The role of HAK/KUP/KT family of transporters and potassium channels in abiotic stress responses in Arabidopsis and rice

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Abstract

Abiotic stress is a worldwide problem that affects the productivity of crops. Salinity stress for example, affects about 950 million hectares of arable lands while about 64% of the global land is affected by water deficit (drought). Drought and salinity are the most deleterious stresses, resulting in nutritional imbalance. Therefore, the main mechanism of improving a plant tolerance to stresses is by improving nutritional acquisition such as K^+ uptake.

The Two Pore K^+ vacuolar Channel (TPK1) was reported to function in K^+ release from guard cell vacuoles, therefore stomatal closure. However, *attpk1* showed weak phenotype, suggesting a good level of redundancy by other transporters predicted to localize in the guard cell tonoplast. The K^+ high affinity uptake permeases HAK/KUP/KT transporters AtKUP12 and AtHAK8 are among these candidates. We test this hypothesis in the model plant *Arabidopsis thaliana*. Loss of function of AtKUP12 mutants showed severe inhibition of germination, seedling establishment and growth of plants during K^+ deficiency resulted from high NH_4^+/K^+ ratio. Therefore, we suggest a potential role for this transporter in K^+ flux during osmotic, salinity and K^+ starvation stresses. We also suggest a possible epistasis relationship between AtKUP12 and AtTPK1. Possibly, they both function in K^+ efflux from vacuoles to cytoplasm during stress. Our results also suggest no functional relationship between AtTPK1 and AtHAK8. AtKUP12 was able to rescue the growth of K^+ deficient yeast strain during K^+ starvation.

In rice, we study the role of the Stelar K^+ Outwardly Rectifying channel (SKOR) in K^+ transport from roots to shoots under different stresses. We aim at improving K^+ transport to shoots therefore the overall plant response to abiotic stress, using different overexpressor lines. Overexpressing the *TPKb* (an isoform of the TPK channel localized in the storage vacuoles) resulted in higher K^+ levels in the transgenic plants during K^+ starvation. We test the possibility of the involvement of other transporters in this phenomenon. Expression levels of the *HAK1* and *KAT1* as well as the *GORK* transporter and channel genes were measured in *TPKb* overexpressor lines. *OsHAK1* showed slight increase in expression while other transporters did not show significant differences.

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Author's declaration

I declare that this thesis is a presentation of original work and I am the sole author. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as References.

I did the RT-PCR analysis of the transporters genes (HAK1, KAT1 and GORK) under the overexpression of the *TPKb* gene in the following paper:

Ahmad, I. et al., 2015. Overexpression of the potassium channel TPKb in small vacuoles confers osmotic and drought tolerance to rice. *The New Phytologist*, 209(3), pp.1040–1048.

Chapter 1. General introduction

1.1 Abiotic stress as a worldwide problem affecting the productivity of plants

Plants are sessile organisms, which forces them to cope with various types of environmental changes they face. These changes can be extreme and negatively affect plants. In this case, it creates a stress on plants such as salinity, water imbalance (flooding-drought), high temperature or freezing, nutrient deficiency or excess and heavy metals toxicity (Wallace et al. 2003; Pereira 2016; Tuteja and Gill 2016). Unfortunately, these stresses reduce the productivity of crops that are vital for human welfare. The limitation of environmental resources is occurring at the same time as an increase in the human world population which is predicted to hit 9.4 billion by 2050 compared to 7.4 billion for now (Census Bureau, world population (https://www.census.gov/popclock/), (Wallace et al. 2003). Meanwhile, FAO reports indicated that only about 3.5% of the land area is not affected by changes in environmental conditions in 2007 and the percentage has increased since (http://www.fao.org/docrep/010/a1075e/a1075e00.htm).

Among all stresses, salinity and drought show the most drastic effects on plants because they affect homeostasis of ions and water status and lead to osmotic stress. Salinity, for example, results in 27.3$ billion loss per year and affects about 950 million hectares of arable lands (Ruan et al. 2010; Qadir et al. 2014). While about 64% of the global land is affected by water deficit (drought) (Cramer et al. 2011). It is also expected that this percentage will increase based on a change towards drier climate (Zhang et al. 2014). The previous facts created a bigger challenge for researchers to improve the tolerance of important crops (rice, maize, wheat, barely) against such harsh conditions.

We cannot deny the impact of the green revolution in improving crop productivity in the last 50 years, where combining investment, infrastructure and developing markets resulted in such increase in crop yield. Despite the fact that the world population has nearly doubled and the agricultural area only increased by 13%. The first green revolution between 1966-1985 and the next revolution in the last two decades made

use of the current scientific knowledge in crop genetics and resulted in such improvement (Pingali 2012). Yield productivity increased in developing countries by 208% for wheat, 109% for rice and 157% for maize between 1960 and 2000 (FAO reports (ftp://ftp.fao.org/agl/agll/docs/salinity_brochure_eng.pdf). However, the complex nature of abiotic stress responses has decreased the efficiency of these improvements. For decades, breeding programmes have been helping in generating strong plants against abiotic stresses but it is slow to cope with the increase in the world population. The molecular techniques in the last decade provided faster and more powerful tools in this aspect by enabling us to understand how stress is being received and transmitted in plants (Ahmad and Prasad 2011).

1.2 K^+ as a major ion in plant physiology

Among all nutrients essential for plants, K^+ remains the most important ion that its deficiency heavily affects the physiology of plants. K^+ is the eighth most abundant element in the earth crust, representing about 2.1% of it (Hans Wedepohl 1995; Ashley et al. 2005). Plants absorb K^+ in an ionic form (H J Evans and Sorger 1966). It is found in soil in four forms, soil solution, exchangeable K^+, fixed K^+ and lattice K^+. The first form is what a plant can take up, and it is affected by the existence of other cations such as NH_4^+, Na^+. Mainly, the concentration of K^+ in soil ranges from 0.1-1 mM (Maathuis 2009).

K^+ is essential for photosynthesis, stomatal movement, phloem transport, photosynthesis, osmoregulation, countercharge of toxic ions and many other vital processes. It is important for many biological processes in the cytoplasm such as enzyme activation. For example, the pyrophosphatase (H^+-PPase) isoforms located in the tonoplast are highly dependent for K^+ and responsible for proton accumulation into vacuoles (Davies et al. 1992; Maathuis 2009). Other enzymes activated by K^+ are the enzymes involved in Carbon metabolism such as pyruvate kinase, phosphofructokinase and ADP-glucose starch synthase (Marschner 1995). In protein synthesis, K^+ is essential and required in high concentrations. It is involved in the synthesis of ribosomes and aminoacyl-tRNA (Blaha et al. 2000).

In osmoregulation, K^+ plays an important role as an osmoticum leading to water uptake into cells and accordingly expansion of cells. For example, the germination of

the pollen grain. The influx of K^+ into non-germinated pollens creates the osmotic pressure that induces the germination of the pollen. SPIK, an inward shaker-type potassium channel, is involved in K^+ influx into pollen grains and in controlling the growth of pollen tube (Mouline et al. 2002). K^+ is also important for anion countercharge (Hastings and Gutknecht 1978; D T Clarkson and Hanson 1980; Leigh and Jones 1984; Maathuis 2009; Szczerba et al. 2009), protecting cells from toxicity and ion imbalance.

A plant needs to maintain a stable level of K^+ in the cytoplasm. This level ranges from 20-200 mM, while in the cell vacuole this level is more subject to changes depending on the K^+ availability and its level in the cytoplasm (Leigh and Jones 1984). K^+ can reach up to 500 mM in vacuoles to create turgor pressure such as during stomatal opening (Ahmad and Maathuis 2014). Plants can accumulate K^+ 1000 fold against its concentration gradient (Grabov 2007).

1.3 Mechanisms of K^+ uptake and distribution in plants

K^+ transport in fungi, bacteria and higher plants is carried out by two major transporter systems; one is operating under micromolar, and another one under millimolar K^+ concentrations (Epstein et al. 1963; Quintero and Blatt 1997). The low affinity uptake of K^+ occurs when K^+ concentrations are 1 mM and above. It is suggested that K^+ channels carry out the low affinity uptake (Grabov 2007). But when plants are exposed to low K^+ levels (10-1000 µM) (Szczerba et al. 2009), high-affinity K^+ uptake occurs (Ashley et al. 2005). High-affinity K^+ uptake -or accumulation- happens against K^+ gradient and is coupled with the transport of H^+ through the H^+/K^+ symporters. These transporters show a conformational change in the protein structure due to this energization (Maathuis and Amtmann 1999) leading to an increase in their ability to take up K^+ at such very low levels. It is suggested that this active transport occurs by coupling the passive influx of H^+ with the active influx of K^+ and is maintained by the H^+-ATPases (proton pumps). However, this mechanism has not been proved yet in plants although it is in bacteria and fungi (Bakker and Harold 1980; Rodriguez-Navarro et al. 1986). The high affinity K^+ uptake is sensitive to NH_4^+ and Na^+ as they compete with K^+ during entry to cell (Spalding et al. 1999). K^+ is also transported to different plant tissues symplastically through the plasmodesmata. Plasmadesmata

connect neighbouring cells and permit transport of nutrients and water, such as K^+ transport from the epidermis to the stele and to seeds. The endodermis plays an important role in this transport as it blocks apoplastic and coupled transcellular transport but permits symplastic flow to be directed to stelar cells (Barberon and Geldner 2014).

1.3.1 Shaker-type channels

The shaker-type family of channels is considered the main family responsible for K^+ transport in plant membranes, it is similar to the shaker–type K^+ channels in animals. It consists of a group of K^+ channels that has a structure of four subunits organized around the core of the channel. Each subunit consists of six transmembrane segments (TMS) or domain (TMD). The fourth segment functions as a voltage sensor (S4). That is, the shaker-type family of channels are voltage-dependent and is affected by the membrane voltage. This is called the P (pore) domain, which is highly conserved in these channels. It consists of the gly-tyr-gly-asp (GYGD) motif which is the main character of K^+ channels (Heginbotham et al. 1992; Lebaudy et al. 2007). The cytoplasmic C-terminal of these channels contains regulatory domains and a cyclic nucleotide-binding site. The interaction with regulatory proteins is suggested to happen through the ankyrin domain (Véry and Sentenac 2003). The shaker family members are voltage-dependent channels and their activity is affected by membrane voltage. There are outward rectifying channels and inwardly rectifying channels. The inward rectifiers are activated by a negative membrane potential (hyperpolarization) and function in K^+ uptake (Lebaudy et al. 2007), while the outwardly rectifying channels are active at positive membrane potential (depolarization) and therefore function in K^+ efflux. KAT1 and AKT1 were the first two shaker family channels to be characterized in plants (Anderson et al. 1992; Sentenac et al. 1992; Lebaudy et al. 2007). They are both inwardly rectifying channels that function in K^+ uptake. They can participate in the passive transport of K^+ from epidermal root cells and then from xylem to parenchymal cells when the plasma membrane is hyperpolarized (Ahmad and Maathuis 2014; Anschütz et al. 2014) . KAT1 was found to confer salt stress in yeast strains as well as rice plants (Obata et al. 2007). KAT1 and KAT2 are also involved in K^+ uptake in guard cells during the stomatal opening.

The AKT1 K^+ channel is essential in low affinity and high affinity uptake under certain conditions. It is predominantly expressed in roots (Hirsch et al. 1998; Spalding et al. 1999; Dennison et al. 2001; Gierth et al. 2005). AKT1 is considered the main uptake mechanism when K^+ is provided in millimolar concentrations. In Arabidopsis, loss of function of AKT1 resulted in negative effects on plant growth in the presence of NH_4^+ (Spalding et al. 1999; Dennison et al. 2001; Pyo et al. 2010). The AtKC1 subunit is not functional by itself, however, it functions in the regulation of the AKT1 channel by forming a complex.

In rice, OsAKT1 is a major K^+ channel that is important in K^+ uptake and stress responses (Obata et al. 2007; Ahmad et al. 2015; Ahmad et al. 2016). *OsAKT1* expressing yeast was tolerant to NaCl salinity by altering ion homeostasis, OsAKT1 was able to increase K^+ uptake in salt sensitive yeast mutants resulting in relieving the harmful effects of NaCl (Obata et al. 2007). *OsAKT1* also confers drought tolerance in rice plants when overexpressed (Ahmad et al. 2016).

AKT2/3 is involved in K^+ transport to phloem (Ketchum and Slayman 1996; Marten et al. 1999; Lacombe et al. 2000), it is considered an inward rectifier as well. And as the previous channels, it is activated by the hyperpolarization of the plasma membrane, however, it has a weak rectification activity. It can mediate both efflux and influx of K^+ and is expressed in the phloem (Marten et al. 1999; Lacombe et al. 2000; Xicluna et al. 2007).

The GORK (Guard Cell Outwardly rectifying K^+) channel, which is mainly expressed in guard cells and roots (Hosy et al. 2003; Fan et al. 2004; Ward et al. 2009), is involved in the efflux of the K^+ during stomatal closure (MacRobbie 1998; Pandey et al. 2007; Osakabe et al. 2013). This channel is activated by the depolarization of plasma membrane. Loss of function mutants of GORK caused disruption in the stomatal closure and an absent outward K^+ current from the guard cells of Arabidopsis (Becker et al. 2003). It was also reported that it functions in K^+ efflux during stomatal closure with other KUP (K^+ uptake permeases) K^+ transporters (KUP2, KUP6 and KUP8) as well as in negative control cell expansion (Osakabe et al. 2013). Moreover, the GORK channel is expressed in the root epidermis and root hairs (Ivashikina et al. 2001) and participates in K^+ efflux from the root (Riedelsberger et al. 2010; Sharma

et al. 2013). The K^+ efflux occurs when plants face different stresses like a pathogen or salinity stress followed by a programmed cell death (PCD) (Demidchik 2014).

The SKOR (Stelar Rectifying Outwardly K^+ Channel) is mainly expressed in the root stelar cells (Gaymard et al. 1998; De Boer and Volkov 2003) and is involved in uploading K^+ into xylem sap. Therefore, it functions in the translocation of K^+ in Arabidopsis (Gaymard et al. 1998; Demidchik 2014). SKOR was the first channel in plants to be described as activated by depolarization. A loss of function mutant of SKOR in Arabidopsis displayed lower shoot K^+ content indicating, the role of this channel in the transportation of the K^+ from roots to shoots (Gaymard et al. 1998; Ward et al. 2009). It was also reported that SKOR is highly selective for K^+ over Na^+ and is inhibited by the stress hormone ABA (Maathuis and Amtmann 1999). The overexpression of the SKOR gene was suggested by (Munns 2005) to improve stress tolerance in plants. This and the fact that keeping a high K^+/Na^+ ratio in shoots is an important mechanism of tolerating stress in plants, recommended the study of this channel by overexpression (Maathuis and Amtmann 1999; Shabala and Cuin 2008). Moreover, it is important to investigate the role of this channel in K^+ homeostasis and the response of plants to abiotic stress in an important crop such as rice.

1.3.2 The two-pore K^+ channels (TPK) family

In *Arabidopsis thaliana*, the TPK family of channels (formerly known K^+ outwardly, KCO), are highly selective for K^+ and voltage-independent. They feature four transmembrane domains/two-pore structure (Figure 1-1). They contain the signature sequence of (GYGD) motif for K^+ selectivity which is located in the P domain of the channel and is considered a hallmark for K^+ channels (Heginbotham et al. 1992; Lebaudy et al. 2007). The TPK family members contain one or two EF-hands, each one consists of alpha helices with a short loop region for Ca^{2+} binding in the C-terminus (Gobert et al. 2007). The family members are activated by Ca^{2+} and by the 14-3-3 proteins. These proteins interact with the TPK channels and phosphorylate them under different conditions (Latz et al. 2013) (Figure 1-1). In Arabidopsis, there are six members of the TPK family. TPK1, TPK2, TPK3, TPK5 and TPK6 are localized in the cell vacuole, meanwhile TPK4 is localized in the plasma membrane and neither contains EF-hands nor is activated by cytoplasmic Ca^{2+} (Schönknecht et al. 2002; Dunkel et al. 2008; Teardo 2014). It is localized in the plasma membrane of

pollen grains and is suggested to be involved in pollen tube expansion. The tonoplast localization of many TPK channels might suggest a redundancy in function among these channels. Latz et al., (2007) suggested a possible overlap in the expression of family members. AtTPK1 overlapped with AtTPK3 in vascular tissues. This indicates that heterodimerization could be happening during different stages of expression in plants in this family.

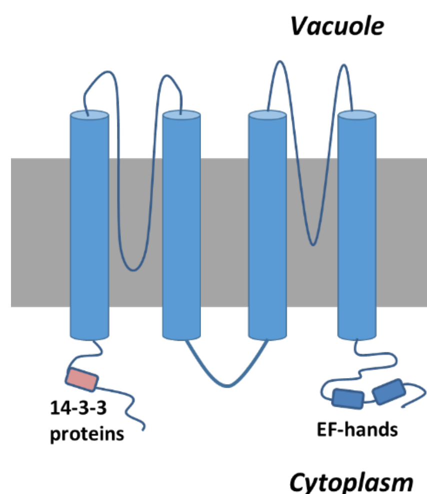


Figure 1-1: Topological structure of the TPK1 sub-unit, a member of the TPK K^+ channels family.

The sub-unit contains four transmembrane domains. The C-terminus contains the EF-hands that bind to Ca^{2+} and regulate the channel activity, while the N-terminus contains the 14-3-3 proteins specific binding sites involved in channel regulation.

1.3.2.1 The AtTPK1 K^+ channel and its role in the abiotic stress responses

Among the TPK family members in Arabidopsis, the AtTPK1 channel shows the highest expression in leaves, seeds, flowers and roots (Voelker et al. 2010). Indicating the importance of this channel compared to other members (Czempinski et al. 1997). TPK1 lacks the S4 voltage sensing domain found in the shaker-like family of K^+ channels, therefore it functions as a voltage-independent channel and is subject to regulation by the 14-3-3 proteins and Ca^{2+} (Latz et al. 2007).

The TPK1 K^+ channel was covered thoroughly by many studies, reported to be predominantly localized in the guard cell tonoplast (Czempinski et al. 2002). Loss of function mutants of this channel displayed higher stomatal conductance under the ABA treatment, possibly suggesting a role for the AtTPK1 in K^+ efflux from the guard cell vacuole during the ABA-induced closure. While the overexpression mutants

exposed the opposite phenotype (Gobert et al. 2007). However, Gobert et al., (2007) indicated there is a potential redundancy that led the guard cells in the *tpk1* mutants to close after ABA treatment. This redundancy is most likely due to the occurrence of other K^+ transporters in the guard cell tonoplast that might participate in the efflux of K^+ during the closure. Therefore, other candidate K^+ transporters should be investigated alongside TPK1 to understand if they participate in this process. Seeds of *tpk1* loss of function mutants also showed slower germination rates under standard K^+ concentrations and higher sensitivity to ABA. Germination of loss of function mutants was inhibited under 80 and 150 mM of NaCl alone or combined with 50 μM of K^+ while the overexpressor lines showed higher germination compared to wildtype (Gobert et al. 2007; Latz et al. 2013). This suggests a role for TPK1 in K^+ movement from the protein storage vacuoles (PSV) of seeds during the early stages of germination. Loss of function *tpk1* mutants in Arabidopsis showed lower fresh weights compared to wildtype when grown over low K^+ (10 μM) or high K^+ (80 mM) media. Overexpressor lines showed the opposite phenotype. However, this was not accompanied by any change in K^+ levels in the tested plants, indicating that AtTPK1 participates in the intercellular and intracellular K^+ homeostasis but not the K^+ uptake (Gobert *et al.*, 2007). These results emphasize that AtTPK1 plays an important role in the response of plants to abiotic stresses by controlling K^+ movement. AtTPK1 also showed a mechanosensitivity, indicating it participates in the cell osmoregulation (Maathuis 2011).

AtTPK1 was found to interact with the general regulatory factor 6 (GRF6), which belongs to the 14-3-3 proteins. It is suggested that Ca^{2+} binds to the EF hands resulting in a conformational change in the channel allowing it to open or close (Latz et al. 2007). From this, we conclude that the 14-3-3 proteins and Ca^{2+} modulate the TPK1 channel. The interaction of the TPK1 and the GRF6 happens after the phosphorylation of the 14-3-3 binding motif. This occurs by the calcium-dependent protein kinases (CDPK). The CPK3 phosphorylates the 14-3-3 binding motif in TPK1 upon salt stress. Indicating a direct role for TPK1 in salt stress response. Indeed, a loss of function mutants of TPK1 and CPK3 showed higher sensitivity to salinity (Latz et al. 2013).

Rice contains two isoforms of the TPK channel, TPKa and TPKb. Both are located in different types of vacuoles. The TPKa channel is located in the lytic vacuole (LV)

which is the largest vacuole, while the TPKb is located in protein storage vacuoles (PSV). It is suggested to participate in seed germination and mobilization of reserves in rice seeds, functions in the movement of K^+ from vacuole to cytoplasm and plays an important role under different stress conditions in rice (Isayenkov et al. 2011; Ahmad et al. 2015).

1.3.3 The HAK/KUP/KT family of transporters

The HAK/KUP/KT family is a group of K^+ transporters functioning in K^+ homeostasis, mineral nutrition and the development of plants (Osakabe et al. 2013). This family was first identified in the *Escherichia coli* bacteria as K^+ uptake permeases (KUPs) (Schleyer and Bakker 1993), then in the *Schwanniomyces occidentails* fungus as high affinity K^+ transporters (HAKs) (Bañuelos et al. 1995), but was not identified in animals (Very et al. 2014). Members of this family are highly selective for K^+ (Whiteman et al. 2008). This family belongs to class 2.A porters, which includes uniporters, symporters and antiporters (Busch and Saier 2002). Antiporters and symporters are activated by a proton or sodium motive force, and so they can transport K^+ against its concentration gradient by coupling it with H^+ (Rodriguez-Navarro et al. 1986). This feature enables class 2 transporters to function in K^+ uptake under high-affinity conditions (Grabov, 2007) when there is K^+ deficiency. There are 913 sequences of this family recognized in 46 genomes (Nieves-Cordones et al. 2016). The number of members of this family varies among species, for example, Arabidopsis has 13 members while rice has 27 and other species like soybean contains 32 members (Nieves-Cordones et al. 2016).

The family is grouped into four clusters (Rubio et al. 2000; Very et al. 2014) by phylogeny (Figure 1-2), clusters I and II transporters are mostly described to function in the high affinity K^+ uptake as in HAK5 (Bañuelos et al. 2002). It is also important to indicate the surprising diversity of cluster II, which also involves participation in cell expansion with multiple phenotypes (Rigas et al. 2001; Elumalai et al. 2002; Osakabe et al. 2013). Cluster III is still in need for more investigation and includes AtKUP5, AtKUP7, AtKUP11 and AtKUP12 (Grabov, 2007). Cluster IV is a small group which contains four transporters but none of them is in Arabidopsis (Very et al. 2014).

Conversely, not all members participate in the high affinity uptake of K^+. Even transporters from the same clusters do not necessarily share the same function or even location in the cell (Nieves-Cordones et al. 2016). Some family members have been reported to be mediating a low affinity K^+ transport. For example, AtKT2 was reported to be mediating low affinity K^+ transport when expressed in K^+ deficient yeast strain (Quintero and Blatt 1997). while AtKUP1 and AtKUP2 were able to complement the growth of a K^+ deficient *E.coli* strain when the medium contained 2 mM of K^+ (Kim et al. 1998). At the same time, (Fu and Luan 1998) reported that AtKUP1 is a dual K^+ transporter that can function under both high and low K^+ uptake affinity in yeast.

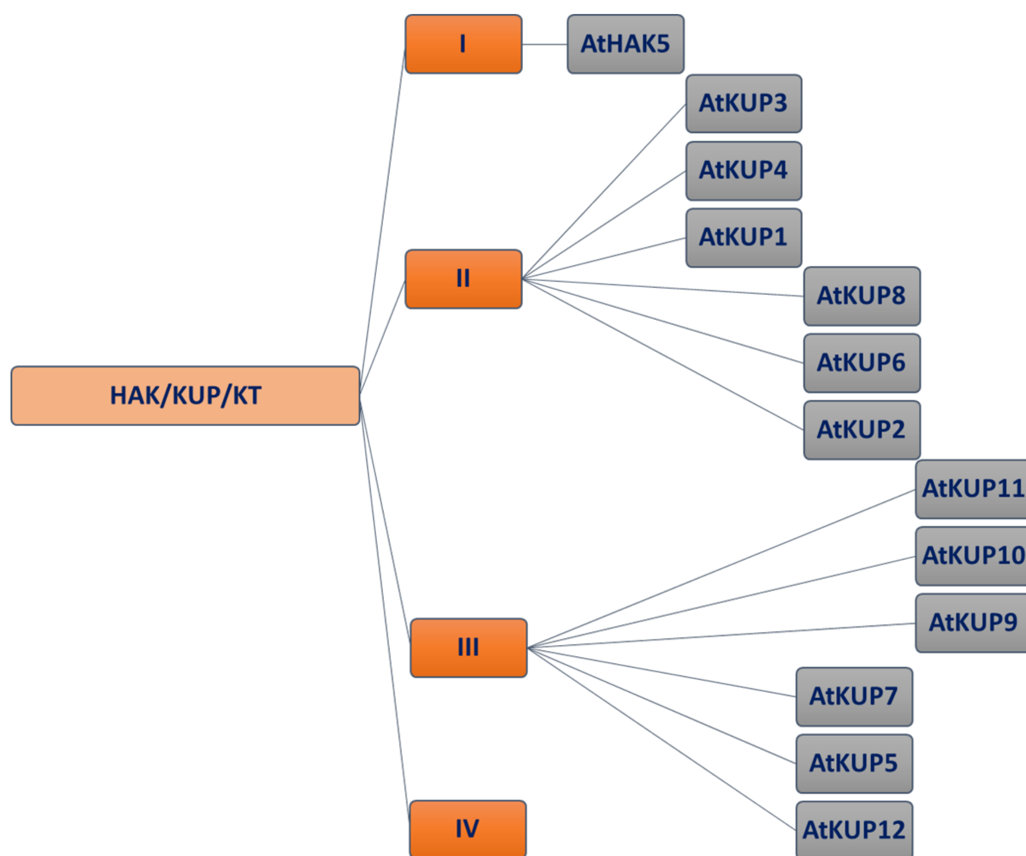


Figure 1-2: Clusters of the HAK/KUP/KT family of transporters in Arabidopsis.

The HAK/KUP/KT family of transporters is categorized into four clusters. In Arabidopsis, all KUP transporters belong to clusters I, II and III, while none of them belongs to cluster IV (Very et al. 2014).

Several members of the HAK/KUP/KT family of transporters have been investigated in previous work to understand the expression pattern of these K^+ transporters in different plant tissues. A well-known member of this family is AtHAK5 with an important role in K^+ uptake under low external concentrations (Gierth et al. 2005). A

study by Shin and Schachtman., (2004) indicated the up-regulation of AtHAK5 as a result of K^+ deprivation in Arabidopsis roots which also resulted in (ROS) metabolism and ethylene synthesis. HAK5 is a plasma membrane transporter that is strongly expressed in root tissues, and was up-regulated during K^+ starvation conditions in Arabidopsis, Tomatoes and Barley (Nieves-Cordones et al. 2008; Pyo et al. 2010; Ragel et al. 2015). Another member of this family is AtKUP7 which is also expressed in roots and has a role in both K^+ uptake and upload to xylem in plants (Ahmad and Maathuis 2014; Han et al. 2016). AtKUP7 was recently confirmed to be a plasma membrane transporter protein (Han et al. 2016). Although for this transporter, it is not yet confirmed that a H^+/K^+ coupling is happening, especially in uploading K^+ to xylem. In rice, OsHAK7 and OsHAK10 were found to function in the low affinity K^+ transport and are expressed in shoots and roots. OsHAK1 is considered the main K^+ transporter in rice during the high affinity K^+ uptake, OsHAK10 was also found to be expressed in the tonoplast of onion epidermal cells (Bañuelos et al. 2002). Disruption of *OsHAK1* resulted in a great loss in rice grain yield (Chen et al. 2015). This author also indicated the importance of OsHAK1 in K^+ uptake during salinity stress with high or low K^+ levels. In the next sections, we explain in detail the functional characteristics of this family and the important family members.

1.3.3.1 The phosphorylation of the HAK/KUP/KT family members

The expression of the HAK/KUP/KT family members is regulated on the transcriptional level as a result of the presence of low K^+ concentrations (Armengaud et al. 2004; Whiteman et al. 2008). For example, AtHAK5 and LeHAK5 (in tomato) were up-regulated as a result of exposing plants to different periods of K^+ starvation (Wang et al. 2002; Gierth et al. 2005). The expression of AtKUP3 was strongly induced by the K^+ starvation as well (Kim et al. 1998). The post-translational activation by phosphorylation is also reported for members of this family. For instance, AtHAK5 was found to be phosphorylated by the CBL-interacting protein kinase 23 (CIPK23) (Ragel et al. 2015; Scherzer et al. 2015). The C-terminal domain of the KUP6 transporter was phosphorylated by the SNF1-related protein kinase 2E (SRK2E) under drought stress conditions (Osakabe et al. 2013).

The HAK/KUP/KT family of transporters featurs 12 transmembrane domains (Figure 1-3) (Very et al. 2014). The family has been predicted for phosphorylation sites. There is a phosphosite in the N-terminus in the KUP7, KUP5 and KUP12 (Figure 1-3). A well-conserved serine residue which is predicted in all of the KUP family members was found in another phosphosite between the second and third predicted transmembrane domains (TMD), KUP12 also contained another phosphosite in the 188 position (not shown in the figure) which is not highly conserved (Whiteman et al. 2008).

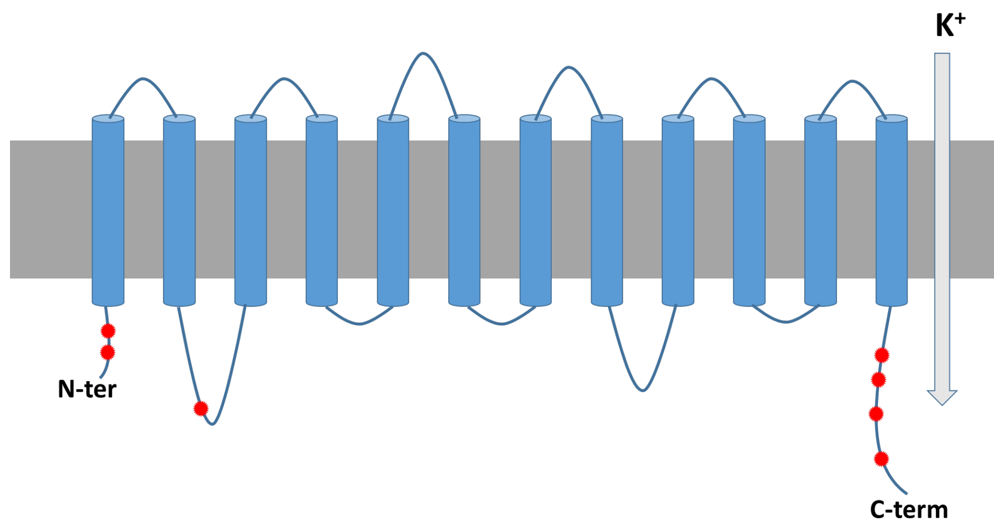


Figure 1-3: Suggested topology and function of the HAK/KUP/KT family transporters.

The diagram shows the 12 transmembrane domains, the longer C-terminus and phosphorylation sites (red dots) of the family and the direction of K^+ movement across the membrane.

1.3.3.2 Physiological roles of family members

The Members of the HAK/KUP/KT family have multiple functions, mainly in the high-affinity K^+ transport (Grabov 2007; Nieves-Cordones et al. 2016). However, transporters of this family have shown different affinity activities as mentioned previously. KUP1, for example, is a dual K^+ transporter. Expression of AtKUP1 in yeast cells increased K^+ uptake under both high and low-affinity K^+ conditions. This suggests a function for AtKUP1 in K^+ uptake in both mechanisms. When external K^+ levels are in the range of 100-200 μM, this transporter transforms from high affinity (44 μM K^+) to low affinity state (Fu and Luan 1998). The change in external K^+

concentrations or in the membrane potential is suggested to result in a conformational change in the protein, which might be responsible for switching the activity.

The AtHAK5 transporter is considered the main high-affinity K^+ transporter when external K^+ concentrations are lower than 10 µM. Under 10-200 µM, both AtHAK5 and AtAKT1 function in K^+ uptake. However, the HAK/KUP/KT family of transporters is inhibited by NH_4^+, while the AKT1 is active in the presence of NH_4^+. Therefore, it is suggested that AtAKT1 carries out this function during the presence of NH_4^+ (Alemán et al. 2011). AtHAK5 is also important for seed germination and establishment under K^+ starvation conditions (Pyo et al. 2010).

The family members are also thought to be functioning in cell development through K^+ homeostasis and osmoregulation of the cell (Nieves-Cordones et al. 2016). AtKUP2 (Elumalai et al. 2002), AtKUP6 (a plasma membrane transporter) and AtKUP8 are involved in the negative control of cell expansion in Arabidopsis plants by the efflux of K^+. The loss of function of these transporters resulted in bigger plant sizes due to larger cells and not an increased numbers of cells (Osakabe et al. 2013). Single mutants of these transporters did not show strong phenotypes, while the double *kup6*8 and triple *kup268* mutants showed it with the triple mutant plants showing the largest plant sizes. The KUP6 was suggested to function in the K^+ efflux across the guard cell plasma membrane during stomatal closure. Research by (Hosy et al. 2003) indicated the involvement of other K^+ transporters during the guard cell closure alongside the GORK channel. Indeed, combining loss of function mutants of *kup6*, *hak8* and *gork* (*kup68g*) resulted in higher stomatal conductance compared to wildtype (Osakabe et al. 2013). The involvement of these transporters which belong to the clade IIc in the classification presented by (Nieves-Cordones et al. 2016), shows the diversity of physiological roles for the HAK/KUP/KT family of transporters. This clade is suggested to be involved in the K^+ efflux and therefore negatively controlling cell expansion.

The *trh1* (tiny root hair) loss of function mutant of the AtKT3/AtKUP4 gene in Arabidopsis had a negative effect on K^+ uptake in roots and stopped the growth of root hairs once initiated. A yeast mutant that is lacking the high-affinity K^+ uptake was rescued by the TRH1 transporter (Rigas et al. 2001), suggesting a role for this family

member in K^+ uptake under starvation conditions in root hairs. TRH1 is also involved in auxin transport which is reflected in a root phenotype (Vicente-Agullo et al. 2004). Another family member AtKUP9, was suggested to participate in the transport of K^+ and Cs^+ (a K^+ channel blocker) by complementing the growth of a K^+ deficient *Escherichia coli* bacteria (Kobayashi et al. 2010). AtKUP5, AtKUP6, AtKUP7, AtHAK5, AtKUP10 and AtKUP11 were able to complement the growth of the K^+ deficient *E.coli* on K^+ limiting medium (Ahn et al. 2004).

Recently, high affinity K^+ transporters have been found to participate in the translocation of K^+. The shoot/root K^+ ratio in *atkup7* loss of function mutants showed significant reduction compared to wildtype under low-K^+ conditions, suggesting that the movement of K^+ from roots to shoots is affected by the disruption of AtKUP7. Xylem sap of mutants contained lower K^+ levels compared to wildtype. AtKUP7 is localized in plasma membrane and is speculated to be involved in K^+ uptake from roots and K^+ translocation from shoots to roots particularly under low K^+ conditions in Arabidopsis (Han et al. 2016). This suggests that SKOR is not the only channel functioning in K^+ upload from roots to shoots in plants.

1.3.4 The high affinity K^+ transporters (HKT)

Isolated from a K^+ starved wheat plant, HKT1 structure showed similarity to the TRK fungal K^+ transporters, and was able to rescue the K^+ deficient yeast growth (Schachtman and Schroeder 1994). The HKT transporters couple K^+ transport with Na^+ or H^+, however, they mostly function in Na^+ transport in plants. Arabidopsis has one HKT transporter that is AtHKT1 and it is localized in the plasma membrane. The disruption of this transporter resulted in salinity hypersensitive plants that showed higher accumulation of Na^+ in leaves and less accumulation of Na^+ in roots (Mäser et al. 2002; Horie et al. 2006). In rice, there are seven transporters of this family. OsHKT1;5 is the ortholog of AtHKT1;1, and it plays a paramount role in rice response to salinity by removing Na^+ from the sap of the xylem to parenchyma where it is less toxic to leaves (Ren et al. 2005).

1.3.5 Other channels/transporters

***Cyclic nucleotide gated channels (CNGC)*:** These channels are similar to the shaker-type K^+ channels except that they lack the K^+ selectivity domain in the P-pore (Finn

et al. 1996). They contain a cyclic nucleotide-binding domain in the C-terminal region and a calmodulin-binding site (Mäser et al. 2001; Kohler 2007). They can be permeable for both K^+ and Na^+. Therefore, it is also suggested to function in the entry of Na^+ into roots. The AtCNGC1 and AtCNGC4 can be permeable for K^+ and Na^+ in equal levels (Hua et al. 2003). While AtCNGC2 have more selectivity for K^+ over Na^+ and therefore is suggested to be directly participating in K^+ uptake.

Glutamate receptors (GLRs)**:** this family is present in plants as well as animals, there are 20 members of GLRs in Arabidopsis. They can participate in K^+, Na^+ and Ca^{2+} transport, and they are expressed in roots (Lam et al. 1998; Lacombe et al. 2001).

The diversity of K^+ channels/transporters indicates the importance of such element in plant physiology during normal and abiotic stress conditions. We here explain its role in abiotic stresses and how a plant responds during low K^+ levels.

1.4 The role of K^+ in plant responses to different stresses

K^+ is a major ion that plants use to react to different types of biotic and abiotic stresses. K^+ deficiency inhibits the ABA function and accordingly the stomatal closure during stress (Tanaka et al. 1999). K^+ helps maintain membrane stability, protect photosynthetic pigments from damage, maintain cell turgidity and water content, induces water retention, improves growth of plants and overall keep plants strong against harsh environmental conditions (Wang et al. 2013).

Our approach to understanding the complicated nature of abiotic stress responses is by studying the multigenetic responses and not only one gene under these stresses. This is achieved by combining different loss of function mutants of candidate genes suggested to improve plant responses to abiotic stresses.

1.5 How K^+ deficiency affect plants

1.5.1 Morphological effects

K^+ is an essential ion that is found in the body of plants in high concentrations. Therefore, when there is shortage in such element plants suffer different morphological changes. The first organ affected by K^+ deficiency and the first contact point with soil K^+ is root. The flexibility that roots exhibit is important to ensure the

adequate uptake of nutrients during different concentrations and forms. The roots of a K^+ starved plant is mostly longer because a plant will induce the root growth to obtain K^+ from a wider soil area to be able to maintain its levels of K^+ (Tian et al. 2008). Many phytohormones are involved in shaping the root architecture during K^+ starvation. Ethylene, was found to be generated 6 hours after K^+ starvation (Shin and Schachtman 2004).

1.5.2 Physiological effects

The most prominent physiological effect of K^+ deficiency is probably the change in the plasma membrane potential. It is reported that the membranes of root cells display hyperpolarization of the plasma membrane after minutes of K^+ deficiency (Spalding et al. 1999; Wang and Wu 2010). This change in membrane potential activates the K^+ uptake channels and transporters (Sentenac et al. 1992), such as AKT1 and HAK5. It also deactivate the K^+ outwardly channels such as GORK and SKOR (Gaymard et al. 1998; Hosy et al. 2003; Demidchik 2014). K^+ deficiency is also known to result in the acidification of the extracellular space. The activity of the H^+-ATPases is essential when K^+ is limited as it helps to pump K^+ into cell and as a result, H^+ out resulting in this change in the H^+ levels. This change in the electrochemical gradient of protons can result in the energization of K^+ uptake by the HAK/KUP/KT K^+ transporters (Maathuis and Sanders 1993; Grabov 2007). Many other physiological responses occur due to K^+ deficiency stress that is explained in the next section in the sensing and signalling.

Ca^{2+}, Mg^{2+} and Na^+ participate in reducing the effect of K^+ deficiency. It was reported that the concentration of these ions was increased when K^+ is replete and increased when it is efficient (Diem and Godbold 1993; Pujos and Morard 1997; Jordan-Meille and Pellerin 2008; Gerardeaux et al. 2009; Hafsi et al. 2011; Hernandez et al. 2012). Na^+ can substitute K^+ in some physiological functions when K^+ is deficient. They both have close chemical characteristics (Benito et al. 2014). In the next sections, we explain in detail the physiological aspects of K^+ deficiency.

