *Arabidopsis*, where the overexpression of *AtTPK1* led to improved growth at both low and high K^+ conditions (Gobert et al., 2007). These results support the earlier findings that that TPKs might be involved in tissue K^+ redistribution to maintain turgor and for cell expansion and K^+ homeostasis at low and high K^+ levels (Walker et al., 1996; Gobert et al., 2007) .

At high NaCl conditions, transgenic lines overexpressing *OsTPKb* showed higher relative growth rates compared to wild type control plants (Figure 2-11) suggesting a role of *TPKb* in rice salt tolerance. However, the *OsTPKa* overexpressing line (TPKa1) showed no significant difference in terms of growth compared to wild type control plants when exposed to salt. These results are supported by expression data available in public databases; *OsTPKb* transcript level is up-regulated under salt stress, while *OsTPKa* transcript level is not affected by salinity stress (Figure 2-15, https://www.genevestigator.ethz.ch/at/index.php).

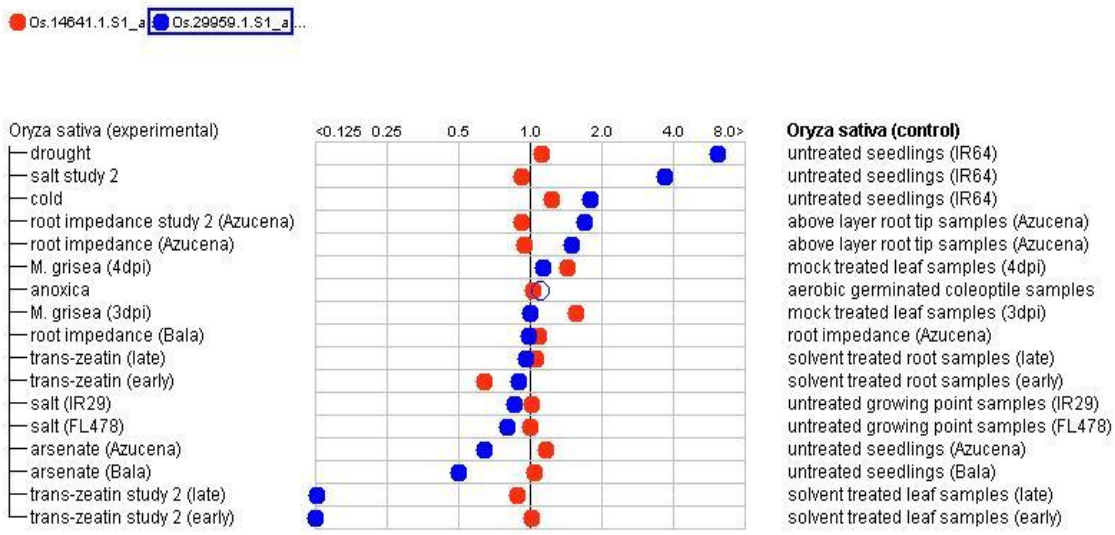


Figure 2-15: Expression of *OsTPKa* (red circles) and *OsTPKb* (blue circles) genes during different stress conditions are generated using the meta-analysis tool at Genevestigator http://www.genevestigator.ethz.ch.

Ion content analyses of the overexpresssors and wild type control plants strengthens the idea of the *OsTPKa* and *OsTPKb* involvement in maintaining K^+/Na^+ homeostasis under various stresss conditions. At control conditions, the *OsTPKa* and *OsTPKb* overexpresssors showed relatively lower K^+/Na^+ ratio and higher Na^+ content in roots which might be the reason for a poor growth rate of the *TPKa* and *TPKb* overxpressors under control conditions. However, at both K^+ depleted (0 K^+) and high K^+ (50 mM KCl) conditions, *OsTPKa* and *OsTPKb* overexpresssors showed increased growth rates compared to wild type control plants(Fig.2-11), suggesting that both OsTPKa and OsTPKb may have a role in K^+ nutrition as rice plants overexpressing *OsTPKa* and *OsTPKb* showed lower Na^+ content in the shoot and maintained higher K^+/Na^+ ratios (Figure 2-12), which is reported crucial for plant growth and development (Yeo, 1998). In high salinity conditions, the overexpresssors show increased Na^+ content in roots (Fig. 2-12D)which is ameliorated by higher levels of K^+ in roots(Fig.2-13D). However, higher Na^+ and lower K^+ content was observed for wild type control plants compared to both the *OsTPKa* and *OsTPKb* overexpresssors (Figure 2-12, 2-13), hence at high salinity rice plants overexpressing *TPKa* and *TPKb* showed higher K^+/Na^+ ratios which may benefit growth. Higher Na^+ content at the root level seems to have little effect on plant growth as was previously reported by (Munns and Tester 2008) while higher K^+/Na^+ ratios seem to play an important role in maintaining cellular metabolism (Zhu 2003).

When compared with controls, the Xylem sap analyses of the transgenic lines showed no significant differences in the Na^+ content, however, for K^+ content, the overexpresssors showed lower K^+ content in xylem sap. This suggests that the *TPKa* and *TPKb* overexpresssors tend to keep more K^+ in the root tissues, possibly to balance the excessive Na^+ content in the roots (Figure 2-14). These results further strengthen the idea that the transgenic lines overexpressing *OsTPKa* and *OsTPKb* maintain higher K^+/Na^+ ratios in roots and shoots which may improve growth under various K^+ and Na^+ stress conditions.

OsTPKa and *OsTPKb* share a large degree of identity at both nucleotide and amino acid level. Moreover, they also have similar electrophysiological properties, such as K^+ selectivity, inward rectification and voltage independent gating in *Arabidopsis* and tobacco TPKs (Gobert et al., 2007; Hamamoto et al., 2008). However, the subcellular localization of *OsTPKa* is predominantly in the tonoplast of the large lytic vacuole and *OsTPKb* in protein storage vacuoles (Isayenkov et al, Unpublished). Most of the important functions like turgor provision, the storage of minerals and nutrients, and cellular signalling are associated with the large lytic vacuole, while protein storage vacuoles are reservoirs for proteins and minerals such as phytate, important for plant growth and development (Isayenkov et al., 2010). *OsTPKa* and *OsTPKb* overexpression might be responsible for the K^+ redistribution in the large lytic vacuole and protein storage vacuole to maintain adequate amount of K^+ during low and high K^+ conditions, crucial for proper functioning of the cell as previously reported by (Gobert et al., 2007).

The results indicated the ability of the transgenic plants overexpressing *OsTPKa* and *OsTPKb* to maintain higher K^+/Na^+ ratio and showed improved growth at different K^+ stress conditions. Despite high Na^+ content in the roots of the transgenic overexpressing plants grown at 0 K^+ and 50 mM KCl, the K^+ content of the transgenic overexpressing line was higher than the K^+ content of the wildtype control plants grown at the above mentioned stress conditions. The role of *OsTPKb* in K^+ homoeostasis in protein storage vacuoles seems very crucial for salt tolerance of the plants. *TPKb* might also be involved in regulation of enzymes responsible for the breakdown of complex minerals such as K-phytate that can facilitate osmotic adjustment by release of K^+ from the vacuole (Isayenkove et al. Unpublished). Moreover influx of K^+ into the vacuole via *TPKb* can encourage the water influx and increase in vacuolar volume during cell growth. This increase in vacuolar volume would facilitate turgor maintenance and was proposed to be an important mechanism in plant salt tolerance (Mimura et al., 2003).

In summary, the results demonstrate that the enhanced K^+/Na^+ ratio in the transgenic lines overexpressing *OsTPKa* and *OsTPKb* enabled them to ameliorate the toxic effects of Na^+ in the roots. This may be important for plant growth and proper functioning of the cell at different stress conditions.

Chapter 3

3 Characterisation of rice OsAKT1, a K^+ inward rectifying channel; its role in K^+ nutrition and salt tolerance

3.1 Introduction

Potassium is the most abundant cation in plant cells and is a vital nutrient for plant growth and development as described in Chapter 1. Plants have the ability to accumulate a considerable amount of tissue K^+ reaching a level of up to 10% of plant dry weight (Leigh and Wyn-Jones, 1984). Earlier studies by Epstein et al., (1963) described the uptake of K^+ from external the environment into the plant system by two uptake mechanisms i.e. a high affinity transport system (HATS) and a low affinity transport system (LATS). The HATS is an active process operating at micromolar level and facilitates the entry of K^+ by coupling the transport of K^+ to the movement of H^+ and ATP hydrolysis, while LATS predominantly functions as a passive process at high external K^+ concentrations and is mediated by various K^+ channels (Maathuis and Sanders, 1994). During recent years many genes encoding K^+ transporters have been identified from higher plants, which are categorized into four families of proteins: Shaker channels TPK channels, KUP/HAK/KT carriers, and HKT carriers type transporters. These transporters have been extensively studied, providing valuable information about various important functions including plant K^+ uptake and transport (Very and Sentenac, 2003).

In the early 1990s, the sequences of the first mineral ion transport systems was identified in *plants* i.e. *KAT1* (Anderson et al., 1992) and *AKT1* (Sentenac et al., 1992) were identified by functional complementation in yeast deficient in K^+ uptake. Both *KAT1* and *AKT1* are activated by hyperpolarisation of the membrane and conduct inward currents (Schachtman et al., 1992; Bei and Luan, 1998). All *AKT* and *KAT* type genes encode proteins with six transmembrane domains, a voltage sensor at the 4^{th} transmembrane domain, a pore region between the 5^{th} and 6^{th} transmembrane domain and a putative cyclic nucleotide binding domain located near the C-terimnus as shown in figure 3-1. Although these channels share similar structure, their electrophysiological features and the regulation of their activities are variable (Zimmermann and Sentenac, 1999).

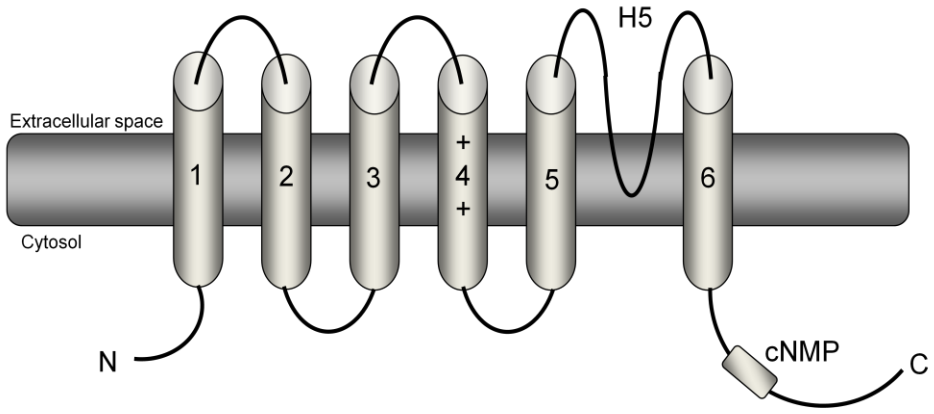


Figure 3-1: Structural model of the AKT1 protein. 1 to 6, transmembrane segments; H5, pore region; cNMP, putative cyclic nucleotide binding site.

In *Arabidopsis* and *Brassica napus*, the *AKT1* promoter activity was shown to be independent of K^+ nutrition and *AKT1* expression was localized to root cells including epidermis, cortex and endodermis that are in close contact with soil solution (Lagarde et al., 1996). Some promoter activity was also detected in specialized hydathode cells that are involved in guttation (Schachtman, 2000). Other members of the AKT gene family in *Arabidopsis*, i.e *AKT2* and *AKT3* are expressed in leaves and leaf phloem respectively (Cao et al., 1995; Marten et al., 1999), suggesting a role of the latter in phloem transport (Su et al., 2001).

Inward rectifying K^+ Channels were shown to be involved in K^+ uptake into many types of plant cells (Maathuis and Sanders, 1995). The physiological role of *AKT1* was first demonstrated using a loss of function mutation with a T-DNA insertion in the *AKT1* gene (Hirsch et al., 1998). The absence of an inward rectifying K^+ channel in this mutant (*akt1*) resulted in poor growth on media with low K^+ concentrations but only in the presence of NH_4^+ (Hirsch et al., 1998). Li et al., (2006) reported an upregulation of *AKT1* at low external K^+ concentrations via a calcium dependent phosphorylation event, further strengthening the role of the AKT1 channel in high affinity K^+ transport. In wheat also, K^+ starvation was shown to upregulate the mRNA level of the AKT orthologue, *TaAKT1* in roots (Buschmann et al., 2000). These studies on the expression of K^+ channels and knockout mutants emphasize the importance of K^+ uptake channels in plant K^+ nutrition.

Environmental stresses such as salinity stress are known to adversely affect K^+ uptake and its transport in the plant. Increased supply of K^+ under stress conditions is one of the

strategies to overcome these stresses (Cakmak, 2005). Beside this, both high and low affinity transport systems have different sensitivities to environmental stresses, such as Na^+, which is an important factor in plant salt tolerance. So far, all potassium channels show high specificity for K^+ over other alkali cations, suggesting K^+ channels are not good candidates for significant inadvertent sodium intrusion even at high external Na^+ to K^+ ratios (Maathuis et al., 1997; Amtmann and Sanders, 1999). However, a role for *AKT1* in Na^+ uptake has also been proposed with an increase in external Na^+ concentration (Amtmann and Sanders, 1999; Blumwald, 2000). For example, Wang et al., (2007) proposed the involvement of *AKT1* type channels in mediating low affinity Na^+ uptake in *S. maritima* under high external Na^+ concentration. In wheat, the mRNA levels of *TaAKT1* were upregulated in roots at low K^+ conditions and K^+ starvatation in the media also led to the enhancement of instantaneous Na^+ currents, suggesting a role of *TaAKT1* in Na^+ uptake during K^+ depleted conditions (Buschmann et al., 2000). In addition to this, animal shaker type K^+ channels, which show high homology to plant AKT1 type channels, have been shown to be permeable to Na^+ at positive potentials (Starkus et al., 2000; Wang et al., 2000).

In rice, OsAKT1, a K^+ channel homologous to *Arabidopsis* AKT1 shares 61% amino acid similarity with AtAKT1 and has been shown to be a salt sensitive K^+ channel, expressed in roots (Fuchs et al., 2005). The expression of *OsAKT1* is regulated differently in salt sensitive and salt tolerant cultivars of rice (Golldack et al., 2003). *OsAKT1* has also been shown to enhance salt tolerance in yeast (Perry et al., 2007). However, the function of AKT1-type channels in plant Na^+ uptake is uncertain. Thus phenotypic analyses of rice lines overexpressing *OsAKT1* and loss of function mutants might give an indication about the permeability properties and the physiological role of *OsAKT1* in rice. Based on the hypothesis that rice *OsAKT1* is involved in K^+ uptake and has a role in rice salt tolerance, the growth and ion contents of *OsAKT1* overexpresssors and T-DNA mutants under different K^+ and Na^+ stress were investigated. This Chapter pertains to the phenotypic and physiological characterisation of the AKT1 channel in rice.

3.2 Materials and Methods

3.2.1 Chemicals, enzymes and oligonucleotides

For general suppliers of chemicals and enzymes see Chapter 2, Section 2.1.1 and 2.1.2. The sequences for the oligonucleotides are given in the respective sections.

3.2.2 Plant material and growth conditions

To study the role of *OsAKT1* in rice, mature seeds of rice plants (*Oryza sativa* L. Japonica cv Nipponbare), obtained from the International Rice Research Institute (IRRI; Laos Banos, Philippines) were used to generate calli for *Agrobacterium* mediated transformation. The rice Tos17 insertion line (NC6423) in the 'Nipponbare' background was provided by the Rice Genome Resource Center of the National Institute of Agrobiological Sciences (RGRC-NIAS), Japan.

3.2.3 Binary vector construction and *Agrobacterium* mediated rice transformation

A full length *OsAKT1* open reading frame (ORF) was amplified with Phusion Hot Start DNA polymerase (New England Biolab, UK) using the cDNA clone of *OsAKT1* (AK120308) as template and primers corresponding to the 5' and 3' ends of OsAKT1 with added Hind3 and EcoR1 restriction sites. The primers used were: 5′CGGGATCCGGCATGGGGCTCGATTT-3′ and 5′-GAACGAGATTAATTTACAGA-3′. The *OsAKT1* ORF was then ligated into the corresponding sites of the35S vector driven by the CaMV-35S promoter. The construct was then restricted with EcoRV and put into the binary vector pGreen as described in Chapter 2, Section 2.2.4. The binary vector pGreen and pSoup were introduced into the *Agrobacterium* strain AGL1 (Chapter 2, Section 2.2.9.6) and rice calli were transformed as described in Chapter 2, Section 2.2.10.

3.2.4 Isolation of homozygous *OsAKT1* overexpressing and mutant lines

For the selection of homozygous overexpressing lines, transgenic plantlets obtained from the transgenic calli were grown to seed setting stage. Seeds from the T2 generation were screened on 1/2 MS hygromycin selection plates and lines showing resistance to hygromycin were selected for further testing as described in Chapter 2, Section 2.3.5. PCR analysis was carried out to confirm the seeds germinating on these plates contained a T-DNA insert using hygromycin primers as described in Section 2.2.11.5. For

characterisation of the *Osakt1* rice mutants, seeds of the Tos17/T-DNA insertion lines from the NIAS, Japan, were grown to seed setting stage and subjected to PCR for identification of homozygous mutants (See 3.3.1).

3.2.5 Analysis of gene expression

Total RNA was isolated by the Qiagen Plant RNeasy kit according to the manufacturer's instructions and cDNA was synthesized as described in Chapter 2, Section 2.2.11.3. Semi-quantitative RT-PCR was performed to check the expression of *AKT1* in overexpressors and mutant plants using gene specific primers (Table 3-1) as previously described in Chapter 2, Section 2.2.12. The rice histone gene was used as an internal control. The primers used for semi-quantitative RT-PCR analyses are listed in Table 3-1.

