The role of OsAKT1 in ammonium uptake and osmotic stress tolerance in relation to nitrogen supply

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Abstract

Nitrogen is an essential plant macronutrient: it not only affects the growth, development and yield of plants, but also contributes to stress tolerance. OsAKT1, a rice homolog of a K^+ channel AKT1 at the plasma membrane of root hair cells, is permeable to ammonium and therefore potentially plays a role in the uptake of this nutrient. Beside taking part mainly in K^+ uptake in the roots, OsAKT1 is also involved in drought and osmotic stress tolerance in rice. Therefore, I investigated the role of NH_4^+ uptake via OsAKT1 and the function of the channel in osmotic stress tolerance related to NH_4^+. Growth experiments which compared the growth of wild-type (WT) and *akt1* knockout rice (KO) in media with different predominant form of nitrogen (either NO_3^- or NH_4^+) under control and osmotic stress of -0.21 MPa (8% PEG 4,000) showed that the OsAKT1 role in osmotic stress tolerance is not associated with NH_4^+. Nevertheless, NH_4^+ depletion experiments illustrated that OsAKT1 functions in NH_4^+ uptake at low external NH_4^+ concentrations of 0.2 mM. Depletion assays at 2 mM NH_4^+ and the complementation growth studies in yeast mutants lacking NH_4^+-specific uptake systems (*Δmep1-3*) also suggested the possibility of NH_4^+ uptake at high NH_4^+ concentrations via OsAKT1. In all, the data imply that OsAKT1 takes up small amounts of NH_4^+ irrespective of external NH_4^+ concentrations.

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Acknowledgements

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Author’s declaration

I hereby declare that the work presented in this thesis is my own work and that I am the sole author. This thesis has not been submitted for examination to this or any other institution. All sources are properly acknowledged.

1 Chapter 1: Introduction and literature review

1.1 General introduction

Plants are the primary food source for animals and humans. Plant growth and productivity thus have a critical role in maintaining complex food chains and webs. Similar to other organisms, plants require nutrients for their building blocks and various biochemical reactions including photosynthesis. Of the essential plant elements, thirteen can be obtained from the soil in the form of inorganic minerals; hence they were named mineral nutrients. These mineral nutrients consist of nitrogen (N), phosphorus (P), potassium (K), sulphur (S), calcium (Ca), magnesium (Mg), boron (B), iron (Fe), manganese (Mn), copper (Cu), zinc (Zn), molybdenum (Mo) and chlorine (Cl). Altogether, the mineral nutrients are responsible for around 5% of the plant biomass. Although all of the mineral nutrients are equally essential for plants to make progress in their vegetative and reproductive phases, some are required in a large amount (macronutrients) while some are only needed in a small quantity (micronutrients) (Roy et al., 2006).

Among the macronutrients, N is the most abundant in plant tissues as it accounts for 2-4% of plant dry matter (Roy et al., 2006). N plays a main role in supplying amino groups in amino acids, incorporating into nitrogenous bases of nucleotides, and the formation of many co-enzymes and metabolites including chlorophyll (reviewed in Maathuis, 2009). N deficiency therefore greatly affects plant growth and subsequently food production. An early sign of N deficiency is pale green leaves which may be accompanied by leaf tip chlorosis. Under severe N stress, narrow and yellowish leaves can be observed followed by stunted growth and reduction in yields. In rice for example, such conditions would eventually lead to the decline of the number of tillers and grains (Dobermann and Fairhurst, 2000). Therefore, sufficient cellular N concentration is important for plants to grow and reach their full productivity potential.

1.2 Nitrogen

1.2.1 Environmental impacts of nitrogen fertilisers

N normally composes 0.1-0.6% of the top layer of soil which is equivalent to 200-12,000 Kg N ha^{-1} (Cameron, 1992). N is one of the limiting plant growth factors, therefore the addition of N fertilisers to agricultural land is a common practice. As there is a higher demand for food production due to the increase in the world's population, N fertiliser application has also increased to supply more food to support the growing population. In fact, the increase in N fertilisers application by around 7-fold was one of the factors which contributed to the growth of global food production by around 2-fold from 1961 to 1996 (Tilman, 1999). Since then, global usage of N fertilisers continued to rise: it was estimated that 93,000-99,000 thousand tonnes of N fertilisers were used in 2004 (Good et al., 2004), however, the figure had risen to 110,000 thousand tonnes in 2014 and was estimated to reach 118,000 thousand tonnes in 2019 (FAO, 2016).

The increase in N fertiliser usage, even though it helps alleviate world hunger, also brings concerns. Not only are commercial fertilisers expensive, which consequently increases the cost of food production, but N fertilisers can also be harmful to the environment. Excess use of the fertilisers can leach into the soil and contaminate fresh and underground water streams as well as coastal marine ecosystems. The leakage of the fertilisers into water reservoirs can make them unsafe for animal and human consumption. N eutrophication of water sources which can lead to "algal blooms" results in the death of aquatic life in polluted water systems. The phenomenon is followed by the occurrence of hypoxic zones, areas with low O_2 level due to the depletion of O_2 by decomposing bacteria after the death of the algae, which turns the area inhabitable (Robertson and Vitousek, 2009). Leaching of N fertilisers also leads to the increase in the emission of NH_3 via volatilization and NO and N_2O via denitrification. While the NH_3 and NO are common air pollutants, N_2O is one of the greenhouse gases contributing to climate change (Mosier et al., 2001).

Therefore, the study to improve N use efficiency (NUE), the efficiency in taking up and using N by plants, will not only provide new means in enhancing crop yields but also prevent the further increase in the cost of food production and harmful environmental impacts of N fertilisers.

1.2.2 Nitrogen cycle

In the biosphere, N is converted from one form to another as is depicted in the N cycle (Figure 1). While the decomposition of living matter and waste is a source of organic N in nature, the majority and primary source of N in the biosphere is in the air in the form of N_2, a gas which constitutes approximately 80% of dry air. N_2 is an inert gas due to the strong triple bond and therefore cannot be used directly by most organisms. As the two major N forms taken up by plants are ammonium (NH_4^+) and nitrate (NO_3^-), the reduction of N_2 allows plants to gain access to the N source.

N-fixing bacteria are able to reduce N_2 to ammonia (NH_3) with the use of an enzyme called nitrogenase. Thus, N-fixing microbes, either residing in the land independently (non-symbiotic types like *Azobacter*) or residing on root structures called nodules (symbiotic types like *Rhizobium*), allow plants to exploit N source from the atmosphere by carrying out N fixation in their niches (Roy et al., 2006). While there is no direct communication between the non-symbiotic microbes and crops, the symbiotic association of N-fixing bacteria with plant species such as legumes is initiated by the signals sent out from plants which attract the bacteria to colonise the roots, allowing the symbiotic relationship to be established (Bernhard, 2010). Despite NH_3 being produced directly from the N-fixation, NH_4^+ is the more prevalent form in which N is taken up due to the fact that NH_4^+ is a weak acid with a pKa of 9.25.

After the reduction of N_2, NH_3 can be oxidised by two groups of aerobic nitrifying bacteria. Ammonia-oxidisers oxidise NH_3 to nitrite (NO_2^-) using ammonia monooxygenase and hydroxylamine oxidoreductase while nitrite-oxidizing bacteria oxidise NO_2^- to NO_3^- (Bernhard, 2010). While NO_3^- in the soil is transported to the plants via transporters at root hair cells, NO_3^- in nodules is sent directly into the stele via nodule vascular tissues.

From the above, it is clear that soil microbes play a significant role in determining soil N availability. Therefore, soil conditions dictate the predominant form of N in the soil by providing a favourable environment to a certain type of microbes. NH_4^+ is the prevalent form in more acidic and anaerobic conditions while NO_3^- is the predominant form in more alkaline and aerobic environments (Maathuis, 2009). Although the assimilation of NH_4^+ requires lower energy than that of NO_3^-, most plants have a preference for NO_3^- instead of NH_4^+ as a N source. This is likely due to the toxic effects of NH_4^+ which can cause several symptoms including stunted growth, burnt leaf tips, small root:shoot ratio and inhibited

seed germination via yet to be identified mechanisms. A well-accepted hypothesis suggests that NH_4^+ toxicity may be the result of a high-energy demanding futile cycling of NH_4^+ across the root plasma membrane to efflux the excess NH_4^+ out of the cytosol of root cells in order to maintain low intercellular NH_4^+ concentration (Britto et al., 2001).

Besides NH_4^+ and NO_3^-, N can also be taken up at the roots in the form of amino acids via specific transporters, for example, the Lysine Histidine Transporter 1 (LHT1). Without a functional LHT1, *Arabidopsis* mutants were found exhibiting lower uptake rates of many amino acids and a concomitant reduction in biomass compared to the wild-type when amino acids are supplied as a dominant form of N (Svennerstam et al., 2007).

Apart from root N uptake, foliar N uptake can also take place either via the cuticle or a stomatal pathway. N compounds like nitric acid (HNO_3) enter plant cells via the cuticular route through the lipid bilayer of plasma membrane. On the other hand, gaseous N pollutants, for example, NH_3 and nitrogen dioxide (NO_2), diffuse into plant cells through stomata. Therefore, stomatal conductance (g_s) can affect N availability in plants (Vallano and Sparks, 2007).

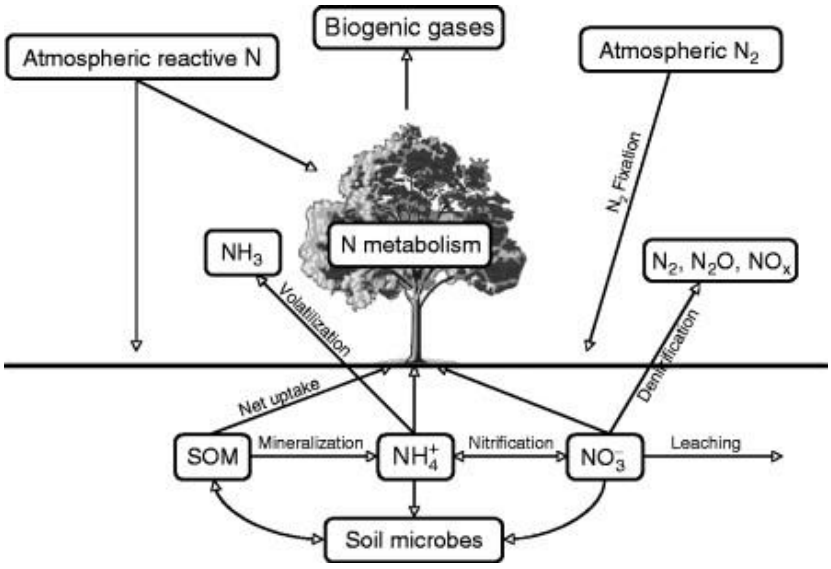

Figure 1: N cycle in the biosphere showing the conversions and movement of N and its recycling in nature (figure from Vallano and Sparks, 2007). N_2 is fixed by bacteria into NH_4^+ which can be taken up by the plants or get nitrified into NO_3^- prior to plant uptake. Soil organic matter (SOM) from dead organisms and waste products is also a N source in the soil. Beside root uptake, atmospheric reactive N enters the plants via foliar uptake. Finally, N returns to the atmosphere in gaseous forms including N_2, NO, NH_3, N_2O and NH_3 via ammonia denitrification and volatilization.

1.2.3 Nitrogen assimilation

After taking N from the external environment, the absorbed N is converted to glutamate and subsequently to other amino acids in a process called nitrogen assimilation (Figure 2).

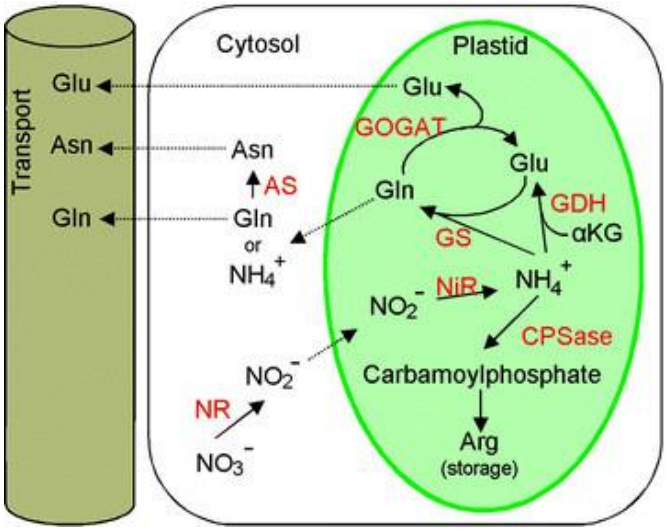

Figure 2: N assimilation metabolic pathway in plants (figure from Miura, 2013). NO_3^- is reduced to NO_2^- by NR in the cytosol and translocated into plastid before being reduced to NH_4^+. NH_4^+ is assimilated in GS/GOGAT cycle which leads to the formation of Glu. On the other hand, Asparagine synthetase (AS) catalyses the formation of Asn and Glu from Asp and Gln. In the plastid, the formation of Arg is catalysed by carbonyl phosphate synthetase (CPSase) with carbamoylphosphate acting as an intermediate from NH_4^+ and CO_2. Also, α-ketoglutarate (αKG) together with NH_4^+ can be converted into Glu by glutamate dehydrogenase (GDH). The amino acids are transported to other parts of the plants via phloem.

NO_3^- reduction into NO_2^- occurs in the cytosol of the root and shoot cells by nitrate reductase (NR). NO_2^- is then translocated into chloroplasts or plastids and is reduced to NH_4^+ by nitrite reductase (NiR). NH_4^+ is assimilated via a cycle called GS/GOGAT which is operated by glutamine synthetase (GS) and glutamine 2-oxoglutarate aminotransferase (GOGAT). GS catalyses the formation of glutamine by fixing NH_4^+ to a glutamate molecule and GOGAT transfers an amide group of the glutamate onto 2-oxoglutarate, resulting in the formation of two glutamate molecules. Both GS and GOGAT have two isoforms. GS isoforms are believed to perform their function in different scenarios under different conditions. GS2 in the chloroplast has a direct role in NH_4^+ assimilation after NO_3^- reduction and the re-assimilation of NH_4^+ from photorespiration. GS1 in the cytosol is involved in the re-assimilation of NH_4^+ during leaf senescence. As for GOGAT, its two isoforms, Fd-GOGAT and NADH-GOGAT, are found predominantly in the chloroplast of the leaf and plastid of non-photosynthetic tissues, respectively. As suggested by the names, the electron donor of Fd-GOGAT is ferredoxin, while NADH acts as the electron donor for NADH-GOGAT (reviewed in Masclaux-Daubresse et al., 2010).

Glutamate molecules from the GS/GOGAT cycle act as a progenitor of other amino acids, for example, proline. The biosynthesis of proline is involved with the activity of pyrroline-5-carboxylate synthetase (P5CS) (Hu et al., 1992), an enzyme with the functions of γ-glutamyl kinase and glutamic-γ-semialdehyde dehydrogenase. P5CS converts glutamate into glutamic-5-semialdehyde which spontaneously cyclises into pyrroline-5-

carboxylate (P5C). P5C is a substrate of pyrroline-5-carboxylate reductase (P5CR), an enzyme which produces proline (Verslues and Sharma, 2010).

1.2.4 Nitrate and ammonium uptake systems

The absorbed N in the root hair cells can be passed on to the leaf via the xylem as inorganic NO_3^- or as organic forms such as amides and amino acids. N can also be transported via the phloem in organic forms when N is re-translocated from older leaves to younger leaves, seeds and fruits during N recycling (Roy et al., 2006). Nevertheless, the transport and usage of N in different organs would be impossible without the uptake of N from the external source. Thus, it is important to pay attention to the root-soil boundaries where N is taken up mostly in the form of either NH_4^+ or NO_3^-.

Intriguingly, a number of channels and carriers are involved in NH_4^+ and NO_3^- uptake. The absorption of NH_4^+ and NO_3^- in the roots is governed by both high-affinity (HATs) and low-affinity (LATs) transport systems which belong to different transporter families. By operating uptake systems with different affinities, the uptake can be performed in both low and high external concentrations of the substrate.

1.2.4.1 Nitrate

NRT1 (PTR) is a family of proton-coupled nitrate transporters, with a protein structure consisting of a hydrophilic loop between domain six and seven out of twelve putative transmembrane spanning regions (Tsay et al., 2007). Large number of *NRT1 (PTR)* genes are found in plants (Figure 3); 53 in *Arabidopsis* and 80 in rice. NRT1. The expression of *NRT1* has been shown to be abundant in high NO_3^- concentrations, however, it can also be found at low concentrations of NO_3^-. For example, the expression of *AtNRT1·1* was induced at NO_3^- concentration as low as 50 μM (Filleur and Daniel-Vedele, 1999). Most of the members of the family perform the uptake of NO_3^- at relatively high concentrations of above 1 mM and are hence classed as LATs (Siddiqi et al., 1990). The only member of the family that does not quite fit the category of LATs is NRT1·1 (previously known as Chl1) as it is a "dual-affinity" transporter acquiring two K_m values corresponding to both low and high NO_3^- concentrations (Liu et al., 1999). The affinity of NRT1·1 is regulated by the phosphorylation status of its threonine 101. While the phosphorylation at the residue results in the transporter functioning as the high-affinity type, the dephosphorylation changes the mode of action to the low-affinity type (Liu and Tsay, 2003). Interestingly, members of NRT1 (PTR) are located in different tissues showing that they not only

contribute to NO_3^- uptake from the soil but also to NO_3^- distribution throughout the plant. For example, in *Arabidopsis AtNRT1·1* and *AtNRT1·2* are expressed in the epidermis of roots, as well as in the cortex and endodermis for *AtNRT1·1*, while *AtNRT1·6* expression is observed in the silique and funiculus (Huang et al., 1996, Huang et al., 1999, Almagro et al., 2008). It is worth noting that beside NO_3^- transporters, the members of the NRT1 family also include peptide transporters (PTRs) due to them sharing sequence similarities to NRT1 (reviewed in Tsay et al., 2007).