1.6 K^+ deficiency sensing and signaling

1.6.1 How a plant senses K^+ deficiency

The signaling and sensing of K^+ deficiency or generally the change in external K^+ concentrations are still largely unidentified despite the extensive studies (Shin and Schachtman 2004; Wang and Wu 2010). While a plant cell can contain up to 400 mM of K^+ (Leigh and Jones 1984; Walker et al. 1996), K^+ is found in soil at very low concentrations that range from 10 µM to 1 M (Maathuis 2009). This indicates that the K^+ uptake in plants is necessarily happening against its concentration gradient. Plants have developed many mechanisms to be able to maintain the K^+ concentration in cells in a way that ensures the fulfilment of the physiological functions. The uptake of K^+ from the soil, therefore, occurs through different plasma membrane channels and transporters, which might sense the change in the external K^+ concentrations and then adjust the appropriate mechanism of acquisition. (See the previous elaboration of the main two K^+ uptake mechanisms).

Roots are the main contact point that senses the change in the external K^+ concentrations. A hyperpolarization in the plasma membrane of root cells occurs when the external K^+ concentration is low (Schroeder et al. 1987; Schroeder et al. 1994; Ketchum and Slayman 1996; Maathuis et al. 1997), this in turn activates the inward K^+ channels such as AKT1 (Hirsch et al. 1998; Spalding et al. 1999; Gierth et al. 2005). The hyperpolarization of plasma membrane also increases the acidification of the extracellular region, which is achieved by the plasma membrane H^+-ATPases. This can activate the K^+/H^+ symporters -such as HAK5- responsible for the uptake of K^+ under even lower concentrations, which in total enables a better K^+ acquisition for plants during deficiency conditions. From this, we conclude that the H^+-ATPases are important in sensing K^+ deficiency. We can also expect that the plasma membrane of root epidermal cells and root hairs might contain K^+ deficiency sensors, however, none of these have been identified molecularly (Wang and Wu 2010; Wang and Wu 2013). Hypothetically, AKT1 can be this sensor, since it shifts its activity from low to high affinity K^+ transporter. Implicating a mechanism of sensing the external K^+ concentrations and adjust its activity accordingly (Kellermeier et al. 2014). This

switch between the two affinities happens by the phosphorylation of the AKT1 channel which occurs by the CIPK23 protein kinase (Li et al. 2014).

1.6.2 Reactive oxygen species (ROS)

ROS are byproducts of plant metabolism and are generated as long as there is oxygen. These can be hydrogen peroxide (H_2O_2), superoxide radical (O^{2-}), hydroxyl radical ($OH^{\cdot}$) and singlet oxygen ($^1O_2$). If produced in higher quantities they can attack cell components such as DNA, RNA, proteins and membranes and cause oxidative damage which is called oxidative stress (Mittler 2002).

It is well established now that the ROS can play a role in signaling as messenger molecules (Hafsi et al. 2014). In Arabidopsis, an increase in the accumulation of hydrogen peroxide (H_2O_2) was observed as a result of the short term K^+ starvation (Figure 1-4). This increase accordingly up-regulates the high affinity K^+ transporter AtHAK5 in roots (Shin and Schachtman 2004). The accumulation of H_2O_2 was realized in a root area that is also responsive to K^+ deficiency. Similar results were obtained by (Hernandez et al. 2012) in tomato. This indicates a direct connection between the production of ROS and K^+ deficiency. The suppression of RHD2, an NADPH oxidase involved in the regeneration of ROS (Apel and Hirt 2004) resulted in hindering the up-regulation of many genes that respond to K^+ deficiency, while it was up-regulated as a result of K^+ starvation. Moreover, the addition of H_2O_2 induced those genes and also AtHAK5 in roots of Arabidopsis (Shin and Schachtman 2004).

Another study showed that the Rare Cold Inducible 3 (RCI3), a type III peroxidase family member, is involved in the production of ROS and the up-regulation of HAK5 during K^+ deficiency (Kim et al. 2010). From this, we can relate the production of the ROS in roots during K^+ deficiency and the up-regulation of other genes such as HAK5. Accordingly, we conclude that the ROS modulates the expression of this K^+ transporter to enable much K^+ uptake to relieve the deficiency effects. ANN1 (a Ca^{2+} permeable transporter in Arabidopsis root epidermis and root hairs) is involved in Ca^{2+} influx. It is activated as a result of ROS production. Loss of function mutants of this transporter showed impaired Ca^{2+} elevation as a result of ROS activation (Laohavisit et al. 2012).

1.6.3 Calcium signaling

Alongside its nutritional importance and regulation of many cell transporters, calcium is the most important signaling component in plant cells. It is involved in many responses to environmental stresses (White and Broadley 2003). As previously discussed, K^+ starvation induces the production of H_2O_2 (Shin and Schachtman 2004). This induction results in the activation of Ca^{2+}-permeable channels such as the nonselective cation channels (NSCCs), and the tonoplast two-pore calcium channel (TPC1) (Peiter et al. 2005), increasing its concentration in the cytoplasm (Demidchik et al. 2003; Demidchik and Maathuis 2007). It is still unclear though how those signals are generated (Wang and Wu 2013).

There are special calcium sensors that perceive calcium signals. In higher plants, four groups were identified that are involved in Ca^{2+} sensing. For example, the Ca^{2+} sensors calcineurin B-like (CBLs) such as CBL1, CBL8, CBL9, CBL10, interact with the CBL-interacting protein kinase23 (CIPK23) and phosphorylate the AtHAK5 K^+ transporter during K^+ deficiency conditions in roots (Ragel et al. 2015). Likewise, the AKT1 K^+ channel is up-regulated by the CIPK23/CBL1 or CIPK23/CBL9 complex (Xu et al. 2006) as a result of K^+ deficiency (Figure 1-4).

1.6.4 Hormones

Ethylene, auxin and jasmonic acid are reported to be generated in plants as a result of K^+ deficiency. Shin and Schachtman (2004) reported an increase in the genes responsible for ethylene synthesis after 6-30 hours of K^+ starvation in Arabidopsis (Figure 1-4). This response was also accompanied by an increase in the production of ROS. While ethylene inhibits lateral roots initiation, it induces the growth of root hairs as a response to low K^+ (Shin and Schachtman 2004). The K^+-dependent transcriptome analysis showed an increase in the transcript levels of JA biogenesis enzymes as a response to K^+ deficiency (Armengaud et al. 2004). Many JA responsive genes were also up-regulated during this study as a response to K^+ starvation. The phytohormone auxin plays an important role in shaping the root under K^+ starvation conditions in Arabidopsis plants as mentioned previously (Vicente-Agullo et al. 2004).

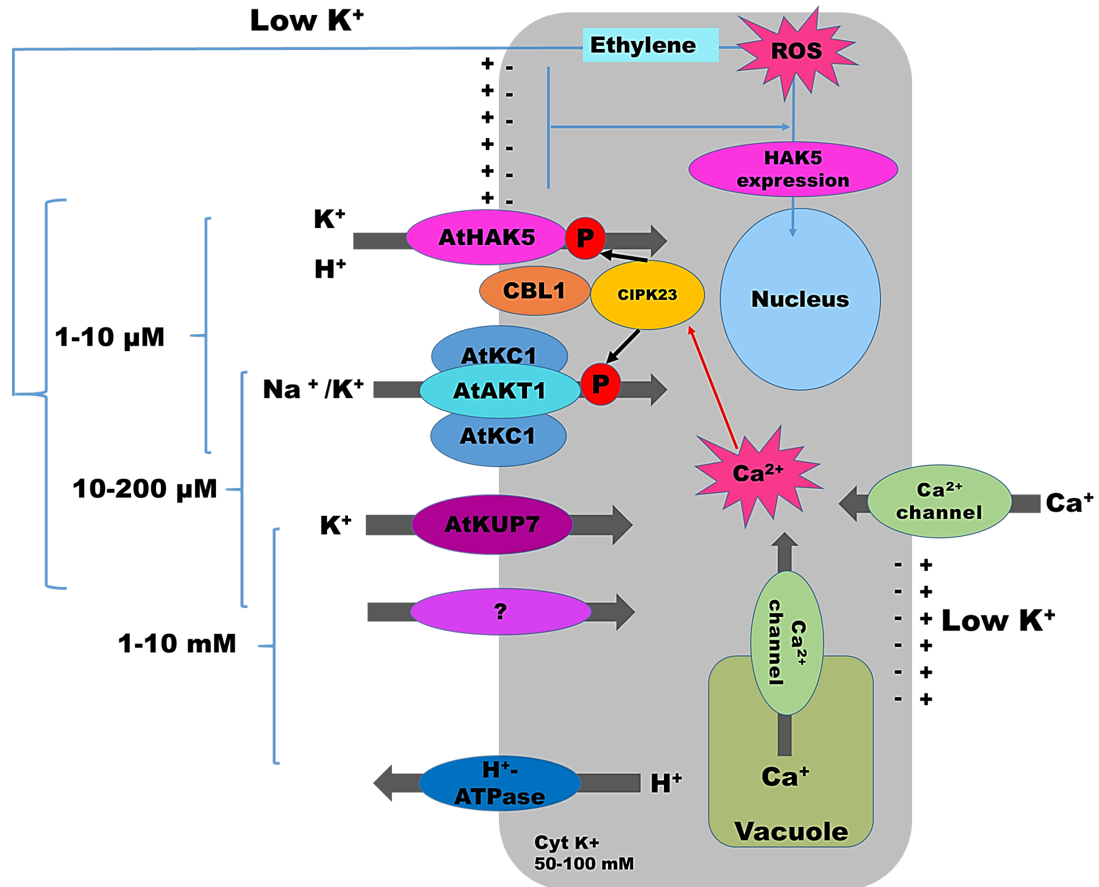


Figure 1-4: Mechanism of low K^+ sensing and signaling in Arabidopsis plants.

When K^+ starvation occurs, the plasma membrane is hyperpolarized, which induces the expression of the high affinity K^+ transporter 5 (AtHAK5). Ethylene and reactive oxygen species (ROS) are generated which induces AtHAK5 as well (Shin and Schachtman 2004). Low K^+ levels might induce the Ca^{2+} channels to flux Ca^{2+} into the cytoplasm, which then forms the CBL1-CIPK23 complex, CIPK23 phosphorylates AKT1 (Alemán et al. 2011; Nieves-Cordones et al. 2014). The AtKC1 silent subunit and interacts with AKT1 channel activating it. AtKUP7 is another KUP family K^+ transporter that functions between 10-200 µM of K^+ (Han et al. 2016). An unidentified transporter is thought to be functioning in the low/intermediate K^+ levels, which is inhibited by NH_4^+ and might be affected by Na^+. This transporter might also be responsible for K^+ uptake at high levels (1-10 mM) (Pyo et al. 2010).

1.7 Salt stress

Salinity is defined as the elevated salt concentrations in soils. When these concentrations are higher than 40 mM of NaCl, soils are called saline (Munns and Tester 2008). Salinity affects about 7% of the area of land (Flowers and Colmer 2008). Soils can be saline for either primary or secondary reason. Primary reasons include naturally topping high salt basic layer of rocks and occurrence of ground water with

high salt concentrations. Arid and semi-arid lands with low rain rates suffer from lack of salt seep through the soil. This -with the difficulty of water drainage- can lead to an increase in soil salts levels. If the sodium is involved in this process, then it is called sodification. Secondary salinization happens as a result of poor irrigated drainage of lands. Here, salts accumulate and cause limitation of micronutrients and water stress. Toxic salts accumulate and affect the natural structure of soils (Kitamura et al. 2014). The next sections explain how Na^+ enters plants and how salinity is sensed. We also indicate the entry, transport and compartmentation of Na^+ in plants as a salt stress tolerance response.

1.7.1 Sodium entry into plants

The HKT transporters were reported to function in Na^+ uptake in many species. Two classes of these transporters were specified, Class I is reported to function in Na^+ uptake while Class II is involved in K^+/Na^+ transport and is only found in monocots (Garciadeblás et al. 2003). There is a difference in the selectivity pore motif due to different polypeptides in these two classes. The second class contains GGGG while the first class contains SGGG polypeptides, where G is substituted with S resulting in different selectivity towards Na^+ (Platten et al. 2006; Benito et al. 2014; Deinlein et al. 2014).

As previously mentioned, AtHKT1 was identified as a Na^+ uptake transporter. AtHKT1 is highly expressed in roots and mediates Na^+ transport in both yeast and *Xenopus* (Uozumi 2001). AtHKT1;1 prevents the accumulation of high Na^+ levels in shoots, therefore, protects leaves from its toxic effects. Loss of function mutants of *athkt1;1* showed a phenotype of leaf chlorosis due to higher concentrations of Na^+ in leaves. In rice, HKT1 was also reported for activity in Na^+ transport (Golldack et al. 2002) and is responsible for the salinity intolerance of rice. While in wheat, HKT1 was reported to function as a K^+/Na^+ symporter and is expressed in the root cortex and leave vasculature. The HAK/KUP/KT family of transporters has also been reported for possible Na^+ uptake although regarded as high affinity K^+ transporters. They can function in the low affinity Na^+ transport. AtHAK5 was reported to mediate Na^+ uptake (Alemán et al. 2009; Alemán et al. 2014; Wang et al. 2015).

Different channels were reported to participate in the uptake of Na^+. Non-Specific Cation Channels (NSCC) such as cyclic nucleotide gated channels CNGC are related to Na^+ uptake from soil. Glutamate receptors (GLRs) function in Na^+ uptake as well (Tapken and Hollmann 2008). The AKT1 channel was reported to mediate Na^+ uptake when it is presented in the medium (Spalding et al. 1999). Together, these transporters result in the accumulation of Na^+ in plants and therefore is important to be considered for salinity improvement studies in glycophytes.

The previous ways of sodium entry through plasma membranes of root cells is called symplast flow. However, this is not the only way Na^+ can enter root cells through. The other way is through the non-living components of root cells, which is called apoplast flow. The apoplast flow occurs through the endodermis passage cells when water loaded with nutrients and Na^+ passes through intercellular spaces and cell wall. This is important in some species such as rice where apoplast flow represents about 50% of the Na^+ that enters roots (Yeo et al. 1987; Kronzucker and Britto 2011).

1.7.2 Long distance Na^+ transport

Once Na^+ enters root, it is then transferred to xylem where it is directed to shoots. The mechanisms of which Na^+ is transferred by are various. The salt overly sensitive Na^+/H^+ antiporter 1 (SOS1) is suggested to contain 10-12 transmembrane domains and a long C-terminus consists of 700 amino acids that face the cytoplasm and thought to sense Na^+ (Shi et al. 2000). Loss of function of the SOS antiporter in Arabidopsis resulted in a salt-sensitive phenotype (Shi et al. 2000b; Shi et al. 2002). SOS1 functions in Na^+ upload to xylem sap during moderate salinity stress while it retrieves Na^+ from xylem under severe salinity stress (Shi et al. 2002). Members of the HKT family of transporters were indicated to be involved in xylem loading of Na^+. The barley HvHKT1;5 was reported to function in Na^+ xylem loading during salinity conditions (Ren et al. 2005).

1.7.3 The compartmentation of Na^+ into vacuoles and removal to the apoplast

The sequestration of Na^+ into cell vacuole is the main tolerance mechanism in plants. A plant can further survive the toxic effects of high Na^+ if it is able to load it into vacuoles where it is then away from cytoplasmic components and physiological functions. This also induces water uptake into cells to maintain osmotic pressure

(Munns and Tester 2008). Many transporters function in this process have been investigated so far. The NHX family of Na^+/H^+ antiporters plays a major role in loading Na^+ into the vacuole. AtNHX1 resulted in salinity tolerance when overexpressed in yeast, Arabidopsis, tomato, rice and cotton (Apse 1999; Zhang and Blumwald 2001; Aharon et al. 2003; He et al. 2005). Interestingly, these antiporters do not only relieve salt stress by loading Na^+ to vacuoles, but also by increasing the uptake of K^+. Indeed, the NHXs are mainly K^+/H^+ exchangers, and the double loss of function mutants of *nhx1/nhx2* showed impaired vacuolar K^+ content and higher accumulation of Na^+ while plants were not sensitive to NaCl (Jiang et al. 2010; Barragán et al. 2012; Maathuis et al. 2014). Indicating that these transporters tolerate salinity by maintaining K^+ uptake rather than Na^+ accumulation in vacuoles.

Removal of Na^+ into the apoplast is another mechanism of salinity tolerance. The SOS1 is suggested to be involved in this process where there are no vacuoles such as in root tip cells. Alongside its other functions, SOS is thought to increase the salinity tolerance in plants by the efflux of Na^+ to the apoplast (Shi et al. 2002).

1.7.4 Salinity sensing and signaling

1.7.4.1 How a plant senses salinity

Despite all progress in understanding how Na^+ is transported, it is still not clear how it is initially sensed by plants (Shabala et al. 2015). The sensing can start after milliseconds and up to hours or maybe days of exposure to NaCl. It can also occur in different tissues rather than one (Shabala et al. 2015). In plants, there are no reports about Na^+ specific channels that only function in Na^+ transport, but it is possible in this case that the Na^+ activated K^+ channels carry out this function (Maathuis 2013). Among these, the CHX family of cation exchangers, such as CHX5, CHX6, CHX7 and CHX16. However, much work still needed to understand the role of these exchangers in Na^+ sensing (Maathuis 2013). The SOS1 is also suggested to play this role, with a 700 amino acids long tail that possibly resides in cytoplasm and might be involved in sensing the Na^+ (Shi et al. 2000; Zhu 2003), yet, no direct evidence on this role has been obtained. The NCX-like (AtNCL) Na^+/Ca^{2+} exchanger is another candidate for Na^+ sensing, being expressed in many tissues as a response to salinity and ABA treatment (Wang et al. 2012).

1.7.4.2 The SOS pathway of salinity signaling

The signalling cascades of salt stress are mainly transduced by the SOS pathway, the AtSOS1 antiporter is located in the plasma membrane and regulated by the CBL-CIPK complex. Once salt stress occurs, it induces Ca^{2+} signalling which is sensed by the myristoylated calcium binding protein SOS3 (CBL4) (Luan 2009), then activated by SOS2. The SOS2 is activated by the CIPK24 protein kinase (Liu et al. 2000), and this complex in the end activates the SOS1 antiporter by the phosphorylation of its C-terminus (Quintero et al. 2011; Maathuis 2013). SOS2 is also interacting with the NHX antiporter in the tonoplast indicating a function in regulating Na^{+} sequestration into vacuoles (Qiu et al. 2004). AtSOS4 was found to function in root hair development while AtSOS5 is important for root architecture during salinity stress (Shi et al. 2002; Shi et al. 2003). Accordingly, we can conclude that the SOS system is paramount in salinity signalling.

The mitogen-activated protein kinases (MAPKs) class has major roles in signalling for many abiotic stress responses in plants (Nakagami et al. 2005). AtMPK4 and AtMPK6 are activated under high salinity conditions (Teige et al. 2004). AtMPK6 also phosphorylated the C-terminal of the SOS1 as a response to salinity stress which indicates a possible cross-talk between signalling pathways (Yu et al. 2010). The overexpression of *OsMAPK5* resulted in higher salinity tolerance (Xiong and Yang 2003). The TPC1 channel was found to function in salt stress responses. It was suggested that the vacuolar channel TPC1 mediated the signalling wave of free cytosolic Ca^{2+} to shoots as a response to root tip NaCl treatment in Arabidopsis. Overexpression of TPC1 resulted in more salt resistant plants, which correlated with faster Ca^{2+} signalling waves compared to wildtype. The overexpressor plants showed better growth and chlorophyll content after 7 days of 100 mM NaCl treatment (Evans et al. 2016)

1.8 Drought Stress

The limitation of water restricts the growth of plants and affects productivity. Drought stress is the number one factor that reduces the yield compared to other stresses affecting the worldwide agriculture (Farooq et al. 2009). The main drought response for a plant is to keep its water content. This is achieved by stomatal closure in the short

term, but at the same time, this reduces the assimilation of CO_2. Accompanied by the disruption of photosynthesis enzymes, a final effect is the reduction of the photosynthesis rate (Griffin et al. 2004). This in the end affects the productivity of the plant, which is the most serious economic effect of drought. Drought stress reduces the yield of the main crops such as in maize, soybean, tomato and cotton (Korres et al. 2016), and this reduction is determined based on the time when the drought happens. Drought causes its severe effect mostly during germination stage and seedling establishment. Rice was negatively inhibited by drought stress during vegetative stage and development of plants (Tripathy et al. 2000; Kaya et al. 2006; Manavalan et al. 2009). Drought affects seed filling as well, in barley, drought can cause up to 57% of yield loss if it happened during seed filling (Samarah 2005), while in maize and rice, the yield loss can go up to 81% and 60% respectively if the drought happens during seed filling (Monneveux et al. 2006; Farooq et al. 2009).

1.8.1 Types of plant responses to drought

When plants face harsh environmental conditions, they have no choice but to cope with it. Therefore, a plant shows the highest levels of modifications during stresses. Mainly, a plant will try to complete its life cycle which is the most important goal. Drought, therefore, affects plants differently. There are four categories of plants according to how they respond to drought, (1) drought avoidance, when plants are able to keep their basic physiological functions under mild drought conditions by modifying its morphological characteristics to avoid the harmful effects of drought mainly by maintaining high water uptake (Hall and Schulze 1980; Mitra 2001; Blum 2005; Luo 2010;). This is achieved by three steps: (a): reduction of water loss by inducing stomatal closure, rolling of leaves (Tardieu 2013) and increasing wax thickness in leaves (Zhang et al. 2005), (b): increasing water uptake by increasing root/shoot ratio through growing deeper roots with more surface area and density and storing water in special organs such as cacti plants (Sawidis et al. 2005; Tardieu 2013). (c): adjusting the conversion of the vegetative stage to reproductive growth according to when drought sets its harmful effects (Mitra 2001; Luo 2010). The second type of drought response in plants is (2) drought tolerance, here, a plant can maintain a number of its essential physiological functions under the harmful effect of drought (Mitra 2001; Manavalan et al. 2009; Luo 2010). This occurs by adjusting the expression of

drought responsive genes and metabolism to reduce or fix the effects of drought. (3) drought escape: a plant modify its growth, planting time or life cycle so it finishes its life cycle without encountering the seasonal or climate drought (Mitra 2001; Manavalan et al. 2009), this adjustment happens either naturally or artificially by human interference, such as growing crops that are able to finish its life cycle and maintain high yield by avoiding the drought stress season. (4): drought recovery: is when a plant can resume its growth even after drought caused damage due to loss of water potential (Luo 2010).

1.9 How drought and salinity affect plants

Drought and salinity stresses share many physiological responses. Because drought resembles the lack of water and salinity lead to a state of water deficit by reducing the availability of water, both stresses restrict the growth of plants. Multiple metabolic alterations occur similarly in both drought and salt stressed plants (Munns 2002; Zhang et al. 2014). Therefore, we explain the effects of both stresses together.

1.9.1 Growth of plants

Salinity results in disturbance of water relations, and a limitation in water in the end which in turn shows water deficit responses (Munns 2002). The root is the main contact point with soil where plants face environmental conditions to obtain water and nutrients. Therefore, when plants face osmotic stress due to drought or salinity roots show different responses. It was reported that drought increases root growth because plants will increase the root surface aiming to obtain the water enough to keep it alive. The roots tend to grow deeper and wider to be able to maintain water uptake in plants. It was found that woody plants grown in dry areas have roots that are 10 times the depth of the parts grown above the soil, which enables plants to maintain water uptake and therefore better growth under such stress (Dixon et al. 1980).

Plants suffering from drought display smaller sizes that is a result of many drought related effects. The water deficit mainly reduces cell division as well as elongation and therefore results in a delay in generating new leaves (Nonami 1998). Other effects can be: reduction in leaf size, shorter stems due to a weak extension, an increase in the root area and a disruption in the water relations of the plants leading to a less efficient use of water (Farooq et al. 2009). Salinity causes a reduction in root and leaf growth

due to the accumulation of salts (NaCl), although plants can recover of this later if the stress was not for long times (Rodriguez et al. 1997).

1.9.2 Germination of seeds

Seed germination is a vital process that needs to be adjusted so it only happens when there are suitable conditions for plant growth. Therefore, seeds can inhibit germination or activate it according to the surrounding factors such as water, temperature, light, and nutrients. This inhibition is called dormancy and results from the phytohormone abscisic acid (ABA). ABA was recently found to be synthesized in the endosperm and then exported to the embryo where it blocks germination (Kang et al. 2015). Meanwhile, when conditions are suitable for germination, seeds will germinate and this happens by other hormones such as gibberellins (GAs). GA antagonize ABA to enable seeds to germinate. In a non-dormant seed, GAs inhibit the action of ABA by the destruction of DELLA factors responsible for the accumulation of ABA therefore promotes germination (Zentella et al. 2007; Piskurewicz et al. 2008; Piskurewicz et al. 2009) . The process of endosperm weakening happens as a result of interaction between GA and ABA (Müller et al. 2006).

When dry seeds uptake water, germination is recognized by the emergence of the radicle from the seed (Weitbrecht et al. 2011). Once the radicle has emerged, the seed is considered germinated and this process ends by the rupture of the testa (Bethke et al. 1998; Daszkowska-Golec 2011; Bewley et al. 2013). The water uptake by seeds during the germination involves 3 phases: phase I starts with the seed imbibition, phase II is a plateau with a steady water content and the beginning of the endosperm rupture and phase III when the radicle emerges from the ruptured endosperm (Daszkowska-Golec 2011). The emergence of the radicle is a result of water uptake and cell expansion. The process of water uptake by the seed activates a chain of metabolic processes as a result of the seed hydration (Bewley and Black 1994; Chen et al. 2002). By the start of phase II of water uptake, small vacuoles are formed in both embryo and endosperm cells (Bethke et al. 1998; Weitbrecht et al. 2011). In barley and maize grains, the aleurone contains several protein storage vacuoles. The globoid crystals in the protein storage vacuoles consist mainly of the phytin which consists of K^+, Mg^{2+} and Ca^{2+} salt of myo-inositol hexakisphosphate (Yupsanis et al. 1990; Mikus et al. 1992; Bethke et al. 1998). Once a seed starts to germinate, these stored proteins are

hydrolysed by pre-existing enzymes, which leads to the mobilization of the stored minerals like K^+. K^+ plays a major role in seed germination alongside with the other ions stored in seed vacuoles. This includes the adjustment of cell osmosis and turgor pressure during germination as well as activation of enzymes (Gobert et al. 2007). The release and movement of K^+ and other ions during early stages of germination is managed by multiple K^+ channels/transporters that can be located in the vacuoles or plasma membranes of the seed endosperm. Any extreme change in the conditions surrounding the seed during the germination process can compromise it by either inhibiting or completely stopping it. These incompatible changes can be salinity, drought or the deficiency of nutrients. Since keeping a balanced content of K^+ in tissues relieves abiotic stress effect, it is important to investigate how K^+ is controlled through the K^+ channels/transporters located in the seed tissues.

As a seed to seed journey starts with germination, improving seed germination under stress conditions becomes substantial for obtaining better growth and yield (Holdsworth et al. 2008). The next section explains how phytohormones work together under abiotic stresses in a germinating seed.

1.9.3 Germination under stress, a harmony of hormones

As many aspects of plant growth, germination is negatively affected by both drought and salinity or other environmental stresses. These stresses do not only affect the seed germination but the later stages of seedling establishment and consequently the growth of the plant (Salas-Muñoz et al. 2016). Osmotic stress caused by both drought and salinity may delay or inhibit the germination along with imbalanced ions and oxidative stress until better conditions occur. Inside the seed, water content, mobilization of the stored reserves and protein structures are also affected (Ibrahim 2016). During salinity, many proteins are involved in transporting and sequestrating Na^+ levels in plants such as SOS1 and NHX1. The occurrence of high NaCl levels in the external medium suppress radicle emergence and also cotyledons and therefore overall germination (Pyo et al. 2010).

Dry seeds contain many stored proteins and among them, the ones involved in ABA signalling are highly presented. Indicating the important role of ABA during seed germination, (Nakabayashi et al. 2005). The ABA was investigated thoroughly in

terms of how it is synthesised, transported and degraded. In germination, (Sucrose Nonfermenting Related Kinase2) SnRK-2 is a kinase that plays a major role in activating the transcription factor ABI5 (ABA insensitive 5) which is vital for seed germination (Fujii et al. 2007; Nakashima et al. 2009). During germination, both ABI3 and ABI5 levels are decreased to allow the seed to germinate (Zhang et al. 2005). However, when seeds are under abiotic stress conditions the decrease in their levels is slower. The *RAP2.6* (Related to Apetala 2) gene is also involved in salt stress tolerance in seeds during germination, its expression was up-regulated as a response to ABA, salt, and osmotic stresses. Overexpression resulted in plants that are hypersensitive to ABA, salt and osmotic stresses during germination (Zhu et al. 2010). This indicates the diversity of ABA responsive genes during germination under different stresses.

GAs have an important role in germination under stress conditions. For example, the destruction of the DELLA proteins by GAs enables seed germination. The RGL2 (Repressor of GA Like1-2) is a Della protein that showed an elevated expression during salt stress germinated seeds (Kim et al. 2008). RGL2 was found to interact with ABAI5 and ABAI4 in salt stress germinated seeds and during early seedling development (Yuan et al. 2011). Confirming the relationship between the two phytohormones ABA and GA during abiotic stress in germinating seeds.

1.9.4 Stomatal conductance and photosynthesis

Stomata are micro-openings on leaves/stems surfaces. They represent the main pathway of water loss through transpiration as well as the main pathway of CO_2 flux that is used in the photosynthesis (Papanatsiou et al. 2016). Stomatal pores are surrounded by a pair of cells (guard cells) that are highly modified to fulfil their unique function under the effect of different external stimuli, such as light, humidity and CO_2 concentrations (Blatt 2016). Therefore, guard cells have gained the attention of researchers for decades, as it became clear that they play a major role in how plants tolerate the abiotic stress and accordingly maintaining a high yield.

Drought and salinity result in water limitation and therefore causing cell components to be viscous and therefore an increase in solutes concentrations that it might become toxic and affect the photosynthesis enzymes (Hoekstra et al. 2001). Drought and salinity induce the closure of stomata which results in a reduction of CO_2 availability

leading to photo-damage (Anjum et al. 2003). Drought also damages the photosynthetic pigments such as chlorophyll and photosynthetic apparatus (Fu and Huang 2001).

1.9.4.1 Stomatal closure under abiotic stress

Both drought and salinity lead to osmotic stress, which induces both the synthesis and inhibition of degradation of the stress hormone abscisic acid (ABA). Different types of genes are related to these two processes (Marin et al. 1996; Tan et al. 1997; Tyerman and Skerrett 1998). ABA has multiple functions in the plant life cycle starting from germination and through growth and development. However, the main task that distinguishes ABA is the protection of water content of plants after osmotic stress occurs, and this is achieved by the stomatal closure (MacRobbie 1998; Zhu 2002; Fan et al. 2004).

When plants face water stress, stomatal closure is a first step to prevent water loss. This response starts with endogenous ABA synthesis by the 9-cisepoxycarotenoid dioxygenase 3 (NCED3) in vascular tissues (Iuchi et al. 2001). Once ABA is accumulated in these tissues, it diffuses passively to guard cells by the ABA transporters ABC25 (which is suggested to export ABA) and ABCG40 (which is suggested to import ABA) and is therefore expressed in guard cells (Kang et al. 2015).

The accumulation of ABA then induces the generation of ROS, which leads to an increase in Ca^{2+} in the cytoplasm. This increase results in the depolarization of plasma membrane which activates the SLOW TYPE ANION CHANNEL-ASSOCIATED1 (SLAC1) activated by Snf1-Related Protein Kinase 2 (SRK2E/OST1/SnRK2.6) (Negi et al. 2008; Vahisalu et al. 2008). SLAC1 is also activated by the calcium dependent protein kinases CPK3, CPK6, CPK12 and CPK23. This in turn leads to the efflux of anions of the guard cells. The depolarization in guard cell plasma membrane also inhibits the K^+ inwardly rectifying channels in *Arabidopsis thaliana* 1 and 2 (KAT1 and KAT2). The KAT1 channel is also inhabited by the SRK2E through phosphorylation (Sato et al. 2014). Meanwhile, the guard cell outwardly rectifying channels like (GORK) and K^+ transporters like the potassium uptake permease 6 (KUP6) are activated (Becker et al. 2003; Hosy et al. 2003; Osakabe et al., 2013). The H^+-ATPases are inhibited by the depolarization of plasma membrane (Figure 1-5).

1.9.4.2 Vacuolar ion channels/transporters involved in the stomatal closure

The guard cell and guard cell vacuole size can both change during closure and opening (Shope et al. 2003; Gao et al. 2005). This rapid change occurs as a result of the ions and anions movement led by a change in water potential across both the plasma membrane and the tonoplast (Figure 1-5). Diverse channels and transporters of different families carry out the vacuolar efflux of ions. The AtTPC1 (Two Pore) channel representing the slow vacuolar ion channels (SV) located in the tonoplast. The crystal structure of the ATPC1 has been recently revealed, the structure shows two 6 transmembrane domains (I and II) each of them contains two pore helices (P1 and P2), with EF-hands per AtTPC1 subunit directed towards the cytosol where the cytosolic Ca^{2+} binds leading to a conformational change in the channel activation (Guo et al. 2016). Loss of function mutants of TPC1 were found to be non-responsive to the extracellular Ca^{2+} and accordingly showed more opened aperture compared to wild type (Peiter et al. 2005). It is important to mention that the role of AtTPC1 in stomatal closure is not yet defined, although it is tightly regulated and shows K^+ and Ca^{2+} currents (Eisenach and Angeli 2017).

As previously mentioned, the two-pore potassium channel 1 (TPK1) representing the vacuolar channels (VK) is involved in the vacuolar efflux of K^+ during stomatal closure (Gobert et al. 2007). *attpk1* loss of function mutants sowed slower stomatal closure compared to wildtype while overexpressor lines showed faster closure. Redundancy of other tonoplast K^+ transporters was suggested by Gobert et al., (2007). Since the phenotype resulted from the loss of function of TPK1 was weak, there is a need to analyse potential candidates that might participate in the stomatal closure alongside TPK1.The third group of vacuolar K^+ channels is the fast-activated (FV) channels. This group is largely unknown and there are no reports on any members that are suggested to belong to it (MacRobbie 1998; Gobert et al. 2007). The vacuolar membrane contains two proton pumps that participate in the energised transport of ions across the tonoplast. These two types are H^+-ATPase and inorganic pyrophosphate H^+-PPase (Davies et al. 1992). They function in the accumulation of ions into vacuoles during stomatal opening by creating an electrochemical gradient (Martinoia et al. 2000; Yoshida et al. 2013).

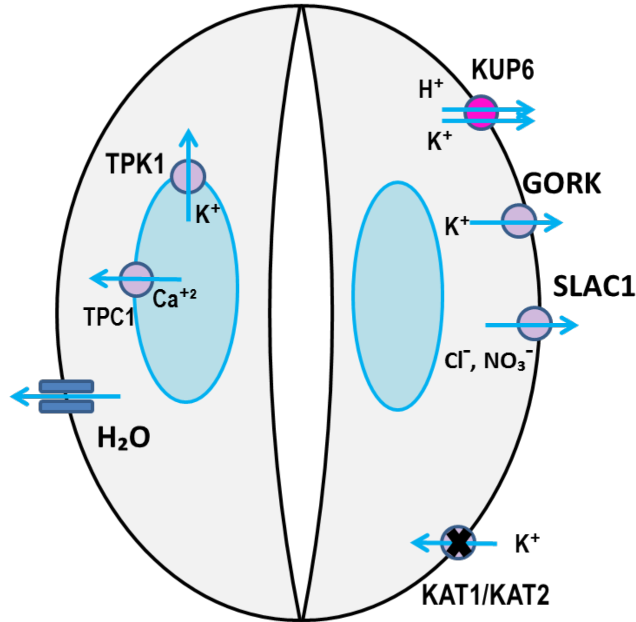


Figure 1-5: Vacuolar channels/transporters functioning in stomatal closure.

ABA induces the efflux of anions through anion channels in plasma membranes (SLAC1) (Negi et al. 2008; Vahisalu et al. 2008). The plasma membrane is depolarized, deactivating K^+ inwardly channels (KAT1, KAT2). The outwardly ion channels/transporters (GORK and KUP6) (Hosy *et al.*, 2003; Osakabe *et al.*, 2013) are activated and K^+ effluxes through tonoplast (TPK1) as well as Ca^{2+} (TPC1) (Peiter et al. 2005). Water moves out the guard cell due to low osmotic pressure causing loss of turgidity and eventually the closure of guard cells.

1.9.5 Reactive oxygen species and oxidative stress (ROS)

As a reaction, plants detoxify their cells of ROS using multiple proteins, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) peroxiredoxin (PRX) and glutathione peroxidase (GPX) (Mittler 2006). Antioxidants such as ascorbate acid and glutathione (GSH) function in this process as well. ROS are important signaling molecules. As previously mentioned, they are generated during K^+ starvation and are responsible for the up-regulation of many genes that function in K^+ deficiency stress (Shin and Schachtman 2004). Salinity and drought also induce the generation of ROS, they are generated in chloroplasts, the apoplast, mitochondria, peroxisomes and other cell components. There must be balancing between the ROS production and scavenging, else, it will cause damage to cells if overproduced which happens during many types of stresses. Salinity and drought result in the generation of ROS. Salt stress-tolerant rice cultivars showed significantly higher levels of SOD, CAT, POX, APX and GR in leaves compared to sensitive ones

(Chawla et al. 2013). In Arabidopsis, NADPH oxidases encoded by genes expressed in guard cells and mesophyll cells were reported to function in the apoplastic generation of ROS which is important during ABA-induced stomatal closure (Kwak et al. 2003; Torres and Dangl 2005).

1.10 Physiological mechanisms of drought and salinity tolerance

1.10.1 Osmotic adjustment

Osmotic adjustment is a major osmotic stress tolerance mechanism in plants. It occurs when plants accumulate different compounds such as sugars, alkaloids, amino acids and inorganic ions to protect cells from osmotic stress. These compounds maintain the osmotic pressure of cells and water retention under osmotic stress conditions (Verslues et al. 2006). Organic compounds can be mannitol, proline, trehalose, betaine, glycine, inositol and fructan which function in osmotic pressure regulation and protect the cytoplasm and its essential physiological processes (Crowe et al. 1984; Ranieri et al. 1989; Thomas and James 1993). Ion and water channels also participate in maintaining the potential of the cytoplasm, inorganic ions such as Ca^{2+} and K^+ are directed to the vacuole to participate in creating turgidity in cells leading to water uptake and therefore reduction of water loss (Fang and Xiong 2014). Proline is an important osmoprotectant that functions in dehydration tolerance and helps to protect membrane structure and scavenging the ROS when synthesized in leaves (Zhu 2002; Wahid and Close 2007).

1.10.2 Aquaporins and water uptake

Aquaporins are water channels that regulate water transportation during many stress responses. It is important for stomatal movement. There are three classes of aquaporins, plasma membrane intrinsic proteins (PIPs), tonoplast-intrinsic proteins (TIPs) and nodulin-26-like major-intrinsic proteins (NLMs) (Maurel et al. 2015; Maurel et al. 2008). The PIPs, are the most important class of aquaporins, they are involved in water transport in roots of plants and they control water movement across the plasma membrane. K^+ starvation induced the expression of K^+ uptake channels as well as *PIPS* in rice plants, while Polyethylene glycol (PEG) resulted in a down-regulation of *PIPs* expression as a mechanism to limit water permeability through these channels and therefore protect water content of cells. Meanwhile, *OsPIP1;1*,

OsPIP1;2, *OsPIP1;4* and *OsPIP2;5* were up-regulated in the long term water deficit in rice to adjust osmotic pressure and water flux in plants (Alexandersson et al. 2005; Liu et al. 2006). In Arabidopsis, HAK5 and PIP1;2 (PIP1b), PIP2;2 (PIP2b) and TIP1;2 (TIP) were inhibited as a response to K^+ channel blocker CsCl (Sahr et al. 2005). From this, we can see the importance of aquaporins under different types of stresses.

1.11 Arabidopsis and rice as model plants

Referred to as *Drosophila botanica*, the *Arabidopsis thaliana* has succeeded in being the most used plant in biology studies. Having a small size, a large number of seeds, short life cycles, identified genes, Arabidopsis is the most suitable plant for studying stress responses such as salinity, drought, heat, cold and biotic stresses. Arabidopsis gained fame when its genetic map was released, and its genome was fully sequenced in 2000 (Meyerowitz 2001). After this, enormous numbers of studies were conducted to unravel the functions of its genes that led to a great knowledge in understanding plant biology and biotechnology. This is then applied in improving the yield of economical crops. Arabidopsis gained the interest of scientists when compared to other model plants such as soybean, maize and even rice, this was reflected by the number of articles. In 2008 only, 3500 papers were published on Arabidopsis on the PubMed database (http://www.ncbi.nlm.nih.gov/pubmed) (Koornneef and Meinke 2010). *Arabidopsis thaliana* is a Brassicaceae family member, a plant that has no agronomic importance compared to other family members such as cabbage, cauliflower, radish and rapeseed. The small genome of Arabidopsis (1/19 of maize and 1/128 of wheat genomes) favored it as a model plant to be able to apply this knowledge on those economical crops. However, the rice genome project indicated that Arabidopsis genome lacks some homologs of the rice genome. Although these homologs are mostly a result of polyploidy of these crops, this indicates that studying Arabidopsis is the point where we start to search for possible solutions to improve crop productivity and not a final goal. For this, studies on agronomic crop plants are essentially benefiting from the genomic knowledge obtained from Arabidopsis.