Table 3-1:Primers used to detect gene expression in control and OsAKT1 mutants, overexpressing and wild type lines using semiquantitative RT-PCR

Gene ID	Primer Sequence	Product Size (bp)
αhistone-F	5'-CGAGAAGCGAAGAGGAGATG-3'	565
αhistone-R	5'-TCAACAAGTTGACCACGTCAC -3'	565
AKT-cDNA-F	5'-TCGACAAGCAGGACGGCAA -3'	660
AKT-cDNA-R	5'-CAGTTATTCCTTAGCTAACCGTT-3'	660

3.2.6 Growth analyses on rice *AKT1* mutants, overexpressors and wild type control lines

To study the effect of *AKT1* expression on growth of rice, the *akt1* mutants, overexpresssors and wild type control plants were grown for 2 weeks on terra green in the green house. After two weeks, the seedlings were transferred to hydroponic growth medium. At the three week stage, three plants each from *Osakt1* mutants, *OsAKT1* overexpressors, and control plants were exposed to different Na^+ and K^+ stress conditions i.e. control, 0 K^+, 75 mM KCl, 35 mM NaCl, 75 mM NaCl and drought stress (10% PEG) for one week (see growth media and conditions). Experiments were repeated three times and relative growth rate (RGR) was calculated as described in Chapter 2, Section 2.2.13.

3.2.7 Na^+ and K^+ content analyses on *OsAKT1 mutants,* overexpressors and wild type control lines

Na^+ and K^+ content of roots and shoots was measured using flame photometry. Wild type rice plants along with *AKT1* mutants and overexpresssors were grown for two weeks on terra green in the green house (see growth conditions) and then transferred to hydroponic control medium for one week. At the three week stage, three plants each from wild type, *AKT1* mutants and overexpressors were exposed to different Na^+, K^+ and drought stress conditions i.e. control, 0 K^+, 75 mM KCl and 75 mM NaCl (see growth media and conditions) for one week. The plants were then separated into roots and shoots and roots were washed with 20 mM $CaCl_2$ solution for 10 minutes. Fresh weights of the samples from roots and shoots were noted after blotting with tissue papers. Na^+ and K^+ content of the samples were recorded as described in Chapter 2, Section 2.2.14. The experiments were replicated three times.

3.2.8 Xylem sap analyses on *OsAKT1 mutants,* overexpressors and wild type control lines

For xylem sap analyses, three week old wild type, *AKT1* mutants and overexpresssors were treated with different Na^+ and K^+ regimes (control, 75 mM KCl, 75 mM NaCl) for one week before collecting the xylem sap. Xylem sap was collected for 30 minutes as described in Chapter 2, Section 2.2.15. Aliquots of 0.1 ml xylem sap were collected from 3 plants in parallel and immediately put on ice. Na^+ and K^+ content of the xylem sap was measured as described in Chapter 2, Section 2.2.15. The experiments were replicated three times.

3.2.9 Statistical analysis

All data shown were derived from experiments carried out across a minimum of three biological replications. The experimental data were subjected to unpaired two-tailed t-tests to identify significance at the $P < 0.05$ level.

3.3 Results

3.3.1 Characterisation of rice *AKT1* overexpresssors and mutants

The rice *AKT1* gene was overexpressed in rice using *Agrobacterium* mediated transformation of the calli under control of a 35S promoter. Seeds obtained from the T3 generation of the transgenic overexpressing rice lines were germinated on ½ MS medium agar plates (Murashige and Skoog, 1962) supplemented with 50 mg l^{-1} hygromycin for selection of homozygous overexpressing lines as shown in figure 3-2.

Figure 3-2:Screening of rice seeds overexpressing *OsAKT1* on 1/2 MS hygromycin selection medium.

A total of 7 independent transgenic *OsAKT1* overexpressing lines was analysed. Among these, only one transgenic lines overexpressing *OsAKT1 was* found to be homozygous, the other 6 lines were all heterozygous with regard to hygromycin sensitivity. In addition to hygromycin germination tests, the presence of the *OsAKT1* construct in the selected *Oryza sativa* overexpressing homozygous lines used in this study was confirmed by PCR analysis of genomic DNA extracted from these plants using hygromycin gene specific primers (2.2.11.5) as shown in in figure 3-3.

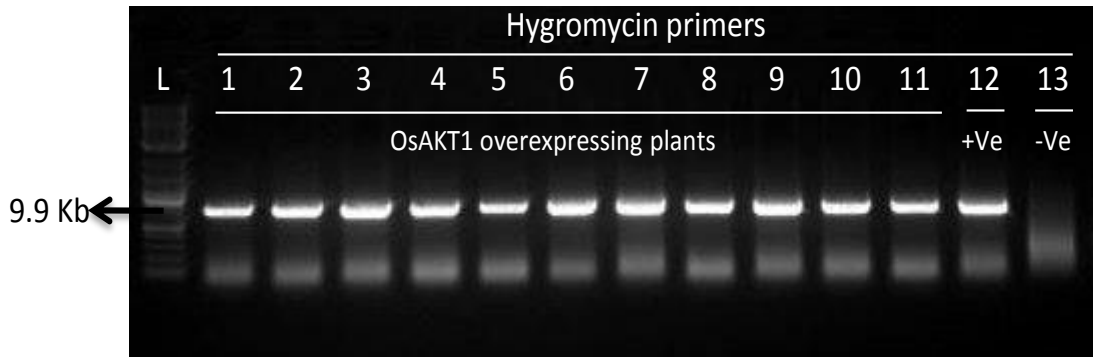


Figure 3-3: PCR analysis of *OsAKT1* overexpressors for selection of homozygous lines and confirmation of germination tests using hygromycin gene specific primers. Lane L contains the ladder. Lanes 1 – 11 contain DNA of different plants from OsAKT1 overexpressing lines. +Ve is the positive control and –Ve is the wild type used as negative control. Arrow indicates band size of the product (9.9 Kb).

For *Osakt1* mutants, seeds of the Tos17/T-DNA insertion line (NC2778), were grown on terra green as described in Chapter 2, Section 2.2.11.1. PCR analyses were carried out to confirm the presence of T-DNA. The absence of *OsAKT1* mRNA transcript was investigated using RT-PCR. The RT-PCR analysis using primer combination spanning the insertion site (Table 3-1), showed no RNA product from the *Osakt1* mutant seedlings. These results clearly demonstrate the absence of the full length *OsAKT1* mRNA in the tos17 homozygous mutant line.

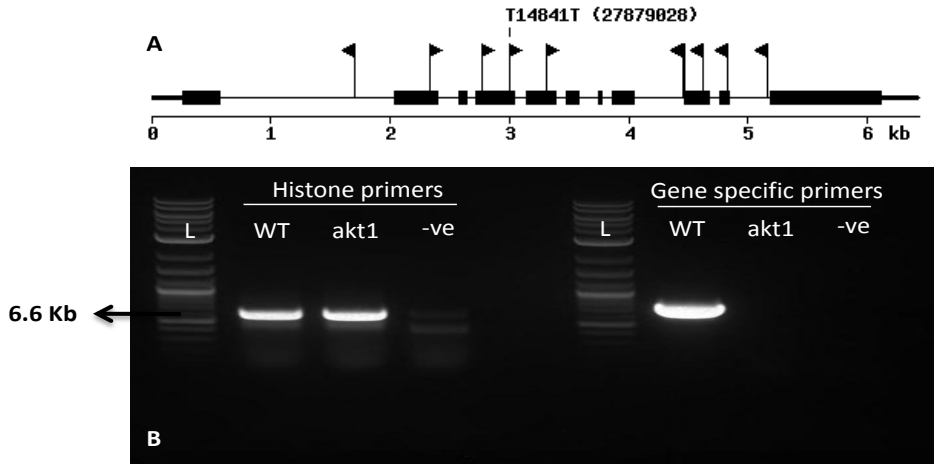


Figure 3-4: Position (A) and expression analysis (B) of *Osakt1* mutants by RT-PCR. The Osakt1 loss of function mutant line (akt1) showed no expression compared to wild type control line. –ve shows the negative control and arrow indicates the band size of the gene product.

3.3.2 Expression levels of *OsAKT1* overexpressing line compared to wildtype plants using reverse transcriptase PCR

Semiquantitative RT-PCR analyses were carried out to investigate the transcript levels in the homozygous *OsAKT1* overexpressing and the *Osakt1* loss of function mutant lines. Samples of cDNA from *Osakt1* mutants and *OsAKT1* overexpressor homozygous lines obtained as described in Chapter 2, Section 2.2.11.4, were semi-quantitatively assayed using RT-PCR. The amplicon of the rice histone gene was used as a control to normalise the data. RT-PCR analyses showed increased expression in the homozygous overexpressing line. The primers used for semi-quantitative RT-PCR analyses are listed in Table 3-1.

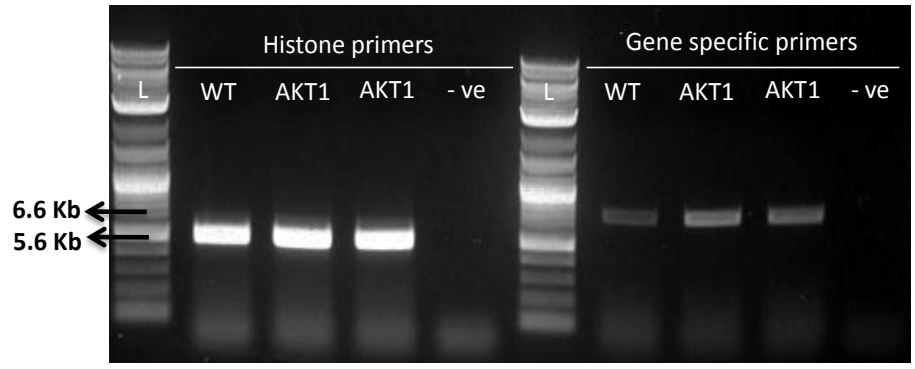


Figure 3-5: Expression analysis of *OsAKT1* overexpressors by RT-PCR. The *OsAKT1* overexpressing line (AKT1) showed increased expression compared to wild type control line (WT). –ve is the negative control and arrows indicates band sizes of gene product.

3.3.3 Growth analyses with *OsAKT1* mutants, overexpressors compared to wild type under different stress conditions

To study the role of OsAKT1 in plant growth and development in different K^+ and Na^+ conditions, relative growth rate of the wild type plants, *Osakt1* mutants and *OsAKT1* overexpressors was recorded at control, 0 K^+, 75 mM K^+ and 75 mM NaCl conditions as described in the Methods section. Under control conditions, *Osakt1* mutants and *OsAKT1* overexpressors showed increased relative growth rates (RGR) compared to wild type plants (Figure 3-6). Due to the apparent differences in growth of the *Osakt1* mutant and *OsAKT1* overexpressors, results from growth experiments are presented in % values, relative to values obtained from corresponding control condition.

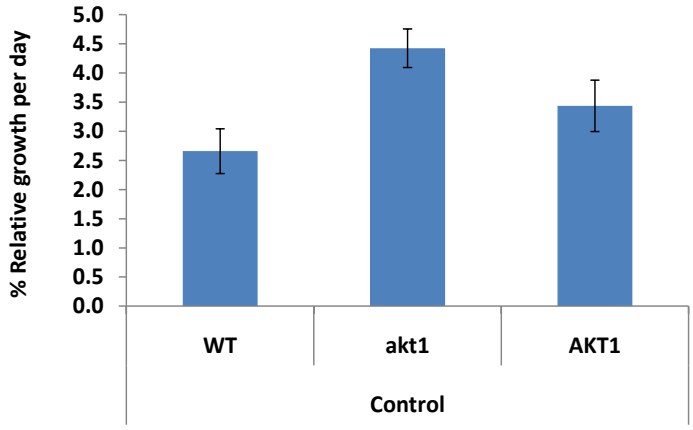


Figure 3-6: Growth of rice AKT1 mutants (akt1), overexpresssors (AKT1) and wild type plants (WT) at control conditions. Twentyone-day-old rice seedlings were exposed to control condition and RGR was measured after 7 day of treatment. Values are the mean ± S.D. (n = 3).

When these data were normalized to that from control conditions, both *Osakt1* mutants and *OsAKT1* overexpressing lines showed significantly reduced relative growth compared to wild type plants under 0 K^+ conditions (Figure 3-7). However, at 75 mM KCl conditions, the loss-of-function mutation in *OsAKT1* gene resulted in reduced growth compared to wild type, while the *OsAKT1* overexpressors responded the same as the wild type (Figure 3-7). At high salt stress (75 mM NaCl) and drought stress (10% PEG) conditions, no significant differences were observed for the growth among the mutants, overexpressors and wild type plants. However, at 35 mM NaCl conditions, *OsAKT1* overexpression had a negative effect on growth compared to wild type plants. These results suggest that the *OsAKT1* gene plays an important role at low and high K^+ conditions, and also can influence salt tolerance of rice at low NaCl concentrations (35 mM NaCl) as shown in (Figure 3-7).

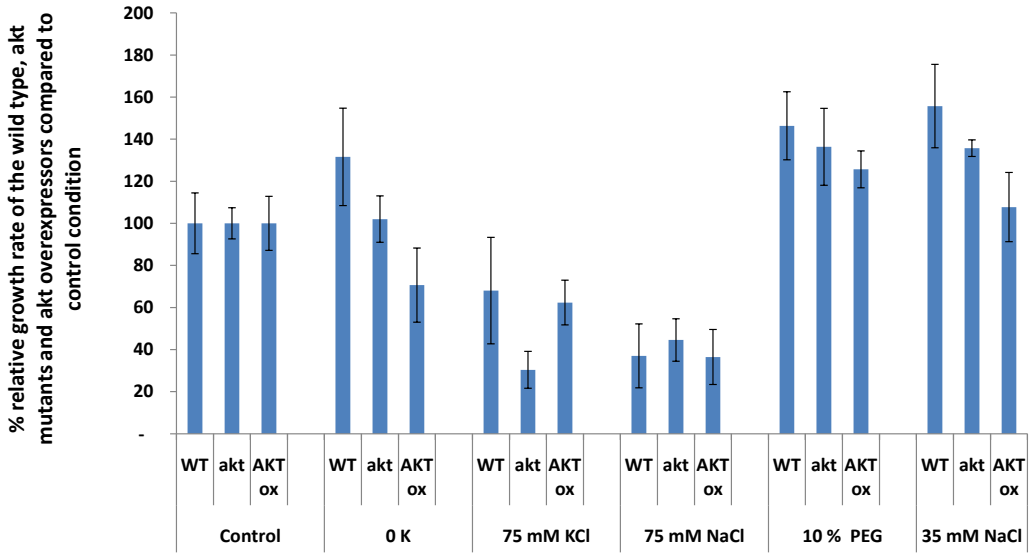


Figure 3-7: Growth of rice lines in different growth conditions. Rice AKT1 mutants, overxpressors and wild type control plants (WT) after exposure to drought and different Na^+ and K^+ treatment. Twentyone-day-old rice seedlings were exposed to control condition, 0 K^+, 75 mM KCl, 75 mM NaCl, 10% PEG and 35 mM NaCl in hydroponic medium. RGR was measured after 7 day of treatment. Values are the mean ± S.D. (n = 3).

3.3.4 Na^+ and K^+ content of rice AKT1 mutants, overexpressors and control plants

Na^+ and K^+ content of the rice AKT1 mutants and overexpressors was recorded using a flame photometer to study the effect of *OsAKT1* on plant nutrition. *OsAKT1* overexpressors showed reduced Na^+ content in the roots compared to wild type and *Osakt1* mutant lines at both control and 0 K^+ conditions (Figure 3-8). However, in shoots, the *OsAKT1* overexpressors showed significantly higher Na^+ content compared to wild type and *Osakt1* mutants at control and 0 K^+ conditions (Figure 3-8). At 75 mM KCl, the *Osakt1* mutants showed lower Na^+ content in roots compared to wild type and the *OsAKT1* overexpressor plants, however, in the shoots, the *Osakt1* mutants showed higher Na^+ content compared to wild type and *OsAKT1* overxpressors (Figure 3-8). Under saline conditions (35 and 75 mM), the *OsAKT1* overexpressors showed increased Na^+ content in the roots while in shoots both AKT1 mutants and overexpressors showed lower Na^+ content compared to wild type plants (Figure 3-8). These results suggest that *OsAKT1* might be involved in Na^+ uptake at high external K^+ (75 mM KCl) and Na^+ (75 mM NaCl) concentrations, but no significant differences were observed for Na^+ uptake at control and low external Na^+ concentration.

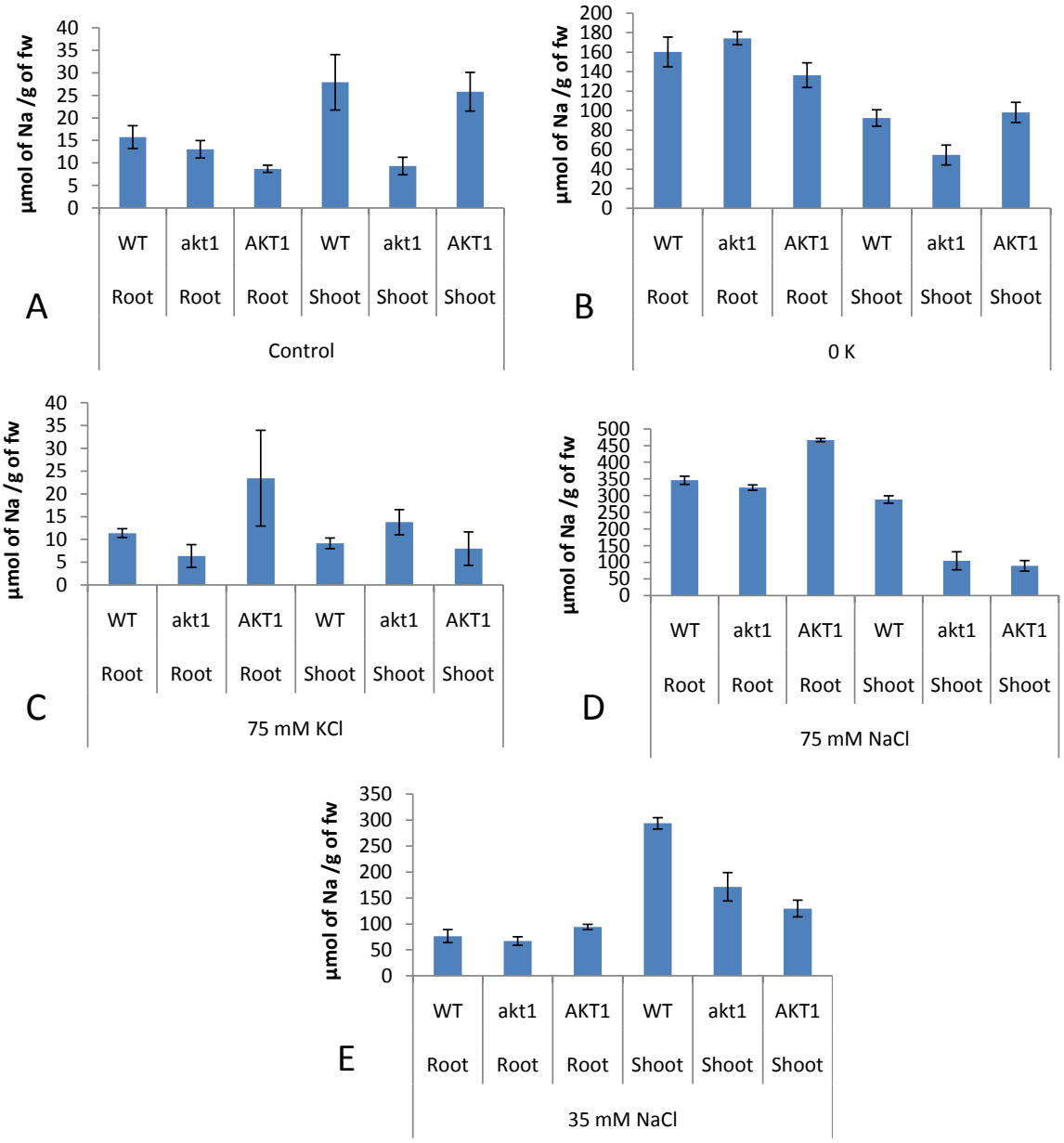


Figure 3-8:Long-term accumulation of Na^+. Fifteen-day-old plants were exposed to different Na^+ and K^+ stress treatments. At the end of 7 days of treatment, Na^+ content in roots and shoots of rice *AKT1* mutants, overexpresssors and control plants was determined at control (A), 0 K (B), 75 mM KCl (C), 75 mM NaCl (D) and 35 mM NaCl (E). Values are the mean ± S.D. (n = 3).

