THE ROLE OF CATION CHANNELS IN ABIOTIC STRESS RESISTANCE IN RICE

Izhar Ahmad

PhD

University of York

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Abstract

The alarmingly increasing human population needs improved food production but this aim is hampered by different abiotic stresses. Osmotic stress and salt stress are the two prominent examples of abiotic stresses and affect up to 50% of the arable land. These stresses severely affect all plants, but glycophytes (e.g. rice) are especially sensitive. During stress, nutrient uptake, such as K^+, is often disturbed. Thus, better K^+ nutrition and distribution play a vital role in plant abiotic stress tolerance. To improve K^+ nutrition, the role of K^+ transporters is likely to be essential. Loss of function and gain of function approaches could help establish the exact function of transporters involved in K^+ nutrition.

Rice TPKs and AKT1 are K^+ channels which are localised to the tonoplast and plasma membrane respectively. The two TPK isoforms, TPKa and TPKb, are localised to the tonoplast of LV and SV respectively. They were characterized in a variety of abiotic stress conditions. The data showed better growth and higher K^+ concentration for the TPKa and TPKb transgenic lines when grown in zero K^+ and osmotic stress conditions suggesting their role in improving in K^+ nutrition. TPKs have no direct involvement in the K^+ uptake, but somehow influence K^+ uptake and improve K^+ nutrition. The higher K^+ concentration in the leaves of overexpressor plants suggested the involvement of TPKs in the distribution of K^+ within the plant body. TPKs play a role in the guard cells' movements and affect the stomatal conductance and therefore showed a better response to the osmotic stress conditions.

The role of rice AKT1 was tested by comparing the knockout and overexpressing lines of AKT1 with the wild type plants. The data suggested that AKT1 is involved in the K^+ uptake in a range of external K^+ concentrations and osmotic stress conditions. The role of AKT1 is obvious in the K^+ deficient conditions where NH_4^+ is present. The leaf K^+ concentration suggested that AKT1 influences K^+ transport into the leaves. The K^+ concentration in the leaf cells showed an effect on the stomatal conductance and in turn an effect on the growth phenotype under zero K^+ and osmotic stress conditions. The data revealed that AKT1 is insensitive to NH_4^+ toxicity.

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Author's declaration

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

I have contributed to the following review papers.

Chapter 1

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Chapter 1

1 General introduction

K^+ is an extremely important nutrient for all living organisms. K^+ fulfils both biophysical and biochemical roles which affect many aspects such as plant growth, tolerance to biotic and abiotic stresses and movement of plant organs. Many of these functions depend on relatively high (~100 mM) concentrations of K^+ and occur in all cells and all parts of the plant. Plants therefore need efficient systems to distribute K^+ to various organs and tissues, often over long distances (Figure 1-4). Furthermore, the partitioning of K^+ within cells, for example between cytoplasm and vacuole, also impacts on many of the above listed functions and this too is under strict control.

Many of the functions that K^+ plays are hampered by drought and salt stress as these tend to lower K^+ acquisition from the surrounding. Therefore, improved K^+ nutrition could help the plant to increase resistance against these abiotic stresses.

1.1 Plant abiotic stresses

Global food production needs to increase by about 50% by 2050 to support the alarmingly increasing human population. This aim is frustrated by a variety of biotic and abiotic stresses which decrease plant growth and productivity. Among the biotic stresses, plants face challenges from pathogens including bacteria, fungi, and viruses as well as from herbivores. Abiotic stress, in fact, is the principal cause of crop failure world-wide, reducing average yields for most major crops by more than 50% (Bray *et al.*, 2000). These abiotic stresses include low temperature, salt, drought, flooding, heat, oxidative stress and heavy metal toxicity. All these stress factors are a menace for plants and prevent them from reaching their full genetic potential and limit the crop productivity worldwide.

1.1.1 Drought stress

Drought is a condition of soil where limited amounts of water are available for the absorption by plants. Drought may be physical when there is deficiency of water for the plants to absorb, for example meteorological drought, or it may be physiological drought where a plant is unable to absorb water because of certain conditions for

example, low temperature, frozen water, higher rate of transpiration than the absorption or available water is held to substances in solution which hinders its absorption by the plant. In the latter case the osmotic stress may be created by salts like NaCl. Drought causes osmotic stress as caused by the salt stress and in response plants suffer from the difficulty to absorb water. It is estimated that about 30% of the world's land is affected by drought (Chaves *et al.,* 2009). In other words, we can say that only 16% of the world's crops get enough water for absorption through irrigation or rainfall and the rest of the crops encounter drought either for short or long periods. Crops growing in sandy soils are more severely affected than those on clay soil because of the difference of water holding capacity. Severe drought in some parts of the world, especially in arid and semi-arid regions, in recent years drastically reduced crop yields and disrupted regional economies. Even in average years, however, many agricultural regions suffer from chronic moisture deficits. Cereal crops (mostly glycophytes) typically attain only about 25% of their potential yield due to the effects of environmental stress, with moisture stress the most important cause. Drought is considered similar to salt stress because responses to both salt and drought stress are related and the mechanisms overlap. However the effects of drought stress are more widespread and damaging for crops (Boyer, 1982).

1.1.2 Salt stress

Salinity is a condition of soil where the concentration of ions of soluble salts (e.g. Na^+, Ca^{2+}, Cl^-, SO_4^{2-} and HCO_3^-) exceeds the normal limits. Salinity is measured as soil electrical conductivity and when it exceeds 4 dS/m (decisiemens per meter), the soil is said to be saline (Salinity Laboratory Staff report, 1954); 4 dS/m is equivalent to approximately 40 mM NaCl.

Salinization of soil is either primary or secondary. The primary sources of salinization include natural ways, e.g. the weathering of rocks and oceanic salts brought by rains. In the coastal salt marshes and inland deserts, weathering of parental rock releases various types of salts, for example chlorides of sodium, calcium, and magnesium, and to a lesser extent, sulfates and carbonates to the soil (Szabolcs, 1989). However, these areas are not really important for agriculture.

Another reason for salinization is the deposition of oceanic salts brought by wind and rain. Rainwater contains 6–50 mg/kg of sodium chloride; the concentration decreases

with distance from the seashore. Rain containing 10 mg/kg of sodium chloride would deposit 10 kg/ha of salt for each 100 mm of rainfall per year (Flowers and Flowers, 2005; Munns and Tester, 2008).

Irrigation and deforestation are the causal agents of secondary salinization and is a substantial threat to farming. Excessive irrigation raises the water table and brings salts to the surface which, after evaporation of the water, leads to salts accumulating on the soil surface, causing salinization of the soil. Out of the approximately 1500 Mha non-irrigated cultivated land, only 2% are affected by salinity whereas 20% of the 230 Mha irrigated, cultivated land is affected by salinity (Munns and Tester 2008).

Every year, approximately 10 Mha land is taking over by salinity (Flowers and Yeo, 1995) and increases in the sea level due to climatic change will also increase salinity. It is calculated that the ocean level will go up by 1 m in the next 500 years as a consequence of planetary warming (Gordon and O'Farrell *et al.,* 1997).

Different plants have different responses to saline conditions. Some are salt tolerant (halophytes), having extensive genetic diversity over a range of taxa (Flowers *et al.,* 1986; Greenway and Munns, 1980). Halophytes have the capacity to accommodate a range of salt concentrations for which they have special anatomical and morphological adaptations (Flowers *et al.,* 1986). However, most species are salt sensitive (glycophytes) and many crops are included in this category. Extensive research through the years has shown that most of the strategies against salt stress for both the halophytes and glycophytes are similar.

The role of Na^+ in salt stress

It has been repeatedly commented that Na^+ (rather than Cl^-) is a main player of the toxic effects of salinity inside the cells. Plants can keep low Na^+ in the cytoplasm and a high K^+: Na^+ ratio and therefore, can alleviate the toxic effects of salinity. The reason for Na^+ being more toxic than Cl^- stems from the notion that Na^+ inhibits enzyme activity, and this is particularly the case for many enzymes that require K^+ for functioning (Maathuis, 2009). For example, the K^+ dependent pyruvate kinase has a Km (for K^+ binding) of around 10 mM. Na^+ can also bind but has only 5-10% of the stimulating effect of K^+ and thus severely inhibits kinase action.

The frequency and severity of such Na^+ toxicity effects depends on the cytoplasmic Na^+ concentration ($[Na^+]_{cyt}$). Unfortunately, accurate measurements of $[Na^+]_{cyt}$ are still relatively scarce and those that have been reported vary greatly. Carden *et al.,* (2003) using microelectrodes, measured 10-30 mM steady state levels of Na^+ in the cytoplasm of barley cells. Kronzucker *et al.,* (2006), using flux analysis, reported $[Na^+]_{cyt}$ values of over 300 mM while measurements with fluorescent dyes yielded estimates from 20-60 mM (e.g. Anil *et al.,* 2007). In all, it is likely that $[Na^+]_{cyt}$ is in the tens of millimols and thus prone to negatively affect enzyme activity.

1.2 Effects of drought and salinity

Both drought and salinity lead to osmotic stress. Drought and salt stress can lead to higher ion and solute concentrations and then ion toxicity within plants. Salinity of soil and water affects the plants in two ways primarily. High concentrations of salts in the root environment make it difficult for the roots to absorb water so causing osmotic stress. Accumulation of toxic ions, either due to the absorption of NaCl from the surroundings or due to the loss of water from the cytoplasm, increases salt concentration that leads to detrimental effects. Salts on the outside of the roots have an immediate effect on cell growth and associated metabolism; toxic concentrations of salts take time to accumulate inside plants before they affect plant function.

1.2.1 Growth

Salinity and drought result in stunted growth of plants and this is primarily due to osmotic stress caused by drought and salinity. After an increase in osmotic stress cells lose water resulting in turgor loss, however this is temporary as cells resume their normal size after making some adjustments. Yeo *et al.,* (1991) reported that an addition of 50 mM NaCl to the culture solution stops leaf elongation of rice genotypes IR2153 and Pokkali for 20 min, but after 24h the elongation rate had returned to the non-salinized control rate. Similar results were shown by Fricke *et al.,* (2004) for barley. Drought causes impaired germination and seedling growth in some plants while in others it affects the vegetative stages of growth. Other than turgor effects, the lower growth might also be because of the slower elongation and division of the leaf cells which results in the slow appearance of new leaves and smaller final size. Under osmotic stress conditions, the water flow from the xylem to the neighbouring elongating

cells is interrupted and thus affects cell elongation (Nonami, 1998). Due to the changes in cell dimension, there is more reduction in leaf area as compared with the thickness so thicker leaves result. Drought affects many complex integrating physiological processes which are responsible for the yield of crops. Drought negatively affects the number of the tillers in barley and in wheat reduces the dry weight of the kernels (Wardlaw and Willenbrink, 2000). Moderate osmotic stress initially affects the development of lateral shoots, but longer exposure ultimately affects the reproductive development, such as early flowering and reduced number of floral leaves. Osmotic stress also leads to barrenness of flowers thus affecting the productivity of the crops. Older leaves may die, but plants continue to produce younger leaves.

1.2.2 Photosynthesis and stomatal conductance

Drought and salinity cause a dramatic decrease in the stomatal pore aperture as a first response to these stresses. The immediate decrease in the stomatal conductance is through lower water availability and then via ABA synthesis (Fricke *et al.,* 2004). Osmotic stress may not affect the rate of photosynthesis per unit leaf area, although the stomatal conductance is reduced (James *et al.,* 2002). This is because osmotic stress results in changes in cell anatomy resulting in smaller and thicker leaves, thus resulting in a higher density of chloroplasts per unit leaf area (Munns and Tester, 2008). Salinity causes reduction in the total leaf area and this reduction always leads to the reduced rate of photosynthesis per plant.

It is hard to explain whether the reduced rate of photosynthesis is the only reason for the lower growth under saline conditions. Salinity affects the leaf size and shape as described earlier and also causes an increase in the unused assimilates which increases storage of carbohydrates (Munns, 2000). Paul and Foyer, (2001) showed that after long exposure to salinity, plants can fine-tune photosynthesis according to demand. In a nut shell, long exposure to salinity reduces the rate of photosynthesis because of reduced demand for photosynthate. The reduced rate of photosynthesis is due to the decline in Rubisco activity (Bota *et al.,* 2004). This reduced activity is because of the more viscous cytoplasm due to dehydration. Increased viscosity leads to an increase in protein-protein interaction, protein denaturation and toxicity having harmful effects on the enzymes including photosynthetic enzymes (Hoekstra *et al.,* 2001).

1.2.3 Oxidative stress

The biological and physiological effects of certain environmental stresses, including salt and drought, induce reduced water potential, ion imbalance, reduced CO_2 assimilation and reduced rate of photosynthesis. The limited CO_2 availability and reduced rate of photosynthesis leads to enhanced production of reactive oxygen species (ROS) including superoxide radical (O^{2-}), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and singlet oxygen ($^1O_2$), leading to oxidative stress. Osmotic stress promotes the generation of superoxide in plant cells because of impaired electron transport in the chloroplasts (Price *et al.,* 1989). The overproduction of ROS triggers toxic reactions such as degradation of lipids and proteins (Jiang and Zhang, 2001; Bor *et al.,* 2003). In response to the production of ROS plant cells produce increasing amounts of enzymatic and non-enzymatic compounds to detoxify the ROS. Superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) are the enzymatic antioxidants while the non-enzymatic antioxidants include water soluble components like ascorbic acid, glutathione, flavonoids and the lipid soluble components such as carotenoids and α-tocopherol. A correlation between antioxidant capacity and osmotic stress has been demonstrated in several plant species (Broetto *et al.*, 2002; Bor*et al.,* 2003; Agarwal and Pandey 2004; DiBaccioetal, 2004; Amor *et al.,* 2005).

Chawla *et al.,* (2013) characterized the role of ROS scavenging systems in combating oxidative stress, such as enzymes and metabolites of the anti-oxidant system in leaves of salt-sensitive and salt-tolerant cultivars of rice. They showed that SOD activity increased progressively with increases in salinity in salt-tolerant cultivars but receded in the sensitive varieties. Increase in SOD activity upon salinization in leaves of all the tolerant cultivars could accelerate dismutation of superoxide ions generated upon salt-treatment, which may allow such varieties to better survive oxidative stress (Desingh and Kanagaraj 2007). In salt-sensitive varieties, reduction in SOD activity would limit their metabolic capacity to withstand oxidative stress. Wang *et al.,* (2005) reported that an increase of about 1.4-fold in total SOD activity in the *pMnSOD* transgenic rice plants was enough to increase oxidative stress resistance and drought tolerance. Indeed, transgenic rice overexpressing a yeast Mn SOD was shown to have improved salinity tolerance (Tanaka *et al.,* 1999).

1.2.4 Nutritional imbalance

Water deficit, either due to drought or salinity, results in limited nutrient uptake and transport to the shoots and thus lowers nutrient concentrations in the tissues. Drought affects the stomatal aperture and lowers the transpiration rate and ultimately absorption of water by the roots. Less water absorption can affect the uptake of nutrients directly but drought can also affect nutrient uptake due to limited availability of energy. Under lower moisture availability, N and K^+ uptake is inhibited (McWilliams, 2003) and P and PO_4^{3-} contents are decreased in the plant tissues (Peuke and Rennenberg, 2004).

As far as the effects of salinity are concerned, osmotic stress is followed by the accumulation of toxic ions. The later causes imbalance of cellular metabolism (Munns *et al.*, 2006), nutritional imbalance and oxidative stress (Alscher *et al.*, 1997). Ion uptake is directly affected when excess Na^+ and Cl^- accumulate and hence increases the ratios of Na^+: K^+, Ca^{2+}: Mg^{2+} and Cl^-: NO_3^-. Among these ions, Na^+ is generally considered to be the most toxic. Na^+ is believed to enter cells passively, but the mechanism is not yet completely known. Among the large number of toxic effects of salinity, ionic imbalance is particularly important where K^+ is concerned. Potassium activity is reduced and it becomes less available for plants because Na^+ directly competes with K^+. This occurs partly because both ions can be transported by the same ion channels on the plasma membrane (Shabala *et al.*, 2005, Demidchik and Maathuis, 2007).

1.3 Strategies to cope with drought and salinity

Plants have certain morphological, physiological and biochemical mechanisms helping them against drought and salt stress conditions. Plants respond to water stress by complex mechanisms, from changes in expression of genes, biochemical metabolism through to individual plant physiological processes to ecosystem levels (Chaves *et al.*,2003; Izanloo *et al.*, 2008; Xu *et al.*, 2009) which may mainly include six aspects: (1) drought escape via completing the plant life cycle before severe water deficit e.g. earlier flowering in annual species before the onset of severe drought (Geber and Dawson, 1990); (2) drought avoidance via enhancing capacity of getting water, e.g., developing root systems or water conservation via reduction of stomata and leaf area/canopy cover (Schulze, 1986; Jackson *et al.*, 2000); (3) drought tolerance, mainly via improving osmotic adjustment ability and increasing cell wall elasticity to maintain

tissue turgidity (Morgan, 1984); (4) drought resistance via altering metabolic pathways to survive under severe stress (e.g., increased antioxidant metabolism) (Bartoli *et al.*, 1999; Penueles *et al.,* 2004); (5) drought abandon by removing a part of the individual, e.g., shedding older leaves under water stress (Chaves *et al.,* 2003); (6) drought-prone biochemical-physiological traits for plant evolution under long-term drought via genetic mutation and genetic modification (Hoffmann and Merila, 1999; Maherali *et al.,* 2010).

Many biochemical and physiological processes are induced by osmotic stress in plants such as stomatal closure, repression of cell growth and photosynthesis, and activation of respiration. Plants store specifically those proteins and osmolytes which are involved in stress resistance as a response to osmotic stress (Shinozaki *et al.*, 2003; Bartels and Sunkar, 2005; Yamaguchi-Shinozaki and Shinozaki, 2005). In the past 10-15 years a number of stress-inducible genes has been identified in *Arabidopsis* and rice. Most of the gene products may function in stress response and tolerance at the cellular level (Zhang *et al.*, 2004; Umezawa *et al*., 2006*a*). The products of stress inducible genes are classified into two groups in rice and *Arabidopsis*. The first group contains proteins which may function in abiotic stress tolerance for example water channel proteins and sugar and proline transporters. The second group contains regulatory proteins for example transcription factors and protein kinases (Shinozaki and Yamaguchi-Shinozaki 2007). These proteins are involved in a variety of processes and help in abiotic stress resistance. For example protein kinases and enzymes involved in ABA biosynthesis, are useful for improving stress tolerance by regulating multiple stress-related genes in transgenic plants. ABA is synthesized *de novo* primarily in response to drought and high salinity stress. Genes involved in ABA biosynthesis and catabolism were identified based on genetic and genomics analyses (Nambara and Marion-Poll, 2005).

1.3.1 Osmotic adjustments

Plants survive under osmotic stress by decreasing their osmotic potential and thus maintaining turgor of the cells. Accumulation of solutes helps to keep the osmotic potential low and to attract more water to enter the cells.

The overexpression of genes responsible for the synthesis of organic osmolytes e.g. proline, mannitol, sorbitol and glycine betaine is an important strategy to improve osmotic stress tolerance. Organic osmolytes can accumulate in the cytosol in high

quantities and are not interfering with the cell metabolic processes. These osmolytes are involved in cell osmotic adjustments and osmoprotection of various membrane structures and proteins. Recent advances in molecular biology have made the osmolytes production toward osmotic stress tolerance more central and convincing. This is largely because of the fact that osmolytes accumulation is often controlled by one gene (Serraj and Sinclair, 2002) so easy to manipulate. Many reports show a positive correlation between osmolytes accumulation and salt tolerance. However other studies do not support this idea especially those using transgenic plants where differences between the wild type and transgenic plants are either small or restricted to a special growth period. There are several other drawbacks in this approach for example high energy consumption, increased susceptibility to fungal diseases etc. (Shabala and Cuin, 2007). Thus the practical applications of this approach are limited (Bajaj *et al.,* 1999) and hardly effective under field conditions (Flowers, 2004). Other than these organic osmolytes, some inorganic ions for example K^+ and Na^+ can also play a role to maintain water potential and help plants to absorb and maintain water within the cells.

1.3.2 Regulation of aquaporins water channels

Transpiration is the driving force for the water absorption especially through the apoplastic pathway (Horie *et al.,* 2011) but osmotic stress leads to reduction in water uptake due to the closure of stomata. However symplastic and vacuolar pathways are under the influence of differential water potentials between the outer and inner environments. Plants minimize the efflux of water upon osmotic stress by lowering the hydraulic permeability (Lp_r) of root cells. This lower efflux of water is achieved either by the accumulation of solutes or due to changes in the expression of relevant genes. The latter process is relatively quick and operates within hours.

Reductions in Lp_r were recorded upon salt stress in *Arabidopsis* and maize (at 100 mM NaCl) and in barley (at 200 mM NaCl) (see review Horie *et al.,* 2012).

Water permeability of the membranes is highly dependent on water channels, aquaporins or major intrinsic proteins (MIPs). Plasma membrane intrinsic proteins (PIPs) and tonoplast intrinsic proteins (TIPs) are two common groups of aquaporins. They are represented by large number of isoforms in plants. PIPs are the most abundant members of the aquaporins and help in water absorption. PIPs are a predominant component of Lp_r. Down regulation of PIPs was reported in the root cells of maize and

Arabidopsis (Maurel et al. 2008) and barley (Horie *et al.*, 2011) under salt stress conditions. Boursiac *et al.*, (2008) suggested that salinity stimulates the accumulation of ROS. These ROS then redistribute PIP from the plasma membrane to internal compartments which could lead to the rapid down-regulation of the Lp_r. Rice on the other hand showed no post-translational regulation of PIPs under salinity stress conditions, however reduced mRNA levels were detected for PIPs and TIPs in rice under osmotic stress conditions (Guo et al. 2006; Li et al. 2008). These results suggest that plant aquaporins could be involved in salt tolerant mechanisms by repressing the activity of water channels under salinity stress (Horie *et al.*, 2012).

1.3.3 Biological membranes

Osmotic stress affects biological membranes therefore affecting many physiological processes. The exact mechanism of how osmotic stress leads to membrane disruption is not clear. However it can be argued that osmotic stress results in lowering the cell volume, congestion, more interaction of the chemicals inside the cell and denaturation of proteins. Wang and Huang, (2004) reported damage to the cell membrane of blue grass when exposed to drought and heat. Many compounds have been identified that can prevent damage to biological membranes during osmotic stress. Some of these compounds are proline, glutamic acid, mannitol, trehalose, etc. (Folk *et al.*, 2001). Better K^+ nutrition also improves the stability of the membranes and ultimately increases drought tolerance (Gnanasiri *et al.*, 1991).

1.3.4 Control of Na^+ transport

Over-accumulation of Na^+ in the tissues during salinity stress negatively affects the essential processes of the plants e.g. protein synthesis, enzyme activity and, in the case of cells that compose the source organ, photosynthesis (Yeo and Flowers, 1986; Glenn *et al.*, 1999; Tsugane *et al.*, 1999; Blaha *et al.*, 2000). The effective strategies for glycophytes to cope with salinity are to keep the cytoplasmic and tissue levels of Na^+ low. Exclusion of sodium from the root uptake (Munns *et al.*, 1999) is not enough to solve the problem of osmotic stress caused by salinity. Water will still leak out of the plant due to higher osmotic potential outside.

Thus Na^+ exclusion on its own would not be the best strategy to cope with salinity. The conviction that the higher Na^+ content is negatively correlated with salt tolerance (Munns, 2005; Tester and Davenport, 2003) is not true in many cases. For example halophytes are capable of accumulating large amounts of Na^+, up to 50% of their dry weights, and still can survive. The argument that the higher content of Na^+ in halophytes may depend on their growth for longer period under saline condition (Shabala and Cuin, 2007) is challenged by the fact that halophytes are showing optimal growth at salinities ranging from 200 to 400 mM NaCl (Khan *et al.*, 2005).

1.3.4.1 Na^+ Uptake and Distribution

The cytoplasmic Na^+ concentration needs to be kept low to avoid Na^+ toxicity. A control system is required for this purpose. Na^+ concentration could be kept low either by removing the excess of Na^+ from the cells to the outer environment or distributing Na^+ to other tissues of the plants. The observed values of cytoplasmic Na^+ concentration are much lower than the thermodynamic equilibrium concentration. This shows that Na^+ extrusion is present. We can compare the Na^+ intake by the roots and net Na^+ concentration within the root cells. A plant which contains ~200 mmol Na^+ per kg FW and has a relative growth rate (RGR) of 10% day^{-1}, requires only a net Na^+ influx of around 800 nmol g^{-1} h^{-1} to stay at this level of cellular [Na^+]. However, experimentally obtained unidirectional Na^+ influx (e.g. measured in roots using radioactive Na^+) is typically 10 times higher than the above (e.g. Maathuis and Sanders, 2001). This implies that ~90% of Na^+ that initially entered the symplast is subsequently removed by Na^+ extrusion into the external medium.

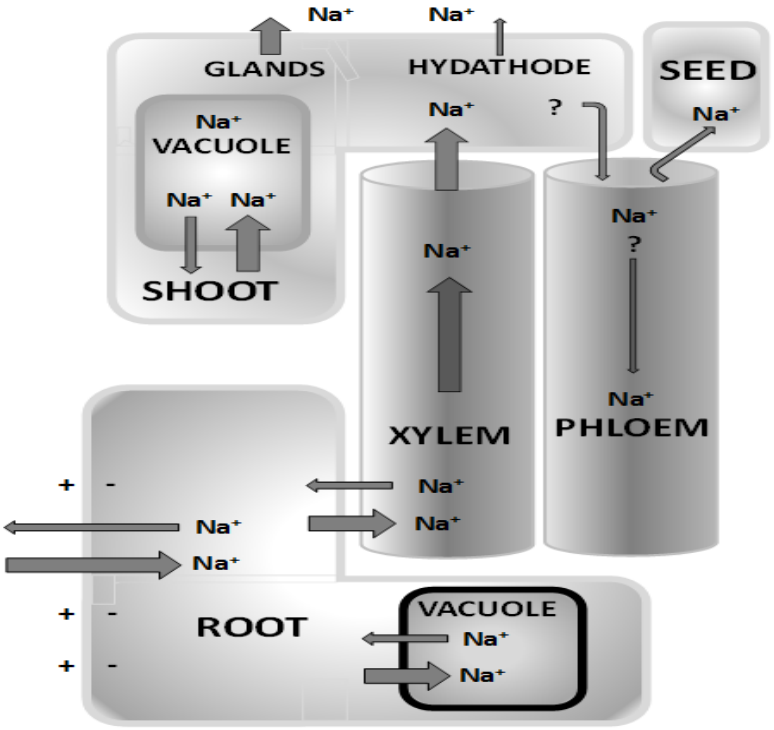

Figure 1-1: Overview of the main Na^+ flux pathways that occur in terrestrial plants

Some mechanisms are still debated such as recycling of Na^+ from root to shoot via the phloem. Other mechanisms are anatomic adaptations found in a limited number of (halophytic) species only, such as extrusion via glands and hydathodes. The size of the arrow provides a relative measure of various fluxes (Maathuis *et al.*, 2014).

Tight control of Na^+ uptake from the soil is proven to be useful strategy against salt stress. Both channels and carriers are involved in the Na^+ uptake from the soil.

Channels: Different channels are involved in the Na^+ absorption from the environment (Figure 1-2). Non selective cation channels (NSCCs) play a significant role in root Na^+ uptake (Amtmann *et al.*, 1999; Tester and Davenport, 2003; Maathuis, 2007). In addition, Golldack *et al.* (2003) reported that K^+ inward rectifying channels (KIRCs) such as OsAKT1 (homologous to *Arabidopsis* K^+ transporter) could mediate Na^+ uptake in rice under saline conditions. Other channels that may play a role include glutamate like receptors (GLRs) (Davenport, 2002) and cyclic nucleotide gated channels (CNGCs) (Assmann, 1995; Bolwell, 1995; Newton and Smith, 2004; Trewavas, 1997).

Carrier transporters: Carrier type transporters involved in Na^+ transport include high affinity K^+ transporters (HKTs) (Golldack *et al.,* 2002; Horie *et al.,* 2001; Mian *et al.,* 2011) and K^+ uptake permease/ high-affinity K^+ transporters (KUP/HAK/KT).
HKT transporters contribute to the Na^+ uptake from the soil (Figure 1-2). Rice contains nine HKT isoforms (Garciadeblas *et al.,* 2003). Some of these isoforms have been studied extensively for example OsHKT2:1 in the Nipponbare rice (Horie *et al.,* 2007), HKT2:2 in the Pokkali rice (Horie *et al.,* 2001)

HAK/KUP/KT transporters are high affinity K^+ transporters and may transport Na^+ with low affinity in the presence of high Na^+:K^+ ratios (Pardo and Quintero, 2002). (Santa-Maria *et al.,* 1997; Fu and Luan, 1998).

1.3.4.2 Removal of Na^+ from the cytoplasm

Na^+ present in the cytoplasm interferes with the cell metabolic processes so plants can either extrude Na^+ into apoplast or store it in the vacuole. The removal of Na^+ into the apoplast is driven by antiporters called Na^+: H^+ antiporter (NHA). SOS1 is a member of the NHA family and has been characterised in detail. SOS1 expression is prominent in root tip cells and also occurs in the xylem parenchyma (Wu *et al.,* 1996). Root tip cells are predominantly evacuolate and hence incapable of vacuolar Na^+ compartmentation. Such tissues therefore must entirely rely on extrusion of cytoplasmic Na^+ into the apoplast which is mediated by SOS1.

However, Na^+ extrusion into the apoplast is assumed to take place in most plant tissues, particularly at the root-soil boundary. Many of these tissues do not show SOS1 expression and it remains unclear which antiporters are involved. Other NHA isoforms may play a role but the NHA gene families in most plants only contain 1 or 2 isoforms. A further alternative is the CHX (cation H^+ exchange) family (Figure 1.2) which probably includes both K^+:H^+ and Na^+:H^+ antiporters and some CHXs have been implied in salinity tolerance (Evans *et al.,* 2012).

Sequestration of Na^+ in the vacuole could be a useful strategy for the cells for many reasons: 1. The cytoplasm gets rid of the toxic concentrations of Na^+, 2. The vacuole does not contain Na^+ sensitive metabolic machinery so is not affected by Na^+, and 3. it lowers the cellular water potential and as such prevents water loss.

One of the studies on plant antiporters discovered NHX1 (Na^+ H^+ exchanger 1) as a major player in the vacuolar cation movement. AtNHX1 overexpression significantly improved salinity tolerance in *Arabidopsis* (Apse *et al.,* 1999) whereas its loss of function yielded the opposite effect (Apse *et al.,* 2003 and Bassil *et al.,* 2011). Manipulation of the expression of NHX1 orthologs in other species such as wheat (Xue *et al.,* 2004), rice (Fukuda *et al.,* 2004) and tomato (Zhang and Blumwald, 2001) showed the fundamental role this protein plays in salt tolerance and explains why it is a major focus for genetic engineering. However, more recent work has thrown some doubt on the molecular details of NHX activity and thus its physiological role. Most NHX isoforms that have been characterised can transport both K^+ and Na^+ and either have a similar Km for these substrates or even prefer K^+ (Jiang *et al.,* 2010). This means that, unless the cytoplasmic Na^+ concentration is considerably higher than that for K^+, NHX exchangers mainly mediate K^+:H^+ exchange rather than Na^+:H^+ exchange (Zhang and Blumwald, 2001; Barragan *et al.,* 2012). Indeed, loss of function of NHX1 and NHX2 in *Arabidopsis* led to impaired vacuolar K^+ accumulation but enhanced vacuolar Na^+ uptake (Barragan *et al.,* 2012). Thus, it seems that the contribution of vacuolar NHX exchangers to salt tolerance is predominantly in maintaining K^+ homeostasis rather than in actual sequestration of Na^+ into the vacuole. Of course this leaves us with the important question how the latter process is catalysed!

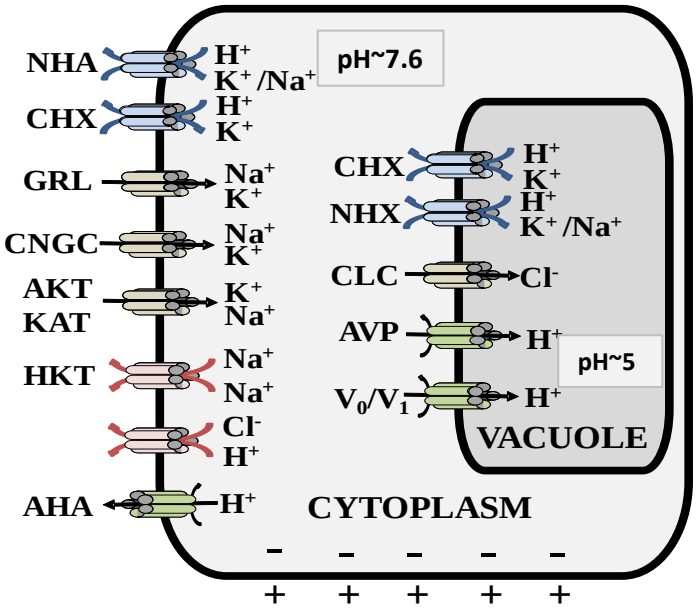

Figure 1-2: overview of the main membrane transporters that contribute to Na^+ and Cl^- uptake and distribution. Not necessarily all depicted proteins are present in one cell

AHA: H^+ pump; AKT/KAT: K^+ inward rectifying channel; AVP: vacuolar pyrophosphatase; CHX; cation-proton exchanger/antiport; CLC: chloride channel; CNGC: cyclic nucleotide gated channel; GRL: glutamate receptor like channel; HKT: K^+:Na^+:symporter; NHA: plasma membrane sodium-proton exchanger/antiport; NHX: tonoplast sodium exchanger/antiport; VoV1: tonoplast H^+ ATPase (Maathuis *et al.*, 2014).

1.3.4.3 Long distance Na^+ transport

Translocation of Na^+ from root to shoot (Figures 1-2 and 1-3) is one of the important strategies in salt stress physiology (Flowers *et al.*, 1977; Epstein, 1998). Glycophytes are mostly classified as salt excluders because they prevent significant accumulation of salts in photosynthetic tissues while most halophytes are includers and actively transport Na^+ from root to shoot (Flowers *et al.*, 1977; Lauchli, 1984). This long distance transport has various points where the plant can exert control over salt distribution such as loading and translocation through the xylem and/or phloem mediated re-translocation from shoot to roots (Figure 1-1).

The bypass flow

Solutes and water can reach the xylem via a symplastic or apoplastic route. The latter catalyses movement of solutes through the cell walls and intercellular spaces to the xylem without crossing plasma membranes and is sometimes called the 'bypass flow' (Yeo *et al.*, 1987; Anil *et al.*, 2005; Krishnamurthy *et al.*, 2009). Casparian strips and suberine layers in the root endodermal and exodermal layers provide morphological

barriers to apoplastic transport but in young roots and initiation sites of lateral roots these structures can be lacking or only partially effective. The efficacy of these anatomical features heavily depends on growth conditions such as the presence of silicon (Yeo *et al.,* 1987) and Ca^{2+} (Anil *et al.,* 2005). In many plants the apoplastic pathway is relatively limited but in other species such as rice the bypass flow can be substantial and therefore is responsible for significant amounts of Na^+ transport to the shoot.

The exact entry site for the Na^+ into the stele is not known. Yeo et al. (1987) proposed that the emerging sites of the lateral roots and cells walls near the root apices are the entry points. In rice, like other monocots, lateral roots arise from the pericycle through the endodermis breaking the casparian bands. Casparian bands are also often absent in the root tip regions. In contrast, Faiyue et al. (2010) showed that the bypass flow significantly increases in the absence of lateral roots, by using mutant lines incapable of making lateral roots. These authors suggested that the higher Na^+ content in the xylem sap and shoots of the mutant lines was caused by the different anatomical architecture and reduced suberine deposition on the exodermal and endodermal walls of the mutant. Recent data from Krishnamurthy et al. (2011) suggested the involvement of lateral root emergence in the leakage of tracer (trisodium-8-hydroxy-1,3,6- pyrenetrisulphonic acid) into the primary roots through the break created by the emergence of lateral roots.

1.3.4.4 Xylem loading of Na^+

Solutes delivered via the symplast have to cross the plasma membrane before they can be released into the xylem apoplast. Plasma membrane localised transporters are proposed to have a role in the xylem loading of Na^+ (Lacan and Durand, 1996) a process that involves the endodermis and xylem parenchyma cell layers (Epstein, 1998). The transport systems located at the xylem-parenchyma boundary may mediate both passive loading via Na^+ permeable channels and active loading through Na^+:H^+ exchangers (Figure 1-3). An example of the latter is SOS1, a plasma membrane antiporter that is expressed in root epidermis and root xylem parenchyma. The exact function of SOS1 is likely to depend on the severity of salinity stress and may include both xylem loading (low or moderate levels of salinity) and removal of Na^+ from the xylem (during high salinity). Members of CHX cation antiporter family are also implicated as playing a role in the loading of Na^+ into the xylem. For example,

Arabidopsis CHX21 is mainly expressed in the root endodermis and loss of function in this protein reduced the level of Na^+ in the xylem sap (Hall *et al.*, 2006).