Rice as a model plant

Half of the world population depends on rice as a main food crop. This crop is grown in all six world continents except for Antarctica where no crops are grown (Awan et al. 2017). Rice is a monocotyledonous plant that belongs to the Poaceae family (formerly Gramineae), subfamily Bambusoideae, tribe Oryzeae and genus Oryzae. The *Oryzae sativa* species has two subspecies: *Oryzae japonica* domesticated in China and *Oryzae indica* domesticated in India and are more close to wildtype than to each other (Gross and Zhao 2014). Rice represents a great model for studying crop genetics for many reasons, 1: its completed sequenced genome, 2: the availability of genomic studies tools such as transposons, T-DNA tagged lines and microarray data, 3: the readiness of generating transgenic plants such as using *Agrobacterium*-mediated transformation in comparison to other cereals (Shimamoto and Kyozuka 2002).

As for its characteristics as a model plant, rice is an important crop that feeds almost half of the world. However, rice is a glycophyte plant that shows sensitivity to salinity stress and negative effects of drought. As mentioned previously that rice is affected by salinity and drought during seedling stage and grain filling as well. Therefore, we need extensive efforts to understand the mechanism of stress tolerance in rice plants to be able to improve the final productivity in order to cover the world demand.

1.12 Aims of the thesis

It is a paramount need to improve plant productivity in the face of the environmental changes. With the population of earth increasing at the same time, pressure doubles to optimize different strategies of generating stress tolerant crops. The efficient supply of plant nutrients and especially K^+ have proved to be an effective approach for more tolerant plants. Consequently, it is equally important to develop our knowledge of K^+ deficiency tolerance mechanisms. This could be achieved through understanding the mechanisms by which a plant can take-up and distribute K^+ in its tissues. Studying the function of K^+ channels/proteins helps achieve this, and by understanding how these proteins function under stress conditions, we can improve plant productivity. However, redundancy is a problem that hinders achieving this goal. K^+ transporters in plants overlap in function even with different structures, enabling them of maintaining K^+ management under different conditions (Schroeder et al. 1994). In this thesis, we

used different approaches of studying K^+ transporters and we investigate new members that have not been tested thoroughly before.

The aims of this thesis are:

- Investigating the role of (HAK8 and KUP12) K^+ transporters in plant responses to abiotic stress. These studies are carried out in Arabidopsis plants.
- Testing the effect of multiple knockout combinations of vacuolar K^+ channel (AtTPK1) and predicted vacuolar transporters (AtHAK8 and AtKUP12) in plant responses to abiotic stress. We aim to understand the possibility of redundancy between AtTPK1 and the other candidates -if any- in K^+ management during the stomatal closure and different abiotic stresses.
- Improving rice stress tolerance by improving K^+ uploading from roots to shoots, using an overexpression approach for the rice SKOR channel.
- Investigating the effect of overexpressing the TPKb rice K^+ channel on the expression levels of other K^+ channels/transporters (GORK, HAK1, KAT1).

Chapter 2. The role of AtKUP12, AtTPK1 and AtHAK8 in the abiotic stress responses

2.1 Introduction

2.1.1 AtKUP12 predicted localization and expression

The majority of the HAK/KUP/KT family of transporters (KUP1, KUP2, KUP3, KUP4, HAK5, KUP6, KUP7, HAK8, KUP9, KUP10, and KUP11) in Arabidopsis and other organisms were investigated in previous studies (see chapter one introduction), except for AtKUP12. Little information is available about this transporter and its role in plant physiology. A study by Armengaud et al., (2004) indicated that KUP12 was down-regulated in Arabidopsis shoots during K^+ resupply after a starvation assay. This might suggest a role for this transporter in K^+ management during K^+ deficiency. Subcellularly, it is predicted to be localized in the chloroplast envelope (Weber et al. 2005; Garciadeblas et al. 2007), tonoplast and plasma membrane (Hooper et al. 2014). A phosphoproteomics study by Whiteman et al., (2008) included AtKUP12 in their study of the tonoplast proteins. They indicated that it is localized in the plastid envelope. On the tissue level, AtKUP12 is mainly expressed in guard cell, seed and seed endosperm, roots, apical shoot tissue and pericycle, according to the data from the Genevestigator database (Hruz et al. 2008). It was also found to be expressed in root hairs (Ahn et al. 2004). The most significant expression of the *AtKUP12* gene appears in the imbibed seeds. *KUP12* is highly expressed in different embryo tissues during different germination stages (Figure 2-1.A). Data from the EFP browser (Winter et al. 2007) indicated that this gene is up-regulated 600-fold 24 hours after the seed imbibed in water (Figure 2-1.B). Other K^+ channels/transporters genes that are highly expressed in embryos such as the *AtTPK1* channel showed significantly lower levels of expression during imbibition compared to *KUP12* (Figure 2-1.C). The predicted localization and expression of *KUP12* suggest a role in K^+ homeostasis during imbibition and germination of seeds.

Highly expressed in guard cells, KUP12 can also suggest a role in stomatal closure –if located in guard cell tonoplast- alongside the TPK1 channel as suggested by Gobert et al., (2007), who mentioned that the AtTPK1 channel is involved in the K^+ efflux from the

guard cell tonoplast during stomatal closure and after ABA treatment. However, Gobert et al., (2007) also indicated a good level of redundancy in this process that led to the stomatal closure despite the TPK1 loss of function resulted in slower closure and higher conductance (See chapter one). The phenotype resulted from the loss of function of AtTPK1 was weak, suggesting that other channels/transporters are participating in this process (Isayenkov et al. 2010; Jarzyniak and Jasiński 2014). AtKUP12 is a good candidate for this role as it is predicted for a possible guard cell tonoplast localization. It can also participate in K^+ movement in guard cell during stomatal movement if located in the plasma membrane. To date, no studies on AtKUP12 have been reported regarding this aspect except the (Armengaud et al. 2004) work on K^+ starvation.

2.1.2 Interactomes and regulation by hormones

AtZAT7 (At3g46090), a transcriptional repressor that belongs to the Cys2/His2-type (C2H2) zinc finger proteins, showed enhanced expression in roots during salt stress in *Arabidopsis thaliana* (Ciftci-Yilmaz et al. 2007). The constitutive expression of this gene resulted in salinity tolerant plants in Arabidopsis. The two-hybrid yeast analysis showed that it is suggested to interact with AtKUP12 during salinity stress. This result increases the importance of studying the role of KUP12 in the plant responses to abiotic stresses such as salinity (Krishnakumar et al. 2015). KUP12 was also found to be regulated by the brassinolide (BL) hormone, a member of the plant group of hormones Brassinosteroids (Goda et al. 2004). This group of hormones is involved in cell elongation, cell division, germination of seeds and differentiation of cells such as stomata and most importantly root growth. It is also involved in different stress responses in plants, such as salinity, drought, and nutrient deficiency (Gudesblat and Russinova 2011; Tang et al. 2016; Wei and Li 2016). Importantly, AtKUP12 was found to be directly connected with the amidase 1 transporter At1g08980 (TOC64-1) in a network. This protein is involved in the biosynthesis process of auxin (Indole Acetic Acid) based on microarrays data. This connection can suggest a role for AtKUP12 in root initiation/growth and many physiological characteristics connected to auxins (Obayashi et al., 2018).

2.1.3 AtHAK8 transporter previous work and expression

Another member of the family is HAK8, was found to be phosphorylated in a tonoplast-enriched membrane fraction (TEF) study of Arabidopsis shoot tissue (Whiteman et al. 2008). This work was based on this information. However, in 2013, Osakabe et al indicated it is a homolog for AtKUP6, which was found to be localized in the plasma membrane, indicating that HAK8 could be expressed in plasma membrane instead. The studies on AtHAK8 and its homologs AtKUP2 and AtKUP6 by Osakabe et al., (2013), indicated that it participates with these two transporters in K^+ efflux from cytoplasm through the plasma membrane. They also indicated its involvement in the negative control of plant growth.

AtHAK8 is highly expressed in guard cell and the root pericycle (Fizames et al. 2004; Winter et al. 2007; Hruz et al. 2008; Hooper et al. 2014). It is also highly expressed in the embryonic and flower tissues (Ahn et al. 2004). The expression of the *HAK8* was induced under salinity stress conditions in the leaves of the halophyte *Helianthus sp*. (Edelist et al. 2009), suggesting a function for this transporter in K^+ homeostasis under salt stress.

As mentioned in chapter one, it is also suggested that AtHAK8 might be involved in the K^+ efflux of guard cells and roots along with AtKUP2 and AtKUP6 (other family members from the same clade (II) (Very et al. 2014; Nieves-Cordones et al. 2016)). See (Figure 1-2) in chapter one. Multiple loss of function mutants of HAK8, KUP2 and KUP6 were studied under different stress treatments in Arabidopsis (Osakabe et al. 2013). Mutants displayed impaired stomatal closure in response to drought stress as well as larger plant bodies due to bigger cells. However, single mutants of HAK8 did not show any strong phenotypes, suggesting a redundancy in function by other proteins (Osakabe et al. 2013). These transporters were suggested to be involved in K^+ efflux during the stomatal closure, a function that is surprisingly different from most studied HAK/KUP/KT family members and indicating a diversity of physiological roles for this family.

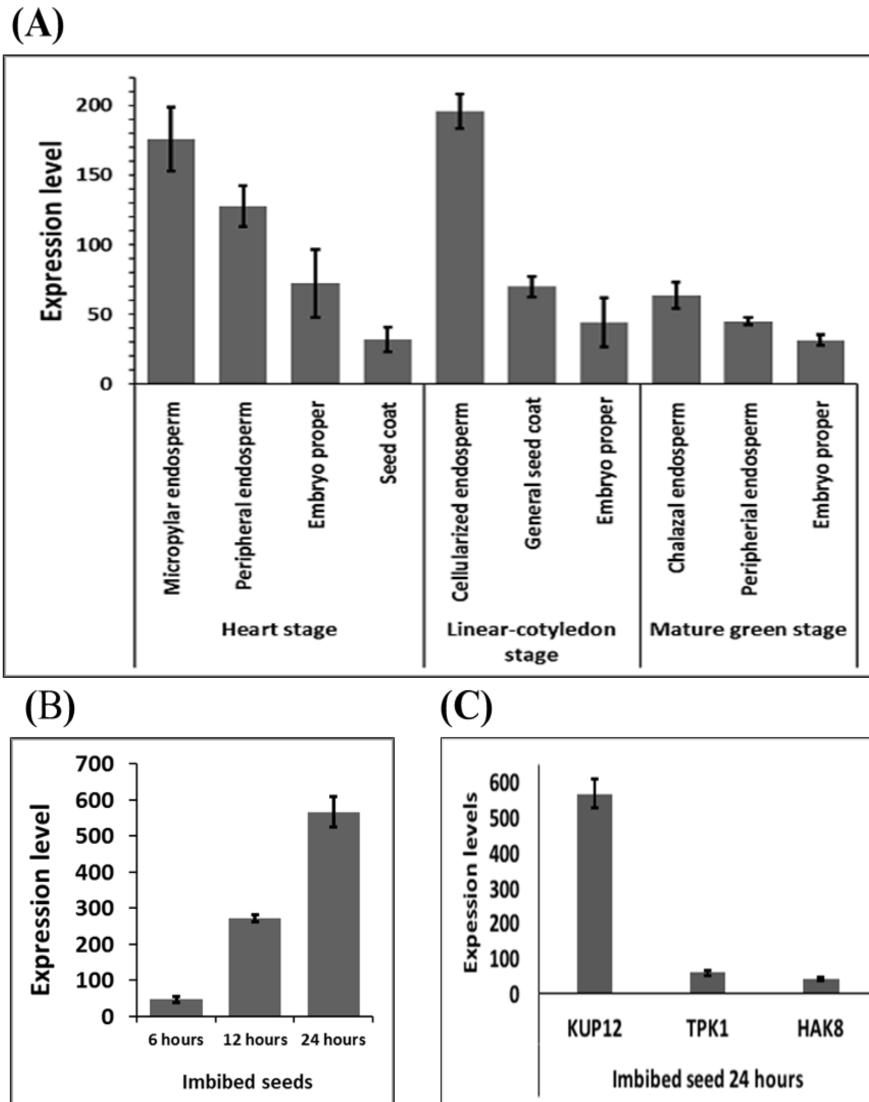


Figure 2-1: Expression levels of *AtKUP12* in different seed tissues and time points and in comparison with *AtHAK8* and *AtTPK1* during imbibition of seeds.

(A): Expression levels of *AtKUP12* in embryo during 3 stages (heart, linear cotyledon and mature green) in Arabidopsis. (B): Expression levels of *AtKUP12* in imbibed seeds 6-24 hours. (C): A comparison of expression levels of *KUP12*, *TPK1* and *HAK8* genes in 24 hours imbibed seeds. Bars are the standard deviation of the mean. Data obtained from EFP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) and Genevestigator database (https://genevestigator.com/gv/).

2.2 Aims of study

When plants are exposed to low K^+ concentrations, there is an urgent need to keep the K^+ levels in the cytosol balanced. Plants will use the K^+ that is stored in vacuoles to maintain its vital physiological functions. When K^+ starvation continues for longer periods, vacuolar K^+ concentrations become lower than in the cytosol (Walker et al. 1996), an active mechanism of K^+ efflux is needed to function in this case (Ahmad and Maathuis 2014). This mechanism is suggested to be carried out by the HAK/KUP/KT family transporters. Low external K^+ concentrations as in soil will also require an active transport (Bañuelos et al. 1995; Santa-María et al. 1997; Quintero and Blatt 1997). This is suggested to be achieved by coupling K^+ and H^+ transport which is hypothetically carried out by HAK/KUP/KT transporters (Maathuis and Sanders 1993; Rodríguez-Navarro and Rubio 2006). K^+ channels are also important for K^+ starvation responses, for example, the TPK1 channel has an important role in K^+ homeostasis between vacuole and cytosol under normal and abiotic stress conditions

Therefore, the aims of this chapter are:

- **Analysing the physiological roles of the potential K^+ transporter AtKUP12 in Arabidopsis during abiotic stress**

  The fact that many members of the HAK/KUP/KT family have been reported to participate in the high affinity (Spalding et al. 1999; Gierth et al. 2005; Qi et al. 2008; Pyo et al. 2010; Alemán et al. 2014; Ragel et al. 2015; Han et al. 2016), or low affinity K^+ transport in Arabidopsis (Fu and Luan 1998), and the down-regulation of *AtKUP12* in Arabidopsis shoots after K^+ resupply (Armengaud et al. 2004), can suggest a potential function for AtKUP12 in the plant physiology. Along with germination, salinity, osmotic stress, water loss and leaf conductance, heterologous expression of *AtKUP12* in yeast is covered in this study under K^+ starvation conditions to understand the role of AtKUP12 in plant physiology. We started by testing a yeast strain that lacks the main plasma membrane transporters to see if the transporter can rescue the growth.

- **Investigating the effect of combining multiple loss of function mutants of candidate genes**

  Despite the novel phenotype that resulted from loss of function of the AtTPK1 K^+ channel, the results indicated a possibility of the involvement of other K^+ channels/transporters in the stomatal kinetics. Candidates for this redundancy included potential K^+ transporters that have not been studied in this aspect, such as AtKUP12 or had no phenotype such as AtHAK8. They are predicted for a guard cell tonoplast localization. These transporters are suggested to play a role in the response of plants to different abiotic stresses. By combining different knockouts, we aim to obtain a stronger phenotype which was either not detectable in single mutants such as *athak8* (Osakabe et al. 2013) or not yet investigated such as *atkup12*. The experimental approaches used are designed according to the suggested expression patterns of the candidate genes. All three candidates (*AtTPK1*, *AtKUP12* and *AtHAK8*) are highly expressed in guard cell and embryos (Hruz et al. 2008). Along with the well-studied K^+ channel AtTPK1, the effect of this approach is analysed through growth under stress treatments, K^+ starvation experiments and stomatal conductance under ABA treatment (Figure 2-2).

  In germination assays, we first examined the *kup12* knockouts, which have not been examined before for any germination phenotype. Secondly, we combined mutants of *tpk1*, *hak8* and *kup12* in doubles and triples investigating the redundancy possibility between these candidate transporters. We based this test on the potentiality of tonoplast localization for both AtHAK8 and AtKUP12. The high expression levels in embryo for the three candidates and the confirmed tonoplast localization of AtTPK1. We also measured water loss from leaves. By this, we can determine if there is any redundancy in the function between these candidates under abiotic stresses applied if any. The expected phenotype could be related to the disruption of K^+ movement across either tonoplast or plasma membrane. (Ahn et al. 2004; Armengaud et al. 2004; Whiteman et al. 2008).
- Finally, conducting this analysis can achieve a better understanding of K^+ uptake/transport of plants under stress and apply this information in improving plant tolerance and productivity during harsh environmental conditions.

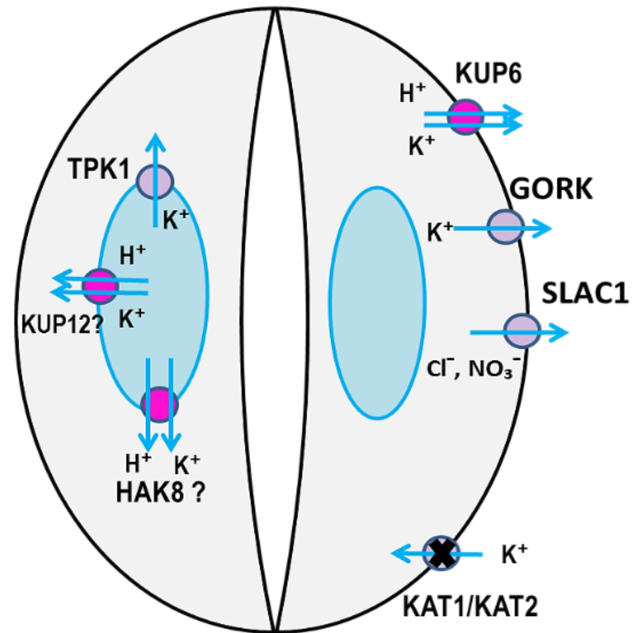


Figure 2-2: Predicted locations and roles of candidate transporters during the stomatal closure.

(HAK8 and KUP12) in Arabidopsis in the guard cell (Hruz et al. 2008). The loss of function of AtHAK8 and AtKUP12 along with the vacuolar AtTPK1 (Czempinski et al. 2002) in the guard cell vacuole, can lead to a stronger phenotype during stomatal movement. Other K^+ channels/transporters are involved in the stomatal movement during closure (AtKUP6, AtGORK) (Hosy et al. 2003; Osakabe et al. 2013). HAK8 and KUP12 have been investigated for potential roles in stomatal closure. Blue ovals represent vacuoles.

2.3 Materials and methods

2.3.1 Genotyping of T-DNA insertion lines

Seeds of *Arabidopsis thaliana* ecotype Columbia-0 were used in this study as wildtype, while the loss of function mutant lines of candidate genes were obtained from the SALK institution (http://signal.salk.edu/cgi-bin/tdnaexpress). T-DNA insertion mutants of AtTPK1 (At5g55630), HAK8 (At5g14880) and KUP12 (At1g60160) were *tpk1* (SALK_146903), *hak8* (SALK_041357.56.00) with the insertion position in the first exon for both, and *kup12-1* (used in all experiments) and *kup12-2* (used in the low K^+ experiment for confirmation) (SALK_045392.55.00, SALK_083613C) with the insertions in the third and eighth exons respectively (Figure 2-4). All mutants were in the Columbia-0 background.

In water loss experiments, the quadruple mutant of *abi1124* was used as a positive control. *abi1124* is a loss of function mutant of 4 ABA receptors (PYR1, PYL1, PYL2, and PYL4) that fails to close stomata under drought conditions, and is insensitive to ABA (Gonzalez-Guzman et al. 2012). Ripened seeds from same patches were stored in Eppendorf tubes at room temperature. Plants were grown in a growth room with 22 °C temperature with 16-h light/8-h dark. Relative humidity was 70% and light intensity was 120 µmol m^{-2} s^{-1}. Generally, plants were 3-4 weeks old during experiments unless differently mentioned.

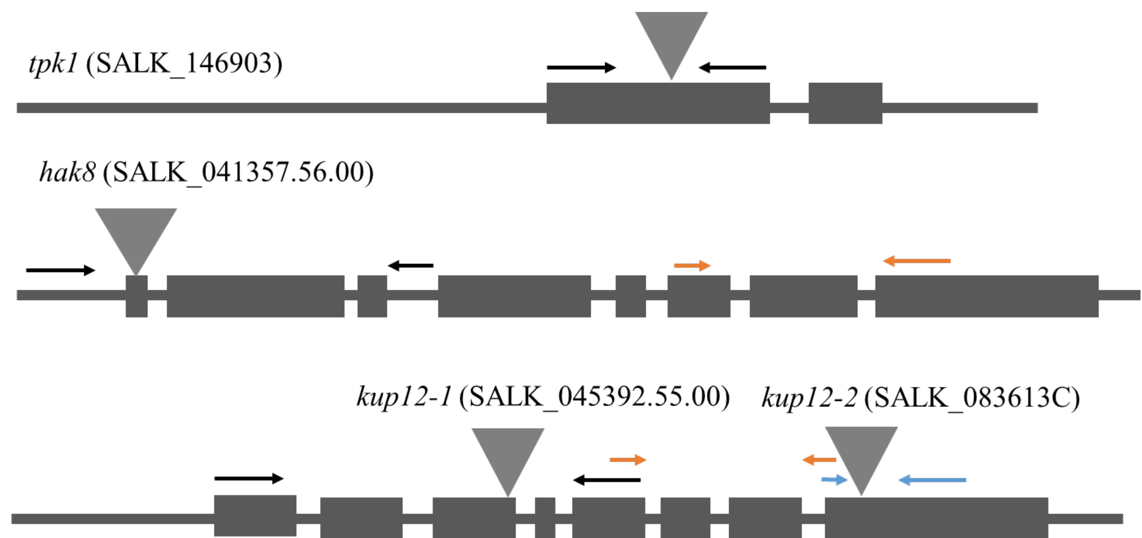


Figure 2-3: Diagram of the mutant lines used in experiments and positions of T-DNA insertions.

One allele for TPK1 and HAK8: (SALK_146903) and (SALK_041357.56.00) with T-DNA insertion in the first exons of both genes. Two mutant lines were used for KUP12 (SALK_045392.55.00, SALK_083613C), insertion in third and eighth exon respectively. In *attpk1*, black arrows indicate the GSP used in standard and RT-PCR, in *athak8* and *atkup12-1*: black arrows indicate GSP used in standard PCR while orange arrows are GSP used in RT-PCR, in *atkup12-2*: blue arrows indicate GSP used in standard and RT-PCR analysis.

2.3.1.1 DNA extraction

DNA was extracted using CTAB buffer method (Doyle 1991). CTAB extraction buffer consisted of 2 % CTAB (2 g/100 ml), 1.4 M NaCl, 100 mM Tris-HCl (pH 8) and 20 mM Na-EDTA. Fresh leaves of 1-4 weeks old plants were ground (approximately 100 mg) after shock-frozen in liquid nitrogen then 450 µl of warm CTAB buffer mix were added to the samples in Eppendorf tubes and incubated on 60 ºC for 45 minutes. A mix of

chloroform and Isoamyl alcohol in a (24:1, v/v) ratio (300 µl) was added to samples and vortexed. Tubes were centrifuged for 5 minutes at 14,000 x g. The clear top supernatant was moved to new tubes, 2 volumes of 96% ethanol and 4% of 3 M Na-acetate (pH 5.2) were added to samples and vortexed briefly then left in room temperature for 1 hour or more to precipitate the DNA. Samples were centrifuged at 14,000 x g for 10 minutes and supernatant was removed. The DNA pellet was washed using 70% ethanol then air-dried. Deionized distilled water (DW) was added to resuspend the pellet (100 µl) and kept in -20 °C until used.

2.3.1.2 Polymerase chain reaction analysis (PCR)

PCR was used to identify the existence of the T-DNA insertions in the single, double and triple mutants. Reagents used in concentrations and quantities as listed in Table 2-1. A PCR analysis mix consisted of 5 µl of the Go-Taq Flexi buffer (5X), 2 µl of $MgCl_2$ (25 mM), 1 µl of dNTPs (10 mM), 1 µl of primers used (10 µM), 0.1 µl Go-Taq polymerase (5 units/µl, 20 ng of tested DNA). Distilled water (DW) was used to complete the reaction to 25 µl. Annealing temperature and extension time were designed according to the primers used and the expected product size. Different sets of primers were designed using the PRIMER3PLUS tool (Untergasser et al. 2012). The LBA1 primer that anneals to the left border of the T-DNA insertion was used to detect the existence of the insertion. Primers used in these reactions are listed in Table 2-2.

PCR products were then resolved in 1% agarose gel (w/v). The buffer mix used -TAE buffer- (Tris-Acetate-EDTA) was prepared as a 50X stock solution consisting of 242 g of Tris base, then dissolved in 750 ml of DW. Then 57.1 ml of acetic acid glacial were added carefully and 100 ml of 0.5 EDTA (pH 8.0). This stock was used to create 1X TAE buffer for electrophoresis. Agarose was added according to the product size using 0.8 % of agarose for a 1000 bp product. Increasing the percentage of smaller product sizes and reducing it to larger. 80-90 V were used for 45 minutes or adjusted according to product size and analyses conditions. Standard PCR conditions were used in all analyses as described in Table 2.3 unless conditions were modified due to different product lengths or primer requirements.

Table 1: Reagents used in PCR analyses.

Reagent	**25 µl reaction**
5X Go Taq green flexi buffer	5 µl
$MgCl_2$ 25 mM	2 µl
dNTPs 10 mM	1 µl
Primers 10 µM	1 µl of each primer
Go Taq polymerase enzyme (Promega) 5 units/µl	0.1 µl
Genomic DNA (Variable)	(usually 2 µl)
Distilled water (DW)	to 25 µl

Table 2: **Primers used in genotyping single, double and triple mutants.**

Primer	**Sequence 5`-3`**	**product size (bp)**
TPK1-for	CAGCTCGTACGCCATTGTTAC	913
TPK1-rev	CCAACAACTCCATCTTCATCG	913
HAK8-for	ACTACCTGATGCAGCAAATCC	1115
HAK8-rev	TCTTGATTTTGTTGGCCAAAC	1115
KUP12-1-for	CATAAGACGTGTTGGTACCGG	1120
KUP12-1-rev	CTGAACAAAGCCACGAGAATC	1120
LBA1	TGGTTCACGTAGTGGGCCATCG	

Table 3: Standard PCR analysis conditions

Step	Temperature	Time
Denaturation	95 °C	2 min
Annealing	55 °C	30 sec
Extension	72 °C	1 min 4 sec
Cycles	30	

2.3.2 Semi-quantitative reverse transcription analysis (RT-PCR) of mutants

2.3.2.1 RNA isolation

Approximately 100 mg of Arabidopsis leaves were ground to a fine powder after shock-frozen in liquid nitrogen using mortar and pestle. Total RNA was extracted using the RNeasy kit (Qiagen, UK) according to manufacturer's manual. Concentrations were then measured using a spectrophotometer.

2.3.2.2 cDNA synthesis

First-strand cDNA was synthesized using Superscript™ II reverse transcriptase kit. Approximately 2 µg of RNA, 1µL of 0.5 µg Oligo (dT), 1µL of 10 mM dNTPs were mixed. The mixture was heated to 65 °C for 5 minutes and quickly chilled on ice. Then 4 µL of 5X first-strand buffer, 2 µL of 0.1 M DTT and 1 µL of RNaseOUT™ were added to the mixture, then it was incubated at 42 °C for 2 min. 1 µL of Superscript™ II RT (reverse transcriptase) was added and mixed gently by pipetting, then again incubated at 42 °C for 45 min. The reaction was deactivated by heating at 70 °C for 15 min. cDNA was then chilled on ice and kept in -20 ºC freezer.

2.3.2.3 RT-PCR of mutants

cDNA was tested for any transcript using gene specific primers (GSP). The PCR mix was prepared by mixing same reagents in table 2-1 except for genomic DNA, which was replaced by 2 µL of 10 times' diluted cDNA. Different PCR conditions were designed according to primers (Table2-3) annealing temperature and size of amplicon expected.

Table 4: Primers used in the semi-quantitative RT-PCR to detect any transcripts in the single, double and triple mutants.

Primer	Sequence 5`-3`	Product size (bp)
TPK1-for-RT	CAGCTCGTACGCCATTGTTAC	913
TPK1-rev-RT	CCAACAACTCCATCTTCATCG	913
HAK8-for-RT	GCACCATGTCCTCCAGAGTG	500
HAK8-rev-RT	TGAAGGAGAGTGCGACTGGA	500
KUP12-for-RT	ACAGGCTTGGTCTGCTCTTG	500
KUP12-rev-RT	TGCTAACCATCATTACACCTACCTC	500
ACTIN- for -RT	ACGAGCAGCAGATGGAAA	490
ACTIN- rev -RT	ACCCCAGCTTTTTAAGCCTTTG	490

2.3.3 Germination and stress experiments

Seeds were surface-sterilized in 70% (v/v) ethanol for 5 minutes, followed by 10 minutes in 10% bleach (v/v), and then washed in sterile water four times. Seeds were stratified in sterile water for four days at 4 °C. For Petri plates experiments, 0.8% agarose (w/v) (Sigma-Aldrich) was used for medium solidification to prevent any K^+ contamination.

Initially, we used Murashige and Skoog (MS) medium (Sigma-Aldrich) in this study (Murashige and Skoog 1962) (See appendix), with modifications to create the desired stress conditions. MS medium was used in half strength for control conditions, which contained 10 mM of K^+. In the K^+ free medium, KNO_3 salts were substituted with $NaNO_3$ salts in equimolar amounts. For salinity and osmotic stress experiments, NaCl and sorbitol were added in the studied concentrations to create stress in the media. The half strength medium contained 10 mM NH_4^+ in both control and K^+ free conditions (See appendix), while the free K^+ medium contained 9 mM of $NaNO_3$.

Subsequently, a test experiment was conducted testing germination over free NH_4^+ / low K^+ medium (Spalding et al, 1999). We used two NH_4^+ levels (0 and 5 mM) combined with 50 µM K^+ added as KCl (see appendix).

All germination experiments were carried out in square Petri plates of 13*13 cm. Seeds used in all experiments were harvested from plants that were growing in parallel and under same growth conditions, they were harvested in the same time and stored under same conditions. Plants were grown in growth rooms at 20 °C, relative humidity 50%, light density 45-100 μmols. All plants were grown in short day conditions. Each experiment contained three replicates per treatment and each replicate contained 50 seeds/genotype. Post-germination, seedling establishment was evaluated by scoring the emerged cotyledons after 7 days of sowing using a binocular.

In abiotic stress experiments of seedlings, mutants were divided into 3 groups, each two single mutants along with their cross (double mutant) have been tested together in same Petri plates. Double mutants of (*hak8kup12*) and triple mutants of (*hak8kup12tpk1*) were tested together in independent experiments. Wildtype plants were tested in same plates with mutants and used as a control. Seedlings were grown at first on a solid half MS medium for 1-2 weeks, and then transferred to treatments before weighed for fresh weights. Agar was used in solidifying the media. The treatments used were 0 K^+, 80 mM KCl, 80 mM NaCl, 80 mM and 120 mM sorbitol. 10-14 days old seedlings of similar sizes were transferred to treatments then plates and placed vertically. Fresh weights were measured 3 weeks after transferring plants. Each treatment had eight plants per each genotype. The experiment was repeated three times.

2.3.4 Generating double and triple mutants

Flowering plants of single homozygous mutants were used for crosses (*tpk1*x*kup12*, *tpk1*x*hak8*, *hak8*x*kup12* and *hak8*x*kup12*x*tpk1*). The *tpk1 and hak8* mutants were used as a donor, no reciprocal crosses were carried out. F1 seeds were collected once ripened, and then grown on soil. Then plants were scanned using both GSP and T-DNA primers for heterozygote plants of the two knockouts of the desired two genes. Double heterozygous plants were kept to set seeds. F2 seeds were collected, grown, and scanned using the same primers. Triple mutants were generated by crossing *kup12tpk1*x*hak8tpk1*. *hak8tpk1* was used as a pollen donor. Double and triple knockouts were identified and kept for seed collection (F3).

A crossing protocol was followed from (Weigel and Glazebrook 2006), where female flowers were chosen before the formation of pollens in anther to avoid self-pollination, while male flowers were chosen with the petals opened with visible pollens. Top two flowers were avoided and young healthy plants were used to increase the success of the crosses. Scissors were used to remove the unwanted flower and leaves. Jeweler's forceps and dissecting microscope were used to remove sepals, petals and anthers from female flowers carefully without hurting the stigma. Then, male flowers with clear pollens were patted on top of the stigma until pollen was seen clearly covering it. Those were wrapped quickly with parchment paper to avoid dryness and cross-fertilization, then labelled with coloured adhesive tape and attached to wooden sticks to keep the stem vertical. Seeds were collected from the successful crosses after siliques turned yellow.

2.3.5 Growth rescue of the K^+ deficient yeast by the AtKUP12 transporter

To test the function of KUP12 in heterologous systems, *AtKUP12* was cloned into a K^+ deficient yeast strain of *Saccharomyces cerevisiae* (pYE52) W delta 3(*trk1, trk2, tok1*) with the genotype of (*mata, ura3 his3 trp1 ade2 trk1D::LEU2 trk2D::HIS3 tok1D::TRP1*). This strain lacks the main K^+ transporters (TRK1, TRK2 and TOK1) in the plasma membrane and cannot grow on low K^+ concentrations (Bertl et al. 2003). It was obtained from Maathuis laboratory, department of biology, York University. Wildtype strain of W303 was used as a control in K^+ deficiency experiments. The wildtype is a parental strain MG100 (MATa {*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11, 15*}).

2.3.5.1 Amplifying full coding sequence

RNA was isolated from leaves of 4 weeks old *Arabidopsis thaliana* ecotype Columbia-0 as mentioned previously in methods under 2.3.2.1 and cDNA was synthesized from this RNA as in 2.3.2.2. The *AtKUP12* full coding sequence was amplified from the cDNA using gene specific primers (Table 2-4). The Q5® High-Fidelity DNA polymerase from New England Biolabs (NEB) was used in the amplification. The protocol of the PCR was as manufacturer's manual for a 50 µl reaction. Two steps PCR was applied using firstly, primers that amplify the full coding sequence. Secondly, desired restriction sites for the BamHI and NotI restriction enzymes were added in another PCR using modified primers

(Table 2-4). These two enzymes do not cut in the insert and are located in the multiple cloning site (MCS) of the used plasmid. Few bases were added upstream the restriction sites to improve the cutting efficiency. Then PCR was run again with same procedures in step one but with a minor change according to the new primers which have higher annealing temperature. The temperature was changed from (30 cycles of 55 °C for 30 seconds) to (5 cycles of 60 °C then 30 cycles of 72 C for 30 seconds), in the annealing step.

Table 5: Primers used in amplifying the full coding sequence of *AtKUP12* and the addition of restriction sites BamHI and NotI.

Primer	Sequence 5`-3`	Product size (bp)
AtKUP12-FS-for	tgcttcttctcgttttggagga	2608
AtKUP12-FS-rev	acagacgtcaaccgcaagaa	2608
AtKUP12-for-BamHI	AGTGGATCCtgcttcttctcgttttggagga	2628
AtKUP12-rev-NotI	TAAGCGGCCGCacagacgtcaaccgcaagaa	2628

2.3.5.2 The plasmid vector pYES2

The pYES2 plasmid (Invitrogen) is a shuttle plasmid that can multiply in both bacteria and yeast. pYES2 is 5.9 kb size and contains a GAL1 promoter that is induced by galactose. It also contains the URA3 gene for uracil selection of transformants. Carbenicillin was used for selection of *E.coli*. pYES2 contains a (MCS) as shown in (Figure 2-3).

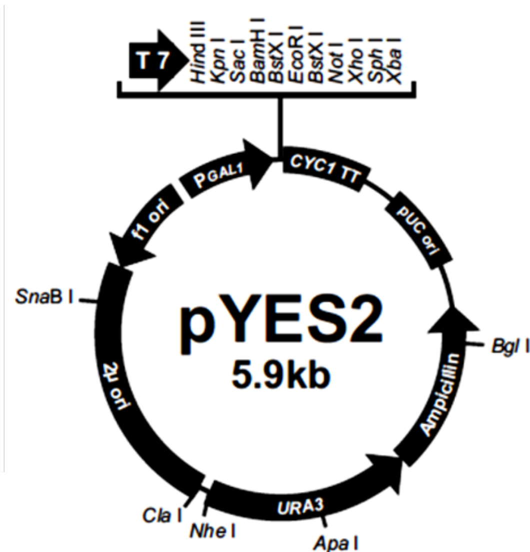


Figure 2-4: The pYES2 vector plasmid (Invitrogen).

GAL1: Galactose induced promoter, T7 promoter, multiple cloning site (MCS), ampicillin resistance gene, URA3 gene for uracil selection.

2.3.5.3 Double digestion (DD) of AtKUP12 and pYES2 using BamHI and NotI restriction enzymes

To create sticky ends in both insert (*AtKUP12*) and plasmid (pYES2), two restriction enzymes were used (BamHI and NotI) from New England Biolabs (NEB). Buffer 3.1 from (NEB) was used for digestion. 1 µg of *AtKUP12* and pYES2 were separately digested at 37 ºC for 4 hours. Digestion mixes were heat inactivated at 65 ºC for 20 minutes then mixtures were purified using MiniElute Reaction Cleanup Kit from QIAGEN according to the manufacturer's manual. Digested samples were resolved on agarose gel 0.7% (w/v) at 100 volts for 30-45 mins. 2 log DNA ladder was used here from (NEB).

2.3.5.4 Purification of the digested plasmid from gel

Cut and uncut pYES2 plasmid were run on gel to confirm linearization of the plasmid by digestion. Cut pYES2 was gel extracted using QIAquick Gel Extraction Kit from QIAGEN following manufacturer's manual. Once plasmids were purified, concentration was measured using the spectrophotometer.

2.3.5.5 Ligation of AtKUP12 into pYES2

After creating sticky ends in the *AtKUP12* insert and the pYES2 plasmid using restriction enzymes, a 20 µl ligation mix was prepared. An insert: vector molar ratio of 3:1 was used. The ligation mix contained 1 µl of T4 DNA ligase enzyme, 2 µl of T4 DNA ligase buffer from (NEB) and the insert and vector DNA. The volume was completed to 20 µl with DW, then gently mixed by pipetting and collected by brief centrifugation. The mix was incubated overnight at 16 ºC then heat inactivated at 65 ºC for 10 minutes and quickly chilled on ice. The ligation mix was then used in transformation.

2.3.5.6 Transformation of Escherichia coli competent cells

Glycerol stocks of the Mach1™ T1R competent cells (Invitrogen) that were kept under -80 °C, were used for transformation of the AtKUP12-pYES2 construct. Transforming the pYES2 plasmid into competent cells was carried out using the manufacturer's manual by the heat shock method.

2.3.5.7 Colony PCR

Single colonies were picked and re-streaked on fresh Luria Broth (LB) plates with carbenicillin. The LB medium consisted of 5 g yeast extract, 10 g tryptone, 10 g NaCl and 950 ml of DW. Single colonies PCR was carried out to detect the insertion in the construct. Sterile toothpicks were used to pick single colonies, colonies were barely touched then sterile toothpicks were dipped into sterile water a few times. Then 2 µl of this were used as a template DNA. The purpose of this practice is to reduce as much as possible the DNA amount from colonies to avoid too much material that can inhibit the PCR. *AtKUP12* primers (Table 2-3) that were designed for RT-PCR were used to amplify the insert.

2.3.5.8 Isolation of plasmids

Colonies with successful ligation that grew on selective carbenicillin LB medium were picked and freshly re-streaked over new LB plates. To isolate the plasmid, NucleoSpin® Plasmid kit (MACHERY-NAGEL) was used. Ten colonies were selected and plasmids were extracted using the kit according to manufacturer's manual.

2.3.5.9 Diagnostic digestion of the construct

To confirm the ligation of pYES2 and *AtKUP12*, a digestion using BamHI and NotI was carried out on isolated plasmids. Diagnostic digestion was done as previously mentioned in the methods, plasmids were checked in this test. Successful digestions that showed two bands corresponding to empty vector size and insert size were confirmed to have successful ligation and were then sequenced.

2.3.5.10 Sequencing of isolated plasmids

To confirm the sequence of the insert ligated to the vector is free from errors, minipreps that successfully showed two bands after diagnostic digestion were sequenced. The sequencing was performed using GATC sequencing service. Using Primer3plus, 4 primers were designed and used in the sequencing (Table2-6).