No significant differences were observed for K^+ content in roots of the wild type, *AKT1* mutants and overexpressors at control, 0 K^+ and 75 mM NaCl conditions. However when grown in 75 mM KCl, the *OsAKT1* overexpresssors showed increased K^+ content in roots, suggesting the involvement of rice *AKT1* in K^+ uptake at higher external K concentrations (Figure 3-9). In shoots, both AKT1 mutants and overexpresssors showed significantly lower K^+ content at control, 75 mM KCl and high 75 mM NaCl conditions (Figure 3-9). However at 0 K^+ conditions, no significant differences in shoots K^+ content were observed for wild type, *Osakt1* mutants and *OsAKT1* overexpressors (Figure 3-9). These results suggest that rice *AKT1* might be involved in Na^+ and K^+ uptake at higher external Na^+ and K^+ concentrations respectively.

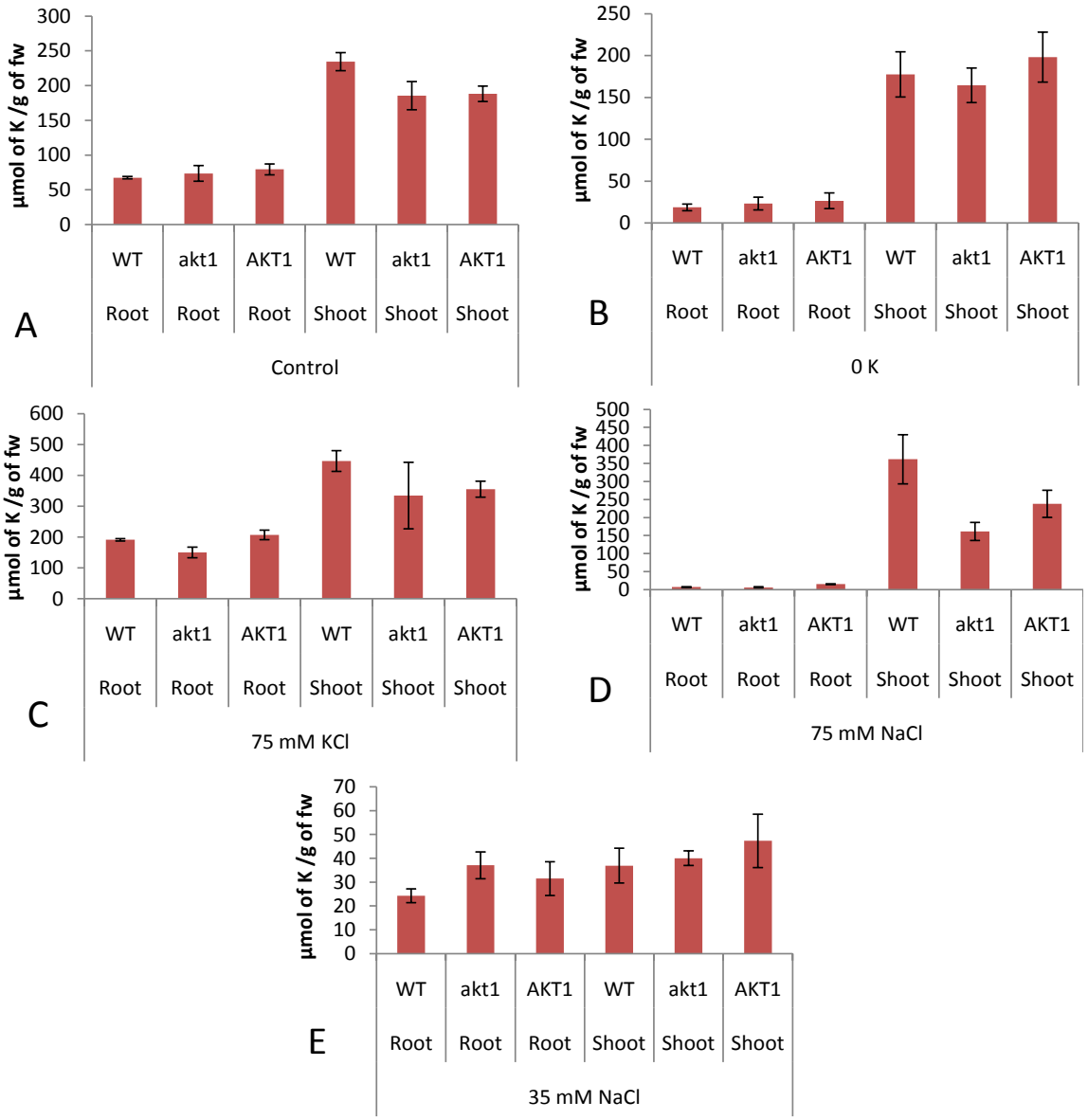


Figure 3-9:Long-term accumulation of K^+. Fifteen-day-old plants were exposed to different Na^+ and K^+ stress treatments. At the end of 7 days of treatment, K^+ content in roots and shoots of rice *AKT1* mutants, overexpresssors and control plants was determined at control (A), 0 K (B), 75 mM KCl (C), 75 mM NaCl (D) and 35 mM NaCl (E). Values are the mean ± S.D. (n = 3).

3.3.5 Xylem sap analysis of rice AKT1 mutants, overexpressors and control plants

To study the role of *AKT1* in Na^+ and K^+ distribution between root and shoots, Na^+ and K^+ content was measured in the xylem sap using a flame photometer as described in the material and methods section. The *Osakt1* mutant xylem sap showed reduced Na^+ content

compared to *OsAKT1* overexpresssors and wild type plants at control and 75 mM KCl condition, but at 75 mM NaCl conditions, the *OsAKT1* overexpresssors showed reduced Na^+ content compared to wild type and *Osakt1* mutant plants. For K^+, the *Osakt1* mutant and *OsAKT1* overexpressors showed lower K^+ in their xylem sap under control conditions. However, higher K^+ content was recorded for both *Osakt1* mutant and *OsAKT1* overexpressors at 75 mM KCl compared to wild type plants. At 75 mM NaCl conditions, the *Osakt1* mutants showed lower K^+ content in xylem sap compared to wild type and *OsAKT1* overexpressors. These results suggest that constitutive expression of AKT1 might enhance Na^+ translocation from root to shoot particularly at low Na^+ conditions.

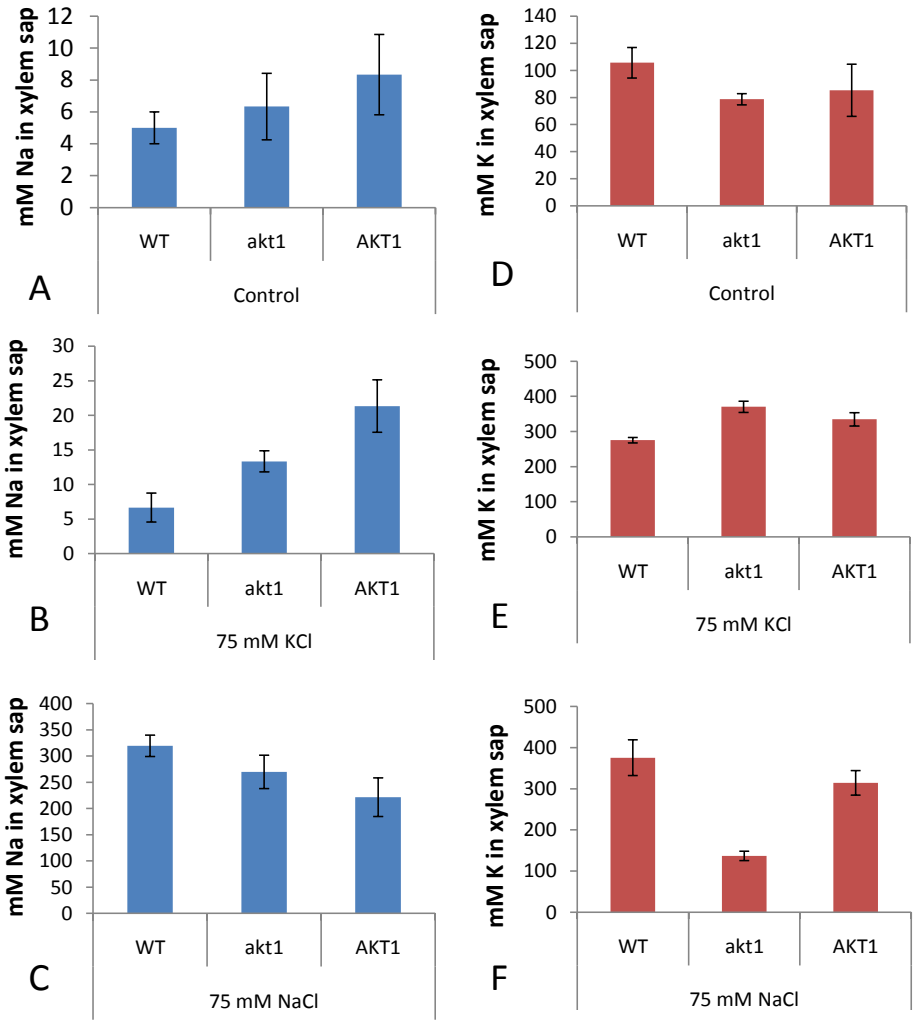


Figure 3-10:Na^+ content (A, B, C) and K^+ content (D, E, F) in xylem sap of rice AKT1 mutants (akt1 mutant), overexpresssors (AKT OX) and wild type control (WT) plants. Fifteen-day-old plants were exposed to different Na^+ and K^+ stress treatment by adding NaCl and KCl to the hydroponic control medium to final concentrations of 75 mM KCl and 75 mM NaCl. At the end of 7 days treatment, xylem sap was collected and Na^+ and K^+ content was measured. Values are the mean ± S.D. (n = 3).

3.4 Discussion

Salinity stress affects plant growth and development mainly by causing osmotic stress and ion toxicity (mainly Na^+) as described in detail in Chapter 1. Plants can achieve ionic balance by regulating uptake and accumulation of minerals like Na^+, Cl^- and K^+ in the system. However, excessive accumulation of Na^+ and Cl^- ions can cause ion toxicity and is the primary reason for growth inhibition and poor performance of plants under salt stress conditions. On the other hand, K^+ being the most abundant cation in a living cell, is crucial to cell metabolism (Marschner, 1995). The effect of salt stress on plant growth and development is to a large extent dependent upon the ability of the plant to control the fluxes of K^+ across cellular membranes and to maintain a high cytoplasmic K^+/Na^+ ratio (Shabala and Cuin, 2008).

Several ion transporter families are known to have some role in plant Na^+ uptake. For example loss of function mutations in AtHKT1 resulted in reduced sodium uptake suggesting AtHKT1 to be a major protein for Na^+ uptake (Rus et al., 2001). However, no specific Na^+ uptake system has been identified in plants (Golldack et al., 2003). Non specific uptake of Na^+ via potassium transport systems such as HKT-type transporters, HAK/KT/KUP-type transporters, inward-rectifying potassium channels, low-affinity cation transporters of the LCT1-type, and voltage-independent channels is suggested (Golldack et al., 2002).

Plant shaker type K^+ channels generally have high K^+:Na^+ selectivity and their function in plant Na^+ uptake is uncertain. Previously it has been reported that high external Na^+ concentrations can reduce K^+ channel expression in rice, *Arabidopsis* and *M.crytallinium* (Su et al., 2001; Golldack et al., 2003; Pilot et al., 2003). In addition, it was also proposed that AKT1 might function as a transporter for Na^+ fluxes (Golldack et al., 2003; Obata et al., 2007; Wang et al., 2007). However, Essah et al., (2003) reported no difference in Na^+ accumulation while comparing *Arabidopsis akt1* mutants with wild type. Similarly, Obata et al., (2007) either found no difference or lower Na^+ accumulation in yeast and rice cells expressing *AKT* compared with non-transformed cells.

The availability of loss-of-function mutants facilitates the analysis of tagged genes to investigate gene functions. For example, in *Arabidopsis*, *Atakt1* and *Atskor* loss-of-function mutations provided useful information about the physiological role of these genes. Gain-of-function is another useful tool that can provide effective information about the role of a specific gene (Kuromori et al., 2009). In this study we used two different approaches:

loss-of-function mutants and gain-of-function transgenic plants to test the hypothesis that the rice inward rectifying K^+ channel AKT1 contributes significantly to the plant Na^+ and K^+ uptake during various K^+ and Na^+ stress conditions.

Growth analyses of wild type control plants, *AKT1* mutants and overexpressors showed a higher relative growth rate for *Osakt1* mutants compared to wild type plants and *AKT1* overexpressors under control and 0 K^+ conditions (Figure 3-6, 3.7). The increase in growth rate of the *Osakt1* mutant is reflected in ion content analyses of the *AKT1* mutants under control and 0 K^+ conditions, which show increased Na^+ content in the roots of *Osakt1* mutants compared to *OsAKT1* overexpressors(Fig.3.8). However in shoots, *Osakt1* mutants showed lower Na^+ content compared to the *OsAKT1* overexpressors and wild type in control and 0 K^+. It is suggested that salt stress affects shoots much more than roots and distribution of Na^+ from root to shoot is considered as an effective strategy for salt tolerance (Munns, 2005). This might explain the improved growth of the *Osakt1* mutants under control and 0 K^+ conditions. These results suggest that rice *AKT1* might not be involved in Na^+ uptake at low external K^+ conditions. Qi et al., (2004) also suggest that entry of Na^+ can proceed in the absence of *AKT1*, making *AKT1* an unfavourable candidate for Na^+ influx. However, these results are in contradiction to the findings of Buschmann et al., (2000), who suggested a role of *TaAKT1* in Na^+ uptake during K^+ depleted conditions. However, the *OsAKT1* overexpressors showed increased Na^+ content in the shoots, suggesting that *OsAKT1* might be involved in Na^+ uptake at the root soil boundary. Maathuis and Sanders, (1999) also proposed a role of *AKT1* in Na^+ uptake. Probably most of the Na^+ content was translocated from root to shoot, leaving lower concentration of Na^+ in the roots of the *OsAKT1* overexpressors. The expression of *OsAKT1* observed in leaveas in meosophyll cells and metaxylem vessels (Golldack et al., 2003) might explain the role of *OsAKT1* in the Na^+ distribution from root to shoot. However, Amtmann and Sanders, (1999) reported no significant Na^+ uptake under conditions of low Na^+ concentrations.

With regard to K^+ the rice *AKT1* mutants and overexpresssors showed no significant differences in K^+ content in roots and shoots under control and 0 K^+ conditions, suggesting *OsAKT1* has no or only a small role in K^+ uptake at low external K^+ concentrations. Previously expression of *AKT1* was reported to be independent of the external K^+ concentration (Lagarde et al., 1996; Su et al., 2001; Pilot et al., 2003). Moreover, cell specificity of *OsAKT1* abundance in WT lines was reported to be independent of K^+ depletion down to 100 μM in the nutrition medium (Golldack et al., 2003). However, (Buschmann et al., 2000) reported increased *AKT1* transcript abundance in K^+ starved

wheat. Similarly, Hirsch et al., (1998) demonstrated growth retardation of *Arabidopsis akt1* mutants in the presence of NH_4^+ at low external K^+ concentrations. These results suggest that the *AKT1* channel has a physiological role at low external K^+ concentrations. Xu et al., (2006) demonstrated, using heterologus expression in oocytes, that K^+ uptake from low K^+ concentrations can occur via *AKT1*. Activation of AKT1 in these conditions happens via phosphorylation by the calcium-dependent proteins CBL-Interacting Protein Kinase23 (CIPK23) and Calcineurin B-Like protein1/9 (CBL1/9).

At high K^+ concentrations (75 mM KCl), the *OsAKT1* overexpressors showed an improved growth rate compared to *Osakt1* mutants (Figure 3-7). This increase in growth rate of the *OsAKT1* overexpressors may be due to a relatively higher K^+ content in the roots and increased K^+/Na^+ ratio in the shoots (Figure 3-8, 3-9). This suggests the involvement of *AKT1* in K^+ uptake at high external K^+ concentration. Moreover the *AKT* overexpresssors also showed increased Na^+ content in the roots suggesting that *AKT1* might also be involved in Na^+ uptake at the root soil boundary.

At drought and high NaCl stress conditions, no significant differences were observed among wild type, *AKT1* mutants and overexpresssors in terms of growth rate, suggesting a limited role of *OsAKT1* on growth of the plants at higher NaCl or drought stress conditions(Fig. 3.7). However, at high NaCl (75 mM NaCl) stress conditions, the *OsAKT1* overexpressors showed higher Na^+ content in the roots compared to wild type and *Osakt1* mutants(Fig. 3.8). The overexpresssors also showed increased K^+ content in the shoots at high NaCl conditions, however this does not improve the growth of the *AKT* overexpresssors at high salt stress conditions. These results suggest that at high external Na^+, *OsAKT1* might be involved in Na^+ uptake at the root soil boundary.