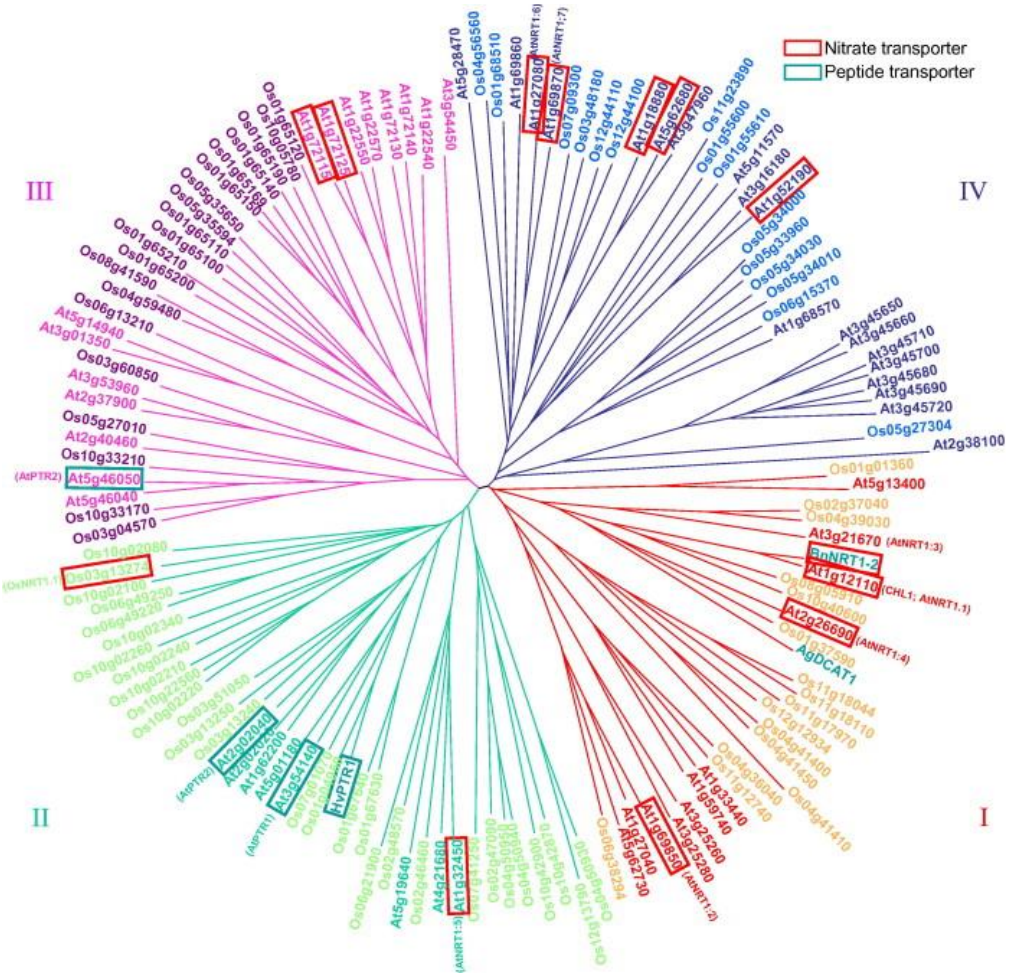

Figure 3: Phylogenetic tree of NRT1 (PTR) transporters in *Arabidopsis* and rice (figure from Tsay et al., 2007). The transporters are divided into four subgroups: I, II, III and IV.

HATs for NO_3^- operate under low NO_3^- concentration, typically in the range of 1 μM to 1 mM (Behl et al., 1988). The HATs are proton-coupled transporters from the nitrate-nitrite porter (NNP) family. It has been predicted that the members of this family in algae and plants, where they are referred to as NRT2s, have protein structures consisting of twelve transmembrane domains with an extended domain consisting of around 70 amino acids at the C-terminal (type III) (Forde, 2000). Type III transporters can be divided into two subgroups: IIIa without N-terminal sequence addition and IIIb with an addition of a sequence with approximately 20 amino acids at the N-terminus (Forde, 2000). The

expression of *NRT2* genes is induced by NO_3^- concentration. For instance, 50 μM of NO_3^- can induce the expression of *AtNRT2·1*, and the magnitude of the induction was found to be much higher for *AtNRT2·1* than *AtNRT1·1* (Filleur and Daniel-Vedele, 1999). On the other hand, *AtNRT2·1* expression was less abundant than *AtNRT1·1* at high NO_3^- concentration (Filleur and Daniel-Vedele, 1999). So far, seven *NRT2* genes were identified in *Arabidopsis* (Orsel et al., 2002) and four putative genes in rice (Cai et al., 2008) (Figure 4). Even though the NRT2 family in higher plants needs to be further characterised, it is now known that NO_3^- HATs of *Arabidopsis* consists of a membrane complex. It was shown that, in *atnrt2.1-1* knockout and *atnar2.1-1* knockout mutants, only 21% and 3% of the NO_3^- influx rate of the wild-type was observed at 0.2 mM NO_3^-, respectively (Orsel et al., 2006). The progeny of crosses of the two mutants also showed similar phenotype to *atnar2.1-1* knockout mutants at low NO_3^- concentration (Orsel et al., 2006). Together with their yeast two-hybrid system study demonstrating that AtNRT2·1 and AtNAR2 interacted with each other, Orsel et al. (2006) suggested that HATs of NO_3^- in *Arabidopsis* is a two-component system consisting of AtNAR2.1 and AtNRT2.1 and their interactions play a crucial role in HATs. Besides NRT2, chloride channels (CLCs) were also reported to be involved in NO_3^- transport at low concentrations. For example, AtCLCa resides in the tonoplast of mesophyll cells and acts as NO_3^-/H^+ antiporter, resulting in the accumulation of the vacuolar NO_3^- (De Angeli et al., 2006).

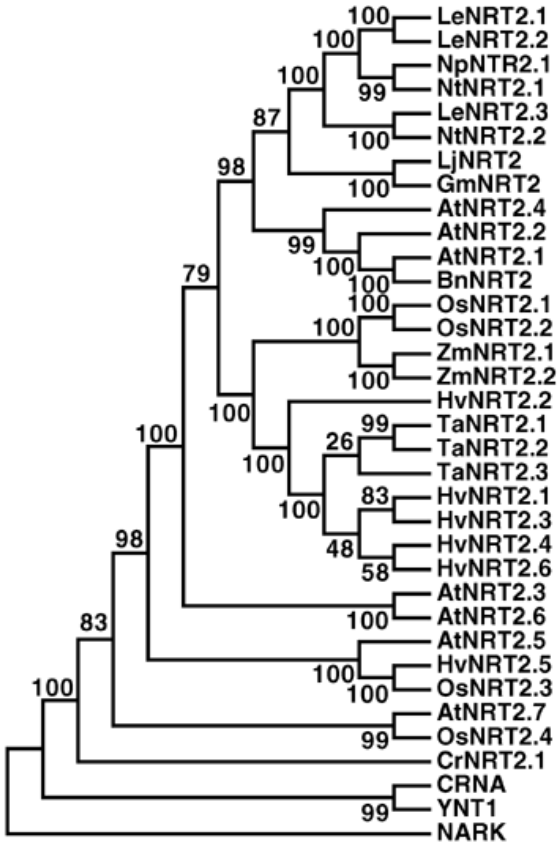

Figure 4: Phylogenetic tree of NRT2 transporters in various plants (figure from Cai et al., 2008).

1.2.4.2 Ammonium

NH_4^+ uptake systems in plants belong to a family of ammonium transporters (AMTs), a protein family with a structure consisting of eleven transmembrane domains with the N-terminus on the exterior side and C-terminus on the interior side of the membrane (Thomas et al., 2000). In *Arabidopsis*, six AMT genes were discovered (Gazzarrini et al., 1999). Interestingly, ten AMTs were identified in rice (Suenaga et al., 2003), a species that is adapted to NH_4^+ environment (Britto and Kronzucker, 2002), suggesting a possibility that the number of AMT genes may imply an evolutionary adaptation for NH_4^+ nutrition.

In *Arabidopsis* AMTs can be divided into two clades; AMT1 and AMT2 with the latter far less extensively studied than the former. While NH_4^+ and the NH_4^+ analogue methylammonium are substrates of AMT1 (Ninnemann et al., 1994), AMT2 can only transport NH_4^+ (Sohlenkamp et al., 2000). Another difference between AMT1 and AMT2 is that the former is highly expressed in both roots and shoots (Ninnemann et al., 1994), while the latter is expressed in low number in the root tissues (Sohlenkamp et al., 2000). Using phylogenetic approaches, it was suggested that the AtAMT1 family members are closely related to each other, while the members of AtAMT2 and NH_4^+ transporters of other

organisms, including methylammonium/ammonium permease (MEP) systems from yeast, form another cluster as they are more similar in terms of structure (Pantoja, 2012). In rice, AMT is divided into four families: OsAMT1 joins the first cluster with AtAMT1 while the other three families namely OsAMT2, OsAMT3 and OsAMT4 join the latter with AtAMT2 (Loqué and von Wirén, 2004) (Figure 5).

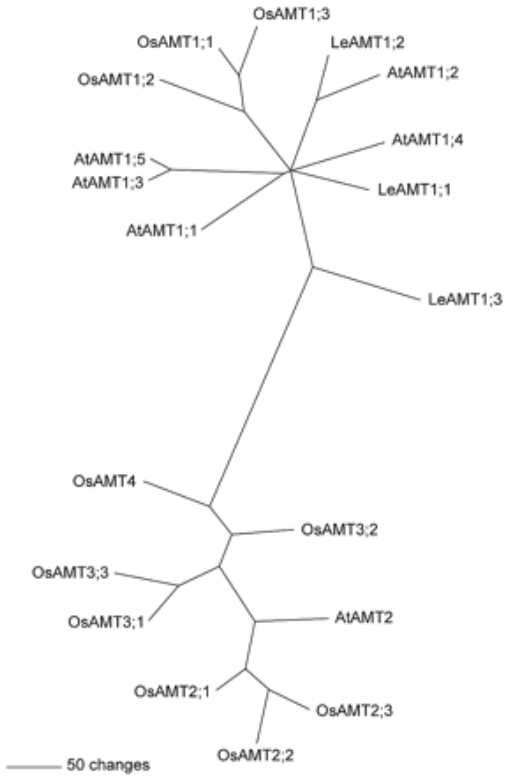

Figure 5: Phylogenetic tree of AMT transporters in *Arabidopsis*, rice and tomato (Le) (figure from Loqué and von Wirén, 2004).

With the use of yeast mutants lacking MEPs, *AMT1* genes in *Arabidopsis* were identified through growth complementation studies (Ninnemann et al., 1994). It was revealed that while *AtAMT1·3* is strictly expressed in the roots, the expression of *AtAMT1·1* is found in both the roots and shoots (Gazzarrini et al., 1999). The expression of the more constitutive *AtAMT1·2* follows the pattern of *AtAMT1·1* but the expression in the shoots is much weaker (Gazzarrini et al., 1999). In rice, *OsAMT1·1* expresses in a more constitutive fashion in both tissues while *OsAMT1·2* and *OsAMT1·3* expressions are root-specific which are induced by the NH_4^+ and limiting N source, respectively (Sonoda et al., 2003a). Altogether, this indicates that the role of AMT is not only confined to NH_4^+ uptake but also in NH_4^+ distribution in plant tissues.

Members of AMT1 family belong to NH_4^+ HAT. For example, AtAMT1·1 which is highly expressed in the roots at low NH_4^+ concentrations of 100 μM (Gazzarrini et al., 1999). After resupplying NH_4^+, the expression of *AMT1* declined at NH_4^+ external concentration of

at least 1 mM (Rawat et al., 1999). It was later revealed that external NH_4^+ is not a regulator of *AtAMT1*, rather the end products of NH_4^+ assimilation are due to the fact that the blockage of NH_4^+ conversion to glutamate by methionine sulfoximine, an inhibitor of GS, leads to the decrease in *AtAMT1* transcript abundance (Rawat et al., 1999). In rice, glutamine was also reported to regulate the mRNA levels of *AMT1*: a positive regulation for *OsAMT1·1* and *OsAMT1·2* and a negative regulation of *OsAMT1·3* (Sonoda et al., 2003b).

From a competition study between methylammonium and NH_4^+ uptake, AtAMT1·1 was found with the highest affinity for NH_4^+ among the three, followed by AtAMT1·3 and AtAMT1·2, respectively (Gazzarrini et al., 1999). The members of AMT1 with higher affinity for NH_4^+ tend to be located in the root hair cells while lower affinity members are situated in the endodermis (Masclaux-Daubresse et al., 2010).

Besides AMTs, other channels were also discovered to possibly be involved in NH_4^+ transport. These candidate channels may participate in LAT as passive NH_4^+ channels. For instance, TIP2, a member of the aquaporin (AQP) subfamily called tonoplast intrinsic proteins (TIPs) from wheat found at vacuolar membranes, was able to restore the growth of yeast without the NH_4^+ uptake systems when NH_4^+ was the only N source (Jahn et al., 2004). K^+ channels like KAT and AKT1 were also found to be permeable to NH_4^+ (Fuchs et al., 2005, Schachtman et al., 1992) which is likely because of the similarity between the ionic radii of K^+ and NH_4^+, a characteristic that is crucial for membrane transport. The possibility that K^+ and NH_4^+ influx shares the same pathway is supported by several findings including the competition between the uptake of the cations (Szczerba et al., 2008, ten Hoopen et al., 2010), the alleviation of NH_4^+ toxicity by the increase in K^+ content in the soil (Britto and Kronzucker, 2002) and the growth restoration of the mutant yeast without high affinity NH_4^+ systems by K^+ channels at low NH_4^+ concentration (ten Hoopen et al., 2010).

1.3 Drought stress

With N being a crucial constituent in amino acids and subsequently proteins, N nutrition has a wide range of impacts on plants and has been extensively studied. One of the lesser studied areas is N's role in conditions such as drought. However, it is known that some amino acids, N compounds and protein channels have a role during osmotic stress which demonstrates that N nutrition is associated with drought tolerance.

1.3.1 Impact of drought on plants

Drought stress is the result of low water availability in the soil and/or high atmospheric vapour deficit. Drought is a cause of osmotic stress, a stress which results in a reduction in the water absorption efficiency and turgor loss in plants. Some common symptoms of drought in plants are stunted growth, rolled leaves and senescing leaves. In general, the severity and the periods of drought have increased worldwide due to climate change: prolonged drought periods lead to fresh water shortage and therefore have a large impact on plant productivity and food security. For example, it was estimated that the national cereal production across the globe dropped by 9-10% from 1964 to 2007 due to the occurrence of drought incidents (Lesk et al., 2016).

One of the primary processes disturbed in C_3 plants by water shortage is photosynthesis. Under water deficit conditions, reduction of leaf turgor, due to high atmospheric vapour pressure deficit or chemical signals from the root, leads to the closure of stomata. This decrease in g_s, along with the decline in mesophyll conductance (g_m), results in less accumulation of intercellular CO_2 (C_i) at the site of rubisco. The decrease in C_i means that there is less C available as a substrate for photosynthetic enzymes, thus the rate of photosynthesis is reduced (reviewed in Chaves et al., 2009). Photosynthesis capacity of plants can be further inhibited by high light and temperature due to photoinhibition. This is because the decline in C_i under drought conditions enhances the formation of reactive oxygen species (ROS). For instance, O_2 becomes more available as an electron acceptor in comparison with CO_2, resulting in the enhanced formation of singlet oxygen at the reaction centre of in photosystem II. A greater electron leakage during drought stress also results in increased production of H_2O_2 ad superoxide in the Mehler reaction in the chloroplast. Moreover, peroxisomal H_2O_2 production in C_3 plants is enhanced due to the relative increase in oxygenation over carboxylation which generates higher rates of photorespiration (reviewed in Noctor et al., 2014). ROS are highly reactive and have the potential to cause deleterious effects on cells due to the peroxidation of membrane phospholipids and fatty acids and damages on DNA. For example, H_2O_2 can inhibit the translation of *psbA* mRNA*,* a gene encoding D1 protein which is involved in the repair of photosystem II, and subsequently disrupt the repair of photosystem II (Takahashi and Murata, 2008). ROS can also mediate cell death by activating ion channels: the activation of K^+ outward-rectifying channel lowers K^+ concentration inside the cell which activates

proteases and nucleases (reviewed in Demidchik, 2014). In addition, ROS can trigger the inactivation and activation of ion transporters at the plasma membrane by lipid peroxidation (reviewed in Stark, 2005). The inactivation of certain ion channels like gramicidin and polyene channel is due to the peroxidation of the lipid-like exterior of the channels which leads to the decrease in hydrophobic property of the channels. Lipid peroxidation is also believed to indirectly inactivate channels by decreasing the channel efficiency due to the damage to the lipid membrane and by secondary reactive species made during the peroxidation. Moreover, some ion channels and carriers at the plasma membrane were found activated by ROS as a result of the increase in the dielectric constant of the membrane interior by the polar products of lipid peroxidation (reviewed in Stark, 2005).

Beside photosynthesis, drought also influences plant nutrition. Water deficit directly decreases the uptake, remobilization and accumulation of nutrients in plants which can lead to repressed growth. This is due to the reduction of the mobility of the nutrients in the soil and plant transpiration flow (Garg, 2003). A study on watermelon, for example, showed that there was a decrease in leaf macronutrients, especially N, in water stress treatment relative to control (Kirnak et al., 2002).

1.3.2 Plant mechanisms against drought

To alleviate water stress, plants have developed internal processes which lead to readjustment of water uptake in plant cells. Drought resistance mechanisms in plants are divided into four components: drought avoidance, tolerance, escape and recovery in which the first two are the major elements.

1.3.2.1 Drought avoidance

Drought avoidance is the ability of plants to maintain high water potential when facing drought. There are three main approaches involved in drought avoidance (reviewed in Fang and Xiong, 2015). First, by acquiring leaf traits that allow the maintenance of sufficient water supply. Leaf responses like leaf rolling and upright leaf configuration reduce the exposed surface area and the exposure to solar radiation, respectively. Both responses lead to a reduction in transpiration rate. Some plants with high resistance to drought have a leaf structure that allows them to decrease transpiration rate even further, for example, smaller leaf, thicker accumulation of wax on the cuticle, thicker palisade tissue, sunken stomata and more epidermal trichomes. Second, by maximising the

efficiency in taking up and storing water. Root depth, density, volume and as well as root and shoot ratio correlate to drought resistance as the increase in these parameters allows plants to obtain more water. The ability to store more water in the plant is also found in arid environments, for example, succulent plants with water-storing tissues such as cacti which store water in the stem. Third, by delaying progress from vegetative to reproductive growth. This is so that the impacts of drought on the development of fruits and seeds are reduced and therefore the damage due to water shortage on plant yield is minimised.

