Role of AtAMT1;2 in nitrogen uptake and plant growth

By

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DECLARATION

I declare that this thesis is a record of original work and contains no material which has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text.

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Preface

This research was performed over 10 months as part of a Masters in Biotechnology (Plant Biotechnology). The literature review was previously assessed. The original research focus has not changed, however due to some unexpected complications in the development and optimisation of an AtAMT1;2 antibody, the last part of proposed research has yet to be completed. Although the research manuscript contained herein will provide the first draft of a future publication in The Plant Journal, the originally intended data set is still incomplete. Additional but incomplete data pertaining to the antibody work and leave confocal microscopy are provided within the appendices.

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Chapter 1-Introduction

Nitrogen (N) is an essential macronutrient required by plants. Nitrogen is often a limiting component in high-input monoculture agricultural systems, despite the extensive use of N-based fertilizers (Raun and Johnson, 1999; Kaiser *et al.*, 2002). A significant proportion of applied N fertilizers is not used by plants due to extensive N leaching and soil volatilization which creates significant environmental problems locally but also direct financial losses to the producer (Raun and Johnson, 1999; Glass, 2003; Gruber and Galloway, 2008). Plants have developed mechanisms to help cope with variable N levels in soils, including the use of integral plasma membrane transporters to enhance the uptake of both inorganic and organic N when available in the soil solution. The uptake of ammonium involves the class of transport proteins called AMT, which are highly similar among almost all living organisms (Ludewig *et al.*, 2007). AMT proteins are considered high-affinity transporters that enable plants to capture N when external ammonium concentrations are low (Shelden *et al.*, 2001). In the model plant *Arabidopsis thaliana* six AMT genes have been identified. Within this gene family, AtAMT1;2 displays some unique features that separates it from the remaining members. This includes a putative biphasic transport property where, in yeast it is capable of transporting methylammonium at both low and high concentrations (Shelden *et al.*, 2001). AtAMT1;2 also contains a predicted plastidic transit peptide and does not respond (gene expression) to N starvation. Loss of activity through the T-DNA mutation method is one of the tools that have been used to characterize individual members of the AMT family and to help understand their compensatory effects on each other (Yuan *et al.*, 2007a; Kaiser *et al.*, 2002). This project aims to further characterize AtAMT1;2 by investigating its role in N (NH_4^+ and NO_3^-) uptake and assimilation.

Chapter 2- Literature analysis

2.1 The significance of nitrogen use efficiency

Despite the apparent role of N-based fertilizers in increasing food production, the environmental problems and financial issues related to their extensive use is a matter of concern (Good *et al.*, 2004). Global acidification, which is a result of oxidization of NO to nitric acid, and its deposition on the ground can lead to eutrophication (fertilization of surface water) of aquatic systems (Gruber and Galloway, 2008). Increased nutrient supply due to eutrophication leads to abnormal growth of algae in many inland and coastal waters causing diversity loss and extensive death of fish and shellfish (Vitousek *et al.*, 1997). In most agricultural crops greater than half of the applied N fertilizers are not utilized by the plant but rather is lost to the environment. Environmental losses can occur through the conversion of NO_3^- to ammonia (NH_3), nitric oxide (NO), N_2O and N_2, which are then transferred to the atmosphere. The gaseous emission of N_2O from the soil makes up the largest source of greenhouse gas from agricultural systems (Beach *et al.*, 2008). Poorly utilized NO_3^- in soil can be subject to leaching causing ground water pollution (Edwards *et al.*, 2006).

Nitrogen use efficiency (NUE) relates to the efficient uptake and utilization of N by plants and can be defined as "the yield of grain per unit of available nitrogen in the soil" (Lea and Azevedo, 2006). Improving nitrogen use efficiency of plants is significant since it is currently as low as 33% for most cereal crops. This low NUE equates to approximately $15.9 billion annual loss of N fertilizers apart from the environmental cost in related pollution events (Raun and Johnson, 1999). Continued characterization of individual ammonium transporters in plants and identifying their contributions to net N uptake and assimilation will be important in identifying mechanisms to enable plants to accumulate and use N more efficiently.

2.2 Sources of N for plant growth and consumption

N is an essential element required for plant growth and development. Often the availability of soil N for plant growth is a limiting factor in most agricultural systems (Miller and Cramer, 2004). Both inorganic (NO_3^-, NH_4^+ and N_2) and organic (urea and amino acids) nitrogen are available in most soils (Crawford and Glass, 1998). NO_3^- and NH_4^+ are the major sources of inorganic nitrogen used by higher plants and depending upon the interactions between soil aeration, pH and temperature their relative availability and utilization by the plant will vary (Marschner, 2002).