Xylem sap analyses of the rice *AKT1* mutants and overexpresssors showed lower Na^+ content under control and high K^+ (75 mM KCl) conditions, confirming the idea of *OsAKT1* involvement in root to shoot partitioning of Na^+ content at low external Na^+ conditions. However, under high Na^+ (75 mM NaCl) stress conditions, the *OsAKT1* overexpresors showed lower Na^+ content in the xylem sap, suggesting a limited role of *OsAKT1* in root to shoot Na^+ partitioning at higher external Na^+ concentrations.

In summary, the results indicate that *OsAKT1* might be involved in Na^+ uptake at low external K^+ concentrations. This would explain the reduced growth that was measured for *OsAKT1* overexpressors at control conditions. Ion content analyses showed increased Na^+ content in the shoots, hence excessive Na^+ is taken up to the shoot, when *AKT1* was constitutively expressed(Fig. 3.8). The involvement of *OsAKT1* in Na^+ transport was

further strengthened by xylem sap experiments, which showed higher Na^+ content in the *OsAKT1* overexpressors at control and 75 mM KCl conditions, while at 75 mM NaCl conditions the *OsAKT1* overexpressors showed lower Na^+ content in xylem sap. The observation that,compared with WT, *OsAKT1* overexpressors did not show any growth phenotype under high salt stress conditions, suggested that *AKT1* only functions at low external Na^+ concentrations.

Chapter 4

4 Physiological characterisation of barley plants overexpressing *HvHKT2;1*

4.1 Introduction

Large areas of cultivated land are affected by salinity worldwide; hence salinity poses a great threat to global agriculture productivity. This has conferred great significance on the development of salt tolerant crop plants, as the majority of crop plants is glycophytic. However, barley (*Hordeum vulgare L*) is a rare exception, capable of completing its life cycle even at 250 mM NaCl (Munns, 2002). Despite many years of intensive research and a reasonable understanding of ion transport regulation in glycophytes such as *Arabidopsis* and rice, the role of many genes and the basic mechanism by which halophytes maintain favourable ion gradients during salt stress is not clear (Horie et al., 2007).

Barley (*Hordeum vulgare L*) is an important cereal crop belonging to the *Poaceae* family and *Triticeae* tribe. In term of production it is the fourth largest cereal crop after wheat, rice and maize (Bengtsson, 1992), grown and cultivated extensively throughout the world. Barley is one of the most salt-tolerant crops and is recognized as being the most salt-tolerant among cereals (Munns and Tester, 2008). Salinity tolerance in *Triticeae* is genetically variable and generally depends on Na^+ exclusion at high saline conditions. For example, wheat restricts Na^+ transport to leaf tissue through Na^+ exclusion and maintains high K^+/Na^+ selectivity, while in barley compartmentalization of excessive Na^+ into the vacuole and the production of organic solutes minimize the adverse effects of salinity (Garthwaite et al., 2005). Glycophytes and halophytes share similar characteristics with the exception of specialized salt excreting glands found in salt tolerant plants. However, generally halophytes show superior Na^+ compartmentation, which increases water use efficiency and may improve ion selectivity. However, high concentrations of Na^+ can negatively affect the functions of vital enzymes, photosynthetic activity, plant nutrition and metabolism (Murguia et al., 1995; Tsugane et al., 1999; Munns and Tester, 2008).

Regulation of sodium, potassium and chloride is vital for plant growth and development during salt stress (Tester and Davenport, 2003). HKT transporters are proposed to play

important roles in these processes. The first HKT (High affinity K^+ Transporter) was cloned from wheat and thought to function mainly as high affinity K^+ uptake systems (Schachtman and Schroeder, 1994) but subsequently it was found that the HKT gene family is functionally quite diverse (Table 4-1) and actually function primarily as Na^+:K^+ symporters or Na^+ uniporters (Haro et al., 2005). HKTs are active at the plasma membrane, expressed mainly in xylem parenchyma cells (Ren et al., 2005; Sunarpi et al., 2005), have been shown to affect salt tolerance in various plant species (Berthomieu et al., 2003; Ren et al., 2005; Byrt et al., 2007) and also improve growth during K^+ starvation conditions (Horie et al., 2007). On the basis of amino acid sequence similarity and differences in Na^+ and K^+ selectivity, HKTs are divided into two subfamilies: subfamily 1 contains HKTs that transport Na^+ only, while subfamily 2 includes HKTs which can transport both Na^+ and K^+ (Table 4-1).

Table 4-1: HKT transporters from *Arabidopsis*, rice and barley, involved with Na^+ specific and K^+-Na^+ co-transport in plants.

Gene	Expression	Function	Reference
AtHKT1:1	Roots xylem parenchyma cells, phloem	Control of both root Na^+ accumulation and retrieval of Na^+ from the xylem	(Uozumi et al., 2000; Berthomieu et al., 2003; Sunarpi et al., 2005; Davenport et al., 2007)
HvHKT1:2	Roots	Na^+-specific transport	(Wang et al., 1998; Garciadeblas et al., 2003; Huang et al., 2008)
OsHKT1:1	Shoots and roots	low-affinity Na^+ uptake	(Garciadeblas et al., 2003)
OsHKT1:2	Roots	Na^+-specific transport	(Garciadeblas et al., 2003)
OsHKT1:3	Shoots and roots	Na^+-specific transport	(Garciadeblas et al., 2003)
OsHKT1;4	Shoots	Na^+-specific transport	(Garciadeblas et al., 2003)
OsHKT1;5 (SKC1)	Roots parenchyma cells and surrounding xylem vessels	Na^+ unloading from root xylem and functions as a Na^+-selective transporter	(Ren et al., 2005)
TaHKT1:2	Shoots	Na^+-specific transport	(Garciadeblas et al., 2003)
TaHKT1:5 (Kna1)	Roots	Na^+ unloading from root xylem and functions as a Na^+-selective transporter	(Byrt et al., 2007)
TmHKT1;5 (Nax2)	Roots	Controls Na^+ unloading from root xylem and functions as a Na^+-selective transporter	(James et al., 2006; Byrt et al., 2007)
TaHKT1:4	Roots and leaf sheath	Removing Na^+ from the xylem of the roots and leaf sheaths	(Lindsay et al., 2004; Huang et al., 2006; James et al., 2006)
HvHKT2;1	Roots	Na^+ and K^+ co transport	(Rubio et al., 1995a; Haro et al., 2005; Huang et al., 2008)
HvHKT2;3/4	Roots	Na^+ and K^+ co transport	(Garciadeblas et al., 2003)
OsHKT2;1	Cortex of roots	Na^+ uptake under K starvation	(Horie et al., 2001b; Garciadeblas et al., 2003; Horie et al., 2007)
OsHKT2;2	Mesophyll	Na^+ and K^+ couple transport	(Horie et al., 2001)
OsHKT2;3/4	Shoots	K^+-Na^+ transport at tonoplast	(Garciadeblas et al., 2003)
TaHKT2;1	Cortex cells of roots	High affinity K^+ and Na^+ transport	(Laurie et al., 2002)
TaHKT2;3/4	Leaves	K^+-Na^+ transport	(Garciadeblas et al., 2003)

HKT members of subfamily 1 have a serine residue in the first pore loop of the protein, which is replaced by glycine in most members of subfamily 2 (Figure 4-1). Moreover, all members of the HKT gene family contain two introns. However, subfamily 1 has longer introns compared to subfamily 2 (Figure 4-2).

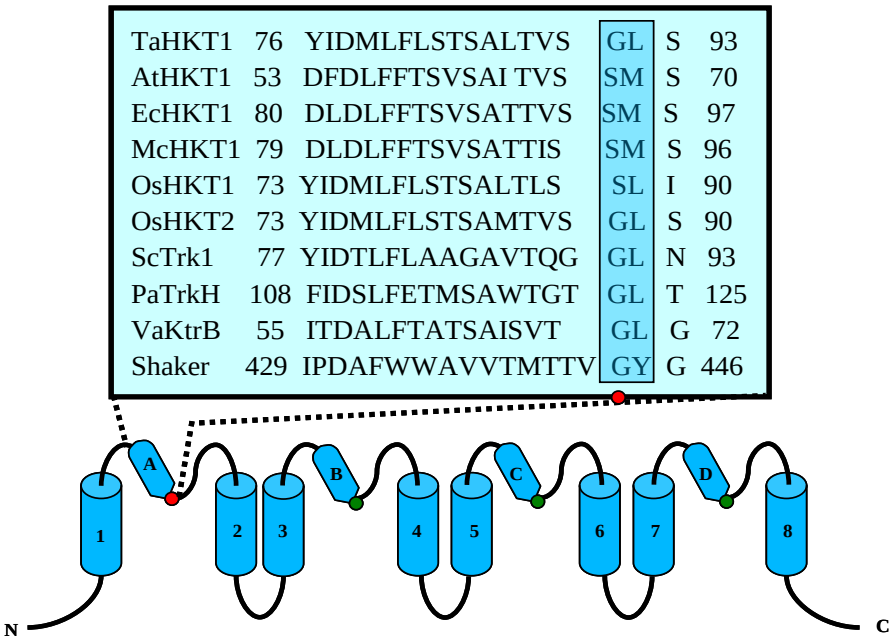


Figure 4-1:Structure of HKT proteins adopted from (Maser et al., 2002a) shows four predicted P-loops, labelled A – D, and eight transmembrane domains labelled 1 – 8. The alignment shows the P-loop A of various plant HKTs compared with Trk1 from *S. cerevisiae*, Trk H from *Pseudomonas aeruginosa*, KtrB from *Vibrio alginolyticus*, and to the P-loop of the *Drosophila* Shaker channel. The residue corresponding to the first glycine of the K^+ channel GYG motif is marked with a red dot.

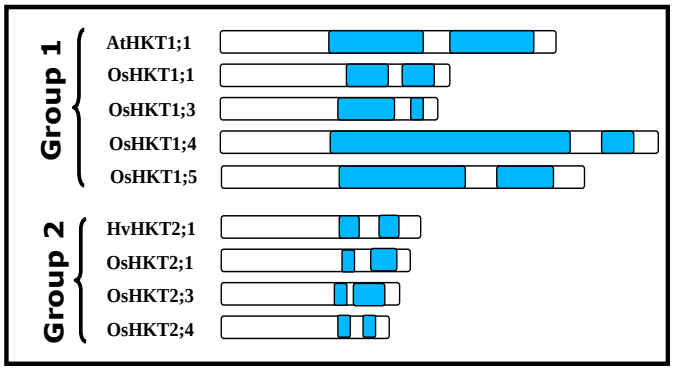


Figure 4-2:Relative intron sizes of members of the HKT gene family (adopted from Platten et al., 2006). Coding sequences (exons) are depicted in white, introns in blue. All HKT genes contain two introns. However, subfamily 2 has smaller introns compared to subfamily 1

In dicots, such as *Arabidopsis*, only 1 HKT gene has been identified which belongs to subfamily 1. Most of the monocots, such as rice and barley, have several HKT genes which are functionally diverse and fall in both subfamilies. Members of the HKT subfamily 1 are Na^+ specific and some of them are located in the plasma membrane of cells in the stele of roots, particularly the xylem parenchyma cells where they regulate Na^+ transport from root to shoot by retrieving Na^+ from the xylem sap (Huang et al., 2008). *Arabidopsis AtHKT1:1* (Figure 4-3) is expressed mainly in xylem parenchyma cells (Sunarpi et al., 2005). *AtHKT1:1* showed high levels of Na^+ uptake activity in both yeast and *Xenopus oocytes* (Uozumi et al., 2000). In *Arabidopsis*, *AtHKT1* plays an important role in the regulation of Na^+ homeostasis (Rus et al., 2001; Maser et al., 2002a). However, the exact mechanism of action of *AtHKT1:1* is still not well understood (Munns and Tester, 2008). Davenport et al., (2007) using $^{22}Na^+$ flux analysis, showed that *AtHKT1:1* contributes to Na^+ accumulation in roots and retrieval of Na^+ from the xylem. Loss of function mutants in *Athkt1:1* showed higher concentrations of Na^+ in xylem sap compared to wild type (Berthomieu et al., 2003; Sunarpi et al., 2005).

In rice, *OsHKT1:5* is a plasma membrane Na^+ transporter expressed in xylem parenchyma cells that retrieves Na^+ from the xylem sap (Ren et al., 2005). The activity of *OsHKT1;5* results in less Na^+ load in shoot tissue and therefore a considerably higher K^+:Na^+ ratio in leaf tissue which may be beneficial to growth. *OsHKT1;5* transcript abundance is responsive to salt treatment (Walia et al., 2005) and interestingly shows allelic variation between cultivars that differ in salt tolerance (Ren et al., 2005). In wheat, members of the gene subfamily 1, such as *TaHKT1:5* (Figure 4-3) are involved in Na^+ transport, resulting in low Na^+ content in leaves either by retrieval of Na^+ from the xylem in roots (James et al., 2006) or by controlling net xylem loading in roots (Gorham et al., 1990). *TaHKT1:4* (Figure 4-3), identified as a quantitative trait locus for Na^+ exclusion in durum wheat (Lindsay et al., 2004), also plays an important role in retrieval of Na^+ from the xylem in roots and leaf bases (James et al., 2006).

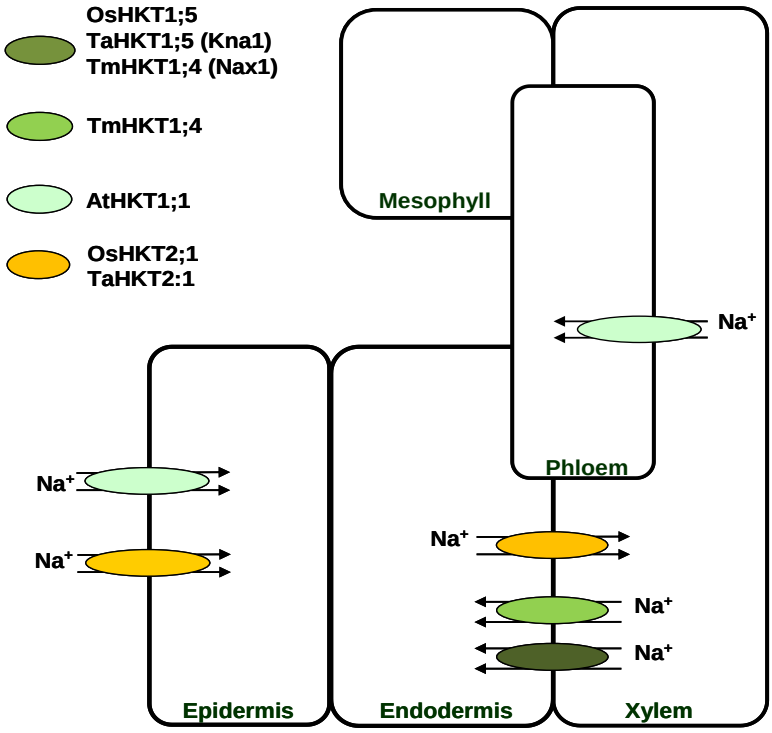


Figure 4-3:Generalised functions and localisation of HKT transporters in controlling Na^+ and K^+ flux in *Arabidopsis*, wheat, barley and rice: HKTs could function as Na^+ uptake system in the epidermal/cortical cells, for example *AtHKT1;1, TaHKT2;1, HvHKT2;1*, and *OsHKT2;1* (Horie et al., 2001; Laurie et al., 2002; Garciadeblas et al., 2003; Haro et al., 2005; Horie et al., 2007). HKTs are also proposed to be involved in Na^+ recirculation through the phloem such as *AtHKT1;1* (Berthomieu et al., 2003; Sunarpi et al., 2005). Some HKTs from rice and wheat are also known to be involved in Na^+ unloading from the xylem of the root such as, SKC1 *(OsHKT1;5*), Nax2 (*TmHKT1;5*), and Kna1 (*TaHKT1;5*) (Gorham et al., 1990; Ren et al., 2005; James et al., 2006; Byrt et al., 2007). Nax1 (*TmHKT1;4*) may unload Na^+ from the xylem of the root and leaf sheath (James et al., 2006).

Members of the HKT gene subfamily 2 are functionally diverse and may co-transport Na^+ and K^+ or act as Na^+ and K^+ uniporters. The members of subfamily 2 are mainly expressed in the root cortex where they scavenge Na^+ in K^+ deficienct conditions and maintain ionic homeostasis. For example *OsHKT 2;1* (Figure 4-3) expression was found to be different in salt tolerant and sensitive rice varieties in response to NaCl stress (Golldack et al., 2002). Moreover loss-of-function mutations in *OsHKT2;1* showed reduced growth in low K^+ conditions and accumulated less Na^+ (Horie et al., 2007b). Members of the HKT2 subfamily were shown to be Na^+ and K^+ permeable when expressed in yeast and Xenopus oocytes, at least at submillimolar Na^+ and K^+ concentrations (Rubio et al., 1995; Horie et al., 2001; Golldack et al., 2002; Jabnoune et al., 2009). However, recent studies suggest that the permeability properties of HKT family are more complex (Corratge-Faillie et al.,

2010). Lan et al., (2010) found rice *OsHKT2;4* to be a Ca^{2+} permeable cation channel, localized at the plasma membrane conducting current carried by a wide range of monovalent and divalent cations, suggesting its role in the uptake and extrusion of cations at the plasma membrane.

In barley, five HKT isoforms are reported (Huang et al., 2008): only *HvHKT2;1* has been studied, which has 92% homology with *TaHKT2;1* (Figure 4-3) at the nucleotide level and both were shown to be Na^+ - K^+ co-transporters in yeast (Rubio et al., 1995; Haro et al., 2005). However, in wheat and barley roots, *TaHKT2;1* and *HvHKT2;1* mediate Na^+ uptake into K^+-starved plants (Wang et al., 1998; Laurie et al., 2002). Wang et al., (1998) reported rapid upregulation of *HvHKT2;1* mRNA levels in the absence of K^+ in the growth medium. These studies suggest an important role of *HvHKT2;1* in the salt tolerance of barley crop.

Understanding plant responses to salinity stress is crucial for genetic engineering of crops with improved tolerance (Wang et al., 2003; Zhang et al., 2004). Halophytes such as barley can grow under high saline conditions by responding at the cellular, tissue and the whole plant level. Hence, to understand salt tolerance in halophytes, the mechanisms at each level must be unravelled. One of the mechanisms to achieve salt tolerance by halophytes is to use Na^+ and Cl^- as osmoticum to adjust water homeostasis while maintaining control over ion influx, compartmentation and translocation to the shoot. Thus, in contrast to glycophytes, halophytes often show a large amount of Na^+ translocation from root to shoot (Flowers and Colmer, 2008).