The presences of non-selective ion channels (NSCs) in the plasma membrane of xylem parenchyma cells provide another pathway for Na^+ entry into the xylem. NSCs have been studied in xylem parenchyma cells of barley roots (Wegner and De Boer, 1997). The molecular identity of these NSCs is as yet unknown but could include members of the glutamate receptor like channels (GLRs) or cyclic nucleotide gated channels (CNGCs) (Demidchik and Maathuis, 2007).

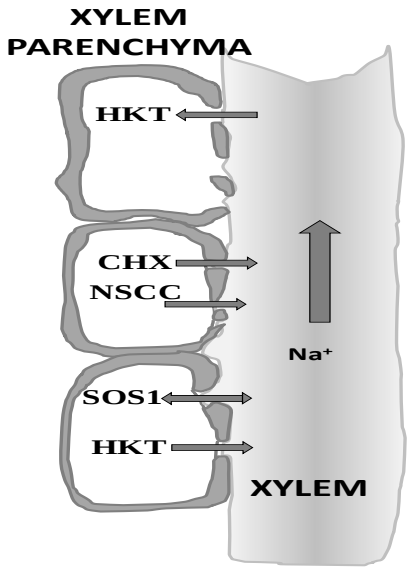

Figure 1-3: Schematic of the xylem and xylem parenchyma

The xylem parenchyma consists of living cells that unload Na^+ across their plasma membrane into the apoplast and xylem lumen (non-living tissue). Once in the xylem, bulk flow under positive and negative pressure ensures transport of minerals to shoot tissues (Maathuis *et al.*, 2014).

1.3.4.5 Na^+ retrieval

Plants can reabsorb Na^+ from the xylem into the root cells as a mechanism to prevent large accumulation of Na^+ in the above-ground tissues (Läuchli 1984; Lacan and Durand 1996). This retrieval mechanism was originally postulated in the 1970s (Lessani and Marschner, 1970) but now has a molecular basis (Figure 1-3). In *Arabidopsis*, disruption of HKT1 leads to hypersensitivity to salinity of the mutant lines with more Na^+ in the leaves (Mäser *et al.*, 2002; Berthomieu *et al.*, 2003; Davenport *et al.*, 2007; Møller *et al.*, 2009). The knockout lines showed higher Na^+ in the shoots but a lower

level of K^+. These results favour the hypothesis that AtHKT1 is responsible for the retrieval of Na^+ from the xylem whilst directly stimulating K^+ loading. This is an ideal mechanism for plants to achieve a higher K^+/ Na^+ ratio in shoots during salts stress (Hauser and Horie, 2010). Similar reabsorption mechanisms were also found in rice and wheat. 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 was significantly more robust in salt tolerant rice cultivars. In wheat, the HKTs NAX1 and NAX2 fulfil similar roles (Lindsay *et al.,* 2004). Shi *et al.,* (2002) suggested a similar role in xylem Na^+ reabsorption for the SOS1 transporter (depending on the level of salinity stress) but the evidence for this is less convincing.

1.3.5 Role of K^+ under salt and drought stress

Abiotic stresses like drought and salinity affect K^+ availability to the plants. In response, plants suffer from K^+ deficiencies and their growth and productivity is affected. Improving K^+ nutrition could increase plants' tolerance to these abiotic stresses. Deep root systems, large absorbing areas and retention of water within the plants are considered effective strategies against osmotic stress (Wang *et al.,* 2013). Provision of K^+ fertilizers together with N and P deep in the soil could enhance deep rooting due to the signalling functions of these nutrients (Kirkby *et al.,* 2011). Adequate K^+ supply increases the surface area of the roots and increases the water uptake (Römheld and Kirkby, 2010). Improved K^+ nutrition helps in water retention by keeping stomata closed under osmotic stress conditions. Low K^+ concentrations under osmotic stress conditions could inhibit the action of ABA. The reduction in ABA then delays stomatal closure and results in greater water loss (Tanaka *et al.,* 2006). Adequate K^+ concentration plays a role as inorganic osmoticum within the cells during osmotic stress conditions, maintaining higher turgor pressure. K^+ also plays a role as a major osmoticum in the cytosol to balance the osmotic gradient between the cytosol and the vacuole (Wang *et al.,* 2013).

Considering the above important roles of K^+ under different conditions, it is important to understand the K^+ nutrition and distribution within the plant.

1.3.5.1 K^+ uptake from the soil

The uptake mechanism for the K^+ involves low and high affinity K^+ transport systems active at a range of external K^+ concentrations (Maathuis and Sanders, 1996; Maathuis *et al.,* 1997; Very and Sentenac, 2003; Rodriguez-Navarro and Rubio, 2006). K^+ channels can proceed through passive transport and is believed to be driven by membrane potential. High affinity K^+ transport systems require energy (Maathuis, 2007). AKT1 is identified as the main contributor of the low affinity K^+ system in *Arabidopsis* (Sentenac *et al.,* 1992; Hirsch *et al.,* 1998) while its homologues were also identified in rice (OsAKT1), potato (StSKT1), carrot (DcDKT1) and maize (ZmKZM1). AKT1 is predominantly expressed in root cortex and in the epidermal cells including the root hairs (Figure 1-5). Its selectivity for K^+ is several times higher than Na^+. AKT1 plays a major role in K^+ absorption at the root soil boundary (Hirch *et al.*, 1998; Spalding *et al.,* 1999; Gierth *et al.,* 2005; Rubio *et al.,* 2008; Alemán *et al.,* 2011). AKT1 is activated by phosphorylation through the CIPK23-CBL1/9 complex (Li *et al.*, 2006; Xu *et al.*, 2006). For instance cipk23 mutants showed less K^+ uptake (Xu *et al.*, 2006) in agreement a role of AKT1 in K^+ uptake at the root level.

At very low external concentrations of K^+, high affinity K^+ transporters from the KT/HAK/KUP group are involved in the K^+ uptake (Figure 1-5). For example, AtHAK5 expression level is upregulated under K^+ deficient conditions (Ahn *et al.,* 2004; Gierth *et al.,* 2005; Qi *et al.,* 2008). Gierth *et al.,* (2005) found that AtHAK5 shows strong and consistent upregulation when the plants are exposed to K^+ starvation either for short time or long time. They found the high expression of AtHAK5 in the epidermal cells of the main root and lateral roots and suggested that AtHAK5 is involved in the K^+ uptake. They also found that the athak5 homozygous mutant line showed less K^+ over 96 hours K^+ starvation. Nieves-Cordones et al., (2008) found that the membrane potential of the K^+ starved plants is more negative than the K^+-sufficient plants. They found a higher expression level of LeHAK5 when the membrane potential becomes more negative. When the K^+ starved plants were treated with 100 μM K^+, depolarization of the root plasma membrane and LeHAK5 expression level were observed. Nieves-Cordones et al., (2009) showed increased AtHAK5 transcription under K^+ starvation, however, the transcription was relatively lower when the same K^+ starved plants were supplied with 30 mM NaCl in the medium (see also Nieves Cordones et al., 2014)). The upregulation of HAK5 was also found in tomato (Wang *et al.*, 2002), and barley (Santa-Maria *et al.,*

1997) under K^+ starvation. Some of the members of the KT/HAK/KUP family have a role in low affinity K^+ transport helping other K^+ transporters (Senn *et al.*, 2001; Garciadables *et al.*, 2002). For example OsHAK7 and OSHAK10 are involved in low affinity K^+ transport (Banuelos *et al.*, 2002). The transporters of KT/HAK/KUP family are involved in the K^+ uptake as well as in the maintenance of K^+ homeostasis.

The non-selective cation channels (NSCCs) are present throughout in the plasma membrane and other membranes. They show high selectivity for cations but do not discriminate between K^+ and Na^+ (Demidchik and Maathuis, 2007). Their physiological role is to function in low affinity transport of K^+. CNGCs are the types of NSCCs and are activated by cAMP and cGMP (Leng *et al.*, 2002), widely present in plants with 20 members in *Arabidopsis* (Maser *et al.*, 2001) and are localised to the plasma membrane (Arzi *et al.*, 1999) (Figure 1-5). All characterised CNGCs are capable of conducting K^+ (Shabala and Cuin, 2007).

1.3.5.2 K^+ transport in the root

Plants absorb nutrients primarily at the peripheral tissues of the root such as the epidermis and hypodermis (or exodermis; Kochian and Lucas, 1983; see also Nieves-Cordones *et al.*, 2014). The root cortex functions less in direct nutrient absorption and mostly acts as a collection system for water and nutrients absorbed by the epidermis. After absorption, radial transport of water and nutrients follows with the ultimate aim of delivery to the root xylem. Radial transport can follow both apoplastic and symplastic pathways (Fig. 1-4). Water and solutes absorbed into the symplast can move from the periphery to the central part of the root (Taura *et al.*, 1988).

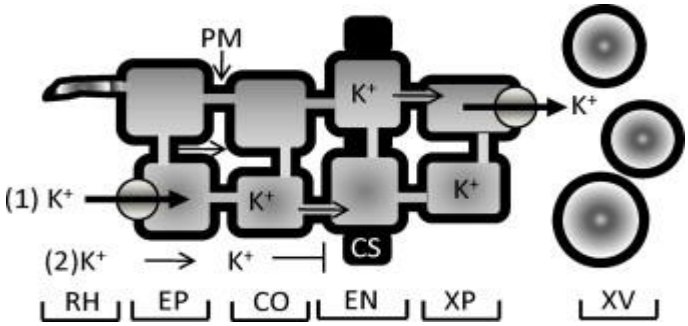

Figure 1-4: Overview of radial root tissues and symplastic and apoplastic pathways for solutes from the root periphery to the xylem

Uptake of nutrients such as K^+ primarily occurs at the root periphery by epidermis (EP) cells, especially those with root hairs (RH). Uptake into the root symplast (1) of K^+ is mediated by diverse K^+ channels (e.g. AKT1 and KAT1), carriers from the HAK/KUP family and possibly non-selective K^+ channels (e.g. from the CNGC family). K^+ is then radially moved within the symplast using interconnecting plasmodesmata (PM). K^+ release into the stele apoplast occurs through K^+ channels such as SKOR and less selective K^+ channels that reside in the plasma membrane of xylem parenchyma (XP) cells. Energised exchangers from the CHX family may also contribute. Nutrients can also cross the outer layers of the root via the apoplast (2) but this pathway is blocked at the endodermis (EN) by the Casparian strip (CS) a highly suberised structure that prevents uncontrolled water and nutrient delivery to the stelar tissues. CO, cortex; XV, xylem vessel

The interconnecting walls of the various root tissues form a highly porous continuum called the apoplast through which water and solutes can freely move (Katou and Furumoto, 1986a; Katou and Furumoto, 1986b). However, the apoplast is blocked at the endodermis, the cell layer that divides the stele from the other root tissues. Endodermal cells are surrounded by the Casparian strip, a highly suberized material which is impermeant to water and solutes. The endodermal block of the apoplast forces the water and dissolved substances to enter into the cytoplasm of the endodermal cells through the plasma membranes, endowing the plant with a high degree of control over the entry of the dissolved solutes into the stele. It also prevents solutes that leak out of the xylem from being lost into the rhizosphere. Some angiosperms also have Casparian strips in the hypodermis providing an additional control point for the entry of solutes.

Overall radial transport is from root periphery towards the stele and then the xylem vessels. The main driving force for this process is transpiration, the evaporation of water from leaves, which reduces pressure in the xylem. This creates a tension that pulls xylem sap upward which is replenished by water and solutes provided by the roots. The other contributor is solute diffusion within the symplast which is facilitated by the plasmodesmata.

1.3.5.3 Xylem loading of K^+

K^+ uptake and subsequent radial transport through the root occurs through mechanisms discussed above. K^+ distribution around the root symplast depends on bulk flow through interconnecting plasmodesmata. However, delivery to non-root tissues requires K^+ loading into the xylem, a process largely controlled by outward-rectifying K^+ channels in the adjacent parenchyma cell membranes. Not all details are known but AtSKOR (stelar K^+ outward rectifying channel), originally identified in *Arabidopsis*, appears a main player (Gaymard *et al.,* 1998). SKOR is a Shaker type K^+ channel activated when the membrane depolarises (Figure 1-5). A SKOR knockout mutant showed a reduced shoot K^+ content and a reduced K^+ content in the xylem sap confirming its significance as a crucial step in delivery of K^+ to the xylem. SKOR transcription is inhibited by abscisic acid (ABA) (Gaymard *et al.,* 1998). The latter is believed to ensure reduced K^+ loading into the xylem to maintain adequate root turgor when soils dry out. All these properties suggest an important role for SKOR in K^+ xylem loading and hence its delivery to shoot tissue. However, using patch clamp studies on xylem parenchyma cells, several other outward rectifying K^+ conducting channels have been observed which may also contribute to xylem K^+ loading (Roberts and Tester, 1995 and Wegner and De Boer, 1999) but further research is needed to establish the molecular identity of these transporters.

1.3.5.4 Translocation of K^+

The movement of solutes from root to shoot is named 'translocation'. The xylem is responsible for root to shoot translocation of K^+. However, long distance K^+ transport is by no means unidirectional and a large proportion of shoot K^+ is recycled to the root through the phloem (Marschner *et al.,* 1997). One reason for this apparently futile cycle is the role K^+ plays as counter ion for root to shoot translocation of NO_3^- in the xylem (which may require much of it to be recycled) while phloem K^+ influx may also be necessary when photosynthetic assimilates are loaded. The amount of K^+ re-translocated from the shoots to the roots via the phloem, also depends on the shoot growth rate and K^+ availability. As such it may be part of a signal to control the amount of K^+ secreted into the root xylem and, ultimately, to help tune root K^+ absorption to the shoot demand for growth (Drew and Saker, 1984; Marschner *et al.,* 1996; White, 1997). The overall

K^+ translocation is likely to be tightly controlled by many factors but it is clear that ABA is an important component. Studies on barley and maize (e.g. Cram and Pitman, 1972; Schaefer *et al.*, 1975; Behl and Dieter Jeschke, 1981; Bassiri Rad and Radin, 1992) showed that the net efflux of K^+ from the stelar parenchyma cells decreases after treatment with ABA. Roberts (1998), using patch clamp, directly demonstrated that K^+ channel activity in xylem parenchyma cells was reduced by ABA but ABA had no effect on similar channels in the cortical cells. Thus, ABA reduces the K^+ permeability of the xylem parenchyma plasma membranes and as such reduces K^+ translocation via the xylem.

1.3.5.5 K^+ transport and distribution in the shoot

Xylem unloading

The ascending xylem sap delivers soil derived nutrients to the above ground parts of the plant. Since K^+ is important for turgor, and therefore expansive growth, large amounts of K^+ need to be unloaded from the xylem sap and distributed across shoot tissues. Leaves have a limited number of major veins but an extensive network of minor veins. This means that effectively no cell is ever further away than a few hundred micrometres from a 'delivery pipeline' (Smillie *et al.*, 2012) to supply the large amounts needed. For example, a 10 g shoot of a plant growing at 10% per day, would require around 100 μmol of K^+ each day, assuming an average K^+ concentration of 100 mM. Because the requirements for K^+ may vary greatly, for example between mature, transpiring leaves and rapidly growing young leaves, this is not a passive process but likely to be controlled by signals and hormones such as ABA, auxin, and cytokinins, many of which are present in the xylem sap itself (Wegner and De Boer, 1997).

Just as they are in the root, the dead leaf xylem vessels are surrounded by xylem parenchyma cells. Many of the latter contain 'pits' (interruptions in the cell wall) which expose the parenchyma cell membrane directly to the xylem content. At the pits, xylem content can be unloaded into the apoplast and, after uptake in the parenchyma cells, this can be passed on (symplastically) to more distant cells and tissues (Botha *et al.*, 2008; but see also Wigoda *et al.*, 2014, for a discussion on the role of the leaf bundle sheath in this distribution process). There is no direct evidence about the molecular mechanisms that mediate this process but it is highly likely that K^+ channels and carriers are

responsible which are similar to those discussed previously: For example, if we assume an average [K^+] in the xylem sap of around 10 mM (Enns *et al.,* 1998), this would equilibrate with the apoplast [K^+]. Passive uptake of xylem K^+ into the parenchyma cells can then proceed through hyperpolarisation activated inward rectifying K^+ channels (e.g. of the *Arabidopsis* (Shaker type) K^+ channel (AKT/KAT) family; s(ee Anschütz *et al.,* 2014 and Véry *et al.,* 2014) and/or non-selective cation channels (Wegner and De Boer, 1997; see also Demidchik, 2014 and Pottosin and Dobrovinskaya, 2014). Whenever xylem K^+ would fall below 0.1–1 mM, active transport in the form of H^+-coupled mechanisms may also contribute (e.g. from the high affinity K^+ transporters (HAK/KUP) family; (see Nieves-Cordones *et al.,* 2014 and Véry *et al.,* 2014). Some authors believe that the high number of membrane vesicles that is observed near the pit membrane suggests that endocytosis could also be important for xylem unloading (Botha *et al.,* 2008).

Phloem loading

While the xylem is responsible for the unidirectional transport of water and solutes from root to shoot, primarily driven by transpiration, the phloem is essential for the transport of assimilates from source to sink. Phloem K^+ concentrations tend to be significantly higher than xylem K^+ and probably range from around 50 to 150 mM (Kallarackal *et al.,* 2012). The reason for this relatively high K^+ concentration is not clear and may be manifold. Loading of both sugars and amino acids is stimulated by apoplastic K^+ (Peel and Rogers, 1982). Just as in the xylem, phloem K^+ requirement may simply be a matter of charge balancing. Phloem sap can contain organic acids and large quantities of amino acids with negative charge (Hayashi and Chino, 1990). In low growth conditions, the amount of K^+ that reaches the shoot through the transpiration stream can outstrip shoot demand and all or a fraction of this may be recycled to the roots via the phloem. Other reports suggest that phloem mediated sugar translocation requires K^+ because of the way sucrose loading itself takes place. At the source, loading of sucrose into the phloem is largely via H^+-coupled sucrose transporters. The influx of H^+ could lead to large depolarisation of the phloem cell, thereby reducing the driving force for sucrose loading. Depolarisation is prevented by K^+ selective channels such as AKT2/3 (Deeken *et al.,* 2002), a shaker type K^+ channel that can switch between inward and non-rectifying states, that ‘clamps’ the phloem cell membrane potential at the K^+ reversal

potential. With several millimolar of K^+ in the apoplast, such a mechanism can only polarise phloem cells effectively if the phloem [K^+] is near or over 100 mM. Clamping is particularly potent when the channel is switched to the non-rectifying mode and when energy provision is compromised (Gajdanowicz *et al.,* 2011). Indeed, this phloem based 'K^+ battery' may constitute a general decentralised energy store that can be used to overcome local energy limitations (Gajdanowicz *et al.,* 2011).

The absolute phloem K^+ concentrations will vary in response to environmental conditions and it has been suggested there is a balance between sucrose and K^+ to maintain the phloem sap osmolality. Thus, when photosynthesis is low and hence sucrose loading is diminished, in several species it was found that phloem K^+ went up (Philippar *et al.,* 2003). Because AKT2/3 is expressed in both source and sinks phloem tissues (Figure 1-5) and is potentially able to mediate both K^+ influx and efflux owing to its weak rectification property, it has been suggested to be involved in both loading and unloading of phloem sap K^+ in sources and sinks respectively (Lacombe *et al.,* 2000 and Marten *et al.,* 1999). Interestingly, the drought hormone ABA reduces the level of *SKOR* mRNA in the xylem (see above) while it increases that of *AKT2/3* in the phloem (Pilot *et al.,* 2003). This dual effect is consistent with the role of ABA during water stress: a reduced K^+ transport to the shoots, and increased delivery of K^+ to the roots via the phloem, both will aid in maintaining a low osmotic potential in the water deprived roots.

Photosynthetic versus non-photosynthetic tissues

Within shoot tissues, K^+ can vary greatly as borne out by several studies comparing K^+ in photosynthetic mesophyll and non-photosynthetic epidermal cells. This variation primarily reflects vacuolar K^+ contents. Grown in control conditions Fricke *et al.,* (1996) recorded around 200 mM K^+ in vacuoles of both mesophyll and epidermal cells. However, growth conditions severely affected these patterns; in saline conditions [K^+] reduced in both types of cells but considerably more so in epidermal cells (Fricke *et al.,* 1996). Another study on barley found considerably higher (~260 mM) K^+ levels in epidermal cells compared to mesophyll cells (~120 mM), while a third paper on barley found the opposite with ~300 mM in the mesophyll and ~170 mM in the epidermis (Dietz *et al.,* 1992). This suggests that partitioning patterns are not fixed and likely respond to growth conditions and developmental stage. For example, saline conditions

typically lead to preferential deposition of Na^+ in non-photosynthetic cell types (e.g. Fricke *et al.,* 1996), effectively exchanging K^+ for Na^+ (for details on the physico-chemical relationship of K^+ and Na^+ (see Benito *et al.,* 2014). However, it is not clear which transport processes underpin this partitioning of K^+. Some studies found that differential uptake of ions might partly explain cell type-specific ion accumulation (e.g. Dietz *et al.,* 1992). For example, inward-rectifying currents are absent from the leaf mesophyll cells of several species (for refs see Karley *et al.,* 2000) which could contribute to differential K^+ uptake. Others found no evidence for differential uptake; Karley et al. (2000) measured comparable membrane potentials in mesophyll and epidermal cells and a much higher $[K^+]$ in epidermal protoplasts in spite of a slightly higher K^+ current density in the mesophyll protoplasts.

1.3.5.6 Cellular partitioning of K^+

Cellular K^+ partitioning into the vacuole

In both roots and shoots, K^+ is normally present at high concentrations in all cellular compartments. In plants, the two major cellular compartments consist of the cytoplasm and the vacuole with the latter by far the largest in terms of volume. Thus, the majority of cellular K^+ is deposited in the vacuole where it is one of the main turgor providers. To provide turgor, vacuoles need high concentrations of K^+ which, depending on growth conditions and cell type, can reach over 500 mM. Vacuolar loading of K^+ may to some extent be mediated by cation channels but must rely on energised mechanisms to reach K^+ concentrations that are equal or higher than those in the cytoplasm, due to the membrane potential across the tonoplast which keeps the lumen at a potential of ~20 mV positive with respect to the cytoplasm. It is generally assumed that K^+:H^+ exchangers, particularly from the cation: proton exchangers (CHX) and Na^+ proton exchanger (NHX) family, drive such fluxes (Pardo *et al.,* 2006, Chanroj *et al.,* 2012 and Mottaleb *et al.,* 2013). Recent studies provide convincing evidence that in particular NHX1 and NHX2 function in vacuolar K^+ accumulation. Double *nhx1/nhx2* loss of function mutants had reduced ability to create a vacuolar K^+ pool, which in turn led to greater K^+ retention in the cytosol. The mutants were also impaired in osmoregulation and turgor generation for cell expansion (Barragán *et al.,* 2012).

The K^+ that is sequestered in vacuoles is probably quite inert in most circumstances. However, it may need to be released in other conditions, for example when cytoplasmic K^+ becomes deficient (Walker *et al.*, 1996), when osmotic adjustment is necessary or when turgor driven movement is required such as during opening and closing of stomata or (nyctinastic) movement of leaves (for further details on the regulation of stomatal movement see Blatt *et al.,* 2014). Release of vacuolar K^+ is largely thermodynamically 'downhill' and thus likely to be through ion channels. Particularly TPK (two pore K^+ channel) type channels are contributors to this process. TPKs show a four transmembrane/two pore structure, and one or two C-terminal EF hands, suggesting that Ca^{2+} signalling may be part of their regulation. TPK1 is expressed in most plant tissues where it forms homomeric channels (Voelker *et al.,* 2006 and Gobert *et al.,* 2007; see also Hamamoto and Uozumi, 2014). Its expression was shown to impact on overall K^+ homeostasis and on stomatal closure in particular giving credence to the notion that it constitutes a major pathway for vacuolar K^+ release (Gobert *et al.,* 2007). In seeds, TPK1 may also be involved in the release of K^+ from protein storage vacuoles during the initial phases of germination (Gobert *et al.,* 2007 and Isayenkov *et al.,* 2011).

Another K^+ conducting tonoplast channel (the fast vacuolar (FV) channel) may also participate in intracellular K^+ distribution. The FV channel has a K^+/Na^+ selectivity of around unity and was originally described in red beet storage tissue (Hedrich and Neher, 1987). The gene(s) encoding the FV channel is not known and this frustrates in depth studies regarding its characteristics and in planta role. But, since both luminal and cytoplasmic K^+ levels impact on FV channel open probability, it has been hypothesised that maintaining cellular K^+ homeostasis is one of the physiological roles of this transporter (Pottosin and Martínez-Estévez, 2003; see also Pottosin and Dobrovinskaya, 2014).

During prolonged K^+ starvation, vacuolar K^+ concentrations may become significantly lower than those in the cytoplasm (Walker *et al.,* 1996). In such conditions, vacuolar K^+ release cannot be passive and needs energised transport. The transporter(s) that is responsible for this process is unknown but interestingly, several proteomics studies show the presence HAK/KUP type transporters at the tonoplast (e.g. Jaquinod *et al.,* 2007 and Whiteman *et al.,* 2008; see also Hamamoto and Uozumi, 2014) which are

energised via coupling to the trans-tonoplast proton motive force. Such systems could facilitate 'uphill' K^+ release from the vacuole into the cytoplasm.

Other endo-compartments

NHX and CHX type K^+: H^+ exchangers may also regulate K^+ homeostasis of other endo-compartments. For example, the tomato LeNHX2 protein co-localises with pre-vacuolar and Golgi markers in both yeast and plants (Rodríguez-Rosales *et al.*, 2008) whereas a translational AtNHX5:GFP fusion localised to pre-vacuolar compartments of onion cells (Pardo *et al.*, 2006). These systems are possibly responsible for loading and unloading of K^+ in cellular endo-compartments and help maintain electrical and pH homeostasis. K^+ provision of chloroplasts may rely on various antiporters: AtCHX23 was found to be targeted to the chloroplast envelope and its K^+: H^+ exchange activity was hypothesised to impact on stromal pH and chloroplast development (Song *et al.*, 2004). More recently Aranda-Sicilia et al. (2012) showed that K^+ efflux antiporter type exchangers may also be important for chloroplast K^+ homeostasis.

1.3.5.7 K^+ delivery to seeds

After flowering, seed development takes place which necessitates a large-scale redirection of metabolites and minerals from vegetative to reproductive tissues and thus a major change in source–sink relationships within the plant. In the seed, K^+ fulfils similar roles as in other tissues, i.e. as an enzyme cofactor in metabolic processes and as an osmoticum to drive turgor driven cell expansion after germination. In addition, the protein storage vacuoles (PSVs) of seeds contain not only protein but also organic forms of phosphorous such as phytate which are complex with inorganic minerals such as Ca^{2+} and K^+.

Delivery of both photosynthates and minerals to seeds occurs primarily through the phloem because seeds are largely non-transpiring tissues. Phloem in the pedicel, the stem that attaches the flower to the main stem (Figure 1-5), delivers water and solutes to the seed which itself consists of both maternal and filial tissues: the seed coat comprises maternal cell layers while the endosperm (storage reserve) and the embryo are composed of filial cells. Maternal and filial organs are isolated from each other through absence of any symplastic continuity which means not only that the main sink shifts

from initially being maternal to being filial in later stages, but also that the phloem-delivered minerals (which move symplastically through the maternal cell layers) have to be unloaded into the seed apoplast and subsequently are taken up by the first filial cell layer, the aleurone cells. Virtually no data are available on the nature of transporters that are involved in mineral loading into developing seed. Phloem unloading of K^+ is likely to be symplastically since the phloem is connected via a large number of plasmodesmata to the maternal seed tissue (Figure 1-5). Preliminary studies on bean seed coats using the patch clamp technique showed that non-selective ion channels might be responsible for K^+ release from the maternal tissue into the seed apoplast (Zhang *et al.,* 2007). The characteristics and function of these channels are analogous to those of non-selective cation channels in the xylem parenchyma (see above) and the genes that encode such channels have yet to be identified. In addition, aleurone (the first filial cell layer) protoplasts also contain AKT-type voltage dependent inward rectifying channels and non-selective cation channels of the cyclic nucleotide gated channel family (Schuurink *et al.,* 1998) both of which may participate in uptake of K^+ into the filial tissues.

1.4 Why rice?

Rice (*Oryza sativa* L.) is a staple food for about half of the world's population. It has both diet and monetary value. About 70% of the world's rice production is in Asia, especially in countries like Thailand, India and Pakistan (IRRI 2009). Currently, cultivated areas in those countries are affected by salinity. Recent studies have estimated that 2.8 Mha in Thailand (Yuvaniyama et al., 2008), 6.7 Mha in India (Singh 2009) and 6.8 Mha in Pakistan (Awan *et al.* 2007) are salt affected. Rice is relatively sensitive to salt stress among the cereals. It tolerates salinities ranging between 1.9-3dS/m (Grattan *et al.,* 2002). Most of the salinity symptoms appear at seedling or panicle induction stage (Akbar *et al.,* 1972). Salinity affects rice in different ways, however, primarily causing osmotic stress and ion disequilibrium (Hasegawa *et al.,* 2000; Zhu, 2001).

There is much evidence that the effects of ion toxicity are more severe than the osmotic stress caused by salinity in rice (Flowers & Yeo 1981; Yeo & Flowers 1982, 1983; Yeo et al. 1985a). Yeo *et al.,* (1985a) tested the rate of photosynthesis in the young and old leaves of the rice IR2153 and reported that the rate of photosynthesis was decreased in

the older leaf by 50% where more Na^+ accumulated while no decrease was found in the younger leaf. They suggested that a reduction in photosynthesis is due to the accumulation of Na^+ in the leaves

Rice is a monocotyledonous plant with a comparatively small genome that has been sequenced (Goff *et al.*, 2002; Yu *et al.*, 2002). Therefore it serves as a model plant for the monocots. Rice, like other glycophytes, responds to salt stress by different mechanisms and strategies. At the whole plant level, tolerant cultivars control Na^+ absorption from the soil, retain more K^+ in the tissue, and minimize Na^+ delivery to the shoot (Kavitha *et al.*, 2012).

Research of more than 40 years confirmed that cellular Na^+ homeostasis is crucial for plant salt tolerance. High external Na^+ negatively affects K^+ uptake. Salinity stress is always associated with interaction between different ions, i.e. Na^+, Ca^{2+} and K^+. Thus, salt tolerance requires not only the adaptation to Na^+ toxicity, but also the acquisition of K^+ as an essential nutrient. A high cytosolic K^+/Na^+ ratio is important for maintaining cellular homeostasis and proper functioning of the cell during salt stress. Since AKT and TPK channels play an important role in K^+ uptake and homeostasis they may positively contribute to plant abiotic stress tolerance.

1.5 Objectives of the thesis

A large number of reports suggested that reducing Na^+ transport into plants and improving K^+ nutrition could improve plant salt tolerance. In other words, improving K^+ nutrition will restrict Na^+ uptake and transport within the plants and will ultimately lead to better tolerance under salt and drought stress conditions. This study is aimed at improving K^+ nutrition and thereby increasing tolerance of rice to abiotic stresses such as salt and drought stress. As it is mentioned above, maintaining comparatively high K^+ concentration in the roots and its distribution to the shoots is vital in the strategies against salinity. K^+ channels are involved in the uptake and distribution of K^+ in the whole plant and in the cells; and therefore it is hypothesized that the characterization of these channels may impact on rice resistance to abiotic stresses.

The objectives of this thesis were:

1. To characterize rice TPKa channels in relation to salt and drought stress. The effects of the overexpression of TPKa were tested for growth, tissue ion concentration, stomatal conductance and rate of photosynthesis and distribution.
2. To investigate and analyse the effects of the overexpression of TPKb on growth and a number of physiological parameters, under a variety of conditions.
3. To investigate the effects of loss of function and overexpression of rice AKT1 channels on the growth, tissue ion concentrations, xylem sap, stomatal conductance and rate of photosynthesis.

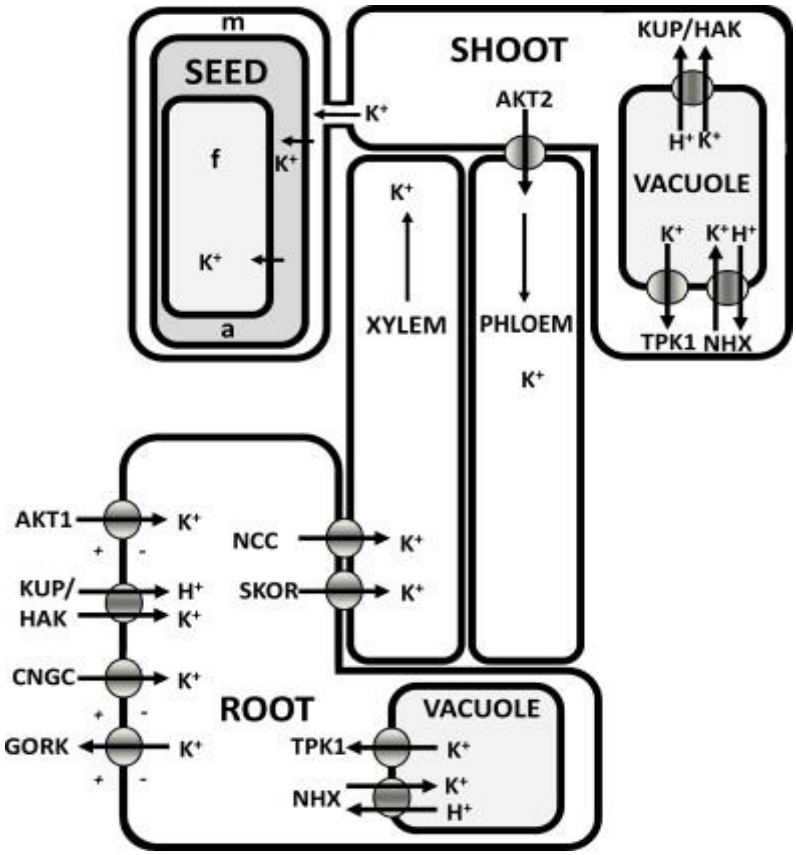

Figure 1-5: Overview of transport processes and proteins that are involved in K^+ uptake, efflux and distribution

Overview of transport processes and proteins that are involved in K^+ uptake, efflux and distribution; At the external soil:root interface transport functions are shown for passive [AKT1 and CNGC (cyclic nucleotide gated channel)] and energised (KUP/HAK) K^+ uptake and channel mediated K^+ release (guard cell outward rectifying K^+ channel; GORK); Xylem loading mainly happens through K^+ selective (SKOR) and non-selective (NCC) cation channels though energised systems may also play a role; Phloem loading of K^+ for recycling and/or sucrose loading may involve the AKT2 channel; K^+ flux to the seed is phloem mediated but K^+ is unloaded into the seed apoplast (a) at the junction between maternal (m) and filial (f) tissues; vacuolar K^+ accumulation is primarily driven by H^+-coupled antiporters such as NHX while vacuolar K^+ release is either passive through TPK type channels or, in K^+ starvation conditions, active through H^+ coupled KUP/HAK transporters.

Chapter 2

2 Materials and Methods

2.1 Plant material

Mature seeds of rice (*Oryza sativa* L.) sub group *Japonica* cv. Nipponbare and cv. Dongjin were obtained from the International Rice Research Institute (IRRI; Laos Banos, Philippines).

Independent transgenic lines of TPKa, TPKb and AKT1 were obtained in the *Oryza sativa* L. sub group *Japonica cv.* Nipponbare background by *Agrobacterium* transformation as mentioned in the relevant section.

Three putative insertion lines of *AKT1* were used in this study to analyse the disruptioonof this gene in rice under different conditions. The putative transposon insertion lines T14884T and NC0227 in the Nipponbare background were obtained from the Rice Genome Resource Centre of the National Institute of Agrobiological Sciences (RGRC-NIAS), Japan. The seeds for the postech T-DNA insertion line PFG_1B-16021 in the Dongjing background were obtained from Crop Biotech Institute, Department of Plant Systems Biotech, Kyung Hee University, republic of Korea. As documented in the Signal database (http://signal.salk.edu/cgi-bin/RiceGE?LOCATION=26121930&CHROMOSOME=chr01&INTERVAL=20) the transposon insertions were in the exon and intron of the T14884T and NC0227 lines respectively while the T-DNA is inserted in the 300UTR region of the line PFG_1B-16021 (for the details see Figure 4-3).

2.2 Chemicals and consumables

The chemicals used in this study were purchased from different companies including Sigma (UK), Fischer Scientific (UK) and Macherey Nagel (UK) and the consumables were purchased from StarLab (UK), Eppendorf (UK). The RNA extraction kits, gel purification and miniprep kits were purchased from Qiagen (UK) and Fermentas (UK).

2.3 Microorganisms

The *Agrobacterium* strain AGl1 was used for the transformation of rice with *TPKa, TPKb and AKT1*. For the amplification and purification of DNA, the *E.coli* strain DH5α was used as a recipient.

2.4 Plasmid vectors

The binary vectors pGreen and pSoup were used for rice transformation (Vain et al., 2004) as shown in Figure 2-1. 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*.