2.2.1 Ammonium

Soil conditions including acidity, low oxygen and cool temperatures can lower the activity of nitrifying bacteria and lead to an increase in ammonium concentrations allowing NH_4^+ to become the predominant form of N available to plants (Britto and Kronzucker, 2002). The concentration of NH_4^+ under these conditions can reach up to 2 mM in contrast to the normal concentrations in aerobic pH balanced soils of around 20-200 μM (Miller and Cramer, 2004). In some agricultural soils NH_4^+ is the predominant source mainly because of human agricultural and industrial activities (pollution) and partially due to immobility of positively charged NH_4^+ within the soil (Britto and Kronzucker, 2002; Miller and Cramer, 2004). Ammonium is a lower energy demanding N resource and thus often preferable to that of nitrate which first needs to be converted to ammonium prior to its assimilation. However, high concentrations of NH_4^+ (~2mM) can be toxic to plants as it can disrupt cellular pH and importantly membrane electrical potentials, which will influence the anion/cation balance in cells. Plants can also expend significant amounts

of energy as they efflux accumulated NH_4^+ back to the soil (Britto and Kronzucker, 2002; Miller and Cramer, 2004).

2.2.2 Nitrate

Nitrate is the predominant form of N in pH balanced aerobic soils (Kaiser *et al.*, 2002) where the activity of soil nitrifying bacteria leads to higher rate of net nitrification than ammonification (Britto and Kronzucker, 2002). The vacuolar storage of nitrate in shoot, root and other storage organs is important for osmoregulation and cation-anion balance in the cell (Marschner, 2002). Nitrate is more mobile in soil than ammonium and can easily leach into ground water and therefore can be less accessible relative to ammonium for plant absorption (Miller and Cramer, 2004).

2.3 Nitrogen transport and assimilation

2.3.1 Ammonium transport and assimilation

2.3.1.1 Physiological systems of ammonium uptake

Two distinct physiological ammonium uptake systems have been identified in higher plants. The high affinity system (HATS) which is active at low external concentration (below 250 μM) and displays Michaelis-Menten saturation kinetics (Rubio *et al.*, 2007). As plant N levels increase, the HATS is subjected to negative feedback (Wang *et al.*, 2000). At higher external concentrations of NH_4^+ (200 μM), the activity of the low affinity system (LATS) contributes to the plant ammonium uptake which is non-saturable (Glass *et al.*, 2001; Glass *et al.*, 2002). The

uptake of ammonium by AMT proteins is thought to consist of a uniport system, based on electrophysiological analysis of AMT expression in *Xenopus laevis* oocytes (Ludewig *et al.*, 2002). Recently, analysis of *L. esculentum* AMT1;1 in yeast and oocytes has suggested that NH_3/H^+ cotransport may also occur (Mayer *et al.*, 2006).

2.3.1.2 Ammonium assimilation

The transport of ammonium to the plant cell is mediated via ammonium transporters (AMT1 and AMT2 families), which will be discussed in detail in later sections. Ammonium transported into the cell cytosol or that produced in chloroplasts or root plastids via nitrite reduction, is assimilated to glutamine, which is then used for transamination reactions to produce further amino acids (Bothe *et al.*, 2007). The GS/GOGAT (glutamine synthetase/glutamate-oxoglutarate aminotransferase) pathway regulates these reactions in the cytosol or plastid. In addition to ammonium which is produced from nitrate and nitrite assimilation, a large amount is produced in leaf mitochondria from the photorespirtory nitrogen cycle, which can be reassimilated by glutamine synthetase (GS) in the chloroplast or by GDH (glutamate dehydrogenase) in the mitochondria (Couturier *et al.*, 2007). It is argued that most of the ammonium that is generated in mitochondria during photorespiration is transported to the chloroplast for assimilation via GS (Lam *et al.*, 1996; Howitt *et al.*, 2000).

2.3.1.3 Enzymes involved in ammonium assimilation

Ammonium is incorporated to organic forms via the activity of three major enzymes GS, GS/GOGAT and GDH (Lam *et al.*, 1996). Glutamine synthetase is involved in primary NH_4^+ assimilation to glutamine, where the ammonium ion is transferred to glutamate via the conversion

of ATP to ADP + Pi. Two classes of cytosolic and chloroplast glutamine synthetase have been identified by ion exchange chromatography (Lam *et al.*, 1996). GOGAT (glutamate synthase) works in association with GS to reduce glutamine and α-ketoglutarate to produce two molecules of glutamate that can be used to further assimilate more NH_4^+ ions. There are two types of GOGAT, one that uses NADH as electron donor with the probable role of primary N assimilation in root and nodules, and another which uses Fd (ferrodoxin) as the electron donor and is more dominant in root tips (Miller and Cramer, 2004). The role of (GDH) is poorly understood but is considered to be involved in the reassimilation of recycled nitrogen in some aspect (Yamaya and Oaks, 1987; Lam *et al.*, 1996).