Members of the HKT gene family are proposed to play a major role in Na^+ uptake and recirculation within salt-stressed plants (Rus et al., 2001; Apse and Blumwald, 2007). Although the HKT gene family has been extensively studied in various heterologous and plant systems, the physiological role of many members of the HKT gene family is still not clear (Maathuis and Sanders, 1996; Hayes et al., 2001; Haro et al., 2005). The present study was undertaken to investigate the role of *HvHKT2;1* in barley salt tolerance and K^+ nutrition by employing an overexpression approach with the rationale being that this strategy would benefit barley salt tolerance because by improving its capability to maintain K^+ /Na^+ homeostasis. To test this hypothesis, analyses were carried out comparing the growth and ion relations of *HvHKT2;1* overexpressors with non transgenic control plants. This chapter will describe and discuss the results that were obtained from the physiological and functional characterization of the barley *HvHKT2;1* overexpressors.

4.2 Materials and Methods

4.2.1 Plant material

Hordeum vulgare cv. Golden Promise was used in all experiments. To study the role of *HvHKT2;1* in barley salt tolerance, a construct for *HvHKT2;1* was made using Gateway cloning technology by Dr. Stanislav Isayenkov. *Agrobacterium* mediated transformation of barley plants was carried out in the John Innes Research Centre, Norwich. Plants selected from a segregating population, showing no resistance to hygromycin during leaf test analysis and no PCR amplification using hygromycin gene specific primers (see below), were used as control plants in all the experiments.

4.2.2 Growth media and conditions

Barley seeds were germinated in sterile conditions at 20ºC, 70% relative humidity and kept in covered black boxes for 3 days. After the emergence of the first true leaf from the coleoptile, the boxes were uncovered and placed in 100 µmol/m^2/s light conditions. After one week seedlings were transferred to a hydroponic culture system consisting of boxes containing 10 litres of aerated nutrient solution and holding up to 30 plants each. Nutrient medium consisting of macronutrients (1.25 mM KNO_3, 0.5 mM $Ca(NO_3)_2.4H_20$, 0.5 mM $MgSO_4.7H_20$, 42.5 µM Fe-EDTA and 0.625 mM KH_2PO_4) and micronutrients (0.16 µM $CuSO_4.5H_20$, 0.38 µM $ZnSO_4.5\ H_20$, 1.8 µM $MnSO_4.H\ H_20$, 45 µM H_3BO_3, 0.015 µM $(NH_4)_2Mo_7O_{24}.4H_20$ and 0.01 $CoCl_2$) as described by Miyamoto et al., (2001), was used as a control medium. The hydroponic containers were placed in a green house set to a light/dark cycle of 16/8 h daily (PAR 300 µmol/m^2 per second), 20-25ºC day/night temperature, and 40-60% relative humidity in a convironment cabinet. Nutrient solution was changed every seven days.

For growth, ion content and xylem sap experiments, Na^+ and K^+ treatments were made by addition of stocks of NaCl (5 M) and KCl (2 M) to the control medium. However, for 0 K^+ treatments, all potassium salts in the control medium were replaced with equimolar quantities of the corresponding sodium salts.

4.2.3 Screening of homozygous *HvHKT2;1* overexpressor lines using a leaf test method

Seeds obtained from the T0 generation were grown to obtain plantlets, which were analysed using a leaf test for screening of homozygous lines. Two healthy green leaf tips about 2 cm long were excised from each plant. Leaf tips were surface sterilized by applying 3% Na-hypochlorite with a drop of Tween-20 for 3 minutes and then rinsed 5 times with sterile water. The sterilized leaf tips were immediately placed on plates with the cut ends embedded in the growth medium for maximum contact. The Murashige and Skoog salts (MS) medium (Murashige and Skoog, 1962) was supplemented with 0.5mg/L 6-benzylaminopurine (6-BA) and 200 mg/L hygromycin. The assays were continued for 7 to 10 days at 24 ^{0}C under a 16/8 h light/dark regime and the bleaching of leaf tips over the period of 7 days was continuously recorded.

4.2.4 PCR analyses for screening of *HvHKT2;1* overexpressor lines

To analyze the integration of the transformed gene in transgenic barley plants and to confirm the leaf test analyses, DNA was isolated and PCR methods were used. Leaf tissues were used for extraction of the DNA. PCR was performed for 30 cycles (30 sec. at 94°C, 30 sec. at 60°C, 60 sec. at 72°C), using the hygromycin phosphotransferase (HPT) gene specific primers and genomic DNA from the transformants as template. The nucleotide sequence of the sense primer HygNfor was 5P'-ATTTGTGTACGCCCGACAGT-3' and the nucleotide sequence of the antisense primer HygNrev was 5P'-GGATATGTCCTGCGGGTAAA-3'. The PCR products were resolved by electrophoresis in a 1.2 % agarose gel.

4.2.5 Expression analyses of *HvHKT2;1* transcript level by semi-quantitative RT-PCR

Total RNA was extracted from leaf tissues of three week old control and *HvHKT2;1* overexpressor lines, using an RNase easy KIT (Qiagen, Valencia, CA), according to the manufacturer's instructions. A total of 1 μg RNA was used to synthesize first strand cDNA using the transcriptor high-fidelity cDNA synthesis KIT (Roche, Mannheim, Germany). PCR was applied to 1 μl of cDNA with gene-specific primers against *HvHKT2;1*. The house-keeping gene, α-tubulin was used as the control. PCR consisted of 35 cycles of 45 s

at 53 °C, 1 min 30 s at 72 °C, and 30 s at 95 °C. The primers used for semi-quantitative RT-PCR analyses are listed in table 4-2.

Table 4-2: Primers used to detect gene expression in control and *HvHKT2;1* overexpressor lines using semi-quantitative RT-PCR.

Gene ID	**Primer Sequence**	**Product Size (bp)**
αTubulin-F	5'-AGT GTC CTG TCC ACC CAC TC-3'	248
αTubulin-R	5'-AGC ATG AAG TGG ATC CTT GG-3'	248
HKT_cDNA-F	5'-AGC GCT CAA ATT GTG GTC TT-3'	678
HKT_cDNA-R	5'-AAT GGC TGT CGA GGA GAG AA-3'	678

4.2.6 Expression analyses of *HvHKT2;1* transcript level by qRT-PCR

Expression levels of *HvHKT2;1* in different transgenic and control lines were also determined by qRT-PCR. RNA was isolated from the young leaves and reverse transcription was performed using the tanscriptor high fidelity cDNA synthesis kit. cDNA samples were diluted to 5 ng/ul. Each cDNA sample was quantitatively assayed in triplicate using SYBR Green master mix in an ABI 7900 sequence detection system. The amplicon of the barley tubulin gene was used as a control to normalise the data. The primers used for qRT-PCR analyses are listed in table 4-3.

Table 4-3: Primers used to detect gene expression in control and *HvHKT2;1* overexpressor lines using qRT-PCR.

Gene ID	Primer Sequence	ProductSize (bp)
αTubulin-F	5'-AGT GTC CTG TCC ACC CAC TC-3'	248
αTubulin-R	5'-AGC ATG AAG TGG ATC CTT GG-3'	248
HvHKTsybr_F	5'-GTTTCTCTCCTCGACAGCCATT-3'	64
HvHKTsybr_R	5'-ATCAACAGCGGAGAACATTGTG-3'	64

4.2.7 Tissue specific expression analyses of *HvHKT2;1* transcript level at different stress conditions in wild type plants using qRT-PCR

Tissue specific expression levels of *HvHKT2;1* in leaves, stems and roots of wild type barley plants were determined by qRT-PCR. Fifteen day old plants were exposed to control, 50 mM KCl, 50 mM NaCl, 0 K^+ and 0 K^+ + 50 mM NaCl conditions for one week. After one week of treatment total RNA was isolated from leaves, stems and roots using the RNase easy KIT (Qiagen, Valencia, CA). A total of 1 µg RNA was used to synthesize first strand cDNA using the transcriptor high-fidelity cDNA synthesis KIT (Roche, Mannheim, Germany). cDNA samples were diluted to 5 ng/ul. Each cDNA sample was quantitatively assayed in triplicate using SYBR Green master mix in an ABI 7900 sequence detection system. The amplicon of the barley tubulin gene was used as a control to normalise the data. The primers for qRT-PCR are listed in table 4-3.

4.2.8 Tissue specific expression analysis of *HvHKT2;1* in root cortex and stele of wild type barley plants using semi-quantitative RT-PCR

Expression levels of *HvHKT2;1* in root cortex and stele of wild type barley plants were determined by semiquantitative RT-PCR. At the three week stage, barley roots were stripped to separate cortex and stele. RNA was extracted from cortex and stele using the RNase easy KIT (Qiagen, Valencia, CA). The transcriptor high-fidelity cDNA synthesis KIT (Roche, Mannheim, Germany) was used for RT-PCR. To allow semiquantitative comparison of transcript levels, RNA starting concentrations were equal for each sample. The house-keeping gene, α-tubulin was used as the control. PCR consisted of 35 cycles of 45 s at 53 °C, 1 min 30 s at 72 °C, and 30 s at 95 °C. The primers used for semi-quantitative RT-PCR analyses are listed in table 4-2.

4.2.9 Growth analyses on HvHKT2*;1* overexpressors and control plants

Barley plants overexpressing *HvHKT2;1* and control plants were grown for 2 weeks on terra green in the green house. After two weeks, the seedlings were transferred to hydroponic growth medium. At the three week stage, three plants each from *HvHKT2;1* overexpressors and control plants were exposed to different Na^+ and K^+ stress conditions i.e. control, 0 K^+, 50 mM KCl, 50 mM NaCl and 0 K^+ + 50 mM NaCl (see growth media and conditions) for one week. Experiments were repeated three times and relative growth rate (RGR) was calculated using the equation described by (Poorter and Garnier, 1996).

4.2.10 Na^+ and K^+ content analyses on *HvHKT2:1* overexpressors and control plants

Na^+ and K^+ content of leaves, stems, and roots was measured using flame photometry. Barley plants overexpressing *HvHKT2;1* and control plants were grown for 2 weeks on terra green in the green house and then transferred to hydroponic control medium for one week. At the three week stage, three plants each from *HvHKT2;1* overexpressors and control plants were exposed to different Na^+ and K^+ stress conditions i.e. control, 0 K^+, 50 mM KCl, 50 mM NaCl and 0 K^+ + 50 mM NaCl (see growth media and conditions) for one week. After one week of treatment, plants were separated into leaves, stems and roots and then roots were washed with 20 mM $CaCl_2$ solution for 10 minutes. Fresh weights of the samples were noted after blotting with tissue papers. Samples were then dried at 80^{o}C for 3 days. Dried samples were treated with 5 ml of 20 mM $CaCl_2$ for 24 hours and Na^+ and K^+ content of the leaves, stems and roots were recorded using a flame photometer (Sherwood flame photometer-410, Cambridge, UK). The experiments were replicated three times.

4.2.11 Short-term Na^+ uptake analyses on HvHKT2*:1* overexpressors and control plants

Barley plants overexpressing *HvHKT2;1* and control plants were grown for 2 weeks on terra green in the green house and then transferred to hydroponic control medium for one week. At the three week stage, three plants each from *HvHKT2;1* overexpressors and control plants were exposed to control and 100 mM NaCl conditions for 3 hours. After 3 hours of treatment, plants were separated into leaves, stems and roots and then washed with 20 mM $CaCl_2$ solution for 10 minutes. Fresh weights of the samples were noted after blotting with tissue papers. Samples were then dried at 80^{o}C for 3 days. Dried samples were treated with 5 ml of 20 mM $CaCl_2$ for 24 hours and Na^+ and K^+ content of the leaves, stems and roots were recorded using a flame photometer (Sherwood flame photometer-410, Cambridge, UK). The experiments were replicated three times.

4.2.12 Xylem sap analysis on *HvHKT2;1* overexpressors and control plants

Three week old *HvHKT2;1* overexpressors and control plants were treated with different Na^+ and K^+ regimes (control, 50 mM KCl, 50 mM NaCl) for one week before collecting the xylem sap. Plants were transferred to a pressure chamber (Digital plant water potential

apparatus, EL540-300), and the shoot was excised about 20 mm above the root/shoot junction. Pressure exceeding the osmotic pressure of the external solution was applied to the chamber. Xylem sap was collected for 30 minutes. Aliquots of 0.3 ml xylem sap were each collected from 3 plants in parallel and immediately put on ice. Na^+ and K^+ content of the xylem sap was measured with a flame photometer (Sherwood flame photometer-410 Cambridge, UK).

4.2.13 Statistical analysis

All data shown were derived from experiments carried out across a minimum of three biological replications. Growth, ion content, and xylem sap experiments were subjected to unpaired two-tailed t tests to identify significance at the $P < 0.05$ level.

4.3 Results

4.3.1 Selection of *HvHKT2;1* overexpressors (*HKT-OX*) homozygous lines using the leaf test method

To screen large numbers of transformed plants obtained from the T0 generation, leaf test analyses were carried out (Figure 4-4) for selection of homozygous lines as described in the methods section. A total of 11 transgenic lines from T2 was analysed. Among these only one transgenic line overexpressing *HvHKT2;1* was found to be homozygous, nine lines were heterozygous and one line was found to be homozygous with regard to hygromycin sensitivity. The latter line was therefore used as control line for all experimentation. PCR analyses using hygromycin phosphotransferase (HPT) gene primers were used to confirm the results of the leaf test (Figure 4-5).

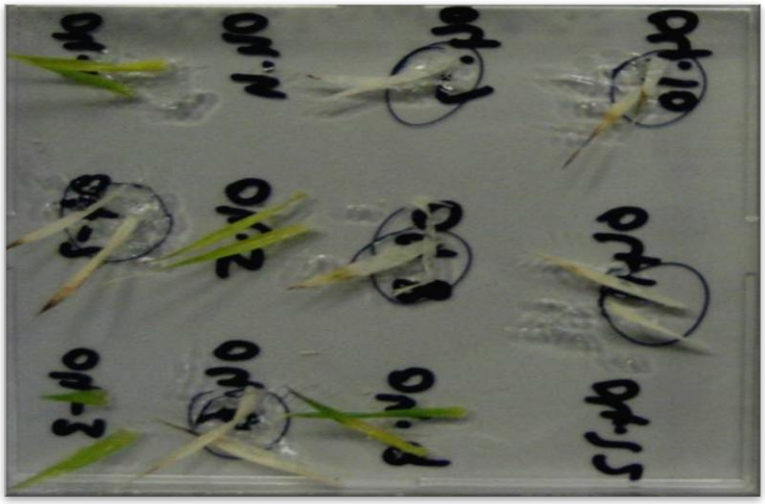


Figure 4-4:Leaf test analysis of barley *HvHKT2;1* overexpressors for selection of homozygous transgenic lines. Leaf tips were excised from three week old plants and inserted in ½ MS medium supplemented with 200 mg/L hygromycin. Leaves from the plants showing bleaching were considered wild type, while leaves showing no signs of bleaching were annotated as transgenic plants.

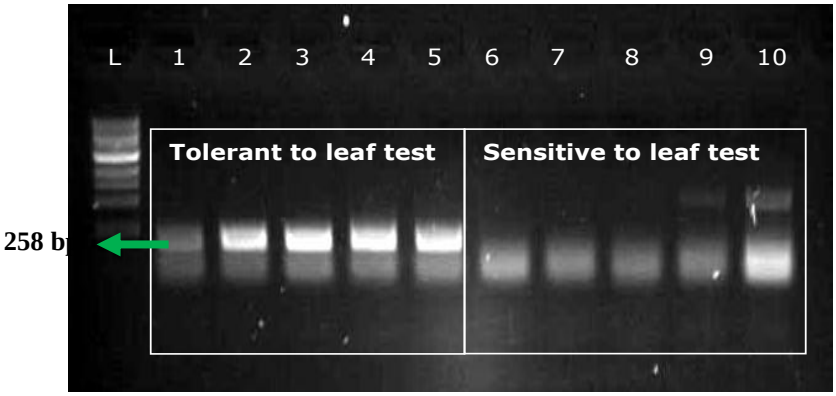


Figure 4-5:PCR analysis of *HvHKT2;1* overexpressors for selection of homozygous lines and confirmation of leaf tests using hygromycin phosphotransferase gene (HPT) specific primers. Lane L contains the ladder. Lanes 1 – 5 contain DNA of different plants that showed hygromycin tolerance while lanes 6 – 10 contain DNA of different plants that showed hygromycin sensistivity during the leaf test. Arrow indicates band size (258 bp) for the hygromycin phosphotransferase (HPT) amplicon.

4.3.2 Expression analysis of *HKT-OX* lines by qRT-PCR

The transcript level of *HvHKT2;1* in two *HKT-OX* lines was studied using qRT-PCR. cDNA samples from control plants, *HKT-OX1* and *HKT-OX2* were obtained as described in the methods section and were quantitatively assayed in triplicate using SYBR Green master mix in an ABI 7900 sequence detection system. The amplicon of the barley tubulin gene was used as a control to normalise the data. Quantitative RT-PCR analyses showed that the *HvHKT2;1* overexpressing line (HKT OX1) has approximately 111 fold, while HKT OX 2 showed 40 fold more expression compared to the control line (Figure 4-6).

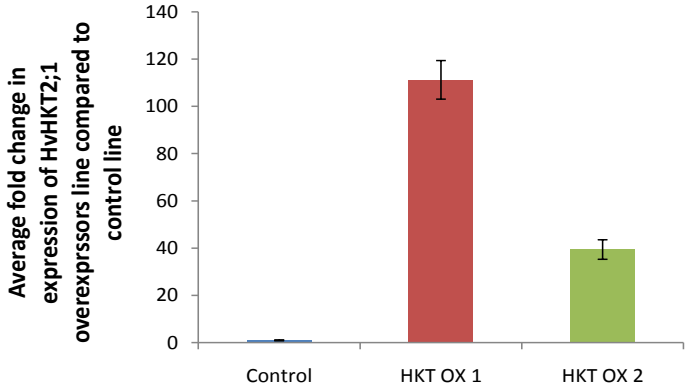


Figure 4-6:Expression analysis of *HvHKT2;1* overexpressors (HKT-OX) by qRT-PCR compared to control plants. The *HvHKT2;1* overexpressing line (HKT OX 1) showed approximately 111 fold while HKT OX 2 showed 40 fold more expression compared to control line. Values are the mean ± S.D. (n = 3).