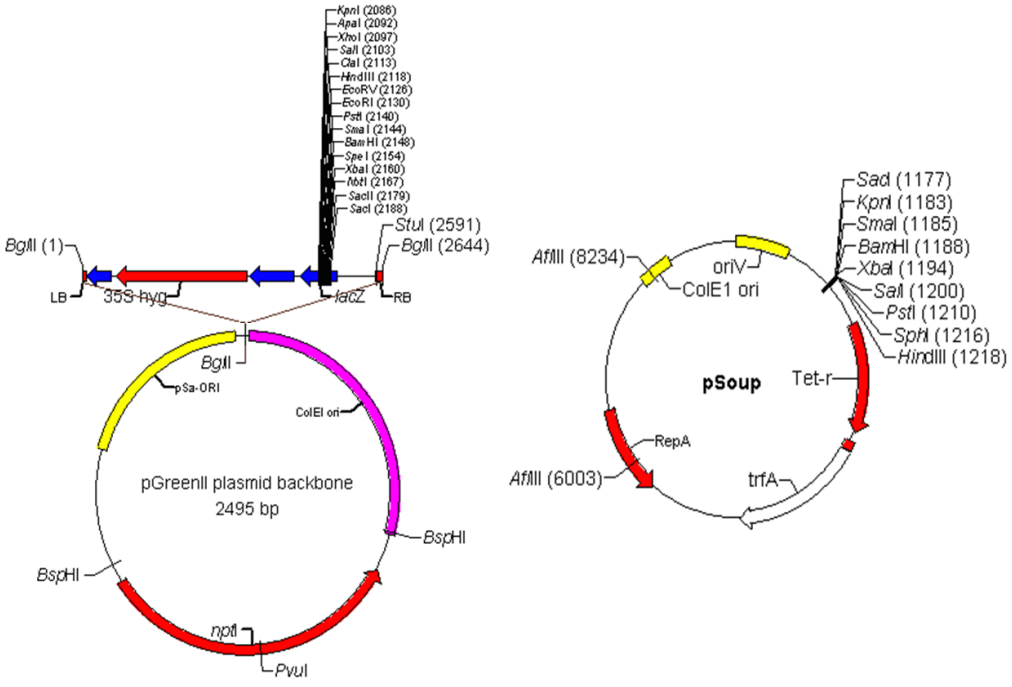

Figure 2-1: pGreen/pSoup based vectors used for cloning of *TPKa, TPKb* and *AKT1* and rice transformation

Binary vectors used for cloning of *TPKa*, *TPKb* and *AKT1* and rice transformation. pGreen and pSoup contain hygromycin and tetracycline as selectable markers respectively.

2.5 Growth medium and growth conditions

Rice seeds were geminated on terra-green and kept for five days in the dark at 28^{o}C and 90% relative humidity. The germinated seedlings were transferred to hydroponics in 2 or 3 litre boxes containing the growth medium. The growth medium consists of macronutrients and micronutrients as mentioned in Table 2-1. K_2SO_4 in the control medium was replaced with the equimolar quantity of Na_2SO_4 (53.48 g/l) for the Zero K^+ condition. The control medium was supplemented with additional KCl and NaCl to increase the concentration of Na^+ and K^+ up to 60 mM in the medium for the salt stress treatments, while for the osmotic stress treatment the control medium was supplemented with 5%, 10% and 15% PEG (Polyethylene glycol-4000). For zero K^+ treatments, all potassium salts in the control medium were replaced with equimolar quantities of the corresponding sodium salts.

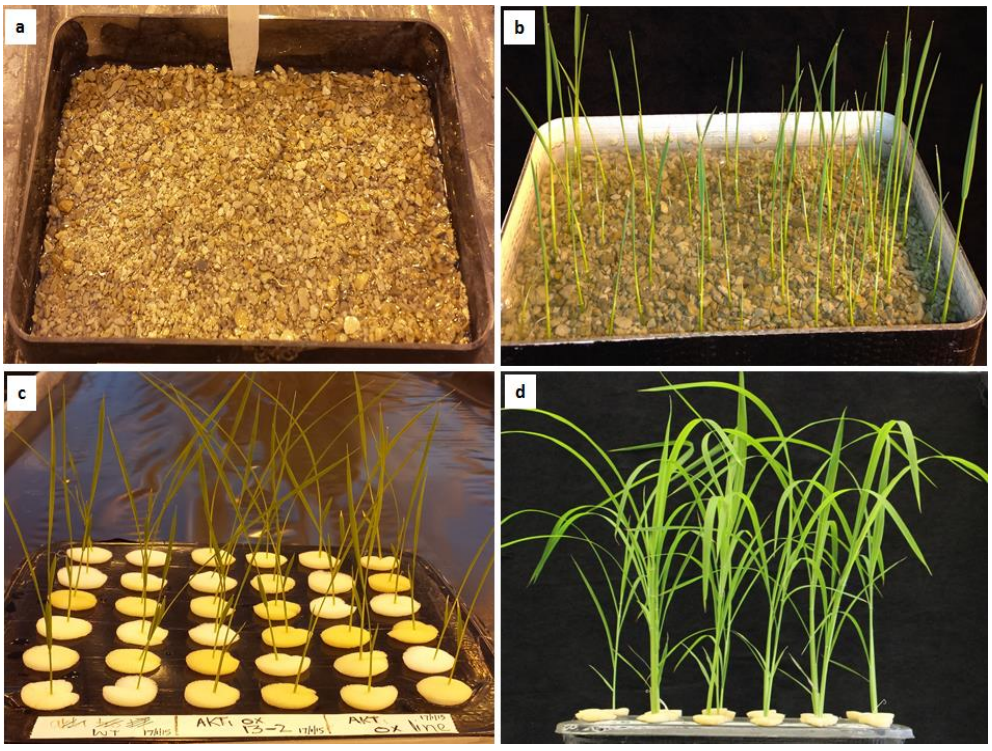

Figure 2-2: Sowing and growth conditions of the rice

a; Rice seeds were sown on terragreen in boxes and were kept in dark by putting a lid on the box, b; one-week old seedlings on the terragreen, c; two- week old seedlings were transferred to hydroponics conditions in the box with 3 L growth medium, d; four-week old plantlets ready for exposure to different media conditions for physiological experiments.

Table 2-1: Composition of plant growth medium

Stock solutions were prepared with deionised water. Micronutrients were dissolved separately, combined with 50 ml of conc. H_2SO_4 and volumes were then made up to 1l. The pH value was adjusted to 5.6 – 5.7 using appropriate amounts of HCl after all components were dissolved in deionised water (Yoshida *et al.*, 1976).

Serial No	*Element*	*Macronutrients*	*Preparation of stock solution (g/l)*	*ml of stock solution/1l medium*	*Concentration of element in medium (mM)*
1	N	NH_4NO_3	*91.4*	*1.25*	*2.9*
2	P	$NaH_2PO_4 \cdot 2\ H_2O$	*40.3*	*1.25*	*0.3*
3	K	K_2SO_4	*71.4*	*1.25*	*1.0*
4	Ca	$CaCl_2$	*88.6*	*1.25*	*1.0*
5	Mg	$MgSO_4 \cdot 7\ H_2O$	*324.0*	*1.25*	*1.6*
		Micronutrients			
1	Mn	$MnCl_2 \cdot 4\ H_2O$	*1.5*	*1.25*	*0.01*
2	Mo	$(NH_4)_6 \cdot Mo_7O_{24} \cdot 4H_2O$	*0.074*		*0.001*
3	B	H_3BO_3	*0.934*		*0.2*
4	Zn	$ZnSO_4 \cdot 7\ H_2O$	*0.035*		*0.0002*
5	Cu	$CuSO_4 \cdot 5\ H_2O$	*0.031*		*0.0002*
6	Fe	$FeCl_3 \cdot 6\ H_2O$	*7.7*		*0.04*
7	Silica	Na_2SiO_3	*0.18*		

The hydroponics boxes were placed in the glass house in the following conditions: (16h light/8h dark; 28/24 ^oC day/ night; 60% relative humidity with light radiation of about 160W/m^2). The growth medium was changed every three days. Three to four week old seedlings were used for growth experiments under different Na^+, K^+ and osmotic stress conditions. At the end of the growth experiments, the exposed plants were used for ion concentration analysis. Non-treated plants were grown in parallel and harvested at the same time and served as a control. The AKT1 transgenic plants and their corresponding wild types were also tested in different NH_4^+ concentrations as listed in Table 2-2.

Table 2-2: Medium concentrations of K^+ and NH_4^+ to test the effect of NH_4^+ on AKT1 genotypes (chapter 5)

Media	NH_4^+	K^+	NO_3^-	Na^+
Control	1.42 mM	1 mM	1.45 mM	0.3 mM
0 K^+ + 0 NH_4^+	0	0	1.45 mM	2.32 mM
0 NH_4^+	0	1 mM	1.45 mM	1.35 mM
0 K^+ + 3 mM NH_4^+	3 mM	0	1.45 mM	1.3 mM

2.6 DNA extraction from rice plants

DNA was extracted from rice plants according to the CTAB method. Plant material was ground to fine powder in liquid nitrogen and was quickly mixed with 450 µl pre-warmed CTAB buffer and incubated at 65 °C for 50 minutes. 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 sterilized water.

2.7 Total RNA isolation from rice tissues

Total RNA was extracted from the rice root and leaf tissues, using an RNase easy KIT (Qiagen, UK). Approximately 100 mg leaves were ground to fine powder in liquid nitrogen with the help of a grinder, while kept frozen. The tissue was transferred to RNase-free 2 ml tubes, allowing the liquid nitrogen to evaporate, but not letting the tissue to thaw. 450 µl of buffer RLT was added and vortexed vigorously. The lysate was added to a Q1Ashredder spin column placed in a 2 ml tube and was centrifuged for 2 min on full speed. The supernatant was transferred to a clean 1.5 ml Eppendorf tube. 0.5 ml of 96% ethanol was added to the lysate and was mixed immediately by pipette. The sample was then transferred to an RNeasy spin column and then centrifuged for 30 Sec at 10,000 RPM. The follow-through was discarded. To wash the RNeasy column 350 µl of buffer RW1 was added and centrifuged for 30 Sec. 10 µl of the DNase I stock solution was added to 70 µl of buffer RDD and was mixed by inverting the tube several times. 80 µl of DNAse I incubation mixture was directly poured over the RNeasy spin column membrane and left on the bench for 15 minutes. To wash the RNeasy column, 350 µl of buffer RW1 was added and centrifuged for 30 Sec and the flow-through was discarded. To wash the RNeasy spin column 500 µl of buffer RPE was added and then centrifuged for 30 Sec at 10,000 RPM. 500 µl of buffer RPE was again added and then centrifuged for 2 minutes at 10,000 RPM. For avoiding any carryover of buffers and contaminations the RNeasy spin column was placed in a new 2 ml collection tube and

was centrifuged for 1 min at 10,000 RPM. The RNeasy spin column was removed and placed in a 1.5 ml Eppendorf tube. For eluting the RNA 50 µl of RNase free water was added to the RNeasy spin column and then centrifuged for 1 minute at 1000 RPM.

2.8 cDNA synthesis

The enzyme used for the synthesis of the first strand of cDNA was the Moloney murine leukemia virus Reverse transcriptase (M-MLV RT). Two micrograms of RNA and 0.5 µg of oligo-dT primer per microgram of RNA was mixed and water was added to make the total volume up to 15 µl. The tube was heated to 70 ^{o}C for five minutes. Then the tube was cooled on ice to prevent secondary structures from reforming and M-MLV 5X reaction buffer (5 µl), dNTP mix (1.25 µl), recombinant Rnasin ribonuclease inhibitor (0.5 µl) and M-MLV RT (1 µl) were added and the final volume was adjusted to 25 µl by adding an appropriate amount of water. The sample was mixed gently by flicking and was incubated at 42 ^{o}C for 60 minutes.

2.9 PCR analyses for the screening of transgenic overexpressor lines

To analyse whether rice plants were transgenic the PCR method was used. DNA extracted from root and leaf tissues of the different lines was used as a template for the PCR. PCR mix was prepared in 2 ml Eppendorf tube by adding and mixing 5 µl Go-Taq flexi buffer (5X), 2 µl of $MgCl_2$ (25 mM), 1 µl of dNTP (10 mM), 1 µl of each primer (10 µM), 0.1 µl of Go-Taq polymerase (5 units/µl, 2-3 µl of the template DNA (10-20 ng) and the final volume was made to 25 µl (per reaction) by adding the required amount of water. PCR conditions varied according to the requirement of the primer pairs (Table 2-3) and the size of the amplicon. The PCR products were resolved by electrophoresis in a 1% agarose gel.

2.10 Identification of homozygous lines and qRT-PCR analyses

Hygromycin resistant primary transformants were selfed and homozygous lines of *TPKa, TPKb* and *AKT1* were identified in the T3 generation. The cDNA from the transgenic lines was prepared and used as a template for the qRT-PCR to analyse the transcript level of the respective genes. The quantitative analyses were carried out in triplicate using the SYBR Green master mix in an ABI 7300 sequence detection system. The amplicon of the rice histone gene was used as a control to normalise the data.

Table 2-3: **Primers used to screen the transgenic lines and analyse the expression level of genes in the transgenic lines**

Gene ID	Primer Sequence	Amplicon size
αHistoneFOs06g04030.1	CGAGAAGCGAAGAGGAGATG	465bp
αHistone-R Os06g04030.1	TCAACAAGTTGACCACGTCAC	465bp
Hygromycin-F	GGATATGTCCTGCGGGTAAA	794bp
Hygromycin-R	ATTTGTGTACGCCCGACAG	794bp
35S promoter-F	AAACCTCCTCGGATTCCATT	1600bp
35S terminator-R	GCTCAACACATGAGCGAAAC	1500bp
TPKa SYBR-F Os03g54100	CAAGGCCCCCCTGAAAAG	65bp
TPKa SYBR-R Os03g54100	CTTGGCCTCATCTCCTTGAATAA	65bp
TPKb-F Os07g01810)	GCTGCACTCGCACACGAT	70bp
TPKb-R Os07g01810	CCCCGCCGTGTAGAGCTT	70bp
Actin-F Os05g36290.2	ATGAAGCTCAGGCAAAAAGGG	322 bp on cDNA and 399 on gDNA
Actin-R Os05g36290.2	ACAGTGTGGCTCACACCATC	322bp on cDNA and 399 on gDNA
Hygromycin- F	TGAAAAAGCCTGAACTCACCG	735bp
Hygromycin-R	TCTGCTGCTCCATACAAGCC	735bp
AKT1 cDNA-F Os01g45990	ATAGTATTAATTAGGCTAGAGCCA	758bp
AKT1 cDNA-R Os01g45990	TGAAGACCTTCTGAATCTGTC	758bp
Transposons specific primer	AGGTTGCAAGTTAGTTAAGA	
AKT1-R Os01g45990	ACGTAGCGAATCCATAAGCTCC	1600bp for *AKT1* transgene
AKT1-F Os01g45990	ACCAACATGGCTTGTTCTTGAC	1002 on gDNA and 638 on cDNA
AKT1-R Os01g45990	TGAAGACCTTCTGAATCTGTC	1002 on gDNA and 638 on cDNA

2.11 Growth analyses of different rice genotypes in hydroponics

Rice plants were grown in different media conditions to study their growth. Plants were grown on terra green for one to two weeks week and were then transferred to hydroponics for two weeks. Three plants of each genotype were then transferred to

different media conditions, i.e. Control condition, 0 mM K^+, 60 mM K^+, 60 mM Na^+ and osmotic stress i.e. 5%, 10% and 15% PEG and the fresh weight (W_i) of each plant was recorded. After two to three week exposure of the plants to the above mentioned media conditions, again the fresh weight (W_f) of each plant was recorded. The percent relative growth rate (RGR) was calculated according to the equation as mentioned here $\left(\frac{W_f - W_i}{N \times W_i}\right) \times 100$ where

W_f: fresh weight of the plant at the end of the experiment

W_i: fresh weight of the plant at the start of the experiment

N: total number of days to which the plants were exposed to different treatments.

The equation is derived from Poorter and Garnier (1996). The experiments were repeated at least three times. The RGR relative to control conditions was calculated using the equation as mentioned here $\left(\frac{\text{RGR in the tested condition}}{\text{RGR in the control condition}}\right) \times 100$.

2.12 Growth analyses of different genotypes in soil conditions

The plants of different genotypes in hydroponics were grown in hydroponics for four weeks. These plants were then transferred to the soil in pots and fresh weight was recorded. The pots were kept in trays and in the control conditions; plants were flooded with water while the drought stress was applied by watering plants twice a week with 500 ml of water per tray. After 6 week exposure, the fresh weight of the plants was recorded and RGR was determined according to the equation as mentioned in the section 2.11.

2.13 Na^+ and K^+ tissue concentration analyses

Na^+ and K^+ concentrations of roots and shoots were measured using flame photometry. All genotypes were grown and treated as described in 2.5. The plants were then separated into roots and shoots and were washed with 20 mM $LaCl_3$ 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 extracted with 10 ml of 20 mM $LaCl_3$ for 24 hours and Na^+ and K^+ concentration of the samples were recorded using a flame photometer (Sherwood flame photometer-410, Cambridge, UK). The root and shoot tissues of individual plant were used separately for the analysis.

2.14 Relative water content analyses

The relative % water content in the tissues was determined by the difference between fresh weights and dry weight using the equation $\frac{FW-DW}{FW} * 100$ where FW is the fresh weight and DW is the dry weight of the plant tissues.

2.15 Xylem sap analyses:

Six week old plants of different genotypes were treated with different media regimes (as mentioned above) for one week before collecting the xylem sap. Plants at their five-six leaf stage were transferred to a pressure chamber (Digital plant water potential apparatus, EL540-300), and the shoot was excised about 15-20 cm above the root/shoot junction. Pressure exceeding the osmotic pressure of the external solution was applied to the chamber. Xylem sap was collected for 10 minutes. Aliquots of 0.3 ml xylem sap were collected from 3 plants in parallel and immediately put on ice. Na^+ and K^+ concentrations of the xylem sap were measured with a flame photometer (Sherwood flame photometer-410 Cambridge, UK).

2.16 Whole-Leaf Conductance Measurements

Four week old plants were grown in different media conditions for one week. Intact leaves of the different genotypes were used to measure leaf conductance and rate of photosynthesis by placing them in the measuring chamber of an Infrared Gas Analyser, Li-Cor 6400 (LI-COR, Cambridge, UK). For each genotype, three leaves (second, third and fourth) per plant (at six-leaf stage) were used and these were derived from three separate plants (n=9). The experiments were repeated three times.

Whole leaf conductance and rate of photosynthesis were also analysed after ABA treatment. Initially, data were recorded in the control condition of the intact leaves of six-week old plants, then the roots were exposed to the contraol medium having 100 µM ABA concentration medium for one hour and data were recorded. Afterwards plants were put back in control conditions and a third set of data was recorded.

2.17 K^+ uptake analyses

Wild type and TPKb overexpressor plants were grown in the control medium for four weeks and then were transferred to small hydroponics boxes containing 200 ml medium. The plants were exposed for 9 hours to low K^+ (50 µM) medium conditions. The samples were collected from the medium after each hour and were analysed for the K^+ concentration using flame photometer as mentioned above. The observed K^+ concentration of the media at each time point was subtracted from the initial total K^+ concentration and was regarded as leaked-out/absorbed K^+ by the plants.

2.18 Energy-dispersive X-ray spectroscopy (EDX or EDS) analysis

The distribution of K^+ in the cytoplasm and vacuole in the wild type and the overexpressing lines of TPKa and TPKb was analysed using EDX method. Three week old rice plants were shifted to the control and osmotic stress (10% PEG) conditions for one week. The leaves of the plants were used to measure the distribution of K^+ in the vacuole and cytoplasm using EDX techniques and these analyses were carried out by J Devonshire at the Rothamsted Research station in Harpenden. Tissue was cut from the 4^{th} leaf of each plant 4 cm from the leaf tip. 2 sections of 2 x 3 mm were cut from this and mounted in slotted cryo pins using OCT compound (Agar Scientific). Samples were cryo-planed using a Leica UC6 cryo-ultramicrotome, with temperatures set for the sample -90^{o}C / knife -120^{o}C / chamber -120^{o}C. For imaging, samples were examined for suitable sites of interest from which spectra were acquired inside and outside the cell vacuole. About 7-10 spectra of each (vacuole and cytoplasm) were collected for all samples from several sites along the planed surfaces. The EDS analysis was performed using the INCA Energy 350 (Oxford Instruments UK) system.

2.19 Statistical analyses

Data were obtained from a minimum of three replicates for all experiments and significance was analysed using unpaired two-tailed Student t-tests. Significance levels were at $p<5\%$ unless indicated otherwise. The error bars in the figures represent standard errors.

Chapter 3:

3 Overexpression of TPKa and TPKb improves abiotic stress tolerance in rice

3.1 Introduction

Plant cell vacuoles are important organelles and comprise up to 90% of the cell volume in the mature cells of the woody parts of plants and in the herbaceous plants (Isayenkov and Maathuis, 2013: Raven, 1987). Vacuoles play vital roles in many crucial physiological processes such as turgor provision, storage of minerals and nutrients, cellular signalling, storage and inactivation of toxic compounds, participation in programmed cell death, accumulation of defence enzymes and thousands of secondary metabolites and storage proteins. It was believed that vacuoles store a variety of proteins; however, the low pH of vacuoles favours protein breakdown and the activity of proteases. This has brought about debate as to how it is possible that proteins and proteases are present in the same vacuoles (Nishimura and Beevers, 1979). Recent studies have now made it clear that plant cells may have two functionally distinct vacuoles, the central lytic vacuole (LV) and small vacuoles (SVs) (Isayenkov *et al*., 2010). The majority of the above mentioned processes has been associated with the LV. The low pH of the LV helps in the degradation of both exogenous and endogenous compounds (Echeverria and Jacqueline, 1989). SVs have some characteristics of protein storage vacuoles (PSVs) which are mainly found in reproductive tissues such as seeds, where large quantities of proteins and minerals are stored for the developing embryo. PSVs of the seeds store globulin and prolamin (Staswick, 1994) while in the leguminous plants they store lectins and its isoforms in the PSVs of the leaves and bark (Herman et al., 1988). All these proteins are deposited in the vacuoles by Golgi-mediated processes (Klauer and Franceschi, 1997).

The functions of PSV-like SVs are not entirely clear but they may be distinct from seed PSVs. SVs of vegetative cells store a variety of proteins in contrast to seed PSVs which store only specific proteins. The proteins in the seed PSV are regulated by developmental programming (Vitale and Hinz, 2005) whereas the storage of proteins in

the SVs of the vegetative cells is regulated by variety of factors, such as developmental and seasonal changes (Nsimba-Lubaki and Peumans, 1986; Wetzel et al., 1989). SVs might have a role as osmoregulatory structures of the cells rather than specific protein storage requirements. The tonoplast of SVs in the seeds and vegetative cells contain different proteins. The vegetative cells contain proteins which might have a role in osmoregulation while the PSVs of the seeds have protein which might be involved in the efflux /influx of water during the maturation and germination of seeds (Herman and Larkins, 1999).

Membrane transporters involved in vacuolar functions

Most of the physiological functions of the vacuoles rely on the presence of membrane transporters in the tonoplast. The tonoplast is energised by two types of proton pumps i.e. V-ATPase and P-Pase. A wide range of transporters is involved in the transport of substances across the tonoplast. Examples include the cation: H^+ exchanger (CHX family), Ca^{2+}: H^+ exchanger (CAX family), heavy metal transporter (CDF family), aquaporins (TIPS), etc.

The tonoplast contains various types of ion channels such as anion channels, ligand gated cation channels and vacuolar cation channels. The anion channels have not been characterised extensively but some of these may have a role in nutrient homeostasis during biotic and abiotic stresses. It has been reported by Pantoja *et al.,* (1989) that anion channels are involved in Cl^- loading into the vacuole during salt stress, and in malate accumulation. AtALMT9 (Kovermann *et al.,* 2007 and VvALMT9 (De Angeli *et al.,* 2013) are reported to be localised in the tonoplast and involved in the malate transport to the vacuole. CLCs is another group of anion channels (Harada *et al.*, 2004), but the picture is cloudy where functions are concerned. They have been suggested to have a role in chloride, nitrate, malate and citrate transport (Harada *et al.*, 2004). Other studies suggested a role of AtCLCa as $2NO_3^-/1H^+$ antiporters (De Angeli *et al.,* 2006).

GLRs and CNGCs are the two families of the ligand gated cation channels in plants. The localisation of these channels in the tonoplast is not confirmed, however, there are reports which suggested the presence of these channels in the tonoplast (Isayenkov *et al.,* 2010) where they may be involved in the Ca^{2+} release from the vacuole (Allen *et al.,* 1995).

Vacuolar cation channels are divided into three subgroups, the fast vacuolar (FV) channels, the slow vacuolar (SV) channels and vacuolar K^+ (VK) channels.

The FV channels have low selectivity with a K^+: Na^+ selectivity ratio of around one and other monovalent cations can also pass through these channels. Not much information is available about the divalent cation conductance of FV channels. FV channel activity decreases when the concentration of Ca^{2+} increases in the cytoplasm beyond 200 nM and its permeability does not depend upon the membrane potential. The genes coding for these channels are not yet known. FV channels have been reported in the storage tissues of red beet (Hedrich and Neher, 1987) and mesophyll tissues of barley (Tikhonova *et al.,* 1997). These channels may be involved in the cellular K^+ distribution between and vacuole and cytoplasm (Pottosin and Martinez-Estevez, 2003)

Slow vacuolar (SV) channels are present in all plant species investigated so far. They can conduct both monovalent and divalent cations across the tonoplast and their activity is dependent on the membrane potential. Channel activity is regulated by cytoplasmic Ca^{2+}, phosphorylation and 14-3-3 proteins (see review Isayenkov *et al.,* 2010). Some reports suggested their role in preventing Na^+ leakage from the vacuole during salt stress (Maathuis and Sanders, 1992). Peiter *et al.,* (2005) identified AtTPC1 as the candidate gene that encodes the SV channel. AtTPC1 has been characterised extensively, but so far contrasting results were achieved for AtTPC1 in different plants and in different conditions.

Vacuolar K^+ channels (VK) are K^+ selective channels. They activate at much lower cytoplasmic Ca^{2+} than the SV channels and are not dependent on the membrane potential. TPKs are members of the VK channels and are characterised by a four transmembrane-two pore structure with GYGD motifs which are highly selective for K^+ and one or two EF hands. 14-3-3 proteins increase the activity of VK channels, and the interaction of 14-3-3 proteins were reported in *Arabidopsis* (Latz *et al.,* 2007), barley (Sinnige *et al.,* 2005) and rice (Isayenkov *et al.,* 2011). These proteins bind to the N-terminus of the TPK proteins. The *Arabidopsis* genome consists of five members of TPK isoforms of which four are localised to the tonoplast while one is localised to the plasma membrane. *Arabidopsis* TPK1 (an isoform of TPKs), localised to the tonoplast and is characterised in detail (Dunkel *et al.,* 2008; Becker *et al.,* 2004; Gobert *et al.,* 2007). It was found that this isoform is involved in K^+ homeostasis especially in K^+

deficient conditions, although not changing the overall K^+ concentration. TPK1 was found to be involved in stomatal closure (Gobert *et al.,* 2007).

Gobert *et al.,* (2007) also found that the expression of TPK1 has a role in the germination of seeds. They found a comparatively better germination rate of the wild type and TPK1 overexpressor seeds as compared with the *Attpk1* mutant, in control and ABA treated seeds. After rupturing of the seed coat and endosperm layer, cellular turgidity is required for the elongation of the radical. This turgidity is achieved by the accumulation of more K^+ in the vacuoles. TPK1 channels are supposed to be involved in this accumulation. This is supported by the increased expression level of TPK1 at this stage of germination. During radicle elongation, up to 10 fold increase in the expression level was observed. The accumulation of K^+ in the vacuole through the TPK1 channels may depend on the vacuolar membrane potential and lower K^+ concentration in the vacuole. The required K^+ for the accumulation in the vacuoles may be available from the K^+ sources in the form of complex molecules, such as potassium phytate, stored in the form of globoids in PSVs. During these conditions, these complex molecules are broken down and K^+ is released in the form of ions. It is then stored in the vacuoles to provide turgor in the cell for the elongation of the radical and hence increased germination rate.

The TPK family has five members in *Arabidopsis thaliana* and at least two members in rice. Out of five AtTPK channels, four are localised in the tonoplast and one, TPK4, is localised in the plasma membrane (Voelker *et al.,* 2006; Schönknecht *et al.,* 2002; Becker et al, 2004; Gobert *et al.,* 2007; Dunkel et al, 2008). Only AtTPK1 has been shown to form functional ion channels. TPK1 activity is voltage independent, and it is regulated by intracellular Ca^{2+} (Gobert *et al.,* 2007), cytoplasmic pH, phosphorylation, and 14-3-3 proteins (Gobert *et al.,* 2007; Latz *et al.,* 2007) whereas the activity of *Nicotiana tabacum* TPK1 is reported to be also sensitive to spermidine and spermine (Hamamoto *et al.,* 2008). AtTPK1 has been functionally characterized using overexpression, loss-of-function, and heterologous expression analyses; it is ubiquitously expressed and has a role in cellular K^+ homeostasis and K^+ release during stomatal functioning (Gobert *et al.,* 2007). At*TPK1* expression also affected seed germination, and its expression is high in embryonic tissues (Czempinski *et al.,* 2002; Gobert *et al.,* 2007). TPKs have a four-trans membrane domain (4-TMD) two pore

structure, and both pores contain the GYGD motif, (Voelker *et al.,* 2006), responsible for K^+ selectivity (Maathuis, 2007) as shown in Figure 3-1a. Most of the TPK channels have two clear 'EF hand' domains localized in the C terminus where Ca^{2+} binds suggesting that Ca^{2+} is involved in TPK regulation (Maathuis, 2007).

The rice genome has two isoforms of TPK, i.e. TPKa and TPKb (Isayenkov *et al.,* 2011). Both isoforms are 63% identical to each other and 57% identical to AtTPK1. However, TPKa and TPKb are localised to different membranes: TPKa is localised to the tonoplast of LVs and TPKb to the tonoplast of SVs (Figure 3-1b, c and d). Isayenkov *et al.,* (2011) showed that the C-terminal region of the TPKb protein is important for the localisation of this protein to the SVs. They changed the C-terminal region of the TPKa with the same region of TPKb and observed a shift of localisation for TPKa from LVs to PSVs. They suggested that TPKb trafficking to the PSVs follows the same model as proposed for α-TIP trafficking (Jiang and Rogers, 1998; Oufattole *et al.,* 2005). The change in the TPKa C-terminus residues, i.e. valine, asparagine and lysine at the specific positions (303, 313 and 326) with three TPKb C-terminus residues, i.e. leucine, serine and asparagine respectively showed effects in the changes of localisation of TPKa from LVs to PSVs. Size of the side chain, charge and phosphorylation is affected in the C-terminus by these changes. Among these three mutations, the change in the asparagine residue of TPKa with the serine residue of the TPKb showed strongest effects in the changes of localisation of TPKa from LVs to PSVs.

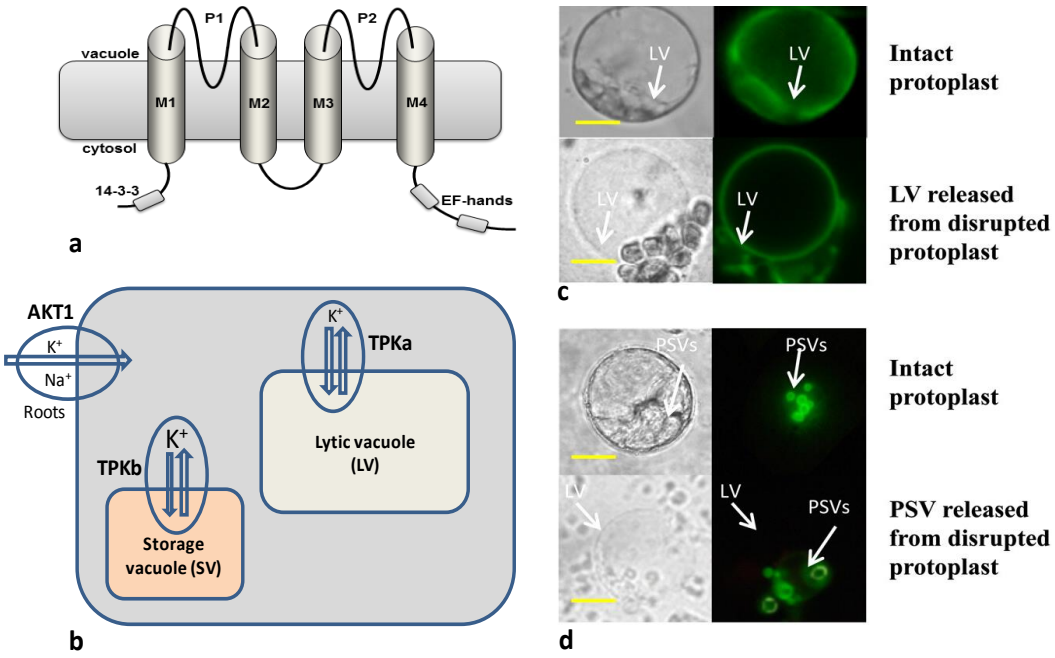

Figure 3-1: Structure and localization of TPKa and TPKb channels in rice

a; 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 carboxy terminal Ca^{2+} binding EF-hands, (b); Vacuolar localization of TPKa and TPKb channels in the tonoplast of LV and SV respectively, (c and d); Fluorescence images of intact rice protoplasts and released vacuoles show TPKa:EYFP expression in the central LV (c) and TPKb:EYFP expression in multiple smaller SVs (d). Bars = 5 µm (reproduced from Isayenkov *et al.*, 2011).

This study is aimed at improving K^+ nutrition and thereby increasing tolerance of rice to abiotic stresses such as salt and drought stress. As it is mentioned above, maintaining comparatively higher K^+ concentration in the roots and its distribution to the shoots is vital in the strategies against salinity. TPKs are involved in the distribution of K^+ in cells and K^+ distribution within the plant body and therefore it is hypothesized that the overexpression of these channels may impact on rice resistance to abiotic stresses.

3.2 Results

PART A: CHARACTERIZATION OF RICE TPKa

3.2.1 PCR analysis to identify *TPKa* overexpressor rice plants

Two putative rice *TPKa* transgenic lines (line-133 and line-212) were screened by PCR at their T3 generation for Os*TPKa* transgene presence and overexpression. Different sets of primers were used; hygromycin primers to test the presence of the hygromycin

resistance marker gene (Figure 3-2a), 35S promoter region "forward" and terminator "reverse" primers (Figure 3-2b) and 35S promoter region "forward" and *TPKa* gene specific "reverse" primers (Figure 3-2c). All 20 plants (not all are represented in the Figure 3-2) of both the *TPKa* transgenic lines gave amplification with the three sets of primers which suggests that these lines are transgenic and homozygous for the *TPKa* transgene. These transgenic lines were then checked by semi quantitative RT-PCR and qPCR to check the level of *TPKa* expression relative to control plants (Figure 3-3 and 3-4). The results showed approximately 5-20 times higher levels of *TPKa* expression in the transgenic lines as compared with the wild type plants. To obtain appropriate control lines for the transgenic lines, progenies of self-crossed heterozygous transgenic plants which lacked the transgene in the T3 generation were identified. These are called wild type throughout and were used as control in all experiments.

In the following text TPKa overexpressor line-133 and line-212 are labelled as TPKa ox-1 and TPKa ox-2 respectively.

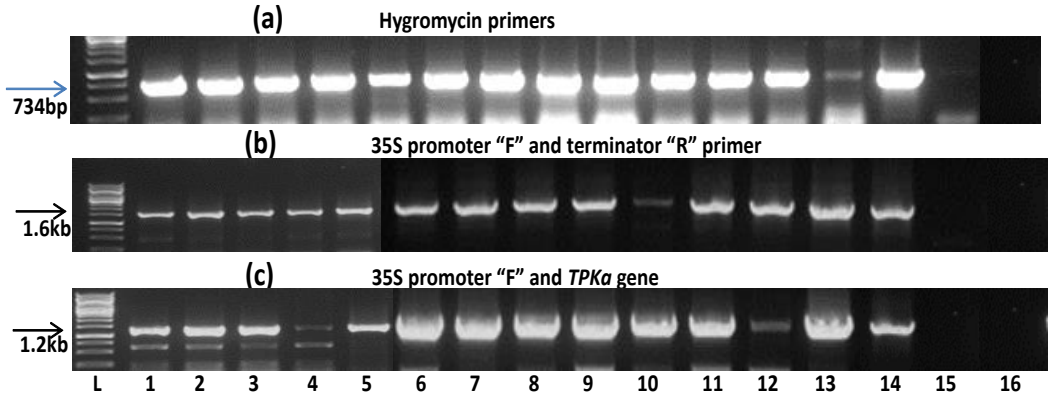

Figure 3-2: Screening of the putative TPKa transgenic lines by PCR

PCR results for TPKa ox-1 and TPKa ox-2; Putative overexpressor lines gave amplification with three sets of primers which suggests that these lines are homozygous transgenic for TPKa. Lane 1-7, TPKa ox-1; Lane 8-13, TPKa ox-; Lane 14, construct (+ive control); Lane 15, wild type; Lane 16, water (-ive control); L is the 2-Log ladder.