2.3.2 Nitrate transport and assimilation

2.3.2.1 Physiological systems of nitrate uptake

Nitrate uptake is the first step in the nitrate assimilatory pathway. Cytoplasmic concentrations of nitrate range from the undetectable up to ≥30 mM. NO_3^- uptake into the cell is primarily an active transport process that utilizes a proton motive force across the plasma membrane in a ratio of $2H^+$ to 1 NO_3^- molecule (Glass, 2003). Three physiological transport systems have been identified for nitrate (Glass *et al.*, 2002). The first is the constitutive high-affinity transporters (cHATS), which operate at low capacity and low external concentrations (Glass *et al.*, 2002). The second high-affinity system is the inducible high affinity system (iHATS), which is involved in the rapid uptake of NO_3^- after exposure to NO_3^- followed by rapid down regulation (Glass *et al.*, 2002). Both cHATS and iHATS show Michaelis Menten saturation kinetics. The third mechanism is the low affinity transport system (LATS), which is responsible for nitrate uptake at high external concentrations (> 200 μM) and does not show saturation kinetics (Bothe *et al.*,

2007). The Nrt1 and Nrt2 gene families are assumed to encode both the LATS and HATS NO_3^- transporters respectively (Gansel *et al.*, 2001). Among Nrt1 members Nrt1;1 corresponds to inducible LATS while Nrt1;2 gene is related to the constitutive HATS system (Cerezo *et al.*, 2001).

2.3.2.2 Nitrate assimilation

Nitrate that enters into a root cells are either stored in the vacuole or subject to further reductions to nitrite and ammonium within the cell. The first reduction step takes place in the cytosol where nitrate reductase (NR) catalyzes the two-electron reduction of nitrate to nitrite. Since nitrite can cause oxidative stress in the cytosol, the second reduction step occurs in the chloroplast involving a six electron reduction via nitrite reductase (NiR) (Galván *et al.*, 2002). There is some uncertainty how nitrite is transferred to the chloroplast. It has been suggested that a saturable plastidic nitrite transporter exists or that NO_2^- simply diffuses as HNO_2 (Rexach *et al.*, 2000) or even through a chloride channel located in the envelope membranes of chloroplasts (Galván *et al.*, 2002).

2.4 Known ammonium transporter families

AMTs belong to the protein super family of ammonium transporter/methylamine permease/rhesus proteins (AMT/MEP/Rh), which have been identified in plants, yeast and animals (Ninnemann *et al.*, 1994; Marini *et al.*, 1997; Gazzarrini *et al.*, 1999; Howitt *et al.*, 2000; Ludewig *et al.*, 2003; Loqué and von Wirén, 2004; Bothe *et al.*, 2007; Couturier *et al.*, 2007). Only a few organisms (such as *Helicobacter pylori* or the parasite *Plasmodium falciparum*) lack ammonium transporters and instead use other mechanisms to regulate their NH_4^+ uptake

(Ludewig *et al.*, 2007). In Arabidopsis there are 6 AMT genes, 14 putative AMTs in *P. trichocarpa* (poplar) (Couturier *et al.*, 2007) and 10 in rice (Loqué and von Wirén, 2004). The number of individual homologs is variable in different plant species sequenced thus far. However, the existence of these genes in almost all known organisms emphasizes their evolutionary importance in nature, however the reason for the large variety in number and potential functions remains unclear (Yuan *et al.*, 2007a).

Plant AMT's were initially identified through yeast complementation assays using defective ammonium uptake yeast strains (Ninnemann et al., 1994). As stated previously, six homologous AMT genes belonging to the AMT1 and AMT2 families have been identified and partially characterized in Arabidopsis (Gazzarrini *et al.*, 1999; Rawat *et al.*, 1999; Sohlenkamp *et al.*, 2000; Shelden *et al.*, 2001; Sohlenkamp *et al.*, 2002; Ramesh *et al.*, unpublished result).

2.4.1 AMT1

Five homologous AMT genes belonging to the AMT1 family have been identified and partially characterized in Arabidopsis (Ninnemann *et al.*, 1994). Members of the AMT1 family in Arabidopsis and tomato are suggested to be high-affinity transporters based on their functional activity when heterologously expressed in yeast (Gazzarrini *et al.*, 1999; von Wirén *et al.*, 2000).

2.4.1.1 Protein structure of AMT1

The AMT1 family in plants and the Mep family in yeast are very similar and encode hydrophobic plasma membrane proteins with an external amino terminus and an internal carboxyl terminus with 11 or 12 transmembrane spanning regions (Ludewig *et al.*, 2003; Ludewig *et al.*, 2001). The crystal structure of plant AMT1 proteins is unknown, however it is considered to function as a

trimer (Loqué *et al.*, 2007). At the posttranslational level it was shown that phosphorylation of the cytosolic C-terminus (which is present in bacteria, fungi and plants) has inhibitory effects on the function of the protein (Loqué *et al.*, 2007). Mutations within this region results in functionally inactive proteins in yeast and fungi and trans-inhibition of other AMT / MEP proteins (Ludewig *et al.*, 2003; Neuhäuser *et al.*, 2007).