4.3.3 *HvHKT2;1* tissue specific gene expression

To study the physiological role *HvHKT2;1*, the expression pattern of *HvHKT2;1* in roots, stems and leaves of three week old barley plants was examined by using qRT-PCR as described in the material and methods. We found that the *HvHKT2;1* gene is highly expressed in roots and at a much lower level in leaves. However, in stems even less expression was found as shown in figure 4-6. To get more insight into the physiological function of *HvHKT2;1*, the expression of *HvHKT2;1* in the root at tissue level was also studied using semi quantitative RT-PCR. Root cortex and stele of 3 week old barley plants were separated as described in the material and methods. RT-PCR analysis using gene specific primers revealed that *HvHKT2;1* was 13 fold more expressed in the root cortex compared to stellar tissue as shown in figure 4-7. The tissue specificity of *HvHKT2;1*

expression in this study is consistent with the pattern previously reported for the HKT gene members of the subfamily 2 in rice plants (Horie et al., 2007).

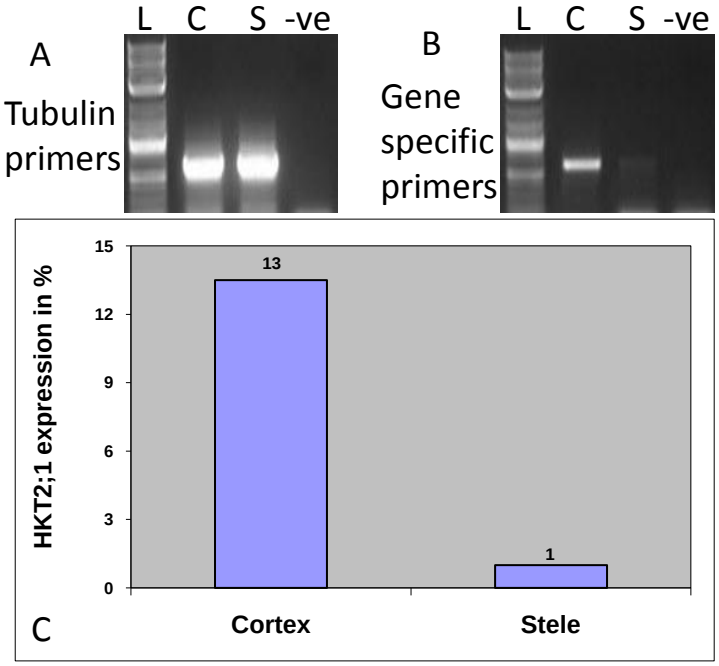


Figure 4-7: PCR analysis of *HvHKT2;1* expression in root stele and cortex using tubulin (A) and *HvHKT2;1* gene specific primers (B). C is the percentage expression of the *HvHKT2;1* gene in cortex and stele, based on the densitometry analysis. L is ladder, C is cortex, S is stele and –ve is negative control (water).

4.3.4 Regulation of gene expression of *HvHKT2;1* in wild type plants in response to different growth conditions

To understand the role *of HvHKT2;1* in response to different Na^+ and K^+ stress conditions, expression profiles of *HvHKT2;1* in 3 week old seedlings exposed to control, 50 mM K^+, 50 mM NaCl and 0 K^+ + 50 mM NaCl were recorded by semi-quantitative RT-PCR using *HvHKT2;1* specific primers (Figure 4-8) as described in the materials and methods section. To be able to compare the *HvHKT2;1* expression in roots, stems and leaves at different conditions, the expression of *HvHKT2;1* at control condition in roots, stems and leaves was normalised to 100% (Figure 4-9).

The *HvHKT2;1* expression in roots was downregulated at 50 mM KCl, 50 mM NaCl and 0 K^+ + 50 mM NaCl conditions while in stems approximately 8 to 12 fold upregulation of *HvHKT2;1* was observed at 50 mM KCl and 50 mM NaCl treatments respectively. In leaves however, at 50 mM KCl the *HvHKT2;1* expression was upregulated 5 times compared to control condition. No or very little upregulation was observed at 50 mM NaCl

and 0 K^+ + 50 mM NaCl in leaves. This further supports the idea of *HvHKT2;1* involvement in Na^+ and K^+ distribution at high KCl and NaCl conditions. However, at 0 K^+ + 50 mM NaCl treatment only minor upregulation was observed suggesting a limited role of *HvHKT2;1* in Na^+ and K^+ uptake and distribution in K^+ starved condition.

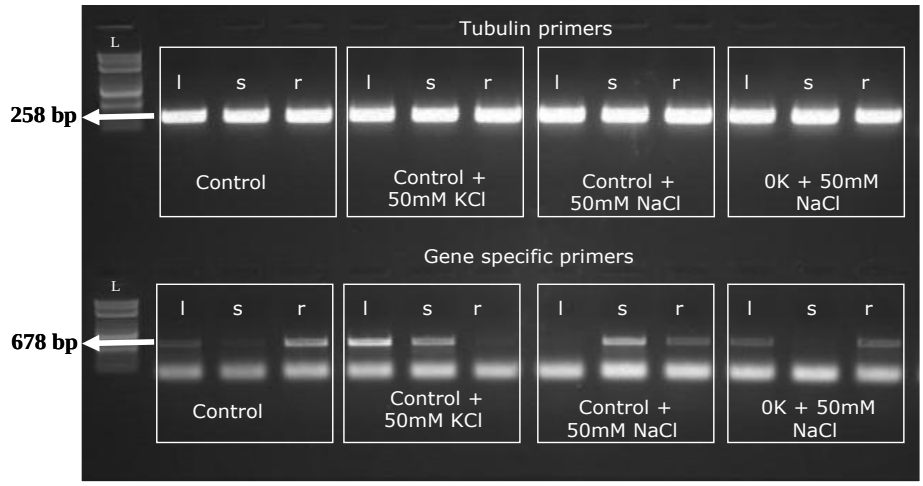


Figure 4-8:Semi-quantitative RT-PCR of *HvHKT2;1* in response to control, 50 mM K+,50 mM NaCl and 0 K^+ + 50mM NaCl conditions. Tubulin was used as an internal control. The total RNAs were extracted from 3 week old seedlings after one week of treatment. Lanes L shows a 2-log ladder; l, leaves; s, stem and r, root. Arrows show predicted sizes of the generated amplicons for tubulin and *HvHKT2;1* gene specific primers.

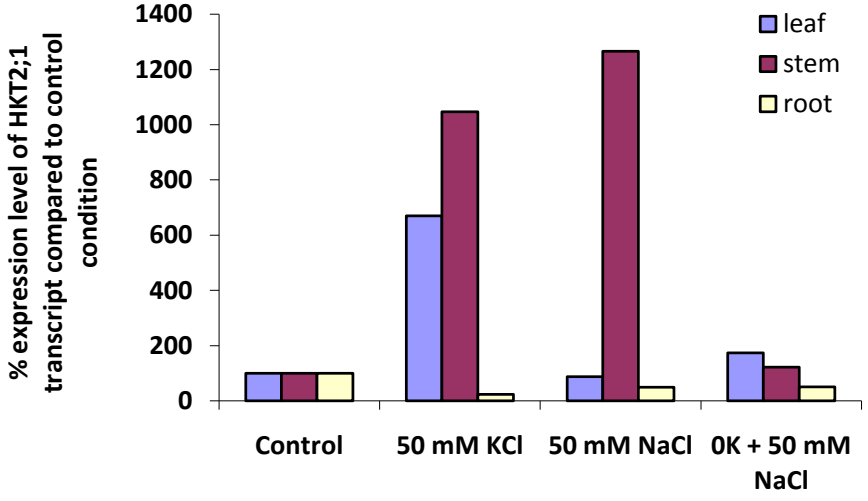


Figure 4-9:Differential expression values are shown, calculated using densitometry for various Na^+ and K^+ stress conditions relative to the control condition for semi-quantitative RT-PCR analysis. The expression of *HvHKT2;1* at control conditions in roots, stems and leaves was normalised to 100%.

4.3.5 Growth of *HvHKT2;1* overexpressor lines in different growth conditions

The relative growth rate of *HvHKT2;1* overexpressors and control plants was recorded at control, 50 mM K^+, 50 mM NaCl, 0 K^+ and 0 K^+ + 50 mM NaCl as described in the methods section. In control conditions, no significant differences were observed between the *HvHKT2;1* overexpressors and control plants. However, at 50 mM KCl control plants showed better relative growth compared to the *HvHKT2;1* overexpressors. In contrast, at 50 mM NaCl the *HvHKT2;1* overexpressors showed more relative growth compared to the control plants (Figure 4-7) in both overexpressing lines. These results suggest that *HvHKT2;1* activity affects both K^+ homeostasis and salt tolerance. At 0 K^+ and 0 K^+ + 50 mM NaCl conditions, no significant difference in the growth rate of control and *HvHKT2;1* overexpressors was observed (Figure 4-10).

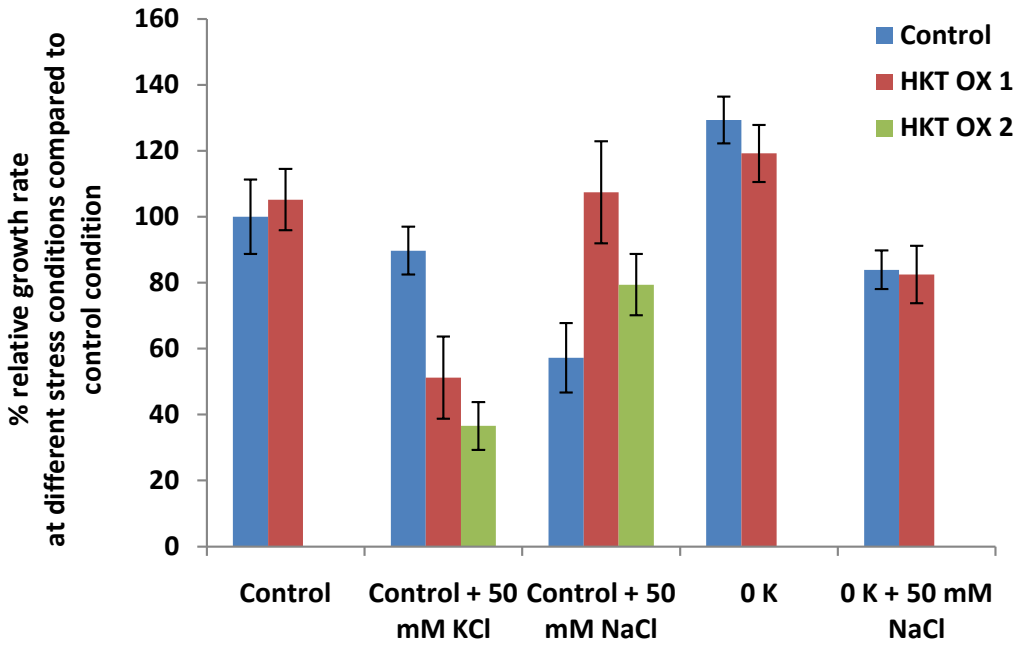


Figure 4-10: Growth of barley lines in different growth conditions. Barley *HvHKT2;1* overexpressors (HKT OX) and control plants after exposure to different Na^+ and K^+ treatment. Fifteen-day-old barley seedlings were exposed to control condition, 50 mM KCl, 50 mM NaCl, 0 K^+ and 0 K^+ + 50 mM NaCl in hydroponic medium. *RGR* was measured after 7 day of treatment. Values are the mean ± S.D. (n = 3).

4.3.6 Na^+ and K^+ content of *HvHKT2:1* overexpressors and control plants

Na^+ and K^+ content of *HvHKT2;1* overexpressors and control plants was recorded using a flame photometer as described in the methods section. For Na^+ content, no significant difference was observed at control conditions in the *HvHKT2;1* overexpressing and control lines as shown in figure 411A. At 50 mM KCl, the *HvHKT2;1* overexpressors showed increased Na^+ content in the roots and stems compared to the control lines, however, increased Na^+ content in the leaves was observed for control lines compared to the *HvHKT2;1* overexpressor lines (Figure 4-11B). The *HvHKT2;1* overexpressors showed significantly increased Na^+ content in the leaves at 50 mM NaCl conditions compared to control lines. However, no significant difference was observed for Na^+ content in the roots and stems at 50 mM NaCl conditions (Figure 4-11C). At 0 K^+ no significant difference was observed for Na^+ content in *HvHKT2;1* overexpressing and control lines (Figure 4-11D). At 0 K^+ + 50 mM NaCl, the *HvHKT2;1* overexpressors showed a higher Na^+ content in the leaves compared to the control plants, but Na^+ content was more or less the same in the roots and stems between the *HvHKT2;1* overexpressing barley and control plants.

For K^+, the *HvHKT2;1* overexpresors and wildtype barley plants showed more or less similar tissue concentrations in roots, stems and leaves at control, 50 mM K^+, 0 K^+ and 0 K^+ + 50 mM NaCl conditions. However, when plants were treated with 50 mM NaCl, plants overexpressing *HvHKT2;1* showed increased K^+ content in stems as compared to the control plants (Figure 4-12). These results suggest that at high Na^+ and K^+ concentrations, barley *HvHKT2;1* plays an important role in Na^+ and K^+ distribution, possibly via Na^+ and K^+ xylem loading, hence maintaining the Na^+ and K^+ homeostasis in the plant system.

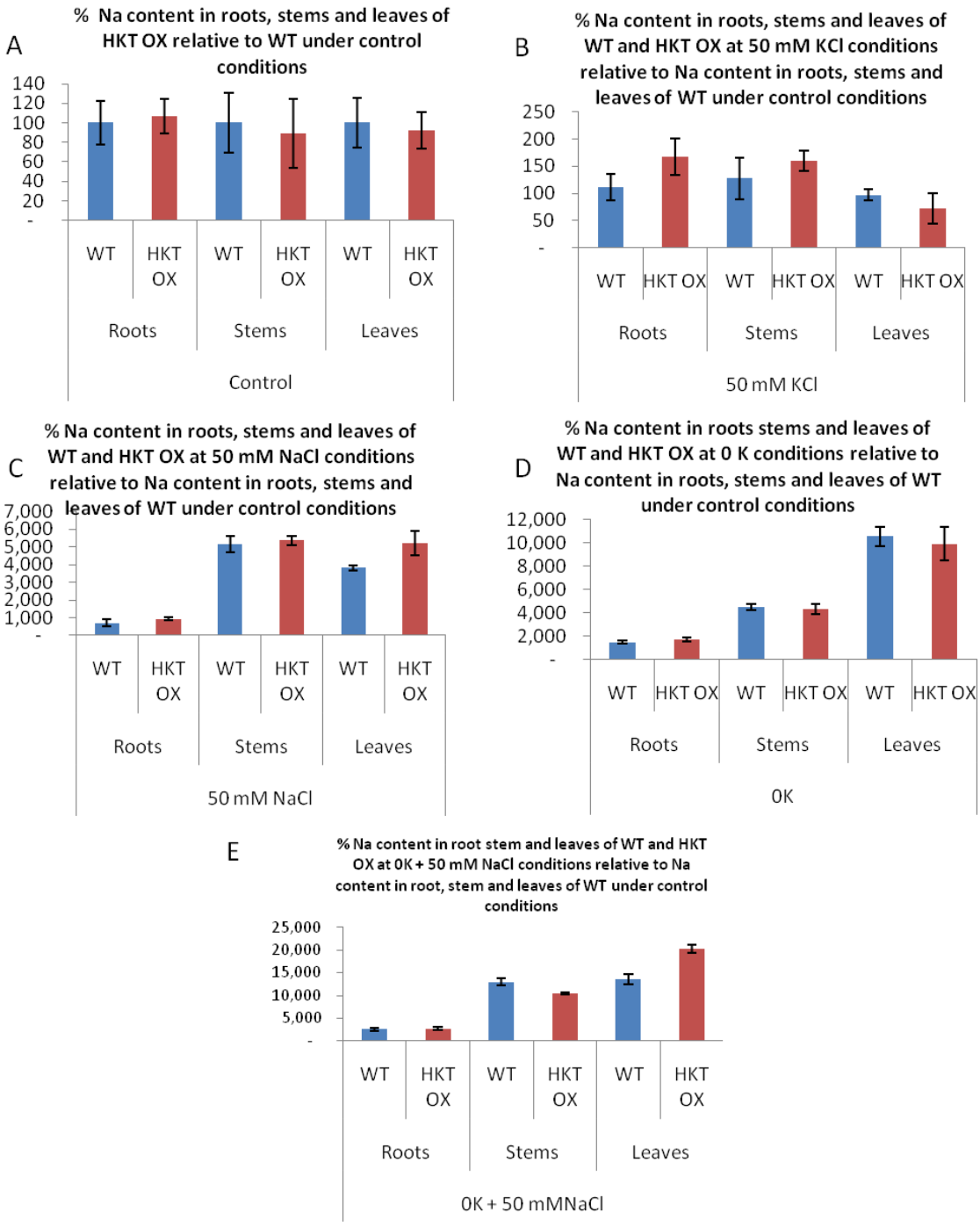


Figure 4-11:Long-term accumulation of Na^+. Fifteen-day-old plants were exposed to different Na^+ and K^+ stress treatments. At the end of 7 days of treatment, Na^+ content in roots, stems and leaves of barley plants overexpressing *HvHKT2;1* (HKT OX) and control (WT) plants were determined at control (A), 50 mM K^+ (B), 50 mM NaCl (C), 0 K^+ (D) and 0 K^+ + 50 mM NaCl (E) conditions. Values are the mean ± S.D. (n = 3).