Figure 3-3: Analyses of the expression of *TPKa* in transgenic lines by RT-PCR

RT-PCR results for *TPKa* ox-1 and *TPKa* ox-2. The results show high level of expression for *TPKa* in the overexpressor lines. Lane 1-2, wild type; Lane 3-4, *TPKa* ox-1; Lane 5-6, *TPKa* ox-2; Lane7, gDNA *TPKa* ox-1; Lane 8-9, water for both *TPKa* and *actin* primers.

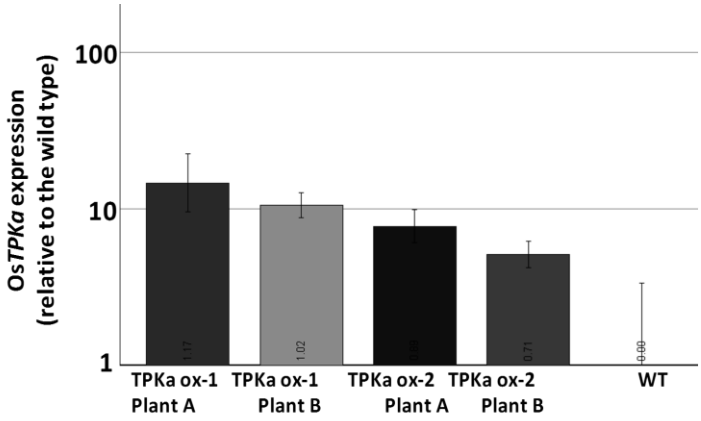

Figure 3-4: Analyses of the expression of *TPKa* in transgenic lines by q-PCR

qPCR for rice TPKa ox-1 and TPKa ox-2, two plants of each line were tested compared to the wild type for *TPKa* gene expression relative to the actin gene. The results showed a high level of gene expression in both overexpressor lines as compared to the wild type. The error bars represent the standard error among the TPKa expression of different lines.

3.2.2 Growth analysis of wild type and rice TPKa overexpressing plants

To investigate the effect of *OsTPKa* overexpression on plant growth, relative growth rates (RGRs) were recorded for plants grown in control, 0 K^+, 60 mM KCl, 60 mM NaCl and 10% PEG (osmotic stress) conditions.

The results show (Figure 3-5) that both the overexpressor lines of *TPKa* showed significantly less growth as compared to the wild type in control conditions while no significant difference was noted in the rest of the media conditions (Figure 3-5a). 25% growth reduction was observed for the wild type plants in the $0K^+$ as compared to the control conditions while not so much reduction was observed for the overexpressing plants in both conditions. However when the data were normalized to control conditions (Figure 3-5b), the results varied between the genotypes. Both the overexpressing lines of TPKa showed higher growth as compared with the wild type in 0 mM K^+ and osmotic stress conditions. Both the overexpressing lines of *TPKa* showed no significant

difference in the growth as compared to the wild type in 60 mM K^+ and 60 mM Na^+ conditions.

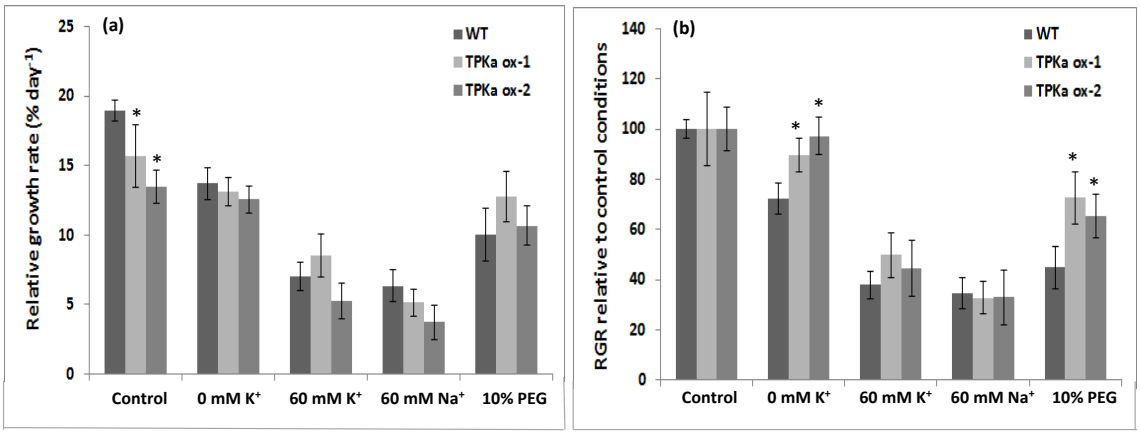

Figure 3-5: Relative growth rate for wild type and TPKa transgenic rice lines in hydroponics exposed to different media conditions

a; relative growth rate (RGR, % day^{-1})) absolute data, b; RGR relative to the growth in control condition (% day^{-1}) after 14 days for the wild type plants and transgenic lines of TPKa. Plants were grown in hydroponics media using control medium, 0 mM K^+, 60 mM K^+, 60 mM Na^+ and osmotic stress (10% PEG) conditions. Data are from three independent experiments, and the bars in the figures represent the standard errors. * denotes a significant differences by T-test at a probability level of $p < 0.05$ between the wild type and overexpressor lines.

3.2.3 Overexpressing lines of *TPKa* showed better growth under drought stress in soil conditions

We observed a better growth phenotype for the hydroponically grown TPKa overexpressing plants as compared with the wild type plants in osmotic stress conditions (Figure 3-5). These results were tested in the more natural drought conditions in soil. Therefore, to study the effect of TPKa expression on drought stress rather than osmotic stress, plants were grown in soil conditions and were tested for growth as described in the method section. The overexpressing lines of TPKa showed lower growth as compared with the wild type plants in control conditions while no growth phenotype was observed in drought condition (Figure 3-6a). About3 fold decrease was observed in the growth of wild type plants in drought as compared to the control conditions. However the normalized data showed higher growth for the TPKa ox-1 line as compared with the wild type plants in drought condition (Figure 3-6b).

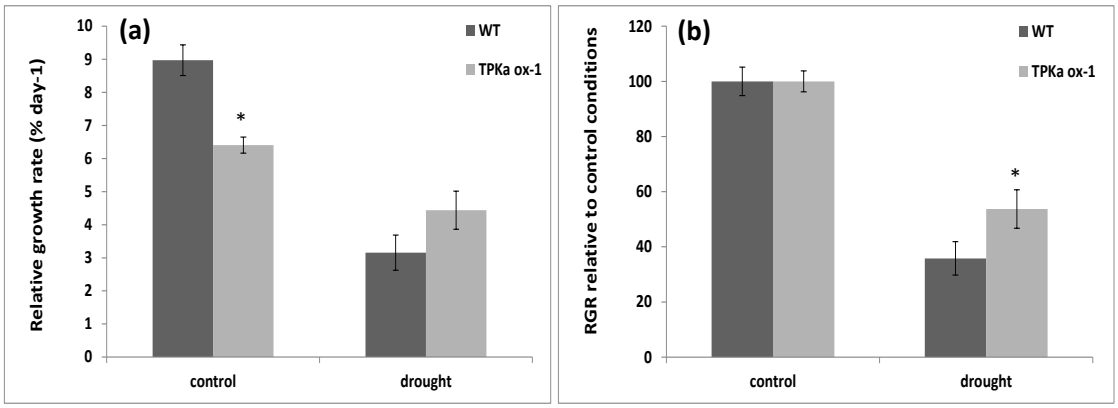

Figure 3-6: Relative growth rate for wild type and TPKa transgenic rice lines in soil exposed to drought and control conditions

a; relative growth rate (RGR % day^{-1}) absolute data, b; RGR relative to the growth in control condition (% day^{-1}). Wild type plants and transgenic lines of TPKa were grown for 6 weeks in control and drought conditions in soil and growth was recorded then. Data are from three independent experiments, and the bars in the figures represent the standard errors. * denotes a significant differences by T-test at a probability level of $p < 0.05$ between the wild type and overexpressor line.

3.2.4 Tissue ion concentration analyses in rice TPKa overexpressor lines and wild type plants

The better growth of the TPKa transgenic lines in K^+ deficient and osmotic stress conditions might be because of improved K^+ nutrition as it is suggested that TPKa is involved in K^+ homeostasis and distribution. To study the effects of TPKa on K^+ nutrition the Na^+ and K^+ concentration of the root and leaf tissues of the *TPKa* overexpressing lines and wild type plants were analysed using flame photometer as described in the method section.

3.2.4.1 K^+ concentration analyses

The roots of both the overexpressing lines of TPKa showed (Figure 3-7a) higher K^+ concentration as compared to the wild type at control, and 0 mM K^+ conditions. Both the overexpressor lines showed less K^+ in roots as compared to the wild type when grown at high K^+ and high Na^+ conditions. TPKa ox-1 has more K^+ while TPKa ox-2 has less K^+ as compared with the wild type in osmotic stress conditions. The leaves of both the overexpressor lines of TPKa showed (Figure 3-7b) higher K^+ concentration as compared to the wild type plants at control, 0 mM K^+ and 60 mM K^+ conditions while no difference was found at high Na^+ and osmotic stress conditions.

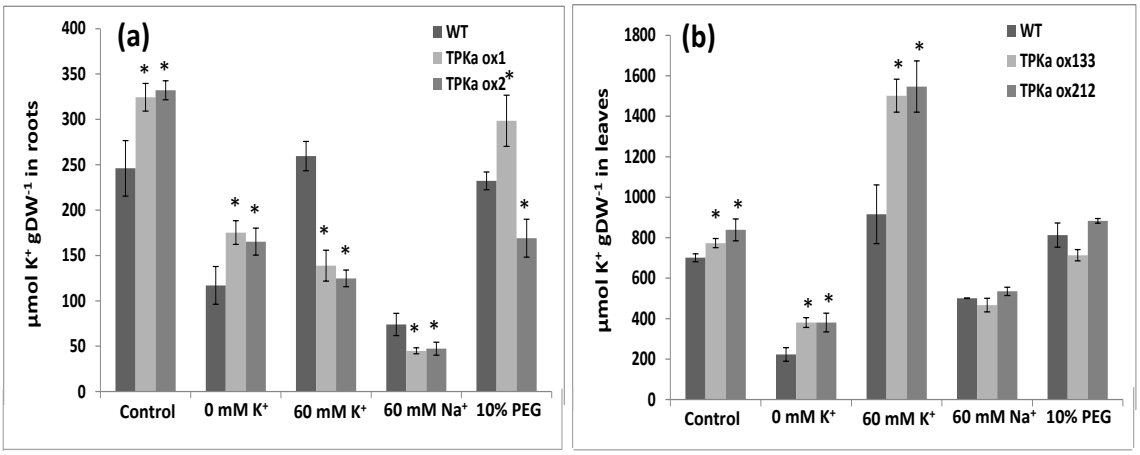

Figure 3-7: K^+ concentration analyses for wild type and TPKa transgenic rice lines in hydroponics exposed to different media conditions

a; root K^+ concentration and b; shoot K^+ concentration of rice wild type and TPKa overexpressor plants. The plants were grown in different media conditions as mentioned in the Figure and root and shoot tissues were analysed for K^+ concentration. Experiment was replicated three times and the bars in the figures represent the standard errors. * denotes a significant differences by T-test at a probability level of $p < 0.05$ between the wild type and overexpressor lines.

3.2.1.1. Na^+ concentration analyses

Both the overexpressing lines of TPKa showed higher Na^+ concentration as compared with the wild type plants in the root tissues at 0 mM K^+ in the medium (Figure 3-8a). During higher Na^+ exposure (60 mM) no difference was observed for TPKa ox-1 while TPKa ox-2 showed significantly less Na^+ in the root tissues as compared with the wild type plants. At 0 mM K^+ in the medium TPKa ox-1 showed less Na^+ in the shoots as compared with the wild type plants while no difference was observed for the TPKa ox-2. At higher Na^+ (60 mM), both the overexpressor lines of TPKa showed higher Na^+ in the shoot tissues as compared with the wild type plants (Figure 3-8b).

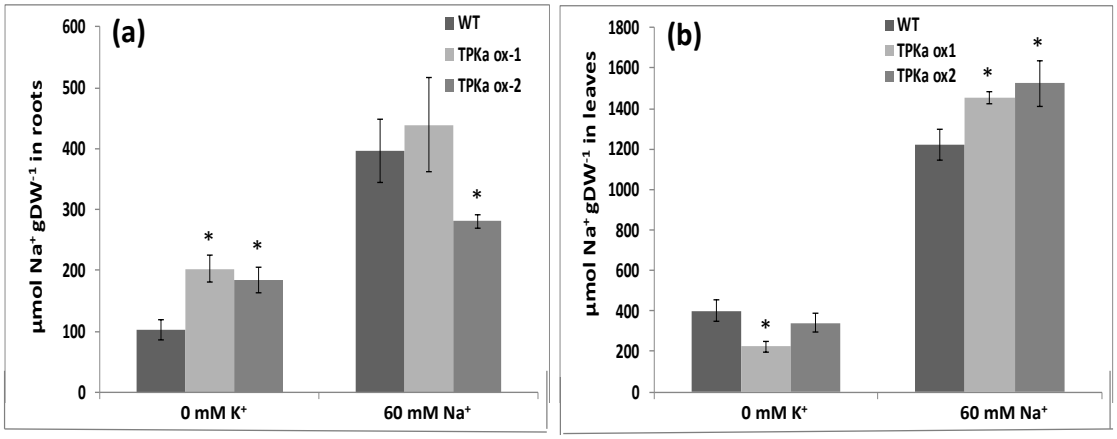

Figure 3-8: Na^+ concentration analyses for wild type and TPKa transgenic rice lines in hydroponics, exposed to different media conditions

A; root Na^+ concentration and b; shoot Na^+ concentration of rice wild type and TPKa overexpressor plants. The plants were grown in 0 mM K^+ and 60 mM Na^+ conditions and root and shoot tissues were analysed for Na^+ concentration. Experiment was replicated three times and the bars in the figures represent the standard errors. * denotes a significant differences by T-test at a probability level of $p < 0.05$ between the wild type and overexpressor lines.

3.2.5 Tissue ion concentration analyses in rice wild type and *TPKa* overexpressor plants grown in soil

The root tissues showed no significant difference in the K^+ concentration for wild type and TPKa ox-1 plants at control conditions while higher K^+ concentration for TPKa overexpressing line as compared with the wild type plants in drought conditions (Figure 3-9a). TPKa ox-1 showed higher K^+ concentration as compared with the wild type plants in the leaf tissues at control and drought conditions (Figure 3-9b).

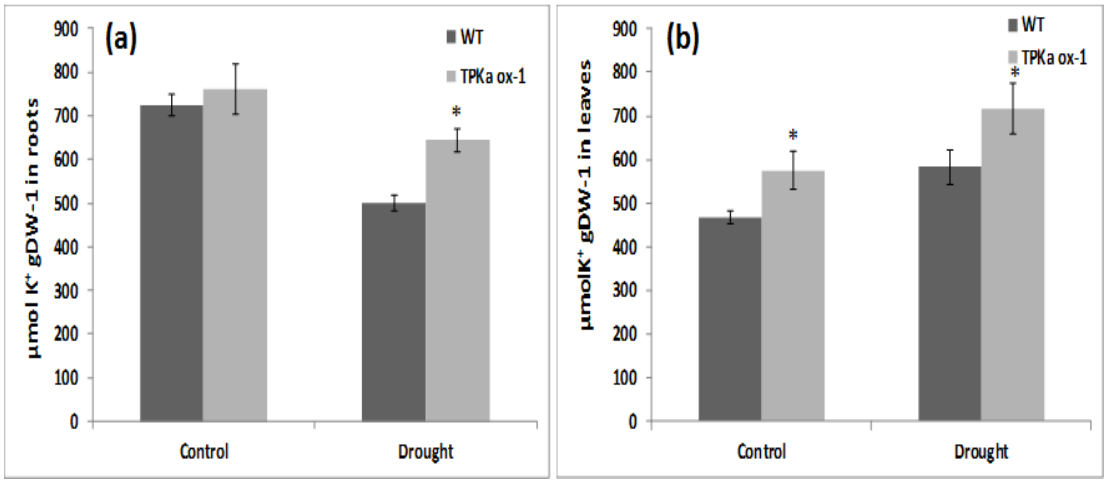

Figure 3-9: K^+ concentration analyses for the wild type and TPKa transgenic rice lines exposed to drought stress in soil

a; root K^+ concentration and b; shoot K^+ concentration of rice wild type and TPKa overexpressor plants. The plants were grown for six weeks in control and drought conditions in soil and root and shoot tissues were analysed for K^+ concentration. Experiment was replicated three times and the bars in the figures represent the standard errors. * denotes a significant differences by T-test at a probability level of $p < 0.05$ between the wild type and overexpressor line.

The root tissues of the *TPKa* ox-1 showed less Na^+ concentration as compared with the wild type plants in both conditions (Figure 3-10a) while there is no significant difference in the shoot tissues of TPKa ox-1 and wild type plants for the Na^+ concentration in both conditions (Figure 3-10b).

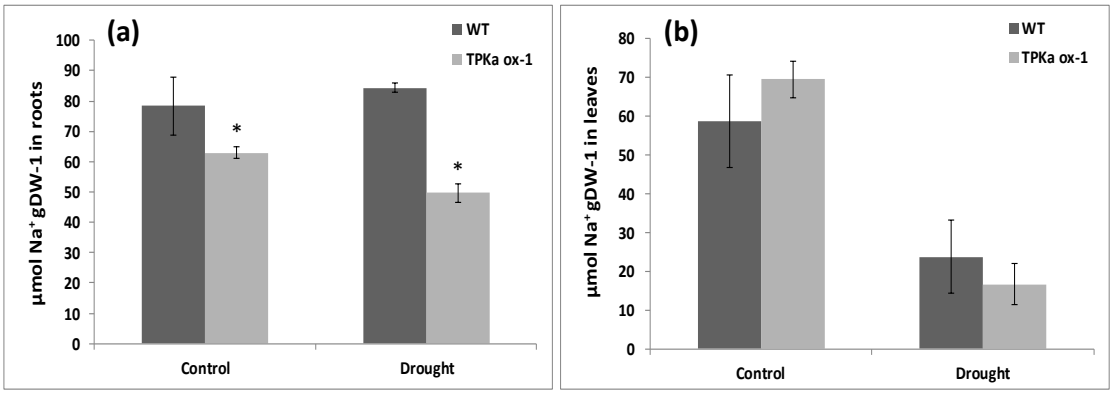

Figure 3-10: Na^+ concentration analyses for wild type and TPKa transgenic rice lines exposed to drought stress in soil

a; root Na^+ concentration and b; shoot Na^+ concentration of rice wild type and TPKa overexpressor plants. The plants were grown for six weeks in control and drought conditions in soil and root and shoot tissues were analysed for Na^+ concentration. Experiment was replicated three times and the bars in the figures represent the standard errors. * denotes a significant differences by T-test at a probability level of $p < 0.05$ between the wild type and overexpressor line.

3.2.6 Distribution of K^+ in the cytoplasm and vacuole

The K^+ distribution between vacuole and cytoplasm for TPKa transgenic and non-transgenic rice plants was analyzed to see the function of TPKa channels in the influx and efflux of K^+ from the vacuole in different media conditions. For this, energy dispersive X-ray (EDX) analysis was used which allows the analysis of the spatial distribution of elements such as K^+ in plant tissues (Figure 3-11) as described in the method section. The leaves of the overexpressing lines of *TPKa* and wild type plants were analyzed by EDX using plants exposed to control and zero K^+ conditions in hydroponics.The results showed that in both wild type and TPKa-ox-1 plants there was no difference in cytoplasmic K^+ in control conditions and $0K^+$ conditions (Figure 3-12a). In both genotypes, $0K^+$led to a reduction in vacuolar K^+ but this was only significantly so in TPKa ox-1 (Figure 3-12b).

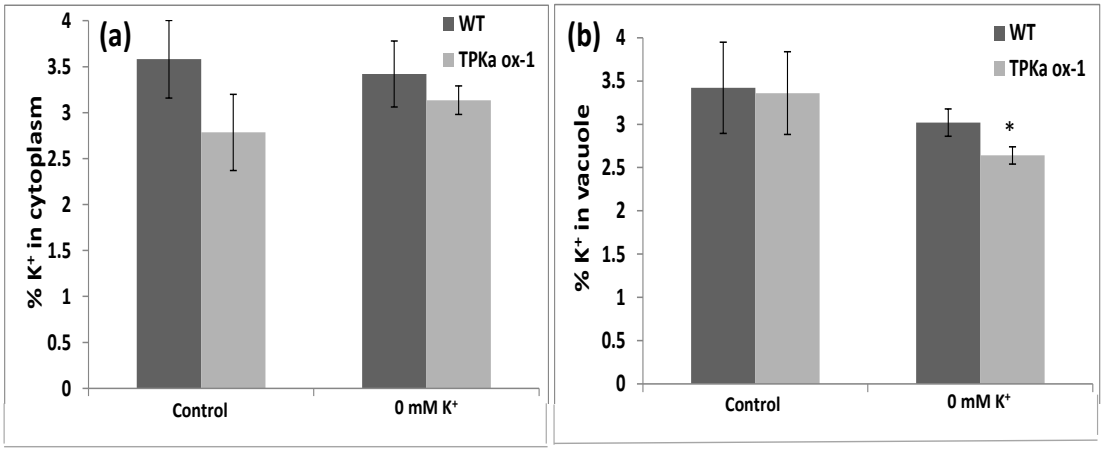

Figure 3-11: EDX analyses for the distribution of K^+ between cytoplasm and vacuole of the leaves of wild type and transgenic lines of TPKa

a; K^+ in cytoplasm, b; K^+ in vacuoles of wild type and transgenic lines of TPKa. Plants were analysed after one-week exposure to the treatments as mentioned in the figures. The bars in the figures represent standard errors. * denotes a significant differences by T-test at a probability level of $p < 0.05$ between the wild type and overexpressor line.

3.2.7 Greater water retention may be a reason for the higher growth of TPKa overexpressing plants in drought

The water content of the TPKa ox-1 plants was analyzed to see whether it was affected by a high K^+ concentration in the root and shoot tissues and therefore could ameliorate drought stress. The data revealed that the TPKa overexpressing plants shows a lower water content in the roots at control conditions (Figure 3-13a) while no difference was

observed in root water content between the genotypes under drought conditions. However, the results showed a far more dramatic reduction in root water content in wild type plants when exposed to drought. In the leaves (Figure 3-13b) of the overexpressing plants, higher water content under drought stress was seen. Again, the relative reduction in water content in wild type plants was considerably larger than that in the transgenic plants. There is a significant reduction in the water content of the wild type while no difference was observed for the TPKa ox-1 plants in the two tested conditions.

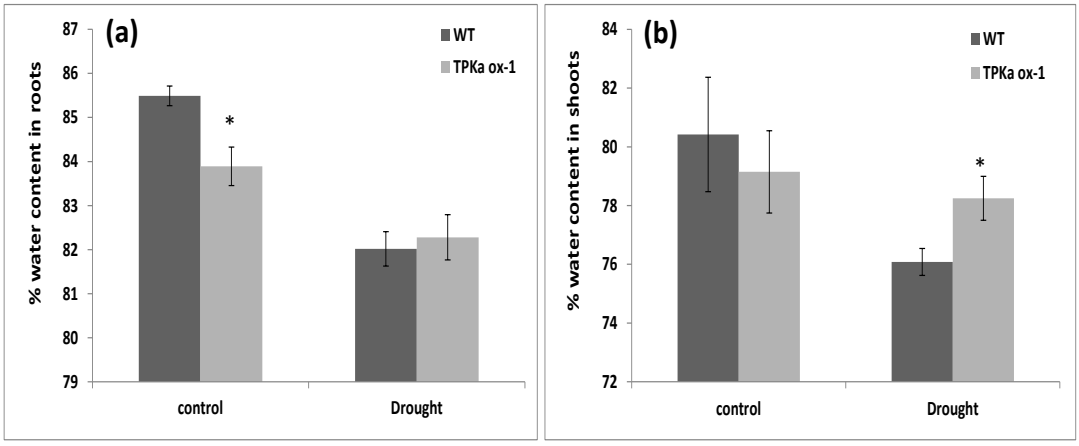

Figure 3-12: Water content analyses for wild type and TPKa transgenic rice plants exposed to drought stress in soil

a; root water content and b; shoot water content of rice wild type and TPKa overexpressor plants. The plants were grown for six weeks in control and drought conditions in soil and root and shoot tissues were analysed for water content. Experiment was replicated three times and the bars in the figures represent the standard errors. * denotes a significant differences by T-test at a probability level of $p < 0.05$ between the wild type and overexpressor line.

3.2.8 Xylem sap analysis for K^+ and Na^+ concentration in rice plants grown in hydroponics

Overexpression of TPKa leads to higher K^+ concentration in the root and shoot tissues (Figure 3-7). An increase in shoot K^+ is likely dependent on its delivery via the xylem. Xylem sap of all three genotypes was therefore analysed for K^+ and Na^+ concentration in control, 0 mM K^+ and 10% PEG conditions. No significant difference was observed for the K^+ concentrations in either of the genotypes in control and 0 mM K^+ conditions (Figure 3-14a). However TPKa ox-1 showed more K^+ in the xylem sap at osmotic stress condition. No difference was recorded for the Na^+ concentrations in the xylem sap for all the three genotypes in either of the media conditions (Figure 3-14b).

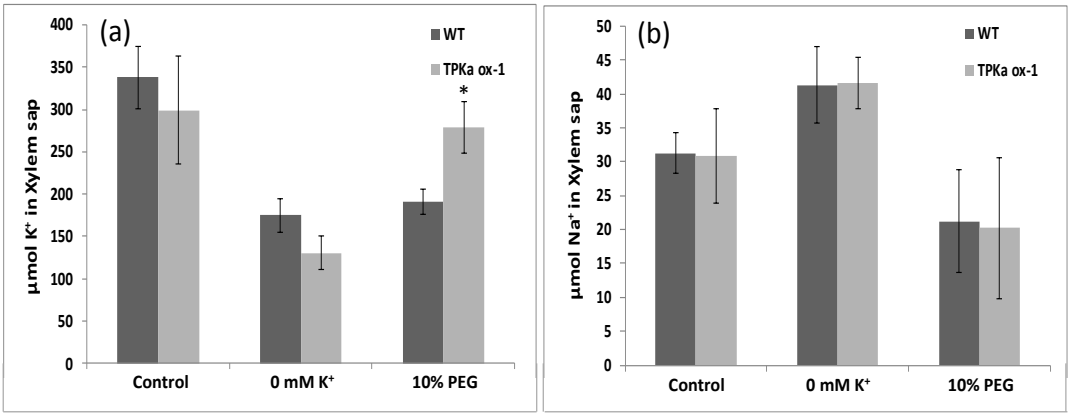

Figure 3-13: Xylem sap analyses for wild type and TPKa transgenic rice lines exposed to different media conditions in hydroponics

a; K^+ concentration and b; Na^+ concentration in the xylem sap of rice wild type and TPKa overexpressor plants. The plants were grown for one week in different media conditions and xylem sap was analysed for K^+ and Na^+ concentration. Experiment was replicated three times and the bars in the figures represent the standard errors. * denotes a significant differences by T-test at a probability level of $p < 0.05$ between the wild type and overexpressor line.

3.2.9 Analyses of the leaf conductance and rate of photosynthesis in *TPKa* overexpressor plants

Gobert et al (2007) showed that *Arabidopsis* TPK1 is involved in the release of K^+ from the vacuoles in the guard cells and has a role in the stomatal movement. We therefore analyzed the effects of the TPKa and TPKb overexpression on rates of photosynthesis and stomatal conductance in rice intact leaves using an infra-red gas analyzer, Li-Cor 6400 (LI-COR, Cambridge, UK).

The data showed that both the overexpressing lines of TPKa have lower rates of photosynthesis as compared with the wild type at control conditions (Figure 3-15a). At 0 mM K^+ and osmotic stress conditions both the transgenic lines showed a higher rate of photosynthesis as compared with the wild type plants.

The results for the leaf conductance revealed that both the overexpressing lines of *TPKa* showed lower conductance at control conditions and higher conductance at osmotic stress condition as compared with the wild type (Figure 3-15b). No difference was observed for both TPKa overexpressor lines as compared with the wild type at 0 mM K^+ conditions.

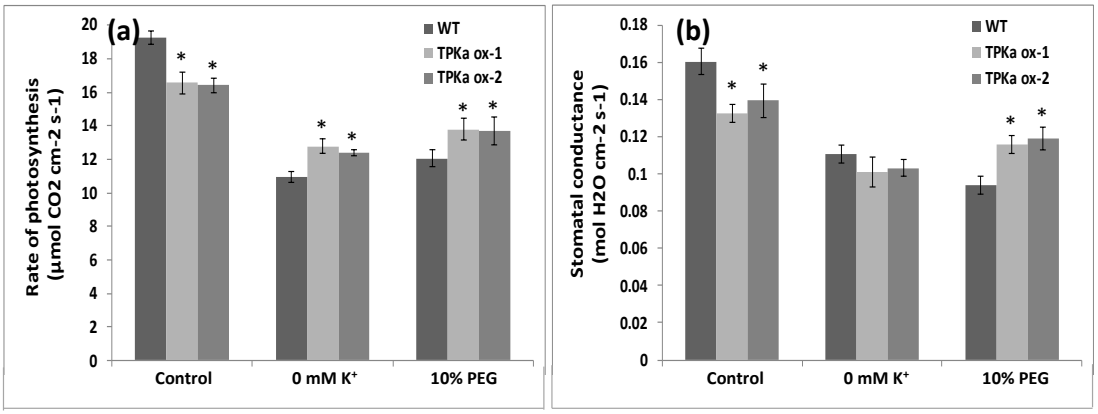

Figure 3-14: Rate of photosynthesis and stomatal conductance of wild type and TPKa transgenic rice lines exposed to different media conditions in hydroponics

Rate of photosynthesis (a) and stomatal conductance (b) of wild type and transgenic lines of TPKa; the plants were grown for two weeks in different media conditions as mentioned in the figure and stomatal conductance was measured. Experiment was replicated three times and the bars in the figures represent the standard errors. * denotes a significant differences by T-test at a probability level of $p < 0.05$ between the wild type and overexpressor lines.

Rate of photosynthesis and stomatal conductance were further tested under ABA conditions as described in the method section. The results showed a lower rate of photosynthesis (Figure 3-16a) and conductance (Figure 3-16b) for the transgenic lines of TPKa as compared with the wild type plants under control conditions, while opposite results were observed when plants were treated with ABA for one hour.

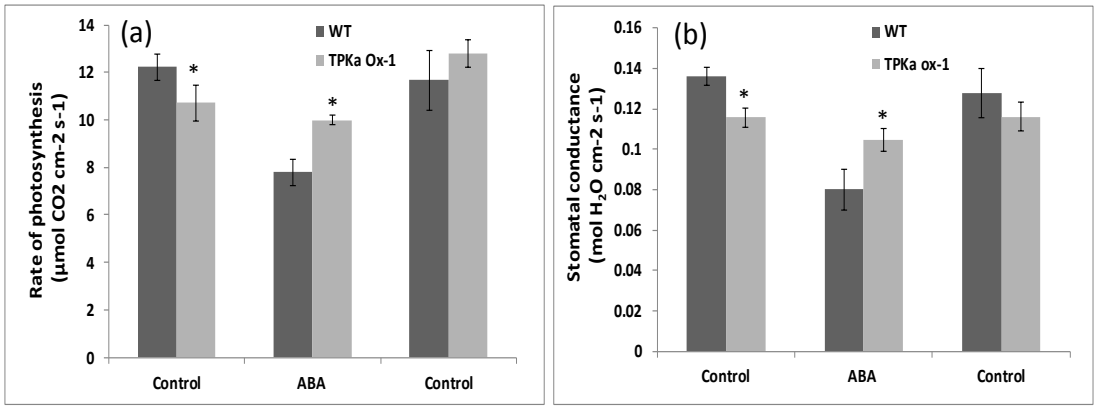

Figure 3-15: Rate of photosynthesis and Stomatal conductance of wild type and TPKa transgenic rice lines exposed to ABA in hydroponics

Rate of photosynthesis (a) and stomatal conductance (b) of wild type and transgenic line of TPKa were recorded in 6 week old plants. Data were recorded when plants were in control conditions. Plants were then exposed to media containing 100 µM ABA for one hour and data were recorded. Plants were shifted back to control conditions and data were recorded after one hour. Experiment was replicated three times and the bars in the figures represent the standard errors. * denotes a significant differences by T-test at a probability level of $p < 0.05$ between the wild type and overexpressor line.

Part B: Characterization of rice TPKb

3.2.10 PCR analysis to identify *TPKb* overexpressor rice plants

Three putative rice *TPKb* transgenic lines (line-13, line-155 and ox-line) were screened by PCR at their T3 generation for the presence of the Os*TPKb* transgene and overexpression. Different sets of primers were used; hygromycin primers to test the presence of the hygromycin resistance marker gene (Figure 3-16b), 35S promoter region "forward" and *TPKb* gene specific "reverse" primers (Figure 3-16c) and 35S promoter region "forward" and terminator "reverse" primers (Figure 3-16d). All 20 plants (not all are represented in the Figure 3-16) of all the *TPKb* transgenic lines gave amplification with the three sets of primers which suggests that these lines are homozygous for the *TPKb* overexpressor transgene. The transgenic lines were then checked by semi quantitative RT-PCR and qPCR to check the level of *TPKb* expression relative to control plants (Figure 3-17 and 3-18). The results showed approximately 5-20 times higher levels of *TPKb* expression in the transgenic lines as compared with the wild type plants. Those progenies of the self-crossed heterozygous transgenic plants which were found with no transgene in T3 generation have been termed as wild type and were used as control plants for all experiments.

In the following text TPKb overexpressor line-13, line-155 and 'overexpressor lines' are labelled as TPKb ox-1, TPKb ox-2 and TPKb ox-3 respectively.

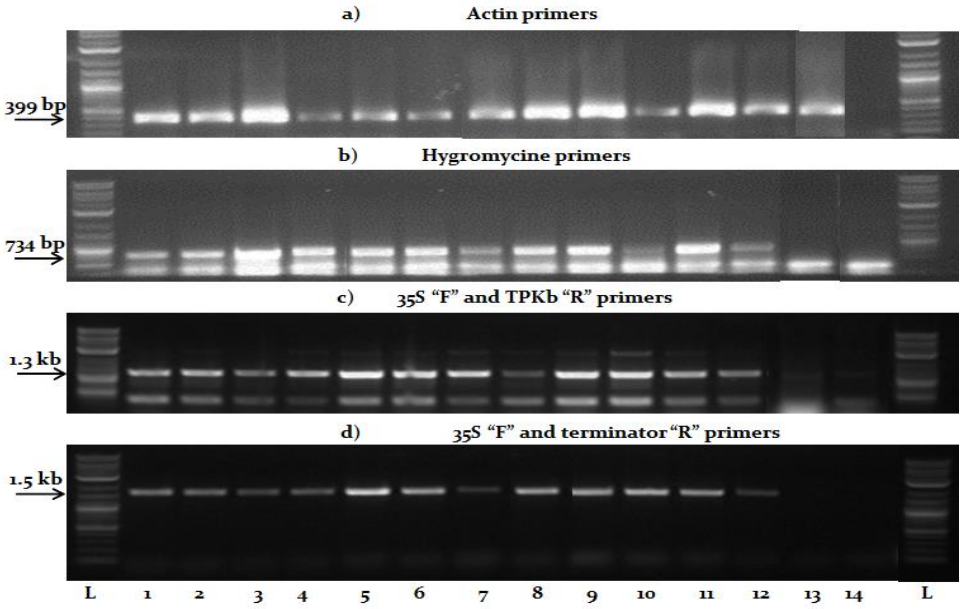

Figure 3-16: Screening of putative TPKa transgenic line by PCR

PCR results for TPKb overexpressor line-13; Putative overexpressor line gave amplification with three sets of primers which suggests that this line is homozygous transgenic for TPKb. Lane 1-12, TPKb overexpressor line-13; Lane 13, wild type; Lane 14, water (-ive control); L is the ladder. The DNA quality was fine for the transgenic and wild type as shown by the PCR with actin primers (a). The same PCRs were used for other putative overexpressing lines (TPKb ox-2 and TPKb ox-3) which were also found to be homozygous for the TPKb transgene (data not shown).

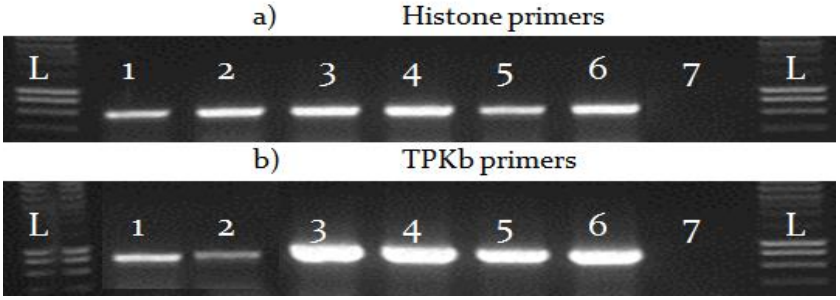

Figure 3-17: Analyses of the expression level of *TPKb* in transgenic lines by RT-PCR

RT-PCR results for *TPKb* ox-1. The results show high levels of expression for TPKb in the overexpressor line. Lane 1-2, wild type; Lane 3-6, TPK ox-1; Lane 7, water for both *TPKb* and *actin* primers, L is the ladder. Similar analyses were done for the two other TPKb transgenic lines (TPKb ox-2 and TPKb ox-3).