1.3.2.2 Drought tolerance

Drought tolerance is the ability to reduce or repair the damage of drought stress via the regulation of genes and metabolic pathways. Drought tolerance is commonly executed by two mechanisms (reviewed in Fang and Xiong, 2015). First, the removal of excess ROS accumulation which is carried out by antioxidant defence systems. These antioxidants consist of enzymes like superoxide dismutase and ascorbate peroxidase and non-enzymes like carotenoids. While superoxide dismutase converts superoxide radicals into H_2O_2 and subsequently to water by ascorbate peroxidase, carotenoids convert singlet oxygen and triplet chlorophyll into the ground state. Interestingly, though ROS at high levels causes cellular damage, at lower levels ROS can serve as a signalling molecules. For instance, H_2O_2 is able to induce the expression of genes in *Arabidopsis* which are related to cellular protection and plant stress response (Desikan et al., 2000), activate Ca^{2+}-permeable channels in the plasma membrane of guard cells that promote the closure of stomata (Pei et al., 2000) and activate protein-kinases involved in biotic and abiotic stress responses including drought (Desikan et al., 1999).

The second mechanism is the accumulation of osmoregulators for osmotic adjustment purpose. The accumulation of these substances maintains high turgor potential and cellular water retention and also protects cellular components. Inorganic ions play a role in osmoregulation. One of the most common known osmoregulators is K^+ which has multiple roles in osmotic adjustment including preserving water content in leaves and mediating the opening and closing of stomata (reviewed in Wang et al., 2013). Organic compounds known as compatible solutes also act as osmoregulators. Compatible solutes are compounds that are small, highly soluble and “neutral” at pH 5-8. They are synthesised and accumulated in response to various stresses including drought. Compatible solutes are classified into five groups (Hamedi, 2015): 1. Disaccharide sugar (for example, trehalose and sucrose), 2. Polyols (for example, sorbitol and mannitol), 3. N-acetylated diamino acids

compounds (for example, N-acetylglutaminylglutamine amide), 4. Betaine compounds (for example, glycine betaine) and 5. Amino acids (for example proline and alanine). In general, compatible solutes provide protections to plants by participating in osmotic adjustment, protein and enzyme stabilisation, protection of membrane integrity and ROS scavenging. Glycine betaine, for instance, is believed to provide protection to Rubisco and Rubisco activase, activates the ROS scavenger genes and/or protects the translation of D1 protein (Chen and Murata, 2011). Besides compatible solutes, there are other organic compounds with osmotic adjustment properties such as late embryogenesis abundant (LEA) proteins and AQPs. LEA are hydrophilic proteins that can be found in the nucleus and cytoplasm. The accumulation of LEA proteins is observed during stress periods including water deficiency. It has been shown that transgenic rice with overexpressing *OsLEA3-1*, a gene encoding for LEA protein, has improved drought tolerance (Xiao et al., 2007). Together with compatible solutes, LEAs function in a possible synergistic manner to stabilise macromolecules and protoplasm (Close, 1996). AQPs are involved in water transport and can take part in drought tolerance by adjusting hydraulic conductance, supplying water to important cells and organs or preparing drought-suffered tissues for rehydration (Maurel, 1997).

1.4 AKT1

Like N, K is a plant macronutrient. K is the second most abundant nutrient in plants after N, making up 1-5% of plant dry matter (Roy et al., 2006). K is taken up in the form of K^+. K^+ mobility is high and it is distributed throughout the plant via xylem and phloem. In general, K^+ is involved processes including photosynthesis, growth and ion homeostasis. The well-known role of K^+ is the activation of K^+-dependent enzymes, such as pyruvate kinase, by binding to the enzymes as a cofactor. Ribosome function in protein synthesis also requires high K^+ concentration. Moreover, K^+ has a crucial role in turgor pressure provision which controls plant movement and growth. As mentioned, K^+ also has a role as an osmoregulator as its accumulation leads to a decrease in cellular water potential, which is essential during drought to prevent water loss from the cells. K^+ accumulation in the guard cells regulates the opening of stomata, therefore K^+ is involved in the regulation of gas exchange and transpiration (Maathuis, 2009). With many involvements in plant processes, the maintenance of sufficient concentration of K^+ in the cells is crucial: about

80-150 mM of K^+ is typically found in the cytosol while the accumulation of K^+ in the vacuoles can range from close to zero to 600 mM (Rouphael et al., 2012). The common symptoms found in K^+ deficient plants are leaf chlorosis and browning tips on the older leaves. In K-starved plants, the plant statue is also often small, internodes are short and stalks are weak relative to healthy plants (Cameron, 1992). Plants also become more susceptible to infection when K in the supply is inadequate. Low plant K status, however, promotes the uptake of other cations like Ca^{2+} and Mg^{2+} due to their antagonistic effect. Nevertheless, K uptake is crucial to maintaining optimal plant growth and metabolism.

1.4.1 Potassium uptake via AKT1

At the root-soil boundaries of plants (Figure 6), there are two uptake systems for K^+. Active K^+ uptake is carried out by KUP/HAK H^+-coupled carriers in the HATs. On the other hand, AKT1 channels play a role in the passive LATs along with a non-selective cation channel, possibly a cyclic nucleotide-gated channel (CNGC) (Ahmad and Maathuis, 2014).

AKT1 is a voltage-dependent, inward-rectifying K^+ channel (Hirsch et al., 1998) which belongs to the Shaker family. The heteromerisation of AKT1 and AtKC1 fine-tunes the properties of AKT1 resulting in negative shifting of AKT1 activation potential which reduces the channel conductance and thus preventing K^+ leakage from the channel at low external K^+ concentration (Geiger et al., 2009). Although AKT1 is mainly expressed in the root plasma membrane, it is also present in the leaf (Lagarde et al., 1996), particularly in the guard cell plasma membrane (Müller-Röber et al., 1995). AKT1 can function in a broad range of external K^+ concentrations but mostly contributes to K^+ uptake at higher external concentrations (Nieves-Cordones et al., 2014). The hyperpolarisation of the membrane potential, to around -100 mV, activates AKT1 via conformational changes from closed to open. This change occurs after sensing shifts of the membrane potential done by a group of positively-charged residues in the fourth transmembrane domain of the channel (Hedrich et al., 2011). Ca^{2+} can also regulate the activity of AKT1. It was proposed that the activation of AKT1 is mediated via Ca^{2+} signalling pathway under low K^+ conditions. Two calcineurin B-like proteins found at the plasma membrane, CBL1 and CBL9, bind to a Ser/Thr protein kinase CIPK23. This interaction activates CIPK23 which in turn phosphorylates and promotes a change in AKT1 conformation, resulting in K^+ uptake enhancement via AKT1 (Xu et al., 2006, Li et al., 2006). AKT1 homologs had been identified in various species, including in *Arabidopsis* (Hirsch et al., 1998) and rice (Fuchs et al., 2005).

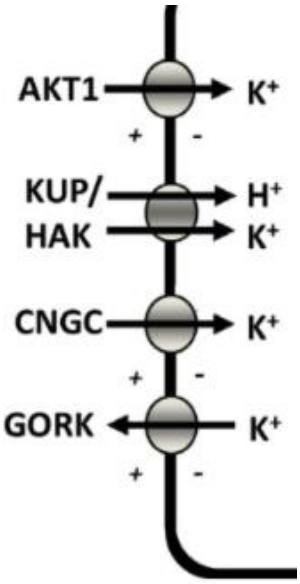

Figure 6: K^+ channels and carriers at the plasma membrane of root cells (figure from Ahmad and Maathuis, 2014). The influx of K^+ in the roots is mediated by AKT1 (passive K^+ influx), CNGC (passive K^+ influx) and KUP/HAK (energized K^+ influx). The efflux of K^+ in the roots is executed by GORK.

1.4.2 OsAKT1 and ammonium uptake

There is speculation that K^+ and NH_4^+ uptake may share some of the same transport pathways because the ionic radii of the two cations are similar. The competition between the two ions was also reported in roots of plants as a negative correlation between K^+ and NH_4^+ influxes could be observed (ten Hoopen et al., 2010). Also, a growth complementation study in *Δmep1-3*, a yeast mutant lacking NH_4^+-specific uptake systems, illustrated that several K^+ channels including AKT1 from *Arabidopsis* partially restored the growth of *Δmep1-3* under low NH_4^+ concentration (ten Hoopen et al., 2010).

Some evidence suggests that AKT1 in rice may have a role in NH_4^+ uptake. First of all, the observation of HEK293 cells expressing *OsAKT1* and patch-clamp analysis of rice root protoplasts showed a current signal in the presence of external NH_4^+, although the current was smaller than that of K^+ (Fuchs et al., 2005). This provides a solid proof that OsAKT1 is permeable to NH_4^+. Moreover, a recent study demonstrated that the growth of wild-type and *akt1* knockout rice mutant in the medium with NH_4^+ as a sole N source was similar to those in NO_3^--based medium (Ahmad et al., 2016). This finding of the rice mutant not suffering from the presence of NH_4^+ in the medium contradicts the previous study in *Arabidopsis* which found deleterious effects of NH_4^+ on growth. In *Arabidopsis*, an *akt1* knockout phenotype with a clear reduction in growth can be observed when a small amount of NH_4^+, in millimolar levels, was present under low K^+ provision (Hirsch et al., 1998). It was later suggested that NH_4^+ blocked the remaining non-AKT1 component of

HAK5 which belongs to the HAK/KUP family in *Arabidopsis* (Rubio et al., 2008, Spalding et al., 1999). As the presence of NH_4^+ did not affect the growth of rice *akt1* mutants, it suggests that the detrimental effect of NH_4^+ is absent in rice, possibly because rice is highly adapted to NH_4^+ nutrition (Britto and Kronzucker, 2002).

1.4.3 OsAKT1 and drought tolerance

AKT1 activity has been reported to affect plant drought tolerance. In *Arabidopsis*, *akt1* knockout plants were found with higher shoot water content and lower g_s relative to wild-type at both low and high K^+ concentrations under water stress for 24 hours in hydroponic cultures. As the shoot K^+ concentration of the two genotypes differed at low K^+ (with the knockout line accumulating less shoot K^+) but similar at high K^+ concentration, it was suggested that the higher water content in the knockout line during the short period of water stress was not due to K^+ shortage but more likely the effects of K^+ on transpiration (Nieves-Cordones et al., 2012). The statement was supported by the abscisic acid (ABA)-hypersensitive characteristics of guard cells in the mutant line which resulted in smaller stomatal aperture (Nieves-Cordones et al., 2012). Therefore, *akt1* knockout *Arabidopsis* were more tolerance to short-term water stress than the wild-type because the mutant line was more efficient in preventing water loss via the stomata. The data also implies that the benefits of K^+ taken up via AKT1 in the roots are outweighed by water loss via transpiration modulated by AKT1-regulated g_s in the guard cells during the short period of water stress. Interestingly, the knockout line was also found with lower g_s than the wild-type when the plants were treated in non-irrigated pots for two weeks, however the plant biomass, K^+ concentration and water content of the two genotypes were not significantly different.

A similar study in rice, however, produced different results. Ahmad et al. (2016) showed that dysfunctional AKT1 led to a significant reduction in growth rate as well as root K^+ concentrations in *akt1* knockout line both during a 2-week period with polyethylene glycol (PEG) and a 6-week period with limited water supply in the soil. They also found that AKT1 overexpressing rice plants grew significantly better than the wild-type as well as exhibiting higher K^+ concentration in both root and shoot tissues during both periods. In contrast to the *Arabidopsis* study, they found no significant difference in the g_s nor water content of wild-type and the knockout line in the 2-week treatment period with PEG, however, a significantly higher water content was found in the overexpressing lines. Thus,

OsAKT1 may contribute to rice drought tolerance by either participating in K^+ accumulation to lower cellular water potential especially in the roots and/or its contribution in guard cells to maintain water homeostasis.

It is also possible that OsAKT1 contributes to rice drought tolerance by mediating NH_4^+ uptake in which products of NH_4^+ assimilation may be used in the production of proteins involved in drought tolerance. Besides proteins that are associated with photosynthesis, there are a number of N-containing osmoprotectants as discussed in 1.3.2.2 such as proline, glycine betaine and polyamines that are important in drought tolerance. While proline is accumulated in the cytosol, glycine betaine can be found in chloroplasts. Their accumulation is generally, but not necessary, correlated with stress tolerance. For instance, proline concentrations and the activity of P5CS were found at a higher abundance in both drought tolerant and drought susceptible genotypes of rice but more so in the tolerant genotype (Choudhary et al., 2005). As for polyamines, it has been reported that these improve water content, water use efficiency and photosynthesis and as well as increasing proline production. Interestingly, the accumulation of polyamines is considered an immediate response towards drought stress (Pandey and Shukla, 2015). In rice, the accumulation of polyamines was associated with the degree of drought tolerance: the tolerant cultivar was found with higher amount of polyamines than the drought-susceptible ones. It was also reported that rice had a large capacity for the biosynthesis of polyamines in the leaf under water stress (Yang et al., 2007).

1.5 Rice

Rice (*Oryza sativa* L.) is a monocotyledonous species with a genome size of approximately 430 Mb. Due to its relatively small genome compared to other major cereals, rice was the first cereal to be sequenced (International Rice Genome Sequencing Project, 2005). Rice along with other major cereals like wheat and maize was found to share a high degree of conserved gene content and gene order (Gale and Devos, 1998). Therefore, rice is a suitable model plant for cereal crops and other grass species.

Other than being a model plant, rice also has an important role as one of the most consumed staple crops in the world. Consumption of rice exceeds 100 kg per capita per year in many countries in Asia and Africa (Seck et al., 2012). Almost all rice production bases situate in Asia: the production from China and India alone amounts to at least 33% of the

global rice output (Seck et al., 2012). As human global population rapidly rises the demand for rice increases, the Food and Agricultural Policy Research Institute (FAPRI) estimates that rice production has to increase from 439 million tonnes (in 2010) to 555 million tonnes by 2035 to support the expected future population (Global Rice Science Partnership, 2013).

As N nutrition is important for achieving high productivity, reaching sufficient N requirement is crucial to obtain optimal rice yields. In rice, leaf N abundance must be at least 1.4 g m^{-2} leaf area for rice to achieve its maximum potential yield (Dobermann and Fairhurst, 2000). However, acquiring the optimal level of N may be hard to achieve in rice because of the unique anaerobic submerged ecosystem in which it is cultivated. The paddy rice field enhances N loss via various methods including denitrification to inaccessible N forms (N_2O and N_2), volatilization of NH_3 and NO_3^- leaching from soil (Ghosh and Bhat, 1998).

Being a crop that grows in a submerged environment, water availability greatly affects growth and productivity of rice. Drought has negative impacts on rice yields, especially on rain-fed rice which dominates cultivation in South Asia and Africa (Dawe et al., 2010), for example, drought was responsible for a yearly 700 to 1,300 kilogram loss in rice productivity per farm in Bangladesh (Mottaleb et al., 2015). Therefore, the improvement in rice drought tolerance is urgently needed.

1.6 Hypothesis and Objectives

There are two hypotheses in this research project: rice AKT1 channel plays a role in NH_4^+ uptake at the root-soil boundary and NH_4^+ improves osmotic stress tolerance of rice via AKT1.

The aims of this research project are as follows.

1. To investigate the role of OsAKT1 in NH_4^+ uptake in rice and yeast.
2. To investigate the effects of NH_4^+ via OsAKT1 on growth and N contents of rice in the presence of osmotic stress.

2 Chapter 2: Materials and methods

2.1 Plant materials

Wild-type (WT) and *akt1* knockout mutant (KO) seeds of rice in the Dongjin background were obtained from China Agriculture University, Beijing. KO rice plants were the result of the insertion of T-DNA at the position 303 bp upstream of the start codon of *OsAKT1* in PFG_1B-16021 line (Figure 7).

Figure 7: T-DNA insertion site in *OsAKT1*. The black boxes and lines represent exons and introns, respectively. The arrow indicates the T-DNA insertion site at -303 bp.

2.2 Growth conditions

Seeds were dehusked and sterilised using 70% ethanol before being incubated at 28^{o}C on 0.25% agar plates. Rice seedlings were transferred to a tray of attapulgite (Oli-Dri Corporation of America, Cambridgeshire, UK) in the glasshouse (day/night temperature 28/24^{o}C, LED lighting / high-pressure sodium lamps, day length of 12 hours with a minimum light intensity of 160 µmol m^{-2} s^{-1}, 55-67% relative humidity) ten days after sowing. After two weeks, rice seedlings were treated with 1.6 L of Yoshida medium (2.9 mM NH_4NO_3, 0.3 mM $NaH_2PO_4{\cdot}H_2O$, 0.5 mM K_2SO_4, 1 mM $CaCl_2.2H_2O$, 1.6 mM $MgSO_4{\cdot}7H_2O$, 0.01 mM $MnCl_2{\cdot}4H_2O$, 0.001 mM $(NH_4)_6{\cdot}Mo_7O_{24}{\cdot}4H_2O$, 0.2 mM H_3BO_3, 0.0002 mM $ZnSO_4{\cdot}7H_2O$, 0.0002 mM $CuSO_4{\cdot}5H_2O$, 0.04 mM $FeCl_3{\cdot}6H_2O$ and 0.87 mM Na_2SiO_3, pH 5.6-5.7) (Yoshida et al., 1976) until the plants reached the desired age for each experiment in the glasshouse. Fresh Yoshida medium was supplied every seven days.