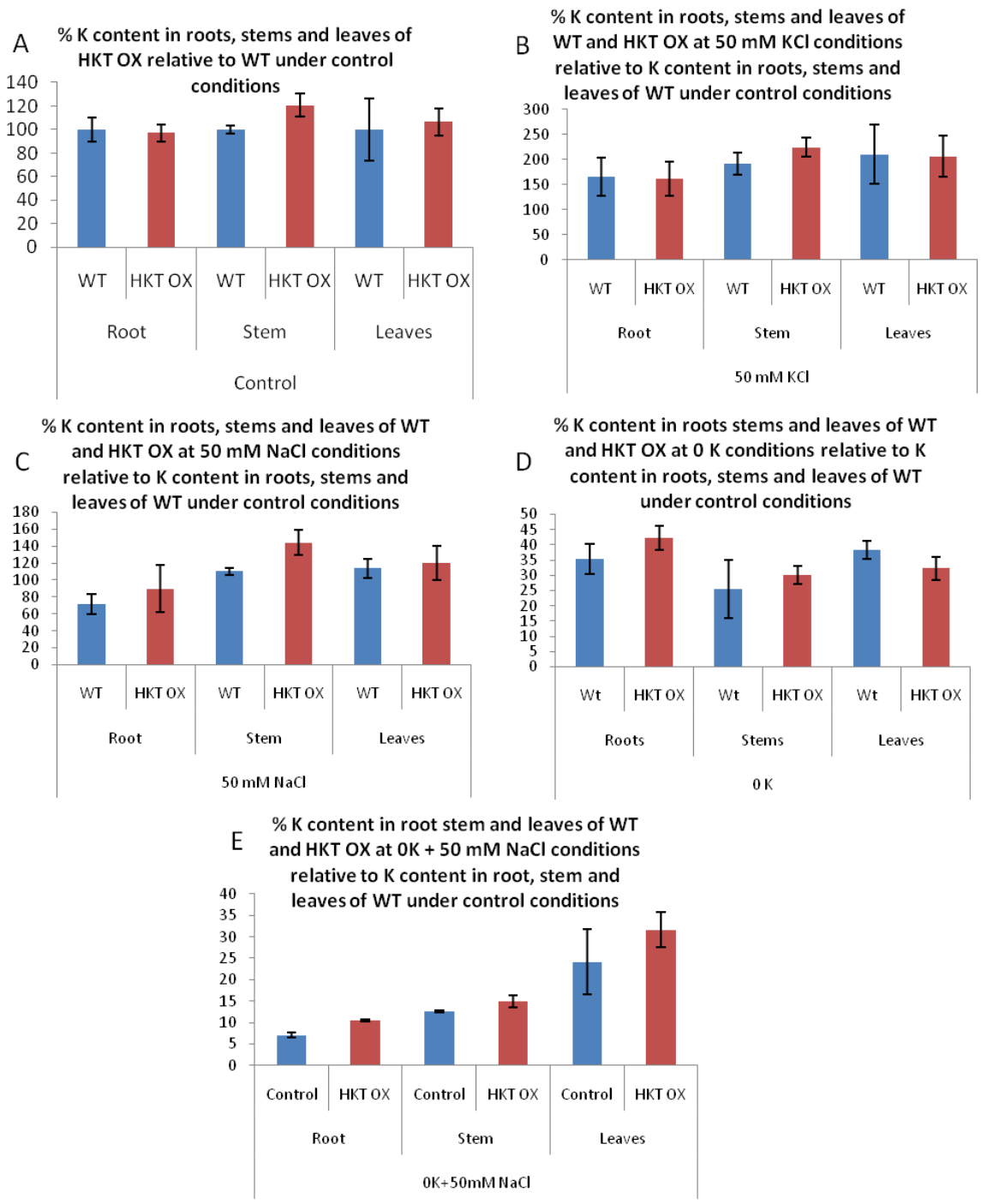


Figure 4-12:Long-term accumulation of K^+. Fifteen-day-old plants were exposed to different Na^+ and K^+ stress treatments. At the end of 7 day of treatment, K^+ content in roots, stems and leaves of barley plants overexpressing *HvHKT2;1* (HKT OX) and control (WT) plants were determined at control (A), 50 mM K^+ (B), 50 mM NaCl (C), 0 K^+ (D) and 0 K^+ + 50 mM NaCl (E) conditions. Values are the mean ± S.D. (n = 3).

4.3.7 Short-term Na^+ uptake in control and *HvHKT2;1* overexpressor lines

Short-term (3h) Na^+ uptake experiments were carried out to see if the trend discussed above was also relevent in shorter period. Figure 4-13A shows that 3 h exposure to 100 mM NaCl resulted in a different pattern of tissue Na^+ in *HvHKT2;1* overexpressors. The *HvHKT2;1* overexpressors have significantly lower Na^+ content in leaves compared to the control line, suggesting that *HvHKT2;1* has a role in Na^+ exclusion from leaves via Na^+ removal from the xylem sap or Na^+ loading into phloem vessels when exposed to high salt stress for relatively short periods. For K^+ content, no significant difference was found in *HvHKT2;1* overexpressing lines compared to the control line at 100 mM NaCl (Figure 4-13B).

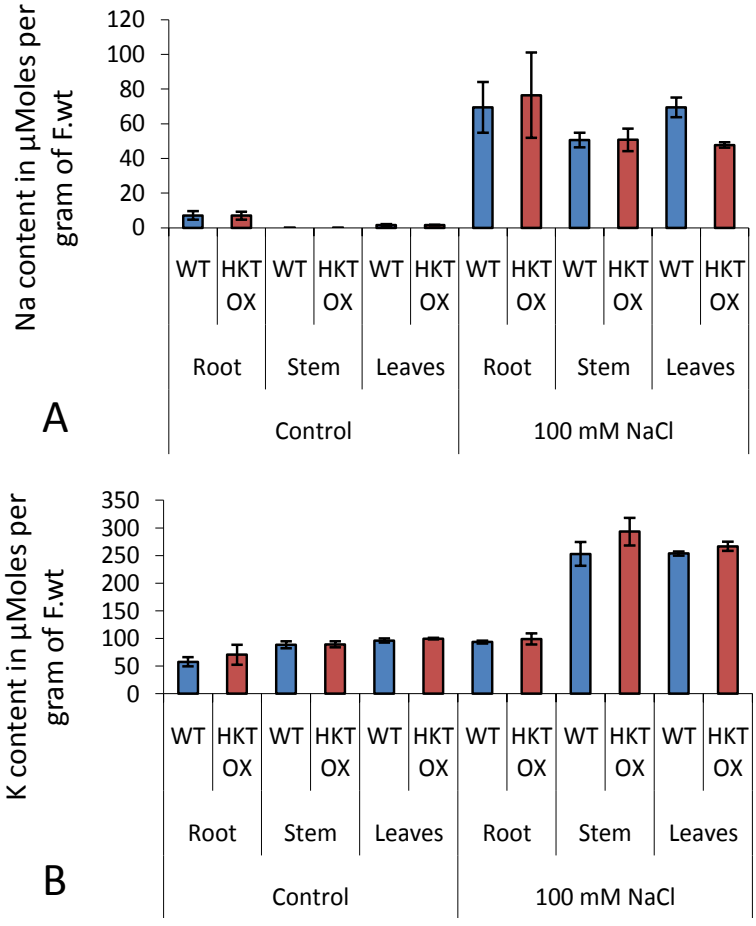


Figure 4-13:Short-term accumulation of Na^+ (A) and K^+ (B). Fifteen- day-old plants were exposed to different Na^+ stress treatment by adding NaCl to the hydroponic medium to final concentrations of 100 mM NaCl. At the end of 3 hours treatment Na^+ (A) and K^+ content (B) in roots, stems and leaves of barley overexpressing *HvHKT2;1* (HKT OX) and control plants was measured at control and 100 mM NaCl. Values are the mean ± S.D. (n = 3).

4.3.8 Xylem sap analysis of HvHKT2*;1* overexpressors and control plants

To study the role of *HvHKT2;1* in Na^+ and K^+ distribution between roots and shoots, Na^+ and K^+ content was measured in the xylem sap using flame photometer as described in the material and methods section. For this, xylem sap was collected from the cut shoot base of *HvHKT2;1* overexpressor and control plants, exposed to control, 50 mM K^+ and 50 mM NaCl conditions. *HvHKT2;1* overexpressors showed increased Na^+ and K^+ content in the xylem sap at high Na^+ and K^+ concentrations compared to the control line (Figure 4-14). These results strengthen the idea of *HvHKT2;1* involvement in Na^+ and K^+ distribution from root to shoot.

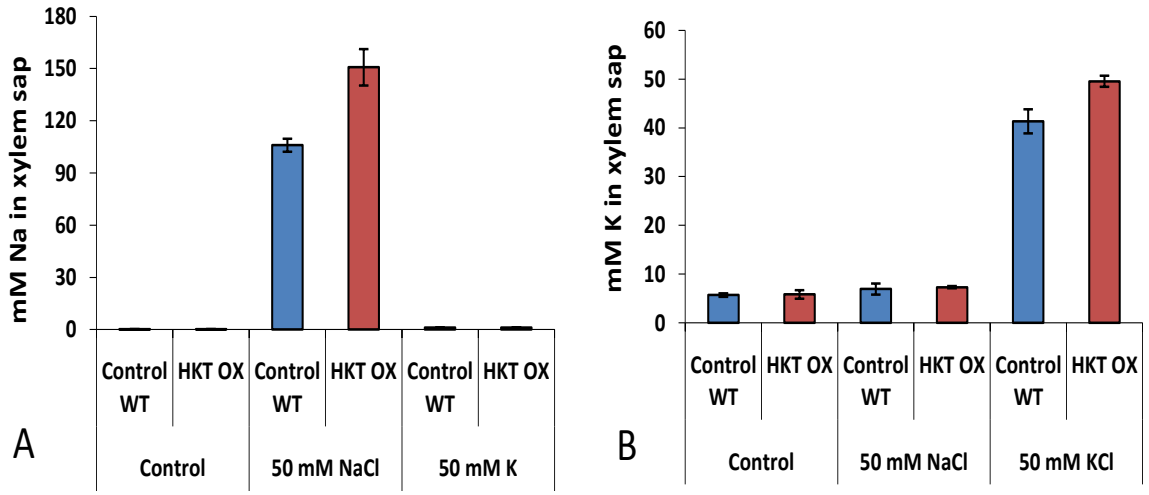


Figure 4-14:Na^+ content (A) and K^+ content (B) in xylem sap of barley *HvHKT2;1* overexpressors(HKT OX) and control (wt) plants. Fifteen-day-old plants were exposed to different Na^+ and K^+ stress treatment by adding NaCl and KCl to the hydroponic control medium to final concentrations of 50 mM KCl and 50 mM NaCl. At the end of 7 days treatment, xylem sap was collected and Na^+ and K^+ content was measured. Values are the mean ± S.D. (n = 3).

4.4 Discussion

Salinity affects plant growth and development by causing various effects, mainly osmotic stress and ion toxicity. Controlled uptake and distribution of Na^+ and K^+ by various plant membrane transporters plays an important role in plant salt tolerance. K^+ uptake and release play critical roles in maintaining turgor and water potential in plant cells, whereas excessive Na^+ accumulation in plants causes ion toxicity. HKTs are active at the plasma membrane and mediate either Na^+ and K^+ or only Na^+ transport. HKTs have been shown to improve salt tolerance in plants (Berthomieu et al., 2003; Ren et al., 2005; Byrt et al., 2007) or improve growth under K^+ starved conditions (Horie et al., 2007). HKTs are divided into two groups, mainly on the basis of Na^+ or K^+ selectivity (Garciadeblas et al., 2003). Among five HKT isoforms reported in barley, *HvHKT2;1*, which shares 92 % similarity with *TaHKT2;1* at the nucleotide level, was reported to be a Na^+-K^+ co-transporter in yeast (Rubio et al., 1995; Haro et al., 2005). However, in roots both *HvHKT2;1* and *TaHKT2;1* are believed to be Na^+ uniporters (Haro et al., 2005). To investigate a putative role of *HvHKT2;1* in barley salt tolerance *HvHKT2;1* expression in wildtype barley and the phenotype of plants that overexpressed this transporter was studied.

Gene expression studies of *HvHKT2;1* in wild type barley plants at different Na^+ and K^+ stress conditions showed downregulation in roots at 50 mM KCl, 50 mM NaCl and 0 K^+ + 50 mM NaCl conditions while in stems, approximately 8 to 12 fold upregulation of *HvHKT2;1* was observed at 50 mM KCl and 50 mM NaCl treatments respectively. This indicated that *HvHKT2;1* may be involved in Na^+ and K^+ distribution at high KCl and NaCl conditions and that its activity is particularly important in stem tissue in the Na^+ and K^+ recirculation through the phloem vasculature. However, these approaches cannot give any detailed functional insights. Therefore transgenic lines were generated that overexpress *HvHKT2;1* under a constitutive promoter.

Comparison of relative growth rates of barley plants overexpressing *HvHKT2;1* with control plants, showed lower RGR for the *HvHKT2;1* overexpressors at 50 mM K^+, while at 50 mM NaCl conditions, the *HvHKT2;1* overexpressors showed increased growth suggesting a role of *HvHKT2;1* in both K^+ nutrition and salt tolerance (Fig 4.7). Ion content analyses showed no significant difference in Na^+ and K^+ content in control and *HvHKT2;1* overexpressors at control condition (Figure 4-8A and B). However, at the 50

mM NaCl conditions, barley plants overexpressing *HvHKT2;1* showed increased Na^+ content in leaves of the transgenic plants suggesting a role of *HvHKT2;1* in the distribution of Na^+ between roots and shoots. In agreement with this notion, Na^+ concentrations in the xylem sap of *HvHKT2;1* overexpressor plants were considerably higher than those measured in xylem sap of control plants when lines were exposed to high Na^+ conditions (Figure 4-11). Taken together these results suggest that at high Na^+ concentration barley *HvHKT2;1* may be involved in xylem loading of Na^+ and therefore overexpressors show increased Na^+ content in leaves. However, during short term Na^+ uptake experiments significantly less Na^+ was found in leaves of *HvHKT2;1* overexpressors compared to control plants. Thus, in addition to xylem Na^+ loading *HvHKT2;1* may also participate in Na^+ exclusion from leaves via Na^+ removal from the xylem sap or Na^+ loading into phloem vessels as proposed previously (Berthomieu et al., 2003; Sunarpi et al., 2005). These different activities may dominate various periods after the onset of salt stress.

A possible role of *HvHKT2;1* in K^+ homeostasis can also be deduced from the obtained data. At high K^+ conditions, i.e. 50 mM K^+, the overexpressors show more K^+ in stems compared to control plants while at 50 mM NaCl conditions the K^+ content was relatively higher in control plants compared to the *HvHKT2;1* overexpressors. This suggests that at high K^+ levels, *HvHKT2;1* might also be involved in xylem loading of K^+ which is a key step for K^+ accumulation in the shoot (Takano et al., 2002). Indeed, after 50 mM K^+ treatment, xylem sap of *HvHKT2;1* overexpressors had a much higher K^+ concentration than control plants. This greater capacity to translocate K^+ to the shoot may be a disadvantage when external K^+ is high, resulting in accumulation of K^+ in shoots to toxic levels. Hence plants overexpressing *HvHKT2;1* showed lower relative growth compared to controls with 50 mM K^+ in the external medium.

In summary, the present study revealed that barley *HvHKT2;1* plays an important role in K^+ and Na^+ distribution at high K^+ and Na^+ concentrations. Moreover, the short term Na^+ uptake experiment also suggests a role of *HvHKT2;1* in the Na^+ recirculation through the phloem vasculature. Moreover the *HvHKT2;1* transporter protects leaves from salinity stress by mediating removal of Na^+ from the xylem sap via Na^+ uptake into xylem parenchyma cells and/or Na^+ loading into phloem vessels (Figure 4-6) as previously reported (Berthomieu et al., 2003). Overall the results reveal that barley *HvHKT2;1* plays an important role in K^+ and Na^+ distribution at high K^+ and Na^+ concentrations, as previously suggested (Uozumi et al., 2000; Horie et al., 2001). Future Studies investigating

the role of other *HvHKT* members will be interesting subject to elaborate the mechanism of salt tolerance in barley, a salt tolerant crop.

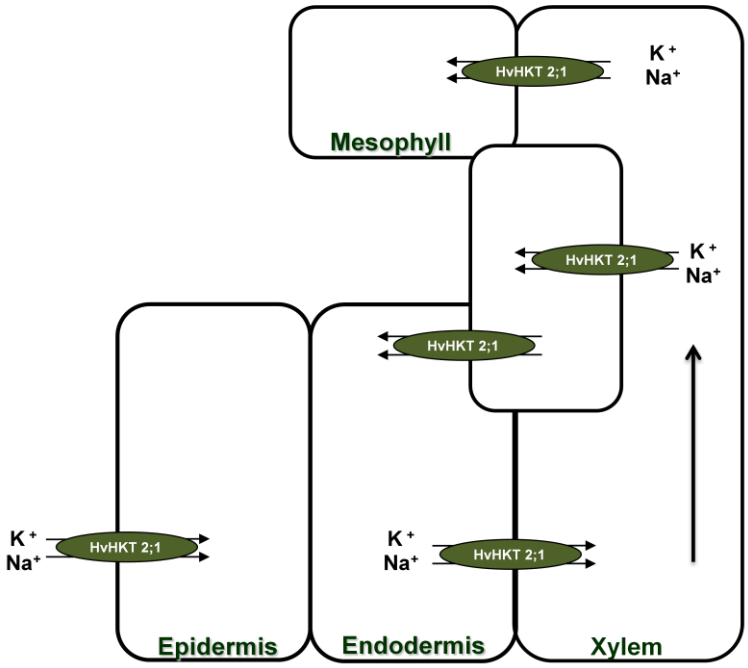


Figure 4-15: Putative functions of HvHKT2;1 transporter in controlling Na^+ and K^+ flux in barley. *HvHKT2;1* may transport K^+ - Na^+ into the system, it may mediate K^+-Na^+ xylem loading and unloading of Na^+ into xylem parenchyma cells and/or participate in the Na^+ recirculation through the phloem vasculature.

Chapter 5

5 Final Conclusions

5.1 The salinity problem

Salinity is a complex environmental stress which affects several aspects of plant physiology. Mainly it hinders the plant's ability to absorb water and nutrients from the soil, which leads to rapid reduction in growth and induces a wide range of metabolic processes (Tester and Davenport, 2003). Accumulation of salts to toxic levels in soil and water can either be from primary sources, which include weathering of minerals and salts from natural salt reservoirs, or secondary sources i.e. human activities, mainly irrigation with poor quality water (Rengasamy, 2006). Soil salinity has become a serious threat to agricultural productivity, especially in arid and semi arid regions of the world (Munns, 2002). It is estimated that almost 800 MHa of land throughout the world is affected by soil salinity or its associated condition of soil sodicity (FAO, 2008). Soil salinity can reduce crop productivity by more than 50 % (Bray, 2000). These facts suggest that reduction of soil salinity and an increase in salt tolerance of agriculturally important crops are vital for meeting the food requirements of the world.