Table 6: **Primers used in sequencing plasmids.**

Uppercase letters are primers in the coding region. Lowercase letters are primers in the 3' UTR.

Primer	Sequence
AtKUP12-seq-1	ttcatggagatatccagaggaga
AtKUP12-seq-2	TTTCCCTGACCTCGCTTTGG
AtKUP12-seq-3	AGAATATGCGAGCTGAGGCC
AtKUP12-seq-4	TGGTGTTAGAATTCCATCGCCA

2.3.5.11 Yeast transformation

The transformation of the K^+ deficient yeast was carried out for AtKUP12/pYES2 and the empty vector according to the high efficient protocol using the LiAc/SS carrier DNA/PEG method (Gietz and Schiestl 2007). The competent yeast cells were aliquoted in 500 µl tubes and then 2 µg of plasmid DNA were added. Transformed yeast was selected on a selective medium without uracil. The selective medium used was Yeast Nitrogen Base (YNB) without amino acids and ammonium sulphate 1.7 g/L (FOREMEDIUM) which contains about 7.3 mM of K^+, 1.92 g/L of Yeast Synthetic Drop-out Medium Supplements without uracil (Sigma-Aldrich), 20 g/L glucose, 100 mM KCl, $(NH_4^+)_2SO_4$ and 20 g/L agar. Plates were incubated at 30 ºC for 3-5 days. Empty vector transformed yeast (used as a control) and the AtKUP12-pYES2 construct plus wildtype

were tested in this assay. The drop-out medium is used to select the plasmids transformed into yeast. Plasmids like pYES2 can grow on a medium that lacks a component like uracil, an amino acid. Therefore, when yeast transformed with pYES2 grow on a medium that lacks uracil-a drop-out medium- it doesn't need to be added to the medium. While untransformed strains cannot grow without supplying the medium with uracil.

2.3.5.12 K^+ starvation assays of yeast on agarose plates

Single colonies of empty vector and plasmid transformants plus wildtype yeast strain were inoculated in the liquid selective medium without uracil or K^+ (same as in transformation plates but without K^+ or agar addition), while uracil was added for the media that were used for growth of the untransformed strains (wildtype). The liquid medium used here is same as transformation medium but with extra 5 mM of K^+, using KCl to enable the K^+ deficient yeast to grow. According to (Bertl et al. 2003), the triple mutant yeast can grow on a 10 mM K^+ medium, which is a weak growth, so the K^+ medium was increased up to 12 mM. Cultures were incubated in 5 ml volume media in a 50 ml falcon tubes, then placed on a shaker adjusted to 200 rpm at 30 ºC for 18 hours or overnight.

Yeast cells were harvested by centrifugation at 4000 rpm for 1-2 minutes. Then cells were washed using sterile double distilled water 3 times and resuspended in water to reach the density OD600 of 1 ± 0.05 (Bertl et al. 2003). Fivefold serial dilutions of each genotype were prepared and 5-7 µl of each dilution were spotted on agarose plates. The medium used for K^+ deficient treatments was arginine-phosphate (AP) medium (Rodríguez-Navarro and Ramos 1984; Alemán et al. 2014). The AP medium consists of 10 mM L-arginine, 8 mM phosphoric acid, 2 mM $MgSO_4$, 0.2 mM $CaCl_2$, 2% glucose or galactose, recommended concentrations of a 100X stock solution of vitamins and 1000X stock solution of trace elements (Rodríguez-Navarro and Ramos 1984), amino acids (leucine, tryptophan and uracil when the untransformed strain was used) and adenine. pH was adjusted to 6.5 using L-arginine. Agarose (Sigma-Aldrich) was used for medium solidification to prevent any K^+ contamination that might occur if agar is used, it was added at 20 g/l. Plates were then scanned to compare the growth of genotypes used. The scanner used was a 1200 dpi resolution. K^+ concentrations tested in experiments were

added to the medium in the concentrations of 50 μM, 100 μM, 2 mM and 100 mM using KCl. The growth was checked after 2, 3 and 5 days.

2.3.6 Stomatal conductance under ABA treatment

The opening buffer was prepared by mixing: 10 mM KCl, 10 mM MES-KOH (pH 6.15) (Gobert et al. 2007). Detached leaves of same sizes from 4-6 weeks old plants (6-10 per genotype) were numbered and floated on the opening buffer for 2 hours under light to enable maximum opening. Leaves were taken of the solution, wiped gently then placed individually in the Infrared Gas Analyser chamber (IRGA) of the Li-Cor 6400 (LI-COR Cambridge-UK). Conductance was measured for all leaves and appointed as 0 time. Once a leaf was measured, it was put back to the opening solution again until all leaves were measured for conductance. Half of the leaves were treated with 10 μM of ABA (Sigma-Aldrich), which was prepared as a stock of 100 mg of the ABA dissolved in 1 ml of methanol. This stock was used to be added to the opening buffer while the rest of leaves were left in the opening buffer only, adding the same volume of methanol to it. These leaves were used as a parallel control for any possible drifts that might be resulted from natural reduction in conductance. Reduction in conductance was measured after 10-15 minutes of ABA treatments and at certain successive time points that were kept through the experiment (0-10-20 minutes or 0-15-30 minutes). The experiment was repeated at least three times unless differently mentioned.

2.3.7 Water loss measurements

Rosettes of 4-5 weeks old soil grown plants of both wildtype and mutants were cut, marked and immediately weighed at the start of the experiments. All rosettes were cut at the same stage. Then rosettes were left at room temperature on either the bench or the flow hood. Fresh weights were measured every 30 minutes until the end of the experiment. Five leaves of each genotype were used and each experiment was repeated at least 3 times. *abi1124* mutants were used as a control. The plants were grown on soil in pots at 20 °C for 16-hours light/ 8-hours dark photoperiod and 60% (RH), and watered well to avoid any drought that might affect the measurements.

2.4 Results

2.4.1 Genotyping of ***tpk1***, ***hak8*** and ***kup12*** single mutants using PCR analysis

Multiple standard PCR analyses were carried out using GSP and *actin* primers as a control for each mutant. We tested the single mutant lines of *tpk1, hak8, kup12-1* and *kup12-2* for homozygosity of the T-DNA insertion by the lack of wildtype copy of the gene.

As shown in Figure 2-5 (Upper panel), GSP were tested first with wildtype DNA and showed the existence of the gene (Figure 2-5), while the four single mutants were tested and showed the lack of the gene product. T-DNA primer (LBA1) and GSP were used to test the existence of the T-DNA insertions in single mutants. All mutants showed positive results with the T-DNA primers. This, with the lack of gene product using GSP indicate that all mutant lines obtained were homozygous for the T-DNA insertion. Plants were kept for seed collection to be used in the physiological analysis. All reactions were repeated at least three times.

We conducted an RT-PCR analysis to confirm the complete loss of function in the single mutants used. cDNA of single mutants was tested with *Actin* primers as a control (Figure 2-5, lower panel). Wildtype showed the expected band sizes, then mutants were tested with the GSP and showed a complete disruption of the gene in the four single mutants under study.

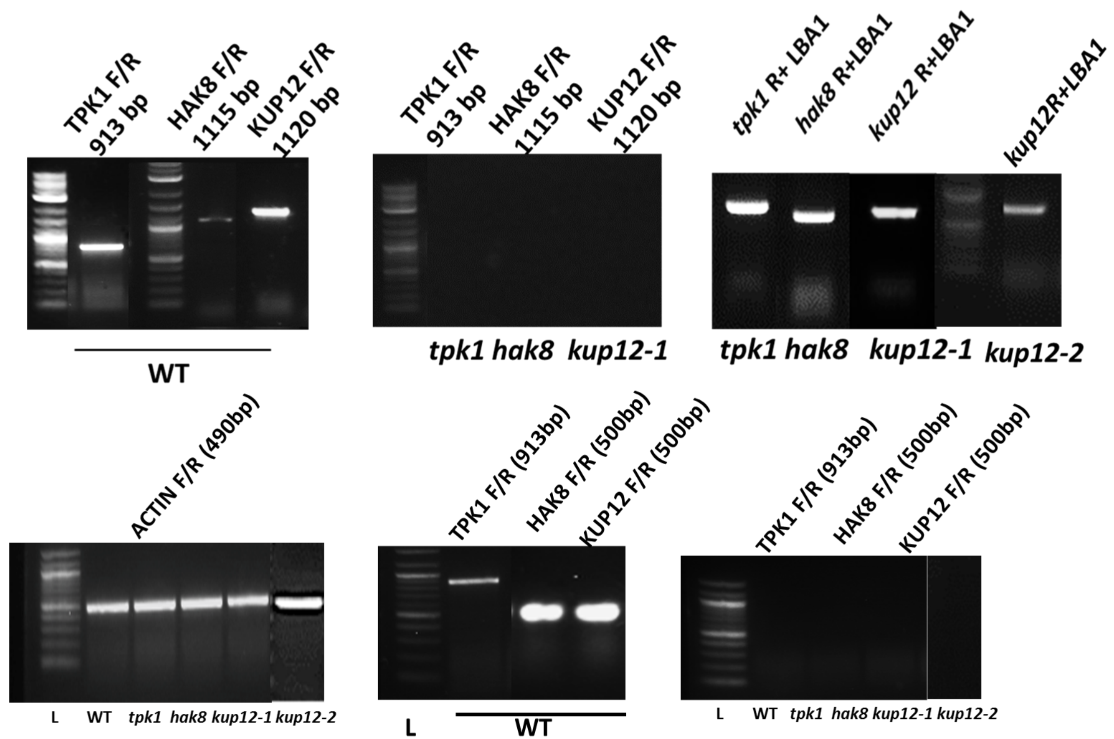


Figure 2-5: Confirming T-DNA insertions in single mutants using standard PCR and RTPCR.

(Upper panel): PCR analysis indicates the existence of specific gene bands in the wildtype, lack of bands in single mutants and single mutants tested with T-DNA primers. **All lines showed existence of T-DNA insertions. (Lower panel):** RT-PCR shows *ACTIN* used as a control, the existence of genes products in wildtype and lack of products in single mutants.

2.4.2 Generation of double and triple knockouts

After generating double and triple mutants using crossing, multiple mutants were tested using GSP of HAK8, KUP12 and TPK1 and T-DNA primers, while the *hak8kup12* and *hak8kup12tpk1* mutants were tested in an RT-PCR analysis (Figure 2-6). First double mutants of *hak8tpk1* and *kup12tpk1* were tested using GSP to show the lack of product (Figure 2-6, A). The figure shows the lack of product when DNA was tested using GSP of HAK8 and TPK1 confirming that it is a double loss of function mutant, while GSP and T-DNA primers showed the existence of two insertions in the double mutant. Same results were obtained for the *kup12tpk1* double mutants (Figure 2-6.B), which confirms it is a double mutant. Later when we generated the double mutants of *hak8kup12* and the triple mutants of *hak8kup12tpk1*, we tested the plants in an RT-PCR analysis using GSP primers (Figure 2-6,C). *Actin* primers were used to check the cDNA with all genotypes WT,

kup12hak8 and *kup12hak8tpk1*, and the GSP of *TPK1*, *HAK8* and *KUP12* to test the complete knockout of the generated mutants. Double and triple mutants were successfully obtained and confirmed after PCR analysis. Plants that showed the required genotype in the PCR analysis were kept for seed harvesting.

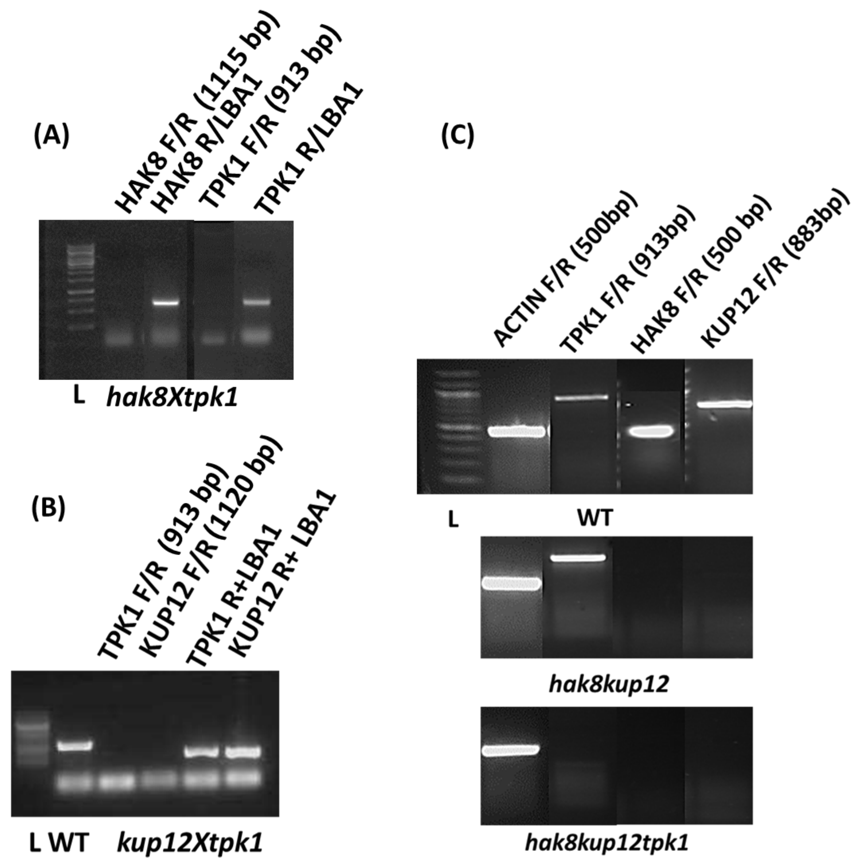


Figure 2-6: PCR analysis of double and triple mutants.

A: Standard PCR was done to test the loss of function *hak8tpk1* with gene specific primers, which showed the lack of product, while the GSP and T-DNA primers showed the existence of the T-DNA insertion. B: *kup12tpk1* double mutant tested with gene specific primers then GSP and T-DNA primers. C: *hak8kup12* and *hak8kup12tpk1* mutants were tested in an RT-PCR analysis to confirm the loss of function of multiple genes (*TPK1*, *HAK8* and *KUP12*). Wildtype was used as control and *Actin* as a control gene. *hak8kup12* showed the absence of product of *HAK8* and *KUP12* genes but the existence of the TPK1. While *hak8kup12tpk1* showed no products for the three tested genes. All samples showed an *Actin* primers specific band. L represents the ladder used (2-log DNA ladder).

2.4.3 *atkup12* germination is severely inhibited by K^+ deficiency and AtTPK1 is epistatic to AtKUP12

To investigate the germination of the mutant seeds under low K^+ concentrations, a K^+ free medium was used first. Sowing seeds on this medium resulted in severe inhibition of germination (data not shown), so a minimum of K^+ was added to the medium to enable seeds to germinate. Germination of loss of function mutant seeds (*tpk1*, *hak8*, *kup12-1* and *2*, *hak8tpk1 (h8t1)*, *kup12tpk1 (k12t1)*, *hak8kup12 (h812), hak8kup12tpk1 (h812t1)*) was tested in comparison with wildtype under different low K^+ levels (10, 30, 50 and 200 μM K^+). It was first tested on 50 μM K^+ medium and resulted in significant differences. Therefore, two lower K^+ concentrations were used to test if the phenotype was stronger (10 and 30 μM K^+) and a higher concentration as well (200 μM). Germination was scored 48, 72 and 96 hours of moving plates from cold room to growth room. A seed was considered germinated when a clear radicle was seen under the light microscope, figure (2-7, A) shows a germinated seed of wildtype.

When seeds were plated on treatments (10, 30, 50 and 200 μM K^+), all genotypes showed significant inhibition in germination compared to control conditions. This inhibition persisted to the fourth day although highly decreased but was still significant. The reduction in germination rates decreased gradually with the increase of K^+ concentrations added to the medium. Under control condition, germination of most genotypes was comparable to wildtype after 4 days of treatments.

The severe inhibition in germination was due to the high NH_4^+/K^+ ratio resulted from removing K^+ salts from medium (see methods), we conducted a test experiment with a medium contained either 0 or 5 mM NH_4^+ combined with 50 μM of K^+ (see Appendix). In this experiment, only *kup12-1* showed same phenotype while *hak8kup12* and *hak8kup12tpk1* showed different responses to the treatment.

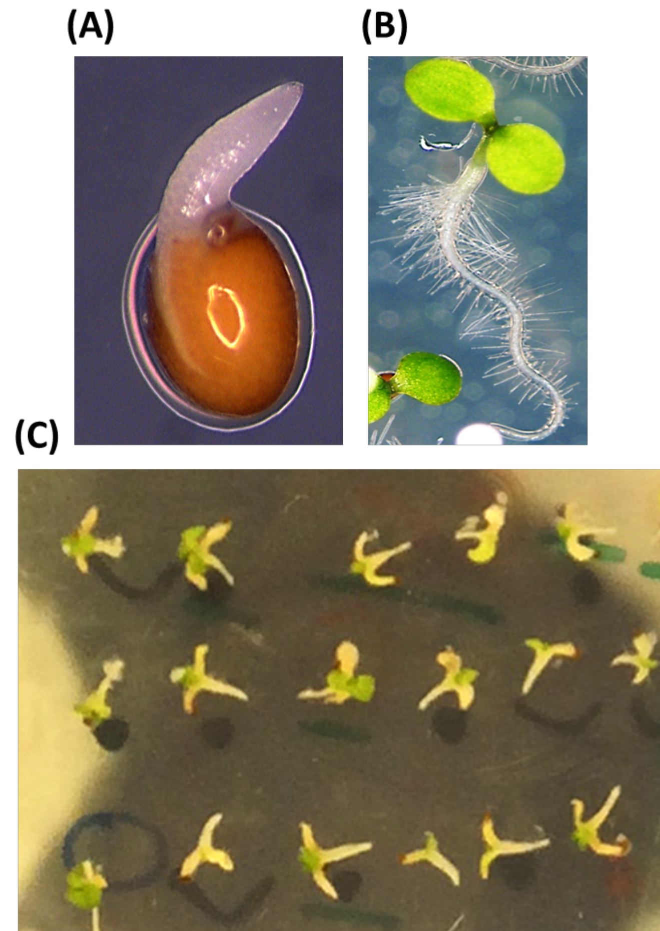


Figure 2-7: Stages of seed germination and inhibition of seedlings during K^+ starvation

(A): Seed germination was counted when radicle was visible. (B): Cotyledons emergence was counted to determine seedling establishment after 7 days. (C): Inhibition of growth root and chlorosis symptoms on 9 mM Na^+, 10 mM NH_4^+ and low K^+ treatment (10 µM K^+) in 7 days old seedlings.

2.4.3.1 Germination rates after 48 hours

After 48 hours, seeds of mutants showed variation in germination rates over 10 mM K^+ (control) treatment when compared to wildtype (Figure 2-8). *hak8* mutants showed higher germination. Osakabe et al. (2013) did not report any phenotypes for the single mutant *hak8* in their published work. However, they also used a different mutant line than what we used in our study. *atkup12-1* showed significant inhibition in germination under control conditions. Furthermore, the other allele (*atkup12-2*) showed same results on control medium (Figure 2-8). We can confirm that the loss of function of AtKUP12 resulted in such phenotype. The double mutants of *kup12tpk1* (*k12t1*) and *hak8kup12* (*h8k12*) showed a significant higher germination compared to wildtype. The post hoc analysis did not indicate any significant differences between wildtype and mutants under control conditions. We normalized data to control condition to exclude the effects resulting from variation of initial germination.

The 10 µM K^+ treatment affected seed germination negatively, all genotypes showed severe inhibition in germination compared to control. *atkup12-1* and *hak8kup12tpk1* single and triple mutants were the most inhibited genotypes.

Under 30 µM K^+ treatment, seeds showed different responses to K^+ starvation. The single mutant of *kup12* and the triple mutant of *hak8kup12tpk1* showed nearly same rates. They showed a significant reduction in germination compared to wildtype (Figure 2-8). Our data here are in accordance with Pyo et al., (2010), who indicated that low K^+ can affect the germination of seeds under the loss of function of certain transporters. They also indicated that the double mutants of *athak5 akt1* were significantly affected by the external K^+ when compared to the single mutants and wildtype. However, the inhibition in our results was very high compared to what was presented in this paper.

Applying post-hoc test revealed a significant difference between both wildtype and *kup12tpk1* in comparison to *kup12*, where at the same time wildtype and *tpk1* did not show any differences between each other and were both significantly higher in germination compared to *kup12*. This indicates that combining *tpk1* with *kup12* mutants resulted in recovering germination (*tpk1* masked the phenotype of *kup12*). We therefore suggest a possible epistasis relationship between KUP12 and TPK1.

Combining *kup12* with *hak8* mutants rescued the phenotype and rendered germination back to wildtype rates. The phenotype of *hak8kup12* was non-significant to wildtype or when compared to the single mutants of *hak8*. It is therefore tempting to suggest a possible epistasis relationship between KUP12 and HAK8. However, giving the previous work on HAK8 indicated it is a homolog for KUP2 and KUP6, such relationship between KUP12 and HAK8 could be irrational. Germination rates of *hak8tpk1* were not significant compared to the single mutants.

Under 50 µM, genotypes showed same trend, however, *hak8* showed significant higher germination compared to wildtype. The increase was non-significant after data normalization and was not present in the previous K^+ two concentrations or control treatment and therefore, we cannot confirm that this phenotype is biologically significant.

The triple mutants of *hak8kup12tpk1* showed phenotype that is similar to *kup12*. Germination was significantly inhibited under 30 and 50 µM K^+, however, post-hoc test indicated that there was no significant differences between treatments for the triple mutants.

2.4.3.2 Germination rates after 72 hours

After 72 hours, all genotypes showed increased germination rates under all treatments, however, the inhibition was still significant in low K^+ treatments when compared to control conditions (Figure 2-9). Germination under control treatment was almost completed for all seeds on the third day. Under 10 µM of K^+ the inhibition was still significant in *kup12, hak8kup12tpk1* mutants. Meanwhile, *hak8* and *hak8kup12* showed higher germination, but only *hak8* was significantly higher when data were normalized. *hak8* mutants did not show any significant differences in the 30 and 50 µM K^+ treatments though, consequently, we cannot consider this a phenotype for the mutant. Under 30 and 50 µM K^+, *kup12* and *hak8kup12tpk1* showed the same phenotype and were significantly inhibited. Other mutants did not show significant results but showed increased germination rates in comparison with 10 and 30 µM K^+.

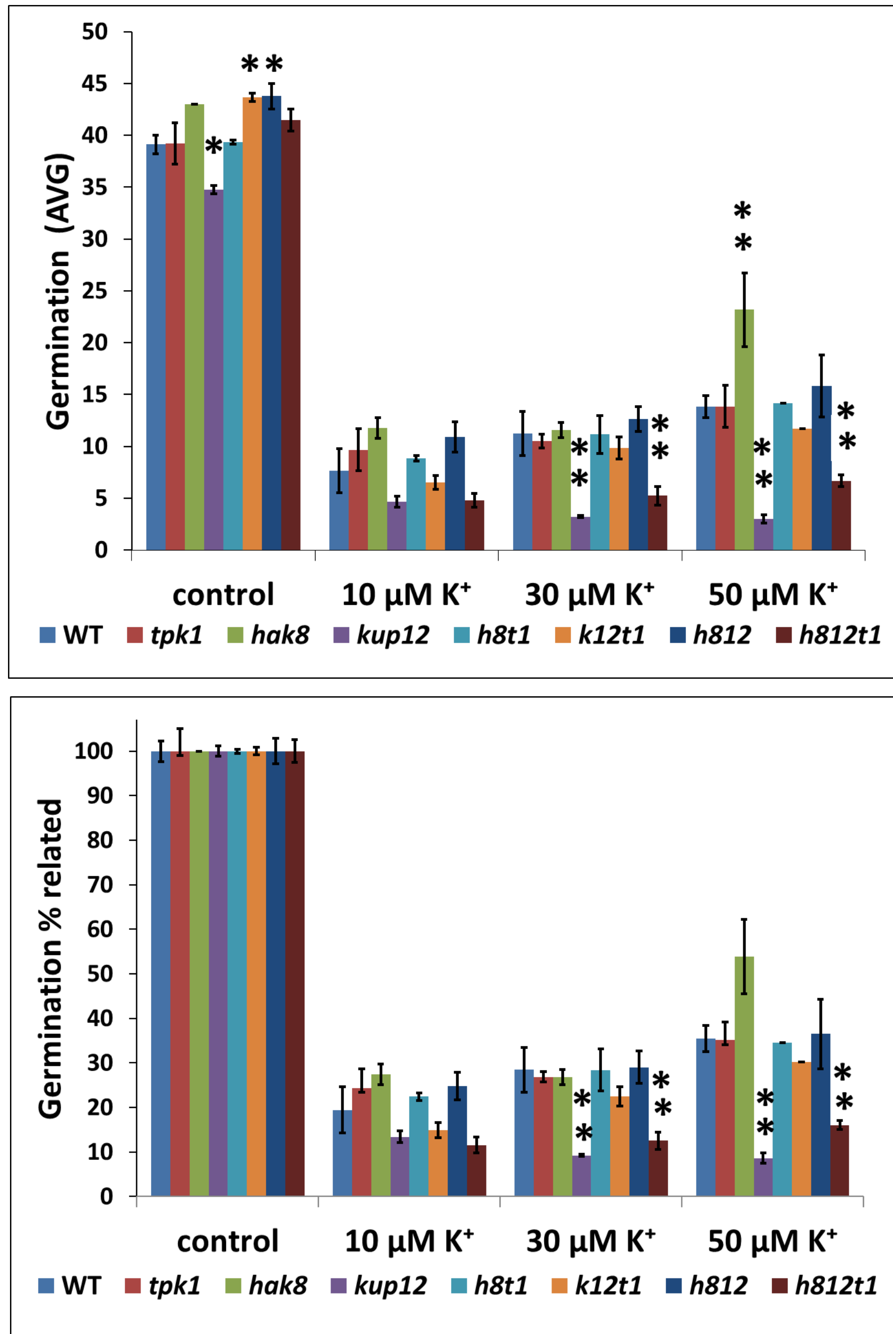


Figure 2-8: Effect of K^+ starvation on the germination of seeds under high NH_4^+/K^+ levels after 48 hours.

Absolute values (upper panel) and normalized to control (lower panel). Error bars are the standard error of the mean. Each experiment is repeated at least three timess. Asterisks represent significant results where $P < 0.05$ and is indicated with *, and $P < 0.01$ is indicated with **. Two way ANOVA was used to analyse data between wildtype and mutants, and between genotypes using Tukey as a post-hoc test.

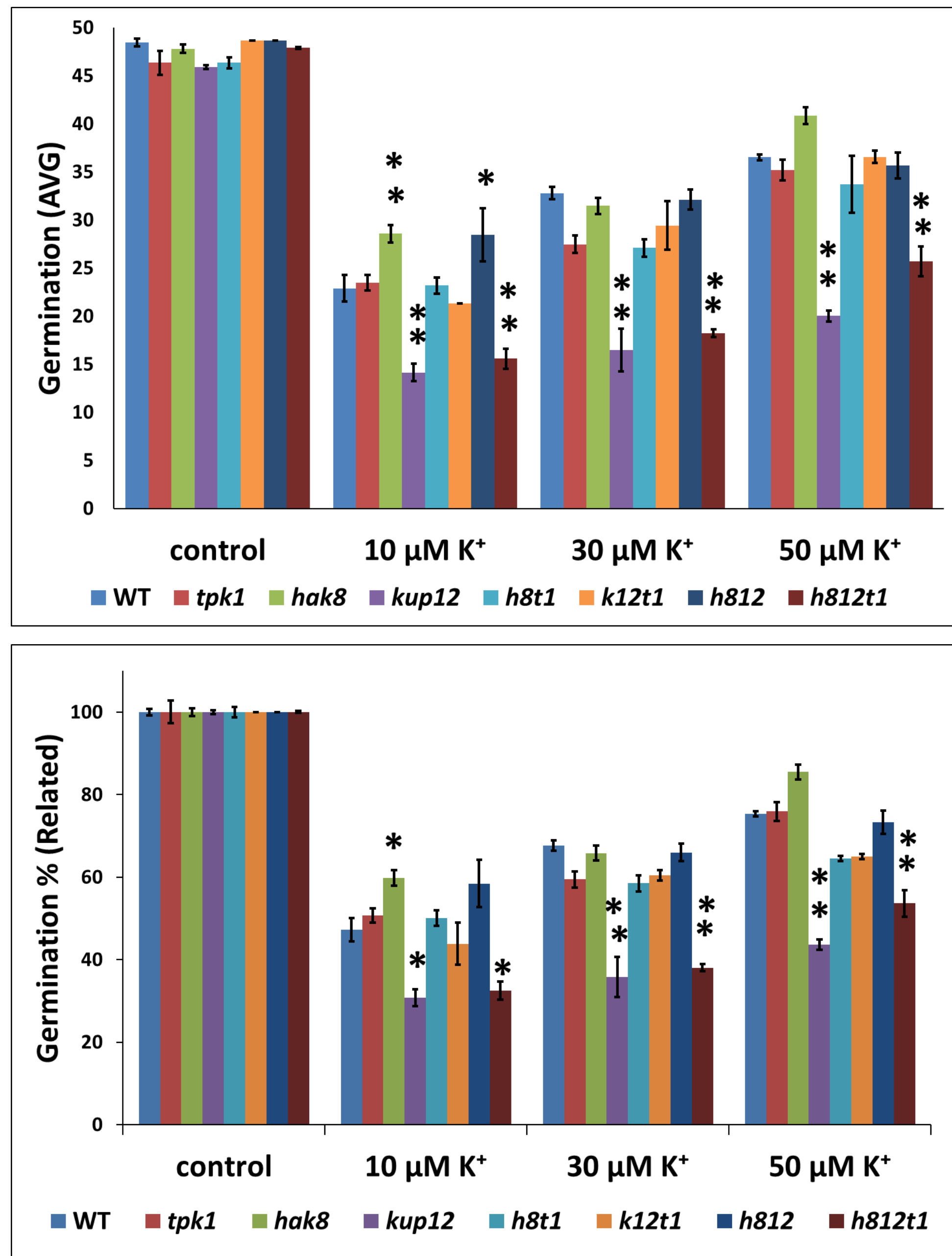


Figure 2-9: Effect of K^+ starvation on the germination of seeds under high NH_4^+/K^+ levels after 72 hours.

Absolute values (upper panel) and normalized to control (lower panel) after 72 hours. Error bars are the standard error of the mean. Asterisks represent significant results where $P < 0.05$ and is indicated with *, and $P < 0.01$ is indicated with **. Two way ANOVA was used to analyse data between wildtype and mutants, and between genotypes using Tukey as a post-hoc test.

2.4.3.3 Germination rates after 96 hours

After 96 hours, germination was less inhibited under low K^+ treatments when compared to control (Figure 2-10). All genotypes recovered germination and were not different compared to wildtype except the *kup12* and *hak8kup12tpk1* mutants, which were still significantly inhibited for the fourth day under all treatments. The double mutants of *hak8tpk1* showed higher germination under 30 µM K^+ compared to wildtype although was not significant after normalization.

Generally, we noticed a severe inhibition in the single mutant of *kup12* that was not recovered even 96 hours after the treatment. Combining *kup12* and *tpk1* mutants resulted in a rescue for *kup12*, indicating a possible epistasis relationship between these two transporters under high NH_4^+/K^+ ratio conditions. On the other hand, we noticed different responses to the other mutants under treatments, *hak8* mutants for example showed higher germination under different treatments/time points, however, the post-hoc analysis indicated that these differences were not significant when we compared treatment/mutants to each other. Consequently, there are two results that might need further investigation, these are the *hak8kup12* and *hak8kup12tpk1* behaviour. We could suggest a possible epistasis relationship between HAK8 and KUP12 as we can see the germination recovered under low K^+ treatments. It is also important to indicate that the statistical analysis showed that *hak8* and *hak8kup12* mutants had almost same response under all treatments and time points, at the same time *kup12* and *hak8kup12tpk1* showed typical responses.

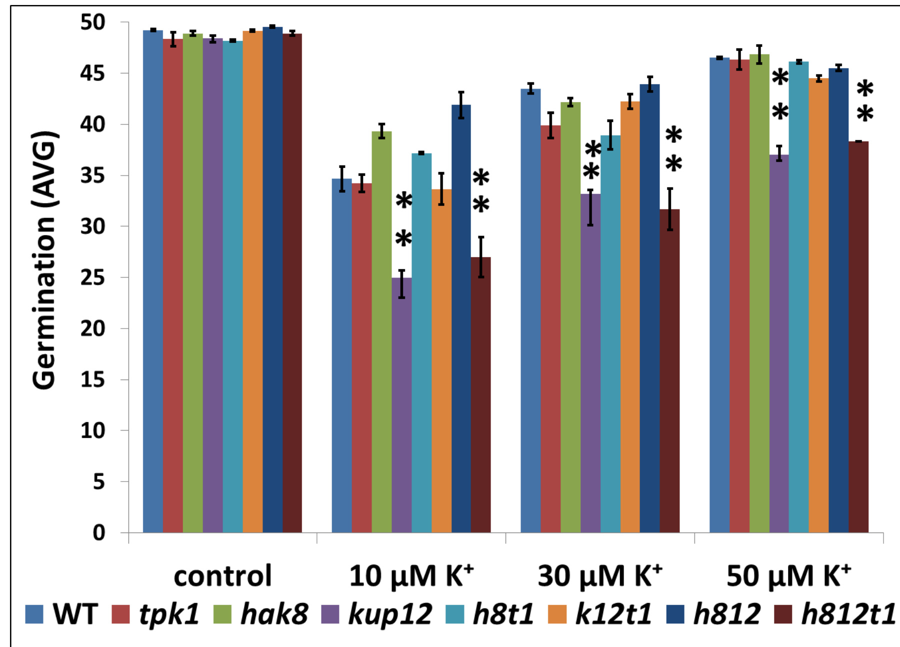


Figure 2-10: Effect of K^+ starvation on seed germination under high NH_4^+/K^+ levels after 96 hours.

Figure show absolute values. Error bars are the standard error of the mean. Each experiment is repeated at least three times. Asterisks represent significant results where $P < 0.05$ and is indicated with *, and $P < 0.01$ is indicated with **. Two way ANOVA was used to analyse data between wildtype and mutants, and between genotypes using Tukey as a post-hoc test.

2.4.3.4 atkup12-2 mutants response resembles atkup12-1

Another loss of function line (*atkup12-2*) was tested to confirm the interesting results obtained in *atkup12-1*. Line *atkup12-2* was investigated under low K^+ treatment in comparison with wildtype seeds (Figure 2-11). The results showed the same effect of K^+ deficiency on mutant seeds, germination was negatively affected under 50 µM K^+ treatment. These results indicate that the loss of function of AtKUP12 results in slower germination during K^+ starvation under high NH_4^+/K^+ ratio, suggesting a role for this transporter during early stages of germination in Arabidopsis seeds possibly during radicle expansion. The reduction in this experiment was lower compared to previous experiments, but was still significant. *atkup12-2* showed the same trend as in *atkup12-1* line confirming the phenotype. We used same experimental conditions in this set of experiments.

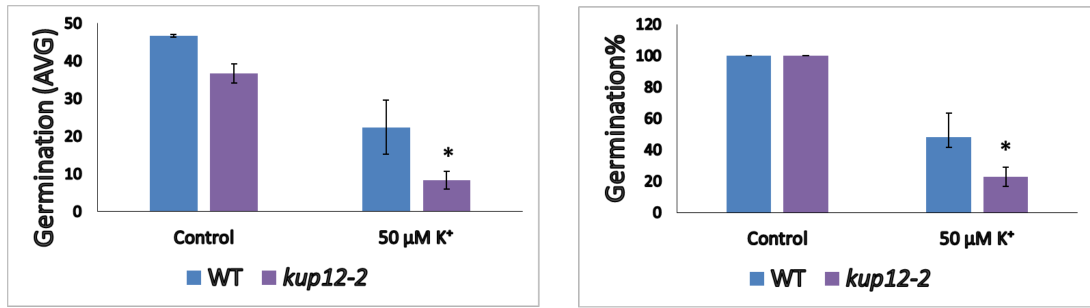


Figure 2-11: Seeds germination of *atkup12-2* after 48 hours of sowing.

Another allele for the *kup12* knockout mutant (*atkup12-2*) was tested in this experiment. Seeds of two different plants of this line were used and averaged compared to wildtype. Bars are the standard error of the mean. Student's t-test was applied to determine significant results. * indicates that $P < 0.05$.

2.4.3.5 Effect of 200 µM K^+ treatment on germination of mutant seeds

A higher K^+ concentration (200 µM) of KCl was used to test the effect of higher K^+ levels in the medium on the germination of mutants. Only mutants with previous phenotypes were used in this experiment (*hak8*, *kup12* and *hak8kup12tpk1*). Less inhibitory effect was expected on both *kup12* and *hak8kup12tpk1* mutants. Using a higher concentration of K^+ in the medium has still reduced the germination rates significantly in the *kup12* mutants. However, this effect lasted for only 48 hours (Figure 2-12.A) and was almost equal to control in the third day of germination. The increase in germination rates in the single mutant *hak8* disappeared here as well (Figure 2-12.B).

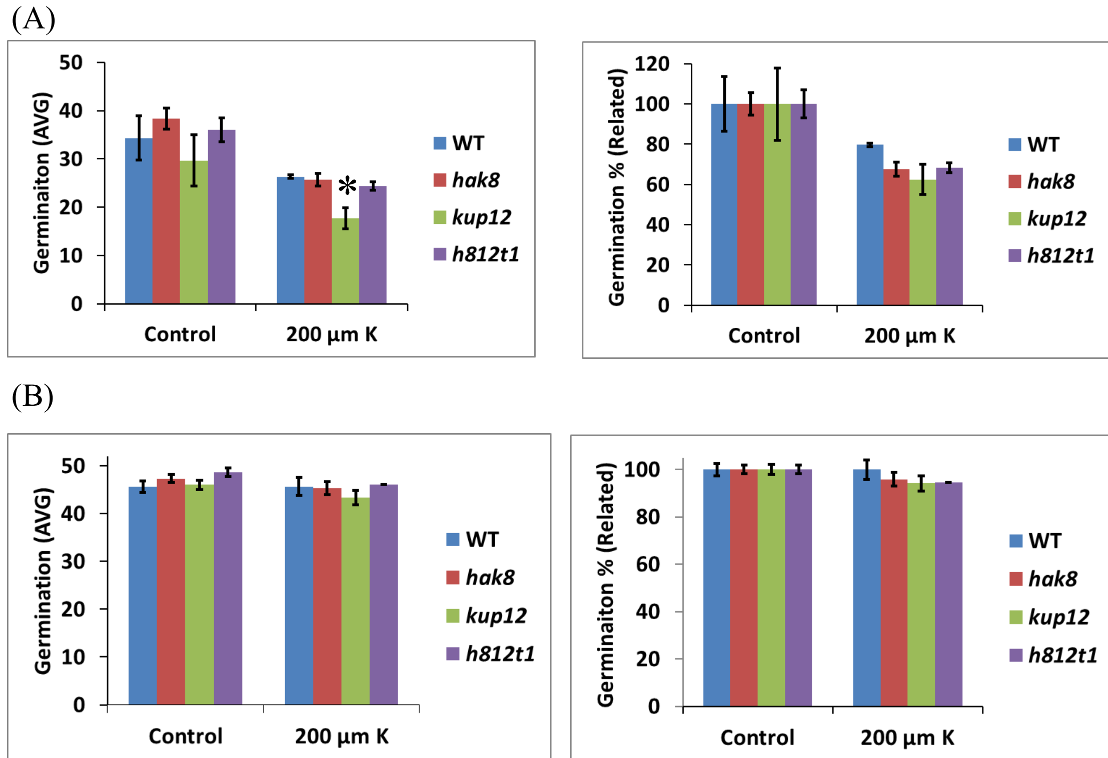


Figure 2-12: Germination under 200 µM K⁺ average and normalized to control (10 mM K+).

(A): After 48 hours, (B): After 72 hours, seeds of four genotypes were plated on modified ½ MS medium, cold-stratified then moved to growth room. Results show one-experiment with three replicates. Bars are standard error of the mean. Student's T-test test was used to identify significant results, $P < 0.05$. Asterisks represent significant results.

2.4.4 Loss of function of AtKUP12 inhibits seedling establishment during K^+ deficiency when combined with 10 mM NH_4^+

To test the effect of the treatments on the establishment of seedlings, we observed the emergence of cotyledons after germination (Figure 2-7). We counted the number of emerged cotyledons 7 days of sowing seeds at a stage when they should be fully opened (Boyes et al. 2001). All seeds grown over control medium containing high NH_4^+ (10 mM) plus high K^+ (10 mM) showed full emergence and establishment in all genotypes (Figure 2-13 a,b) and achieved 100% of cotyledons emergence as well as root growth. All low K^+ treatments (10, 30 and 50 µM) resulted in severe inhibition of seedling establishment compared to control conditions. Moreover, the loss of function mutants of *atkup12-1* and the triple mutants of *hak8kup12tpk1* showed maximum inhibition in the seedling establishment under 10, 30 and 50 µM K^+.