2.3 Verification of *akt1* knockout rice mutant plants

2.3.1 Using Genomic DNA

Leaves from three individual rice plants in each genotype were collected and pooled to extract genomic DNA using a modified CTAB method (Rogers and Bendich, 1985) as follows: the leaf tissues were added to 200 μl of 2xCTAB buffer (2% CTAB, 1.4 M NaCl, 100 mM Tris-HCl pH 8 and 20 mM Na-EDTA) and homogenised. The homogenised tissues were incubated at 65^{o}C for 30 minutes and vortexed briefly. After the temperature of the leaf mixture reduced to room temperature, 200 μl of chloroform was added and mixed vigorously to the homogenised samples. The samples were centrifuged at full speed for 5 minutes followed by transferring 100 μl of the top aqueous layer to the new 1.5-ml tube to mix with 250 μl of ethanol. Genomic DNA pellets were retrieved after placing the solution in the freezer for 10 minutes and centrifuging at full speed for 10 minutes. The dry pellets were dissolved in 50 μl of elution buffer (EB) (10 mM Tris-Cl, pH 8.5)

PCR was performed using the extracted genomic DNA as a template. Each PCR mixture consisted of 0.13 μl of *Taq* DNA polymerase (New England Biolabs, Hertfordshire, UK), 2.5 μl of 10X ThermoPol® buffer (New England Biolabs, Hertfordshire, UK), 0.5 μl of each primer with 10 μM (forward primer = 5'-CCCTCCCCTCGCATCCATA-3' and reverse primer = 5'-CACCTCGCTGGCCTCGAA-3') in which the primer pair spans the T-DNA insertion site, 0.5 μl of 10 mM dNTPs and the rest of the volume was made up with deionised to 25 μl. PCR products were run along with 2-log DNA ladder (New England Biolabs, Hertfordshire, UK)on 1% agarose gel with SYBR® Safe (ThermoFisher, Leicestershire, UK) and viewed under blue light, the condition used to observe DNA bands throughout this research project.

2.3.2 Using mRNA

Roots from three individual rice plants in each genotype were collected and pooled to extract root RNAs using E.Z.N.A.® Plant RNA Kit (Omega Bio-tek, Norcross, USA). cDNAs were synthesised from the extracted RNAs as follows: to 500 ng of the extracted RNAs, 250 ng of oligo dT primer (Invitrogen, Leicestershire, UK), 0.5 μl of 10 mM dNTPs and deionised to bring the solution to 6 μl were mixed on ice. The mixture was heated at 65^{o}C for 5

minutes and quick-chilled on ice. To the mixture, 2 μl of 5X first strand buffer (Invitrogen, Leicestershire, UK), 1 μl of 100 mM DTT (Invitrogen, Leicestershire, UK), 0.5 μl of SuperScript® II Reverse Transcriptase (Invitrogen, Leicestershire, UK)) and 0.5 μl of RiboLock RNase Inhibitor (ThermoFisher, Leicestershire, UK) were added and mixed by pipetting. The mixture was heated at 42°C for 60 minutes and 70°C for 15 minutes

With rice root cDNAs, the amplification of *OsAKT1* was carried out using the same PCR recipe as described in 2.3.1 except with a change in a primer pair: forward primer = 5'-AGCTAGCATACTCTCTATCTCCTTT-3' and reverse primer = 5'-CTCAATATCTGTTCTTCCATCTGG-3' were *OsAKT1* specific.

2.4 Effects of different nitrogen supplies under osmotic stress

WT and KO rice plants that reached four weeks old were treated with modified 2.4 L of Yoshida medium using a mixture of NH_4Cl and $NaNO_3$ as N sources with the final N concentration of 2.9 mM; either under NH_4Cl: $NaNO_3$ ratio of 0.1: 0.9 (NO_3^--N) or 0.9: 0.1 (NH_4^+-N). Medium with only NH_4^+ or NO_3^- was not used in the experiment because rice grows better when both N forms are present rather than either alone under normal conditions (Guo et al., 2008, Ta and Ohira, 1981). Into the medium, 8% polyethylene glycol (PEG) with the average molecular weight of 4,000 (Alfa Aesar, Lancashire, UK) was used to introduce osmotic stress. The osmotic pressure of PEG-added treatments was -0.21 MPa: Roebling Automatic Osmometer (Camlab, Cambridgeshire, UK) was used to measure the osmolality of the treatment and the osmotic pressure was calculated using the equation osmotic pressure = osmolality*-2.479 (Jones et al., 2007). Note that 10 mM NaCl was added to all treatment combinations to bring up Na^+ and Cl^- concentrations from NH_4Cl and $NaNO_3$ to approximately the same values. The plants were treated in the glasshouse and fresh medium was supplied to the rice plants every seven days. The course of the experiment lasted for three weeks. The experiment was carried out independently three times. In each repeat, 2-3 plants of each genotype were included in each combination of the treatments.

2.4.1 Growth

Relative growth rate (RGR) (Hoffmann and Poorter, 2002) was calculated using the equation RGR = (ln fFW – ln iFW)/($d_2 – d_1$) where fFW is final fresh weight (FW) and iFW is initial FW between day d_2 and d_1 (gFW day^{-1}). In addition, the total FW of roots and shoots were collected three weeks after the start of the experiment to determine root and shoot biomass ratio (root:shoot).

2.4.2 Shoot potassium and sodium concentrations

After three weeks of treatment, the root and shoot tissues from the highest node of each plant were collected and dried at 80 ^oC for 72 hours. Dry weight (DW) of the tissues was collected before the tissues were suspended in 8-10 ml of 20 mM $CaCl_2.2H_2O$ solution in capped tubes for 72 hours. Flame photometry was conducted using a model 410 flame photometer (Sherwood, Cambridgeshire, UK) to determine the concentration of K^+ and Na^+ in the shoot solutions, and subsequently to calculate the ion concentrations in the tissues based on DW (μmol gDW^{-1}).

2.4.3 Shoot carbon and nitrogen contents

After final FW was collected, the shoot tissues from the second highest node of each plant was collected and dried at 80 ^oC for three days. The dry shoot tissues from each repeat of the growth experiment were pooled according to the genotype and treatment and ground using Plant Grinding and Preparation System (Labman, North Yorkshire, UK). The amount of each ground samples used was 10 mg (± 0.1 mg) which was contained in a tin foil cone and folded into a compressed ball. C:N analysis was performed using Vario MACRO cube (Elementar, Langenselbold, Germany) to determine N and carbon (C) percentage in the shoots. “Plant 200” mode was selected while glutamic acid was used as a standard and Birch leaf standard (Elemental Microanalysis, Devon, UK) was used as a reference material.

2.5 Effects of elevated ammonium concentration

WT and KO rice plants that reached 12-14 weeks old were treated with 4.8 L of modified Yoshida medium using 8 mM NH_4Cl as the only source of N for three weeks. The plants were treated in the glasshouse and fresh medium was supplied to the rice plants

every seven days. The experiment was carried out independently three times. In each repeat, 1-2 plants of each genotype were included in the experiment.

2.5.1 Growth

RGR was calculated using the equation as shown in 2.4.1. Root:shoot biomass ratio was also calculated based on the total FW of roots and shoots collected three weeks after the start of the experiment.

2.5.2 Root and shoot potassium and sodium concentrations

The concentration of root and shoot K^+ and Na^+ was determined using the same protocol as described in 2.4.2 from the shoots and roots collected after three weeks of treatment. Note that root tissues were soaked in 20 mM $CaCl_2.2H_2O$ solution for 10 minutes before drying at 80 ^oC for three days.

2.6 Ammonium depletion experiments

WT and KO rice plants were treated in modified Yoshida medium with K^+ concentration of 0.2 mM and without N source for three days. N-starvation was established before the start of the assays to induce the rapid, detectable uptake of NH_4^+. Before the start of the depletion assay, each of the plants was weighed to collect FW. The assays were carried out using external NH_4^+ concentration of 0.2 mM and 2mM. The experiments were done in the glasshouse and the determination of NH_4^+ concentration in the medium was done at room temperature. It is worth noting that 0.2 and 2 mM NH_4^+ were selected to represent the concentration in which the HATs and LATs of NH_4^+ would dominate, respectively (Wang et al., 1993). To ensure that OsAKT1 is functional and to minimise the competition between K^+ and NH_4^+ uptake via the channel, 0.2 mM of K^+ was used in the medium during the assays. The experiment at each NH_4^+ concentration used 2-3 plants for each genotype and was repeated independently three times.

2.6.1 Ammonium depletion at low concentration

After N starvation, each of the 10-to-12-week-old WT and KO rice plants was transferred into 30 ml of modified Yoshida medium with 0.2 mM NH_4Cl as the N source and 0.2 mM K^+. At 1, 2 and 3-hour intervals after the transfer, 0.2 mM K^+-N free Yoshida medium was added to the plant medium to make up the original volume and 0.2 ml of the well-

mixed medium was collected for analysis. NH_4^+ concentration in the collected medium samples was determined using a colorimetric procedure (Baethgen and Alley, 1989) as follows: to each of the 0.2 ml of the collected samples, 1.1 ml of buffer solution (0.1 M Na_2HPO_4, 5% Na-K tartrate and 5.4% NaOH), 0.8 ml of salicylate/nitroprusside solution (0.94 M $C_7H_5NaO_3$ and 1mM $Na_2[Fe(CN)_5NO]\cdot 2H_2O$) and 0.4 ml of 0.32% NaOCl were added to the mixture. After every addition, the mixture was vortexed vigorously. The final mixture was kept at room temperature for 45 minutes before the absorbency of the solution was read using an Ultraspec 2,000 UV/VIS spectrophotometer (Pharmacia Biotech, Cambridgeshire, UK) at the wavelength of 650 nm. The concentration of NH_4^+ accumulated in the plants at each interval was calculated based on the decrease of NH_4^+ concentration in the medium and FW (μmol gFW^{-1}).

2.6.2 Ammonium depletion at high concentration

WT and KO rice plants used in the experiment were 6-to-8-week-old. After N starvation, each of the plant was transferred into 10 ml of modified Yoshida medium with 2 mM NH_4Cl as the N source and 0.2 mM K^+. The rest of the procedures was as specified in 2.6.1.

2.7 Studies on yeast

2.7.1 pYES2-*OsAKT1* constructs

The preparations of rice root cDNAs were done as described in 2.3.2 using root mRNAs extracted from rice in Nipponbare background. The amplification of full-length *OsAKT1* cDNA was performed using a high-fidelity DNA polymerase: to the template rice root mRNAs, 0.5 μl of Q5® High-Fidelity DNA Polymerase (New England Biolabs, Hertfordshire, UK), 10 μl of 5X Q5 high GC enhancer (New England Biolabs, Hertfordshire, UK), 10 μl of 5X Q5 reaction buffer (New England Biolabs, Hertfordshire, UK), 1 μl of 10 mM dNTPs, 2.5 μl of each 10 μM primer (forward primer = 5'-AAAAGAATTCAAAAATGGCGAGGTGGGGCGCTCGGATGGC-3' and reverse primer = 5'-AAAAGCGGCCGCCTAGCTCTTGCCTTTCATCTTCTC-3') and deionized water to make the solution up to 50 μl were added. Note that forward and reverse primers were designed to add *Eco*RI and *Not*I restriction site to 5' and 3' ends of *OsAKT1*, respectively, and that these primers have the addition of yeast consensus A-riched sequence found at the translational

initiator site to enhance the expression of *OsAKT1* in yeast (Hamilton et al., 1987). The amplified *OsAKT1* cDNA then was purified using Wizard® SV Gel and PCR Clean-Up System (Promega, Hampshire, UK) for PCR amplification product.

The amplified full-length cDNA of *OsAKT1* and pYES2 empty vector (EV) (Invitrogen, Leicestershire, UK) were double-digested with 2 μl of *Eco*RI (New England Biolabs, Hertfordshire, UK), 2 μl of *Not*I (New England Biolabs, Hertfordshire, UK), 8 μl of buffer H (Sigma-Aldrich, Suffolk, UK), 0.8 μl of BSA (New England Biolabs, Hertfordshire, UK) and deionised water to make up the volume to 80 μl. The digestion lasted for four hours at 37°C. Note that pYES2 was selected as the plasmid to carry the *OsAKT1* cDNA because the plasmid allows the transformation in both *E. coli* and yeast with appropriate selection markers. As for the restriction enzymes for digesting DNA, *Eco*RI and *Not*I were selected as each restriction enzyme only cuts the plasmid once at the multiple cloning site. Also, *Eco*RI and *Not*I do not digest *OsAKT1* cDNA without the addition of the restriction enzyme sites introduced during RT-PCR, thus ensuring that full-length cDNA was intact in the construct.

After confirming the completion of the digestion on a gel, the double-digested products were purified by centrifugation using Wizard® SV Gel and PCR Clean-Up System before proceeding to ligation: *OsAKT1* cDNA was purified directly from its double-digested PCR amplification product while double-digested pYES2 EV was ran on 1% agarose gel and extracted from a gel slice before being purified. Ligation of the double-digested *OsAKT1* (90 ng) and pYES2 EV (128 ng) was carried out using 0.13 μl of T4 DNA ligase (Promega, Hampshire, UK), 1 μl of T4 DNA ligase buffer (Promega, Hampshire, UK) and deionised water to make up the volume to 25 μl at 14°C overnight.

The ligation product was transformed into *E. coli* using a One Shot® Mach1^{TM}-T1^{R} (genotype: F- ϕ80(*lac*Z)ΔM15 Δ*lac*X74 *hsd*R(rK-mK^{+}) Δ*rec*A1398 *end*A1 *ton*A) chemically competent cell (Invitrogen, Leicestershire, UK). The transformation was carried out in a water bath at 42°C for 50 seconds. The transformed *E. coli* was plated on LB agar plates (in 1 L, LB agar consisted of 10 g of tryptone (Formedium, Norfolk, UK), 10 g of NaCl, 5 g of yeast extract micro granulated (Formedium, Norfolk, UK) and 20 g of agar) with 0.05 μg/ml carbenicillin and incubated at 37°C overnight. *E. coli* colonies from the LB plates were selected for colony PCR by using 25 μl of *E.coli*-suspended deionised water as a template, 0.25 μl of *Taq* DNA polymerase, 5 μl of 10X ThermoPol® buffer, 1 μl of each primer with 10 μM in which the primers for the colony PCR were *OsAKT1* specific as described 2.3.2, and deionised water to make up the volume to 50 μl. Out of the positive colonies from colony

PCR, five were selected for miniprep preparations using NucleoSpin® Plasmid DNA Purification (Macherey-Nagel, Düren, Germany). The minipreps were double-digested with *Eco*RI and *Not*I using the conditions specified above. Minipreps found with DNA bands consistent with the size of linearised pYES2 and full-length *OsAKT1* cDNA insert were sent for sequencing at the University of York: 3130*xl* genetic analysers (Applied Biosystems, California, USA) performed the sequencing with the use of T7 promoter-specific primer (5'-TAATACGACTCACTATAGGG-3') and *OsAKT1* specific primers (5'-TTACTTCTGGGTTCGATGTG-3', 5'-GCGTGGTATGGATCCAAATG-3', 5'-ACAGCGAGCCCATGATGAAG-3' and 5'-CTCAATATCTGTTCTTCCATCTGG-3'). The sequencing results were compared with cDNA *OsAKT1* in Rice Genome Annotation Project database (coding sequence of LOC_Os01g45990.1).

2.7.2 Yeast transformations

Three strains of yeast (*Saccharomyces cerevisiae*) were used for this study; *WΔ3TOK1Δ* (*MAT*a, *ura3 his3 trp1 ade2 trk1Δ::LEU2 trk2Δ::HIS3 tok1Δ::TRP1*), Δ*mep1-3* (*MAT*a *ura3 mep1Δ mep2Δ::LEU2 mep3Δ::KanMX2*) and WT (*ura3*). pYES2 EV, pYES-*OsAKT1* constructed in 2.7.1 and pFL61-*AtAKT1* obtained from the Technische Universität Darmstadt, Germany, were transformed into the yeast using a modified lithium acetate (LiAc) protocol with the use of single-stranded carrier DNA and PEG (Agatep et al., 1998) as follows: yeast was inoculated into 5 ml of liquid YPD (1 L of YPD consisted of 10 g of yeast extract micro granulated (Formedium, Norfolk, UK), 20 g of bacteriological peptone (Oxoid, Hampshire, UK) and 20 g of glucose) with the addition of 100 mM KCl and 50 mM of $(NH_4)_2SO_4$ and shaken at 200 rpm overnight at 30°C. To the inoculated YPD, 50 ml of fresh liquid YPD was added for OD_{600} to reach 0.2-0.3 before being grown further at 30°C and 200 rpm to an OD_{600} of approximately 0.6. To harvest the yeast cells, 50 ml of the inoculated culture was spun at 2,400 rpm for 10 minutes at room temperature and washed with 25 ml of deionised water. The harvested cells were resuspended in 1 ml of 100 mM LiAc and 50 μl of the yeast suspension was transferred to 1.5-ml tubes and pelleted to discard the LiAc solution. To the yeast pellet, 240 μl of 50% PEG 4,000, 36 μl of 1 M LiAc, 10 μl of pre-boiled 10 mg/ml single stranded salmon DNA (Sigma-Aldrich, Suffolk, UK), 5 μl of plasmids and 69 μl of deionised water in this order. The cell solutions were vortexed vigorously and incubated at 30°C for 30 minutes, following by the addition of 36 μl of 100% DMSO which was mixed gently into the cell solutions before the yeast suspensions were heat shocked

at 42°C for 45 minutes. The cell solutions were then centrifuged at 6,000 rpm for 15 seconds to discard the supernatant. To resuspend cell pellets, 600 ml of deionised water was added. Cell suspensions were plated on synthetic defined (SD) plates without uracil (SD-ura) which composed of the following ingredients: in 1 L, it contained 1.9 g of yeast nitrogenous base (YNB) without $(NH_4)_2SO_4$ and without amino acid (Formedium, Norfolk, UK), 1.92 g of yeast synthetic drop-out medium supplements without uracil (Sigma-Aldrich, Suffolk, UK), 15 g of agar and 20 g of glucose. SD-ura plates was also added either with 100 mM KCl (for *WΔ3TOK1Δ* transformants), 100 mM $(NH_4)_2SO_4$ (for Δ*mep1-3* transformants) or none (for WT transformants). The plates were incubated for four days at 30°C.