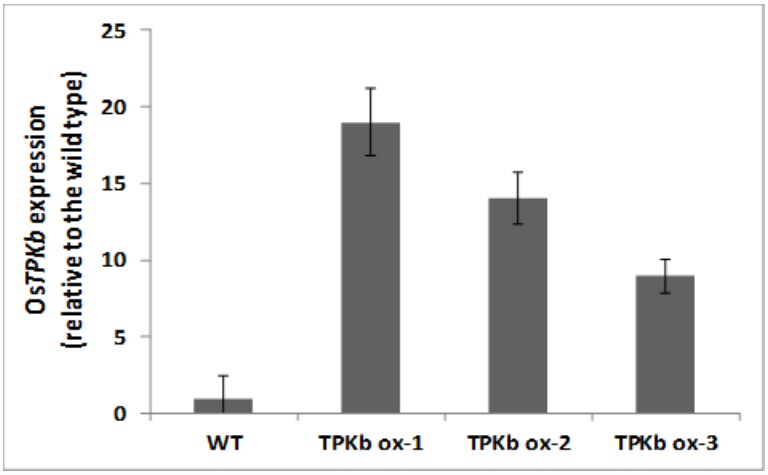

Figure 3-18: Analyses of the expression of *TPKb* in transgenic lines by q-PCR

qPCR for TPKb ox-1, TPKb ox-2 and TPKb ox-3. Three plants of each line were tested compared to the wild type for *TPKb gene expression* relative to the actin gene. The results showed a high level of gene expression in the three overexpressor lines as compared to the wild type.

3.2.11 Overexpression of TPKb has a positive effect on the growth of rice plants in abiotic stress conditions grown in hydroponics

To investigate the effect of Os*TPKb* overexpression on plant growth, RGRs were recorded for plants grown in control, 0 K^+, 60 mM KCl, 60 mM NaCl and osmotic stress (5%, 10% and 15% PEG) conditions.

All the three overexpressing lines of Os*TPKb* (ox-1, ox-2 and ox-3) showed varied growth in different media conditions except in the osmotic stress (5% and 10% PEG) conditions where all the three overexpressing lines showed higher growth as compared with the wild type plants (Figure 3-19a). Absence of K^+ in the medium reduced the growth of all genotypes but a comapratively larger growth reduction (100%) was observed in the wild type plants as compared to the control conditions whild lower growth reduction (30-40%) for the TPKb overexpressing was observed. However, when the data were normalized to control conditions, all the three overexpressing lines of TPKb showed better growth, as compared with the wild type plants, in zero K^+ conditions while no growth phenotype was observed under salt stress conditions (60 mM K^+ and 60 mM Na^+) (Figure 3-19b).

The growth phenotype of rice *TPKb* overexpressor plants was also tested under different osmotic stress (5%, 10% and 15% PEG) conditions (Figure 4-4a). The three overexpressing lines showed better growth as compared with the wild type in 5% PEG

and 10% PEG condition while no difference was observed in the growth phenotypes for all the genotypes in 15% PEG conditions. At 10% PEG conditions, 4, 2, 3 folds increased growth was observed for TPKb ox-1, TPKb ox-2 and TPKb ox-3 respectively than the wild type plants (Figure 3-20a). The normalized data showed the same results for the overexpressor line in these media conditions (Figure 3-190b).

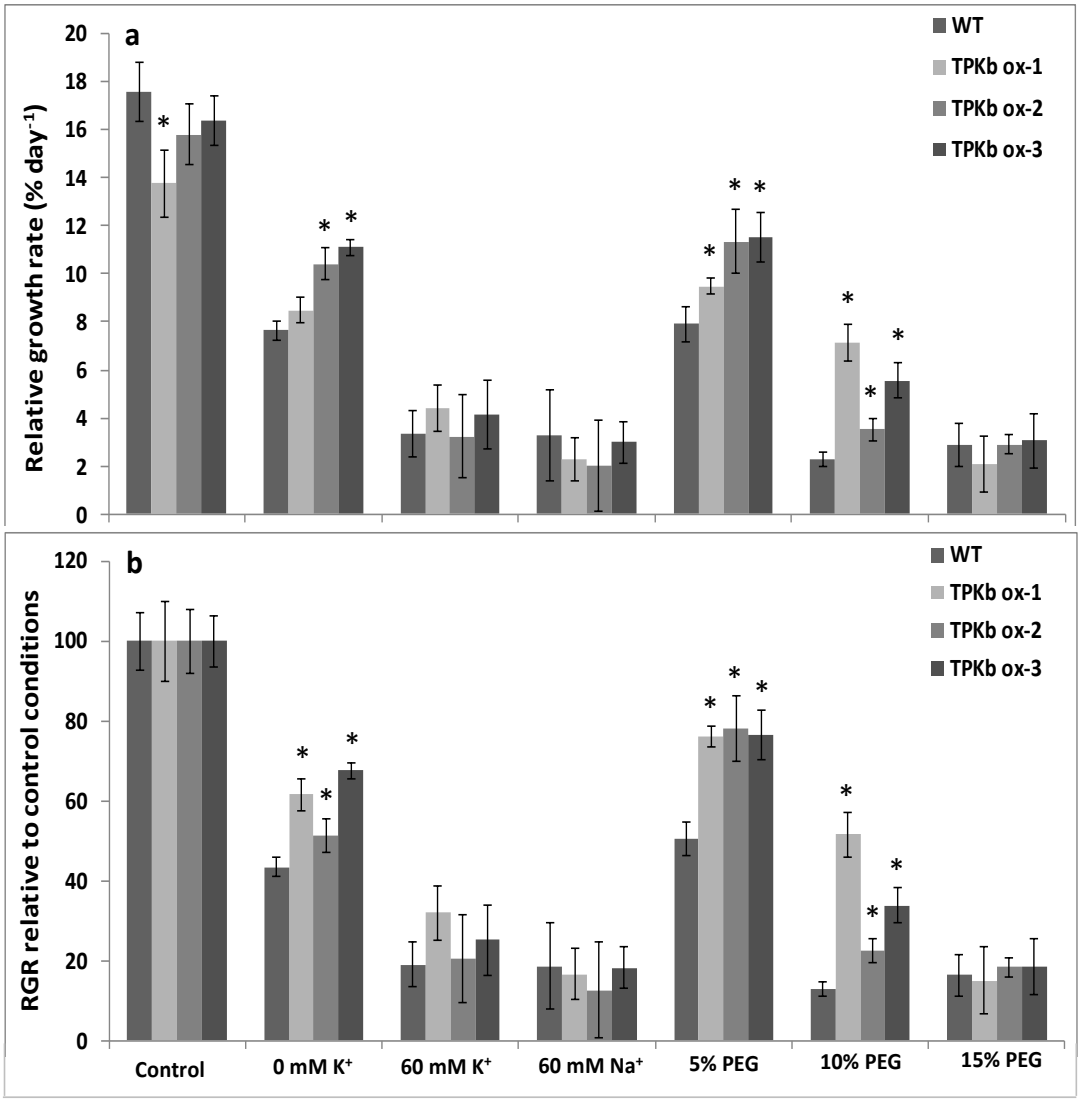

Figure 3-19: Relative growth rate for transgenic rice lines and wild type in hydroponics exposed to different media conditions

a; relative growth rate (RGR, % day^{-1}) absolute data. b; RGR relative to the growth in control condition (% day^{-1}) for the wild type plants and transgenic lines of TPKb. Plants were grown in hydroponics media for 14 days using control medium, 0 mM K^+, 60 mM K^+, 60 mM Na^+ and osmotic stress (5%, 10% and 15% PEG) conditions. Data are from three independent experiments, and the bars in the figures represent the standard errors. * denotes a significant differences by T-test at a probability level of $p < 0.05$ between the wild type and overexpressor lines.

3.2.12 Overexpressing lines of *TPKb* showed better growth under drought stress in soil

To study the effects of TPKb expression on drought stress in the soil rather than osmotic stress in hydroponics, plants were grown in soil conditions and were tested for growth as described in the method section.

Both the overexpressing lines of TPKb showed no growth phenotype in the control conditions. However, better growth was observed for the overexpressing lines as compared with the wild type plants under drought stress conditions (Figure 3-20a). The same trend was observed in the normalized data. TPKb ox-1 showed 2 fold increase in the growth than the wild type in drought conditions (Figure 3-20b).

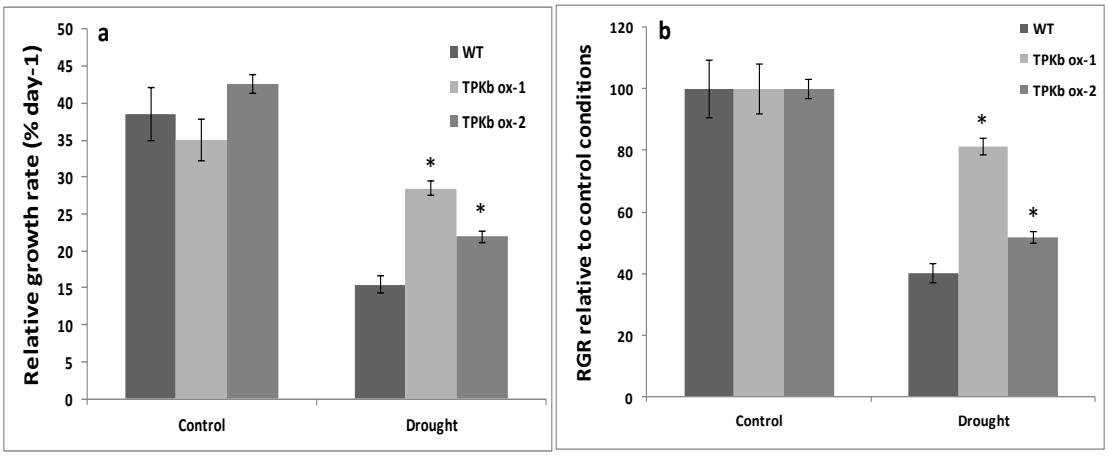

Figure 3-20: Relative growth rate for transgenic and wild type rice plants in soil exposed to control and drought conditions

a; relative growth rate (RGR % day^{-1}) absolute data. b;, RGR relative to the growth in control condition (% day^{-1}). Wild type plants and transgenic lines of TPKb were grown for 6 weeks in control and drought conditions in soil and growth was recorded. Data are from three independent experiments, and the bars in the figures represent the standard errors. * denotes a significant differences by T-test at a probability level of $p < 0.05$ between the wild type and overexpressor lines.

3.2.13 Tissue ion concentration analyses in rice *TPKb* overexpressor lines and wild type plants

The overexpression of TPKb may be involved in better growth under stress conditions through the improvement of K^+ nutrition and homeostasis. Analyses of the K^+ and Na^+ concentration in the tissues of the rice plants could be important for determining the role of TPKb in K^+ homeostasis under stress conditions. K^+ and Na^+ concentration of the root

and leaf tissues of the *TPKb* overexpressing lines and wild type plants was analysed using a flame photometer as described in the method section.

3.2.13.1 K^+ concentration analyses

The roots of all the tested transgenic lines of TPKb showed higher K^+ concentration under control, zero K^+ and, 60 mM Na^+ and osmotic stress (5% and 10% PEG) conditions (fig 3-21a). However, no significant difference was observed in the root K^+ of all the genotypes in 60 mM K^+ and 15% PEG conditions. The shoots of the different genotypes showed variable K^+ concentration in different conditions (Figure 3-21b). In control conditions, no difference was observed in the leaf K^+ concentration of TPKb ox-1 and TPKb ox-2 as compared with the wild type plants, however TPKb ox-3 showed higher K^+. All the tested transgenic lines of TPKb showed higher K^+ in leaves as compared with the wild type plants at zero K^+ conditions. No difference was observed in the K^+ concentration of wild type and TPKb ox-2 at 60 mM K^+ conditions however TPKb ox-3 showed lower K^+ concentration but not significantly. No difference was observed for the K^+ concentration in the shoots of transgenic and non-transgenic plants at high Na^+ conditions. Osmotic stress (5% and 10% PEG in the medium) resulted in significantly higher K^+ in transgenic shoots, and this was true for all the TPKb overexpressing lines. However, at a 15 % PEG this trend was not observed.

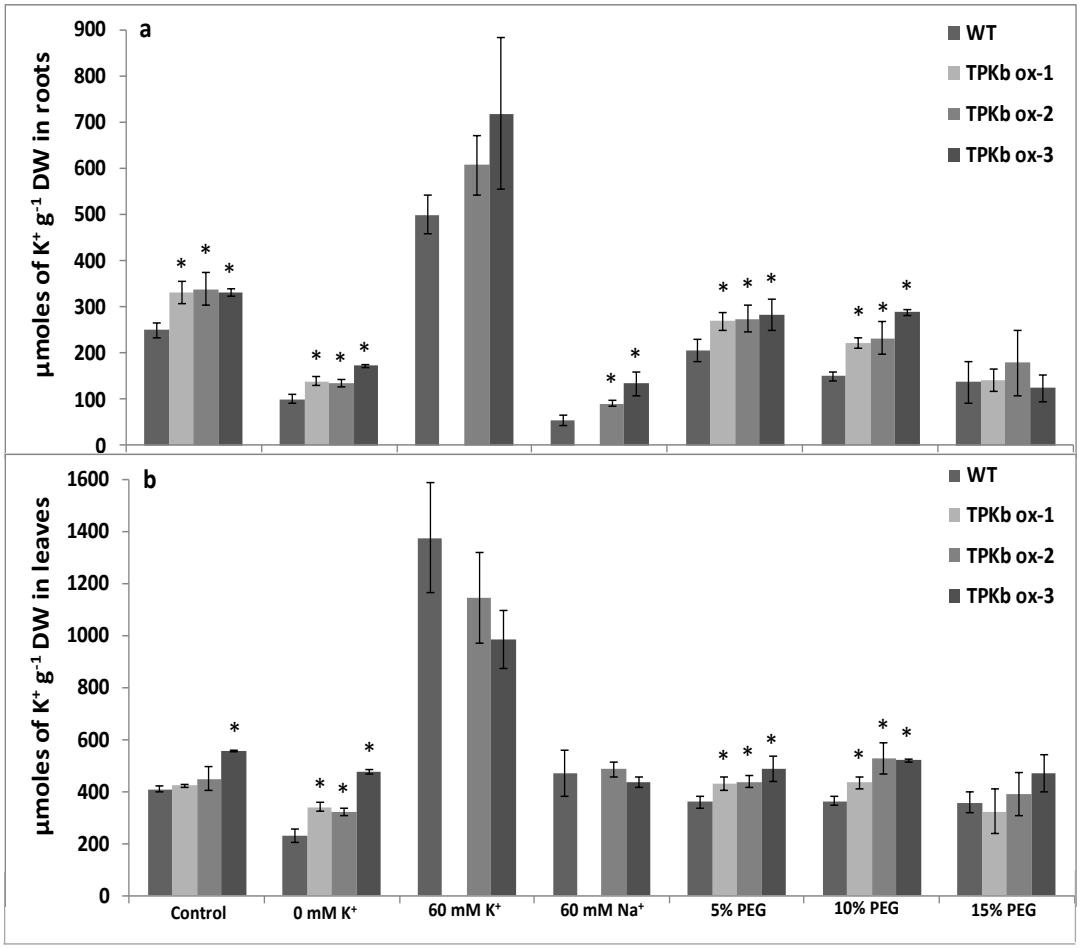

Figure 3-21: K^+ concentration analyses for transgenic rice lines and wild type in hydroponics exposed to different media conditions

a; root K^+ concentration and b; shoot K^+ concentration of rice wild type and TPKb overexpressor plants. The plants were grown in different media conditions as mentioned in the figures and root and shoot tissues were analysed for K^+ concentration. TPKb ox-1 was not tested in 60 mM K^+ and Na^+ conditions. Experiment was replicated three times and the bars in the figures represent the standard errors. * denotes a significant differences by T-test at a probability level of $p < 0.05$ between the wild type and overexpressor lines. TPKb ox-1 plants were not tested for ion concentration analyses under 60 mM K^+ and Na^+ condition due to the shortage of plants.

3.2.13.2 Na^+ concentration analyses

The root tissues of TPKb ox-1 and TPKb ox-3 showed higher Na^+ as compared with the wild type plants while no difference was observed for TPKb ox-2. Interestingly, lower Na^+ concentration were observed in the leaf tissues of TPKb ox-1 while no difference was observed for TPKb ox-2 and TPKb ox-3 as compared with the wild type plants. (Figure 3-22).

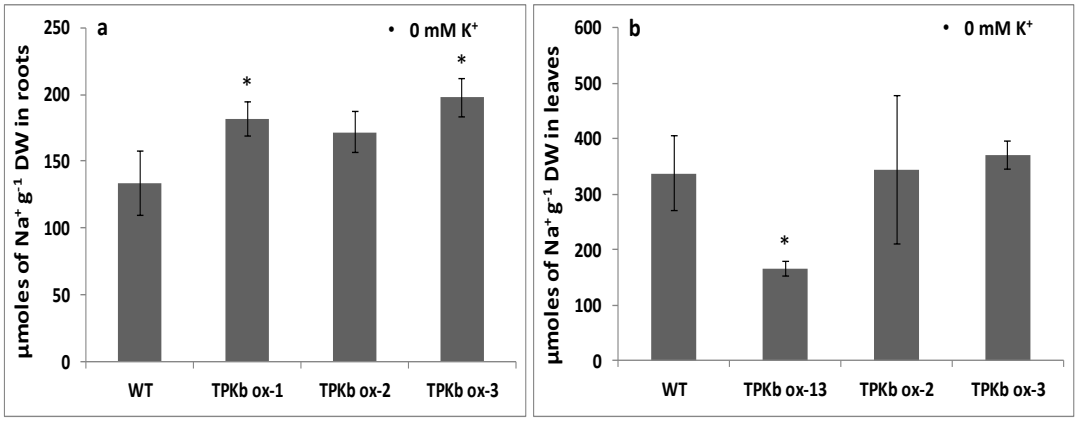

Figure 3-22: Na^+ concentration analyses for transgenic rice lines and wild type in hydroponics exposed to different media conditions

a; root Na^+ concentration and b; shoot Na^+ concentration of rice wild type and TPKb overexpressor plants. The plants were grown in 0 mM K^+ conditions and root and shoot tissues were analysed for Na^+ concentration. Experiment was replicated three times and the bars in the figure represent the standard errors. * denotes a significant differences by T-test at a probability level of $p < 0.05$ between the wild type and overexpressor lines.

3.2.14 Tissue ion concentration analyses in rice wild type and *TPKb* overexpressor plants grown in soil

The soil grown wild type and TPKb overexpressing plants under control and drought conditions were tested for ion concentrations. In the control conditions, the roots and leaves of the wild type and TPKb overexpressor plants showed no differences with the wild type plants in their K^+ concentrations (Figure 3-23). However, drought leads to a difference in the root and leaf K^+ concentration of the transgenic and non-transgenic lines. Here both the TPKb overexpressing lines showed higher K^+ concentration in the roots and leaves as compared to the wild type plants (3-23).

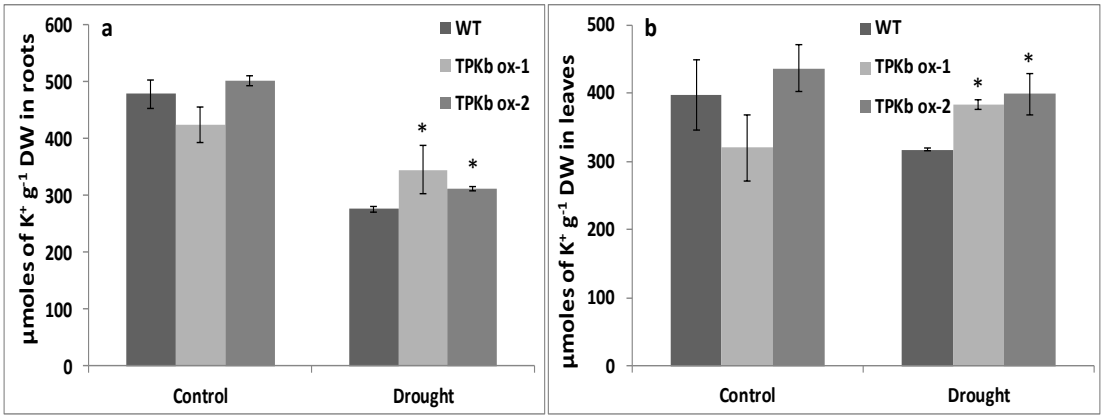

Figure 3-23: K^+ concentration analyses of the wild type and transgenic rice lines exposed to drought stress in soil

a; root K^+ concentration and b; shoot K^+ concentration of rice wild type and TPKb overexpressor plants. The plants were grown for six weeks in control and drought conditions in soil and root and shoot tissues were analysed for K^+ concentrations. Experiment was replicated three times and the bars in the figures represent the standard errors. * denotes a significant differences by T-test at a probability level of $p < 0.05$ between the wild type and overexpressor lines.

In the control conditions, the roots and leaves of both the transgenic lines of TPKb showed lower Na^+ as compared with the wild type (Figure 3-24). However, when the plants were grown in the drought, the roots of the overexpressing lines of TPKb showed lower Na^+ concentrations as compared with the wild type plants while the leaves of the overexpressing lines showed higher Na^+ as compared with the wild type plants (Figure 3-24).

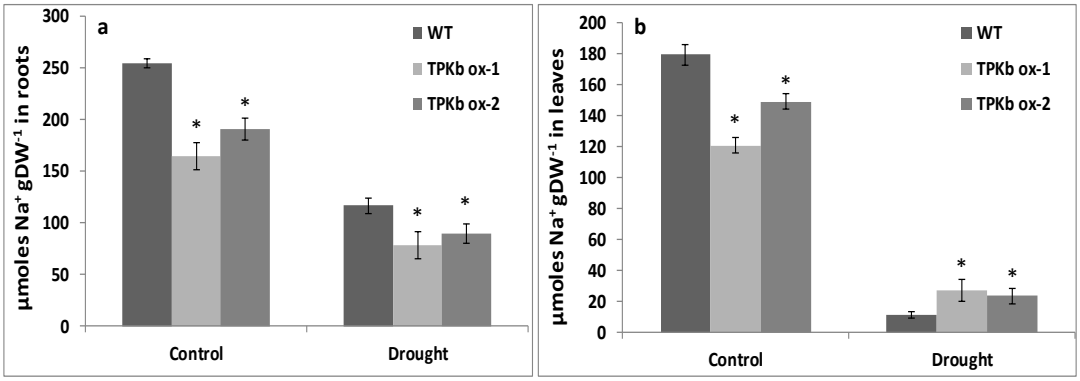

Figure 3-24: Na^+ concentration analyses of wild type and transgenic rice lines exposed to drought stress in soil

a; root Na^+ concentration and b; shoot Na^+ concentration of rice wild type and TPKb overexpressor plants. The plants were grown for six weeks in control and drought conditions in soil and root and shoot tissues were analysed for Na^+ concentration. Experiment was replicated three times and the bars in the figures represent the standard errors. * denotes a significant differences by T-test at a probability level of $p < 0.05$ between the wild type and overexpressor lines.

3.2.15 Distribution of K^+ in the cytoplasm and vacuole

The K^+ distribution between vacuole and cytoplasm of transgenic and non-transgenic rice plants was analyzed by EDX method to see the function of TPKb channels in the influx and efflux of K^+ from the vacuole in different media conditions. The leaves on the overexpressing lines of TPKb and wild type plants were analyzed in plants exposed to control and zero K^+ conditions.

The results showed that the cytoplasm of TPKb ox-1 contains less K^+ as compared with the wild type plants in control conditions. There was no difference in cytoplasmic K^+ in zero K^+ conditions (Figure 3-25a). No difference was observed in the vacuolar K^+ content of both the genotypes in control and zero K^+ conditions (Figure 3-265b).

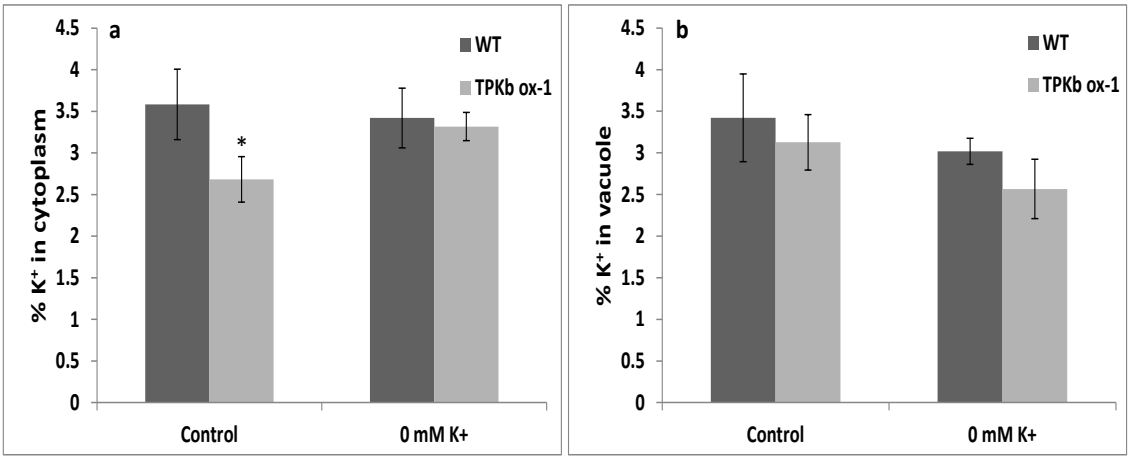

Figure 3-25: EDX analyses for the distribution of K^+ between cytoplasm and vacuole of the leaves of wild type and transgenic lines of TPKb

a; K^+ in cytoplasm, b; K^+ in vacuoles of wild type and transgenic lines of TPKb. Plants were analysed after one-week exposure to the treatments as mentioned in the figures. The bars in the figures represent standard errors. * denotes a significant differences by T-test at a probability level of $p < 0.05$ between the wild type and overexpressor lines.

3.2.16 Water content analyses of the rice TPKb overexpressor plants in soil conditions

The roots and leaves of the overexpressing lines of TPKb showed higher K^+ concentration as compared with the wild type plants under drought stress (Figure 3-23). Therefore the water content of these tissues was analyzed to see the effect of the higher K^+ content on the water content. The data revealed that the root and leaf tissues of all the tested genotypes showed no difference in the water content under control conditions

(Figure 3-26). However, a higher water content in the root and shoot tissues of both the TPKb overexpressing plants was observed in drought conditions. About 1.5 and 2 folds higher water content was in the root tissues of TPKb ox-1 and TPKb ox-2 respectively as compared with the wild type plants.

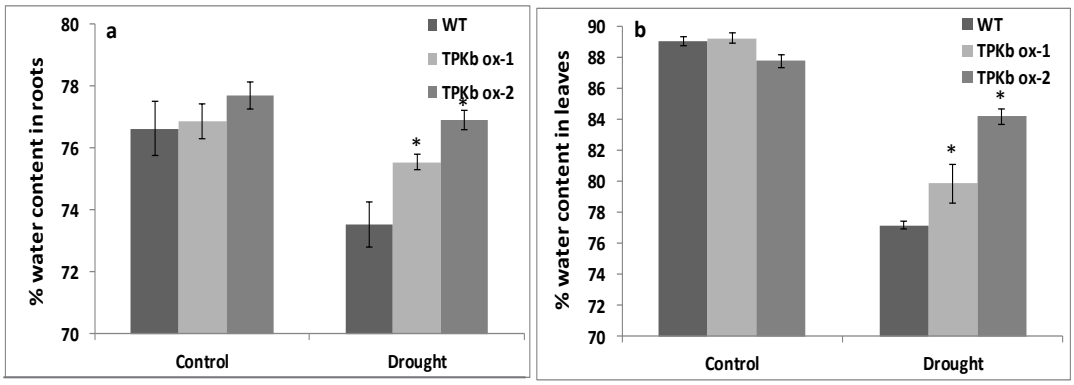

Figure 3-26: Water content analyses of wild type and transgenic rice plants exposed to drought stress in soil

a; root water content and b; shoot water content of rice wild type and TPKb overexpressor plants. The plants were grown for six weeks in control and drought conditions in soil and root and shoot tissues were analysed for water content. Experiment was replicated three times and the bars in the figures represent the standard errors. * denotes a significant differences by T-test at a probability level of $p < 0.05$ between the wild type and overexpressor lines.

3.2.17 Xylem sap analysis for K^+ and Na^+ concentration in rice plants grown in hydroponics

Our data showed higher K^+ concentration in the root and shoot tissues of the TPKb overexpressing plants (Figure 3-21). Xylem sap of the wild type and two overexpressing lines of TPKb was, therefore, analysed for K^+ in control, 0 mM K^+ and 10% PEG conditions and for Na^+ concentrations under zero K^+ conditions. The data showed variable results. A large, but non-significant difference was observed for the K^+ concentrations in all genotype in all conditions (Figure 3-27a) except for TPKb ox-2 in the zero K^+ condition where less K^+ in the xylem sap was found compared with the wild type plants. Both overexpressing lines showed a trend towards lower K^+, although not significant, in the xylem sap during osmotic stress.

Both the overexpressing lines of TPKb showed lower Na^+ concentrations in the xylem sap as compared with the wild type plants under zero K^+ conditions (Figure 3-27b).

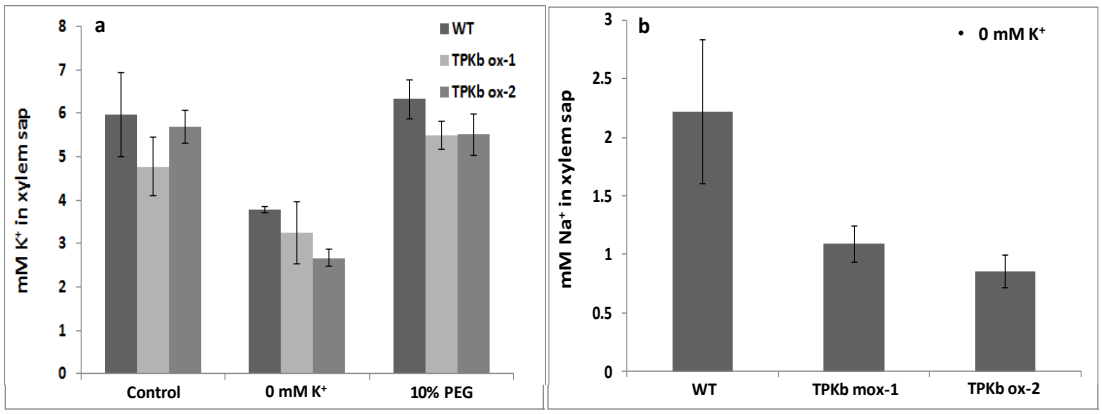

Figure 3-27: Xylem sap analyses for wild type and transgenic rice lines exposed to different media conditions in hydroponics

a; K^+ concentration and b; Na^+ concentration in the xylem sap of rice wild type and TPKb overexpressor plants. The plants were grown for one week in different media conditions as mentioned in the figure and xylem sap was analysed for K^+ and Na^+ concentrations. Experiment was replicated three times and the bars in the figures represent the standard errors. * denotes a significant differences by T-test at a probability level of $p < 0.05$ between the wild type and overexpressor lines.

3.2.18 Overexpression of TPKb improves K^+ uptake from the root environment

The higher K^+ concentration in the root and shoot tissues of TPKb overexpressing lines (Figure 3-21) as compared with the wild type plants in different media conditions suggested improved K^+ uptake from the root environment. To analyze whether TPKb overexpression affects K^+ uptake, wild type and one transgenic line (TPKb-ox1) were grown in 50 µM K^+ media solution and were analyzed as described in the method section.

The results revealed that when wild type and TPKb ox-1 plants were exposed to K^+ deficient (50 µM K^+) medium, the roots of both the genotypes initially showed net leakage of K^+ into the medium (Figure 3-28). However TPKb ox-1 showed less leakage as compared with the wild type plants. After an initial interval of 2-3 hours, both the genotypes showed net K^+ uptake from the nutrient solution. However TPKb ox-1 showed an earlier change from efflux to influx than the wild type plants. In addition, the uptake rate of K^+ from the nutrient solution for the TPKb ox-1 was higher than that in wild type plants.

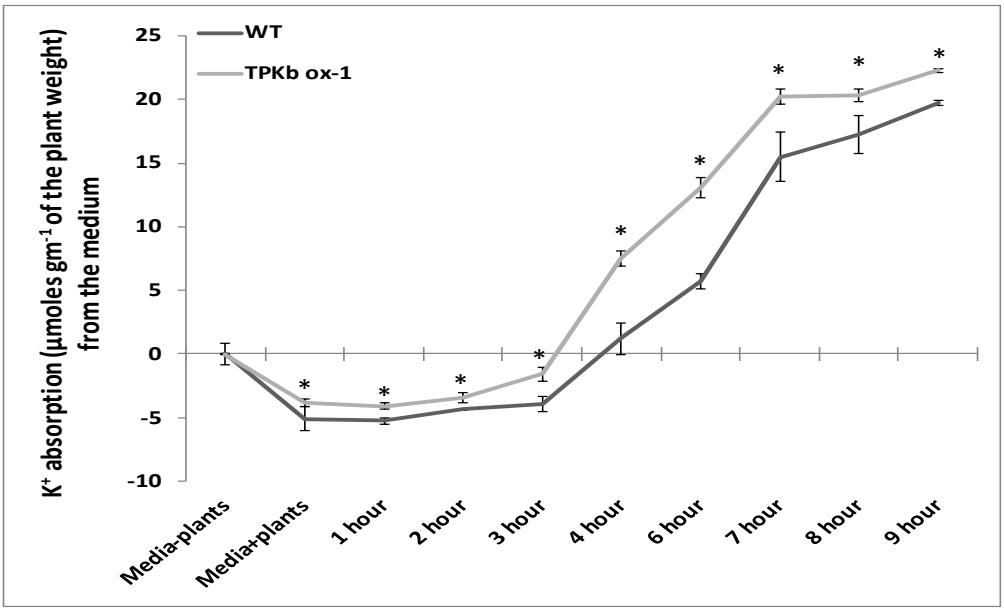

Figure 3-28: K^+ uptake from the medium

K^+ uptake from the medium for rice wild type and TPKb overexpressor plants; the plants were kept for up to 9 hours in medium containing 50 µM K^+. Medium samples were collected after each hour for nine hours and were analysed for the K^+ concentration. The K^+ concentration of the medium at each time point was subtracted from the initial total K^+ concentration and was regarded as leaked-out/absorbed K^+. The experiment was replicated three times and the bars in the figure represent the standard errors. * denotes a significant differences by T-test at a probability level of $p < 0.05$ between the wild type and overexpressor line.

3.2.19 Analysis of the rate of photosynthesis in wild type and *TPKb* overexpressor plants

AtTPK1 was shown to have a role in stomatal movements by releasing K^+ from guard cell's vacuole (Gobert *et al.*, (2007), we therefore, analyzed the effects of TPKb overexpression on the rate of photosynthesis and stomatal conductance in rice intact leaves using an infra-red gas analyzer (LI-COR, Cambridge, UK).

The results showed no difference in the rate of photosynthesis of the TPKb ox-1 and wild type plants while TPKb ox-2 showed a lower rate of photosynthesis in control conditions. At 0 mM K^+, TPKb ox-1 showed a higher rate of photosynthesis while no difference was observed for the TPKb ox-2 as compared with the wild type plants. No difference was observed under osmotic stress conditions for wild type and overexpressing plants (Figure 3-29a).

No difference was observed in the rate of photosynthesis between genotypes when the plants were treated with ABA, and rates were also comparable when plants were put back in control medium after ABA treatment (Figure 3- 29b).

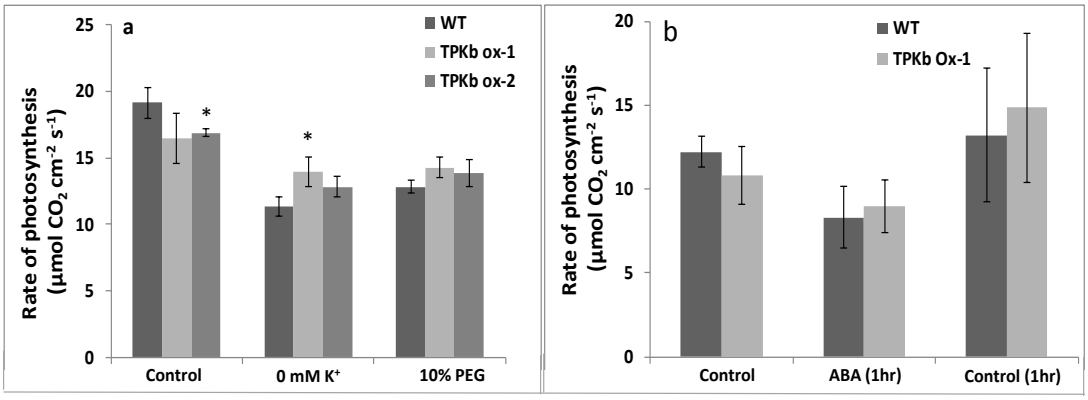

Figure 3-29: Rate of photosynthesis of wild type and transgenic rice lines exposed to different media conditions in hydroponics

Rate of photosynthesis of wild type and transgenic TPKb lines; a; the plants were grown for two weeks in different media conditions as mentioned in the figure. b; the rate of photosynthesis was measured at three time points; In control conditions before ABA treatment ('control'); after 1h 50 µM ABA treatment ('ABA(1hr') and 1h after plants were transferred back to control medium after ABA treatment ('control(1hr)). Experiment was replicated three times and the bars in the figure represent the standard errors. * denotes a significant differences by T-test at a probability level of $p < 0.05$ between the wild type and overexpressor lines.