It is important to confirm that the seedlings failed to grow roots under both 10 and 30 µM K^+ treatments during high NH_4^+/K^+ ratio inhibition (Figure 2-7, C) which is a typical NH_4^+ toxicity symptom in Arabidopsis (Cao et al. 1993; Li et al. 2010). Seedlings also showed chlorosis as a symptom of K^+ deficiency due to the interference of NH_4^+ with the high affinity uptake of K^+. After 10 days, under control and 50 µM K^+, seedlings succeeded in establishment and were able to grow roots, however, this was slower compared to wildtype. This suggests that AtKUP12 have a role in plant responses during high NH_4^+/K^+ ratio as reported for other HAK/KUP/KT family members (Shin and Schachtman 2004; Gierth and Mäser 2007; Pyo et al. 2010) possibly, through relieving NH_4^+ toxicity by K^+ release from vacuole to cytoplasm. It might as well function in root growth during such stresses.

The results show that mutants are not only sensitive to high NH_4^+ concentrations at low K^+ levels during germination, but also during the post-germination stage. It suggests that the high concentration of NH_4^+ in the medium inhibits the growth of seedlings under K^+ starvation conditions (Dennison et al. 2001; Pyo et al. 2010). We also confirm that the increase in K^+ levels relieves NH_4^+ toxicity. Our results are in accordance with Pyo et al., (2010) and Cao et al., (1993), who indicated that increasing K^+ during NH_4^+ toxicity improves the establishment of seedlings and can reverse the inhibition. The effect of high NH_4^+ in the medium was stronger on seedling establishment compared to seed germination, especially under the 10 µM K^+ treatment, where all genotypes showed slow cotyledons emergence compared to control and other K^+ concentrations tested.

Seedlings grown on 50 µM K^+ were able to form roots despite the growth inhibition that reflected on plant size. Seedlings were able to establish fully after 10 days of sowing for all genotypes (Figure 2-14). It might be interesting to test fresh weights and ion contents of these plants under these conditions to understand if the internal K^+/Na^+ levels were affected in these genotypes.

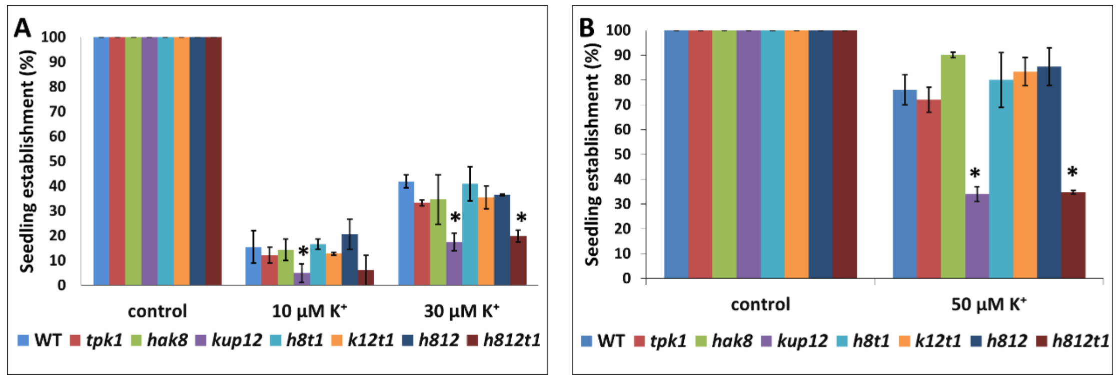


Figure 2-13: Effect of high NH_4^+/K^+ ratio on seedling establishment. Cotyledons emergence was counted 7 days of sowing.

(A): Experiments were repeated twice in each experiment included three replicates with 50 seeds/replicate of each genotype. (B): Data show one experiment of three replicates with 50 seeds/replicate for each genotype. Significant results were determined using Student's t-test, $P < 0.05$. Asterisks represent significant values.

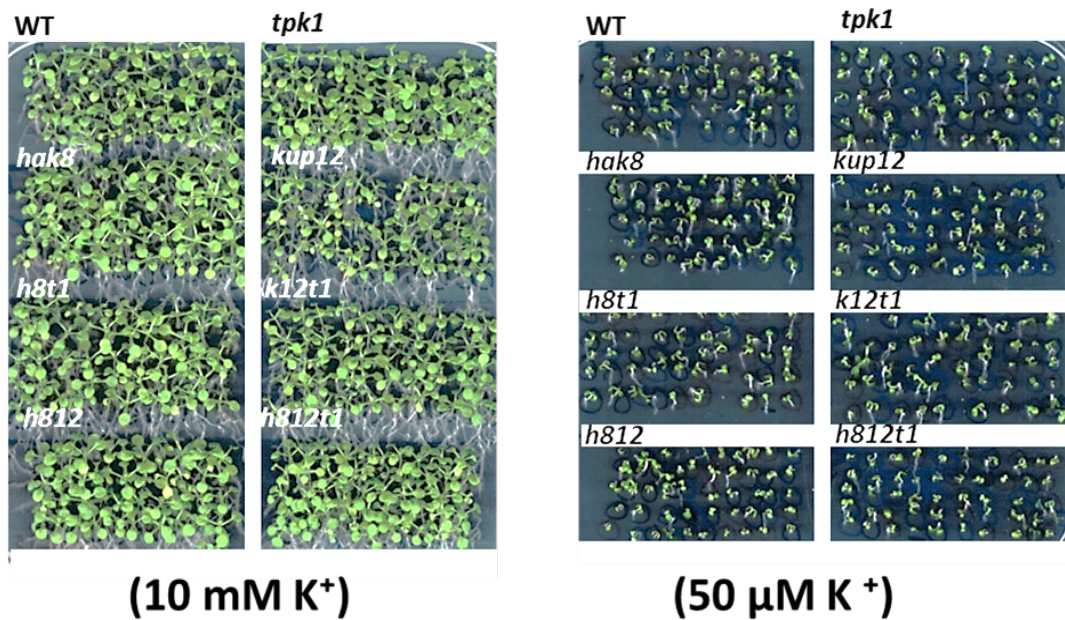


Figure 2-14: Seedling establishment under high K^+ (10 mM) and low K^+ (50 µM) combined with 10 mM NH_4^+.

Seedlings of 10 days old showing normal growth in all genotypes under control conditions (Left), and seedlings showing severe growth inhibition under 50 µM K^+ (right). *hak8tpk1=h8t1*, *kup12tpk1=k12t1*, *hak8kup12=h8k12*, *hak8kup12tpk1=h8k12t1*.

2.4.5 Analysis of the role of AtKUP12 transporter in the K^+ deficient yeast strain

Previous results suggested a possible role for the AtKUP12 in K^+ transport during seed germination under low K^+ concentrations. *AtKUP12* was cloned into *Saccharomyces cerevisiae* K^+ deficient yeast strain (pYE52) Δ *trk1, trk2, tok1*. This strain lacks the main K^+ transporters and cannot grow in low K^+ concentrations (Bertl et al. 2003). The idea was to express the KUP12 transporter in this mutant strain and analyse its activity in K^+ transport if any. The full coding sequence of *AtKUP12* was amplified from Arabidopsis wildtype cDNA, the amplification was checked on the gel (Figure 2- 15.A) and showed the expected size 2608 bp. The *AtKUP12* PCR product was analysed in an electrophoresis and showed bands corresponding to the right size (Figure 2-15.B). The sequencing of transformed plasmids showed errors that did not result in a change in the protein encoded.

(A) (B)

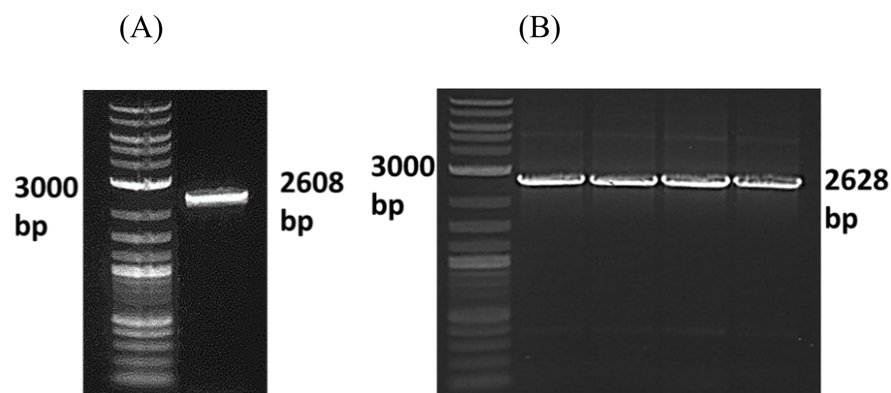


Figure 2-15: Amplifying AtKUP12 full coding sequence using PCR.

A: Step 1 PCR using primers that amplify the full coding sequence (2608 bp). B: Step 2 PCR adding restriction sites to the amplicon and few bases to increase cleavage efficiency (4 reactions were carried out and are shown in 4 lanes. First lane contains 2-log DNA ladder.

The *AtKUP12* was digested using BamHI and NotI enzymes, and purified to be used later in ligation. While the pYES2 was digested using same enzymes, run on gel and gel extracted. The gel analysis shows the linearized pYES2 compared to the uncut plasmid. The cut plasmid should appear as a single linear band at the expected size (Figure 2-16). While the uncut plasmid shows the typical three bands, nicked, linear and supercoiled (Figure 2-16). The plasmid was then gel extracted as in methods.

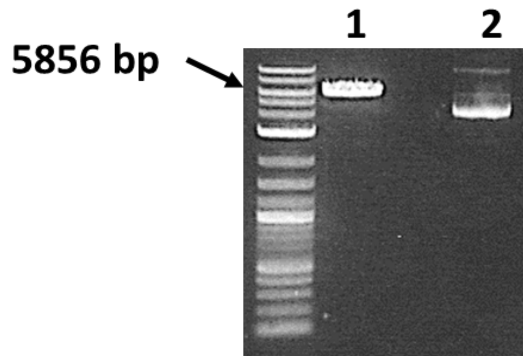


Figure 2-16: **Double digestion of the pYES2 plasmid using BamHI and NotI restriction enzymes.**

1: Cut pYES2 showing linearized plasmid with 5856 bp size. 2: Uncut pYES2 plasmid showing typical three bands representing (nicked, linear and supercoiled) plasmid. First lane contains 2-log DNA ladder.

Ligation of insert and plasmid was conducted as in methods, and then ligated product was transformed into *E. coli* competent cells. The transformed colonies were then tested for the occurrence of the insertion using single colonies PCR. The *AtKUP12* primers used previously were used here. These primers amplify a 500 bp portion of the gene. All colonies checked showed the existence of the insert (Figure 2-17).

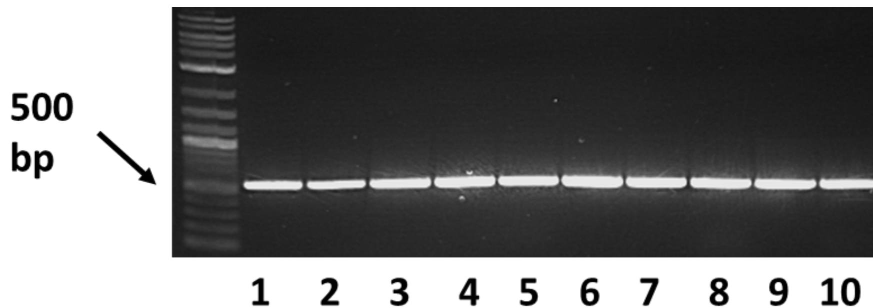


Figure 2-17: **Colonies PCR. Transformed colonies were selected on LB medium supplied with 100 mg/L carbenicillin.**

Primers were used from table 2-3 to amplify the insert. Ten colonies were scanned in this analysis (1-10). PCR shows positive results, indicating the existence of the insert at this stage. First lane contains 2–log DNA ladder.

Plasmids were isolated as described in methods, and to further confirm successful ligation, diagnostic double digestion was conducted to release the insert from the construct. Digested constructs were then run on gel (Figure 2-18), the figure shows that only 4 colonies contained the insert, which means that other colonies were only showing false positives in the previous PCR. This can happen due to contamination during the

procedure and hence it is important to re-digest samples again to exclude these false results. Digestion of positive colonies showed two distinctive bands of both the pYES2 plasmid and the *AtKUP12* insert. These plasmids were later used for yeast transformation.

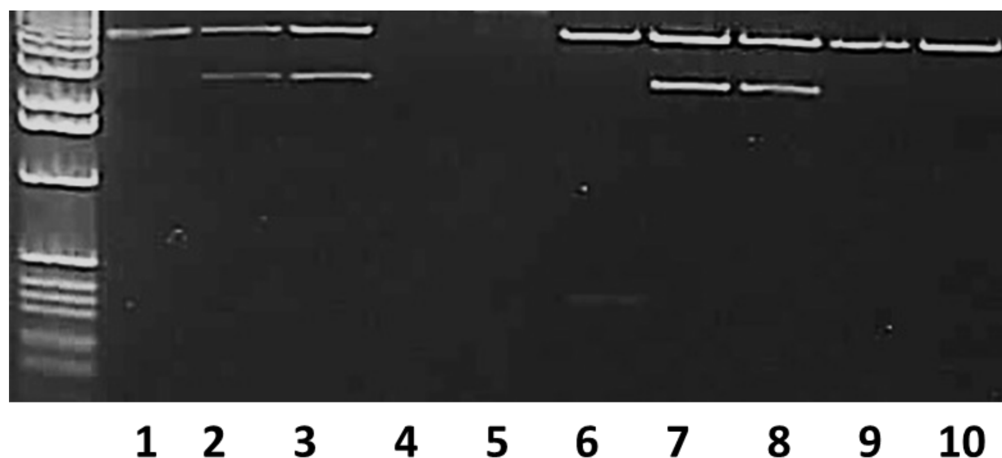


Figure 2-18: **Diagnostic double digestions of the isolated AtKUP12-pYES2 construct.**

Figure indicates that only 4 plasmids (2, 3, 7 and 8) contained the insert. First lane contains 1 kb DNA ladder.

2.4.5.1 Yeast transformation

The construct was then introduced to yeast strains used in this study. Successful transformants were selected on selective medium and restreaked over fresh selective plates, then used in physiological experiments.

2.4.5.2 AtKUP12 complements yeast growth under low K^+ conditions

To test if the AtKUP12 functions K^+ transport, heterologous expression in yeast was used. The yeast cell contains a vacuole which resembles the plant cell and therefore it is an excellent system to analyse the functions of channels/transporters (Bagriantsev and Minor 2013). Complementation assays were conducted on both glucose and galactose AP containing medium using different K^+ concentrations. The AP medium is a minimal medium that does not contain K^+ or Na^+ and is widely used in K^+ complementation assays in yeast. In a glucose-supplied medium, the growth of yeast was affected by the different K^+ concentrations. The growth was minimal at 50 µM K^+ and 100 µM K^+, for both AtKUP12-pYES2 and pYES2 genotypes (Figure 2-19). The growth was observed for the lowest three dilutions (1-3). Generally, the vector control yeast showed weaker growth

under low K^+ concentration compared to the AtKUP12-pYES2. This is a typical response of the K^+ deficient yeast mutant under K^+ starvation conditions (Bertl et al. 2003). Last two dilutions (4-5) showed single colonies under both 50 and 100 µM K^+ for the AtKUP12-pYES2 only. The control yeast failed to grow on 50 and 100 µM K^+ but showed single colonies growth under 2 mM K^+ treatment. This suggests that AtKUP12 improves mutant growth under low K^+ concentrations with glucose in the medium. These results, therefore, are novel and never been reported for the AtKUP12 transporter before.

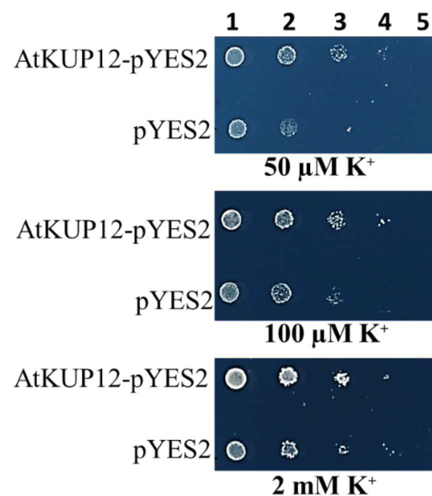


Figure 2-19: **AtKUP12 complements the growth of K^+ deficient yeast in glucose plates.**

(*trk1*, *trk2*, *tok1*) K^+ deficient mutant was transformed with both AtKUP12-pYES2 and pYES2 only. Spot plates were prepared and incubated at 30 ºC for three days.

2.4.5.3 Growth of K^+ deficient yeast on galactose contained medium

pYES2 contains a galactose induced promoter that activates protein expression. To induce the expression of *AtKUP12* under low K^+ concentrations, an assay was conducted on AP plates containing galactose instead of glucose. The results showed better growth for the AtKUP12-pYES2 yeast on all K^+ conditions used after 2 days (Figure 2-20.A). The vector control did not show any growth after this time indicating that AtKUP12 could complement mutant yeast growth under all three K^+ concentrations tested (50 and 100 µM, 2 mM). This confirms the results obtained on glucose plates. It also indicates that the growth of mutants complemented with AtKUP12 was seen at higher dilutions. This explains the inductive effect of galactose in the medium and confirming that the KUP12

mediates a K^+ transport under low K^+ concentrations. The growth on galactose can be slower and accordingly plates were left further to grow (Figure 2-20.B). The plates showed that the vector control yeast grew weakly in its lowest dilutions (1 and 2) under both 100 µM and 2 mM of K^+ in the medium. However, a limitation in data here is the lack of control plates that contain all genotypes growing over 100 mM K^+, this should indicate that the pYES2 transformed yeast can actually grow on high K^+ concentrations but not on low K^+ medium and that the lack of growth is K^+-dependent.

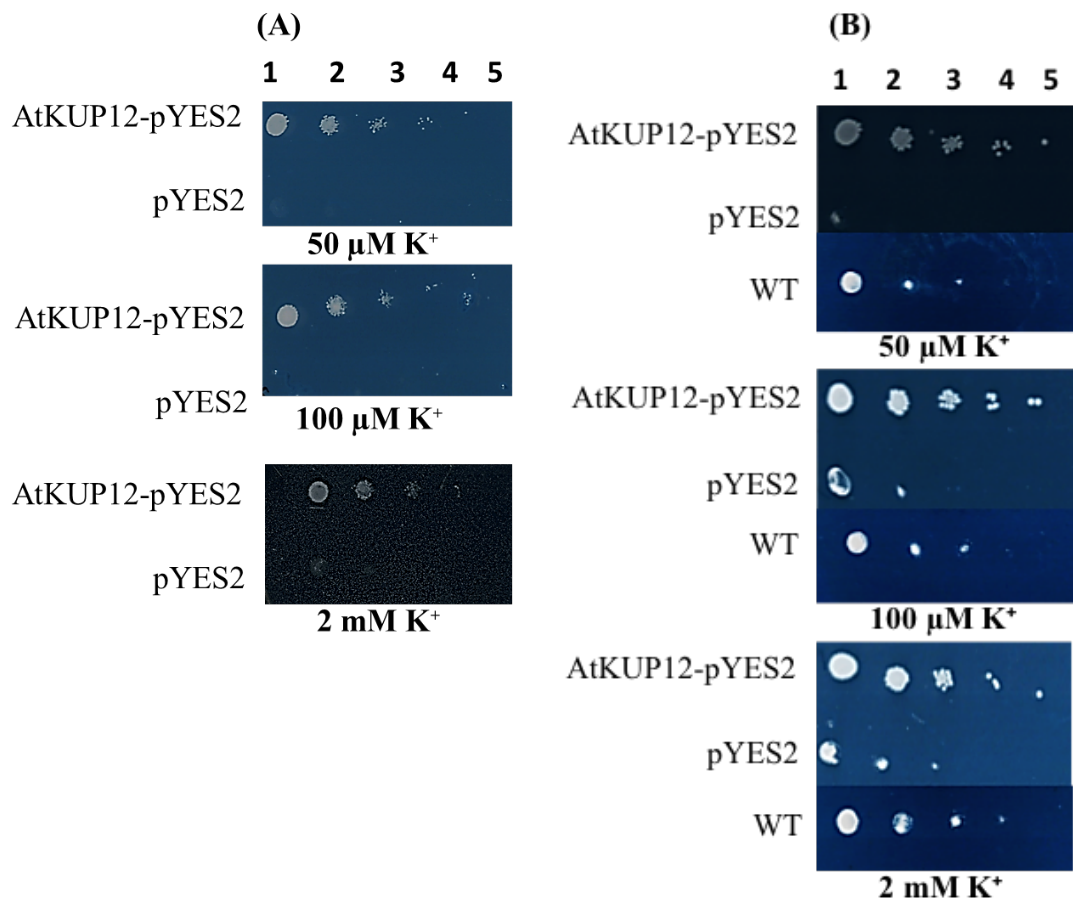


Figure 2-20: AtKUP12 complements the growth of K^+ deficient yeast in galactose plates.

(A): Growth of yeast after 2 days of incubation. (B): Growth of yeast after 5 days of incubation, the figure shows the growth of the wildtype strain after 5 days compared to empty vector and AtKUP12-pYES2.

2.4.6 Effect of different stresses on seedlings of mutants

To test the potential role of candidate transporters in the response of plants to stress during different growth stages, we treated seedlings with different stress treatments. We previously indicated the role of KUP12 in the germination and seedling establishment during K^+ starvation and high NH_4^+ and Na^+ levels, which considered early stages of growth. At these tests, seedlings of 10-14 days old were grown on ½ MS medium modified to create stress in media. The main point to note here is that the inhibition due to stress treatments was not as high as germination and seedling establishment. Two reasons could explain this difference. First: the difference in the medium used here which contains high K^+ concentrations. Second: the previous growth of young seedlings with full medium that can lead to accumulation of nutrients such as K^+ to be used later when plants are stressed

2.4.6.1 Effect of abiotic stress treatments on tpk1, hak8 and hak8tpk1 mutants

Results indicated that *hak8* mutants showed significantly higher growth under control, high K^+ and high sorbitol (120 mM) conditions compared to wildtype (Figure 2-21). These results, are in line with the germination results obtained previously which also indicated a faster germination under different conditions (Figures 2-8) and faster seedling establishment (Figure 2-13,B). It is possible that the loss of function of AtHAK8 improves growth of plants under stress conditions due to less K^+ efflux from cells. However, the mutant seedlings were initially bigger in sizes, and therefore, this effect might be the reason of higher fresh weights under treatments. In this set of experiments, high Na^+ treatment did not show higher reduction when compared to high K^+ treatment. We think that experimental conditions resulted in bigger plants in the plates of the 80 mM Na^+ treatment, possibly by being exposed to more light. Previous work by Osakabe et al., (2013) reported that the loss of function of AtHAK8 did not show phenotypes except when combined with other mutants of its homologs KUP2 and KUP6. However, the mutant line used by Osakabe et al., (2013) was different than the one used in our study.

On the other hand, *tpk1* showed sensitivity to high sorbitol treatment and this phenotype continued when combined with the loss of function of HAK8. This could be due to the loss of function of TPK1 under such conditions, which resulted in less K^+ release to the

cytoplasm during such stresses. This is important to maintain the turgidity of the cell under such osmotic stress. It is also possible that AtTPK1 plays a role in maintaining K^+ levels in the cytoplasm to maintain the turgidity of cells under such high osmotic stresses. This in accordance with previous work on AtTPK1 channel indicating its importance in the response of plants to abiotic stress (Gobert et al. 2007; Latz et al. 2013).

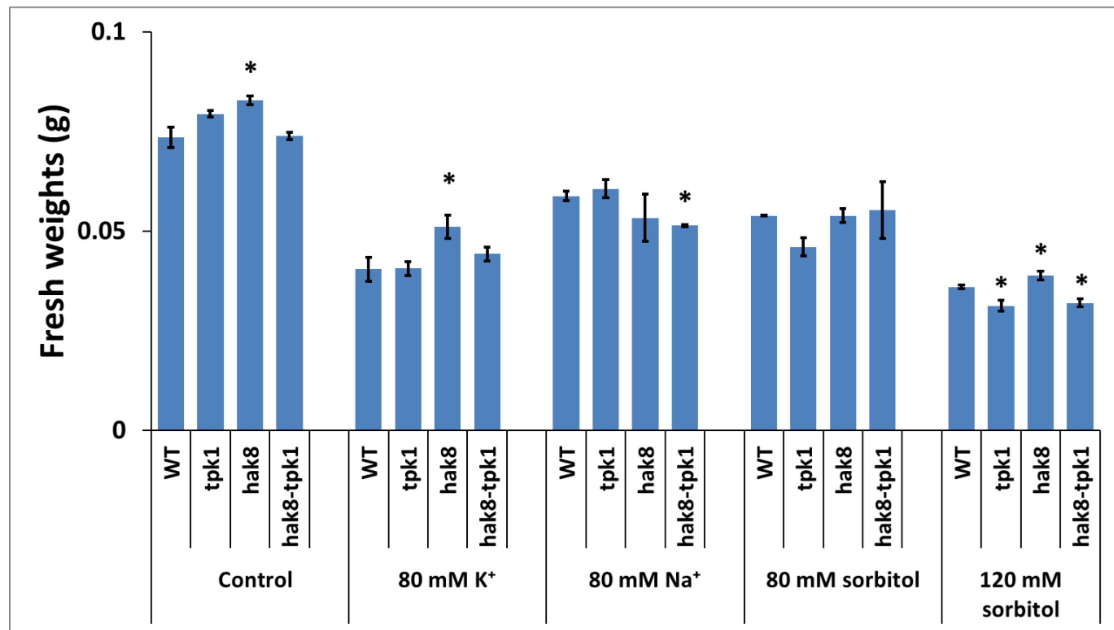


Figure 2-21: **Effect of different stress treatments on fresh weights of Arabidopsis wildtype *tpk1*, *hak8* and *hak8tpk1* seedlings**.

Error bars are standard error of the mean of three experiments. Student's t-test was applied to test significant results, $P < 0.05$. The experiment was repeated 3 times.

2.4.6.2 Effect of abiotic stress treatments on tpk1, kup12 and kup12tpk1 mutants and a role for AtKUP12 in salinity stress response

tpk1 showed lower growth under both 80 mM NaCl and 120 mM sorbitol although was not significant compared to wildtype (Figure 2-22). In the previous experiment, *tpk1* did not show any significant responses either (Figure 2-21). However, seedlings of *tpk1* were probably slightly bigger in sizes initially. Loss of function of *kup12* showed a significant reduction in fresh weights under the high salinity treatment either alone or combined with *tpk1* mutant. The sensitivity of *kup12* seedlings indicates a possible role of AtKUP12 during K^+ deficit perhaps resulted from high concentrations of NaCl. We think that the differences are not significant overall, since the decrease in weights is very small despite

being statistically significant. There were no significant differences for the other genotypes under both high K^+ and 120 mM sorbitol when compared to wildtype. Although *tpk1* showed lower fresh weights under 120 mM this was not significant as in the previous set of experiments.

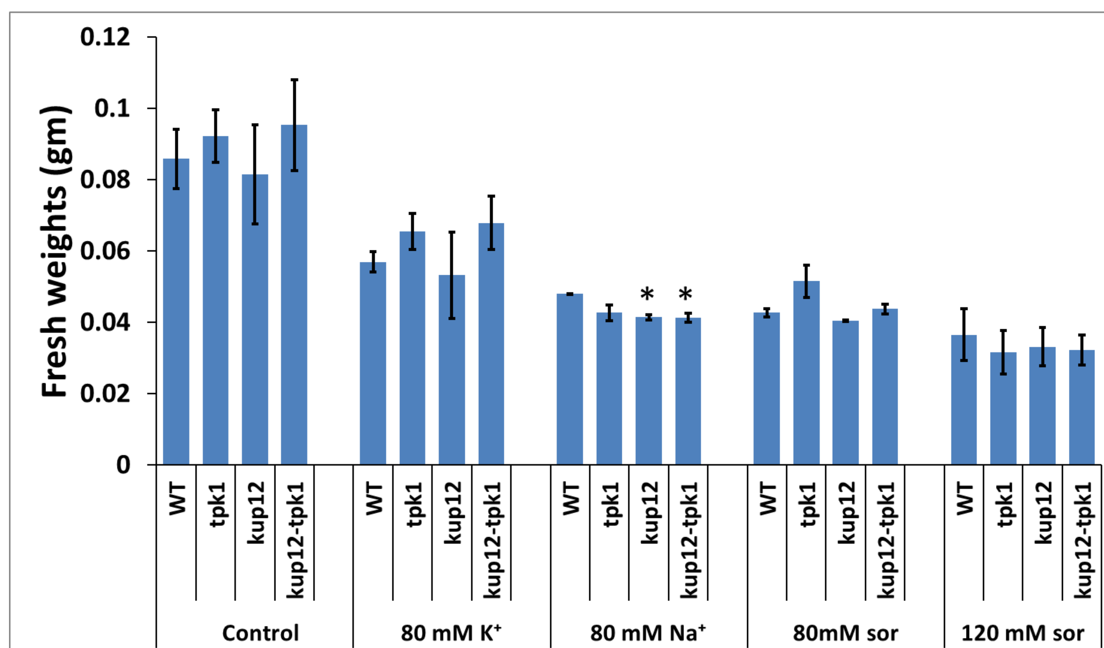


Figure 2-22: Effect of different stress treatments on fresh weights of Arabidopsis wildtype and *tpk1*, *kup12* and *kup12tpk1* seedlings.

Error bars are standard error of the mean of three experiments. Student's t-test was applied to test significant results, $P < 0.05$. * represents significant values. The experiment was repeated three times.

2.4.6.3 Effect of abiotic stress treatments on hak8kup12 and hak8kup12tpk1 mutants

Multiple mutants were generated to further understand the role of HAK/KUP/KT family of transporters and the TPK1 channel in the response of plants to abiotic stress. High K^+ and Na^+ treatments reduced fresh weights in all genotypes compared to control. Both treatments did not show any significant differences in fresh weights between mutants and wild type (Figure 2-23). Applying sorbitol in two concentrations did not show any significant results in all genotypes compared to wildtype. *hak8kup12* (*h812*) showed higher fresh weights than the *kup12* single mutants (Figure 2-22). Although these are different two sets of experiments, but it is in line with germination (Figure 2-9, -10, -11). However, here, the triple mutants did not show the same inhibition as in germination.

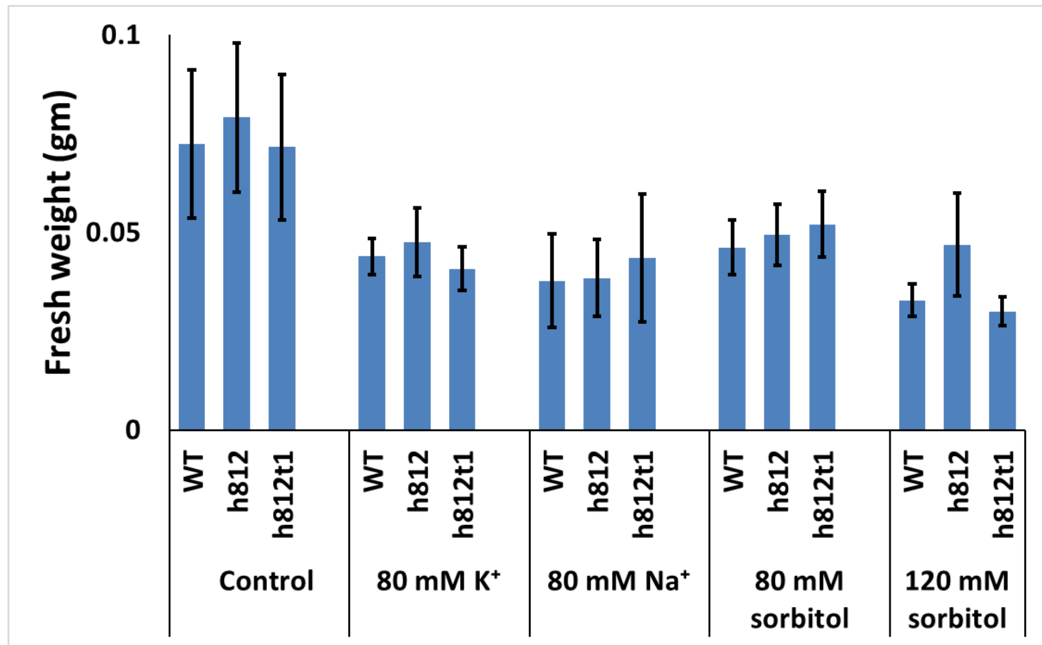


Figure 2-23: Effect of different stress treatments on fresh weights of Arabidopsis wildtype and *hak8kup12* and *hak8kup12tpk1* seedlings.

Error bars are standard error of the mean of three experiments. Student's t-test was applied to test significant results, $P < 0.05$. * represents significant values. The experiment was repeated three times.

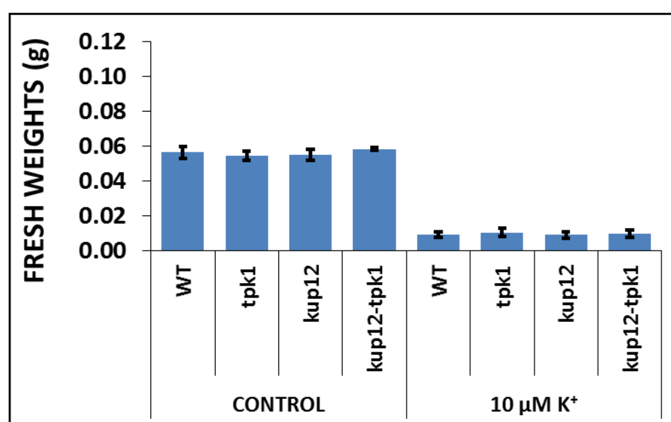
2.4.7 Effect of K^+ starvation on seedlings

It is important to test the response of multiple loss of function mutants of these candidates under K^+ starvation conditions which have not been tested yet. WT, *tpk1*, *kup12* and *kup12tpk1* were tested for growth under 10 µM of K^+. Pyo et al., (2010) treated Arabidopsis seedlings with different low K^+ levels. This resulted in smaller plants as well as slower growth, the response differed according to genotypes tested. For example, loss of function of AtHAK5 and AtAKT1 resulted in significant loss of fresh weights and root length in seedlings. The most reduction in weight resulted from the lowest K^+ concentration used (10 µM), so we started with this concentration.

As expected, K^+ starvation resulted in a severe reduction in the growth of all genotypes. Data showed no significant differences between mutants and wildtype under both control and K^+ starvation treatments. Results indicated that the inhibitory effect of growing seedlings on low K^+ medium caused 80% reduction in fresh weights when compared to seedlings growing on control medium (Figure 2-24). From this, we conclude that the 10 µM of K^+ was possibly too low that the plants responded the same way. All genotypes

showed the same percentage of weight loss under this treatment compared to wildtype. Loss of function of candidate genes did not affect the response of plants under this concentration.

(A)



(B)

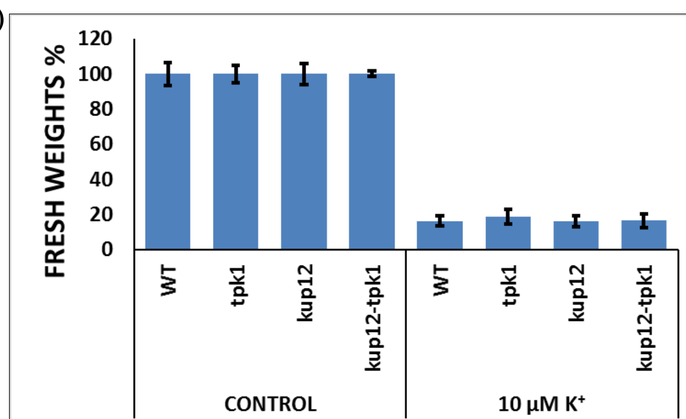


Figure 2-24: **Effect of K^+ starvation on fresh weights of Arabidopsis wildtype and mutant seedlings.**

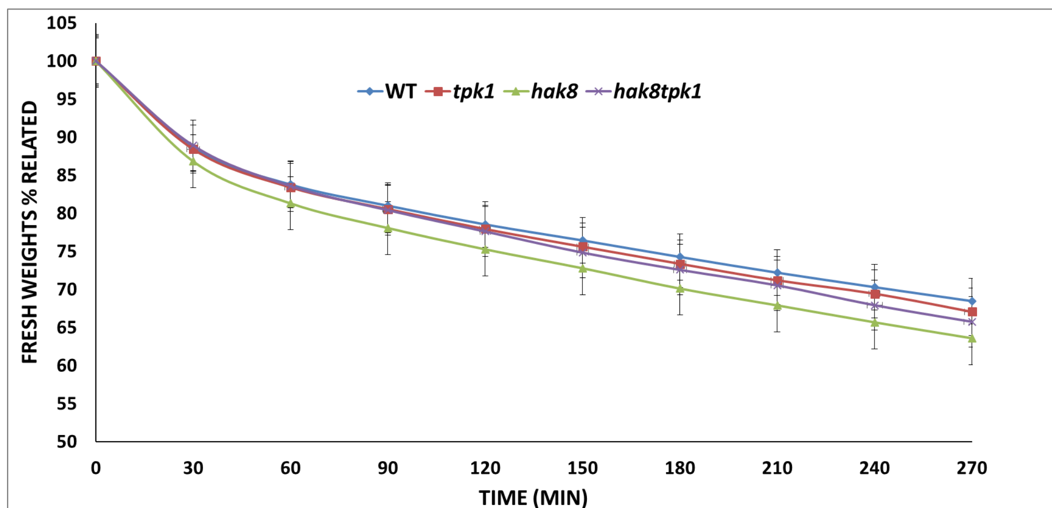
(A): Average of three experiments, (B): Normalized. 7 days old seedlings of similar sizes were transferred to treatments. Fresh weights were measured 2 weeks after transferring plants. Each treatment had eight plants/mutant. Error bars are standard error of the mean of three experiments. Student's t-test was used to test significant results, $P < 0.05$.

2.4.8 Transpirational water loss of mutants

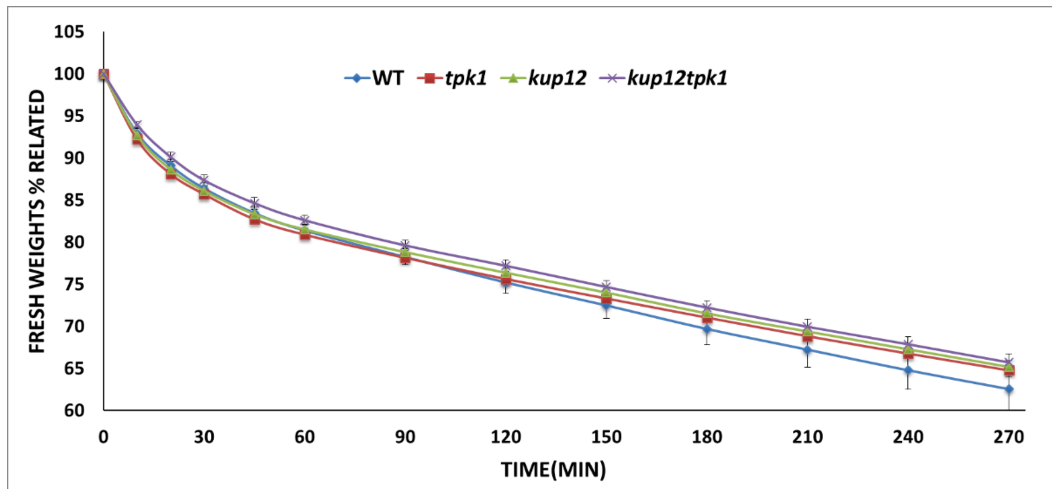
tpk1 loss of function mutants showed slower stomatal closure under ABA treatment (Gobert et al. 2007). It is important to understand the role of other channels/transporters potentially located in the tonoplast of guard cells in the short-term response to drought stress. TPK1, is localized in guard cell tonoplast, while HAK8 and KUP12 are suggested

for this localization as well (Hruz et al. 2008; Whiteman et al. 2008; Hooper et al. 2014). This suggests a possible role of these proteins in stomatal closure/opening under different environmental conditions by controlling K^+ homeostasis. To investigate potential roles of these candidates in short-term stress response (hours), transpirational water loss was tested. Loss of fresh weights in detached rosettes of wildtype, *tpk1*, *hak8* and *hak8tpk1* showed that there were no significant differences between wildtype and mutants in fresh weights loss (Figure 2-25.A). Fresh weights of rosettes decreased by about 45% over 5.5 hours. Rosettes of WT, *tpk1*, *kup12* and *kup12tpk1* showed comparable loss of fresh weights (Figure 2-25.B). None of the mutants tested in these experiments showed significant differences in transpirational water loss when compared to wildtype.