Yeast colonies from the SD-ura plates were re-streaked on new SD-ura plates consisting of compositions as described above, and incubated at 30°C for two days. pFL61-*AtAKT1* and *pYES2-OsAKT1* transformants were subjected to colony PCR using 3 μl of transformant-inoculated deionised water that was heated to 98°C for 15 minutes as templates while the rest of the recipe except for the primers was as specified in 2.3.1. The forward (5'-TGCGGACAAGTCCAAGATGAG-3') and reverse (5'-GAAGACGCCAAAGACGAAGC-3') primers (10 μM each) were specific to *AtAKT1* and used for pFL61-*AtAKT1* transformants colony PCR. The primers used for *pYES2-OsAKT1* were the *OsAKT1* specific primers described in 2.3.2.

2.7.3 Complementation growth assays

Yeast transformants were inoculated in liquid SD with proline as the only N source: in 1 L, it composed of 1.9 g of YNB without $(NH_4)_2SO_4$ and without amino acid, 100 mM KCl, 20 g of glucose and 0.1% proline. The inoculated liquid SD was shaken at 200 rpm at 30°C overnight. The culture was harvested at 2,400 rpm for 10 minutes at room temperature and washed twice with 5 ml of deionised water before the cell pellets were resuspended in deionised water.

For *WΔ3TOK1Δ* transformants, 5 μl of the cell suspensions with OD_{600} of 0.01 was grown on SD-ura plates with the following compositions: in 1 L, the medium composed of 1.6 g of translucent K^+-free YNB (without $(NH_4)_2SO_4$ and without amino acid) (Formedium, Norfolk, UK), 1.92 g of yeast synthetic drop-out medium supplements without uracil, 15 g of agar, 20 g of galactose and the addition of either 10 mM or 100 mM of K^+ from KCl. K^+ concentration from agar was not taken into account in any growth assays. The growth was characterised after the plates were incubated at 30°C for two days.

For WT and Δ*mep1-3* transformants, 5 μl of the cell suspensions with OD_{600} of 0.01 and 0.001 was grown on SD plates consisting of the following compositions: in 1 L, it contained 1.9 g of YNB without $(NH_4)_2SO_4$ and without amino acid, 15 g of agar, 20 g of galactose and 0.1% proline or NH_4^+ from $(NH_4)_2SO_4$ (0.5 mM, 1 mM, 5 mM, 10 mM, 25 mM, 50 mM, 75 mM and 100 mM). K^+ concentration of the plates was approximately 7.35 mM based on KI and KH_2PO_4 from the YNB. In addition, for Δ*mep1-3* transformants, 5 μl of the cell suspensions with OD_{600} of 0.01 was grown SD plates prepared as follows: in 1 L, it composed of 1.6 g of translucent K^+-free YNB (without $(NH_4)_2SO_4$ and without amino acid), 15 g of agar, 20 g of galactose, K^+ from KCl (0.15 mM, 1 mM, 10 mM and 100 mM) and NH_4^+ from $(NH_4)_2SO_4$ which was used to make up the NH_4^+ concentration of the SD plates to 30 mM, 70 mM and 120 mM. Note that $(NH_4)_3PO_4$ (20 mM) in the K^+-free YNB was taken into account. The plates were incubated at 30°C for three days before the growth was observed.

2.8 Statistical analyses

All statistical analyses were carried out using SPSS Statistics 23 (IBM Corp., New York, USA). Data obtained from each experiment was analysed using either independent-samples t-test (one-tailed), paired t-test (one-tailed) or ANOVA (one-way) followed by Duncan post hoc test. Significance level was at $P = 0.05$. The data in the figures represent the mean while the error bars represent standard error of the mean (SEM). The number of biological replicates (n) is stated in the corresponding figures.

3 Chapter 3: Results

3.1 Genotype verification of *akt1* knockout rice plants

To verify the absence of *OsAKT1* transcript in KO rice plants, root RNAs and shoot genomic DNA of WT and KO genotypes were used as templates in PCR. The PCR results from the amplification of genomic DNA (Figure 8a) did not occur when using the primer pair spanning the T-DNA insertion site 303 bp upstream of *OsAKT1* in KO while the amplification was observed in WT. The result is consistent with the report that KO rice plants were produced via the insertion of T-DNA at that site (Li et al., 2014). Also, RT-PCR result (Figure 8b) illustrates that WT contained abundant *OsAKT1* mRNA in the roots while such activity of OsAKT1 was absent in KO. A faint band, however, was found at the expected band size for *OsAKT1*. The weak band was likely the result of amplification of contaminating genomic DNA in the mRNA sample due to the primers specificity to the exons of *OsAKT1*. Thus, the PCR results verified the genotype of KO rice plants.

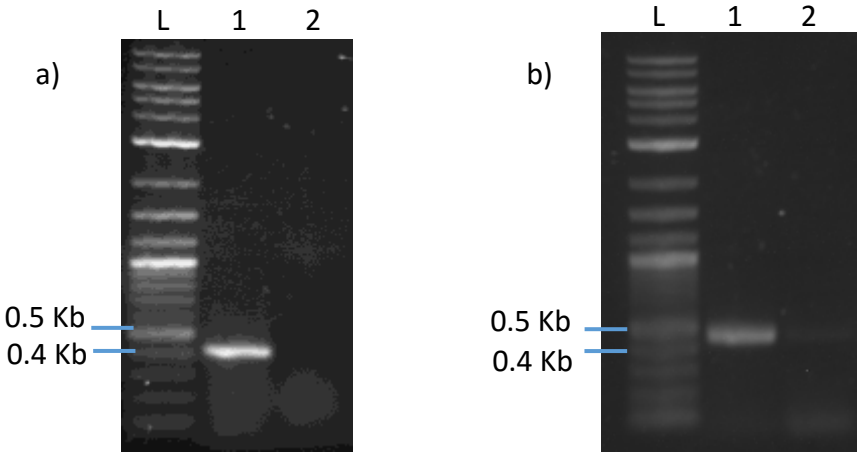

Figure 8: Genotype verification of *akt1* knockout rice plant by a) the amplification of T-DNA insertion site at 303 bp upstream of OsAKT1 using genomic DNA as a template and b) the amplification of *OsAKT1* using root cDNA. L = 2-log DNA ladder; 1 = WT PCR product; 2 = KO PCR product. The expected band size of a) and b) in WT is 414 bp and 464 bp, respectively.

3.2 Nitrogen supply and osmotic stress tolerance in wild-type and *akt1* knockout rice plants

An experiment was conducted to investigate the effects of NH_4^+ via OsAKT1 on osmotic stress tolerance of rice. To do so, WT and KO rice plants were treated in the modified Yoshida medium with either NO_3^--N (2.61 mM NO_3^-, 0.29 mM NH_4^+) or NH_4^+-N (2.61 mM NH_4^+, 0.29 mM NO_3^-) under control or osmotic stress conditions.

3.2.1 Growth

The weekly observations of the physiological effects of the treatment on WT and KO rice plants showed that the plants in PEG-added medium suffered osmotic stress. Leaf chlorosis on rice plants could be observed since the first week of applying PEG while the leaves of the plants grown without PEG were mostly green throughout the course of the experiment (Figure 9). The severity of the leaf damage on PEG-treated plants increased as the treatment progressed to the second and third week.

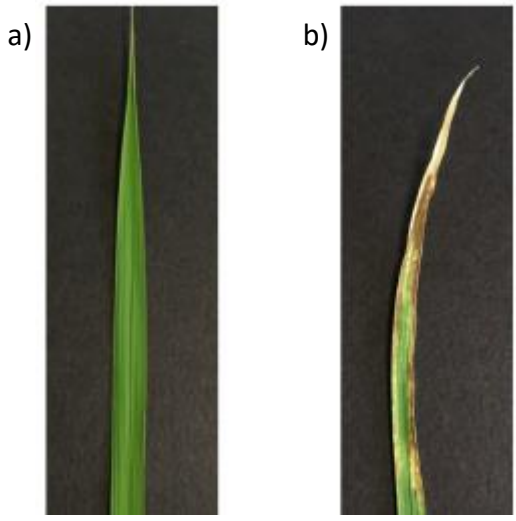

Figure 9: Leaf comparison of a) WT and b) KO rice plants. The leaves were collected at the end of the experiment from 7-week-old rice plants (three weeks in the treatment).

The comparison between RGR of WT and KO rice plants in different treatment combinations (Figure 10) illustrates that, under non-osmotic stress, (a) the growth of WT was significantly higher than that of KO and (b) both WT and KO had a significant greater RGR in NO_3^--N than in NH_4^+-N. In the presence of PEG, RGRs were similar between genotypes and treatment. Both WT and KO rice plants showed growth reduction when osmotically stressed, significant in both N regimes for WT and only in NO_3^--N for KO. The reduction in RGR due to osmotic stress was overall higher in rice treated with NO_3^--N than

NH_4^+-N. Figure 11 illustrates that WT and KO shared similar root:shoot ratios in all treatment combinations. The ratios for both genotypes were significantly higher in NO_3^--N than in NH_4^+-N under both control and osmotic stress conditions. A significant difference in root:shoot ratio between the control and PEG-treated rice plants was only found in NH_4^+-N in which the ratio of both WT and KO was greater under water stress.

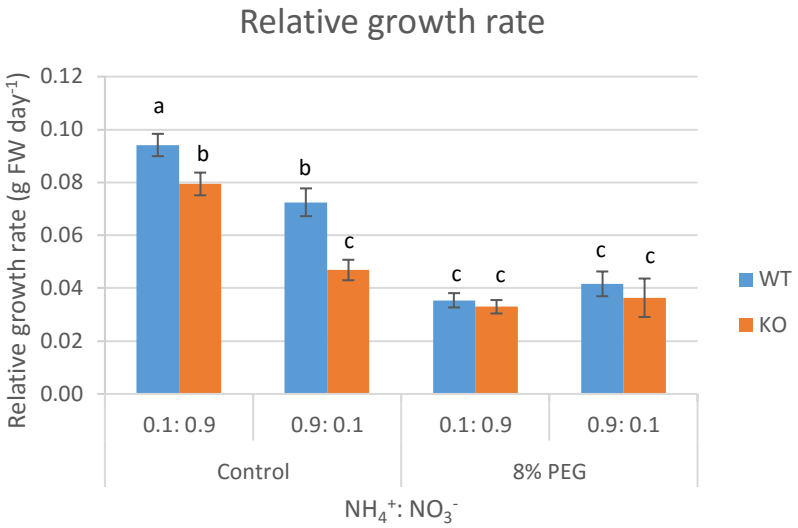

Figure 10: Relative growth rate of WT and KO rice plants in NH_4^+: NO_3^- ratio of either 0.1: 0.9 or 0.9: 0.1 under control and osmotic stress conditions after three weeks. Data are from three independent experiments (n = 8-9), represented in means ± SEMs. Different letters indicate significant differences at $P < 0.05$ as determined by ANOVA (one-way) followed by Duncan post hoc test.

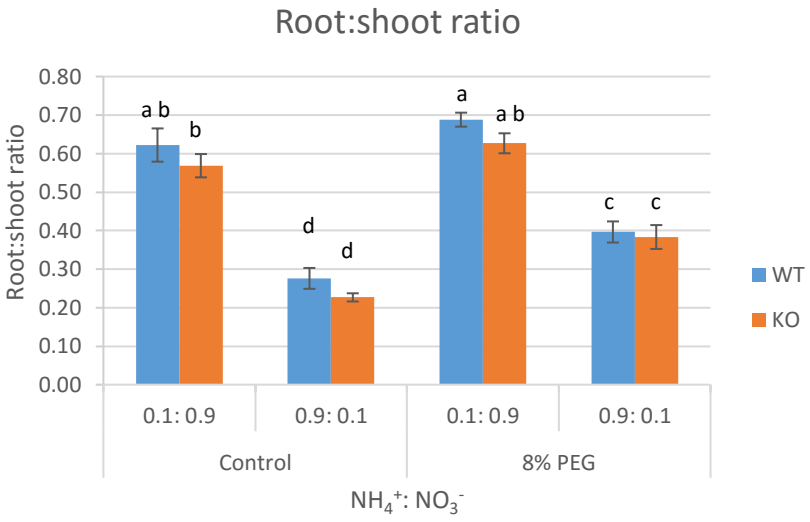

Figure 11: Root:shoot biomass ratio of WT and KO rice plants in NH_4^+: NO_3^- ratio of either 0.1: 0.9 or 0.9: 0.1 under control and osmotic stress conditions after three weeks. Data are from two independent experiments (n = 5-6), represented in means ± SEMs. Different letters indicate significant differences at $P < 0.05$ as determined by ANOVA (one-way) followed by Duncan post hoc test.

3.2.2 Shoot potassium and sodium concentrations

Figure 12a shows that K^+ concentration in the shoots was significantly higher in KO than WT under control conditions in both NH_4^+-N and NO_3^--N and under osmotic stress in NH_4^+-N. No significant differences were seen between shoot K^+ content of the control or PEG-treated rice plants, irrespective of the genotype and N supply. In general, N regime did not affect shoot K^+ concentration, though samples collected from KO grown in NH_4^+-N under control conditions obtained higher shoot K^+ concentration than rice grown in NO_3^--N. Shoot Na^+ concentration (Figure 12b) of KO was significantly higher in the control condition than the osmotic stress condition in both NH_4^+-N and NO_3^--N while WT was found with such pattern only in NH_4^+-N. No significant differences in shoot Na^+ between WT and KO and rice of the same genotype between NH_4^+-N and NO_3^--N were detected. Compared to shoot K^+ concentration, shoot Na^+ concentration was much lower showing that the plants did not suffer from salt stress.

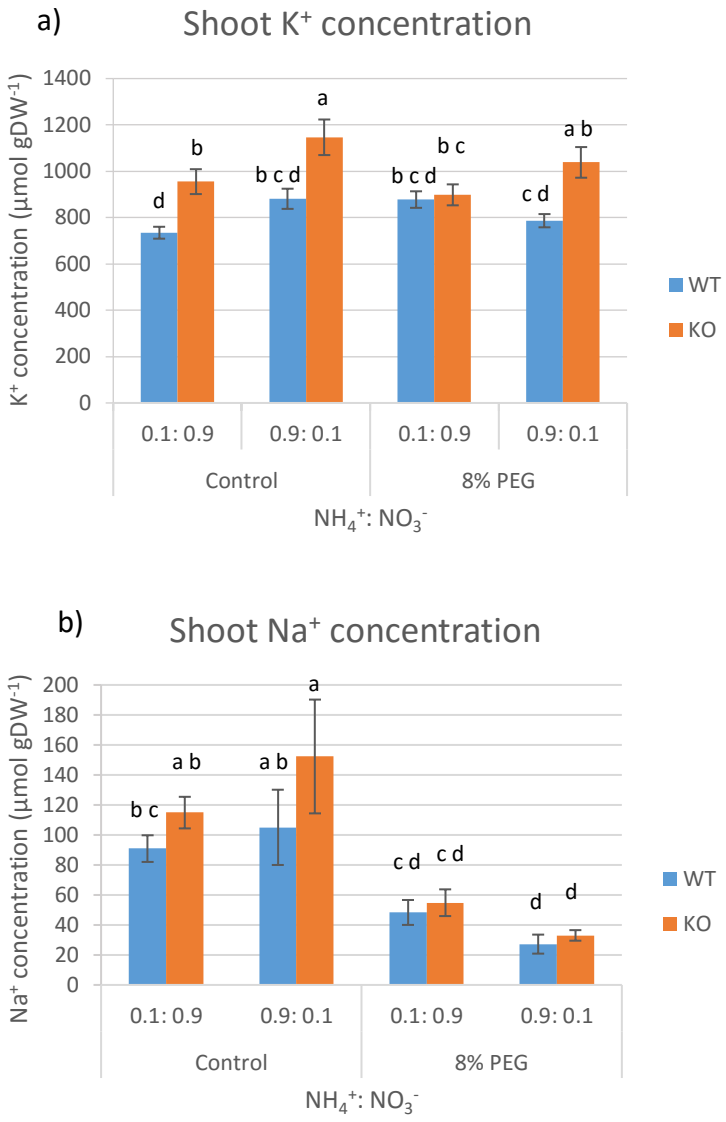

Figure 12: Shoot a) K^+ and b) Na^+ concentrations of WT and KO rice plants in NH_4^+: NO_3^- ratio of either 0.1: 0.9 or 0.9: 0.1 under control and osmotic stress conditions after three weeks. Data are from three independent experiments (n = 7-9), represented in means ± SEMs. Different letters indicate significant differences at $P < 0.05$ as determined by ANOVA (one-way) followed by Duncan post hoc test.

3.2.3 Shoot nitrogen and carbon contents

The comparison of shoot N percentage (%N) (Figure 13a) between the two genotypes revealed that WT had similar shoot %N to KO in NO_3^--N. Interestingly, shoot %N of WT tended to be higher than KO in NH_4^+-N irrespective of osmotic stress conditions. There was a significant decrease in shoot %N of both WT and KO in NO_3^--N when osmotically-stressed while no change in shoot %N was found in NH_4^+-N. Figure 13b illustrates that shoot C percentage (%C) showed a similar trend to that of shoot %N.