2.4.1.2 Characteristics of AtAMT1;1 *in planta*

The best characterized member of the AMT1 family is AtAMT1;1. It is expressed in both shoot and root tissues and disruption of its activity using T-DNA mutagenesis decreased high-affinity NH_4^+ influx by 30% compared to wild type plants (Kaiser *et al.*, 2002). AtAMT1;1 expression is sensitive to N availability, where expression increases quickly with N removal and disappears when N is supplied (Rawat *et al*., 1999). Heterologous expression of AtAMT1;1 in tobacco (*Nicotiana tabacum*) using the cauliflower mosaic virus 35S promoter has shown that both N status and plant organ dependent posttranscriptional events may regulate the activity of this gene (Yuan *et al.*, 2007b). As far as chlorophyll and total plant N concentration was concerned, Yuan *et al*. (2007b) did not detect any growth advantages of AMT1;1 overexpression in tobacco relative to wild-type plants (in soil), although an improved capacity of these lines for N capture had been show in hydroponically grown plants. AtAMT1;1 has been detected in both epidermal and cortical root cells using a GFP/AMT1;1 fusion protein under the control of its endogenous promoter and is predicted to be specifically located on the plasma membrane (Neuhäuser *et al.*, 2007).

2.4.1.3 Characteristics of AMT1;2

Among the Arabidopsis AMT family members, AMT1;2 displays some distinctive characteristics. First of all, there is no visible response at the RNA level when plants are transferred to minus N solutions. Unlike AtAMT1;1 and AtAMT1;3 its level of mRNA expression does not increase in response to N starvation (Figure 1 a) (Gazzarrini *et al.*, 1999). Secondly AtAMT1;2 mRNA expression has been shown to change when plants are transferred from 1 mM NH_4NO_3 to 1 mM KNO_3^- as a sole N source (Figure 1 b) which could be indicative of nitrate dependent regulation of this gene.

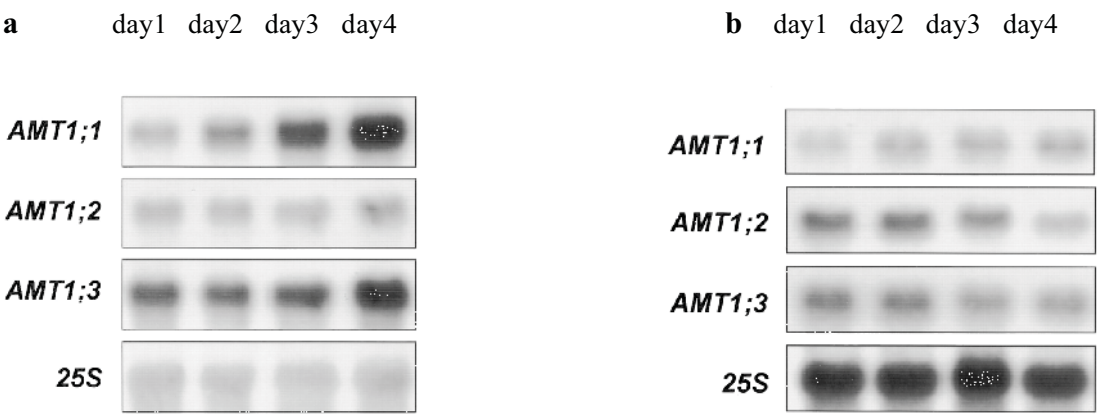

Figure 1.Response of AMTs at RNA level to different N treatment for 4 days (Gazzarrini *et al.*, 1999).

(A) No visible response of AMT1;2 gene expression when plants transferred from 1 mM NH_4NO_3 to minus N environment for 4 days. (B) The enhancement of expression when plants transferred from 1 mM NH_4NO_3 to 1 mM KNO_3.

The third interesting feature of AtAMT1;2 is the existence of a predicted putative plastid transit peptide, the first 40 amino acids are rich in serine which is a feature of plastidic peptide targeting sequences. This is a unique feature amongst the Amt family in Arabidopsis; however the protein has been suggested to be primarily localized to the plasma membrane as choloroplast import studies in spinach failed to show any choloroplastic import (Shelden *et al.*, 2001). Never the less,

if the plastidic targeting motif is functional, one probable role of AtAMT1;2 could be transport of ammonium in or out of plastids or choloroplasts.