3.2.20 Analyses of the stomatal conductance in wild type and *TPKb* overexpressor plants

The results showed lower stomatal conductance for both the overexpressing lines of TPKb as compared with the wild type plants in control conditions. In zero K^+ conditions, a higher stomatal conductance for TPKb ox-1 was recorded while no difference was observed for the TPKb ox-2. Both overexpressing lines showed higher stomatal conductance during osmotic stress (Figure 3-30a). ABA treatment had no significant effect on either genotype (Figure 3-30b)

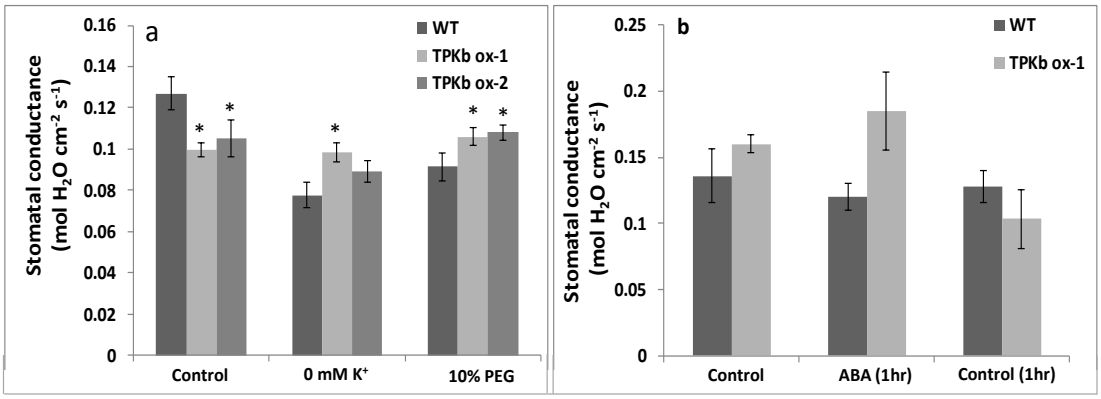

Figure 3-30: Stomatal conductance of wild type and transgenic rice lines exposed to different media conditions in hydroponics

Stomatal conductance of wild type and transgenic lines of and TPKb. a; the plants were grown for two weeks in different media conditions as mentioned in the figure. b; the stomatal conductance was measured at three time points; 1. In control conditions 2, Plants were shifted to media containing 50 µM ABA and were analysed after one hour and 3. When plants were shifted back to the control conditions and were analysed after one hour. Experiment was replicated three times and the bars in the figure represent the standard errors. * denotes a significant differences by T-test at a probability level of $p < 0.05$ between the wild type and overexpressor lines.

3.3 Discussion

3.3.1 Plant vacuoles

Plant vacuoles are important organelles. They store both beneficial and harmful compounds and many essential minerals such as K^+ (Gobert *et al.*, 2007). Excess K^+ is stored in the vacuoles when it is in abundance and released into the cytoplasm when needed (Leigh and Wyn-Jones., 1984, Walker *et al.*, 1996; Gobert *et al.*, 2007). The movement of K^+ is controlled and regulated by different membrane proteins e.g. Shaker like K^+ channels, K^+ inward rectifier K_{ir} like channels and tandem pore channels (Lebaudy *et al.*, 2007 ; Szczerba et al ., 2009). Among these proteins TPKs are important and highly selective for K^+. There are two TPK isoforms in rice i.e. TPKa and TPKb. TPKa is localised in the lytic vacuole (LV) and TPKb in protein storage vacuole (PSVs) and small vegetative vacuoles (SVs), suggesting specific roles of these different homologues (Isayenkov *et al.*, 2011).

3.3.2 TPKa and TPKb overexpression causes increased growth and higher K^+ in the tissues

Here the effects of TPKa and TPKb overexpression are shown on a number of physiological parameters. The data revealed that both lines of TPKa and one line of TPKb overexpressing plants showed less growth as compared with the wild type plants in standard conditions (Figure 3-5a and 3-19). The observation that some overexpressor lines showed slower growth agrees with Kurusu *et al.*, (2004) who showed less growth of the *OsTPC-1* transgenic lines. They suggested that the overexpression of stress relieving proteins in unstressed conditions might be the cause for reduced growth of transgenic plants

When the data were normalized to the growth in control conditions, all the overexpressor lines of TPKa and TPKb showed higher growth rates as compared to the wild type plants in 0 mM K^+ and osmotic stress conditions (Figure 3-5b and 3-19b). Gobert *et al.*, (2007), showed the same phenotype for *Arabidopsis AtTPK1* overexpressor plants at 0 mM K^+. These results suggest a positive role of TPKs in both low K^+ and osmotic/drought stress conditions which may be due to changes in homeostasis of K^+, K^+ concentration and rates of photosynthesis.

Efficient K^+ homeostasis and K^+ translocation within plants plays a positive role in plant tolerance to many abiotic stresses. Our results showed that the K^+ concentration of the root and leaf tissues is higher in the TPKa transgenic lines (Figure 3-7a, b and 3-21a, b) as compared with the wild type plants in several of the tested conditions. This increase in K^+ concentration may be an important factor in the observed growth phenotypes and is likely a direct consequence of the overexpression of the TPKa and TPKb proteins. It is generally believed that the main function of TPKs is in vacuolar K^+ release (Latz *et al.*, 2007, Gobert *et al.*, 2007). Thus it is not immediately obvious how TPKa and TPKb overexpression would lead to increased tissue K^+. However, the increased vacuolar K^+ release is likely to raise the cytoplasmic K^+ concentration. In turn the higher cytoplasmic K^+ causes a hyperpolarisation of the plasma membrane (i.e. the membrane potential is more negative). The hyperpolarisation of the membrane increases the activity of the AKT1 channel while decreasing the activities of GORK and SKOR (refer to Figure 1-5). AKT1 is a hyperpolarization-activated, inward rectifying K^+ channel while SKOR and GORK are depolarization-activated outward rectifying K^+

channels (Shabala and Cuin, 2008). Thus, TPK transgenic lines might have higher AKT1 activity and lower SKOR and GORK activities due to comparatively higher membrane polarisation. Enhanced AKT1 activity might be responsible for the increased uptake of K^+ at the root soil boundary, while reduced activity of the GORK and SKOR channels might prevent the loss of K^+ from the root to soil and xylem respectively. A combination of these factors may explain the observed higher K^+ concentration in the root tissues. Further experiments are needed to see the effects of TPK overexpression on the membrane polarization and to see whether overexpression causes any relative increase in the K^+ concentration of the cytoplasm.

In shoot tissues too, the K^+ concentration of transgenic lines was higher in most conditions. This means translocation is up-regulated. Xylem sap analyses (Figure 3-12a and Figure 3-28) show comparable K^+ concentrations between transgenic and non-transgenic plants. However, leaf conductance (and therefore transpiration) is generally higher in transgenics (Figure 3-13b and Figure 3-30a). The latter would increase K^+ loading to shoot tissues. The larger xylem K^+ flux would need to be sustained by xylem loading systems. The activity of the SKOR channel, which is a main player in xylem K^+ loading, would be reduced if cells of the transgenic lines are generally more hyperpolarised. However, non-selective cation channels (NCCs) also might be involved in the loading of K^+ into the xylem (Ahmad and Maathuis, 2014). Demidchik *et al.*, (2002) reported that NCCs can be activated by a large number of factors including hyperpolarization.

3.3.3 TPK overexpression relieves K^+ deficiency stress

Higher K^+ concentration was observed in the root and shoot tissues of the transgenic lines of TPKa and TPKb as compared with the wild type plants in 0 mM K^+ conditions. These results are different from Gobert *et al.,* (2007) who showed no significant difference in the K^+ concentration s of the transgenic lines and wild type plants at low K^+ conditions. The explanations given above for the higher K^+ concentration in the root and shoot tissues of the transgenic lines may also be applicable here. The overall K^+ levels in this condition are lower in both genotypes because of the leaking out of the K^+ in to the surrounding medium. However, leaking of K^+ for the non-transgenic lines might be higher as compared with the transgenic lines due to lower activity of outward rectifying K^+ channels as explained above. Our K^+ uptake data for the TPKb

overexpressing in the K^+ deficient conditions confirm that the overexpressing lines showed more K^+ uptake and less leakage (Figure 3-30). The net effect is a higher tissue K^+ concentration in the transgenics which may help relieve K^+ deficiency. This would especially help in maintaining cytoplasmic K^+, one of the most important parts of K^+ homeostasis (Maathuis, 2006). The higher K^+ in the vacuoles would help in increased turgidity. The lower level of K^+ deficiency stress is borne out by the observed higher stomatal conductance and rate of photosynthesis in the transgenic lines at 0 mM K^+ conditions. Higher stomatal conductance in the transgenic lines also suggests a higher rate of transpiration which might be evidence for the higher K^+ translocation in the transgenic lines as transpiration is one of the driving force the upward movements in the plant body.

The higher Na^+ concentration in the roots of the TPKa transgenic lines in the zero K^+ conditions may also help in relieving stress during K^+ deficient conditions (Horie *et al.*, 2007). At 0 mM K^+ conditions higher release of K^+ might occur from the vacuoles in the transgenic lines through TPKa channels. This might have negative effects on the turgidity and growth of the roots. To overcome these adverse effects due to the loss of K^+ from the vacuole, NHX antiporters might be involved to transport Na^+ into the vacuoles to provide turgor and restore growth. On the other hand, at K^+ deficient conditions, AKT1 channels might be involved in the uptake of Na^+ from the medium (Buschmann *et al.*, 2000) which contains mM concentrations of Na^+. However no difference was observed in the shoot Na^+ concentration of the transgenic and non-transgenic genotypes which suggests a limited role of TPKs in the transport of Na^+ and this also strengthens the idea that TPKs are highly selective for K^+ (Gobert *et al.*, 2007). It has been reported repeatedly that plants have the ability to tolerate Na^+ toxicity by controlling its long distance transport (Gorham, 1990; Schachtman *et al.*, 1992; Munns and James, 2003; Garthwaite *et al.*, 2005; Munns and Tester, 2008). This might be a further reason for the better growth of the TPKa transgenic lines in the K^+ deficient conditions where on one hand Na^+ provides osmoticum for the better root growth while on the other hand limited distribution of Na^+ to the shoot tissues prevents toxic effects of Na^+.

3.3.4 Overexpression of TPKs has positive effects on growth during osmotic and drought stress

The overexpressing lines of TPKa and TPKb showed better growth, as compared to the wild type, at osmotic stress in hydroponics and drought stress in soil, suggesting a positive role of this vacuolar channel during osmotic stress whether it is during short term (hydroponics) or long term (soil) exposure. The root and shoot tissues of the transgenic lines contained more K^+ during osmotic stress which would help lowering the cellular osmotic potential and thus reduce the drought stress.

The higher K^+ concentration in the roots of the transgenic lines in these conditions may result from factors discussed above. Nevertheless, there may be further aspects involved that are specific to water stress. For example, it has been reported that water stress and ABA modify the activity of K^+ channels to favour enhanced K^+ storage in roots and reduced K^+ loading to xylem (Cram and Pitman, 1972; Roberts and Snowman, 2000). ABA signalling includes Ca^{2+} as a second messenger (McAinsh *et al.*, 1990, 1992; Schroeder and Hagiwara, 1990; Gilroy *et al.,* 1991; Irving *et al.,* 1992; Allan *et al.,* 1994; Staxen *et al.,* 1999) and TPKa channel activity is sensitive to Ca^{2+} (Latz *et al.,* 2007; Isayenkov *et al.,* 2011). Thus, ABA could directly increase TPKa activity. ABA signalling might also be involved in the regulation and activation of TPKs via kinases and 14-3-3 proteins such as GRF6, both of which have been shown to modulate TPK activity (Latz *et al.,* 2007). With more TPK channels available, similar ABA stimuli could therefore cause more K^+ release into the cytoplasm from root vacuoles. Increasing K^+ concentrations in the cytoplasm during osmotic stress conditions is an important strategy of plants to maintain root growth (Sharp and Davies, 1979; Saab *et al.,* 1990).

Roberts and Snowman, (2000) reported that ABA increases the membrane potential of the maize root stelar cells and hyperpolarised membrane increases the driving force for the uptake of K^+ from the medium. It is reported that ABA lowers the transcription level of AtSKOR (Gaymard *et al.,* 1998) while the expression level of AtAKT1 is not affected (Roberts, 1995) which means that on the one hand the absorption of K^+ from the soil by AKT1 is not affected (however channel activity is increased by hyperpolarization of the membrane) while loading of K^+ to the xylem through SKOR is reduced during osmotic stress conditions which might be a reason for the increased K^+ concentration in the roots. These different factors may explain the higher K^+

concentration in the roots of the transgenic lines in osmotic and drought stress conditions.

In most circumstances K^+ of the vacuoles is metabolically inert. To fulfil the needs of the cytoplasm for metabolic activities, more K^+ can be released from the vacuoles (Walker *et al.*, 1996). The overexpression of TPKa might be loading more K^+ into the cytoplasm increasing polarization of the plasma membrane and this activates K_{in} activity while decreasing K_{out} activity in the guard cells as discussed above. Release of K^+ from the vacuole may activate other K^+ channels and transporters (for example AKT, KAT, HAK and KUP) to transport more K^+ to shoot.

Higher concentrations of K^+ in the shoot of the transgenic lines increases cell turgor which would include turgor in guard cells and hence could cause an increase in leaf conductance (Figure 3-14b and Figure3-30a). The data from the water content analyses (Figure 3-26) and stomatal conductance support the idea that the transgenic lines transport more water to keep the cells turgid and to maintain higher transpiration rates. Higher transpiration rates draw more K^+ from the root tissues to shoot. Thus water stress induces increased K^+ accumulation in the roots and shoots which maintains a water potential favouring the uptake of water and promoting cell turgor pressure necessary for growth. Indeed we can see the evidence for this in the form of increased water contents in TPKa overexpressors when plants were grown in soil. The higher water contents in the roots and leaves confirm that *TPKa* overexpression helps in accumulating comparatively higher K^+ concentrations and thus helps to relieve water stress. These results are comparable to the results from the hydroponic media experiments. Public expression data (https://www.genevestigator.ethz.ch/at/index.php) show that OsTPKa is upregulated in osmotic stress conditions which agrees with our model. Similarly, Hamamoto *et al.*, (2008) showed increased expression of the tobacco TPK1 channels during salt and osmotic stress conditions and suggested that the expression of NtTPK1 is involved in transporting K^+ into the cytosol during osmotic stress conditions.

3.3.5 How can TPKb function in different environments?

TPKb is localised in the tonoplast of SVs (Isayenkov *et al.*, 2011). SVs store a variety of organic and inorganic molecules and are distributed in different types of cells in the plant body. They may store K^+ in the form of complex molecules such as potassium phytate in seeds. Under normal K^+ conditions in the surrounding environment, plants

absorb K^+ and may store it in the ionic form as well as in the form of complex molecules. SVs may serve as repository to store K^+ in the form of complex molecules. Storage of K^+ in the form of complex molecules would be a good strategy as it is more stable. Plants could use this strategy to store excess of K^+ for unfavourable conditions. Abiotic stresses may trigger enzymatic changes within the cells. This could result in the break-down of the complex molecules and release K^+ in the ionic form. TPKb might be important in the subsequent K^+ release. Other K^+ transporters like TPKa or NHX may also transport K^+ from the cytoplasm into the LV where it can provide turgidity to the guard cells. Gobert *et al.,* 2007 suggested this role for AtTPK1 and reported up to 10 fold increase in expression level of AtTPK1 in the embryonic and endosperm tissues.

Chapter 4

4 The role of the inward rectifying K^+ channel OsAKT1 in abiotic stresses

4.1 Introduction

Potassium is one of the most important nutrients and fourth among the most abundant minerals, making up about 2.5% of the lithosphere. The actual soil concentration of this nutrient varies between 0.04-3% (Sparks and Huang, 1985). Soil K^+ is present in different pools, but plant available K^+ is dissolved in the soil solution. The solution concentration ranges from 0.1 to 6 mM (Adams, 1971). All plants store considerable amounts of K^+ which constitutes 0.8% to 8% of the plant dry weight (Maathuis, 2009). The storage of K^+ in the plant body is mostly in the cytosol and vacuoles (Szczerba *et al.*, 2009). K^+ is essential because it plays vital roles in many physiological and biochemical processes. It is involved in cellular osmo and turgor regulation, functions as an activator for about 50 metabolic enzymes that include those involved in photosynthesis, respiration, protein synthesis and other important metabolic processes (Marschner, 1995). It is important for charge balancing, plant cell movement, pH regulation (Cuin and Shabala, 2005) and cell and leaf expansion (Maathuis and Sanders, 1996; Elumalai *et al.*, 2002).

4.1.1 Potassium acquisition

According to Epstein (1961), K^+ transport consists of two systems in plants, the low and high affinity systems. These activities are assigned to channels and carriers at the molecular level (Maathuis and Sanders, 1994, 1997). The functions of K^+ transport proteins are diverse and the boundaries between carriers and channels are not clearly defined (Fu and Luman, 1998; Hirsch *et al.*, 1998; Spalding *et al.*, 1999). Channel mediated K^+ transport has been studied in great detail because of the availability of advanced electrophysiological techniques and the relative ease with which channels can be expressed in heterologous systems (Ashley *et al.*, 2006).

Among the transport systems, Shaker-type K^+ channels constitute important pathways for K^+ influx and efflux in guard cells (Hosy et al. 2003, Lebaudy et al. 2008a). They are

similar in sequence and structure to the animal shaker type channels. The name shaker comes from the first member of this family initially identified in *Drosophila*. Ion channel activity can be dependent or insensitive to membrane polarization but shaker channels are sensitive to membrane polarization. The 4^{th} TMS of the α-subunit hydrophobic core contains basic amino acid residues (R and K) which allow it to act as voltage sensor. The secondary structure of Shaker channels has six trans-membrane domains and a pore domain between the 5^{th} and 6^{th} trans-membrane domains as shown in Figure 4-1. Movement of the voltage sensor in response to changes in the transmembrane potential results in conformational changes in the channel protein, leading to its activation. Shaker type channels are classified into three groups depending on the range of voltage where they show their activity. These groups are inward, weekly inward and outward rectifying channels. The inward rectifiers e.g. *Arabidopsis* K^+ transporter (AKT1) are activated by hyperpolarization while outward rectifiers (e.g. SKOR) are activated by depolarization of the membrane. Studies of their functional properties in heterologous systems suggest that Shakers are predominantly expressed in the plasma membrane (Schroeder *et al.*, 1984; Moran and Satter, 1989; Maathuis and Sanders, 1995) where they are involved in K^+ transport across the membrane.

Shaker type K^+ channels such as AKT1 (Sentenac *et al.*, 1992) and KAT1 (Anderson *et al.*, 1992) were the first K^+ transporting proteins cloned from plants (Sentenac *et al.*, 1992). The inward rectifying K^+ channels, of the AKT family, are present throughout the plant kingdom (Anderson *et al.*, 1992; Sentenac *et al.*, 1992; Lebaudy *et al.*, 2007). AKT1 is involved in K^+ uptake from the soil over a range of micro to millimolar concentrations (Golldack *et al.*, 2003).

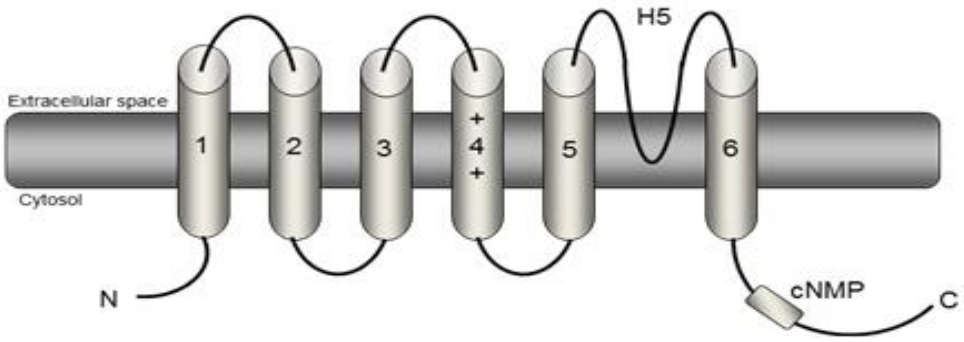

Figure 4-1: The proposed topology of Shaker type AKT1 channel

The structure comprises 6 TMD with a voltage sensing region in the S4 domain that controls channel gating, and a 'GYGD' motif (H5 region) that confers K^+ selectivity; cNMP is Cyclic nucleotide-monophosphate binding domain; N is N terminus; C is C terminus.

4.1.2 Regulation of AKT1 channels

Channel ion selectivity, sensitivity to voltage, pH, K^+ or Ca^{2+} are the prerequisites for a better understanding of the channel activity (Lebaudy *et al.,* 2007). Electrophysiological analysis using heterologous expression in animal systems is one of the sources to get such information (Lebaudy *et al.,* 2007). Xenopus oocytes provide one of the active and suitable systems for such analyses. But not all the plant K^+ channels are active in oocytes. For example, AKT1 channels cloned in this system did not show any activity. Recent studies have shown that the lack of AKT1 activity in Xenopus oocytes is due to the absence of regulators involved (Xu *et al.,* 2006). The *Arabidopsis* genome contains a number of AKT1 regulators. These regulators belong to the calcineurin B-like calcium (CBL) sensor proteins and CBL -interacting protein kinases (CIPKs). CBL targets CIPK and forms a complex network of signalling. This signalling complex regulates many physiological processes in plants (Luan, 2009; Luan *et al.,* 2009) such as channel activity. More than one CBLs may be involved in the regulation of one CIPK activities (Shi *et al.,* 1999; Albrecht *et al.,* 2003; Luan *et al.,* 2002. Conversely there may be one CBL regulating more than one CIPKs. These observations indicate that CIPK-CBL interactions are both specific and overlapping.

Xu *et al.,* (2006) showed a regulatory pathway for AKT1 in *Arabidopsis.* In *Xenopus oocytes*, no AKT1 activity was recorded in the absence of either CIPK23 or CBL1 (or CBL9); however, in the presence of both CIPK23 and CBL1 (or CBL9) normal activity was recorded. According to these findings, it was suggested that CIPK23 phosphorylates AtAKT1 and increases K^+ uptake especially in K^+ deficient conditions.

Six members of the CBL family showed interaction with CIPK23 but only two (CBL1 and CBL9) showed strong interaction. The double mutants of cbl1 and cbl9 showed the same phenotypes as the mutant of *akt1* at low external K^+ concentrations. In addition, overexpression of CBL1 and CBL9 had the same growth phenotype and tissue K^+ concentration as the AKT1 overexpressor plants. Based on the above observations, Xu *et al.,* (2006) concluded that the CIPK23 is a positive regulator for AKT1 and CBL1 and CBL9 are positive regulators of CIPK23. Under low external K^+ conditions, a cytosolic Ca^{2+} signal is triggered which activates the plasma membrane localised CBL1 and CBL9. These two CBLs interact and bring CIPK23 to the plasma membrane. AKT1 in the plasma membrane is phosphorylated by CIPK23 and as a result AKT1 is activated for more efficient K^+ uptake from the external environment. Lee *et al.,* (2007) showed that ankyrin repeat domains which are present in the cytoplasmic region of AKT1, serve as the site of attachment for the CIPK23 proteins. This part of AKT1 is sufficient for the interaction with CIPKs and then mediates AKT1 regulation (Figure 4-2).

When similar approaches were used to study AKT1 like channels in barley and grapevine, it was found that they are also activated by *Arabidopsis* CBL1 and CIPK23 in Xenopus oocytes (Boscari *et al.,* 2009; Cuellar *et al.,* 2010). Furthermore, Li *et al.,* (2014) showed that OsCBL1 and OsCIPK23 are involved in the regulation and activation of OsAKT1. They cotransfected HEK293 cells with OsCBL1 and OsCIPK23 and OsAKT1 and found a remarkable increase in the AKT1 activity as compared with the cells transfected with only OsAKT1. Two other rice CIPKs (OsCIPK3 and OsCIPK19) were also found interacting with AKT1 but only CIPK19 was involved in the activation of AKT1 in the presence of CBL1 when tested in HEK293 cells. However, the activation was much weaker than the activation caused by CIPK23. Based upon their observations, Li *et al.,* (2014) concluded that the OsAKT1 activity is regulated by OsCBL1 and CIPK23 in rice.

AKT1 is activated by phosphorylation through the CBL-CIPK network after Ca^{2+} signalling during K^+ deprivation. This short term activation can be reversed by dephosphorylation. In search of the protein that dephosphorylates, Lee *et al.,* (2007) focussed on PP2C (2C-type protein phosphatases) and found a member of the PP2C

family named as AIP1 which interacts with AKT1. Thus, AIP1 may be a negative regulator of AKT1 (Figure 4-2).

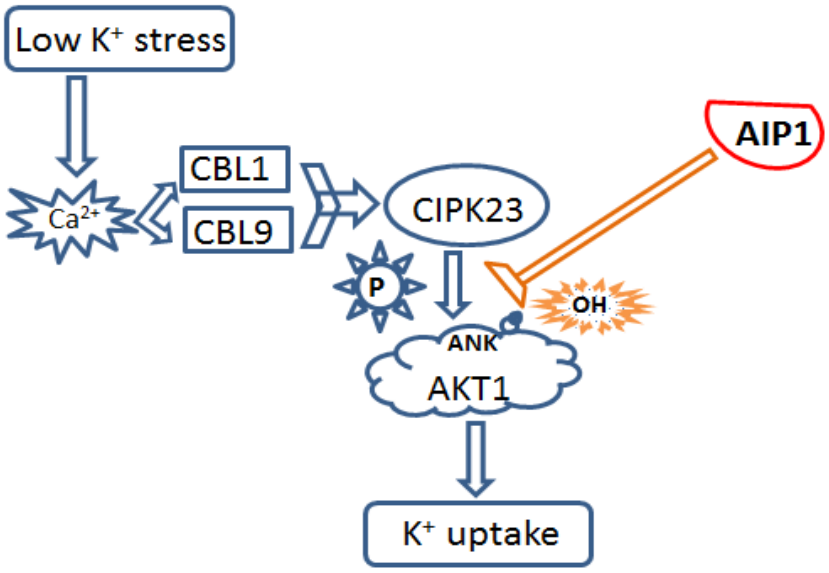

Figure 4-2: A model for the regulation of the AKT1

Under low external K^+ conditions, a cytosolic Ca^{2+} signal is triggered which activates the plasma membrane localised CBL1 and CBL9. These two CBLs interact and bring CIPK23 to the plasma membrane. AKT1 in the plasma membrane is phosphorylated by CIPK23 and as a result AKT1 is activated for more efficient K^+ uptake from the external environment. ANK is the ankyrin domain in the AKT1 protein and is the binding site for the CIPK23. AIP1 is a type of PP2C proteins and dephosphorylate the AKT by binding to the ankyrin domain and inhibit the CIPK23 to bind to AKT1. "P" represents the phosphorylation, "OH" represents the dephosphorylation (adopted from Xu *et al.*, 2006; Lan *et al.*, 2011).

4.1.3 Functions of AKT1

Inward rectifying K^+ channels were shown to be involved in K^+ uptake into many types of plant cells (Maathuis and Sanders, 1995). AKT1 is highly expressed in epidermal and cortical cells of the root throughout the planta and is involved in the uptake of K^+ from the external environment. Direct evidence for this was given by Hirsch *et al.*, (1998) who used *akt1* mutant lines and found that the knockout line has less K^+ in its tissues and showed less growth as compared to the wild type plants. Hirsch *et al.*, (1998) also suggested that AKT1 can significantly contribute to the K^+ uptake at very low external K^+ concentrations. They also found that AKT1 activity is essential when NH_4^+ is present in the medium because the non-AKT1 component of K^+ absorption mediated by KUP/HAK transporters, is sensitive and inhibited by NH_4^+ (Santa-Maria *et al.*, 2000). 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 AKT1 ortholog *TaAKT1* in roots (Buschmann *et al.*, 2000). Li *et al.,* 2014 transformed the yeast mutant strain R5421 (defective in K^+ uptake) with rice AKT1 and tested growth on different external K^+ concentrations (from 50 mM to 50 µM). The wild type strain R757 and the OsAKT1 transformed strains showed better growth at lower external K^+ concentration than the mutant strain R5421, while no difference was observed at higher external K^+ concentration among these lines. These results suggest that OsAKT1 too absorbs K^+ from a wide range of external K^+ like AtAKT1. *Osakt1* mutant plants also showed a phenotype comparable to *Atakt1* (Hirsch *et al.*, 1998; Spalding *et al.*, 1999) in K^+ deprived conditions. Like *Arabidopsis akt1* mutants *Osakt1* mutants showed significantly lower K^+ concentration in the tissues. The inward K^+ fluxes of the mutant lines were also reduced significantly as compared with the wild type plants. This lower K^+ uptake might be responsible for the growth inhibition in the rice *akt1* mutant lines (Li *et al.*, 2014).

These studies on the expression of K^+ channels and knockout mutants emphasize the importance of K^+ uptake channels in plant K^+ nutrition. There are contrasting reports about the dependence of AKT1 expression on external K^+ concentration. For example, Lagarde *et al.,* (1996) reported that AKT1 expression is independent of external K^+ concentration in *Brassica napus* ranging from micro to mill molar concentrations. Pilot *et al.,* (2003) reported unchanged expression of AtAKT1 in roots and shoots upon K^+ deprivation and salt stress. However Buschmann *et al.,* (2000) reported that the expression of TaAKT1 increases upon K^+ deprivation.

All K^+ channels studied so far are highly specific for K^+ over other alkali cations, suggesting that K^+ channels are not good candidates for significant sodium intrusion even at high Na^+ to K^+ ratios (Maathuis *et al.*, 1997; Amtmann and Sanders, 1999). However, there are reports which suggested that AKT1 may play a role in Na^+ uptake during high external Na^+ concentrations (Amtmann and Sanders, 1999; Blumwald, 2000). In *Sueda maritima* AKT1 type channels may be involved in Na^+ uptake under high external Na^+ concentrations (Wang *et al.*, 2007). The mRNA levels of *TaAKT1* were upregulated in roots on K^+ deprivation and also led to the enhancement of instantaneous Na^+ currents, suggesting a role of these channels in Na^+ uptake during K^+ depleted conditions (Buschmann *et al.*, 2000). The animal shaker type K^+ channels have

also been shown to transport considerable amounts of Na^+ at positive potentials (Starkus *et al.,* 2000; Wang *et al.,* 2000).

4.1.4 The function of AKT1-type channels in plant Na^+ uptake is uncertain

AtAKT1 has been shown to be a salt sensitive K^+ channel, highly expressed in roots (Fuchs *et al.,* 2005). Golldack *et al.,* (2003) suggested that OsAKT1 could mediate Na^+ uptake in saline conditions. They analysed the expression level of AKT1 in three cultivars of rice (salt-sensitive rice cv. IR29, and salt-tolerant rice Pokkali and BK) under high salts conditions (150 mM Na) for 48 hours and found that the AKT1 transcripts disappeared in the roots of Pokkali and BK cultivars while they remained present in IR29. They concluded that the expression of *OsAKT1* is regulated differently in salt sensitive and salt tolerant cultivars of rice. *OsAKT1* has also been shown to enhance salt tolerance in yeast (Perry *et al.,* 2007).

Aims of the study

Better K^+ nutrition and maintaining a higher K^+: Na^+ ratio have been shown to play a positive role in different abiotic stresses as discussed in chapter 1 in detail. AKT1 is an inward rectifier K^+ channel and is expressed throughout the plant body but most highly in the roots. It plays a role in the acquisition and then distribution of K^+ within the plant and so could improve plant K^+ nutrition during different abiotic stress conditions. The aim of this study was to analyse the role of rice AKT1 channels under different stress conditions. Different parameter such as growth, K^+ and Na^+ concentrations in the tissues and in the xylem sap, stomatal conductance and rate of photosynthesis were recorded.

4.2 Results

4.2.1 Testing of rice *akt1* knockout mutant lines

The rice *akt1* transposon insertion lines (i.e. line T14884T and NC0227) in the "Nipponbare" background and T-DNA insertion line PFG_1B-16021 in the "Dongjin" background (as shown in Figure 4-3) were characterised by PCR and RT-PCR using different sets of primers (see chapter 2). PCR results showed that line-T14484T (Figure 4-4b and 4-4c) and line-PFG_1B-16021 (Figure not shown) were homozygous for the transposon and T-DNA insertion respectively while the line-NC0227 (Figure not shown) was heterozygous. Line T14884T (Figure 4-5) and line-PFG_1B-16021 (Figure

4.6) were subsequently analysed using RT-PCR and both lines showed absence of any *AKT1* mRNA which confirms that these are genuine loss of function mutants. In the further discussion the line- T14884T and line-PFG_1B-16021 are named as *akt1-1* and *akt1-2* for convenience.

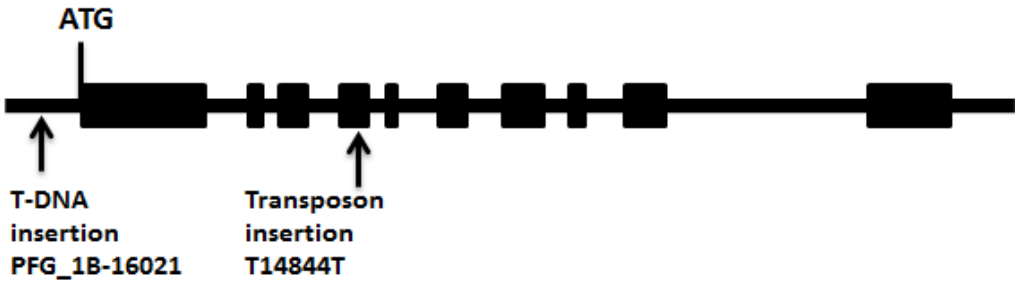

Figure 4-3: Model showing the insertions in Os-AKT1

The black boxes indicate exons and the lines represent introns. The T-DNA and transposon insertion site in the Os-*akt1* mutant is shown using an arrow.

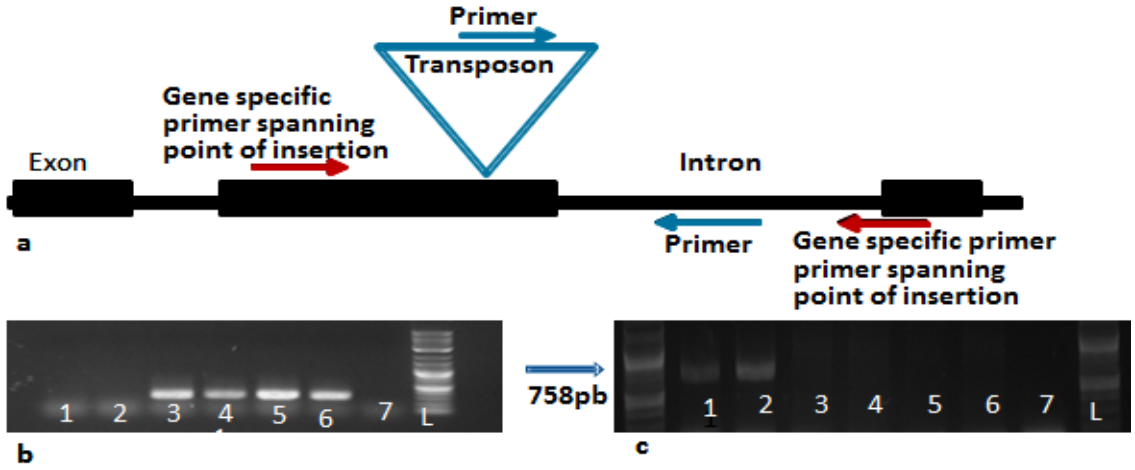

Figure 4-4: Model of the primers positions and PCR for the *akt1* knockout line

a; Gene model showing transposon and primer positions for line T14484T, b; PCR screening of *akt1* knockout mutant line T14484T, with transposon primer and a gene specific primer. Amplification in the putative knockout mutants shows that a transposon is inserted in the gene, c; PCR using gene specific primers spanning the transposon insertion. The lack of amplification in the putative knockout line shows that the line is homozygous for transposon insertion. Lane 1-2, wild type; Lane 3-6, knockout mutants; Lane 7, water, L is the ladder for both b and c.

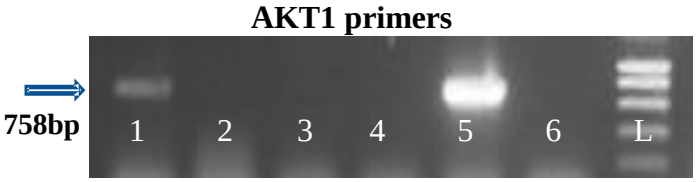

Figure 4-5: RT-PCR for *akt1* knockout mutants line T14484T

Lack of amplification in the knockout line suggests that the line is a genuine knockout line. Lane 1, cDNA from wild type; Lane 2-4, cDNA from the knockout mutants; Lane 5, gDNA from wild type; Lane 6, water. L is the ladder.