(A)



(B)



(C)

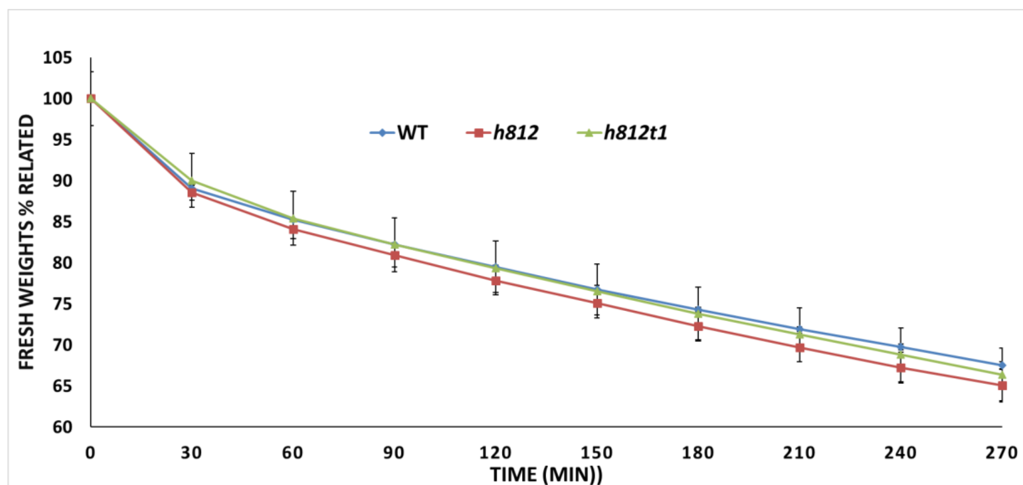


Figure 2-25: Transpirational water loss in rosettes.

Data were normalized based on the weight at time zero. Bars are standard error of the mean, n=3. Student's t-test was used to test significant results, $P < 0.05$.

Single and double mutants did not show any significant water loss compared to wildtype, so it was tested in double *hak8kup12* (*h812*) and triple *hak8kup12tpk1* (*h812t1*) mutants as it might result in a clear phenotype. Results were similar to those described in the previous water loss assays (Figure 2-25 A and B), there were no significant differences between wildtype and mutants (Figure 2-25.C). With the lack of phenotype under previous experimental conditions, a trial to challenge plants further with drier conditions was carried out, rosettes were placed in a flow-hood exposed to the airflow.

ABA is a stress hormone that is synthesized once plants are exposed to drought stress resulting in both stomatal closure and activation of stress-related genes (Shinozaki and Yamaguchi-Shinozaki 2007; Lawson and Blatt 2014). It is then perceived by several receptors (PYR1, PYL1, PYL2, and PYL4). We used the quadruple mutant of *abi112* as a positive control. We increased the stress by exposing plants to an airflow hood.

Analysing the data showed no significant loss of weight in the first 60 minutes of the experiment between the wildtype and the mutants tested except for the *abi1124*, which was expected. The *abi1124* mutant showed the highest loss of weight during the experiment, which was about 60% of their fresh weights by the end of the assay compared to wildtype (Figure 2-26). However, after 90 minutes, differences became significant for both *hak8kup12* and *hak8kup12tpk1* mutants which showed lower water loss.

The comparison with wildtype plants indicated that the difference increased with time to reach the highest significance at 270 minutes for both double and triple mutants. The *tpk1* did not show any significant differences up to 210 minutes of the experiment, after this, the difference became significant and increased with time as well, which is in accordance with the function of the TPK1 channel in stomatal closure (Gobert et al. 2007).

We presume that placing plants in the hood might have induced the loss of transpirational water and formed a stronger stress on leaves. Under these conditions, genotypes might have behaved differently and showed these significant results. From this, we can suggest a possible role of candidate genes during stomatal movement under drought conditions.

We can use the previous conclusion in germination and stress treatments to explain the results obtained here. For example, the loss of function of HAK8 may have resulted in better retention of K^+ in plants, which should probably result in more opened stomata. However, when this was combined with the loss of function of KUP12, the stored K^+ was less, resulting in more closure of the stomata, therefore, higher fresh weights under drying conditions. Measuring K^+ in mutants can help explain this more.

However, it is also important to indicate that sometimes stressing plants during the experiment results in the generation of ABA and therefore the closure of stomata regardless of the genotype.

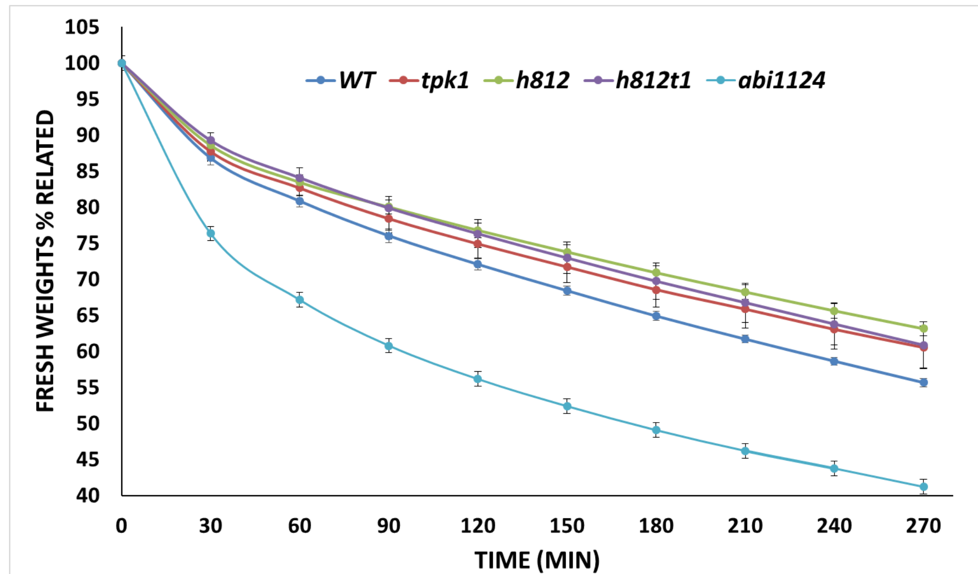


Figure 2-26: Transpirational water loss in rosettes.

4-5 weeks old rosettes were detached and placed in plates in a flow hood for 270 minutes after scoring the initial fresh weight. Measurements of fresh weights were carried out at 30 minutes intervals. Bars are standard error of the mean. The experiment was repeated 3 times, n=3. Student's t-test was used to test significance at the $P < 0.05$ level.

2.4.9 Stomatal conductance under ABA treatment

It was previously reported that *tpk1* showed higher conductance under both control and ABA treatments (Gobert et al. 2007), but was suggested to function alongside other transporters in this process. Our results showed that the *hak8* and *kup12* single mutants did not show any significant differences in stomatal conductance when compared to wildtype plants. Data indicated that the stomatal conductance of leaves did not respond differently under 10 µM ABA in mutants compared to wildtype (Figure 2-27). Accordingly, the double mutants of *hak8kup12* did not show any differences in their ABA-mediated stomatal closure when compared with wildtype.

The triple mutants of *hak8kup12tpk1* displayed a higher conductance under ABA treatment (Figure 2-27). This difference was significant when compared to wildtype. The triple mutant phenotype however might be a result of the TPK1 loss of function. *tpk1* single mutant showed higher conductance after 20 minutes of treatment compared to wildtype (Figure 2-28). This result is in accordance with Gobert et al. (2007), despite this was not significant statistically.

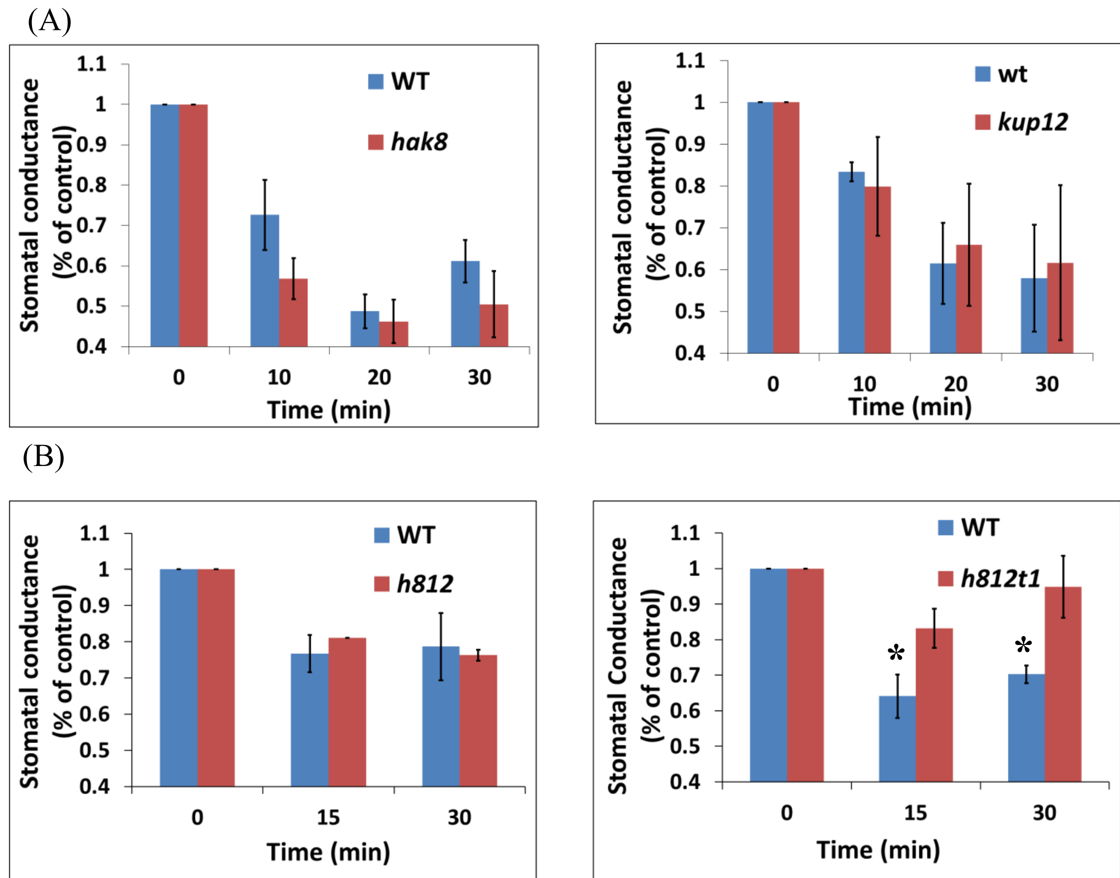


Figure 2-27: Stomatal conductance of whole leaves in mutants

Leaves treated with 10 µM ABA were placed in a LI-COR chamber. (A): Single mutants, (B): Double and triple mutants. Each experiment is an average of three times. 6-10 leaves of each genotype were used in each experiment.

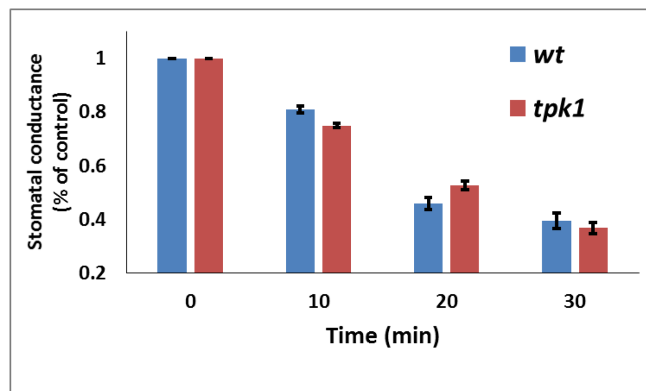


Figure 2-28: Stomatal conductance of *tpk1* leaves.

Leaves treated with 10 µM ABA were placed in a LI-COR chamber. Results showed represent the average of two experiments.

2.5 Discussion

Abiotic stress is an obstacle in the way of producing enough food for the world. These stresses differ in its types and effects on plants. A strong healthy plant is required to tolerate such harsh conditions and obtain a good productivity. This is achieved by maintaining a balanced nutrition, which is a key component in this process. Potassium is essential ion in plant physiology and cannot be replaced by any other ion especially for long periods. Therefore, understanding the mechanisms of which by K^+ is managed in plants during stress, is paramount to enable us to further understand how to produce a plant that is stress tolerant and with a high yield.

Vacuolar K^+ transporters such as TPK1 plays an important role in K^+ movement during salinity, drought and osmotic stresses. Mainly by releasing K^+ from vacuole to cytoplasm to maintain K^+ levels in cytoplasm enough for physiological processes and during the stomatal closure as a response to stress (Gobert et al. 2007; Latz et al. 2013; Hartley and Maathuis 2016). The involvement of other transporters during stomatal closure alongside AtTPK1 is under analysis in this thesis, as well as investigating new roles for other transporters which have not been under study before such as AtKUP12, or partially tested such as AtHAK8.

Our results indicated that the loss of function of the AtKUP12 K^+ transporter affected germination and seedling establishment rates under K^+ starvation conditions. The germination of the two *kup12* knockout lines was inhibited under K^+ starvation conditions. This inhibition resulted from the combined stresses of NH_4^+ levels and Na^+ in the media. The inhibition was also reported in post-germination affecting seedling establishment. This occurred by the blockage of root growth due to high NH_4^+ levels. This suggests a role for AtKUP12 in plant responses to salinity and K^+ starvation when combined with high NH_4^+ levels (high NH_4^+/K^+ ratio) possibly alongside AtHAK5 and other KUP transporters. AtKUP12 was also able to rescue the growth of K^+ deficient yeast mutants under low K^+ concentrations. This is in line with the other results obtained for other family members (KUP1, KUP4, HAK5 and KUP7) (Fu and Luan 1998; Rigas et al. 2001; Gierth et al. 2005; Han et al. 2016). No previous studies were reported on the AtKUP12 transporter to understand their role in seed germination and seedling

establishment, especially under low K^+ concentrations. We therefore discuss this point in the next section.

2.5.1 Germination of seeds during K^+ starvation is significantly inhibited by the presence of NH_4^+ and Na^+

Once dormancy is broken and conditions are suitable for germination, an array of physiological processes is activated. The efflux of minerals like K^+ from protein storage vacuoles is a major characteristic of seed germination carried out by tonoplast channels/transporters (Bethke et al. 1998). External K^+ is suggested to be none essential for the early stages of germination (Gobert et al. 2007), as a seed can rely on the vacuolar storage. However, the composition of the external medium can either improve the germination process by inducing the radicle emergence or reduce it by blocking the emergence of radicle or cotyledons. Later, and during seedling establishment, external K^+ is essential for the growth, which occurs by the uptake of K^+ from the medium by different mechanisms and transporters.

Loss of function of important K^+ transporters such as AKT1 and HAK5 in Arabidopsis resulted in slower germination under K^+ starvation conditions (Pyo et al. 2010). Also, loss of function of some K^+ transporters may result in lower K^+ concentrations in seeds as these transporters can participate in the accumulation of K^+ in vacuoles, or else, the mobilization of vacuole stored K^+ during germination, as suggested in *tpk1* mutants. Knockout mutants of *attpk1* showed generally slower germination under control conditions and even slower germination when grown on ABA contained medium (Gobert et al. 2007). TPK1, HAK8 and KUP12 are highly expressed in imbibed seeds (Figure 2-1.B). Therefore, we tested if they play a role in seed germination and seedling establishment through K^+ homeostasis.

The results of germination assays under control medium (10 mM K^+) conditions showed that, germination percentage ranged from 75-90% after 48 hours of stratification for the eight genotypes tested, with wildtype displaying 75% (Figure 2.8). Previous work by Pyo et al., (2010) showed slightly higher germination at the same stage for the wildtype. It was approximately 90% two days after the stratification period when seeds were exposed

to 1 mM of K^+. The germination of seeds we tested increased and approached the normal rates starting from day three until germination is fully achieved in day four (Figure 2.10).

Under potassium starvation conditions, our results indicated a significant reduction in germination for all genotypes compared to control conditions (Figure 2-8). The results showed a severe reduction in germination on 10, 30 and 50 µM K^+ media. This reduction in germination decreased with the increase of K^+ concentration in the medium from 10 µM to 50 µM and almost disappeared when 200 µM were added to the medium. This inhibition was very high compared to other work such as presented by Pyo et al., (2010). For example, Pyo et al., (2010) indicated that germinating seeds on 10 µM K^+ medium resulted in slightly lower germination percentage for wildtype especially in the first two days of germination. However, the double mutants of *athak5akt1* showed a very low germination percentage compared to single mutants of *athak5*, *atakt1* and wildtype, despite the recovery of germination in the third day. Their results also showed that full germination on 10 µM K^+ was never achieved even after 7 days of treatment. From this, we can suggest that external K^+ can still affect germination especially when we test a mutant that is deficient in important K^+ transporters such as AtHAK5 and AtAKT1.

The differences in germination rates are due to differences in the media used in ours and in Pyo et al., (2010) assays. This can effectively result in different germination rates. In their study, NH_4^+ concentrations were adjusted to avoid the interference with the high affinity uptake of K^+. Therefore, an NH_4^+ free medium was used by substituting $NH_4H_2PO_4$ with H_3PO_4 then K^+ was added as KCl in tested concentrations. In our medium, we substituted K^+ with Na^+ in same levels, while NH_4^+ was left in the medium (see methods). The final concentration of NH_4^+ in the medium was approximately 10 mM, while it was 9 mM for Na^+. This indicates that high levels of both ammonium and sodium compared to K^+ occurred in medium and resulted in osmotic stress and maybe toxicity. This explanation is likely because when we increased the K^+ concentration in medium from 10 to 30, 50 and then 200 µM, the inhibitory effect of NH_4^+ decreased. We also conducted a test experiment using an NH_4^+ free medium and the inhibition was far lower when compared to our results (see Appendix). In this test, we used a medium that is both NH_4^+/Na^+ free by Spalding et el., (1999). Removing NH_4^+ from medium reduced the

inhibition in germination although was still seen, but was not significant. Adding 5 mM of NH_4^+ rendered seeds sensitive to 50 µM K^+. It is important to test different combinations between K^+, NH_4^+ and Na^+ to see the effect on germination. This suggests that the toxic effect of NH_4^+ is more prominent during K^+ starvation, because it is assumed that NH_4^+ competes with K^+ on same transporters resulting in deficiency symptoms.

2.5.2 K^+ relieves NH_4^+ toxicity and Na^+ cannot substitute K^+ in high NH_4^+/K^+ conditions

Increasing K^+ concentration in the medium did improve germination and seedling establishment, because K^+ can reverse the inhibitory effects of NH_4^+ toxicity (Cao et al. 1993). However, the concentration required of K^+ to elevate this effect varies depending on the NH_4^+ concentrations presented in the medium. For example, Cao et al., (1993) indicated that to elevate the toxic effect of 4 mM NH_4^+, 0.03 mM of K^+ are needed. Accordingly, to elevate the inhibitory effect of 10 mM NH_4^+, 0.09 mM of K^+ are needed. In our experiments, we noticed that the addition of 50 µM of K^+ still showed inhibition in the seedling establishment when compared to wildtype despite improving germination rates (Figure 2-8). However, when 200 µM of K^+ were added to the medium, the inhibitory effect almost disappeared after 3 days of treatment (Figure 2-9, 2-10). This explains the persistence of the inhibitory effect of NH_4^+ even with the increase of K^+ levels in the medium. This effect is mainly reversed by the recovery of root growth.

However, it is important to mention that this inhibitory effect did not result from NH_4^+ alone, because under 50 µM K^+ seedlings did not grow normally as in wildtype and displayed a severe inhibition in plant size and developed no roots (Figure 2-7 and 2-21) even after 10 days of treatment where all seeds germinated. Cao et al., (1993) indicated that whatever the toxicity resulted from, it could be reversed. Therefore, and since the inhibition was continuous, in our medium we had a 9 mM of Na^+ which might have affected plants negatively in the lack of important ion such as K^+. We suggest that this might have caused osmotic stress on seedlings that was noticeable only under low K^+ conditions, while the control treatment contained enough K^+ to oppose the toxic effect of NH_4^+ and no Na^+.

Since our medium contained NH_4^+, we can suggest that any phenotypes obtained here are in the lack of other functional HAK/KUP/KT family members. Other HAK/KUP/KT members that were previously found to function in root growth and K^+ uptake are AtHAK5, AtTRH1, and AtKUP7 (Rigas et al. 2001; Shin and Schachtman 2004; Han et al. 2016). It is previously confirmed that the members of this family of transporters are inhibited by NH_4^+ (Spalding et al. 1999), therefore, they represent the non-AKT1 mechanism in K^+ uptake when NH_4^+ is absent. In conclusion, we had phenotypes that resulted from NH_4^+ toxicity combined with low K^+, and the possible inhibition of other KUPs transporters in the background. This is interesting, because it leads to new explanations regarding transporters tested and how they reacted differently under such stresses.

The medium we used resembled the medium used in this paper (Cao et al. 1993), where they substituted K^+ salts with Na^+ salts but kept NH_4^+ in a concentration that goes up to 10 mM. From this, we can suggest that the recovery of germination that happened in the seeds in the third and fourth days was due to the uptake of K^+ from the external medium. The internal K^+ stored in seeds may not be enough to relieve this inhibition, because it is limited. The higher the concentration of K^+ in medium, the higher the recovery. They also indicated that other alkali ions could relieve NH_4^+ toxicity by recovering root growth, such as Cs^+, Rb^+ but interestingly, not Na^+, which we noticed in our experiments. Our medium contained 9 mM of Na^+ but this did not lead to better germination rates under NH_4^+ toxicity in the absence of sufficient K^+ levels.

2.5.3 The AtKUP12 is important in the early stages of germination during K^+ starvation resulted from high NH_4^+/K^+ ratio

Our data showed that germinating seeds over low K^+ concentrations (10, 30, 50 and 200 μM) caused significant inhibition in all genotypes. Many genotypes showed impaired germination and non-significant results at different time points and treatments. However, the two mutant lines of *atkup12-1* and *atkup12-2* exposed significant inhibition under all treatments and at all time points (Figure 2-8, -9, -10, -11, -12). The loss of function of AtKUP12 resulted in a very sensitive seed germination phenotype. We explained previously that the high NH_4^+ levels in the medium meant that most likely all

HAK/KUP/KT family members are inhibited under such conditions in all genotypes tested. And therefore, any phenotype we obtained here in the knockout mutants of *atkup12* might suggest a role for AtKUP12 in K^+ uptake during NH_4^+ absence, or else balancing the K^+/H^+ in the cytoplasm by K^+ release from the vacuole.

However, since even under control treatment seeds of *atkup12* showed lower germination rates (Figure 2-8 and 2-11), we suggest that AtKUP12 plays a role in K^+ transport even under high external K^+ levels such as 10 mM, specifically in the occurrence of high NH_4^+ levels.

Pyo et al., (2010) indicated that there is an unidentified transporter that functions in K^+ acquisition in the intermediate concentrations such as 400 µM but not at 100 µM. This transporter is likely to be affected by the existence of Na^+ in the medium. They also indicated that the loss of function of *athak5akt1* double mutants was sensitive to NH_4^+ during seedling establishment. This is in line with our experimental conditions, where we had a high NH_4^+ medium and 10 mM K^+ in the control treatment with an inhibition in germination of *atkup12-1,2* mutants. Although we did not find this phenotype during seedling establishment under control treatment, maybe the reason is that there might be functioning AKT1 channel that enabled plants to establish or possibly other transporters in roots that replaced AKT1 and HAK5 such as NSCC (Non-Specific Cation Channels) in roots. These transporters are not sensitive to NH_4^+ and therefore can probably substitute the main K^+ acquisition mechanisms in roots (AtHAK5 and AtAKT1) while they are blocked by NH_4^+ or Na^+. Therefore, further investigation for a triple mutant of AtHAK5, AtAKT1 and AtKUP12 where we can completely terminate K^+ uptake is suggested. This can lead to a better understanding for AtKUP12 role in germination and seedling establishment. Since we obtained the most significant results for the *atkup12* mutants under concentrations between 10-200 µM of K^+, which is the range that both AtHAK5 and AtAKT1 function at (Alemán et al. 2011), it is also interesting to combine these three mutants and see if the phenotype changes.

We can conclude that AtKUP12 is important for germination under high NH_4^+/K^+ ratios, the predicted vacuolar localization of AtKUP12 can reinforce this conclusion. For example, it can participate in the release of K^+ from the vacuole to the cytoplasm during

these conditions to maintain the K^+ content in cytoplasm. Indeed, we noticed a possible epistasis relationship between AtKUP12 and AtTPK1 in our results, which can suggest a similar pathway for these two proteins. This is discussed in the next sections.

Another KUP transporter showed involvement in K^+ uptake during K^+ starvation conditions, AtKUP7 have recently been indicated to affect K^+ uptake under 10-200 µM external K^+ (Han et al. 2016). It is important to indicate that both AtKUP12 and AtKUP7 are in the same clade in the phylogeny of the HAK/KUP/KT family (Nieves-Cordones et al. 2016) (Figure 1-2). This suggests possible redundancy or functional relationship between these two transporters. It would be interesting to study plants that have loss of function of both transporters under different stress conditions.

2.5.4 Behavior of *tpk1*, *hak8*, *hak8kup12* and *hak8kup12tpk1* under treatments

Under control conditions, our results indicated no significant differences in germination between wildtype and *tpk1* mutants (Figure 2-8). Which contradicts the results obtained by Gobert et al., (2007). We can explain this by the difference in the media used in both studies. However, as mentioned before, we had high NH_4^+/Na^+ levels that resulted in different germination rates. It is possible that the medium composition resulted in different or lack of phenotypes previously obtained. It is also important to indicate that Latz et al., (2013) did not show a significant reduction in germination after 3 days under control conditions for the two *tpk1* mutant lines they tested which is in accordance to our results.

The treatments did not show significant results for the *tpk1* single mutant after 48 hours of germination. However, after 72 hours, an inhibition of germination rates existed but only under 30 µM K^+ treatment (Figure 2-9). However, it was not significant according to the statistical analysis. When data were normalized to control, we could still see lower germination under this concentration in *tpk1* mutant seeds.

hak8 mutants showed higher germination under different treatments and time points (Figures 2-8, -9, -10), this increase was significant when compared to wildtype. The increase only appeared in two treatments (30 µM and 50 µM K^+), while no significant differences were found under 30 µM K^+. A post-hoc test could not find any significance

when we compared treatments to each other. We therefore suggest an experimental variation rather than an actual difference due to treatments. Generally, we think that *hak8* responded similarly to wildtype under all treatments.

The double mutants of *hak8kup12* showed some increase in germination rates under 10 μM of K^+, but there was no differences under other treatments. Instead, we noticed that *hak8kup12* response was typical to the *hak8* single mutants. Therefore, there is a possibility that segregation occurred in the double mutants of *hak8kup12* that resulted in such phenotype. Possibly, *hak8kup12* exposing the single mutant of *hak8* rather than a double mutant of *hak8* and *kup12* due to segregation. Consequently, further investigation is needed here to indicate if *hak8kup12* is a loss of function of *KUP12* and *HAK8* mutant. We would expect to see an effect of loss of function of *kup12* here, but we did not. Only if this analysis indicated that *hak8kup12* is a double mutant we can suggest a possible epistasis relationship between HAK8 and KUP12 transporters. Moreover, the statistical analysis revealed no such relationship between the two transporters.

Similarly, the triple mutants of *hak8kup12tpk1*, showed a typical *kup12* responses under all treatments. It would be difficult to analyze the phenotype of the triple mutants because we cannot determine where the effect comes from. Presumably, since we did not see any effect for the treatment in the test experiment (See Appendix), the segregation possibility still occurs. Therefore, the phenotype of the triple mutant could be simply the result of the loss of function of *KUP12*. The statistical analysis did not indicate any differences between *hak8kup12tpk1* and *kup12* under any treatments or time points. Finally, further analysis for these two mutant combinations is needed to enable us to explain the physiological effects under our treatments.

2.5.5 Combining knockout mutants suggests epistasis in function between AtTPK1 and AtKUP12

Analysing single mutants under stress treatments indicated the involvement of AtKUP12 in the response of plants to high NH_4^+/K^+ ratio. The loss of function of AtKUP12 negatively affected germination under all low K^+ treatments. Meanwhile, loss of function of AtTPK1 did not affect germination and showed a typical wildtype response. Interestingly, combining the two mutants resulted in a rescue for germination and the

atkup12 phenotype disappeared. The statistical analysis indicated that *tpk1* mutants were not significantly different from *kup12tpk1*, while it was significant compared to *kup12*. From this, a possible epistasis relationship between AtTPK1 and AtKUP12 can be suggested. We can benefit from the AtKUP12 predictions as a vacuolar transporter in this result and suggest that AtKUP12 might participate in relieving the stress resulting from high NH_4^+/K^+ ratio. AtKUP12 might function in K^+ release from vacuoles to compensate low K^+ resulted from such conditions using the coupled transport mechanism we explained in chapter 1. Moreover, being a HAK/KUP/KT family transporter it could be functioning in adjusting the pH of cytoplasm if affected by external high NH_4^+ concentrations.

The epistasis between TPK1 and KUP12 may indicate that TPK1 is epistatic to KUP12 (masks its effect), therefore, TPK1 functions downstream of KUP12 while KUP12 functions upstream of TPK1 (Figure 2-29). AtKUP12 therefore negatively regulates AtTPK1. This kind of epistasis relationship is considered a (switch regulation pathway). Both TPK1 and KUP12 can function in K^+ release from vacuoles during K^+ deficiency.

The possible epistasis relationship is important as it helps understand the complex interactions between different channels/transporters during the response of plants to abiotic stresses, which was the initial hypothesis of this thesis. There are multiple scenarios here regarding the localization of KUP12 according to its predicted function.

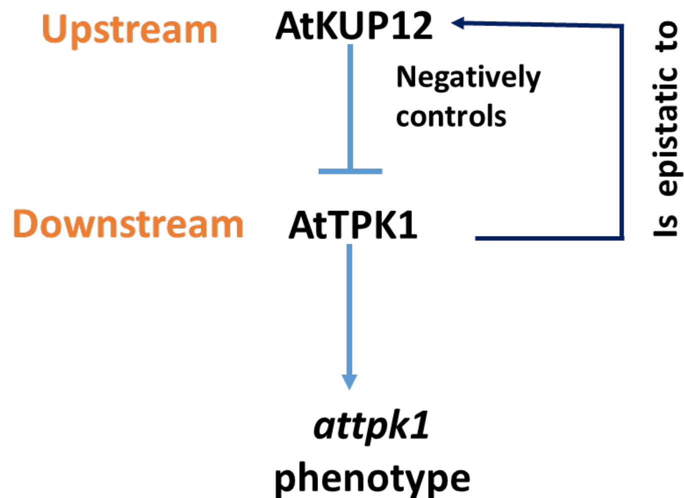


Figure 2-29: Suggested model for the epistasis relationship between AtKUP12 and AtTPK1

AtKUP12 and AtTPK1 both function in the same pathway (possibly K^+ release), where KUP12 negatively controls TPK1. TPK1 is epistatic to KUP12 (Masks its phenotype), TPK1 functions downstream of KUP12 while KUP12 functions upstream.

2.5.6 AtKUP12 is important for seedling establishment as for germination under low K^+ treatment

Members of HAK/KUP/KT family of transporters are involved in the high affinity K^+ uptake in roots (Santa-María et al. 1997; Quintero and Blatt 1997; Shin and Schachtman 2004; Gierth et al. 2005). AtHAK5 is essential for plant growth under very low K^+ concentrations such as 10 µM, loss of function of this transporter caused root growth defect under such concentration in the absence of NH_4^+ (Pyo et al. 2010). Our results indicated that at this K^+ concentration resulted in severe inhibition in seedling establishment occurred due to high NH_4^+ in media. Despite we do not have the data from the seedling establishment in the test experiment, we can conclude from the stress experiments of K^+ starvation (Figure 2-24), that the seedling of *kup12* grew indifferently to wildtype or the *kup12tpk1* double mutants where seedling were able to establish despite the severe inhibition in sizes under 10 µM of K^+.

The important note here is that single mutants of *kup12-1* were the most affected by NH_4^+ under 10 µM of K^+ (Figure 2-13). From this, we can suggest a possible role in relieving

K^+ starvation effects that is carried out by AtKUP12, and due to NH_4^+ toxicity, no other KUP transporter was able to compensate this role. This is a new result for this transporter, as it was not tested before under these conditions. The media used in low K^+ treatments contained high NH_4^+ concentrations known to inhibit root growth, and there is a possibility that K^+ and NH_4^+ share same transports in roots. Plants need to utilize its metabolites to cope with the NH_4^+ toxicity, and efflux NH_4^+ out of cells, which is reflected on the growth of plants negatively. The epistasis between TPK1 and KUP12 could be seen in the establishment of seedlings (Figure 2-13) as the double mutants of *kup12tpk1* showed recovered establishment.

As we mentioned previously, the conditions of the media resulted in the inhibition of KUP transporters due to the high NH_4^+ levels, therefore, the same suggestion might be applied here. AtAKT1 K^+ channel is insensitive to NH_4^+ (Dennison et al. 2001), and therefore, when NH_4^+ exists in the medium AKT1 is expected to function in K^+ uptake when HAK/KUP/KT family of transporters is inhibited. However, with higher NH_4^+ concentrations in the medium, we can expect that even AKT1 is blocked and therefore the total K^+ uptake is hindered affecting growth negatively. Increasing K^+ in medium from 10 to 30, 50 and 200 μM improved the growth of plants as previously happened in germinations of seeds.

2.5.7 AtKUP12 rescued the growth of the mutant yeast on K^+ deficient medium

To test if AtKUP12 rescues the growth of a K^+ deficient yeast strain over low K^+ media, heterologous expression of the protein in yeast was carried out, using a yeast strain that lacks the main K^+ transporters (TRK1, TRK2 and TOK1) in the plasma membrane and cannot grow in a low K^+ medium.

Our results showed that AtKUP12 rescued the growth of the mutant yeast on low K^+ concentrations (Figure 2-20). This complementation was present under the last three dilutions (3, 4 and 5) with the number of single colonies higher in the AtKUP12-pYES2 compared to the vector control. However, the growth of yeast under 2 mM K^+ was similar in both genotypes, indicating that AtKUP12 could only rescue the growth under low K^+ concentrations. This result only occurred using glucose as a carbon source in the medium. It is also important to mention that the yeast grew better and faster when glucose was used

in the medium. Contrarily, galactose caused slower growth especially for the vector control strain. The yeast uses glucose as an energy source directly, but when galactose occurs in the medium, it has to convert it first through a different pathway into glucose then consume it (Stockwell et al. 2015). This process explains the slower growth on galactose. Moreover, the lack of K^+ creates more difficulty for the yeast to grow.

When galactose was used instead, the growth of the vector control was inhibited under the three K^+ concentrations tested (including the 2 mM K^+), while both wildtype and AtKUP12-pYES2 strains grew normally and better than the growth on glucose contained medium through the five dilutions tested. This indicates that AtKUP12 rescued the growth in both low and high K^+ concentrations.

This could be either explained by two means, first: that the phenotype is not K^+ dependant and possibly another component in the medium could be the reason of rescuing the yeast growth. We cannot define this component as not enough experiments have investigated this, however, if we considered the previous results in seed germination and establishment, we can suggest a role for AtKUP12 in pH adjustment or relieving osmotic stress. Being a HAK/KUP/KT family member, KUP12 might be functioning as an H^+/K^+ symporter and help adjust proton concentration during K^+ deficiency. Other members of this family showed dual activity such as AtKUP1, which increased K^+ uptake in yeast under both high and low affinity conditions (Fu and Luan 1998). This suggests that various members of this family can function under different external K^+ concentrations. Ragel et al., (2015) indicated that the yeast co-expression of AtHAK5 and its protein kinase CIPK23, affected the transporter affinity. This could be applied in our case, once proper phosphorylating proteins of AtKUP12 are known.

Second: the galactose induces the expression of *AtKUP12* in yeast because the pYES2 is a galactose induced vector. This might explain the better growth of AtKUP12-pYES2 compared to the empty vector even after 2 days of incubation. However, different treatments and K^+ measurements in media can also explain more about the role of AtKUP12 in K^+ transport.

The rescue of yeast growth by AtKUP12 can enable a space to speculate the localization of this transporter. Since the K^+ transporters deleted in the yeast mutant are plasma membrane transporters, a suggestion can be that KUP12 is located in the plasma membrane of Arabidopsis cells. Han et al., (2016) speculated the localization of the KUP7 transporter after being able to rescue the growth of mutant yeast. The localization analysis they carried out in plants confirmed it is a plasma membrane transporter, even though it was previously predicted as a tonoplast protein (Whiteman et al. 2008). However, for AtKUP12, the localization must be confirmed in the plant system. Moreover, there is still a possibility for the vacuolar localization of KUP12 based on germination results.

However, we must ask, why does a potential vacuolar K^+ transporter substitute plasma membrane K^+ transporters? Therefore, an explanation might be that AtKUP12 is a plasma membrane transporter based on the previous results, or the fact that the expression of plant proteins in yeast can face difficulty due to the difference in directing proteins in the yeast system. This can be due to the lack of other regulatory proteins responsible for the direction of a protein in a plant cell, which urges the need of studying AtKUP12 expression along with its regulatory proteins once identified. It is also important to test the function of AtKUP12 in a yeast mutant that lacks vacuolar K^+ transporter to fully determine its role and have more inputs to explain the results.

The results emphasise that multiple members of the HAK/KUP/KT family members are functioning in K^+ transport and can carry out a high affinity K^+ uptake function in heterologous systems. Overall, it is tempting to suggest a plasma membrane localization of AtKUP12 as a member of a cluster where other transporters such as AtKUP7 were confirmed as plasma membrane K^+ transporters (Han et al. 2016). Further analysis for this transporter is necessary to understand how it functions under different types of stress and how we can benefit from this in improving the productivity of crops.

2.5.8 Role of transporters in seedlings under stress treatments

It was suggested previously that the KUP6 subfamily of K^+ transporters are expressed in roots and participate with the GORK channel in K^+ efflux, as a response to stresses (Osakabe et al. 2013; Demidchik 2014). They also analysed the role of these transporters in the response of roots to auxin. KUP2, KUP6 and KUP8 were found to function in

lateral root regeneration negatively by the efflux of K^+ during this process. The loss of function of mutants of these transporters and GORK channel (*kup268* and *kup68g*) showed enhanced lateral root growth in response to auxin (Indole-Acetic-Acid [IAA]) treatment. While the single mutants of *kup6* and *kup8* showed same response but with less stronger phenotypes (Osakabe et al. 2013).

The higher growth of *hak8* single mutants under high levels of K^+ (80 mM) treatment could be due to a higher K^+ efflux that led to a less negative effect of stress on plants (Figure 2-21). The loss of function of *HAK8* under such conditions might lead to the up-regulation of other K^+ channels/transporters involved in K^+ efflux. These transporters are mostly its homologues (KUP2 and KUP6) as well as the GORK channel. When plants were treated with higher concentration of sorbitol (120 mM), *hak8* mutants showed better growth. We suggest that under these conditions loss of function of HAK8 helped in prevention of K^+ loss, which improved water retention under such osmosis.

On the other hand, *tpk1* mutants showed sensitivity to 120 mM sorbitol (Figure 2-21), indicating a role for this channel in osmotic stress possibly by K^+ release from vacuole to cytoplasm. AtTPK1 is suggested to function in K^+ accumulation to vacuoles during germination of seeds. *attpk1* mutants showed sensitive phenotype to both low and high K^+ treatments. Gobert et al., (2005) suggested that AtTPK1 functions in K^+ movement internally to adjust the osmosity of cells under stress conditions but it does not change K^+ levels in tissues. In our results, TPK1 might play a role under 120 mM sorbitol treatment in adjusting cell turgidity by controlling K^+ flux across tonoplast.

Comparing *hak8tpk1* with single mutants indicated that it was not significantly affected by the treatments. Although *hak8* showed higher fresh weights, but it was initially higher than wildtype in control conditions which indicates a variation in the initial weights of plants. Comparing *tpk1* and *hak8* to the double mutants did not indicate significant differences, which might indicate that the difference in fresh weights is due to experimental conditions variations but not the treatments.

The loss of function of KUP12 resulted in lower fresh weights compared to wildtype when treated with 80 mM of NaCl (Figure 2-22). Moreover, the double mutants of

kup12tpk1 showed same inhibition in fresh weights. We think that this is due to the mutant in *AtKUP12*, however, the reduction in weights is very small when compared to all genotypes under treatment, and could be biologically not important.

The double and triple mutants of *hak8kup12* and *hak8lup12tpk1* did not show any significant results under stress treatments (Figure 2-23). The treatments were significantly different when compared to control conditions. Combining mutants did not result in different phenotypes such as in germination. The lack of phenotypes again suggest the possibility of segregation in the multiple mutants between *kup12*, *hak8* and *tpk1*.