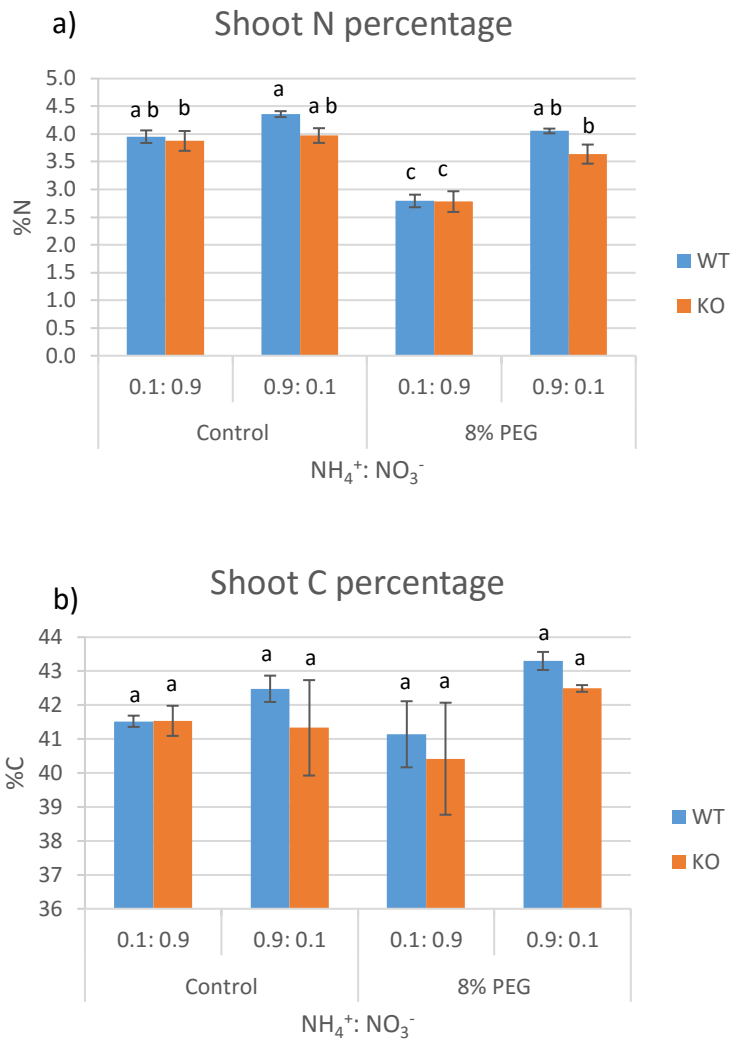

Figure 13: The percentage of a) N and b) C in the shoots of WT and KO rice plants in NH_4^+: NO_3^- ratio of either 0.1: 0.9 or 0.9: 0.1 under control and osmotic stress after three weeks. Data are from pooled samples from each of the three independent experiments (n = 3), represented in means ± SEMs. Different letters indicate significant differences at $P < 0.05$ as determined by ANOVA (one-way) followed by Duncan post hoc test.

3.3 The effect of elevated ammonium concentration on wild-type and *akt1* knockout rice

To observe the effects of high NH_4^+ concentration on growth via OsAKT1, an experiment was conducted to observe the effects of 8 mM NH_4^+ on WT and KO.

3.3.1 Growth

WT and KO rice plants both displayed symptoms which indicated that they suffered from high NH_4^+ content such as leaf chlorosis and the appearance of brown patches on the stem. The severity of the damage increased from week-1 to week-3 of the experiment. Both genotypes did not differ regarding the severity of the symptoms at the end of the experiment. The extent of the symptoms was not quantified.

The calculation of RGR after three weeks (Figure 14) showed that WT growth was significantly greater than that of KO at high NH_4^+ concentrations. The root:shoot ratio of WT, however, did not indicate a significant difference in comparison to KO (Figure 15).

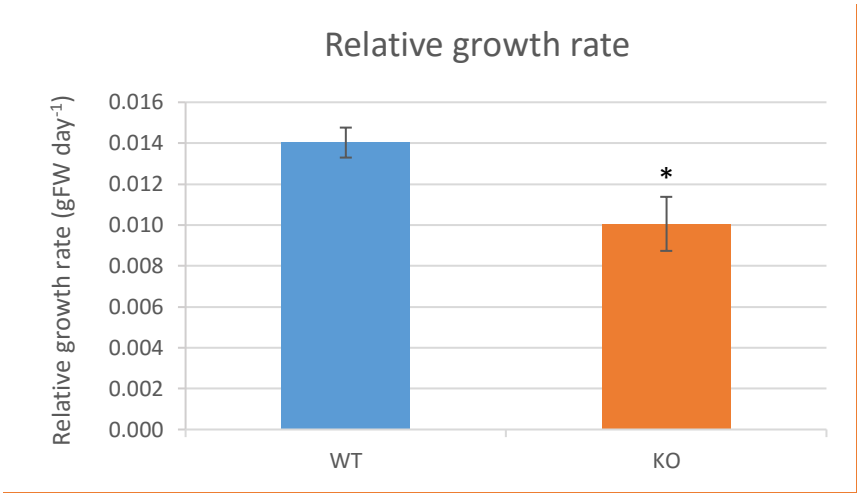

Figure 14: Relative growth rate of WT and KO rice plants in 8 mM NH_4^+ treatment after three weeks. Data are from three independent experiments, represented in means ± SEMs (n = 4-6). * indicates significant differences between KO and WT at $P < 0.05$ by independent-samples t-test (one-tailed).

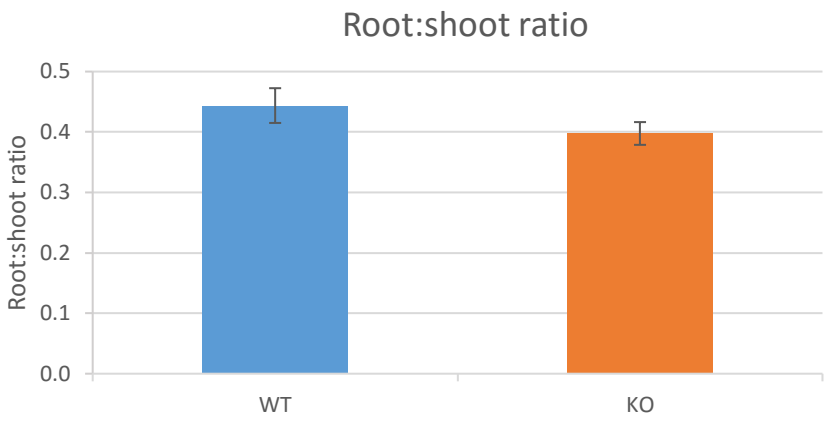

Figure 15: Root:shoot biomass ratio of WT and KO in 8 mM NH_4^+ treatment after three weeks. Data are from three independent experiments, represented in means ± SEMs (n = 4-6).

3.3.2 Potassium and sodium concentrations in roots and shoot

Figure 16 illustrates that there was no significant difference between K^+ concentration of WT and KO in the root tissues when treated in high NH_4^+ environment. On the other hand, K^+ concentration of KO shoot tissues was significantly higher than that of WT (Figure 10b). No significant differences were found between the two genotypes for Na^+ concentration in the roots and shoots (data not shown).

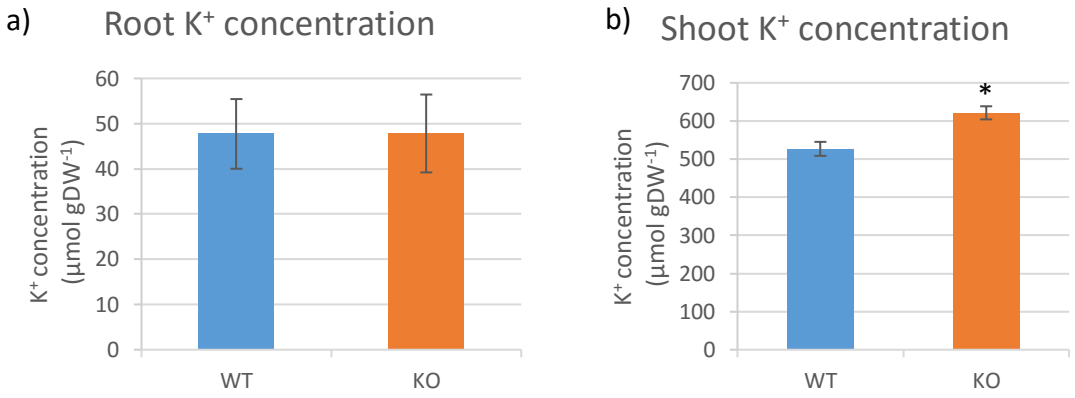

Figure 16: K^+ concentration in the a) root tissues and b) shoot tissues of WT and KO in 8 mM NH_4^+ after three weeks. Data are from three independent experiments, represented in means ± SEMs (n = 4-6). * indicates significant differences between KO and WT at $P < 0.05$ by independent-samples t-test (one-tailed).

3.4 Ammonium depletion experiments

NH_4^+ depletion assays were conducted to test for potential NH_4^+ uptake via OsAKT1 at 0.2 and 2 mM NH_4^+. By observing the decrease in NH_4^+ concentration in the medium at different time intervals the NH_4^+ accumulation of each genotype, based on their FW, was

determined. I tested two different methods of measuring the remaining NH_4^+ in the medium by using an ammonium-specific electrode and a colorimetric approach. Unlike the electrode, the colorimetric method could detect a wide range of NH_4^+ concentrations without the disturbance of other cations. Thus, the colorimetric approach was selected for this experiment.

At external NH_4^+ concentration of 0.2 mM (Figure 17a), there was a significant difference in NH_4^+ accumulation between WT and KO one hour after the start of the assay. Although insignificant, there was a trend showing higher accumulation of WT than KO at 2-hour and 3-hour intervals. At external NH_4^+ concentration of 2 mM (Figure 17b), the difference in NH_4^+ accumulation between WT and KO was not significant at all time points. However, a trend showing that WT accumulated more NH_4^+ than KO can be observed but not at 3-hour time point where the average NH_4^+ accumulated in KO was approximately the same to that of WT.

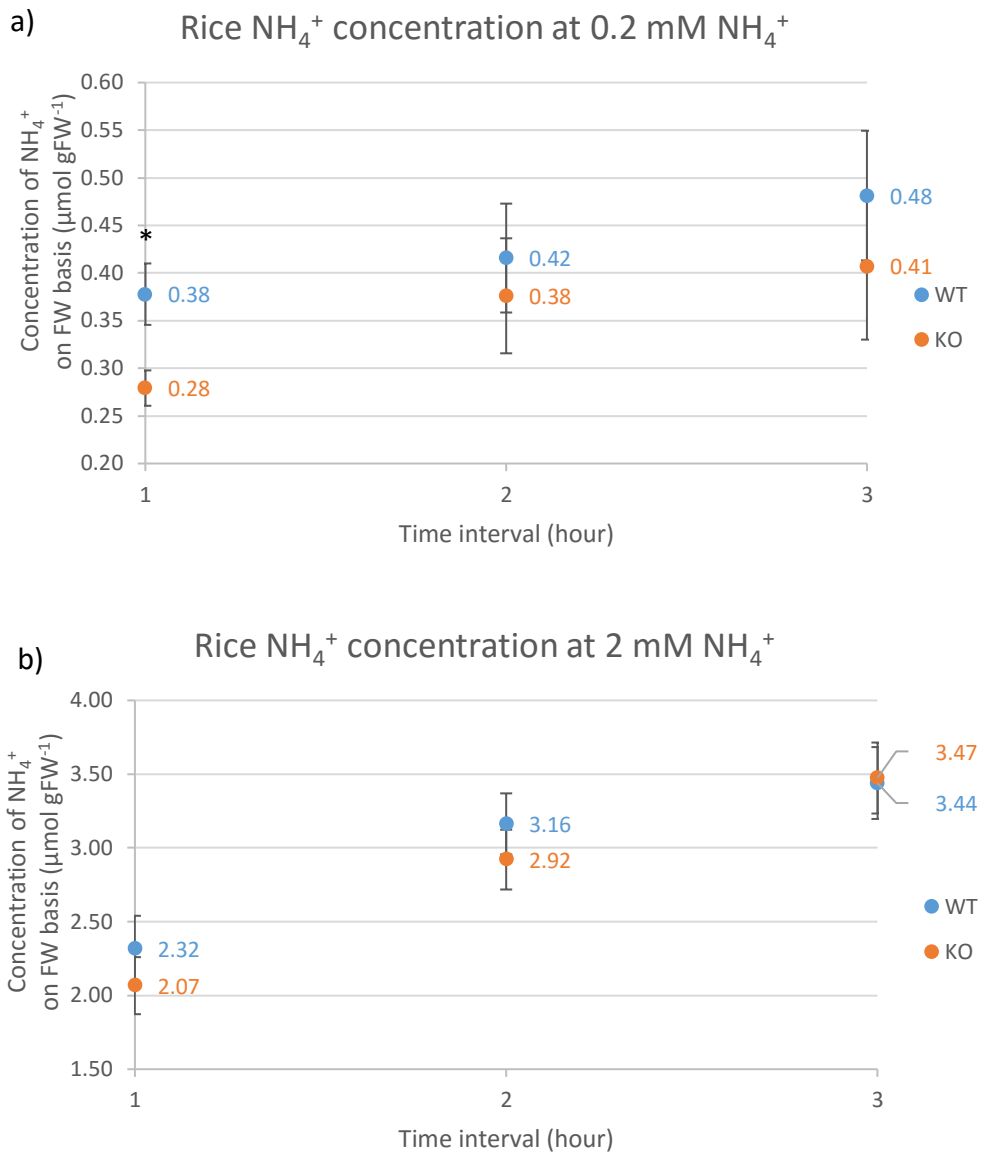

Figure 17: NH_4^+ concentration on FW basis of WT and KO rice plants at a) 0.2 mM and b) 2 mM external NH_4^+ after 1, 2 and 3 hours. Data points are three independent experiments, represented in means ± SEMs (n = 8-9). * indicates significant differences between KO and WT at $P < 0.05$ by paired sample t-test (one-tailed).

3.5 Studies of *OsAKT1* in yeast

The investigation of *OsAKT1* in yeast lacking NH_4^+ uptake systems was conducted to further characterise the potential role of *OsAKT1* in NH_4^+ transportation at the molecular level. Growth complementation assays were carried out at various NH_4^+ and K^+ concentrations. Besides pYES2-*OsAKT1*, a construct containing *AtAKT1* referred to as pFL61-*AtAKT1* was also included in the experiment as it was previously studied in yeast (ten Hoopen et al., 2010) and it represents an AKT1 isoform from an NH_4^+-sensitive species.

3.5.1 Constructing *OsAKT1* plasmid

To make a construct containing full-length *OsAKT1* cDNA, cDNA of *OsAKT1* was amplified using RT-PCR from rice root mRNA (Figure 18). The PCR product size was approximately 3.0 Kb, consistent with the size of *OsAKT1* which is 2,808 bp. *OsAKT1* cDNA along with pYES2 EV were then double-digested with *Eco*RI and *Not*I (Figure 19) to produce sticky ends for ligation of the pYES2-*OsAKT1* construct. After the ligation and *E. coli* transformation, colonies were selected for colony PCR to identify possible positive transformants (Figure 20). Colonies detected with amplified *OsAKT1* were identified from the PCR products with the amplicon size between 400 bp and 500 bp, consistent with the expected product size of *OsAKT1* which was 464 bp. Among the colonies found with *OsAKT1* amplification, five were selected for miniprep preparations and double-digested with *Eco*RI and *Not*I to confirm the successful ligation of pYES2-*OsAKT1* (Figure 21). Out of the five minipreps, two had two bands at around 6 Kb and 3 Kb which were consistent with the size of the linearised pYES2 EV and *OsAKT1* cDNA insert, respectively. Sanger sequencing confirmed that both minipreps were a 100% match with *OsAKT1* cDNA sequence (coding sequence of LOC_Os01g45990.1) on the Rice Genome Annotation Project database.

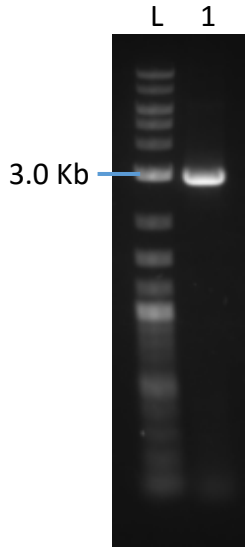

Figure 18: Amplified full-length *OsAKT1* cDNA using rice root cDNA as a template. L = 2-log DNA ladder; 1 = amplified *OsAKT1.* The expected size of full-length *OsAKT1* cDNA was 2,808 bp.

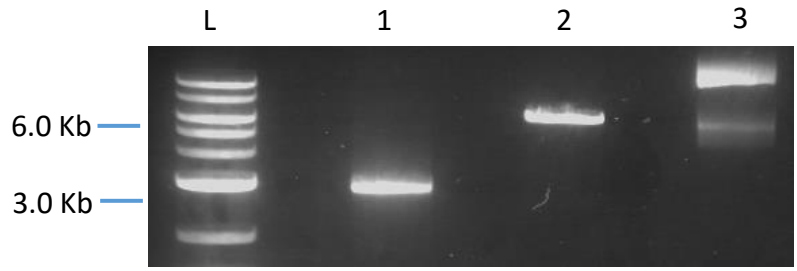

Figure 19: Double-digested *OsAKT1* and pYES2 EV using *Eco*RI and *Not*I. L = 2-log DNA ladder; 1 = digested *OsAKT1I*; 2 = digested pYES2 EV; 3 = undigested pYES2 EV. The expected size of double-digested *OsAKT1* and pYES2 EV was approximately 2.8 Kb and 5.9 Kb, respectively.

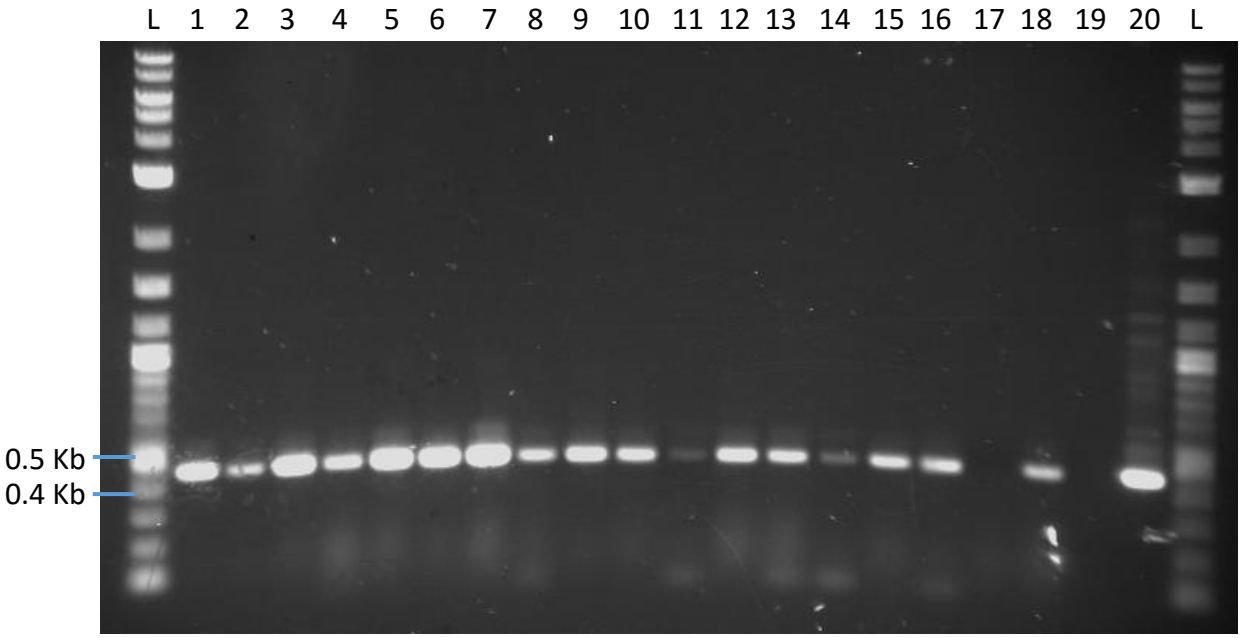

Figure 20: Colony PCR using *E. coli* transformants as templates. L = 2-log DNA ladder; 1-18 = *E. coli* transformants; 19 = pYES2 EV; 20 = *OsAKT1*. The expected size of amplified *OsAKT1* was 464 bp.