In a study investigating the functional activity of AtAMT1;2, silencing of the gene using T-DNA mutagenesis decreased the uptake of NH_4^+ by approximately 18-26% in high-affinity range (NH_4^+ < 200 μM) and no change in low affinity range (200μM to 5mM) (Yuan *et al.*, 2007a). There was no visible growth phenotype with the loss of AMT1;2 either on agar plates or in soil.

2.4.1.3.1 Regulatory effects of glutamine

The endogenous level of glutamine could be an index of N status of plants. In a study examining the activity of the rice AMT1;2, a positive feedback regulation of OsAMT1;2 by glutamine was detected. This indicates a putative regulatory role of glutamine for AMT activity in rice (Sonoda *et al.*, 2003). In another study of Sonoda *et al.* (2003), a positive correlation between increasing glutamine levels and the induction of OsAMT1;2 mRNA was observed. The negative regulatory effect of glutamine pools on N uptake has also been observed elsewhere (Gazzarrini *et al.*, 1999; Rawat *et al.*, 1999).

2.4.1.3.2 Protein characterization of AMT1;2

AtAMT1;2 and AtAMT1;1 have highly similar protein sequences which includes 17 identical residues in the cytoplasmic C-terminus (Neuhäuser *et al.*, 2007). A highly conserved Gly and partially conserved phosphorylated Thr have been detected in this shared region, the Thr could be a site for posttranslational modification in planta (Neuhäuser *et al.*, 2007). In the study of

Neuhäuser *et al.*(2007) it was shown that the replacement of Thr468 by Asp in AtAMT1;2 inactivated transport of ammonium in ammonium transport deficient yeast strains.

2.4.1.3.3 Protein localization of AMT1;2

The subcellular localization of AtAMT1;2 by two phase partitioning confirms the plasma membrane allocation of protein (Yuan *et al.*, 2007a). At a root cellular level, using a GFP fusion construct under AtAMt1;2 endogenous promoter, AtAMT1;2 was detected in the endodermis, cortical and root hair cells (Neuhäuser *et al.*, 2007; Yuan *et al.*, 2007a). However, in the section of emerging lateral root zone, AMT1;2/GFP signal was only detected in the cortical cells (Yuan *et al.*, 2007a).

2.4.1.4 Characteristics of AMT1; 3

AtAMT1;3 transcript levels increase when plants are transferred to a growing environment without nitrogen (Gazzarrini *et al.*, 1999; Kaiser *et al.*, 2002). Using T-DNA insertion lines the contribution of AMT1;3 to overall N uptake was obtained and estimated at approximately 25% (Loqué *et al.*, 2006). A double knock out of AtAMT1;1 and AtAMT1;3 showed a further reduction of NH_4^+ uptake to 70% revealing their additive contribution to NH_4^+ uptake in the high affinity range (Loqué *et al.*, 2006). The expression of AtAM1;3 under the activity of its endogenous promoter was detected (using GFP fusion) in root tips and cortical cells of nitrogen-deficient plants which was increased relative to that of N-sufficient plants (Loqué *et al.*, 2006).

2.4.1.5 Characteristics of AMT1;4 and AMT1;5

The complete sequencing of Arabidopsis genome and screening of DNA libraries lead to recognition of two other AMT genes, notably *AtAMT1;4* and *AtAMT1;5* (von Wirén *et al.*, 2000). In an unpublished study of Ramesh *et al.* (2007) it was shown that both AtAMT1;4 and AtAMT1;5 were capable of accumulating ^{14}C-methylammonium when expressed in the yeast ammonium transporter mutant strain 31019b. The affinity of AtAMT1;4 for methylammonium was higher (K_M:70±4μM) than that of AtAMT1;5 (K_M:104±0.01 μM). Gene expression patterns were varied as *AtAMT1;4* mRNA expression increased within the first 24 hours of N starvation but then declined to an initial expression level (Ramesh *et al.*, unpublished results). In contrast, the expression of *AtAMT1;5* increased when plants were grown under low N concentrations. Interestingly, the expression of *AtAMT1;5* in plants was shown to increase when the activities of AtAMT1;1, AtAMT1;2, AtAMT1;3 and AtAMT2;1 were silenced, which is indicative of a compensatory role of this gene while other members are absent (Yuan *et al.*, 2007a). Using GFP fusion construct under the native AtAMT1;5 promoter, AMT1;5 GFP signal has been detected in root hairs and the root tip in nitrogen deficient plants (Yuan *et al.*, 2007a).

2.4.2 AMT2

AtAMT2 is more closely related to bacterial ammonium transporters than that of plants. It has been functionally characterized in yeast and shows limited capacity for methylammonium transport and the capacity to rescue cell growth of an ammonium transport mutant 31019b (Sohlenkamp *et al.*, 2000). Unlike other AMT1 family members it was shown by Sohlenkamp *et al.* that AtAMT2 was able to discriminate between ammonium and its toxic homolog methylammonium. AtAMT2;1 has a K_M of 20 μM for NH_4^+ and is predicted to be on the plasma membrane of leaf epidermal cells. Together these two features could be indicative of its high

affinity nature with a possible role in symplast-apoplast ammonium movement (Sohlenkamp *et al.*, 2002).