2.5.9 Water loss and conductance measurements

The water loss experiments and stomatal conductance indicated no differences in the response of single mutants compared to wildtype (Figure 2-25, -26). The water loss experiments were done first in room conditions, which led to no phenotypes in rosettes. However, putting plants in hoods resulted in some significant differences for *tpk1*, *kup12hak8* and *hak8kup12tpk1* when compared to wildtype 90 minutes of the start of the experiment. We did not test the single mutants in these conditions due to time limitation, therefore we cannot determine if this effect was initiated in the in single mutants first. However, comparing the two experiments results can suggest that these differences might have happened due to exhausted leaves of genotypes that led to faster closure of the stomata in the leaves of mutants compared to wildtype. The insertions might have led to leaves of mutants of being much sensitive to cutting and handling and therefore the synthesis of ABA ending with closure of stomata.

The stomatal conductance measurements showed various responses of the leaves of mutants to 10 μM ABA, while *hak8* showed lower conductance when compared to wildtype after ABA treatment. The reduction was not significant when compared to wildtype. *kup12* single mutants did not show any significant differences in conductance when compared to wildtype (Figure 27-A). We would expect a conductance phenotype for *kup12* mutants, since we obtained clear phenotype in germination. However, it would be possible that we get this phenotype if plants were under K^+ stress such as in germination experiments.

tpk1 single mutants showed higher conductance as a response to ABA and was less sensitive to this addition when compared to wildtype in the first 20 minutes of treatments (Figure 2-28). This is in accordance with Gobert et al., (2007) who indicated that the loss of function of AtTPK1 reduces the K^+ efflux from guard cell vacuole resulting in slower closure kinetics. However, after this time point, the conductance was impaired in both wildtype and *tpk1* mutants. We have conducted many experiments to test the conductance of *tpk1* mutants as a response to ABA and it was not possible to reproduce the results in line with Gobert et al., (2007) (data not shown). We suggest that this might be due to difference in experimental conditions.

The double mutants of *hak8kup12* showed comparable results to wildtype when treated with ABA, perhaps this is in line with the lack of phenotypes in *hak8* and *kup12* single mutants. Meanwhile, the triple mutants showed a response that is closer to *tpk1* single mutants after treatment (Figure (2-28). The triple mutants exposed higher conductance as a response to ABA. We think that this is due to the loss of function of the TPK1 channel, however, it could be difficult to analyse the phenotype of the triple mutant due to the possibility of the involvement of various genes in this phenotype.

Chapter 3. The role of potassium channels in rice responses to abiotic stress

3.1 Introduction

3.1.1 The SKOR channel and its role in abiotic stress tolerance

The K^+ level in a plant is an indicator of its health. It is well known that higher K^+ concentrations help relieve stress. A study on the salinity stress in rice cultivars has indicated that the salt tolerant cultivars were the ones with the highest K^+/Na^+ ratio in leaves while the susceptible cultivars showed lower K^+/Na^+ ratios (Asch et al. 2000). This indicates that maintaining a high K^+/Na^+ ratio in leaves is an important mechanism of salinity tolerance. Multiple K^+ transporters are involved in the uptake and distribution of K^+ in plants (See chapter one introduction). The SKOR channel is functioning in K^+ upload into xylem in Arabidopsis plants and loss of function of this channel causes lower K^+ content in shoots and xylem sap (Gaymard et al. 1998). SKOR is localised in the plasma membrane of the root stelar cells. It was down-regulated as a response to K^+ starvation in Arabidopsis plants, alongside the other shaker-type family channel AKT2. This is a possible mechanism of K^+ starvation tolerance in plants where K^+ recirculation is restricted by decreasing the activity of the channels involved in K^+ transportation in xylem sap and phloem (Maathuis et al. 2003; Pilot et al. 2003). These important notes indicate a role for the SKOR channel in controlling K^+ transport to shoots under different stress conditions, and probably participating in the stress tolerance in plants.

SKOR belongs to the shaker type K^+ channels family, which plays a role in K^+ management in plants. The plant family members resemble the animal members, they feature four α-subunits where each subunit consists of a polypeptide with N-terminus and C-terminus presented in the cytosolic side and six transmembrane regions with a pore between the S5 and S6. Johansson et al., (2006) conducted an interesting study on the sensitivity of the Arabidopsis SKOR K^+ channels in K^+ efflux. They used site-directed mutagenesis to investigate the domains responsible for K^+ selectivity. The study revealed a protein domain in the membrane-spanning region that functions in the K^+ and voltage

sensitivity of the SKOR channel in Arabidopsis. Johansson indicated that the SKOR channel is regulated by the external K^+ and not the internal, he reported the channel being regulated internally in a non-voltage dependent way (Riedelsberger et al. 2010).

The interesting findings for this channel showed it has a potential role in abiotic stress responses. We wanted to transfer this knowledge to an economical crop such as rice, aiming at improving its productivity during different stress conditions. It is a recommended candidate for the overexpression studies due to its role in K^+ upload from roots to shoots through xylem sap (Munns 2005). Using multiple potential overexpressor lines, we analysed the effect of overexpression of *SKOR* in rice growth under different stress conditions.

3.1.2 The TPKb vacuolar channel and its role in abiotic stress tolerance in rice

Vacuolar K^+ channels may also participate in relieving the harmful effects of stress on plants. The overexpression of the *AtTPK1* of the TPK family for example resulted in better growing plants under K^+ starvation conditions (Gobert et al. 2007) as well as faster germination under ABA treatment (see chapter one introduction). This indicates a role for TPK1 channel in stress relief possibly by releasing K^+ from vacuoles during K^+ starvation. This is to maintain the essential physiological functions in the cytoplasm. In rice, there are two isoforms of TPK channels, TPKa localized in the tonoplast of the lytic vacuole (LV), and TPKb localized in the tonoplast of the protein storage vacuoles (PSV) (Isayenkov et al. 2011). The overexpression of the TPKb channel in rice resulted in an increase in both shoots and roots K^+ content and a relief of K^+ starvation effects (Ahmad et al. 2015). This relief was reflected in the higher relative growth rate of overexpressor lines compared to azygous lines. The accumulation of K^+ in shoots and roots was noticed under both control and K^+ starvation treatments. This result was reported in hydroponics and soil growth conditions. In addition, different overexpressor lines were tested in this study. The increase in K^+ levels could be explained by two possibilities. First: the release of K^+ from vacuoles in the overexpressor lines resulted in hyperpolarization of the plasma membrane and therefore the activation of K^+ inwardly channels and K^+ transporters such as OsAKT1, OsKAT1 and OsHAK1. Second: the hyperpolarization at the same time

resulted in the deactivation of the K^+ outwardly channels such as GORK, which led to a reduction in K^+ efflux through this channel, although experiments did not show the difference in K^+ leakage between wildtype and overexpressor lines. In this study, we aim to analyse the expression levels of these transporters under the overexpression of *TPKb* during K^+ starvation conditions. This will be achieved by RT-PCR analysis of rice roots.

3.2 Aims of the chapter

In this chapter, we investigate the possible approaches to improve rice growth under different stress conditions through an overexpression approach. As previously mentioned, AtSKOR channel functions in uploading K^+ into xylem sap where it is transported to shoots, while OsTPKb, is functioning in the release of K^+ from the vacuole to the cytoplasm of rice plant cells. High K^+ levels were found in the overexpressor lines of *TPKb*, and other candidates were investigated for potential roles in this phenomenon.

Therefore, the aims of this chapter are:

- Analysing the effect of abiotic stresses on growth and ion content of *SKOR* overexpressor lines.
- Identifying the possible role of different K^+ transporters (GORK, KAT1, HAK1) in the high K^+ levels obtained in *TPKb* overexpressor rice lines during K^+ starvation, by analysing the difference in expression levels of these transporters.

3.3 Materials and methods

3.3.1 Plant material

Potential overexpressor lines of the *SKOR* gene (Os06g14030) (*12, 13, 19, 23,* and *31*) with the background of (*Oryza sativa*. L) sub group *japonica* cv. Nipponbare were obtained from previous transformation work by Mohammed Younousee in the laboratory of Maathuis. Lines were a T3 generation, and seeds were grown from each line to be checked for *Hygromycin* resistance. Homozygous lines were used in physiological experiments. Two overexpressor lines of the *TPKb* gene (Os07g01810) (*OX1* and *OX2*) and one azygous line that was used as a control were obtained from Izhar Ahmed,

Maathuis Laboratory. The transformation was conducted as mentioned in the paper (Ahmad et al. 2015). Plants were a T3 generation. Seeds were grown over hygromycin-contained medium to select homozygous lines, which were used in physiological experiments. Experiments were repeated three times unless differently mentioned.

3.3.2 Hygromycin media selection

Seeds of potential overexpressor lines of *OXTPKb* were gown first over water agar plates containing 0, 50, 75 and 100 mg/L of the antibiotic hygromycin. As the transformed lines contained the hygromycin resistance gene so we can select it from the non-transformed lines. Seeds were surface sterilized using 10% Sodium hypochlorite (v/v) for 15 minutes then washed in sterile DW four-times. Seeds were then soaked in DW and incubated at 28 °C for one day in darkness then placed over water agar plates. The water agar plates were prepared using 8 gm of agar per liter of DW. The hygromycin antibiotic was added when the medium cooled to 60 °C and plates were kept in darkness to avoid the degradation of hygromycin. The line of which all the seeds were able to grow over hygromycin was selected as a homozygous line.

3.3.3 Growth medium and conditions

First, germination was initiated by growing rice seeds on a water-saturated terra-green substrate in the darkness at 28 °C for 10-14 days. Then, seedlings were transferred to a full growth liquid medium (Yoshida et al. 1971) (see Appendix) in five liters dark boxes for two weeks. Boxes were placed in the glasshouse under these conditions: 12h light/12h dark, 28 °C day and 24 °C night temperature, 140-180 W/m^2 light radiation and 60% relative humidity. The control medium was modified to obtain the required stress treatments. K_2SO_4 was replaced with an equimolar amount of Na_2SO_4 to obtain a K^+ free medium in the experiments, while KCl and NaCl were added to the control medium to obtain different treatments in the *OXSKOR* lines experiments.

3.3.4 Extraction of DNA and RNA

DNA and RNA were extracted from shoots of a two weeks old *OXSKOR* plants and from roots of *OXTPKb* lines. The DNA extraction procedure was as previously described in

chapter two methods with the exception of flash freezing tissues using liquid nitrogen and then samples were ground using an electric grinder. Extracted DNA and RNA pellets were suspended in 100 µl of sterile water; samples were stored at -80 °C until used.

3.3.5 PCR analysis of overexpressor *SKOR* lines

DNA of rice seedlings of overexpressor *SKOR* lines was used in the PCR analysis. *Hygromycin* primers were used (Table 3-1) in this analysis to identify the existence of the hygromycin resistance gene in transgenic lines. PCR mix and conditions were prepared as mentioned in chapter two methods.

3.3.6 RT-PCR and qPCR analysis

An RT-PCR analysis was conducted to test the expression levels in the *OXSKOR* lines. Primers used in the analysis are presented in (Table 3-1). The *OXTPKb* two overexpressor lines (*OX1* and *OX2*) were previously validated as overexpressor lines by Izhar Ahmad (Ahmad et al. 2015) and showed that the overexpressor lines had 5-20 times higher expression levels of *TPKb* compared to wildtype. RT-PCR for *OXTPKb* lines tested the expression levels of *OSsHAK1* (Os04g32920), *OsKAT1* (Os01g55200) and *OsGORK* (Os04g36740) genes in roots. QPCR was conducted to determine the expression levels of the SKOR gene in the potential *OXSKOR* lines. Three samples of each line were tested in this reaction. CDNA was prepared for the qPCR and we used it as a template. We conducted the analysis with three replicates for each cDNA template. We used the SYBR green mix. We used the histone gene as a control for data normalization. To measure the expression levels of rice transporters in *OXTPKb* lines, we used ImageJ software. We related the expression of the genes to the control (*Histone*).

Table 7: Primers used in PCR and RT-PCR analysis of *OXSKOR* and *OXTPKb* lines.

Primer	Sequence 5-3	Product size (bp)
ACTIN-FOR	TATCCTCCGGTTGGATCTTG	399
ACTIN-REV	CCATGTTTCTGGAATTGCT	399
HYGROMYCIN-FOR	GGATATGTCCTGCGGGTAAA	785
HYGROMYCIN-REV	ATTTGTGTACGCCCGACAG	785
SKOR-FOR	GATGCTGCAGTACGAGAGCA	581
SKOR-REV	TTGGAGTAAACAATGCTGCAT	581
HISTONE 3-FOR	CGAGAAGCGAAGAGGAGATG	406
HISTONE 3-REV	TCAACAAGTTGACCACGTCAC	406
GORK-FOR	TGCAGGAGCAGATCCGAGTA	435
GORK-REV	GGCGTGTTTCCCCACCTATC	435
HAK1-FOR	ACTGCATCCTGTTCCCATCG	406
HAK1-REV	GTCGTCACCACACAGATCCC	406
KAT1-FOR	GCGATGCGATCCAGCATTTC	496
KAT1-REV	TCACTTGCTGAAGGTTGCTTCT	496

3.3.7 Relative growth rate of *OXSKOR* lines under stress treatments

The growth of the *OXSKOR* lines was tested under (0 K^+, 50 mM K^+ and 50 mM Na^+) treatments along with the standard medium grown plants. Initial fresh weights of plants were measured, and then plants were weighed again after 2 weeks of treatment. Relative growth rate (RGR) was calculated based on the formula of Poorter and Garnier (1996).

3.3.8 Measurement of ion contents in rice tissues

Fresh weights of separated shoots and roots were measured then tissues were washed with 20 mM $CaCl_2$ solution for 10 minutes. Samples were placed in an oven at 80 ºC for 3 days until weights of tissues were stable. Dry weights were measured then were extracted using 10 ml of 20 mM $CaCl_2$. Samples were left for 1 day in the solution then the concentration of K^+ in shoots and roots was measured using a flame photometer (Sharewood flame photometer 410, Cambridge, UK).

3.3.9 Statistical analysis

Student's t-test was used to analyse data. Results were considered significant when the P value was < 0.05, bars on data are the standard error of the mean.

3.4 Results

3.4.1 Hygromycin selection of *OXTPKb* homozygous lines

Seeds of transgenic lines of *OXTPKb* were grown over water agar Petri plates supplied with hygromycin to select the homozygous lines. All seeds were able to grow on these plates indicating that they contain the hygromycin resistance gene. We had two overexpressor lines *OX1* and *OX2* and both were homozygous for the hygromycin resistance gene (Figure 3-1).

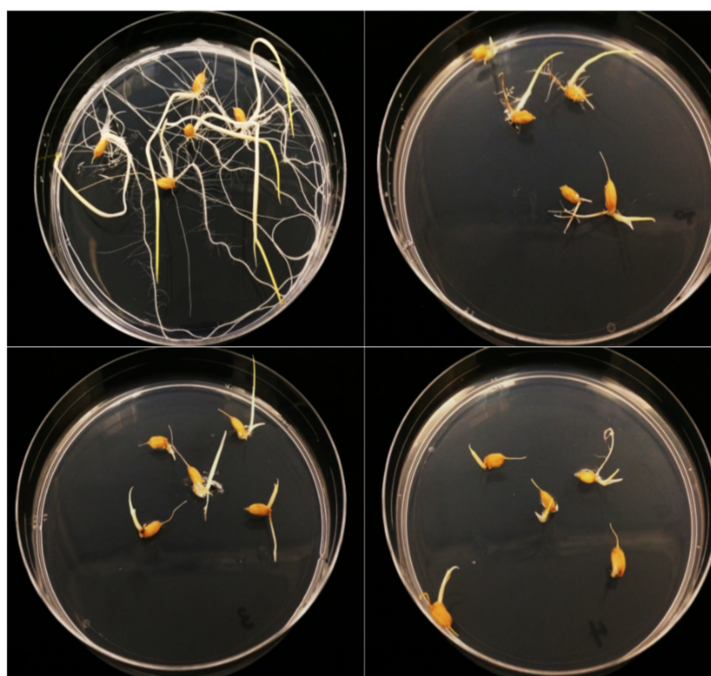


Figure 3-1: Selection of homozygous lines of *OXTPKb* seeds.

Up: 0 hygromycin medium right: 50 mg/L. Down: 75 mg/L and 100 mg/L. Surface sterilized seeds were placed over 0, 50, 75 and 100 mg/L hygromycin. The seeds grew on all concentrations indicating the existence of hygromycin resistance gene, and that this line was homozygous.

3.4.2 Identification of transgenic *OXSKOR* lines using *hygromycin* primers

Hygromycin primers were used in a PCR analysis to confirm the existence of the resistance marker gene (Figure3-2, A). The *SKOR* Lines tested showed they are not homozygous for the presence of the hygromycin gene. Lines that showed positive results were (*SKOR23, SKOR27,* and *SKOR36*) (Figure 3-2, B). Line 13 was the only line that showed no product using *hygromycin* primers for all the tested plants, indicating that line 13 is an azygous line. Accordingly, it was used as a control in the experiments. Actin gene was used as a control.

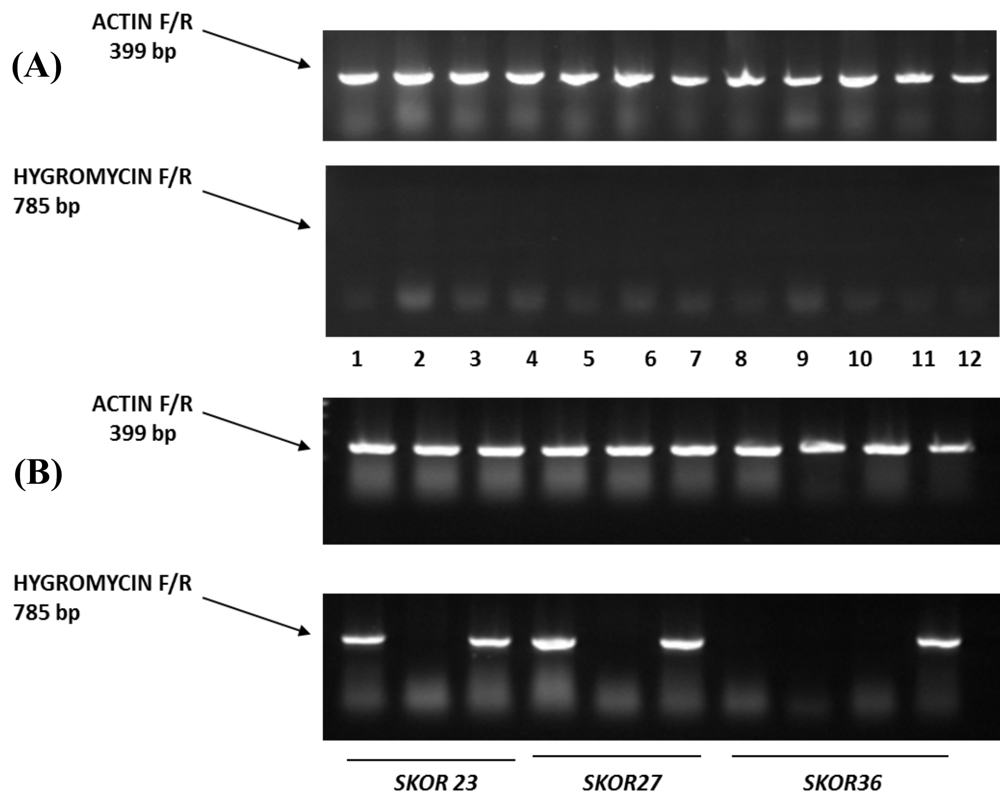


Figure 3-2: PCR analysis of *OXSKOR* lines using hygromycin.

(A): The analysis of line 13 for plants 1-12 showed clear bands with Actin gene primers at the expected size and the lack of *hygromycin* bands. These plants were used as a control line. (B): Analysis of different *SKOR* lines (*SKOR23, SKOR27* and *SKOR36*) with *actin* and *hygromycin* primers.

3.4.3 RGR of *OXSKOR* lines under different abiotic stress treatments

To understand the role of the SKOR channel in K^+ homeostasis in rice, the growth of the *OXSKOR* lines was tested under different stress treatments. *OXSKOR* lines were treated with (0 K^+, 50 mM KCl and 50 mM NaCl). These lines are obtained from plants that were previously tested with *hygromycin* primers, however, we do not know if it have the transgene or not. Under control conditions, all lines showed some differences when compared to wildtype and the azygous line *SKOR13* (Figure 3-3). Lines 12, 23 and 31 showed better growth, none of the differences was significant compared to wildtype.

Under 0 K^+ treatment, all lines showed a significant decrease in RGR compared to control condition. The RGR of all *OXSKOR* lines was higher compared to both wildtype and *SKOR13*, but this was not significant.

Increasing KCl and NaCl concentrations in the medium to 50 mM reduced RGR of plants compared to control treatment. However, this reduction was far lower than published work on rice plants, where it mostly resulted in a much significant reduction (Ahmad et al. 2015). We think this is due to the older age of our plants during these experiments, which might have reduced the inhibitory effect of these treatments. Statistical analysis did not show any significant differences between wildtype and *OXSKOR* plants. As a conclusion, we do not see a great effect for the treatments on the *OXSKOR* lines

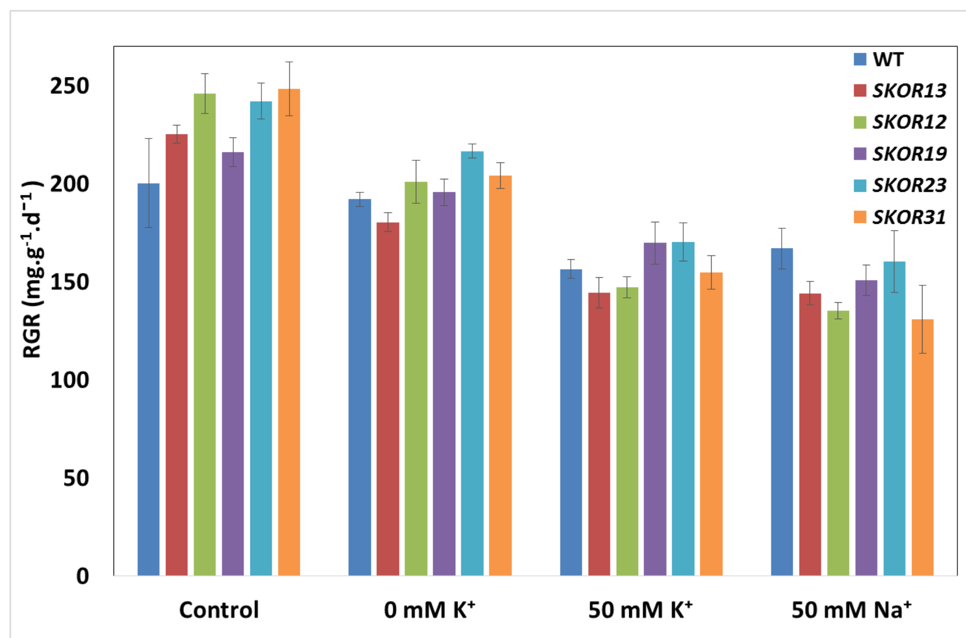


Figure 3-3: Relative growth rate of rice plants under different treatments.

Different *OXSKOR* lines were tested under different stress treatments (control, 0 mM K^+, 50 mM K^+ and 50 mM Na^+). Data were analysed using the Student's t-test. $P < 0.05$. There were no significant differences between the wildtype and *OXSKOR* plants.

3.4.4 Ion contents of *OXSKOR* lines under different abiotic stress treatments

The SKOR channel is involved in the upload of K^+ into xylem sap in Arabidopsis (Gaymard et al. 1998). Given that the *SKOR* knockout lines showed lower K^+ content in this study, overexpression was suggested to increase K^+ content under both K^+ limitation and salinity conditions improving the overall plant growth (Ahmad et al. 2015; Munns 2005). An ion analysis was carried out to test if the overexpression of the *SKOR* gene has affected the K^+ levels in both shoots and roots.

In shoots, the analysis of ion contents in transgenic plants was conducted under both standard and stress conditions. The control treatment showed comparable K^+ levels in the shoots of treated rice *OXSKOR* lines compared to the wildtype and the azygous line (Figure 3-4). The K^+ content in shoots corresponded to the treatment applied as it decreased under 0 K^+ treatment and increased under 50 mM K^+ treatment, while was comparable under control and high Na^+ conditions.

3.4.4.1 Ion contents of shoots

Under 0 K^+ treatment, the K^+ content was reduced by 50% compared to the control in all plants (Figure 3-4). The relatively high K^+ content under the 0 K^+ treatment can be explained by the period of plant growth in the control medium previously. Plants might have accumulated K^+ in their tissues and this is why we can still see relatively high K^+ in tissues although significantly reduced. The results also showed that all *OXSKOR* lines contained K^+ levels that were not significantly different from its levels in the wildtype and *SKOR13* line plants under the 0 K^+ treatment. However *SKOR13* showed lowest K^+ concentrations in shoots, but this was non-significant compared to other lines.

On the other hand, the high K^+ treatment (50 mM KCl) resulted in higher K^+ contents in the shoots. All lines displayed a comparable K^+ content in shoots, except for the *OSKOR12* line, which showed a significant increase in the K^+ concentration compared to other lines. It is not clear why this line in particular had this higher K^+ content. It could be related to the expression levels of the *SKOR* gene in this line.

The 50 mM NaCl treatment resulted in a slight reduction of K^+ content in all lines when compared to the control treatment, which can be explained by the competition of Na^+ with K^+ when presented in high concentrations in the medium. All lines showed comparable K^+ concentrations under high Na^+ treatment.

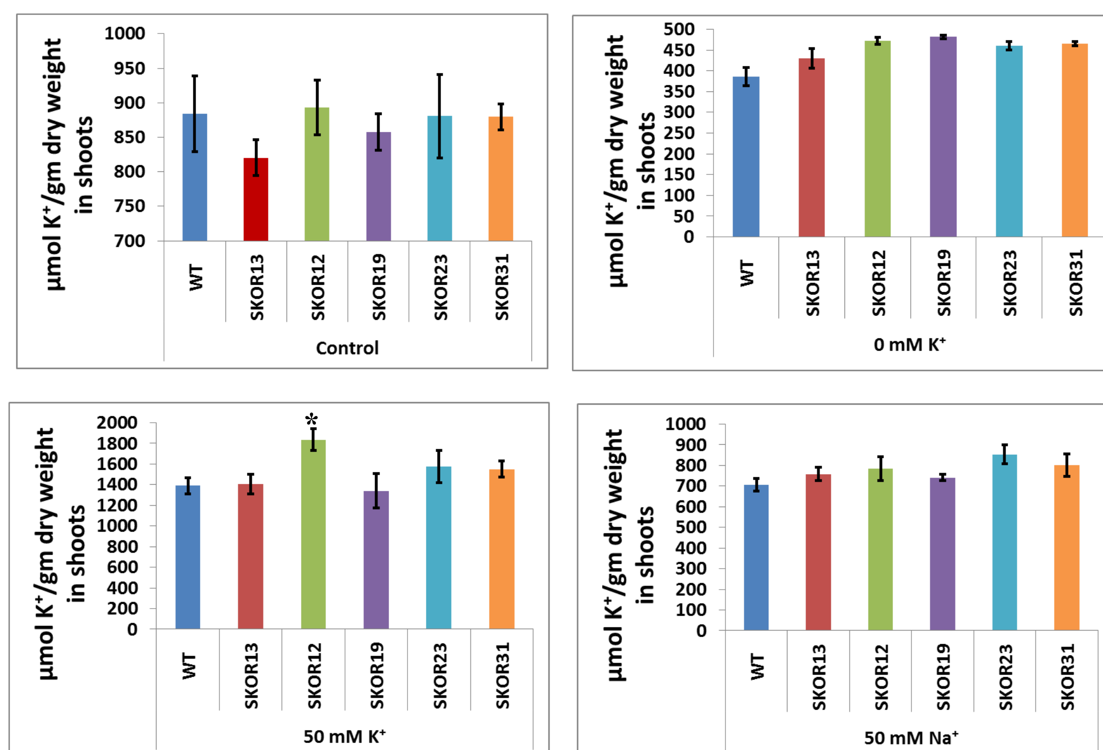


Figure 3-4: Effect of stress treatments on the K^+ content in the shoots of the *OXSKOR* lines compared to wildtype and *SKOR13*.

Plants were exposed to 0 K^+, 50 mM KCl and 50 mM NaCl treatments in a hydroponics system. K^+ content was calculated based on the dry weights of the shoots. Bars are the standard error of the mean, Student's t-test was used to test the significance of the results. $P < 0.05$.(*) represent significant results between wildtype and *OXSKOR* lines.

3.4.4.2 Ion contenets of roots

Roots showed a comparable K^+ content among all lines except line 19 which showed lower content but was not statically significant (Figure 3-5). Hypothetically, we should expect lower K^+ contents in the *OXSKOR* roots, as it is suggested that more K^+ will be transferred to shoots. But we did not see this effect in any of the lines. Treating plants with 0 K^+ resulted in a significant decrease in the K^+ content in the roots of all lines. K^+ levels in roots were about 20% of control under starvation treatment. The decrease in K^+ content in roots was greater than in shoots. This is expected as plants tend to maintain higher K^+ content in shoots to enable the physiological processes like photosynthesis to continue under stress conditions.

Under high K^+ (50 mM), plants showed increased root K^+ content in a comparable amount in all lines. The K^+ content was lower compared to control accordingly under 50 mM NaCl treatment but there was no significant difference between wildtype, azygous and *OXSKOR* lines (Figure 3-5).

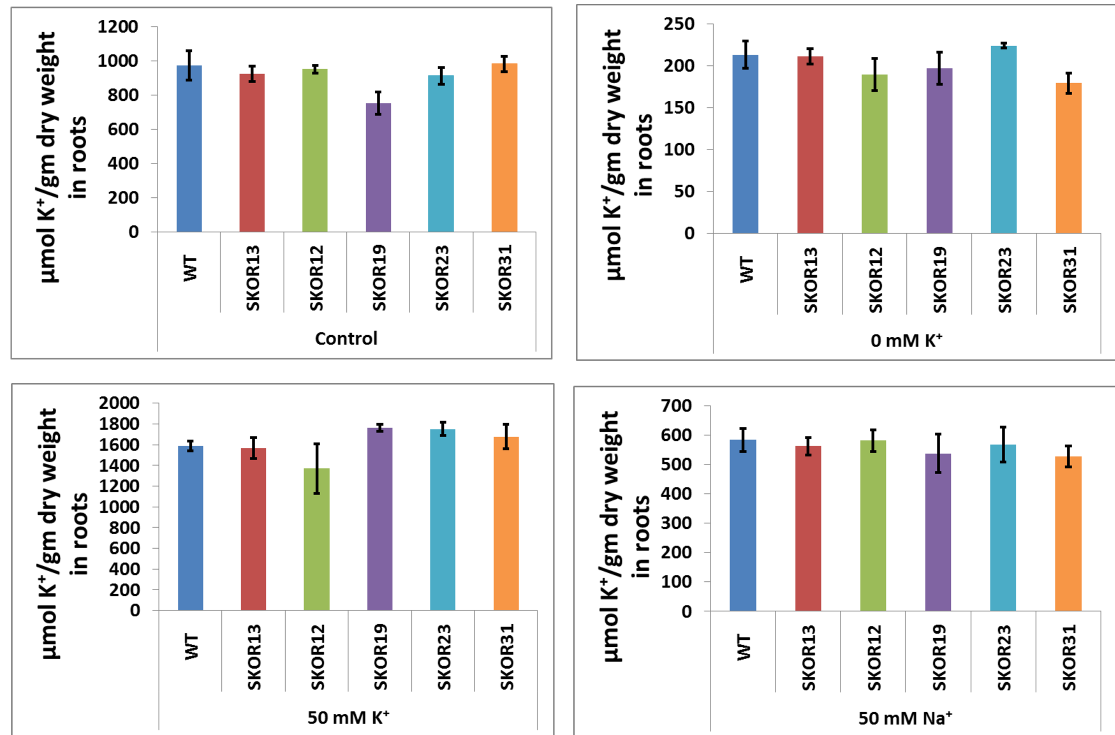


Figure 3-5: Effect of stress treatments on the K^+ content in the roots of the *OXSKOR* lines compared to wildtype.

Plants were exposed to 0 K^+, 50 mm KCl and 50 mM NaCl treatments in a hydroponics system. K^+ content was calculated based on the dry weights of the roots. Bars are the standard error of the mean, Student's t-test was used to test the significance of the results. $P < 0.05$. There were no sigificant differences between wildtype and *OXSKOR* lines.

3.4.5 RT-PCR analysis of *OXSKOR* lines:

The physiological analysis of the overexpressor *SKOR* lines under different stress treatments did not result in significant differences as expected as indicated in the previous experiments when compared to wildtype and SKOR13 (Figure 3-3,-4,-5). To assess the expression levels of these lines, a RT-PCR analysis was conducted (Figure 3-6). We tested plants that were previously checked with *Hygromycin* primers and showed the

existence of the product, however, we could not confirm that they are transgenic. All lines showed bands at expected size (399 bp) using actin primers. The second plant of *SKOR12* line did not show an *Actin* band. Relating the *SKOR* gene expression to the *Actin* showed no significant differences in the expression in any of the transgenic lines analysed (*SKOR12*, *19*, *23* and *31*) compared to the wildtype and the azygous line 13. This indicates that these lines are not overexpressors of the *SKOR* gene.

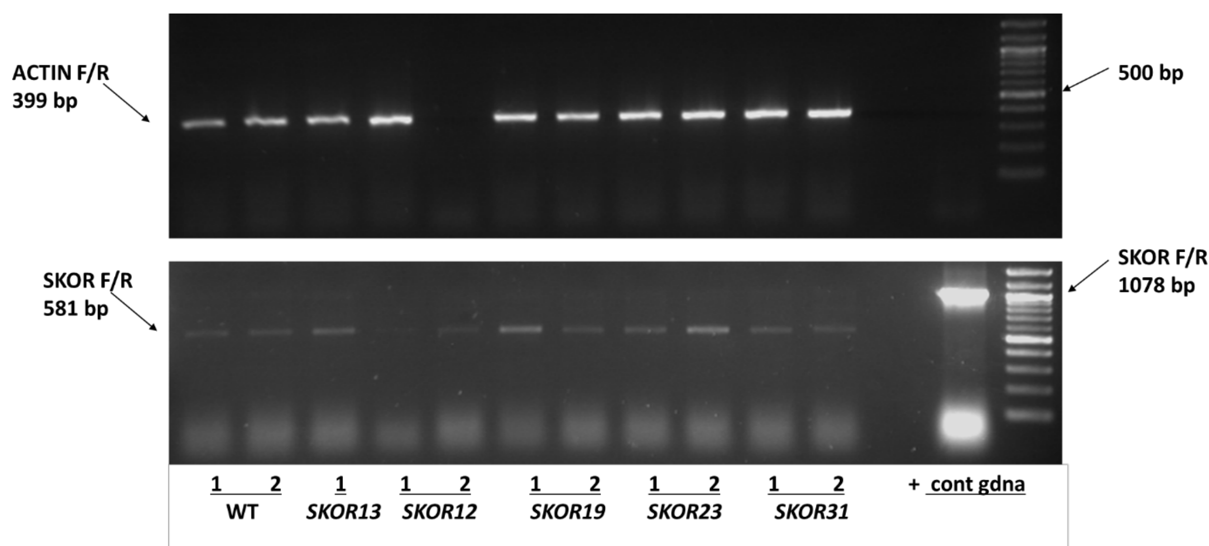


Figure 3-6: RT-PCR analysis of some overexpressor SKOR lines.

Two plants of wildtype and each *SKOR* line (S*KOR12*, *19*, *23* and *31*) were used except for line 13 where only one plant was tested. The *SKOR* forward and reverse primers were tested with a positive control using genomic DNA of rice plants and indicated the absence of genomic DNA contamination. *Actin* was used as a control in this analysis. The RT-PCR analysis showed no higher expression in the *SKOR* lines used in the study compared to wildtype and azygous line 13.

3.4.6 qPCR analysis of the *OXSKOR* rice lines

qPCR analysis of the *SKOR* lines was conducted to accurately analyse the expression levels of potential overexpressor lines. Two plants of *OXSKOR* lines were tested in this analysis and compared to wildtype. These two plants were previously tested using *Hygromycin* primers in a PCR analysis. The analysis showed that the two *SKOR* lines 19 and 23 were not overexpressing the *SKOR* gene (Figure 3-7). In fact, they showed lower expression when related to *actin* and compared. This suggested that these lines do not exhibit any overexpression of the *SKOR* gene, and confirms the RT-PCR results. We,

therefore, suggest that the previous lack of phenotypes might have resulted from the fact that these lines might have segregated and lost the transgene or became non-overexpressors. The 35S promoter (used in the transformation of lines) can sometimes lead to a down-regulation of the gene. Rice transformed lines might also suppress the gene because its overexpression might actually lead to reverse effects in non-stress conditions.

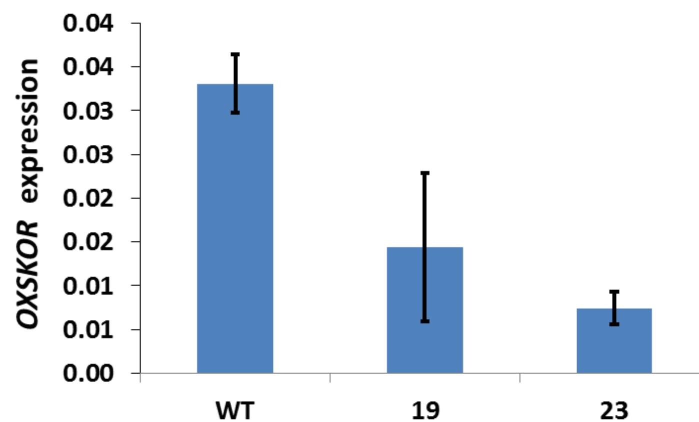


Figure 3-7: qPCR analysis of two rice *SKOR* lines in comparison

One plant of each line was tested in comparison with wildtype for the expression of *SKOR*. The expression was related to actin gene. The analysis showed that the two lines are not overexpressors. Error bars are standard error of the mean.

3.4.7 Expression levels of the major K^+ channels/transporters under the overexpression of *TPKb* in rice

Overexpressing *TPKb* increases the release of K^+ from the vacuole to the cytoplasm. *OXTPKb* lines contained higher shoot and root K^+ levels. Since TPKb is a tonoplast channel that is not involved in K^+ uptake from the external medium (Isayenkov et al. 2011), we investigated the possibility of the activation of other K^+ inwardly transporters such as AKT1 and KAT1, or possibly the inhibition of K^+ channels involved in leakage such as GORK. These high K^+ levels can increase the hyperpolarization of the plasma membrane, activates other K^+ inward channels and transporters and inhibits the outward channels. Expression of these transporters was analysed under the overexpression of *TPKb* in a RT-PCR analysis in control and low K^+ conditions.

ACTIN and *HISTONE* were used as constitutive controls. However, *ACTIN* showed no or faint bands for tested samples (Figure 3-8), where only few showed faint bands. Therefore, we used *HISTONE* as a control here. *KAT1* primers did not result in any bands either and probably needed to be re-designed (Not shown). Under control conditions, the expression of the *GORK* and *HAK1* did not show any significant differences between wildtype and overexpressor lines (Figure 3-8, A).

Under 0 K^+ treatment, we noticed no differences for all the transcripts analysed except for the HAK1 (Figure 3-8, B). We still could not obtain bands for *KAT1* (Not shown). *HAK1* displayed a slight increase in the expression when related to *Histone* under 0 K^+ conditions, then related to control conditions (Figure 3-8, A). This increase was estimated by 0.4-0.7 fold because of the overexpression of the *TPKb* under the K^+ depletion treatment. Which might be related to the role of OsHAK1 in the high affinity K^+ uptake. This is in accordance with (Chen et al. 2015), who reported that OsHAK1 transporter was up-regulated under the K^+ deficiency conditions. Therefore, also indicating the importance of K^+ uptake through OsHAK1. Although, this increase is regarded as slight and not enough to result in such greater K^+ levels in *OXTPKb* lines. We, therefore, suggest the involvement of other transporters in this increase. Indeed, Ahamad et al., (2015) reported an up-regulation in AKT1 transcript under K^+ starvation conditions. However, we still cannot relate this to the overall K^+ increase in tissues because AKT1 is not mainly a high affinity K^+ transporter.