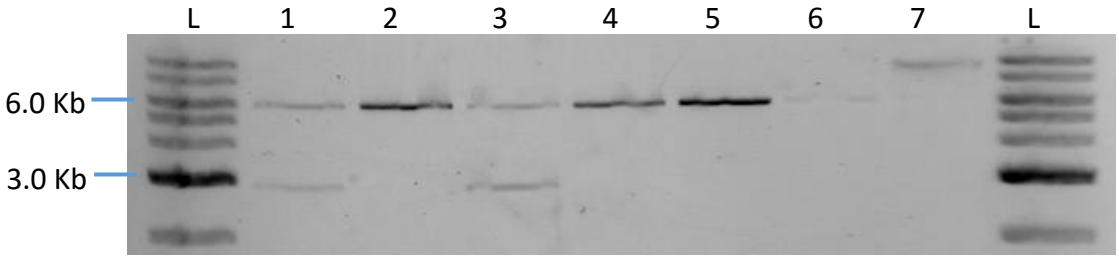

Figure 21: Double-digestion of minipreps using *Eco*RI and *Not*I. L = 2-log DNA ladder; 1-5 = digested minipreps; 6 = digested pYES2 EV; 7 = undigested pYES2 EV. The expected product size from the double-digestion of pYES2-*OsAKT1* was approximately 2.8 Kb and 5.9 Kb

3.5.2 Complementation growth assays

To carry out a study of OsAKT1 in yeast, pYES2-*OsAKT1* was transformed into two different strains of yeast; *WΔ3TOK1Δ* (deficient in high affinity K^+ uptake) and Δ*mep1-3* (deficient in high affinity NH_4^+ uptake). In addition, *AtAKT1* cDNA insert in pFL61 vector (pFL61-*AtAKT1*) obtained from Technische Universität Darmstadt was also transformed into the two yeast strains to compare the activity of *AtAKT1* and *OsAKT1* on NH_4^+ transport.

Colony PCR after yeast transformation (data not shown) confirmed that the transformations were successful.

3.5.2.1 Verification of functional AtAKT1 and OsAKT1

To verify that *AtAKT1* and *OsAKT1* in the constructs were functional, *WΔ3TOK1Δ* was transformed with pFL61-*AtAKT1* and pYES2-*OsAKT1* along with pYES2 EV as a control.

Figure 22 illustrates that pFL61-*AtAKT1* and pYES2-*OsAKT1* can complement growth of *WΔ3TOK1Δ* yeast on low K^+ concentration (10 mM K^+) as significantly more growth of those transformants can be observed compared to pYES2 EV transformed yeast. Note that no significant difference regarding growth was found when compared the transformants at high K^+ concentration (100 mM K^+). Thus, *AtAKT1* and *OsAKT1* carried on the plasmids were able to produce functional K^+ channels.

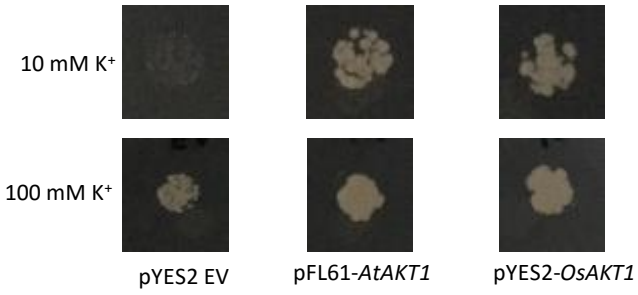

Figure 22: Growth complementation of pFL61-*AtAKT1* and pYES2-*OsAKT1* in *WΔ3TOK1Δ* at 10 mM K^+ and 100 mM K^+ after two days on SD-ura plates with galactose as the source of C and 0.1% proline as a N source. OD_{600} = 0.01.

3.5.2.2 Growth complementation of Δmep1-3 at different ammonium concentrations

Δ*mep1-3* was transformed with *OsAKT1, AtAKT1* and pYES2 EV for growth assays. WT transformed with pYES2 EV was used to compare the growth between WT and the mutant.

At two different starting dilutions, WT yeast transformed with pYES2 EV could grow on 0.1% proline and all concentrations of NH_4^+ ranging from 0.5 mM to 100 mM (Figure 23a). However, little growth of WT was observed at 0.5-1 mM NH_4^+ while moderate and high growth was observed at 5-10 mM and 25-100 mM NH_4^+, respectively. When comparing the growth of WT and Δ*mep1-3* at OD_{600} of 0.01 (Figure 23b), it was clearly shown that transformed Δ*mep1-3* growth was much smaller than WT across NH_4^+ concentrations. When transformed with pYES2 EV, Δ*mep1-3* could not grow at NH_4^+ concentration lower than 10 mM, proving the mutant had the growth suppression phenotype at low NH_4^+ concentrations. Note that very small, and very few colonies could

be found at 10 mM NH_4^+. Growth of Δ*mep1-3* improved as the concentration of NH_4^+ increased, however the improvement was not dramatic. When Δ*mep1-3* was transformed with pFL61-*AtAKT1*, a small growth complementation of Δ*mep1-3* was shown at NH_4^+ concentrations of 25 mM, 50 mM, 75 mM and 100 mM at OD_{600} of 0.01. Similar to pFL61-*AtAKT1*, pYES2-*OsAKT1* complemented the growth of Δ*mep1-3* to a small degree at NH_4^+ concentrations of 50 mM, 75 mM and 100 mM. However, unlike pFL61-*AtAKT1,* pYES2-*OsAKT1* did not complement the growth of Δ*mep1-3* at NH_4^+ concentrations of 25 mM. The comparison between pFL61-*AtAKT1* and pYES2-*OsAKT1* transformants showed that OsAKT1 was slightly more effective in restoring growth compared to AtAKT1, though it was unlikely to represent any significant differences in NH_4^+ transport between the two channels. Note that no significant differences could be detected among Δ*mep1-3* transformants at OD_{600} of 0.001.

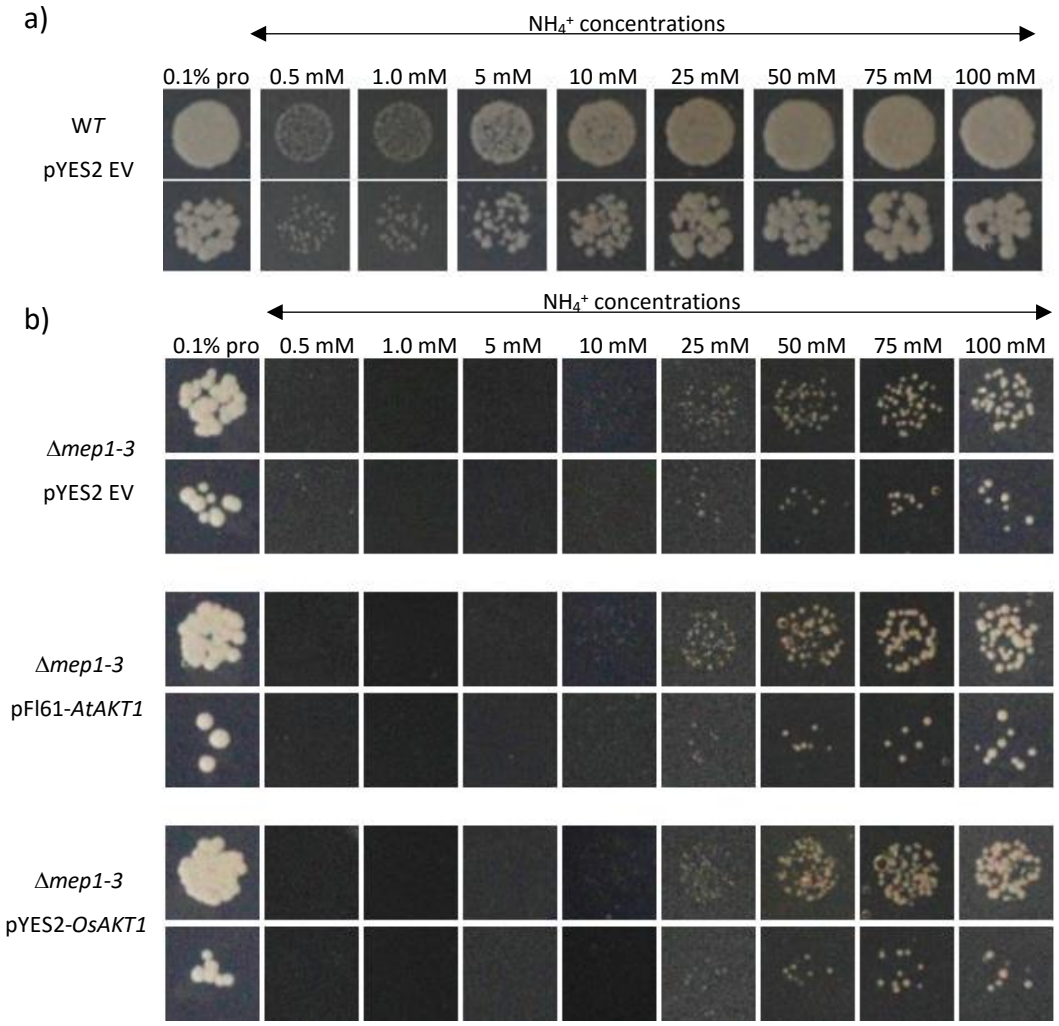

Figure 23: Growth complementation assay of a) WT transformed with pYES2 EV and b) Δ*mep1-3* transformed with pYES2 EV as a control, pFL61-*AtAKT1* and pYES2-*OsAKT1* in different NH_4^+ concentrations (K^+ concentration is approximately 7.35 mM) on SD-ura plates with galactose as C source after three days. Top row = OD_{600} of 0.01, bottom row = OD_{600} of 0.001.

3.5.2.3 Growth complementation of Δmep1-3 at different ammonium and potassium concentrations

To further characterise OsAKT1, interactions between NH_4^+ and K^+ transportation via *OsAKT1* were studied.

Figure 24 demonstrates that Δ*mep1-3* transformants grew at all combinations of NH_4^+ and K^+. pYES2 EV transformants show characteristics of Δ*mep1-3* under different K^+ and NH_4^+ concentrations: growth at 0.15 mM K^+ was slightly better than at 1 mM K^+ while growth at 10 mM and 100 mM K^+ was much reduced compared to that at 0.15 mM. Also, Δ*mep1-3 grew* better at higher NH_4^+ when K^+ was no more than 10 mM in the medium. When K^+ concentration reached 100 mM, the growth of Δ*mep1-3* was unaffected by NH_4^+. The growth of pFL61-*AtAKT1* and pYES2-*OsAKT1* transformants followed the same pattern as mentioned for pYES2 EV yeast. The comparison between the growth of the transformants shows that the activity of AtAKT1 and OsAKT1 could complement Δ*mep1-3* growth to a small degree at all NH_4^+ concentrations when K^+ concentration was between 0.15 mM to 10 mM. No distinct differences in the complementation could be observed between pFL61-*AtAKT1* and pYES2-*OsAKT1* transformants. However, it is worth noting that in 0.15 mM K^+ medium with 70 and 120 mM NH_4^+, the transformants with pYES2-*OsAKT1* grew slightly better than pFL61-*AtAKT1* transformants. Similar observation was also found in the medium with 70 mM NH_4^+ and 1 mM K^+.

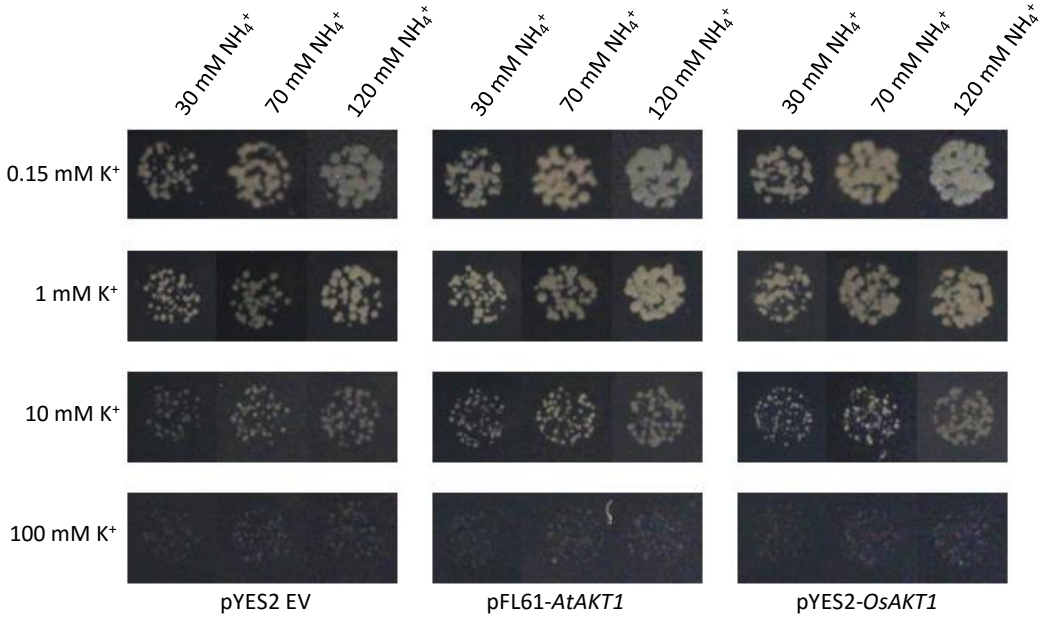

Figure 24: Growth complementation assay of Δ*mep1-3* transformed with pYES2 EV as a control, pFL61-*AtAKT1* and pYES2-*OsAKT1* in different NH_4^+ and K^+ conditions on SD-ura plates with galactose as C source at OD_{600} of 0.01 after three days. pYES2 EV transformants act as a control.

4 Chapter 4: Discussion

The effects of N on drought stress responses are species-dependent (Dziedek et al., 2016, Fusaro et al., 2016) but have been reported to be positive in various species. For example, in winter wheat, plant growth, yield and water use generally increase with the addition of N fertiliser application as NH_4NO_3 (Nielsen and Halvorson, 1991). Adding N fertiliser during drought stress also improves the net photosynthetic rate and water use efficiency in *Abies fabri* (Yang et al., 2012b). Different N forms also have different impacts under drought stress conditions in different species. For instance, NO_3^- improves maize drought tolerance via the enhancement of dry mass and net photosynthesis rate (Zhang et al., 2011, Zhang et al., 2012). In contrast, NO_3^- fails to maintain photosynthesis rate in rice as the rate decreases in NO_3^- treatment under drought stress condition (Guo et al., 2008).

Unlike Arabidopsis and barley which are sensitive to NH_4^+ and suffer even from low NH_4^+ concentrations, rice may have a preference in taking up N in the form of NH_4^+ due to its ability to maintain energy expenditure and growth despite an increased NH_4^+ concentration in the environment. Britto et al. (2001) demonstrated that there was no additional energy cost in transporting NH_4^+ in rice when there was an increase in NH_4^+ provision, possibly due to the activity of passive channels. This assumption is consistent with NH_4^+ being the predominant form of N found in paddy rice fields which are anaerobic (Iqbal, 2011). Thus, it is possible that rice may be able to benefit more from NH_4^+ nutrition than NH_4^+-sensitive species. In addition, NH_4^+ nutrition has been proven to strengthen rice seedling tolerance to drought in various ways including reducing negative impacts of the stress, in comparison to NO_3^- nutrition, on water uptake by inducing the expression and activity a subfamily of AQP called plasma membrane intrinsic proteins (PIPs) and photosynthetic rate (Gao et al., 2010, Ding et al., 2015). With OsAKT1 being permeable to NH_4^+ (Fuchs et al., 2005) and reported to take part in rice drought tolerance (Ahmad et al., 2016), there is a possibility that OsAKT1 may fulfil a role in NH_4^+ uptake which is associated with plant tolerance against water stress.

4.1 Rice prefers nitrate to ammonium nutrition in the absence of osmotic stress

The observation on the growth rate of WT rice under non-osmotic stress revealed that rice preferred NO_3^- over NH_4^+ (Figure 10). This contrasts with the common notion that rice prefers NH_4^+ as a N source (Britto et al., 2001). Kronzucker et al. (1999) showed that NO_3^- uptake was inhibited by NH_4^+ and NH_4^+ uptake was stimulated, as was NH_4^+ metabolism, by NO_3^-. This could possibly lead to rice seedlings showing a faster absorption of NH_4^+ when both NO_3^- and NH_4^+ are present (Kronzucker et al., 1999, Sasakawa and Yamamoto, 1978). However, when providing each N form alone, NO_3^- was found to have a greater influx and cytosolic accumulation in rice than NH_4^+ (Kronzucker et al., 1999). Thus, the higher growth rate of rice in NO_3^--N under control conditions may be due to the limited inhibition by NH_4^+ on NO_3^- metabolism. On the other hand, the lower growth rate of rice in NH_4^+-N in our study may due to an insufficient amount of NO_3^- in the NH_4^+-N medium. As greater rice growth was achieved in a mixture of both N forms (Guo et al., 2008, Ta and Ohira, 1981), it is possible that NO_3^- concentration in NH_4^+-N does not reach the minimal requirement of rice to support optimal growth. Such insufficiency in NO_3^- supply was previously postulated as the cause for leaf growth inhibition in tobacco (Walch-Liu et al., 2000). It is also possible that N preference may depend on other factors and these may vary between cultivars. For example, reports have shown that there were no significant effects of N form on dry mass of Shanyou 63 rice cultivar while NH_4^+ nutrition induced lower production of dry mass than NO_3^- in the Yangdao 6 cultivar (Li et al., 2009, Guo et al., 2008).