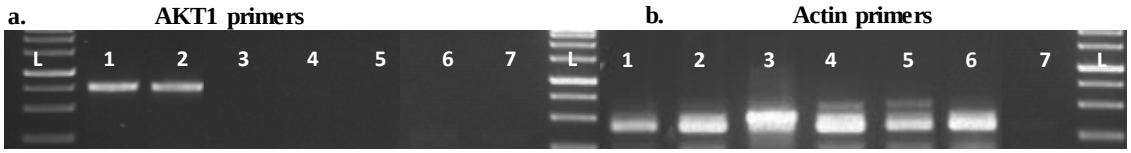

Figure 4-6: RT-PCR for *akt1* knockout mutant line- PFG_1B-16021

cDNA of the wild type and water were used as positive and negative control respectively. Actin primers were used as control for the quality of DNA. The lack of amplification for the knockout mutants shows that they are genuine mutants. Lane 1 & 2 wild type: Lane 3-6, akt1 knockout mutants; Lane 7, water. L is the ladder.

4.2.2 Screening of putative AKT1 overexpressing lines

The putative *AKT1* overexpressor lines were tested by PCR. The amplification with hygromycin primers showed that the plants are transgenic (Figure 4-7). To further characterise, primer sets which are cDNA specific and spanning introns, were used . The PCR was expected to give us two bands on the genomic DNA of the AKT1 putative transgenic plants (as shown in Figure 4-8a). The larger size amplicon was expected for the *constitutive AKT1* gene while the smaller amplicon was for the *AKT1* transgene. All the 20 plants tested (not all shown) gave two bands which shows that the plants are transgenic for AKT1 (Figure 4-8b). gDNA and cDNa of the wild type plants were used as a positive control. To further investigate, PCR was carried out with 35S promoter region forward primer and *AKT1* reverse primer (as shown in Figure 4.9ab). Again, all the plants gave amplification with the mentioned set of primers (Figure 4-9b) which shows that the AKT1 line 13-2 is a genuine transgenic line for the AKT1 transgene. The same PCRs were carried out for one other line (AKT1 ox-line) and the results were the same as those for AKT1 overexpressor line 13-2. Both lines were tested with q-PCR to quantify the level of overexpression. The AKT1 ox 13-2 and AKT1 ox

line were named as AKT1 ox-1 and AKT1 ox-2 respectively in the coming text for convenience.

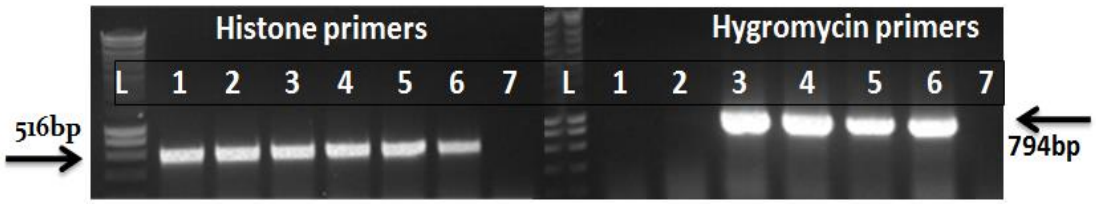

Figure 4-7: Characterization of *AKT1* transgenic plants by PCR using hygromycin primers

Lane 1-2, wild type; Lane 3-6, *AKT1* transgenic plants; Lane 7, water (Negative control); L is the ladder. The amplification suggests that these plants are transgenic for hygromycin resistance. Size of amplicon is 794 base pairs. Histone primers were used as a control for the quality of DNA.

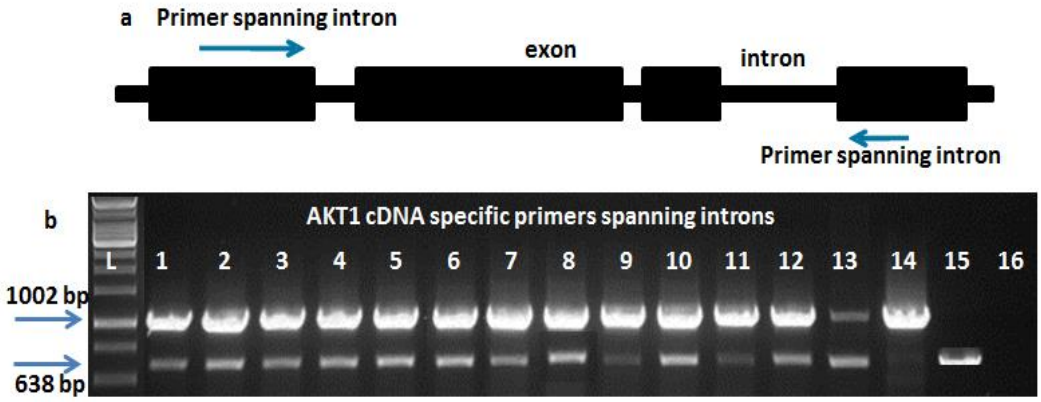

Figure 4-8: Model of the primers position and PCR for the AKT1 transgenic line 13-2

a; Model of the PCR with AKT1 primers spanning intron where two bands are expected for the transgenic lines. b; Screening of putative AKT1 transgenic line 13-2 with *AKT1* cDNA specific primers spanning introns, Lane 1-14 gDNA from the putative AKT1 transgenic plants; Lane 14 gDNA from WT and Lane 15 cDNA from WT (as controls); Lane 16, water (negative control); L is the ladder. The results showed two bands for the putative *AKT1* transgenic plants. The larger size band corresponds to the endogenous AKT1 gene, while the small band corresponds to the transgene AKT1.

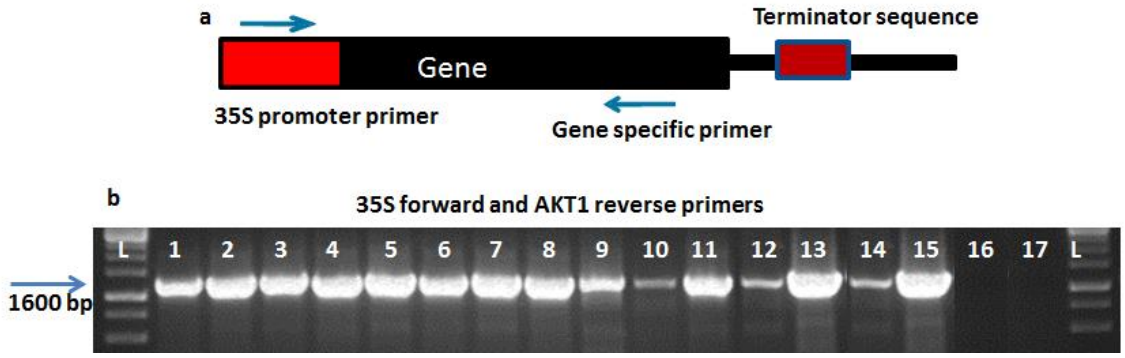

Figure 4-9: Model of the primers positions and PCR for the AKT1 transgenic line 13-2

a, model of the PCR with 35S forward primer and AKT1 gene specific reverse primer; The DNA is expected to give amplification in case the plants are genuinely transgenic. b, Screening of putative AKT1 transgenic line with 35S forward primer and *AKT1* reverse primer. Lane 1-14; cDNA from the AKT1 Transgenic plants, Lane 15 Plasmid (as a positive control), Lane 16 gDNA from WT and Lane 17 water (as negative controls), L is the ladder. The results showed that the line is transgenic for *AKT1* and homozygous.

4.2.3 Growth analysis of wild type, *Osakt1* mutants, and AKT1 overexpressor rice plants

Rice AKT1 is responsible for the uptake of K^+ and may be important for the growth in a range of environmental conditions. To see the effects of AKT1 on growth, the knockout and overexpressing lines were analysed for growth against their corresponding wild type plants.

The results showed no difference in the growth for all the tested genotypes in control conditions (Figure 4-11). At zero K^+ conditions, both the *akt1* knockout lines showed less growth as compared to their corresponding wild type plants while no growth phenotype was observed for both the overexpressing lines. When the zero K^+ medium was supplemented with 100 µM K^+, the growth phenotype of the *akt1* knockout lines was lost, however, both the AKT1 overexpressing lines showed better growth as compared with the wild type plants. No growth phenotype was observed for all the genotypes when either of KCl or NaCl was present in the medium in higher (60 mM) concentrations. Both the knockout lines showed less growth while both the overexpressing lines showed better growth as compared to their corresponding wild type plants under mild osmotic stress (5% PEG). Under 10% PEG conditions, we observed a trend of lower growth, although not significant, for the knockout lines, however the overexpressor lines showed better growth as compared with the wild type

plants. Higher osmotic stress (12% PEG) conditions led to a comparable growth phenotype for all the tested lines.

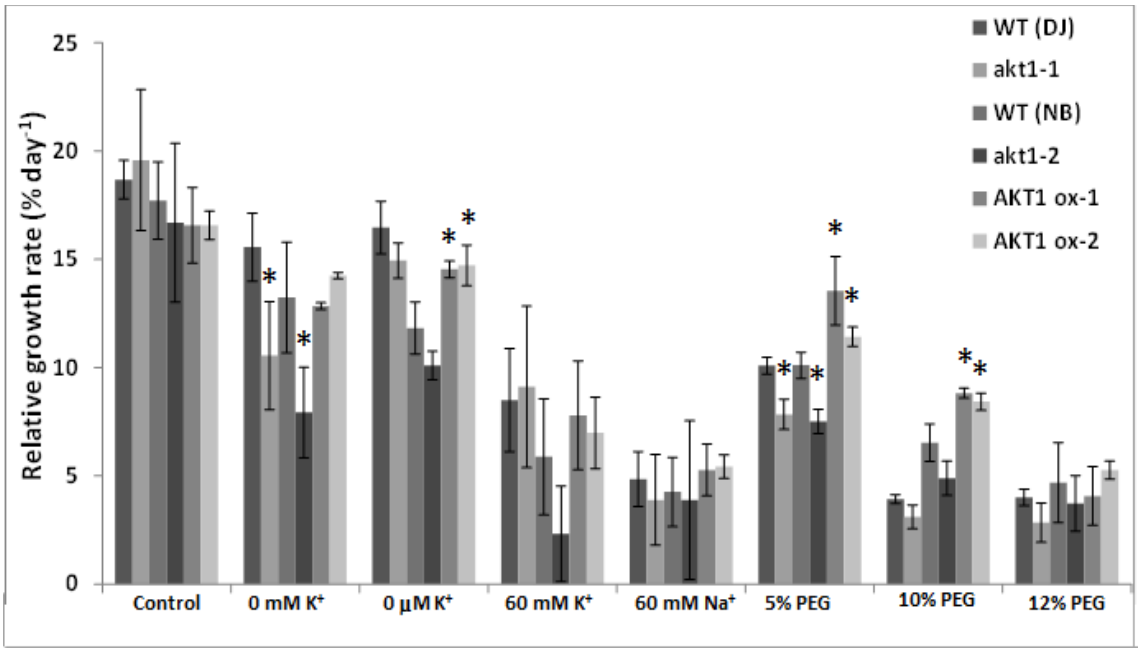

Figure 4-10: Relative growth rate for wild type and transgenic rice lines exposed to different media conditions

Relative growth rate (RGR, % day^{-1}), after 14 days for the wild type, *akt1* knockout and AKT1 overexpressor lines. Plants were grown in hydroponics media using control medium, 0 mM K^+, 60 mM K^+, 60 mM Na^+ and osmotic stress (5%, 10%, 12% PEG) conditions. Data are from three independent experiments, and the bars in the figure represent the standard errors. * denotes a significant differences by T-test at a probability level of $p < 0.05$ between the wild type and knockout lines or wild type and overexpressor lines.

4.2.4 Rice AKT1 plays important role in better growth under K^+ deficient conditions

Hirsch *et al.,* (1998) showed that the presence of AKT1 is essential for the K^+ uptake in a K^+ deficient medium where NH_4^+ is present. NH_4^+ inhibits KUP/HAK K^+ uptake transporters which become essential in the *akt1* mutant in K^+ deficient conditions (Figure 4-11). To see whether this pattern is similar in rice, plants were grown at different combinations of NH_4^+ and K^+ in the medium and were analysed for growth.

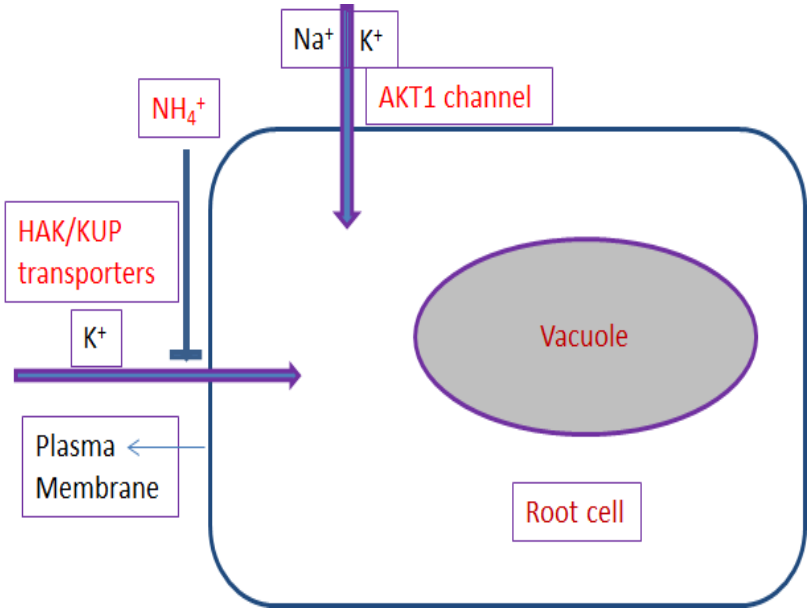

Figure 4-11: A model showing NH_4^+ toxicity to HAK/KUP transporters

In the presence of $NH4^+$, AKT1 channels are the only functional K^+ transporters while the HAK/KUP transporters are inhibited by $NH4^+$, especially in K^+ deficient conditions.

The *akt1* knockout lines showed less growth while the overexpressing lines showed more growth as compared to their corresponding wild type in the absence of both NH_4^+ and K^+ in the medium. The knockout lines showed less growth while no difference was observed in the overexpressing line as compared to the wild type at 0 mM NH_4^+ in the medium. In the absence of K^+ and presence of 3 mM NH_4^+ in the medium, the knockout plants showed less growth while the overexpressing plants showed higher growth as compared to the wild type plants (Figure 4-12).

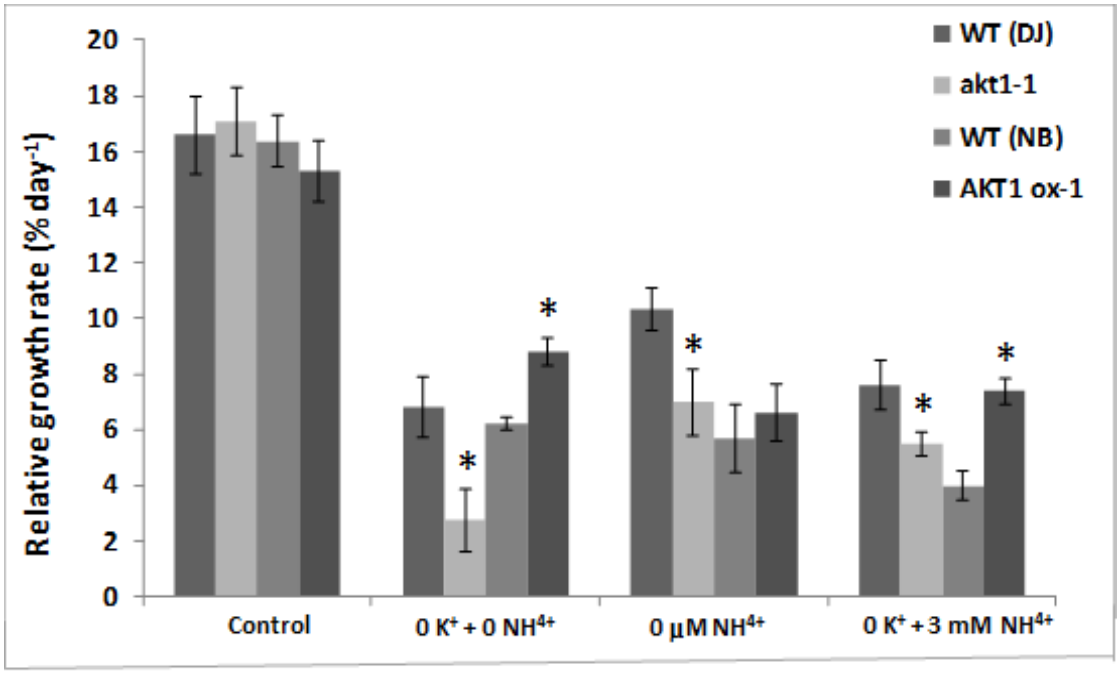

Figure 4-12: Relative growth rate for rice wild type and transgenic lines exposed to different media conditions

Relative growth rate (RGR, % day^{-1}) after 14 days for the wild type, *akt1* knockout and AKT1 overexpressor lines. Plants were grown in hydroponic media using control medium, 0 mM K^{+} + 0 mM NH_4^+, 0 mM NH_4^+ and 0 mM K^{+} + 3 mM NH_4^+ conditions. Data are from three independent experiments, and the bars in the figure represent the standard errors. * denotes the significant differences by T-test at a probability level of $p < 0.05$ between the wild type and knockout lines; and wild type and overexpressor lines. Refer to chapter 2 for the concentrations of NH_4^+, K^+, NO_3^- and Na^+ in the media used here.

4.2.5 AKT1 plays a positive role during drought stress

The growth phenotype observed during osmotic stress conditions in hydroponics was further analysed using soil grown plants

In fully watered conditions, there was no significant difference between the growths of all the tested genotypes. However, both the knockout lines showed less growth while both the overexpressing lines showed better growth as compared to their corresponding wild type plants under drought stress conditions (Figure 4-13).

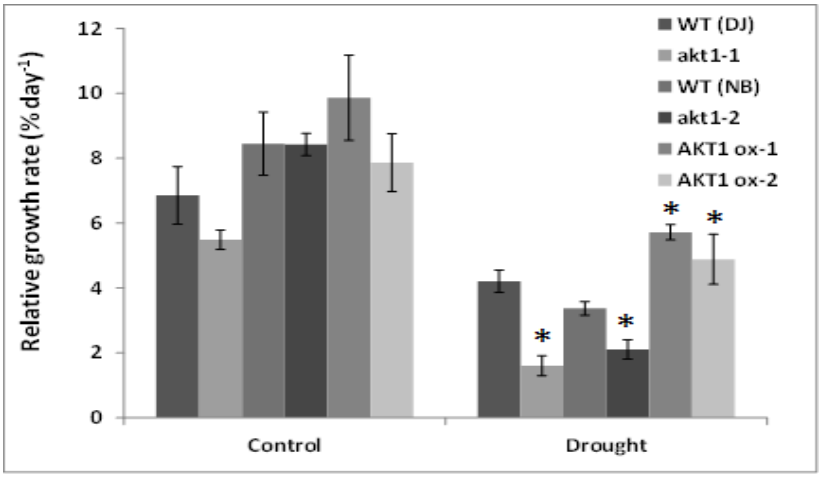

Figure 4-13: Relative growth rate for wild type and transgenic rice lines exposed to control and drought conditions

Relative growth rate (RGR % day^{-1}) of Wild type, *akt1* knockout and AKT1 overexpressor lines which were grown for 6 weeks in control and drought conditions in soil. Data are from three independent experiments and the bars in the figure represent the standard errors. * denotes the significant differences by T-test at a probability level of $p < 0.05$ between the wild type and knockout lines; and wild type and overexpressor lines.

4.2.6 Tissues K^+ concentration analyses of the transgenic and non-transgenic rice plants

4.2.6.1 Root K^+ concentration analyses

AKT1 is involved in the K^+ uptake in a range of external K^+ concentrations, therefore the K^+ concentration of the wild type and transgenic lines were analysed in the roots and leaves. The results showed no differences in the root K^+ concentration of all the genotypes in control conditions. At zero K^+ and 100 µM K^+ conditions, both the *akt1* knockout lines contained less K^+ while both the overexpressing lines showed a higher K^+ concentration as compared with the wild types. The decrease in the root K^+ concentration was about 2 fold in both the knockout lines as compared with their wild type plants in the $0K^+$ conditions. At 60 mM K^+, the *akt1* knockout lines showed no difference with the wild type while the overexpressing lines showed higher K^+ in the roots as compared with the wild type plants. At high Na^+ in the medium, no significant difference was observed in all the genotypes for the K^+ concentration, except for the *akt1-1* which showed higher K^+ in the roots as compared with the wild type plants. Under osmotic stress (5% PEG and 10% PEG) conditions, both the knockout lines

showed lower K^+ while the overexpressing lines showed higher K^+ in the roots as compared with the wild type plants (Figure 4-14).

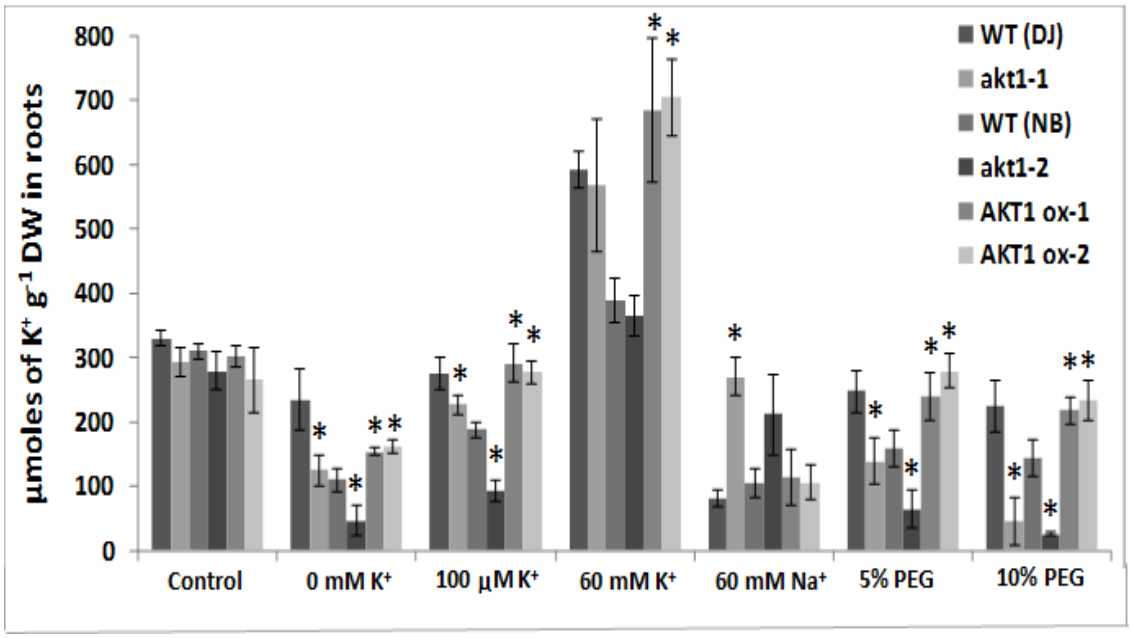

Figure 4-14: Root K^+ concentration analyses for wild type and transgenic rice lines exposed to different media conditions

Root K^+ concentration of rice wild type, *akt1* knockout and AKT1 overexpressor plants. The plants were grown in different media conditions as mentioned in the figure and root tissues were analysed for K^+ concentrations. The experiment was replicated three times and the bars in the figure represent the standard errors. * denotes the significant differences by T-test at a probability level of $p < 0.05$ between the wild type and knockout lines; and wild type and overexpressor lines.

4.2.6.2 Leaf K^+ concentration analyses

The shoot data showed no difference in the K^+ concentration in the leaves of all the tested genotypes under control, 60 mM K^+ and 10% PEG conditions. At zero K^+ and 100 µM K^+ conditions, the knockout lines showed less K^+ while the overexpressing lines showed higher K^+ concentration in the leaves as compared with the corresponding wild type plants. The *akt1*-1 line showed higher K^+ concentration in the leaves while no difference was observed for the *akt1*-2 line at 60 mM Na^+ in the medium, however, both the overexpressing lines showed less K^+ concentration in the leaves. Mild osmotic stress (5% PEG) led to lower K^+ in the leaves of both the knockout lines while higher K^+ in the leaves of both the AKT1 overexpressing lies as compared to the wild type plants. All the genotypes showed comparable K^+ concentration in the leaves under higher osmotic stress (10% PEG) conditions (Figure 4-15).

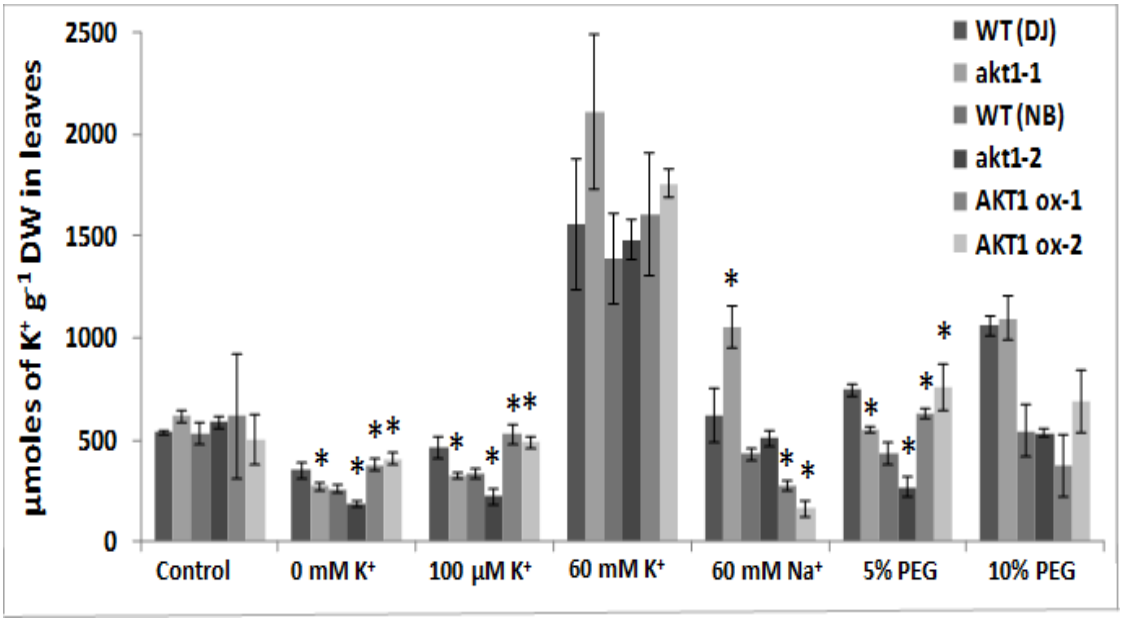

Figure 4-15: Leaf K^+ concentration analyses for wild type and transgenic rice lines exposed to different media conditions

Shoot K^+ concentration of rice wild type, *akt1* knockout and AKT1 overexpressor plants. The plants were grown in different media conditions as mentioned in the figure and shoot tissues were analysed for K^+ concentration. The experiment was replicated three times and the bars in the figure represent the standard errors. * denotes the significant differences by T-test at a probability level of $p < 0.05$ between the wild type and knockout lines; and wild type and overexpressor lines.

4.2.6.3 Tissue ion concentration analyses of the wild type and transgenic plants grown in soil

When plants were grown in fully watered soil, root K^+ concentrations were similar in all the genotypes. However, under drought stress, the *akt1* Knockout plants showed less K^+ in the roots while the AKT1 overexpressing plants had more K^+ in the roots as compared to the wild type plants (Figure 4.16a). The leaves of the *akt1* knockout plants showed no differences with the wild type while both the overexpressing lines contained more K^+ in their leaf tissue in both control and drought stress conditions (Figure 4-16b).

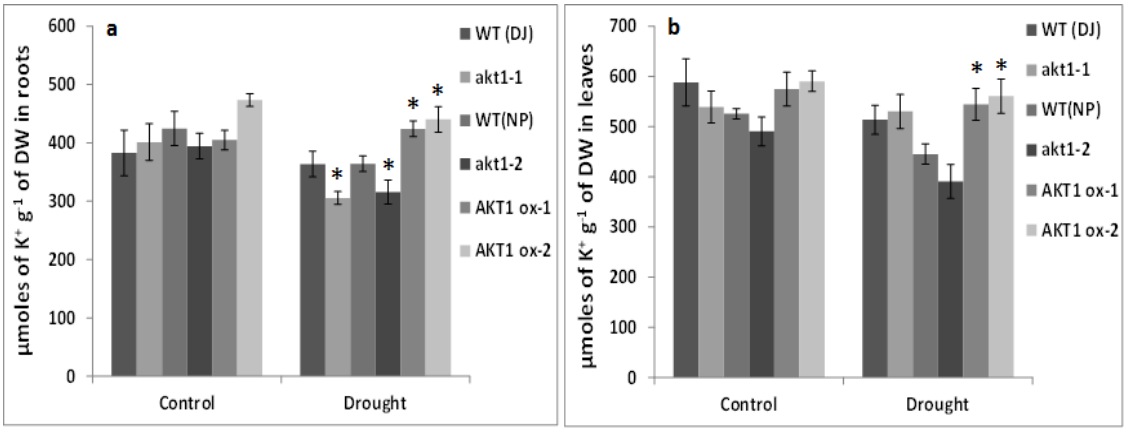

Figure 4-16: K^+ concentration analyses of the wild type and transgenic rice lines exposed to drought stress in soil

a; root K^+ concentration and b; shoot K^+ concentration of rice wild type, *akt1* knockout and AKT1 overexpressor plants. The plants were grown for six weeks in control and drought conditions in soil and root and shoot tissues were analysed for K^+ concentration. The experiment was replicated three times and the bars in the figure represent the standard errors. * denotes the significant differences by T-test at a probability level of $p < 0.05$ between the wild type and knockout lines; and wild type and overexpressor lines.

4.2.7 Tissues Na^+ concentration of the transgenic and non-transgenic rice plants

It has been suggested that AKT1 plays a role in the Na^+ uptake (Maathuis, 2006) especially in K^+ deprived conditions to provide turgor to the cells. We therefore analysed the Na^+ concentration of the tissues to see the effect of AKT1 on the tissue Na^+ concentration.

4.2.7.1 Root Na^+ concentration

The results showed that both the knockout lines showed less Na^+ while both the overexpressor lines had more Na^+ in the root tissues as compared with their corresponding wild type plants at zero K^+ in the medium. All genotypes showed comparable Na^+ concentration in the roots when grown at 60 mM Na^+ in the medium (Figure 4-17a and b).

4.2.7.2 Leaf Na^+ concentration

For plants grown at 0 K^+, the leaves of the knockout lines showed less Na^+ while that of overexpressor lines showed higher Na^+ concentration as compared with the corresponding wild type plants. However, no difference was observed in the leaf Na^+

concentration for all the genotypes when plants were grown at 60 mM Na^+ in the medium (Figure 4-17c and d).

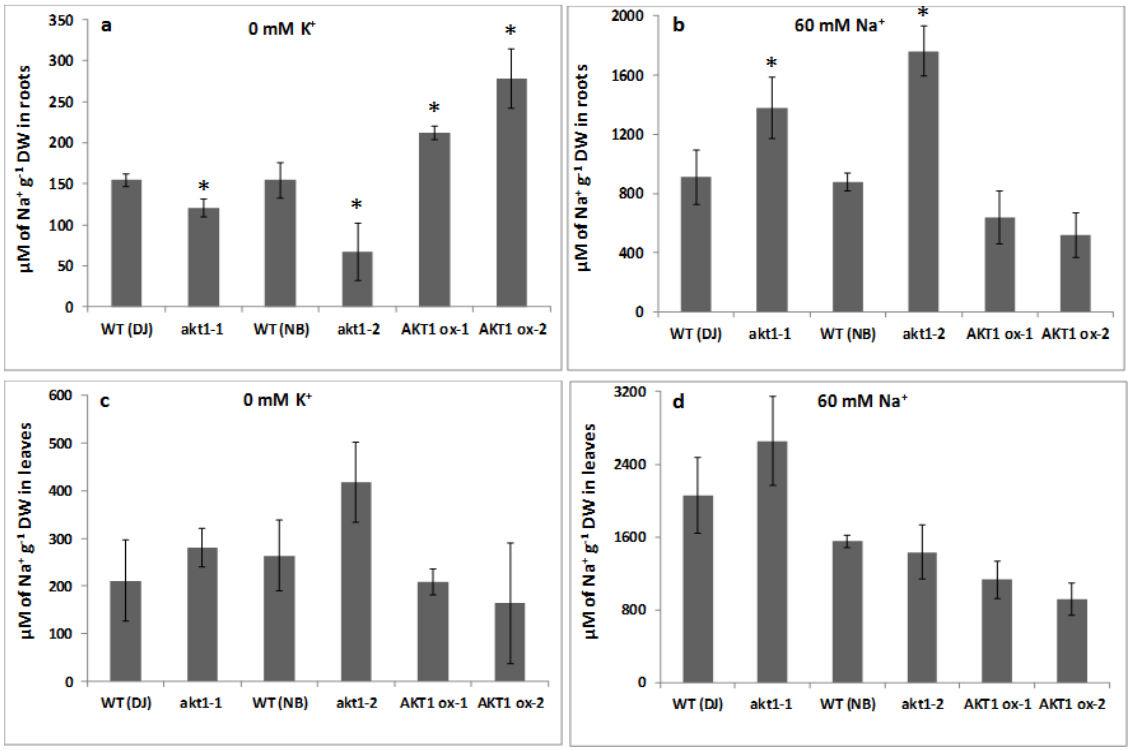

Figure 4-17: Na^+ concentration analyses for wild type and transgenic rice lines exposed to different media conditions

a; root Na^+ concentration of plants grown at zero K^+ conditions, b; root Na^+ concentration of plants grown at 60 mM Na^+. c; leaf Na^+ concentration from plants grown at zero K^+ conditions, d; leaf Na^+ concentration from plants grown at 60 mM Na^+ conditions. The experiment was replicated three times and the bars in the figures represent the standard errors. * denotes the significant differences by T-test at a probability level of $p < 0.05$ between the wild type and knockout lines; and wild type and overexpressor lines.

4.2.8 Xylem sap analysis of the wild type and transgenic plants

4.2.8.1 K^+ concentration analyses

The xylem sap was analysed for the K^+ concentration to see whether uptake through the AKT1 channels had any effect on the loading of K^+ into the xylem.

The data revealed that both the *akt1* knockout lines showed less K^+ while both the overexpressing lines showed higher K^+ in the xylem sap as compared with the wild type plants in control, 0 mM K^+ and 10% PEG conditions. At 60 mM K^+ conditions, no significant difference was observed among all the genotypes. The *akt1*-1 line showed no difference with the wild type while the *akt1*-2 line showed less K^+ and the AKT1

overexpressor line showed higher K^+ in the xylem at 60 mM K^+ in the medium (Figure 4-18).

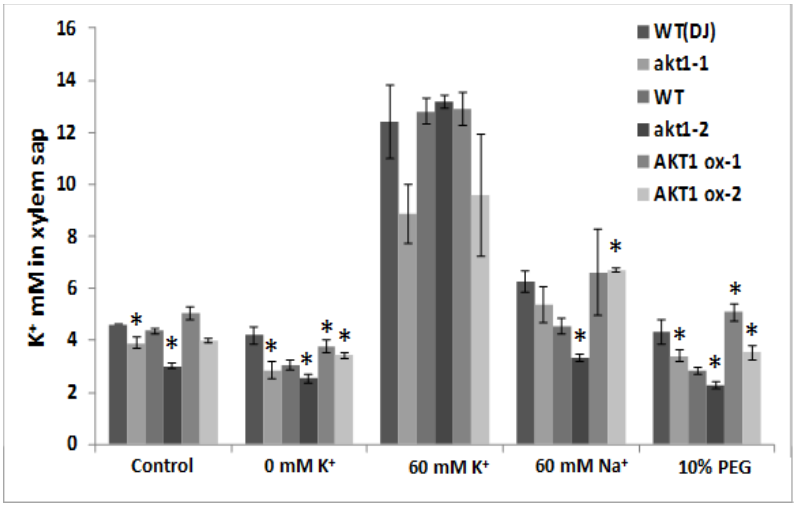

Figure 4-18: Xylem sap analyses for wild type and transgenic rice lines exposed in different media conditions

K^+ concentration in the xylem sap of rice wild type, *akt1* knockout and AKT1 overexpressor plants; the plants were grown for one week in different media conditions as mentioned in the figure, and xylem sap was analysed for K^+. Experiment was replicated three times and the bars in the figure represent the standard errors. * denotes the significant differences by T-test at a probability level of $p < 0.05$ between the wild type and knockout lines; and wild type and overexpressor lines.

4.2.8.2 Na^+ concentration in xylem sap

The data showed varied results for the Na^+ concentration in the xylem sap of different genotypes in various conditions. The *akt1*-1 line showed no difference in Na^+ concentration with the wild type while the *akt1*-2 showed less Na^+ in the xylem sap as compared to the wild type plants in 0 mM K^+ conditions in the medium. AKT1 ox-1 showed higher Na^+ while AKT1 ox-2 showed no difference with wild type plants in 0 mM K conditions (Figure 4-19a).