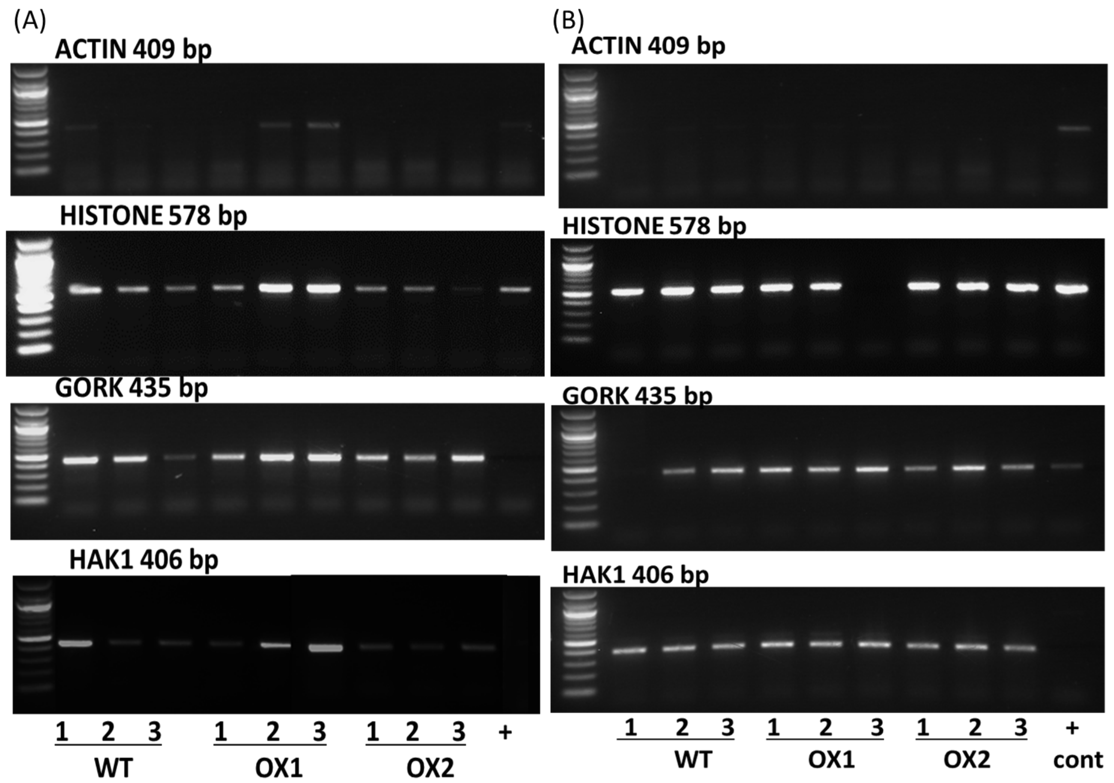


Figure 3-8: RT-PCR analysis of root K^+ channels/transporters under the overexpression of *TPKb*

(A) Control conditions: The expression of *HAK1* and *GORK* were measured in the overexpression lines of *TPKb*. The expression was related to *Histone* as we saw faint or no bands for *Actin* in this assay. (B) 0 K^+ conditions: The expression of *HAK1* and *GORK* were measured in the overexpression lines of *TPKb*. Expression was tested in roots of the plants. The expression was related to *HISTONE* as we did not see bands for *ACTIN* in this assay. Expression of genes that showed bands under 0K^+ conditions was related to control (*Histone*) using ImageJ software.

3.5 Discussion

3.5.1 The SKOR channel: is overexpression always recommended?

We chose the SKOR channel as a candidate to improve the plant responses to abiotic stress based on previous work by Gaymard et al., (1998), who indicated the involvement of the AtSKOR channel in K^+ upload from roots to shoots through xylem sap. Loss of function of this channel resulted in lower K^+ levels in shoots. Therefore, *SKOR* was recommended in the overexpression studies by Munns et al., (2005) for stress tolerance studies. *SKOR* was inhibited by the stress hormone ABA, which indicates that the reduction of K^+ transport from roots to shoots during stresses is a mechanism that helps in maintaining high K^+ levels in roots for turgidity and osmotic potential adjustment. From this, we conclude that overexpressing SKOR can be against the favor of plants during drought where the root needs to keep high K^+ levels.

We faced problems during this study started with the limited number of seeds and segregation in transgenic lines. The limited number of seeds usually affected the experiments by reducing the number of replicates and therefore, the accuracy of data. We used different *OXSKOR* lines and we did not obtain significant results for most of the lines. After testing the expression levels in a RT-PCR analysis, we found that these lines are not showing overexpression of function. This could probably be due to segregation of lines or that they were not initially overexpressors. We performed a qPCR analysis to accurately determine the expression levels in the two lines, and it was not showing any overexpression. It actually showed lower expression when compared to wildtype. We cannot confirm that the lines tested actually contained the transgene. We could still see significant increase in K^+ contents in *OXSKOR12* shoots under 50 mM K^+ treatment (Figure 3-4), this was not accompanied with higher RGR (Figure 3-3).

3.5.2 TPKb relieves K^+ deficiency by increasing K^+ release from PSV

It is suggested that when K^+ levels in the cytoplasm are below the critical level, the vacuole stored K^+ will be released to compensate this deficiency (Walker et al. 1996). As a main character for the TPK family of K^+ channels, the release of K^+ from vacuoles during different stresses is important to maintain physiological processes in the

cytoplasm. The AtTPK1 channel was reported to function in K^+ release during different stresses such as drought and salinity (Gobert et al. 2007; Latz et al. 2013).

The higher K^+ levels in cytoplasm increase the hyperpolarization of the plasma membrane and accordingly might activate the inwardly rectifying K^+ channels (AKT1 and KAT1). This might inhibit the outwardly K^+ channels (GORK, SKOR) and result in an increase in the activity of K^+ transporters like HAK1.

Investigating the expression levels of the candidate genes (*KAT1*, *HAK1*, *GORK*) using RT-PCR resulted in no significant differences when compared to wildtype in both control and 0 K^+ conditions. The expression level of *KAT1*, which is an inwardly K^+ channel was expected to be up-regulated due to the increase in the K^+ content in roots and shoots of the overexpressor TPKb lines (Ahmad et al. 2015). However, the lack of product in most samples is probably due to the primers incompatibility or reaction conditions, which might lead to difficulty in explaining results. Therefore, we cannot be sure of what was the response of *KAT1* under the overexpression of *TPKb*. Also, the *KAT1* is mainly expressed in the internodes and rachides and hardly expressed in roots (Obata et al. 2007) and maybe this is why we did not obtain bands from root tissues. It might be recommended to repeat this analysis on samples extracted from the shoots instead.

Under low K^+ conditions, the expression of the *HAK1* was slightly up-regulated to 0.4-0.7 folds. This is in agreement with the fact that the K^+ deficiency activates many high affinity K^+ transporters like OsHAK1, which was suggested to have a major role in K^+ uptake during low K^+ conditions (Chen et al. 2015). OsHAK1 was up-regulated during K^+ deficiency and two knockouts in this gene resulted in a significant decrease in the K^+ levels in both shoots and roots. (Cabrera et al. 2012) also indicated that the yeast *HpHAK1* expression was up-regulated due to K^+ starvation conditions. This slight increase in OsHAK1 expression might not be enough to explain the increase in K^+ levels in *OXTPKb* lines.

Ahmad et al., (2015) reported that the expression of *OsAKT1* was significantly increased under the overexpression of *OsTPKb*. The increase in *AKT1* expression is in line with other work that indicated the importance of the AKT1 channel in K^+ uptake. As

previously mentioned, AKT1 is the main channel functioning in K^+ uptake during low affinity conditions but can change its activity when K^+ is found in low external levels and function in the high affinity K^+ uptake. AKT1 is the only K^+ uptake system when K^+ is presented in 500 µM and higher (Alemán et al. 2011). However, AtAKT1 was able to compensate the absence of AtHAK5 under 30 µM K^+ which indicate its role in high affinity K^+ uptake as well (Pyo et al. 2010). Here we see an increase in *OsAKT1* expression during K^+ starvation (Figure 3-9). We can conclude that the change in vacuolar K^+ levels can activate root K^+ transporters if it is low under K^+ starvation conditions. It is also important to investigate other transporters that could participate in this increase such as OsHAK5, NSCC or SKOR and the possibility of the post-transcriptional activation of these transporters.

On the other hand, the K^+ outwardly rectifying channel (GORK) gene did not display any significant differences in the expression levels under both control and 0 K^+ treatments. The GORK channel is mainly expressed in the root epidermis, root hairs and the guard cells (Ache et al. 2000; Ivashikina et al. 2001; Becker et al. 2003) functioning in the efflux of the K^+ from guard cells (Kim et al. 2010; Demidchik 2014). It is a main player in the stomatal closure (Hosy et al. 2003; Osakabe et al. 2013) which is activated by depolarization of plasma membrane (Shabala and Cuin 2008; Ahmad et al. 2015) and ABA (Becker et al. 2003) resulting in K^+ efflux. It is expected to be inhibited under 0 K^+ treatment due to the higher K^+ concentrations in the overexpressor lines of *TPKb*. It would be expected that the expression of the *GORK* is down-regulated in the overexpressor lines, which was not shown by our data. Indicating that it might not be affected by the overexpression of the *TPKb* in rice plants or by the increase in the K^+ concentrations.

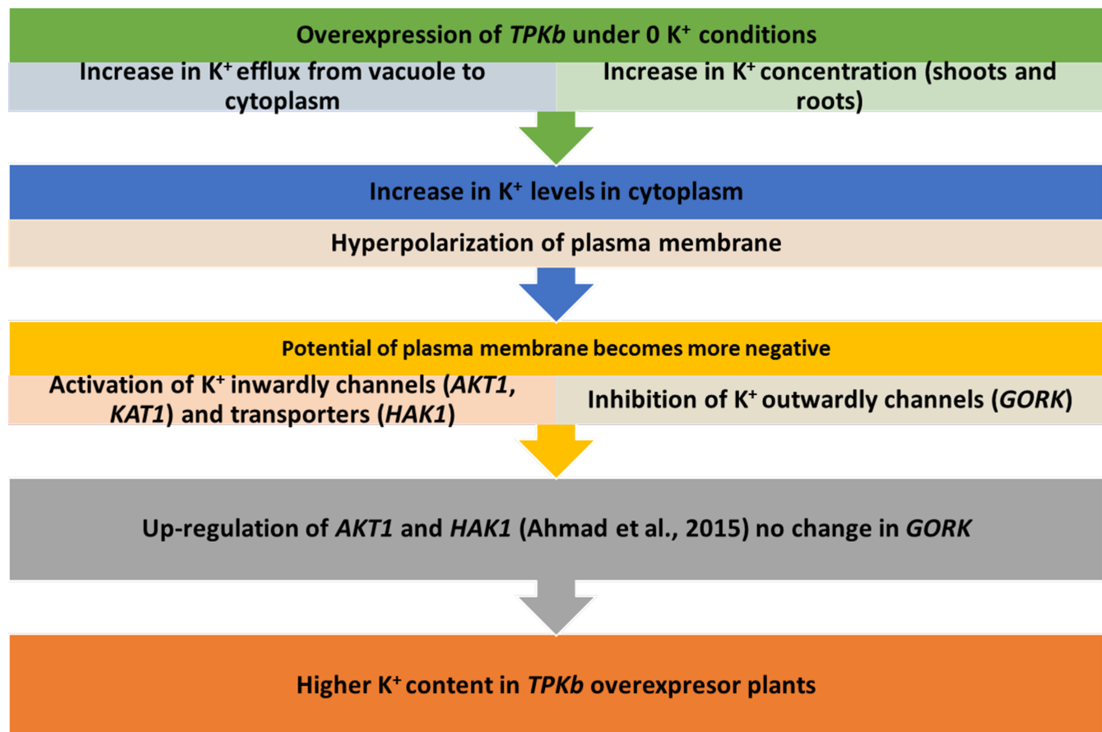


Figure 3-9: Effect of the overexpression of the *TPKb* on the major K^+ transporters in rice roots (AKT1, KAT1, GORK and HAK1).

A model of possible different effects of the overexpression of the *TPKb* in rice and its effect on other K^+ channels/transporters.

Chapter 4. Final conclusions

4.1 Abiotic stress: the challenges, the achievements and the ambitions

The agriculture sector is negatively affected by different abiotic stresses. These stresses can be drought, salinity, cold and high temperature (Tuteja and Gill 2016). Drought and salinity cause the highest reduction in the total productivity of important crops such as rice, maize and wheat. These crops are considered glycophytes and naturally non-tolerant plants. Cereal crops can lose up to 80% of yield if stress occurs during seed filling stage (Monneveux et al. 2006; Farooq et al. 2009). With the world population increasing, the problem of yield loss due to stress became more deleterious. The impact of the green revolution and the huge knowledge obtained had a great effect on improving the productivity of crops. This was reflected by an increase in yield productivity in developing countries by 208% for wheat, 109% for rice and 157% for maize between 1960 and 2000 (FAO reports (ftp://ftp.fao.org/agl/agll/docs/salinity_brochure_eng.pdf). However, with the world population doubled, more effective and fast techniques were needed to cope with such huge demand. Researchers have devoted many studies to improve plant tolerance against abiotic stress through decades. Breeding programs have been used for improving crops tolerance against stress but were always hindered by the complexity of abiotic stresses.

Molecular techniques improved in the last decade and were used for manipulation of crops towards more tolerant cultivars (Ahmad and Prasad 2011). It has increased the speed of studying genes and improving crops as it is much faster and easier than breeding. Moreover, it can be done across different species. Reverse genetics for example, allowed the study of a gene function either by knockdown/knockout or overexpressing it. This is possible to be applied in both model plants and important crops (Pereira 2016). The knowledge gained from studying stress tolerant plants was used in improving crop productivity, such as rice in tolerating salinity (Ren et al. 2005). There are still many problems that hinder the improvement of crops in response to stresses, such as redundancy. Because a stress response does not rely on individual genes, but a complicated network. Creating multiple mutants of genes suggested to participate in

stress response can help better understand how to improve the overall plant response to this stress. We therefore used this approach in our study aiming to obtain stronger phenotypes that we did not see in the single mutants.

The new challenging Genome-Wide Association Studies (GWAS) allowed to field screen genotypes under stress treatments (Kumar et al. 2015). Salinity for example, affects crop yield negatively therefore, salinity tolerance is a favorable characteristic in cultivars. As salinity is a complex trait, GWAS can help study the different salinity-responsive genes by identifying different quantitative trait loci (QTLs). This has led to identifying numerous QTLs in response to salinity. For example, Koyama et al. (2001) were able to identify 11 QTLs related to K^+ and Na^+ levels during salt stress tolerance. The Saltol locus was related to Na^+ and Na^+/K^+ ratios in leaves (Bonilla et al. 2002), while 25 QTLs related to Na^+, K^+ and Cl^- that were measured by Ammar et al., (2009). The enormous bulk of data that GWAS analysis can handle to identify the Single-Nucleotide Polymorphisms (SNPs) where we can connect between genotypes and phenotypes, makes it a great tool for identifying important natural variation in the QTLs (Si et al. 2015). Many studies have been conducted using the GWAS analyses helped in identifying more genes with high potentiality of functioning in stress tolerance.

4.2 Aims of the thesis and research outcomes

In this thesis, we are aiming at improving the response of plants to different types of stresses by using reverse genetics. We have different candidate genes that are suggested to play a role in plant physiology during drought, salinity, K^+ deficiency and osmotic stresses. We chose the TPK1 K^+ channel in Arabidopsis as previously recognized for its role in ABA-mediated stomatal closure, which is a short-term response to drought. Meanwhile, Gobert et al., (2007) suggested that there is a good chance of redundancy between this channel and other channels predicted to be located in the tonoplast of guard cells and might participate in K^+ movement during the stomatal closure.

The candidates for this study were AtKUP12 and AtHAK8, HAK/KUP/KT family members. They are highly expressed in guard cells, embryos and roots. They showed

interesting responses to K^+ deficiency and salinity in previous studies, and they are predicted to be localized in the tonoplast as well as plasma membrane. We started by analysing the single mutants then generated double and triple mutants. We conducted this study in the model plant Arabidopsis.

In chapter three, we investigated the possible role of the K^+ channel SKOR in improving rice responses to abiotic stresses. We based our hypothesis on a suggestion that overexpressing *SKOR* can help increase K^+ transport to shoots and therefore improve the plant response against stresses (Gaymard et al. 1998; Munns 2005).

Another work by Izhar Ahmad, showed that the overexpression of the TPKb rice K^+ channel resulted in better growth of plants under drought and K^+ starvation conditions. Interestingly, the plants showed an increase in K^+ levels under low K^+ treatment. The question was: why did the overexpression of a tonoplast K^+ channel result in such an increase and what are the transporters behind this increase? We tested two TPKb overexpressor lines under K^+ starvation conditions and compared them to wildtype. We chose different candidate rice K^+ transports that might participate in this increase (HAK1, KAT1), or decrease the K^+ efflux (GORK).

We used different approaches to study the response of the studied mutants under different stresses. We started from germination rates, seedling establishment, fresh weights of seedlings, stomatal conductance, water loss, heterologous expression in yeast and expression levels by using RT-PCR analysis.

4.3 AtKUP12 functions in seed germination and seedling establishment during K^+ starvation and a possible epistasis between AtKUP12 and AtTPK1

In our study for potential K^+ transporters that might play an important role in plant physiology during abiotic stresses, we tested the loss of function mutants of AtKUP12 under different stress treatments. AtKUP12 was suggested to play a role in the response of Arabidopsis plants to K^+ starvation being down-regulated after K^+ resupply (Armengaud et al. 2004).

Our results indicated that the loss of function of the AtKUP12 K^+ transporter resulted in seeds sensitive to germination over low K^+ levels when combined with 10 mM of NH_4^+. We have obtained significant inhibition in germination under 10, 30, 50 µM K^+, for the *atkup12-1* mutants. The inhibition was reported after 48 hours of sowing and lasted for 96 hours (Figures 2-8, -9, -10). The phenotype was noticed under 200 µM K^+ after 48 hours, and was significant, but not after normalization (Figure 2-12). The second allele (*atkup12-2*) showed significant inhibition under 50 µM K^+ treatment as well (Figure 2-11). We understand that the external K^+ levels do not affect the early stages of germination as a seed mobilizes its reserves to obtain K^+. However, when low external levels are accompanied by high NH_4^+/Na^+ levels, the effect of K^+ starvation is severe.

We reported that the inhibition in germination was significantly higher than published work under same conditions, such as by Pyo et al., (2010). This was due to the high content of NH_4^+ in the medium as well as Na^+. We have used a medium that contained 10 mM of NH_4^+ and 9 mM of Na^+. These two factors increased the inhibitory effect of K^+ deficiency. Our results in this regard are in accordance with Cao et al., (1993), who referred to the role of high NH_4^+ levels in the inhibition of root growth in Arabidopsis. A test experiment indicated that the removal of NH_4^+ from the medium resulted in better germination over 50 µM of K^+ (See Appendix).

The inhibitory effect of loss of function of *AtKUP12* was present during the seedling establishment as well, where plants showed slower or totally absent cotyledons emergence (Figure 2-7 and 2-13). We suggest that AtKUP12 might be important in K^+ uptake during later stages of plant life and not only seed germination. We noticed that the seedling establishment was significantly affected by the adverse conditions in the medium. The cotyledons emerged under both control and 50 µM K^+ treatments. This is because seedlings were able to develop roots and complete the growth process as the K^+ content in the medium relieved the NH_4^+ toxicity.

We think it is important to measure the K^+ content of seeds and seedlings under different treatments to see if there is a relationship between this and the response of the genotypes. AtKUP12 is a potential K^+ transporter and we think it plays a role in K^+ transport under

K^+ starvation treatments. If this is true, then different K^+ levels in *atkup12* seeds and seedlings could be expected.

AtKUP12 showed possible epistasis relationship to AtTPK1, suggesting a role in K^+ management through the vacuolar membrane. Since we had osmotic stress created in the medium due to the high NH_4^+ ratio plus 9 mM of Na^+, KUP12 might help relieve this toxicity or stress by releasing K^+ from the vacuole to the cytoplasm under such conditions. The K^+ starvation resulted from the interference of NH_4^+ with the high affinity uptake of K^+ could be compensated by AtKUP12 and AtTPK1. It is also important to indicate that the KUP family members can transport Na^+ as well as K^+. Possibly the existence of the Na^+ in the medium could be utilized by KUP12 in the absence of K^+, leading in the end to such phenotype of *kup12* mutants.

The epistasis relationship indicated that *attpk1* masked *atkup12* phenotype, a type of epistasis relationships which indicates that AtKUP12 might be negatively regulating (suppress) AtTPK1 during combined stresses. We initially based the thesis on a suggestion that TPK1 might be redundant to other K^+ transporters predicted to be located in the tonoplast, however, our results interestingly indicates a possible epistasis rather than redundancy. A novel result for both TPK1 and KUP12 that can lead to further understanding of the complex nature of the abiotic stress response in plants. Therefore, based on our results we have two scenarios of the role of KUP12 in the response of Arabidopsis to abiotic stresses explained in the next section.

4.4 Proposed model of AtKUP12 role in K^+ transport and epistasis relationship

First scenario: *AtTPK1* is suggested to be epistatic to *AtKUP12*, they both function in the same pathway and participate in the plant response to K^+ starvation resulted from high NH_4^+/K^+ by managing K^+ across vacuolar membrane. This scenario is reinforced by the various predictions of the vacuolar localization of *KUP12* and its up-regulation in seeds and embryos during seed imbibition. We suggest a role for *AtKUP12* during osmotic stress. In this case, *AtTPK1* is called (epistatic to) *AtKUP12*, and it masks its phenotype. It also indicates that *AtKUP12* is negatively controlling *AtTPK1* during this response (see Figure 2-29). *AtKUP12* is suggested to function upstream *AtTPK1*.

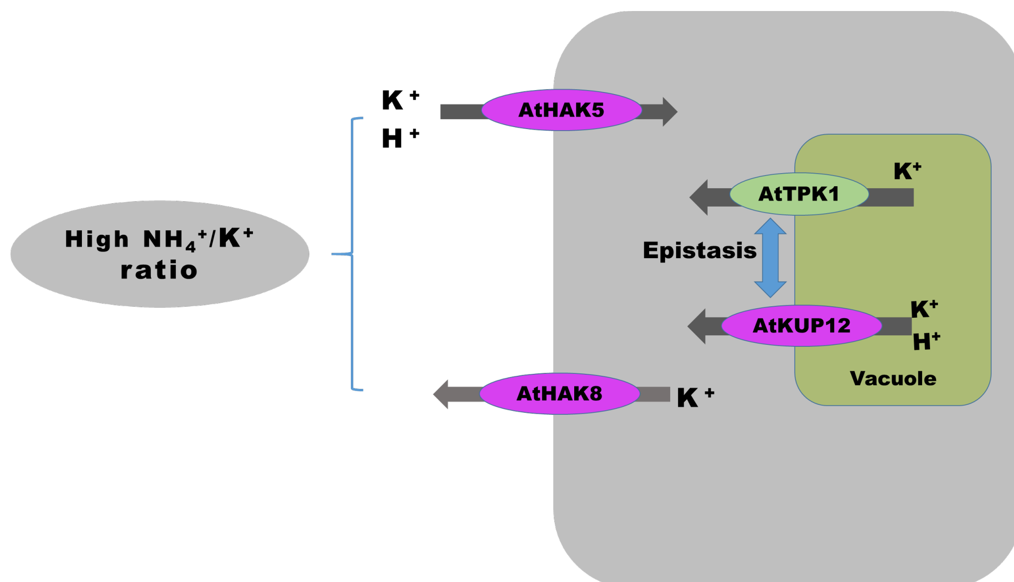


Figure 4-1: Suggested model of AtKUP12 during K^+ starvation resulted from high NH_4^+/K^+ ratio.

AtKUP12 is suggested to be localized in the tonoplast and functions in K^+ release to cytoplasm during K^+ starvation resulted from high NH_4^+/K^+ ratio and NH_4^+ toxicity. AtKUP12 and AtTPK1 could be epistatic to each other under such conditions.

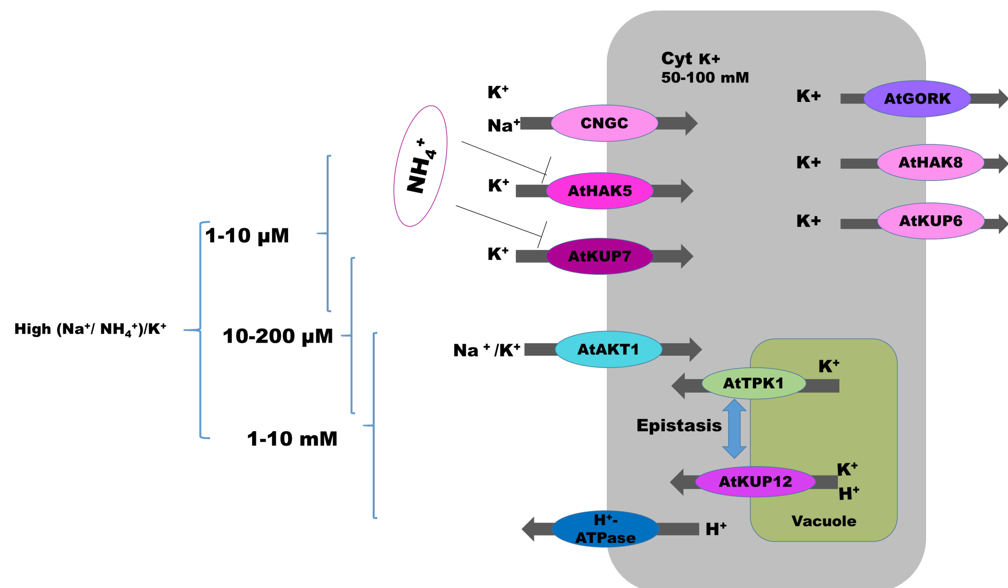


Figure 4-2: Model for AtKUP12 role in K^+ homeostasis during K^+ starvation

Various channels/transporters function in K^+ uptake from roots during different external K^+ levels. AtHAK5 and AtKUP7 function in the high affinity K^+ uptake from soil. AtAKT1 functions mainly in the low affinity K^+ uptake, it can switch to high affinity uptake through a conformational change in the channel by phosphorylation. AtAKT1 is not sensitive to NH_4^+, while members of HAK/KUP/KT family of transporters are inhibited by NH_4^+. The H^+-ATPases function in pumping H^+ through plasma membrane to create the chemical gradient necessary for high affinity K^+ uptake. Other channels are involved in K^+ uptake such as CNGCs. K^+ efflux from roots occurs thought the GORK channels and KUP6 subfamily of transporters. AtTPK1 channel functions in K^+ release from vacuoles. AtTPK1 is epistatic to AtKUP12 and masks its phenotype, Not all transporters are presented in this figure.

Second scenario: Assuming that there is no segregation in the *hak8kup12* and *hak8kup12tpk1* double and triple mutants, AtKUP12 maybe be epistatic to AtHAK8. However, this scenario is not confirmed unless further investigation is conducted to the double and triple mutants. Moreover, physiologically, it is difficult to explain the epistasis relationship between KUP12 and HAK8 without knowing their location in the cell.

4.5 AtKUP12 complements the growth of the K^+ deficient yeast during K^+ starvation

Previous results of AtKUP12 in germination and seedling establishment showed a potential role for this transporter under K^+ starvation conditions. We tested the expression of *AtKUP12* in a heterologous system such as yeast, to further analyse its function under K^+ starvation. AtKUP12 was able to complement the growth of the mutant yeast (Figure 2-20). We suggest that AtKUP12 is involved in K^+ transport during deficiency periods. This is a common phenotype for many members of the HAK/KUP/KT family, such as AtKT3/AtKUP4 (Rigas et al. 2001), KUP1and KUP7 (Fu and Luan 1998; Han et al. 2016).

The puzzling issue here might be, where is AtKUP12 expressed in yeast? We certainly do not know. Because the yeast cell contains large vacuole such as a plant cell, and both the possibility of plasma membrane or tonoplast localization occur. We presume that AtKUP12 is a plasma membrane localized transporter such as most family members studied so far. We can relate this assumption to the previous results, which suggests a role for AtKUP12 in K^+ uptake during K^+ starvation conditions. However, testing the localization of the transporter in a plant system is the most straightforward procedure to confirm its subcellular location in a cell. Another issue is, the fact that KUP12 was able to complement the growth under both high and low K^+ concentrations. This can indicate that the phenotype is basically not K^+-dependent and rather is resulted from another component in the medium used.

It is also important to note the defects in a yeast system when studying a plant protein, such as the lack of regulatory proteins found in plants, the lack of plant cell conditions and the difference in how a yeast cell will direct protein expression according to its need even if it is a plasma membrane transporter.

4.6 *atkup12* mutants showed salt sensitive and possibly a stomatal phenotype

Our results showed that *atkup12-1* plants expose a salt sensitive phenotype when treated with 80 mM NaCl (Figure 2-22). The 4 weeks old plants showed a decrease in fresh weights was significant when compared to wildtype. We suggest that the germination phenotype that probably resulted from a high Na^+/K^+ ratio in the medium is also present in older plants. This adds more importance to the potential role of AtKUP12 under different stress conditions. However, since the reduction in fresh weights was very small, this result might not be an actual phenotype.

We did not find a stomatal phenotype for *atkup12*, however, water loss assays showed higher fresh weights of rosettes when exposed to hood drying conditions. This was noticed when *kup12* was combined with *hak8* and *tpk1*. We cannot be certain of the reason behind this difference since we do not have the single mutants tested under same conditions, but we can still suggest a possible relationship between KUP12 and HAK8, which led to such difference.

4.7 AtHAK8 did not play a role in seed germination or seedling establishment

Our results did not show an increase in seed germination (48-96 hours) or seedling establishment due to the loss of function of *AtHAK8*. This was reported by previous work as Osakabe et al., (2013) did not report any phenotypes for the loss of function mutants of AtHAK8. Also, in a personal communication with Dr. Yurkio Osakabe (the author), it was confirmed that there were no germination phenotypes for *athak8* mutants. Possibly, the function of HAK8 was compensated by its homologs KUP6 and KUP2. Osakabe et al. (2013) indicated that these transporters regulate K^+ efflux, and the loss of function in their genes results in bigger plant sizes due to larger cells.

athak8 mutants showed better growth under both high K^+ (80 mM) and sorbitol (120 mM). These mutants could be able to maintain better K^+ levels than others under such

stresses. Probably, this state induces K^+ efflux and reduces K^+ uptake, and with the loss of function of HAK8, KUP2, KUP6 and GORK could be up-regulated. We cannot confirm that this is due to better K^+ retention in plants though until K^+ levels are measured.

We did not report significant results for *hak8* mutants during water loss assays, although we noticed a slight reduction in the fresh weights of rosettes (Figure 2-25, A). It was previously reported that AtHAK8 participates with its homologs (KUP2 and KUP6) in stomatal movement during drought stress (Osakabae et al. 2013). But alone, HAK8 loss of function did not show any stomatal phenotypes. The triple mutant of these three transporters displayed more opened stomata under different ABA treatments. The *kup268* mutants showed higher stomatal conductance and more sensitivity to water loss reflected in lower fresh weights due to a higher loss in transpirational water. We, therefore, can conclude that HAK8 can function under many stresses but this probably is significant when combined with its homologs.

4.8 Combining *tpk1*, *hak8* and *kup12* mutants

The main aim of this thesis was to analyse the effect of combining different knockouts of candidate genes and study this under different stresses. This approach was mainly to investigate the possibility of any redundancy between TPK1 and other potential K^+ transporters HAK8 and KUP12. We, therefore, generated double and triple mutants of these genes to test this hypothesis. We conducted multiple experiments and we obtained interesting results for the single mutants such as *atkup12*. At the same time we analysed the multiple mutants (double and triples) under different stress treatments. The results suggest different relationships between the transporters under study.

The behavior of *hak8kup12* and *hak8kup12tpk1* multiple mutants did not indicate possible effects for combining mutants. We noticed inconsistent responses for these mutants under different treatments. Moreover, the statistical analysis indicated no significant differences between *hak8* and *hak8kup12* or between *kup12* and *hak8kup12tpk1*. We therefore, suggest a possible segregation occurred during generation of these mutants that resulted

in genotypes of single mutants. The germination experiments clearly indicate a typical response for *hak8kup12* compared to *hak8* single mutant, while *hak8kup12tpk1* was similar to *kup12* under all treatments. Moreover, the previous work by Osakabe et al., 2013 indicated that AtHAK8 is likely to be a plasma membrane transporter functioning in K^+ efflux together with its homologs AtKUP2 and AtKUP6. They all belong to the subfamily of KUP6 transporters. Accordingly, it could be physiologically unsuitable to combine loss of function of HAK8 and KUP12 in one plant. Same explanation applies for combining HAK8 and TPK1 mutants. Finally, we suggest further analysis for these mutants to indicate the segregation percentages in the double and triple mutants and if it is as expected.

4.9 The SKOR channel and its role in abiotic stress responses in rice

We analysed the effect of the overexpression of the *SKOR* under different stress treatments. The idea of the overexpression of this channel was recommended by previous studies by Munns et al., (2005). The suggestion was based on improving the responses of rice plants to different stresses by improving the K^+ acquisition, and movement from roots to shoots. We were not able to determine if this hypothesis is right or not, due to segregation in lines and the fact that they did not overexpress the gene. We could see a down-regulation in the expression of the *SKOR* in two lines we tested in comparison to wildtype. We do not particularly know why did this happen. However, it can be due to the transformation using the 35S promoter, which sometimes results in down-regulation or silencing the gene instead of overexpressing it. It could also be due to the fact that, sometimes plants will down-regulate stress responsive genes if in normal conditions because they result in growth inhibition if overexpressed during non-stress conditions.

Another observation was reported for this gene, SKOR was inhibited by the synthesis of ABA during drought stress (Gaymard et al. 1998). Although SKOR might be important in K^+ transport from roots to shoots, it can negatively affect roots turgidity during other stresses such as drought. In this case, overexpressing the gene can be harmful rather than useful.

4.10 The role of TPK family of channels under K^+ deficiency in rice

In rice, there are two isoforms of TPK (TPKa and TPKb) localized in lytic vacuole and storage vacuole respectively (Isayenkov et al. 2011). The overexpression of *OsTPKa* and *OsTPKb* showed better growth under K^+ starvation conditions. It increased K^+ contents as well. It was suggested that other transporters might participate in this increase under low K^+ conditions, either by an increase in K^+ uptake, such as OsHAK1 and KAT1 or a decrease in K^+ efflux, such as GORK. These transporters were tested for a change in expression levels under the overexpression of *OsTPKb*. Our data showed a slight increase in the *HAK1* expression in the *OXTPKb* lines. We could not detect a clear expression bands for KAT1 therefore, we cannot determine if any changes have happened to it. While GORK did not show any changes in its expression.

Other work indicated that AKT1 was significantly up-regulated under the overexpression of *TPKb*, confirming that the AKT1 is a substitute mechanism of high affinity K^+ uptake (Ahmad et al. 2015), and can be responsible for this increased noticed in the *OXTPKb* lines. We must indicate, that other transporters can participate in this increase in K^+ levels under 0 K^+ treatment, such as NSGC, OsHAK5, and perhaps other unidentified mechanisms.

4.11 Future work

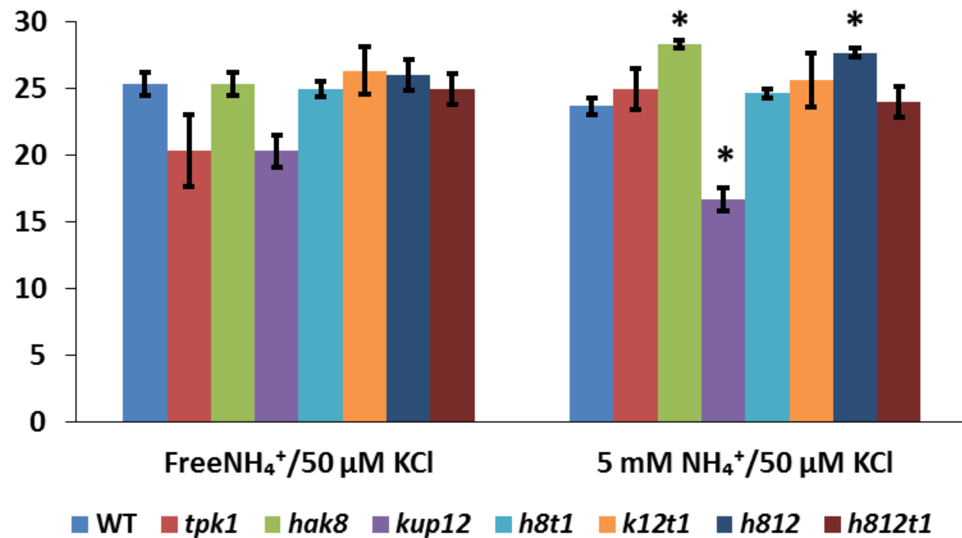
Here, we suggest that AtKUP12 is participating in the response of plants to K^+ deficiency when combined with other stresses such as salinity or high NH_4^+. Our results indicated that germination and seedling establishment were highly inhibited by the loss of function of this transporter especially in the early stages and during imbibition. We can relate this to the rapid increase in the expression of *AtKUP12* during imbibition of seeds. Our results are interesting and novel, moreover, many studies could be conducted to further understand the role of AtKUP12 in the plant physiology.

- We suggest more physiological experiments to further understand the role of AtKUP12 in K^+ transport under normal and stress conditions. This could be by measuring K^+ and Na^+ levels in seeds and plants during normal and stress

conditions. This can help explain how the existence of this transporter is important during salinity or K^+ deficiency. Growth parameters are good indicators of the physiological importance of this transporter.

- Root architecture is a paramount characteristic of K^+ deficiency and might help increase the knowledge about how roots respond to K^+ starvation in loss of function mutants of AtKUP12.
- Further epistasis analysis for AtKUP12 and AtTPK1 is recommended. This will help understand the mechanism of this relationship and how a plant responds to stresses.
- Yeast expression using different yeast mutants, such as a mutant that lacks vacuolar K^+ transporters. K^+ uptake by yeast in growth curves as well will directly indicate the involvement of AtKUP12 in K^+ transport. Another complementation assay can confirm the extent of growth rescue by AtKUP12 by including a high K^+ medium where all genotypes grow in one plate as well as osmotic stress conditions.
- The localization of the AtKUP12 transporter using plant system will greatly help explain the role of this transporter in plant physiology, moreover, the functional relationship with other transporters such as KUP7.
- The H^+-coupled K^+ transport that occurs through this transporter during K^+ deficiency could be investigated using patch clamp techniques.
- Generating overexpressor lines of *AtKUP12*, to test if this approach will result in an opposite phenotype to the loss of function.
- Generating multiple loss of function mutants of AtKUP12 to further confirm the obtained phenotypes.
- For *TPKb* overexpression, testing other transporters and if they are affected by the overexpression and function in K^+ uptake during 0 K^+ treatments.

Appendix



Supplementary data 2.1: Germination rates after 48 hours under different NH_4^+ concentrations (0 mM and 5 mM) combined with low K^+ concentration (50 µM).

30 seeds were sown a K^+/NH_4^+ free medium (Spalding et el, 1999), K^+ was added at 50 µM while the medium was supplied with (0 or 5 mM NH_4^+). Error bars are the standard error of the mean. Asterisks represent significant results where $P < 0.05$ and is indicated with *. Analysis of variance (ANOVA) was used to analyse data between wildtype and mutants, and between control and treatments**.** This is one experiment with three replicates.

- **Table 1: Murashige and Skooge medium composition**

Murashige and Skooge Medium stock solutions			
Macronutrients K^+			
Salt	Mol. Wt	1X mg/L	Final conc mM
NH_4NO_3	80.04	1650.00	20.61
KNO_3	101.10	1900.00	18.79
$CaCl_2.2\ H_2O$	147.02	440.00	2.99
$MgSO_4.7H_2O$	246.48	370.00	1.5012
KH_2PO_4	136.09	170.00	1.25
Micronutrients			μM
KI	166.00	0.83	5
H_3BO_3	61.83	6.20	100
$MnSO_4.H_2O$	169.000	16.90	100
$ZnSO_4.7H_2O$	287.55	8.60	29.91
$Na_2MoO_4.2H_2O$	241.97	0.25	1.03
$CuSO_4.5H_2O$	249.69	0.03	0.1
$CoCl_2.6H_2O$	237.93	0.03	0.11
Ferum			μM
$Na_2.EDTA$	372.24	37.20	100
$FeSO_4.7H_2O$	278.0157	27.80	100

- **Table 2: Yoshida medium used in rice hydroponics**

Macronutrients	Element		Stock solution concentration (g/l)	Volume of stock ml/l	Final concentration
	N	NH_4NO_3	91.4	1.25	2.9
	P	$NaH_2PO_4 \cdot 2H_2O$	40. 3	1.25	0.3
	K	K_2SO_4	7 1.4	1.25	1.0
	Ca	CaCl2	88.6	1.25	1.0
	Mg	$MgSO_4 \cdot 7H_2O$	324.0	1.25	1.6
Micronutrients				1 ml stock/l	
	Mn	$MnCl_2 \cdot 4H_2O$	1.5		0.01
	Mo	$(NH_4)_6 \cdot Mo_7O_{24} \cdot 4H_2O$	0.074		0.001
	B	H_3BO_3	0.934		0.2
	Zn	$ZnSO_4 \cdot 7H_2O$	0.035		0.0002
	Cu	$CuSO_4 \cdot 5H_2O$	0.031		0.0002
	Fe	$FeCl_3 \cdot 6H_2O$	7.7		0.04
	Silica	Added as Na_2SiO3	0.18		

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