4.2 The effects of osmotic stress on rice growth and root:shoot ratio depend on nitrogen form

A number of evidence showed that NH_4^+ nutrition was more beneficial to rice seedlings than NO_3^- during water stress. For example, rice water uptake in NH_4^+ nutrition was higher than that of NO_3^- under water stress because NO_3^- nutrition induced a decrease in water potential in leaves which resulted in lower g_m and g_s and NO_3^- also increased root aerenchyma formation which was the cause of suppressed root hydraulic conductivity by obstructing water radial transport (Li et al., 2012, Yang et al., 2012a). Also, AQP activity and

total FW in rice during osmotic stress were lower in NO_3^- compared to those in NH_4^+ medium (Gao et al., 2010). Therefore, one might expect NH_4^+ nutrition to be more beneficial to rice growth than NO_3^- under osmotic stress. Consistent with the expected results, my data with Dongjin rice indicated that a reduction in RGR due to osmotic stress was greater in NO_3^--N than NH_4^+-N for WT (Figure 10), suggesting that NH_4^+ was more capable of mitigating water stress than NO_3^-. Similar findings to my results were also reported on the Yangdao 6 rice cultivar under osmotic stress (Li et al., 2009, Guo et al., 2008). It is worth taking note that NH_4^+ nutrition was also found maintaining or even increasing the biomass of rice plants under water stress condition (Guo et al., 2007, Gao et al., 2010). One of the possible factors contributing to the difference in NH_4^+ effects is likely the length of the experiment. Nevertheless, it is consistent in every report, including this present study, that NO_3^- is more deleterious than NH_4^+ nutrition in the presence of water stress. Based on the previous studies above, NH_4^+ nutrition may allow higher water uptake than NO_3^- nutrition in rice during water stress period and so water availability and growth rate in NH_4^+-treated plants may be subsequently higher. It is also possible that the lower g_m and g_s in NO_3^--treated rice during the stress period decreases the cellular concentration of CO_2 which enhances photorespiration and reduces photosynthesis comparing to rice in NH_4^+ treatment, resulting in higher growth rate in NH_4^+-treated plants.

Root:shoot data in this study suggested that the ratio was dependent on the N source and also on water stress conditions in NH_4^+-N. I found that NO_3^- nutrition was able to induce a high root:shoot ratio relative to the ratio of NH_4^+-treated rice under both control and osmotic conditions (Figure 11). As there was no difference in the ratio between the control and PEG-added treatments of NO_3^--N, the high root:shoot in NO_3^--N was not due to water stress conditions but possibly the effects of NO_3^- on rice root morphology. It was previously reported in other plants that NO_3^- nutrition can induce the change in root structure. For example, NO_3^- was shown to affect root architecture by enhancing lateral root development in *Arabidopsis* and maize (Zhang and Forde, 1998, Granato and Raper, 1989) and as well as the primary root growth in *Arabidopsis* (Walch-Liu and Forde, 2008). Moreover, It was found that, unlike NO_3^- nutrition, NH_4^+ nutrition promoted an increase in root:shoot ratio under water stress (Figure 11). The increase in root:shoot was possibly to enhance water uptake under water stress (Fang and Xiong, 2015), suggesting that NH_4^+-N nutrition may allow rice to adjust water uptake system to alleviate water stress. This is supported by the water absorption data from Gao et al. (2010): they found that more water

absoprtion based on root FW was conducted in rice treated with NH_4^+ nutrition than NO_3^- while the trend of root:shoot from their study was similar to my findings. Note that the ratio values in this present study were smaller and the increase in the ratio due to water stress in NH_4^+ treatment was not as dramatic as the data reported by Gao et al. (2010), which may be due to the difference in growth conditions and treatments.

4.3 Ammonium nutrition maintains nitrogen content in rice shoots during osmotic stress

The provision of NH_4^+ during drought stress can increase chlorophyll content while NO_3^- nutrition resulted in the chlorophyll content decreasing in some species (Mihailovic et al., 1992, Mihailovic et al., 1997). This suggests that drought may have different effects on protein abundance depending on N-regime. This present study illustrated that rice treated with NO_3^--N had a decrease in shoot %N during osmotic stress (Figure 13a). The results of this present study imply that osmotic stress of -0.21 MPa affects plant accessibility to N when grown on NO_3^- nutrition, consistent with findings of a few previous studies. For example, NO_3^- uptake was decreased in the presence of PEG compared to control condition, leading to the smaller amount of translocated NO_3^- in the leaves in ryegrass (Ourry et al., 1992). NO_3^- reductase activity in leaves also declined during water stress in maize and tomato (Foyer et al., 1998, Brewitz et al., 1996) and so the capacity of NO_3^- assimilation is downgraded in the period of water stress. On the other hand, the results of my study also showed that osmotic stress did not have negative effects on the shoot N abundance when grown in NH_4^+-N (Figure 13a). The maintenance of shoot N in NH_4^+-treated rice suggests that osmotic stress at 8% PEG does not negatively affect rice accessibility to NH_4^+. There are two possible effects of NH_4^+ nutrition on shoot N under osmotic stress that could explain the observation in this study. First, NH_4^+ enables the maintenance of enzymes, photosynthetic apparatus and other functional proteins as implied by a finding which showed a maintenance of chlorophyll content in NH_4^+ treatment, but a reduction in NO_3^- treatment, under water stress (Guo et al., 2008). Based on this assumption, photosynthetic rate and subsequent sugar production may be higher in NH_4^+-treated rice than NO_3^--treated rice when being osmotically stressed which may be implied from a tendency of having higher shoot %C in NH_4^+-N under osmotic stress (Figure 13b). Second, NH_4^+ enables the

accumulation of N-containing osmoprotectants like compatible solutes for osmoprotective purposes which compensates a decrease in leaf proteins and enzymes due to water stress. The increase in the soluble protein content in rice as reported by Guo et al. (2007) due to the increase in NH_4^+ nutrition under water stress compared to control condition may imply the increase in the accumulation of the compatible solutes by NH_4^+. The absolute amount of proline was previously found, under salt stress, to be increased in NH_4^+ nutrition but not in NO_3^- nutrition in *P. simonii* (Meng et al., 2016). Though the experiment was done using salt stress, the similarity between water and salt stress may lead to the similar proline response in NH_4^+-treated plants as well under water stress condition. Further experiments should be conducted due to limited resources.

4.4 OsAKT1 has a role in ammonium uptake

Under normal circumstances, plants keep concentrations of mineral nutrients near an optimum. Surprisingly, an increase in NH_4^+ concentration does not lead to the downregulation of its LATs (Britto and Kronzucker, 2002): in fact, the influx was shown to increase LATs in such conditions, implying that certain LAT transporters were being constitutively expressed. Interestingly, they proposed K^+ channels as one of the candidates for NH_4^+ LAT transporters, possibly due to similar physicochemical properties between K^+ and NH_4^+. As mentioned, OsAKT1 is the K^+ channel which has the permeability to NH_4^+ (Fuchs et al., 2005), allowing the possibility for AKT1 mediated NH_4^+ uptake.

NH_4^+ depletion experiments showed a trend where WT accumulated more NH_4^+ than KO at 2 mM NH_4^+ external concentration (Figure 17b). The trend slowed down and ceased at 3-hour time point, possibly due to higher cytosolic NH_4^+ concentration which lowers the driving force for NH_4^+ uptake across the membrane. In addition, experiments with yeast showed that OsAKT1 could slightly restore the growth of *Δmep1-3* at certain NH_4^+ and K^+ concentrations (Figure 23b, Figure 24). Judging by the NH_4^+ concentrations used in these experiments, OsAKT1 may mediate NH_4^+ absorption in the range of NH_4^+ LATs. Note that the degree of growth complementation in yeast was smaller when K^+ concentration increased in the medium pointing to competition between K^+ and NH_4^+ for entry via AKT1 as reported by ten Hoopen et al. (2010). Nevertheless, the assumption that OsAKT1 takes up NH_4^+ at low NH_4^+concentration is correlated to higher shoot %N in WT compared to that of the KO in NH_4^+-N grown plants (Figure 13a). We also explored the role

of OsAKT1 in NH_4^+ uptake in HAT range: the depletion experiment showed uptake of NH_4^+ at 0.2 mM which was significantly higher in the WT than KO one hour after the start of the assay (Figure 17a). The data suggest that the channel takes part in the initial NH_4^+ uptake at low NH_4^+ concentration and possibly persists its NH_4^+ uptake function after the early NH_4^+ uptake phase. Altogether, these data suggest that OsAKT1 is involved in NH_4^+ uptake in the HAT and LAT range. It is noticeable that the amount of NH_4^+ taken up by the channel was small in both LAT and HAT ranges, which was consistent with a slightly higher shoot %N of WT than KO (Figure 13a) and the small growth complementation in *Δmep1-3* by OsAKT1 (Figure 23b, Figure 24).

In addition, it is worth taking note that, in comparison to AtAKT1, OsAKT1 improved the growth of *Δmep1-3* slightly better (Figure 23b, Figure 24). The isoforms from different species may have varying properties, possibly due to the different NH_4^+ tolerance traits from the parent species. However, the difference in complementation efficacy is too small to make any firm conclusions.

4.5 Ammonium toxicity does not impact rice growth via OsAKT1

Several species of plants have been described as being highly adapted to NH_4^+ nutrition including blueberry, onion and rice (Britto and Kronzucker, 2002). Nevertheless, rice can still suffer from NH_4^+ toxicity in a high NH_4^+ environment especially when K^+ is low (Szczerba et al., 2008).

Previously, Ahmad et al. (2016) showed that, when NH_4^+ was present in the medium, a millimolar level of NH_4^+ resulted in similar growth and K^+ uptake for WT and KO. Their findings suggested that NH_4^+ did not affect the growth of KO like it was found in *Arabidopsis* (Hirsch et al., 1998). I further tested the effects of NH_4^+ at high concentration to observe the effects of NH_4^+ toxicity on different genotypes. Figure 14 illustrates that WT grew significantly better than KO at high NH_4^+ concentration, nevertheless, RGR of both genotypes was small compared to NH_4^+-N treatment (Figure 10). Suppressed K^+ accumulation in the both WT and KO shoots (Figure 16b) was found in the high NH_4^+ environment in comparison to NH_4^+-N (Figure 12a). Such adverse effects caused by high NH_4^+ concentration suggest it is toxic to rice. Compared the growth in non-toxic (Figure 10)

and toxic level of NH_4^+ (Figure 14), it is noticeable that the reduction of RGR in the high NH_4^+ concentration of WT and KO was similar: RGR reduction was 80.7% in WT and 78.7% in KO. Such similarity in RGR reduction between the genotypes implies that OsAKT1 does not impact on NH_4^+ toxicity on growth. The similar RGR reduction at elevated NH_4^+ concentration supports the evidence that NH_4^+ uptake capacity by the channel is small (as discussed in 4.4). According to the 'futile NH_4^+ cycling' hypothesis for NH_4^+ toxicity proposed by Britto et al. (2001), the fact that loss of OsAKT1 function is not reflected in a different RGR reduction suggests an only slight decrease in cytosolic NH_4^+ concentration and hence loss of AKT1 is unlikely to reduce significant energy expenditure in removing NH_4^+.

4.6 Ammonium does not contribute to osmotic stress tolerance via OsAKT1

Ahmad et al. (2016) demonstrated a role of OsAKT1 during water deficit periods: OsAKT1 increased K^+ concentration, especially in the roots, under water stress which likely contributed to rice growth as K^+ is an osmoregulator. Besides contributing to rice drought tolerance by mechanisms associated with K^+, I explored possible osmotic stress tolerance function of OsAKT1 due to NH_4^+ nutrition.

As shown in Figure 10, WT and KO grew at the same rate under osmotic stress in NH_4^+-N. This shows that NH_4^+ nutrition was not able to promote or maintain growth under via OsAKT1 at this level of osmotic stress which may be explained as follows: first, the quantity of the absorbed NH_4^+ via the channel may be too small to be reflected in growth enhancement. For example, previously, Ding et al. (2016) showed an increased in ABA accumulation of root and shoot tissues in rice treated with NH_4^+ during osmotic stress: root ABA accumulation, induced by root NH_4^+, was reported to enhance hydraulic conductivity and AQP activity of both PIPs and TIPs while shoot ABA, induced by NH_4^+ in the symplast, was associated with the induction of stomatal closure during water deficit period. Though OsAKT1 may contribute to the NH_4^+ reservoirs, the small amount of N contribution via the channel is unlikely to affect ABA concentration. Second, NH_4^+ supplied by the channel could participate in a pathway which does not have a significant impact on growth. NH_4^+ is known to not only act as a nutrient but also as a signalling molecule. For instance, NH_4^+ regulates NO_3^- assimilation pathway of an alga (Prieto et al., 1996). However, it is still not well-

understood how NH_4^+ functions as a signalling molecule and what other pathways are under the regulation of NH_4^+. Lastly, it is possible that 8% PEG-imposed osmotic stress is not in the range where OsAKT1 plays a role in drought tolerance. Thus, additional experiments using different levels of osmotic stress, (e.g. 5% PEG, as used by Ahmad et al. (2016)) are needed.

Interestingly, the growth rate of KO plants under control and osmotic stress conditions in NH_4^+-N were very similar suggesting that KO plants are resistant to osmotic stress in this medium. The mechanism behind this tolerance is not known. However, it may relate to N-K interactions in NH_4^+-N which only occur when OsAKT1 is absent.

4.7 Conclusions and future work

N is the most abundant macronutrient found in plant tissues. It is required for the production of amino acids, proteins, enzymes and nucleotides. Therefore, N nutrition contributes greatly to plant growth, development and productivity. While the knowledge regarding N assimilation is relatively well-established, the information on N uptake at root hair cells is an area that needs improvement. AKT1 is potentially involved in NH_4^+ uptake which may be significant in species such as rice. In addition, OsAKT1 has a known role in drought tolerance and although this has been interpreted as being a consequence of its K^+ transport capacity, a role of OsAKT1 in NH_4^+ uptake opens the possibility that the OsAKT1-dependent drought tolerance is due to NH_4^+. This investigation suggests that OsAKT1 does participate in NH_4^+ uptake, especially in the high affinity range. However, OsAKT1-mediated NH_4^+ uptake is not involved in the osmotic stress growth response.

The investigations in this present study not only should be repeated with different levels of osmotic stress but also with OsAKT1 overexpressors. As NH_4^+ was reported to contribute to water status in rice during water stress (Gao et al., 2010), an investigation on AQP expression and activity and ABA concentration should also take place to observe whether the small contribution of NH_4^+ by OsAKT1 takes part in these regulatory processes. Collecting data on root hydraulic conductance, transpiration rate and water content in WT and KO may also provide more useful insights related to water status of rice.

Abbreviations

ABA:	Abscisic acid
AMT:	Ammonium transporter
AQP:	Aquaporin
At:	*Arabidopsis thaliana*
Bp:	Base pair
CBL:	Calcineurin B-like protein
cDNA:	Complementary DNA
CIPK:	CBL-interacting protein kinase
CLC:	Chloride channel
CNGC:	Cyclic nucleotide-gated channel
C_i:	Intercellular CO2
CTAB:	Cetyltrimethyl ammonium bromide
DNA:	Deoxyribonucleic acid
dNTP:	Deoxyribonucleotide triphosphates
DW:	Dry weight
E. coli:	*Escherichia coil*
EB:	Elution buffer
EDTA:	Ethylene diamine tetra acetic acid
EV:	pYES2 empty vector
FW:	Fresh weight
g_m:	Mesophyll conductance
GOGAT:	Glutamine 2-oxoglutarate amino transferase
GORK:	Guard cells outward rectifying K channels
g_s:	Stomatal conductance
GS:	Glutamine synthetase
HAT:	High-affinity transport system
KO:	*akt1* knockout mutant
KUP/HAK:	K^+ uptake permease/high-affinity K^+
LAT:	Low-affinity transport system
LB:	Luria-Bertani media
LEA:	Late embryogenesis abundant proteins

LHT1:	Lysine Histidine Transporter 1
LiAc:	Lithium acetate
MEP:	Methylammonium/ammonium permease
mRNA:	Messenger RNA
n:	number of biological replicates
NH_4^+-N:	Yoshida medium with NH_4^+: NO_3^- ratio of 0.1: 0.9
NO_3^--N:	Yoshida medium with NH_4^+: NO_3^- ratio of 0.1: 0.9
NNP:	Nitrate-nitrite porter
NRT:	Nitrate transporter
NUE:	Nitrogen use efficiency
Os:	*Oryza sativa*
P5C:	Pyrroline-5-carboxylate
P5CR:	Pyrroline-5-carboxylate reductase
P5CS:	Pyrroline-5-carboxylate synthetase
P:	Probability level
PCR:	Polymerase chain reaction
PEG:	Polyethylene glycol
PIPs:	Plasma membrane intrinsic proteins
PRT:	Peptide transporter
RGR:	Relative growth rate
RNA:	Ribonucleic acid
ROS:	Reactive oxygen species
Root:shoot:	Ratio of root and shoot fresh weight
RT-PCR:	Reverse transcriptase PCR
SD:	Synthetic defined media
SD-ura:	SD without uracil
T-DNA:	Transferred DNA
TIPs:	Tonoplast intrinsic proteins
ura3:	*URA3* gene deficiency
WT:	Wild-type
YNB:	Yeast nitrogenous base
YPD:	Yeast extract peptone dextrose media

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