2.5 Summary

Both physiological and molecular techniques are required to investigate the role of each Arabidopsis ammonium transporter in plant N uptake and assimilation. Unlike other AMTs, *AMT1;2* protein expression patterns do not change during nitrogen starvation (Gazzarrini *et al.*, 1999) and it has some unique features including a predicted plastidic transit peptide. Some preliminary evidence (Kaiser et al, unpublished results) has indicated that the loss of *AtAMT1;2* (T-DNA KO) improves growth of Arabidopsis plants when grown on NO_3^- containing media. This suggests AMT1;2 may be involved in the NO_3^- assimilation pathway, possibly in root plastid ammonium transport. The aim of this project is to investigate other probable roles of AMT1;2 in N uptake and assimilation.

Chapter 3- Research Proposal

3.1 Introduction

Extensive use of N-based fertilizers world-wide has resulted in significant environmental problems associated with high-input agricultural production systems. The pollution of water resources and the cross fertilization of surface water have resulted in algae blooms along costal ecosystems and subsequent mass fish death (Vitousek *et al.*, 1997). Secondly, rising costs of N fertilizers has also focused greater attention to methods to improve their use in agriculture. These problems have created renewed interest in the development of improved nitrogen use efficient crop plants (Raun and Johnson, 1999; Good *et al.*, 2004). Despite the increasing rate of fertilizer application to meet the plants demand, the efficiency of plant use is low where near half of the applied fertilizers is used by crop plants (Edwards *et al.*, 2006). Genetic, physiological and molecular studies of N uptake and assimilation will improve our understanding on how to improve plant nitrogen use efficiency (Glass, 2003; Kaiser *et al.*, 2002). Plants regulate their ability to take up inorganic N (ammonium and nitrate) into root cells using integral plasma membrane proteins. An example of this is the AMTs (ammonium transporters), which belong to the protein super family of ammonium transporter/methylamine permease/rhesus (AMT/MEP/Rh). AMTs can be found in almost all living organisms and are believed to be the primary mechanisms involved in ammonium transport in plants (Marini *et al.*, 1997; von Wirén and Merrick, 2004).

Six genes which code AMT proteins in Arabidopsis have been identified and named AMT1;1 to 1;5 and AMT2;1. Most of these AMT genes have been characterized using heterologous expression systems and *in planta* forward and reverse expression analysis (Ninnemann *et al.*,

1994; Shelden *et al.*, 2001; Ludewig *et al.*, 2002; D'Apuzzo *et al.*, 2004; Ramesh *et al.*, 2007(unpublished results) ; Yuan *et al.*, 2007a; Yuan *et al.*, 2007b; Kaiser *et al.*, 2002; Loqué *et al.*, 2006). A better understanding of the function of each gene and its contribution to overall N uptake in different growing situations will help in reaching the end goal of producing more efficient plant N uptake and utilization. Reverse genetic approaches in this field have contributed significantly to understanding the probable role of each member of the family to whole N uptake by plants (Yuan *et al.*, 2007a; Kaiser *et al.*, 2002; Loqué *et al.*, 2006). This project aims to provide a better understanding of the role AMT1;2 in N uptake, and assimilation using knock out, RNAi and GFP tagged protein localization experiments.

3.2 Proposed Research

Three sets of experiments have been designed to give a better understand of the probable role of AMT1; 2 in N uptake and assimilation.

Part I. Comparative functional analysis of AMT1;2 activity in T-DNA/RNAi and wild type lines of the model plant *Arabidosis thaliana* in order to understand its contribution to N uptake over time and its probable efflux or intercellular role.

Proposed Outcome(s):

(1) A better understanding of probable role of AMT1; 2 in the NO_3^- assimilation pathway and N uptake over the time

(2) Comparison of the biomass of T-DNA and wild type plants with different designed pretreatments in order to verify AMT mediated plant growth in response to N provision.

Method:

-Flux experiments

T-DNA lines along with wild type parents will be grown hydroponically in a temperature (21°C) and light (8 hrs light/16 hrs dark) controlled growth room. Six week-old plants will be used for flux experiments using ^{15}N isotope tracer analysis to follow N uptake of the lines. Plants will be treated in different ways (4 day minus or plus N provision) before the flux experiments in order to measure ammonium or nitrate uptake. Dried root samples will be used for mass spectrometry analysis. Different T-DNA lines will be analyzed in the experiment for net nitrate and ammonium uptake over time (10, 15, 30, 60 and 90 minutes). Total replication of six plants from each line will be used for 5 different designed time courses.