At 60 mM Na^+ conditions, *akt1*-1 showed less Na^+ concentration in the xylem sap while no difference was observed for the *akt1*-2 as compared to wild type. AKT1 ox-1 showed higher Na^+ while AKT1 ox-2 showed no difference in the Na^+ concentration of the xylem sap (Figure 4-19b).

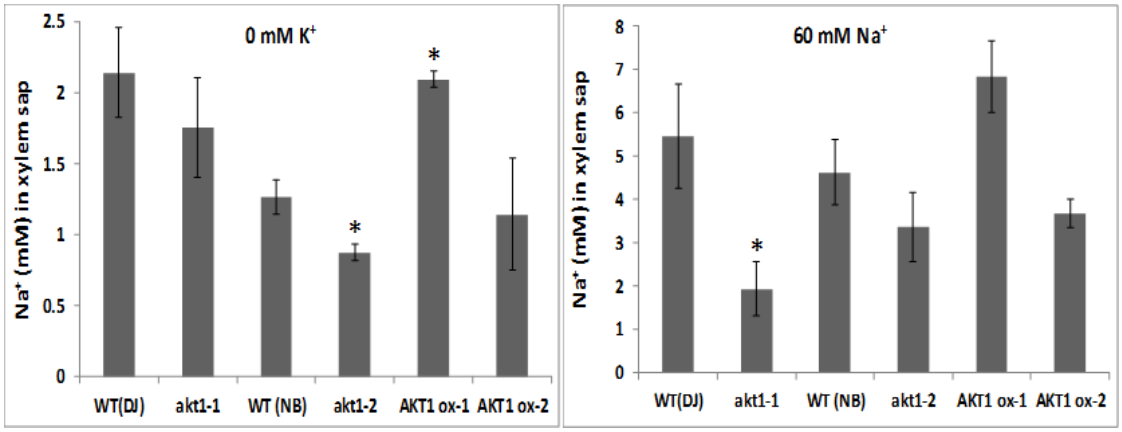

Figure 4-19: Xylem sap analyses for wild type and transgenic rice lines exposed to different media conditions

a; K^+ concentration and b; Na^+ concentration in the xylem sap of rice wild type, *akt1* knockout and AKT1 overexpressor plants. The plants were grown for one week in different media conditions as mentioned in the figures and xylem sap was analysed for K^+ and Na^+ concentration s. The experiment was replicated three times and the bars in the figures represent the standard errors.

4.2.9 Analyses of the stomatal conductance and rate of photosynthesis

Rice AKT1 has a role in K^+ uptake and distribution within the plant. But, AKT1 channels are also expressed in guard cells, thus there is a possibility that these channels play a role in the guard cell movement and therefore affect the stomatal conductance and rate of photosynthesis. We analysed these two parameters to see the role of AKT1 under different conditions. In control grown plants, the data showed no difference for all the genotypes except AKT1 ox-1 which showed higher stomatal conductance. At zero K^+ and osmotic stress (10% PEG) conditions, both the knockout lines showed lower stomatal conductance while the overexpressing line showed higher stomatal conductance as compared to their corresponding wild type plants (Figure 4-20a). All the tested lines showed no significant difference in the rate of photosynthesis under any of the media conditions (Figure 4-20b).

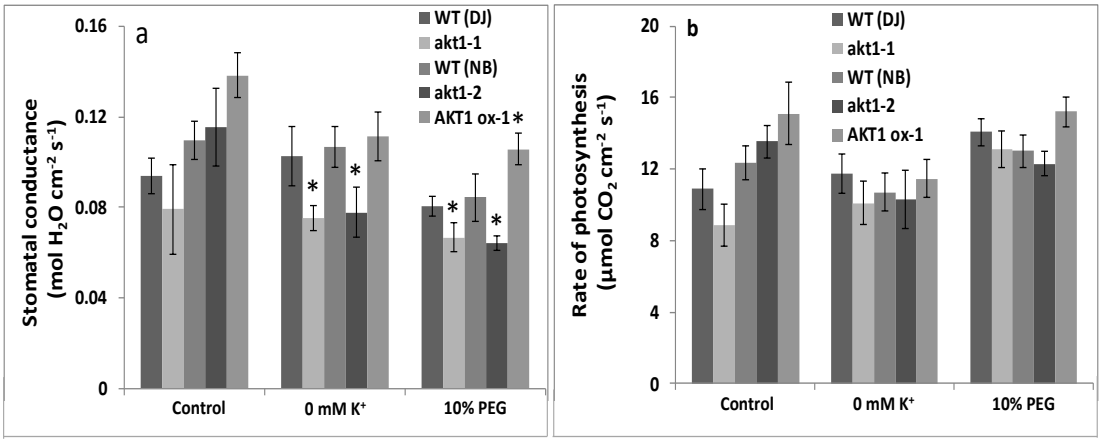

Figure 4-20: Stomatal conductance and rate of photosynthesis of wild type and transgenic rice lines exposed to different media conditions

The stomatal conductance (a) and rate of photosynthesis (b) of wild type, *akt1* knockout and AKT1 overexpressor lines; The plants were grown for two weeks in different media conditions as mentioned in the Figure and stomatal conductance and the rate of photosynthesis was measured. The experiment was replicated three times and the bars in the figures represent the standard errors. * denotes the significant differences by T-test at a probability level of $p < 0.05$ between the wild type and knockout lines; and wild type and overexpressor lines.

4.3 Discussion

Potassium is required for the growth and many other physiological processes of plants. Its deficiency may lead to low productivity of the plants. The trafficking of K^+ into the plant and within the plant is controlled by transporter proteins. These proteins are localised in the plasma membrane, tonoplast of the vacuoles and other membranes. Transporter proteins like AKT1 play an important role in rice as well as in other plants.. AKT1 is localised in the plasma membrane and it is suggested that AKT1 plays a role in salt stress (Maathuis, 2006) and osmotic stress conditions (Nieves-Cordones *et al.*, 2011). To analyse the role of rice AKT1 channels in different abiotic stress conditions, rice wild type, *akt1* knockout and AKT1 overexpressor lines were characterised for different parameters such as growth, ion concentrations in the tissues and xylem sap, stomatal conductance and rate of photosynthesis in different media conditions.

The results showed that all the tested genotypes showed no growth phenotype in the control conditions (Figure 4-10), which suggests a limited role of rice AKT1 in the normal conditions. The K^+ concentration in the root and leaf tissues of all the genotypes also showed no difference when plants were grown in control medium which suggests that other K^+ transporters may be active and fulfil the K^+ requirements of the plants.

4.3.1 AKT1 is important during K^+ deficient conditions

Rice *akt1* knockout lines showed less growth as compared to the wild type in the absence of K^+ in the medium (Figure 4-10). Spalding *et al.,* (1999) reported similar reduced growth for *Arabidopsis akt1* mutants in the absence of K^+ in the medium. The reduced growth of the knockout lines might be the effect of the loss of AKT1 activity. AKT1 is an important K^+ uptake system in the root soil boundary and loss of the AKT activity could reduce K^+ uptake. We observed a lower level of K^+ in the roots of the *akt1* knockout lines as compared with the wild type plants in K^+ deprived conditions (Figure 4-14), which supports the above notion.

AKT1 overexpressor lines showed comparable growth to the wild type plants in the zero K^+ medium. Thus, at this very low level of K^+, overexpression of AKT1 did not affect the growth. However, under the same conditions, the overexpressing plants did show higher concentrations of the K^+ in their tissues as compared with the wild type plants (Figure 4-14). It is not straightforward to explain the higher K^+ concentration since at these external K^+ concentrations it is unlikely that AKT1 plays a major role in K^+ uptake. Three scenarios can be suggested; 1. Before exposing the plants to the zero K^+ condition, they were growing in the normal K^+ conditions and the overexpressing plants may have absorbed more K^+ during this phase 2. The roots of the AKT1 overexpressor plants leak less K^+ into the surrounding than the wild type plants. This could be true if the overexpressor plants have a more negative membrane potential because the latter would reduce open probability of outward rectifying channels such as GORK (Shabala and Cuin, 2008) 3. K^+ leakage from the roots into the surrounding environment (shown in chapter 3 for TPKsb) could bring external K^+ into the range where AKT1 can be active. Some studies suggested upregulation of AKT1 in the K^+ deficient conditions (Li *et al.,* 2006; Buschmann *et al.,* 2000; Li *et al.,* 2014) which supports the idea that AKT1 can function as high affinity transport system and may be involved in the K^+ uptake in a very low external K^+ concentrations.

When the medium was supplied with 100 µM K^+, we observed better growth for the AKT1 overexpressor plants. The higher K^+ concentration in the roots and shoot of the overexpressor lines may be a reason for the better growth of these plants. AKT1 may be responsible for the improved K nutrition. The leaves of the overexpressor plants also showed higher K^+ concentration than the wild type plants (Figure 4-15). The presence

of more K^+ in the xylem sap of the overexpressor plants suggests higher loading of K^+ into the xylem. It is difficult to argue about the loading of K into the xylem here. SKOR channels are the principle K^+ loading channels in the xylem and they are depolarisation-activated (Gaymard *et al.,* 1998; Shabala and Cuin, 2008). The presence of more K^+ in the roots cells could lead to plasma membrane hyperpolarisation. In that case, SKOR activity would be reduced rather than upregulated. Alternatively, non-selective K^+ channels might be responsible for augmented loading of K^+ into the xylem. GORK are the depolarisation-activated outward rectifying K^+ channels and may be less active in the cells of overexpressing plants. It is more likely that the overexpressing cells leak-out less K^+ than the wild type plants and thus having more K^+. The higher K^+ concentrations may be helpful in loading more K^+ into the xylem.

4.3.2 AKT1 is insensitive to NH_4^+ toxicity

The reduced growth of the *akt1* knockout lines in the zero K^+ conditions might be due to NH_4^+ toxicity. The medium contained NH_4^+ and that may have toxic effects in the absence of K^+. Dennison *et al.,* (2001) reported better growth for the *Arabidopsis akt1* knockout mutants at low external K^+ concentration in the absence of NH_4^+, however, by the addition of NH_4^+ into the same medium; the growth of the knockout mutants became reduced. NH_4^+ and K^+ are both univalent cations and it is suggested that they share common transporters (Szczerba *et al.,* 2008) and in the absence of K^+, NH_4^+ causes toxicity (Wang *et al.,* 1996; White, 1996; Howitt and Udvardi, 2000; Kronzucker *et al.,* 2001; Nielsen and Schjoerring, 1998; Hess *et al.,* 2006; Szczerba *et al.,* 2008 a,b). AKT1 is insensitive to NH_4^+ and is the only major K^+ uptake system in the presence of NH_4^+as this cation is toxic to the K^+ transporters of HAK/KUP family. Therefore, in the absence of AKT1 activity and toxification of HAK/KUP transporters, K uptake may be considerably hampered and so may a reason for the lower growth of *akt1* knockout lines. In the absence of K^+, more NH_4^+ enters into the roots passively (Sczebra *et al.,* 2006) for charge balancing. The increasing NH_4^+ may be toxic for the plants. Plants have the strategy to remove NH_4^+ from the cells actively by the expenditure of energy (Britto *et al.,* 2001). The *akt1* knockout lines may have more NH_4^+ and use more energy for the efflux of NH_4^+; therefore it is more likely that they are losing more metabolites, which may be a cause of lower growth rate as compared to the wild type plants.

The lower growth of the *akt1* knockout lines observed at zero K^+ conditions was eliminated when 100 uM K^+ was added to the medium. This shows that the addition of K^+ alleviates the toxic effects of NH_4^+. These results are in agreement with the notion that K^+ can alleviate NH_4^+ toxicity (Barker *et al.,* 1967; Cao *et al.,* 1993). HAKs/KUPs are the high affinity K^+ transporters and are involved in K^+ uptake at micro molar concentrations. Many studies have shown that AtHAK5 is activated in K^+ deficient conditions (Ahn *et al.,* 2004; Armengaud *et al.,* 2004; Hampton *et al.,* 2004; Shin and Schachtman, 2004; Gierth *et al.,* 2005). Elevation of the transcript level of the HAKs/KUPs may be transient (Shin and Schachtman, 2004) or may be for longer duration (Gierth *et al.,* 2005). The activation and elevation of the HAKs/KUPs expression in the *akt1* knockout lines may be involved to transport K^+ enough for the alleviation of NH_4^+ toxicity and restoring growth.

4.3.3 AKT1 is involved in Na^+ uptake

It has been suggested that AKT1 channels are involved in Na^+ uptake from the root environment, especially in K^+ deficient conditions (Amtmann and Sanders, 1999; Blumwald *et al.,* 2000; Buschmann *et al.,* 2000; Maathuis, 2006). The better growth of the wild type and overexpressor plants as compared with the knockout lines in the zero K^+ conditions may be due to the presence of Na^+ in the medium as the medium contained 1.3 mM Na^+ to replace K^+. Several studies have suggested that AKT1-type channels may contribute to Na^+ uptake. For example, Buschmann et al (2000) reported that *TaAKT1* might be involved in Na^+ absorption in K^+ deficient conditions. Golldack *et al.,* (2003); Obata *et al.,* (2007); Wang *et al.,* (2007) also suggested a role for AKT1 in Na^+ transport. Thus, the absence of *AKT1* in the knockout mutants would reduce Na^+ uptake and hence growth. Indeed, the Na^+ concentration in the roots of the wild type and overexpressor plants is higher than that in the knockout mutants (Figure 4.17a). This higher Na^+ concentration might be the cause of the better growth of the wild type and overexpressor lines as compared with the knockout lines. Since many studies have shown that moderate level of Na^+ promote plant growth in the absence of K^+ in the medium (Horie *et al.,* 2007).

4.3.4 AKT1 improves tolerance to water stress

Osmotic stress affects plant productivity due to many reasons (details in chapter 1). Osmotic stress considerably affects K^+ acquisition from the soil (Shabala, 2000) and

therefore disturbs many of the biological processes within the plant in which K^+ is involved. Therefore, it is important to develop strategies which can improve K^+ nutrition/ acquisition, especially pertaining to osmotic stress. Here we show a role for rice AKT1 during osmotic stress. The *akt1* knockout lines showed less growth while the AKT1 overexpressor lines showed better growth as compared with their corresponding wild type plants in osmotic stress conditions (Figure 4-10) in both hydroponics and soil. K^+ is a major osmoticum and its uptake often increases during osmotic stress (Wang *et al.,* 2013). Thus increased uptake through AKT1 could reduce the negative effects caused by water stress. Our data showed lower K^+ concentration in the *akt1* knockout plants while it was higher in the AKT1 overexpressor plants as compared with the wild type plants (Figure 4.14). The same trend was observed for the ion concentrations in the root tissues for plants grown in the drought stress (Figure 4.16a). Together, these findings provide evidence that AKT1 is a major uptake route for K^+ during water stress in spite of the fact that expression of the AKT1 channel in the roots is independent of ABA in *Arabidopsis* (Gaymard *et al*., 1998) and grapevine (Cuellar *et al.,* 2010).

The leaves of the knockout lines showed less K^+ while that of the overexpressor lines showed higher K^+ concentration in 5% PEG and drought stress conditions. The K^+ concentration in the roots may have an important effect on the loading of K^+ into the xylem to translocate K^+ into the leaves. The xylem sap data is in agreement with the K^+ concentration of the leaves. Cuellar *et al.,* (2010) suggested an increase in the expression level of the VvK1 (a homolog of AKT1) in drought stress. This may also be a reason of higher level of K^+ in the overexpressing lines. Nevertheless the differential stomatal conductance in the wild type, knockout and the overexpressing lines also supports the differences in the xylem K^+ loading and leaves K^+ concentration the tested genotypes.

4.3.5 AKT1 is functional in guard cells

Shaker type K^+ channels are expressed throughout the plant including guard cells and are involved in influx and efflux of K^+ in these cells (Hosy *et al.,* 2003; Lebaudy *et al.,* 2008a). These channels play important roles in the stomatal movement, for example to reduce the water loss during osmotic stress. AKT1 is not only involved in the uptake of K^+ in roots but also plays a role in the regulation of stomatal movements (Nieves-Cordones *et al.,* 2011). Our results showed lower stomatal conductance for the *akt1*

knockout lines while higher stomatal conductance for the AKT1 overexpressor lines as compared with the wild type plants in zero K^+ and osmotic stress conditions (Figure 4.20a). These results suggested that AKT1 plays a role in the stomatal movements during abiotic stress conditions by changing K^+ concentration in the guard cells. Lack of *akt1* activity in the knockout lines might be responsible for the comparatively flaccid guard cell and lower stomatal conductance while vice versa stomatal conductance in AKT1 overexpressor lines.

The data from the xylem K^+ loading (Figure 4-18) and leaf K^+ concentration (Figure 4-15) agreed to the differential K^+ in the guard cells of the wild type, knockout and overexpressor lines. GORK channels may be more actively involved in the knockout lines in K^+ efflux and may be responsible for the lower stomatal conductance. The higher stomatal conductance in the wild type and AKT1 overexpressor plants may enhance the diffusion of CO_2 into the plants and therefore increasing the rate of photosynthesis. Although we did not observe any difference in the rate of photosynthesis of these genotypes under different conditions (Figure 4-20b). This may be because the rate of photosynthesis is not reduced per unit leaf area (James *et al.*, 2002) but at the whole plant level (as explained in chapter 1). Abiotic stresses may result in a higher density of chloroplasts as the leaves become thickened and this could be an explanation for why we observed no difference in the rate of photosynthesis (Munns and Tester 2008).

Chapter 5

5 Final Conclusions

5.1 Abiotic stress

The productivity of most crops is affected by different types of biotic and abiotic stresses. Drought, salinity, heat, cold and nutrient stresses are a few examples of abiotic stresses and they reduce the average yield of most crop plants by >50% (Wang et al., 2003). Cereal crops (mostly glycophytes) typically attain only about 25% of their potential yield due to the effects of environmental stresses, such as osmotic and salt stress (Boyer, 1982). Osmotic and salt stresses have almost similar effects and therefore they evoke similar responses (Boyer, 1982). Potentially, a large number of strategies to cope with salinity and drought is available. Changing the plant's surrounding, reclamation of the soil, provision of clean water, proper use of fertilizers, for example, could be used to deal with drought and salinity but all these are costly and may be operative only for a short time. Therefore, we need to apply more cost effective and longer lasting strategies. Genetic manipulation of plants and characterization of transporter proteins could be an effective approach to deal with salinity and drought because they are economical and durable (Epstein, 1983). Important to mention also is the range of responses that different species use to counter abiotic stress. From these we can identify the most appropriate to reduce stress sensitivity in various crops.

Plants adopt a range of strategies to cope with the problems of drought and salinity. Improving plant nutrition could be one of the more effective strategies to reduce drought and salinity stress, especially K^+ nutrition. K^+ is one of the most abundant and an important nutrient for plants for different biochemical and physiological processes and increased K^+ has been shown to relieve drought and salinity stress. To improve K^+ nutrition, uptake and distribution/translocation within the plants are important. The role of membrane transporters is obvious from various studies in plant responses to drought and salinity by controlling influx and efflux of Na^+, and enhancing uptake and accumulation of K^+ and water (Figure 5-1). Therefore, in this study, we focussed on K^+ channels (TPKa, TPKb and AKT1) which are localised to the tonoplast and plasma membrane and we found that these channels can improve K^+ nutrition and growth.

5.2 Growth during abiotic stresses and the role of K^+ channels

Abiotic stresses such as drought and salinity negatively interfere in a plants' nutrients uptake mechanisms especially of K^+. Therefore, improving plant nutrition either by additional supply of nutrients or by improving the uptake could help plants in tolerance against these stresses. K^+ transporters are involved in the uptake and distribution may be the main players (Figure 5-1). Our results showed a better growth phenotype for the TPKa, TPKb and AKT1 overexpressing plants as compared to their corresponding control plants while the *akt1* knockout lines showed lower growth than the wild type plants at K^+ deficient conditions. AKT1 is localised to the plasma membrane and highly expressed in the root epidermis and cortical cells where it is involved in K^+ uptake (Figure 5-1). Hirsch *et al.,* (1998) reported a role of AKT1 in K^+ uptake in *Arabidopsis*. AKT1 absorbs K^+ in a range of external K^+ concentrations (Golldack *et al.,* 2003) and there is a large number of further reports about the role and characterization of AKT1 channels in different plants for example, Maathuis and Sanders, 1995; Lagarde *et al.,* 1996; Hirsch *et al.,* 1998; Spalding *et al.,* 1999; Buschmann *et al.,* 2000; Pilot *et al.,* 2003; Li *et al.,* 2014. These studies on the regulation, activation and expression of AKT1 channels emphasize the importance of K^+ uptake channels in plant K^+ nutrition. Besides AKT1, other K^+ channels like TPKa and TPKb may also have a role in the uptake and distribution of K^+ in different stress conditions. TPKa and TPKb are localised to the tonoplast of LV and SV respectively (Figure 5-1; Isayenkov *et al.,* 2011), and mainly involved in the K^+ release from the vacuoles. Gobert *et al.,* 2007 showed a better growth for *Arabidopsis* under K^+ deficient conditions in the AtTPK1 overexpressing plants. Hamamoto *et al.,* (2008) and Isayenkov and Maathuis, (2013) transformed *E. coli* strain LB2003 with NtTPK1 and AtTPK1 respectively, and found better growth in the transformed lines as compared with the strain lacking the TPK1 transgene. They concluded that the better growth of the transgenic *E. coli* was caused by improved K^+ uptake. We also observed a better K^+ uptake for the OsTPKb overexpressing lines in the K^+ depleted conditions.

Efficient K^+ uptake and distribution through AKT1 and TPK channels and other transporters may be important for growth of rice, especially in stress conditions. This may be due to direct effects of these transporters but there is also the possibility that their overexpression alters the expression and/or the activities of other channels and

carriers (Figure 5-1). For example, TPKs are involved in the release of K^+ from the vacuole and may cause higher K^+ concentration in the cytoplasm. The higher K^+ in the cytoplasm may cause hyperpolarization of the membrane and increase the activities of AKT1 because AKT1 is hyperpolarization-activated. To test this idea, it will be useful to carry out membrane potential measurements in the root cells of the TPK overexpressing lines. Analysing the transcript abundance and activities of other transporters, for example HAK, SKOR, GORK and NSCCs, (Figure 5-1) may also help to understand how TPK overexpression leads to increased K^+ uptake. Furthermore, loss of function mutations in *TPKa* and *TPKb* would be informative in this respect.

5.3 K^+ channels improve translocation of K^+ in rice

Better K^+ uptake can improve K^+ translocation to the shoot. Our data showed a higher K^+ in the shoot tissues of the overexpressing lines of TPKa, TPKb and AKT1 and less K^+ in *akt1* knockout lines as compared with the wild type plants during K^+-deficient and osmotic stress condition. The better growth of the overexpressing lines for these channels could have resulted from this differential distribution of K^+ to the shoot. It has been reported (Gobert *et al.,* 2007) that improved K^+ distribution could improve growth and tolerance to abiotic stresses. There is no evidence that TPKs and AKT1 directly enhance K^+ loading to the xylem but several possibilities could explain indirect effects. For example, AKT1 or TPK overexpression may enhance the expression of the SKOR channel (Figure 5-1), the principle system for loading K^+ into the xylem, and therefore increase translocation of K^+. Other K^+ channels, for example NSCCs, may also be involved in the loading of K^+ into the xylem. Future study should test this idea by measuring expression levels of SKOR and NSCC type channels in AKT1 and TPKs overexpressing lines (Figure 5-1).

Increased shoot K^+ may also derive from altered water flux to the leaves. We observed a higher transpiration rate in the AKT1, TPKa and TPKb overexpressing lines in zero K^+ and osmotic stress conditions. Moreover, the overexpressing lines contained more K^+ in the root tissues as compared with the wild type plants under the same conditions. The comparatively higher transpiration rate and higher root K^+ concentration in the overexpressing lines may both positively affect loading of K^+ into the xylem.

The differential K^+ concentrations in the xylem sap of the wild type, *akt1* knockout and AKT1 overexpressing lines support the above idea. However, no differences were observed in the xylem K^+ concentration of the TPKs transgenic lines and wild type plants under various conditions. To analyse the xylem sap in case of TPKs transgenic plants, it would be informative to expose the plants for a brief duration to zero K^+ and osmotic stress conditions and analyse the K^+ concentration in the xylem sap.

5.4 Stomatal movement and K^+ channels

We observed a higher stomatal conductance in the overexpressing lines of AKT1 and TPKs. It was reported that AtTPK1 (Gobert *et al.*, 2007) and AtAKT1 (Nieves-Cordones *et al.*, 2011) are expressed in the guard cells (Figure 5-1) and are involved in the stomatal conductance under varied external conditions. The higher stomatal conductance in the TPKa, TPKb and AKT1 overexpression lines may be because of a generally higher K^+ concentration in shoot tissue. AKT1 might be loading more K^+ into the guard cells while NHX antiporters (Venema *et al.*, 2002; Rodriguez-Rosales *et al.*, 2008) may be involved in accumulating more K^+ in the vacuoles. The overexpression of one gene may change the activity or the abundance of other genes, so it is possible that the AKT1 or TPK overexpression may be responsible for altered expression or activity of NHX antiporters. Higher K^+ concentration in the vacuole is helpful for the turgidity of the guard cells and will keep stomata open, ultimately resulting in higher transpiration rate.

5.5 AKT1 is important under NH_4^+ toxicity

We observed that lack of AKT1 activity in the *akt1* knockout lines caused less growth as compared with the wild type plants in the zero K^+ conditions. Lower growth rates may be due to a lack of K^+ but also because of NH_4^+ toxicity which occurs especially in K^+-depleted conditions. Improved K^+ availability and nutrition can rescue plants from NH_4^+ toxicity. NH_4^+ causes toxicity through different ways as described in chapter 4. The HAK/KUP (Figure 5-1) transporters are sensitive, but AKT1 is insensitive to NH_4^+ toxicity. Addition of K^+ resulted in comparable growth between the wild type and *akt1* knockout plants. These results confirmed that the presence of K^+ may be helpful for the alleviation of NH_4^+ toxicity.

NH_4Cl and $(NH_4)_2SO_4$ are commonly used fertilizer for rice. These fertilizers can increase the productivity of rice considerably. However, they increase the concentration of NH_4^+ in the root environment and may cause toxicity. The effects may be more severe for rice as rice is grown in the paddy fields and NH_4^+ could be released in the form of ions which are potentially more damaging. Therefore, it is beneficial for rice to be supplied with extra K^+ when external NH_4^+ is high. Our results confirm the preceding statement because when the medium was supplied with extra K^+ the AKT1 overexpressor lines showed better growth as compared with the wild type plants. Measurement of NH_4^+ fluxes and analyses of the nitrogen content in the roots of the *akt1* knockout, AKT1 overexpressor and wild type plants would help to further clarify the picture.

5.6 K^+ channels are involved in Na^+ uptake

Our data revealed that the *akt1* knockout lines showed lower growth and less Na^+ concentration and AKT1 overexpressing lines showed better growth and higher Na^+ concentration as compared with the wild type plants in the zero K^+ conditions. The differences in the growth of the knockout and overexpressing genotypes might be because of the differences in the turgor or because of disturbance in the cations ratio in these genotypes. At zero K^+ conditions, K^+ was replaced by equimolar concentration of Na^+. Provision of turgor and charge balancing are among the important functions of K^+ but other cations can also perform these functions, especially in the absence of K^+. Na^+ uptake from the environment may be important in such conditions. There are many reports (Amtmann and sanders, 1999; Blumwald *et al.,* 2000; Buschmann *et al.,* 2000;; Golldack *et al.,* 2003; Maathuis, 2006; Obata *et al.,* 2007; Wang *et al.,* 2007; Horie *et al.,* 2007) which suggested a positive role of Na^+ and the involvement of AKT1 in Na^+ uptake under K^+-deficient conditions. Our data are in agreement with the previous reports about the role of AKT1 channels in Na^+ uptake and the positive role of Na^+ in the absence of K^+.

In summary, we found a significant role of TPKa, TPKb and AKT1 channels during various abiotic stress conditions for physiological parameters like growth, K^+ uptake and translocation, control of stomatal conductance, water content, etc. Interestingly, we observed almost similar phenotypes for these cation channels although they are different from each other on the basis of structure, regulation and localization. However, these

three K^+ channels impact on K^+ uptake and distribution. It appears that their overexpression improves overall K^+ nutrition of rice plants and therefore improves tolerance to abiotic stresses.

5.7 Proposed model of the role of TPKs and AKT1 in rice under various external conditions

Rice TPKa, TPKb and AKT1 are K^+ channels and are localised to the tonoplast and plasma membrane respectively. TPKa and TPKb are localised to the tonoplast of LV and SV respectively (Figure 5-1) and are involved in the release of K^+ from the vacuole to the cytoplasm. This role may be highly important during K^+ deficient conditions to maintain the K^+ concentration in the cytoplasmic. Drought may affect K^+ uptake from the soil and may cause K^+ deficiency in the cytoplasm. Efficient K^+ release from the vacuoles during osmotic stress may help the plants to tolerate K^+ deficiency by maintaining K^+ concentration in the cytoplasm. The comparatively higher K^+ concentration may affect the K^+ loading into the xylem through other systems (Figure 5-1) and therefore may improve K^+ distribuition to the shoot.

AKT1 is localised to the plasma membrane (Figure 5-1) and it has been reported that these channels are involved in K^+ uptake from the surrounding medium from micro to milli molar concentrations. The increased AKT1 activities may improve K^+ nutrition for the plants especially in abiotic stress conditions where K^+ uptake is negatively affected. AKT1 may also play a role in the Na^+ uptake during K^+ deficient conditions. TPKs and AKT1 are expressed in the guard cells (Figure 5-1) and there they may play a role in the K^+ transport, and thus there are chances they may affect the stomatal movement especially is the osmotic stress conditions.

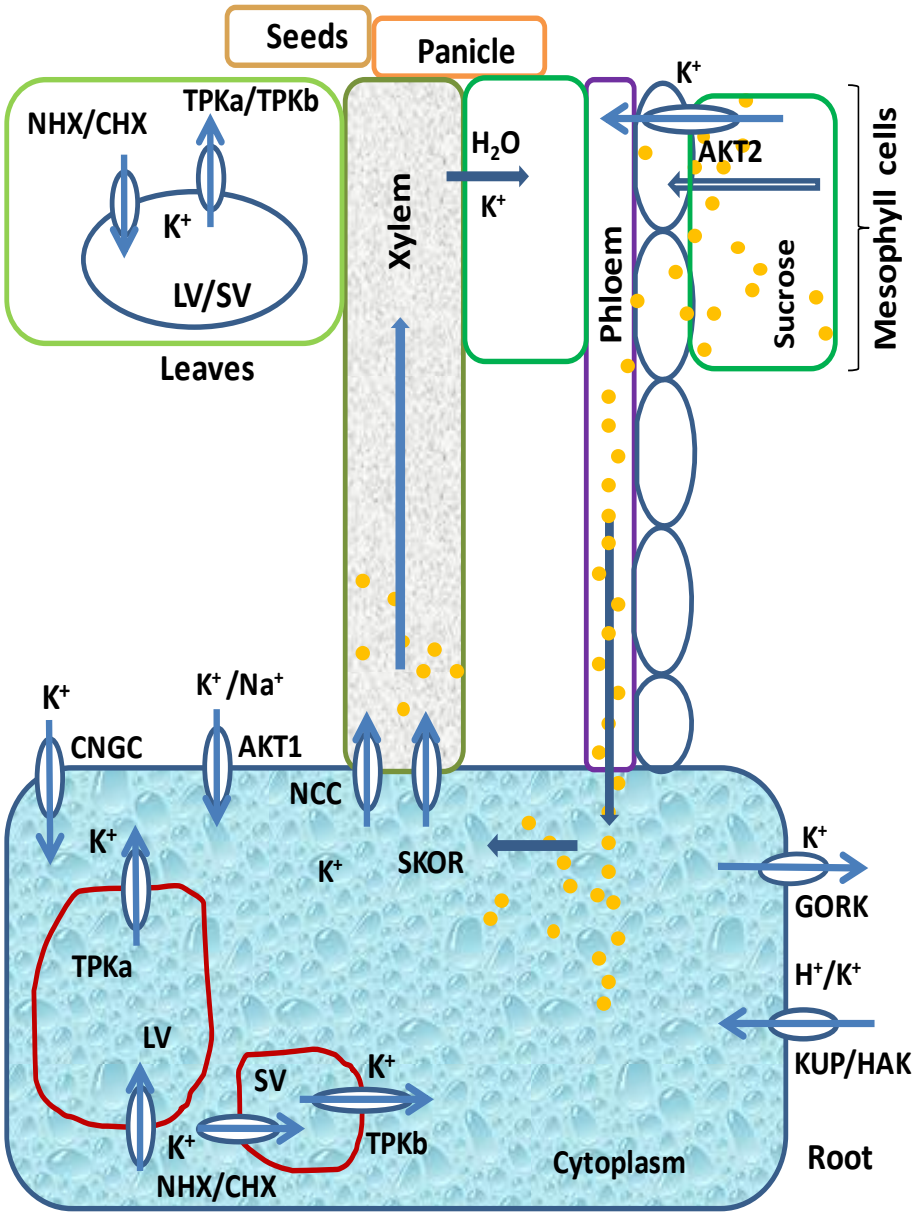

Figure 5-1-1: Overview of transport processes and proteins that are involved in K^+ uptake, efflux and distribution

At the external soil: root interface transport functions are shown for passive [AKT1 and CNGC (cyclic nucleotide gated channel)] and energised (KUP/HAK) K^+ uptake and channel mediated K^+ release (guard cell outward rectifying K^+ channel; GORK); Xylem loading mainly happens through K^+ selective (SKOR) and non-selective (NCC) cation channels though energised systems may also play a role; Phloem loading of K^+ for recycling and/or sucrose loading may involve the AKT2 channel; K^+ flux to the seed is phloem mediated but K^+ is unloaded into the seed apoplast (a) at the junction between maternal (m) and filial (f) tissues; vacuolar K^+ accumulation is primarily driven by H^+-coupled antiporters such as NHX while vacuolar K^+ release is either passive through TPK1 type channels or, in K^+ starvation conditions, active through H^+ coupled KUP/HAK transporters.

5.8 Future Work

We observed a range of phenotypes for the TPKa and TPKb overexpressing plants in comparison to the wild type plants in various growth conditions. It would be a good strategy to use the knockout lines of TPKa and TPKb in comparison to the wild type under various growth conditions. It will provide us with a clearer picture about the role these TPK proteins under various conditions.

We suggested that TPKs might be releasing more K^+ from the vacuole to the cytoplasm in the overexpressing lines and therefore may change the membrane potential of the cells. The hyperpolarized membrane potential may activate AKT1 channels and so AKT1 may efficiently absorb more K^+ from the surrounding medium. To confirm the effect of the TPK overexpression on the membrane potential, it would be useful to measure the membrane potential of the TPKs overexpressing plants in comparison to the wild type.

Overexpression of TPKs and AKT1 may affect the internal K^+ concentration and therefore may affect the expression of other transporters. Analysing the expression of transporter proteins like HAK, AKT1, SKOR, GORK and NCC may give us an idea how TPKs influence the uptake and distribution process of the plant.

Drought and salinity reduce crops yield severely by affecting seed setting. We observed a better growth for the overexpressing lines of TPKs and AKT1 under drought conditions. It will be useful to test these genotypes for seed setting and measure the effects of overexpression on the grain number and weight and the nutritional value of the grains.

Abbreviations

2,4-D: 2,4-Dichlorophenoxyacetic acid

ABA: Abscisic acid

AKT: *Arabidopsis* K^+ transporter

ALMT: Aluminium-activated malate transporter

At: *Arabidopsis thaliana*

Bp: Base pair

CBL: Calcineurin B-like protein

cDNA: Complementary DNA

CIPK: CBL-interacting protein kinase

CNGC: Cyclic nucleotide gated channels

Cryo-SEM: Cryo-scanning electron microscopy

DNA: Deoxyribonucleic acid

dNTP: Deoxyribonucleotide triphosphates

E.coli: *Escherichia coil*

EDTA: Ethylene diamine tetra acetic acid

EDX: Energy Dispersive X-Ray Analysis

GLR: Glutamate like receptors

GORK: Guard cells outward rectifying K channels

HKTs: High-affinity K^+ transporters

KIRC: K^+ inward rectifying channels

KUP/HAK/KT: K^+ uptake permease/high-affinity K^+/K^+ transporters

Lp_r: Hydraulic permeability

LV: Lytic vacuoles

Mha Million hectares

NHX: Na^+/H^+ exchanger or Na^+/H^+ antiporter

NSCC: Non -selective cation channels

P: Probability level

PCR: Polymerase chain reaction

PEG:	Polyethylene glycol
PP2C:	Protein phosphatase 2C
PSV:	Protein storage vacuoles
qRT-PCR:	Quantitative reverse transcriptase PCR
RGR:	Relative growth rate
RNA:	Ribonucleic acid
ROS:	Reactive oxygen species
SKOR:	*S*telar K^+ *o*utward *r*ectifier channels
SOD:	Superoxide dismutase
SV:	Storage vacuoles
T-DNA:	Transferred DNA
TPC:	Two-pore K^+ channels
TPK:	Tandem pore K^+ channels
WT:	Wild type

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