5.2 Approaches to improve salt tolerance in crops

Improved crop production can be achieved either by changing the plant environment, making it suitable for normal growth, or by manipulating the genetic potential of plants allowing them to grow under high saline conditions. Soil reclamation, proper drainage and irrigation with good quality water are indeed effective strategies, however, these are costly and not a permanent solution to the problem (Singh and Singh, 2000). On the other hand, genetic manipulation of plants to improve their ability to tolerate high salt concentrations is considered effective, economical and a lasting approach to improve salt stress resistance (Flowers and Yeo, 1995). Different crops respond differently to salt stress (Marschner, 1995). Most agriculturally important plants are glycophytic but salt tolerant crops such as barley can potentially be exploited to improve the tolerance of sensitive crops. Genetic diversity within crops may also be used for improvement of salt tolerance via various breeding and selection schemes (Kingsbury and Epstein, 1984; Pecetti and Gorham, 1997;

Munns and James, 2003; Dasgupta et al., 2008; Lee et al., 2003). However, the complex and polygenic nature of salt stress tolerance has contributed towards the difficulties in breeding for salt tolerant crops (Flowers, 2004). The use of genetic engineering for salt tolerance by manipulating the expression of different categories of genes involved in cellular, physiological and biochemical mechanisms has been considered an efficient approach (Munns, 2006). Examples include genes that code for various membrane transporters that are involved in Na^+ exclusion at root soil boundary (SOS1, shi et al., 2002), Na^+ compartmentation in vacuole (NHXs, Zhang and Blumwald, 2001) or maintenance of higher K^+/Na^+ ratio (SKC1, Lin et al., 2004) under salt stress conditions. Understanding how ion transport at the cellular and whole plant levels contributes to salt tolerance will help in developing more resistant crops. Furthermore, by comparing these processes between glycophytes and halophytes we may identify specific transporters that form ideal targets for genetic engineering. Therefore, this research was focussed on characterizing various membrane transporters to improve salt stress resistance of important cereal crops like glycophytic rice and halophytic barley.

5.3 Na^+ and K^+ interaction and salinity stress

The maintenance of ion homeostasis and low water potential under salt stress conditions is crucial for a plant's ability to take up water and maintain turgor. Under salt stress conditions, Na^+ competes with K^+ uptake at the root soil boundary (Zhu, 2003), which leads to high Na^+ and low K^+ concentrations in the cytosol (Uozumi et al., 2000). K^+ is the most abundant and an essential inorganic macronutrient, crucial for numerous cell processes, such as enzyme activation, maintenance of osmotic homeostasis and regulation of stomatal movements (Shabala et al., 2008). Due to the chemical similarity of Na^+ and K^+, toxic levels of Na^+ in the cytosol inhibit the activity of enzymes and affect plant metabolism (Tester and Davenport, 2003). Under high salt conditions, efflux of Na^+, while maintaining a high K^+ concentration, is considered as an effective strategy for plant salt tolerance (Blumwald, 2000). Plants use both low- and high- affinity K^+ transport systems for adequate K^+ uptake and distribution. However, both these K^+ uptake systems, particularly the low affinity K^+ uptake system, are influenced by external Na^+ concentration (Kronzucker et al, 2006). Previous studies have reported that at least three different low affinity K^+ channels mediate passive Na^+ uptake into the cell, which include inward rectifying K^+ channels (Sentenac et al., 1992), outward rectifying K^+ channels (Maathuis and Sanders, 1995) and non selective cation channels (Maathuis and Amtmann,

1999). Moreover, high affinity K^+ transporters (HKTs) have also been reported to mediate Na^+ uptake and transport (Uozumi et al., 2000; Berthomieu et al., 2003). The present study focussed on the role of low affinity K^+ uptake channels and high affinity K^+ carriers and assessed their effect on salt tolerance in cereals using gain and loss-of-function approaches.

5.4 Gain-of-function approach for generating stable transformed lines using *Agrobacterium* mediated rice transformation

Gain-of-function is an important tool that can provide useful information about the role of a specific gene (Kuromori et al., 2009). Genetic modification of gene expression involved in various salt tolerance pathways has significantly improved salt tolerance in various plant species (Blumwald et al., 2000, Shi et al., 2003). Improvement in the Ti-plasmid based vectors and plant transformation techniques facilitated the use of *Agrobacterium* for generating stable expression of transgenes in a number of monocotyledonous plants including rice, wheat, sorghum and barley (Veluthambi et al., 2003). In the present study we used the *Agrobacterium* strain AGL1, with the pGreen and pSoup binary vector system to overexpress rice TPKs and the AKT1 gene in Japonica rice cultivar, Nipponbar. A Japonica rice cultivar was selected for its increased transformation efficiency as previously reported by Kumar et al., (2005).

In the present study, we found that three factors greatly influenced rice transformation efficiency. These include: selection and stage of explant (calli), concentration of the *Agrobacterium* suspension culture and the selection marker. Co-cultivation of three week old calli with an *Agrobacterium* suspension of OD 0.5 was found to be the best condition for infection. It was observed that washing of the calli after infection, greatly affected the transformation efficiency, while blotting of the calli after cocultivation on Whatman filter paper significantly reduced the browning of the calli. In the present study, hygromycin was found to be a more effective selective agent for the transformed rice calli compared to kanamycin, as previously reported by Maneewan et al., (2005).

As far as the practicability of the visual marker gene was concerned, the GUS reporter gene was found to be more effective compared to GFP, as strong autofluorescence was detected for calli for GFP. *Agrobacterium* mediated transformation is an effective, simple and efficient strategy for genetic modification of rice, however, somaclonal variation during tissue culturing, frequency of non-transgenic plants escaping the selection process and albinism, are several issues that need to be addressed.

Although these methods generate transgenic plants suitable for characterisation, the presence of antibiotic or herbicide resistance genes in genetically modified organisms can provoke public concern and hence should be avoided in food crops. Alternative screening method are now available such as systems that utilize native plant genes including the anthranilate synthase alpha-subunit gene and positive selection systems, such as benzyladenine N-3-glucuronide, mannose and nitrite reductase (Ozawa, 2009).

5.5 Role of rice TPK channels in K^+ nutrition and salt tolerance

Plant vacuoles are versatile organelles that are crucial to physical and metabolic functions of cells. They are also involved in cellular responses to environmental and biotic stresses (Rosado and Raikhel, 2010). In vegetative cells, vacuoles regulate the turgor while in seeds and specialized storage tissues, they serve as reservoirs for protein and soluble carbohydrates. Vacuoles are considered as the major subcellular organelles for storage of K^+ in plant cells (Very and Sentenac, 2002). Recent reports suggest that accumulation of K^+ in vacuoles is mediated by K^+/H^+ antiporters (Leidi et al., 2010; Rodruuez-Rosales et al., 2010). K^+ content in the vacuole is variable, however the cytoplasmic K^+ concentration is maintained around 100 mM (Cuin et al., 2003). Different Vacuolar channels like SV, FV and VK channels at the tonoplast membrane are proposed to mediate K^+ exchange across the tonoplast (Isayenkov et al., 2010). TPKs represent the VK channels, found in guard cells and also in other cell types derived from root and shoot tissues (Gobert et al., 2007; Latz et al., 2007).

Rice has multiple TPK genes and localization of *OsTPKa* and *OsTPKb* has been studied in our laboratory at The University of York. It was observed that *OsTPKa* is localized in lytic vacuoles while *OsTPKb* is found in protein storage vacuoles (Isayenkove et al., Unpublished). To study the role of TPK channels in K^+ distribution and salt tolerance, we generated stable transgenic lines overexpressing rice *TPKa* and *TPKb* under control of a 35S promoter using an *Agrobacterium* mediated transformation system.

Although screening for the homozygous lines was a time consuming process, it was possible to identify stable transgenic lines showing variable levels of *OsTPKa* and *OsTPKb* expression. The localization of *OsTPka* and *OsTPKb* in plant lytic and protein storage vacuoles respectively (Isayenkove et al., Unpublished) suggests that rice TPKs might be involved in K^+ regulation across the tonoplast under stress conditions as previously reported for *AtTPK1* (Gobert et al., 2007). Growth, ion content, and xylem sap analyses with transgenic lines revealed that rice *TPKa* has an important role in the plant's ability to

tolerate various K^+ stress conditions by maintaining higher tissue K^+ content and redistribution of K^+ in shoots and roots to maintain turgor and facilitate cell expansion at low and high K^+ levels as described in Chapter 2. Similar results were reported for *Arabidopsis AtTPK1* (Gobert et al., 2007). However, *TPKa* overexpressors did not show any growth phenotype under salt stress conditions. In contrast, transgenic lines overexpressing *TPKb* showed improved growth under all K^+ and Na^+ stress conditions, suggesting that TPK channels play crucial roles in K^+ nutrition and in maintaining a higher K^+/Na^+ ratio under different K^+ and Na^+ stress conditions. This confirms the previous idea that an increased level of K^+ over Na^+ in the shoot is an effective strategy for maintaining cellular metabolism (Zhu, 2003). TPKb might also play an important role in regulation of enzymes responsible for biosynthesis of osmolytes that help the plant to maintain ion homeostasis (Isayenkov et al., 2010). Moreover influx of K^+ into the vacuole via *TPKb* can encourage water influx and increases in vacuolar volume during cell growth. This increase in vacuolar volume would facilitate turgor maintenance and was proposed to be an important mechanism in plant salt tolerance (Mimura et al., 2003). It will be interesting to analyze the total ion content in the seeds of rice *OsTPKb* overexpressing transgenic lines and study the role of *OsTPKb i*n K^+ and Nitrogen transport as K^+ has been reported to significantly affect NO_3^- uptake and translocation (Ruiz and Romero, 2002). The effect of TPKb activity on seed germination, nutritional value and seed morphology are other factors that need studying.

5.6 The role of the rice inward rectifying K^+ channel OsAKT1 in plant nutrition under different K^+ and Na^+ stress conditions.

Although potassium channels show high specificity for K^+ over other alkali cations (Maathuis et al., 1997; Amtmann and Sanders, 1999), a role for *AKT1* in Na^+ uptake has also been proposed when increases in external Na^+ concentration occur (Amtmann and Sanders, 1999; Blumwald, 2000). In the present study, the role of *OsAKT1* was investigated under different K^+ and Na^+ stress conditions, using loss of function mutations and overexpression. Growth analyses revealed improved growth rates for AKT overexpressors in high external K^+ conditions. Ion content analyses revealed higher K^+ content in the roots and higher K^+/Na^+ ratios in the shoots of the overexpressors at high external K^+ concentrations. Previous studies on inward-rectifying K^+ channels suggested the involvement of K^+ channels in K^+ uptake in both low-affinity and high-affinity range

under particular experimental conditions (Schroeder and Fang, 1991; Schroeder et al., 1994; Duby et al., 2008). Rice AKT1 overexpressors also showed higher Na^+ uptake at low K^+ and higher external Na^+ concentrations compared to wild type. Buschmann et al., (2000) also reported the involvement of *TaAKT1* in Na^+ uptake in K^+ depleted conditions. In addition, results suggest the involvement of *OsAKT1* in uptake of Na^+ at high external Na^+ concentrations. Similar results were reported by Wang et al., (2007), who reported the involvement of *AKT1* in Na^+ uptake at higher external concentration while studying *AKT1* in *S. Maritime*. Overall, the experimental data suggest that rice *AKT1* might be involved in Na^+ uptake and pose a negative effect on salt stress tolerance of the plants under 0 K^+ or salt stress conditions. However, AKT1 overexpressors showed improved growth under high K^+ stress conditions suggesting that AKT1 channel might be involved in K^+ homeostasis and distribution under these conditions.

5.7 Proposed model of the physiological role of TPKs and AKT1 in rice

The data suggest that AKT1 is also involved in K^+ uptake at high external K^+ concentrations and in Na^+ distribution from root to shoot as discussed in Chapter 3. Rice TPKs are expressed in plant vacuoles and likely involved in K^+ release from the lytic and protein storage vacuoles under salt stress condition. In this manner TPKs may facilitate ion homeostasis in the cytoplasm (Figure 5-1).

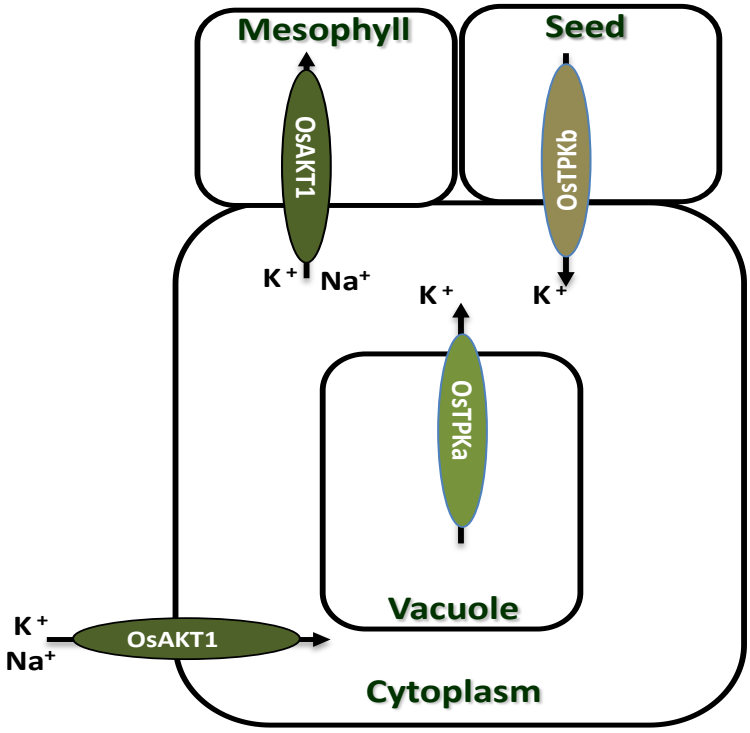


Figure 5-1: Putative functions of rice *TPKa*, *TPKb* and *AKT1*. *TPKs* may be involved in K^+ release from vacuoles and maintain K^+/Na^+ homeostasis during stress conditions while *AKT1* may transport K^+ and/or Na^+ into the system. Moreover AKT1 may also mediate K^+-Na^+ redistribution from root to shoot.

5.8 Physiological characterisation of HvHKT2;1 and its role in salt tolerance of barley

The HKT (high affinity K transporter) family has many members and is functionally quite diverse as discussed in Chapter 4. In barley, five HKT isoforms are reported (Huang et al., 2008): only *HvHKT2;1* has been studied, which has 92% homology with *TaHKT2;1* at the nucleotide level and both were shown to be Na^+ - K^+ co-transporters in yeast (Rubio et al., 1995; Haro et al., 2005). The role of *HvHKT2;1* in barley salt tolerance and K^+ nutrition was determined in the present study by employing an overexpression approach. Data from our experiments showed improved growth of the *HvHKT2;1* overexpressors under salt stress conditions, suggesting HKT2;1 has a significant physiological role in salt tolerance of barley. Expression analyses of the *HvHKT2;1* from our experiments suggest that barley *HvHKT2;1* is localized in the cortex of the roots. The tissue specificity of *HvHKT2;1* expression in this study was consistent with the pattern previously reported for the HKT gene members of the subfamily II in rice plants (Horie et al. 2007). Moreover, the expression analyses under different stress conditions revealed upregulation of *HvHKT2;1* under high K^+ and high Na^+ conditions in the stems consistent with the notion for the involvement of *HvHKT2;1* in Na^+

and K^+ redistribution through the phloem. The role of *HvHKT2;1* in Na^+ and K^+ redistribution was confirmed by short term uptake experiments which showed significantly lower Na^+ content in leaves. Berthomieu et al., (2003) reported similar results showing overaccumulation of Na^+ in the loss-of-function mutants in *AtHKT1* and suggested a role of *AtHKT1* in Na^+ redistribution through the phloem. However when plants were exposed to high levels of salt for longer periods, ion content analyses showed increased Na^+ content in the leaves suggesting that *HvHKT2;1* might be involved in xylem loading of Na^+. This was confirmed in the xylem sap analyses which showed increased Na^+ content in the xylem sap under salt stress conditions. These results suggested that *HvHKT2;1* might be involved in Na^+ transport to older leaves, which is a useful salt tolerance strategy and halophytes can also use Na^+ as osmoticum against the external toxic environment.

5.9 Conclusions

The importance of K^+ and Na^+ transport systems and their role in maintaining cytosolic ion homeostasis under saline conditions has increased considerably in recent years. Prevention of Na^+ entry, efflux or compartmentation of Na^+ from the system while maintaining higher K^+ content are effective strategies commonly used by plants to maintain a desirable $K^{+/}Na^+$ ratio in the cytosol (Hasegawa et al., 2000; Tester and Davenport, 2003; Chen et al., 2007). In the present study, rice plants overexpressing *TPKa* and *TPKb* showed higher K^+/Na^+ ratios and improved growth under different K^+ and Na^+ stress conditions. Although rice *TPKa* overexpressing lines did not show any growth phenotype under salt stress conditions, the performance of *TPKa* overexpressors at different K^+ stress conditions was better compared to control lines. Moreover, transgenic line overexpressing *TPKb* showed improved growth under all K^+ and Na^+ stress conditions, suggesting TPK channels play a crucial role in K^+ nutrition and maintain higher K^+/Na^+ ratio under different K^+ and Na^+ stress conditions. The data from characterisation of rice AKT1 mutants and overexpressors showed the involvement of AKT1 channels in Na^+ uptake at low $[K^+]_{ext}$ or high $[Na^+]_{ext}$ concentrations and resulted in reduced growth under these conditions. On the other hand, data from experiments with barley HKT2;1 overexpressing lines showed improved growth under salt stress conditions via Na^+ redistribution or accumulation of excessive Na^+ in the shoots. These results confirm that prevention of salt uptake or maintenance of higher K^+/Na^+ ratios in the plant to maintain ion homeostasis is crucial for salt tolerance of glycophytic rice. However, the halophytic crop barley showed improved salt tolerance in spite of increased Na^+ accumulation in leaves.

Overall, the use of *Agrobacterium* mediated transformation to generate stable overexpressing lines proved to be an effective strategy to study the role of various important genes in salt tolerance of plants. These studies may be further refined by the use of tissue specific promoters rather than the constitutive 35S promoter used here. The transgenic approach not only helped us understand how individual channels and carriers influence crop salt tolerance but is also ideal for the generation of multiple lines to further increase beneficial and synergistic phenotypes.

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