-Growth analysis

Shoot/root fresh and dry weight of different media grown plants will be measured and used for comparative analysis of different lines.

Part II.

Localization of AMT1;2 protein in plant cells using promoter::AMT1;2::GFP constructs

Proposed Outcome(s):

(1) Detection of protein location via GFP expression

(2) Finding the relationship of protein location and the probable function of the gene

(3) Detecting GFP expression and consequent protein expression over different developmental stages of plant growth

Method:

Plants will be grown hydroponically or on agar plates and samples will be taken for confocal microscopy at different stages of plant growth (old and young tissues).

Part III. Defining intercellular localization in root and shoot tissues

Proposed outcome(s) :

(1). Finding intercellular site where AMT1; 2 protein is expressed and interpreting its probable function.

Method:

A recently generated anti-AtAMT1;2 antibody and pre-immune serum control will be used in immunolocalisation studies, in which root and shoot tissues will be processed and fixed for electron microscopy and incubation with both primary antibodies and gold labeled secondary antibodies. This will lead to detection of protein localisation within the cell.

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Chapter 4-Manuscript

Role of AtAMT1;2 in nitrogen uptake and plant growth

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4.1 Summary

In Arabidopsis, six ammonium transporters mediate the movement of ammonium in or out of cells. Uncertainty exists about their individual location and role, either in primary ammonium uptake or in intercellular translocation of ammonium. Using the endogenous AMT1;2 promoter to drive an AMT1;2:GFP fusion construct, it was shown that AMT1;2 is primarily localised to root endodermal and cortical cells, while in shoots natural fluorescence in the leaves prevented definitive localisation in cells and internal organelles (e.g. chloroplasts). A growth analysis of *amt1;2* and its corresponding wild type parental line revealed improved growth of an *amt1;2* T-DNA insertion line in media containing 2 mM KNO_3. No difference in growth was observed in media containing 1 mM NH_4NO_3. Using ^{15}N to measure net NH_4^+ and NO_3^- uptake suggested the growth response in the *amt1;2* line was not a result of improved nitrogen uptake. Furthermore, examination of high affinity ammonium transport in the *amt1;2* and WT lines revealed no detectable difference in ammonium uptake in either N-starved or N-sufficient grown plants respectively.

4.2 Introduction

The limited availability of nitrogen in most agricultural systems due to its heterogenous distribution in soils and the high demand by most plants necessitates the development of multiple strategies by plants to absorb and assimilate nitrogen (Ludewig *et al.*, 2003; Ludewig *et al.*, 2007; Glass *et al.*, 2002). In most situations, ammonium and nitrate are the predominant inorganic N sources available and used by plants. Whether ammonium or nitrate predominates will depend on the physical and chemical properties of the soil. Soils which are cool, acidic, and or anaerobic, often are ammonium rich as the activity of nitrifying bacteria is often reduced (Britto and Kronzucker, 2002). Furthermore, the lower mobility of positively charged ammonium within the soil solution can lead to increased accessibility of ammonium in many agricultural soil systems (Howitt *et al.*, 2000; Miller and Cramer, 2004). Alternatively, in warm, aerobic, pH neutral soils, nitrifying bacteria are active and nitrate pools tend to dominate.

Physiological studies have revealed two distinct ammonium uptake systems present in higher plants. At low external concentrations of ammonium (below 250 μM), the high affinity transport system (HATS), is active and shows Michaelis-Menten saturation kinetics. HATS activity is subject to a negative feedback mechanism when plant nitrogen levels increase (Wang *et al.*, 2000; Shelden *et al.*, 2001; Rubio *et al.*, 2007). The uptake of ammonium by the HATS may occur passively when external concentrations of ammonium are high > 1 mM. However, it was shown in Lemna that electrogenic uniport was not considered strong enough for uptake of ammonium at concentrations below 67 μm, therefore HATS activity should be thermodynamically active (Ullrich *et al.*, 1984; Glass, 2003). Electrophysiological studies of ammonium uptake via the high-affinity AMTs in *Xenopus laevis* oocytes and yeast are suggestive of uniport system or NH_3/H^+ co transport. The low affinity transport system (LATS) contributes

to overall ammonium uptake of plants while external ammonium concentration is high and follows a non-saturable trend (Glass *et al.*, 2002). The current understanding about the mechanism controlling the LATS system is limited (Glass *et al*., 2002).

The protein super family of ammonium/methylamine permease/rhesus (AMT/MEP/Rh) which mediates the transport of ammonium through membranes have been identified in plants, yeast and animals (Ninnemann *et al.*, 1994; Marini *et al.*, 1997; Howitt *et al.*, 2000; Ludewig *et al.*, 2001). Their existence in almost all organisms demonstrates their evolutionary importance (Loqué *et al.*, 2006; Ludewig *et al.*, 2007; Loqué *et al.*, 2007). In plants, the number of genes can vary widely across genera and species. Unfortunately, the individual role of each within a given species is poorly understood.

Two families of ammonium transporters have been identified in higher plants. In Arabidopsis six homologous genes belonging to AMT1 (AtAMT1;1, AtAMT1;2, AtAMT1;3, AtAMT1;4 and AtAMT1;5) and AMT2 (AtAMT2;1) families have been identified and characterized partially (Gazzarrini *et al.*, 1999; Rawat *et al.*, 1999; Sohlenkamp *et al.*, 2000; von Wirén *et al.*, 2000a; Shelden *et al.*, 2001; Ramesh *et al.*, unpublished results; Yuan *et al.*, 2007a). Like other large multigene families, it is unclear what their individual responsibilities/function(s) related to ammonium transport are in the plants. Heterologous expression of the AMT1 family in yeast has demonstrated that in both Arabidopsis and tomato, AMT1 proteins behave as high-affinity ammonium transporters (Gazzarrini *et al.*, 1999; von Wirén *et al.*, 2000b). Using T-DNA mutagenesis it was shown that AtAMT1,1 is responsible for high affinity ammonium uptake in roots by 30% (Kaiser *et al.*, 2002) and is post-transcriptionally regulated in a nitrogen and organ dependent manner (Yuan *et al.*, 2007b). It has been previously demonstrated that AtAMT1;1 and 1;3 both contribute to high affinity ammonium uptake in an additive manner (up to 70% net

ammonium uptake) when plants have been previously starved of nitrogen (Loqué *et al.*, 2006). Transformed Arabidopsis lines containing either GFP tagged AtAMT1;1 or AtAMT1;3 driven by their endogenous promoters respectively, revealed that AtAMT1;1 was primarily in epidermal and cortical root cells, while AtAMT1;3 was detected in root tips and cortical cells. GFP expression levels for both lines were found to be enhanced in nitrogen deficient plants (Neuhäuser *et al.*, 2007; Loqué *et al.*, 2006). An increase in activity of both transport systems (HATS and LATS) *in planta* has been shown to respond to N starvation. This is mirrored in their mRNA expression patterns, which increase when N is removed from the growing media. In the presence of N, mRNA expression is repressed and there is no obvious response to alternative nitrogen forms (NH_4NO_3 vs. KNO_3) (Gazzarrini *et al.*, 1999). AMT1;4 and 1;5 were identified after complete sequencing of the Arabidopsis genome (von Wirén *et al.*, 2000a). Preliminary data by Ramesh *et al*. (unpublished results) has shown that the expression level of AtAMT1;4 increases transiently after 24 hours of nitrogen starvation while that of AMT1;5 continues to increase over extended N starvation periods.

Among the AMT1 family AMT1;2 displays some considerable features. Unlike AMT1;1 and 1;3 the mRNA expression level of the gene does not increase in response to nitrogen starvation (4 days) (Gazzarrini *et al.*, 1999). On the other hand the level of expression is slightly changed in response to the supply of KNO_3 (Gazzarrini *et al.*, 1999). AtAMT1;2 is the only AMT in Arabidopsis to contain a long serine-rich domain in its N terminus, which is a characteristic plastidic peptide targeting sequence (Gazzarrini *et al.*, 1999; Shelden *et al.*, 2001). Hypothetically, there should be an ammonium transporter within the inner or outer chloroplastic membrane to support the chloroplastic assimilation of ammonium released during photorespiration (von Wiren *et al.,* 2000a; Howitt *et al*., 2000). There is doubt however, whether the sequence peptide identified on AtAMT1;2 is able to target the protein to chloroplasts, as a

study by Shelden *et al.* (2001) failed to show import of AMT1;2 into isolated spinach chloroplasts. This work needs to be reconfirmed using Arabidopsis chloroplasts in case the spinach model was not suitable, as well as organelle localisation using AMT1;2 antibodies or GFP tagged AMT1;2 protein.

In previous unpublished work (Rmaesh *et al*.) a growth advantage was observed with an *amt1;2* mutant over its related parental wild type when grown in media containing 2 mM KNO_3. This finding is of particular interest as it suggests AMT1;2 may be important in the utilisation of nitrate, and that the loss of AMT1;2 has improved either the capture or assimilatory capacity of reduced nitrogen within the plant.

In this study we have used a reverse genetic approach to re-examine the functional role of AMT1;2. In particular, we have focussed on a re-examination of the growth phenotypes associated with the loss of *AtAMT1;2* that develop when grown in the presence of 2mM KNO_3. Furthermore, using mass spectrometry, we have examined the contribution of AtAMT1; 2 to overall ammonium or nitrate uptake in both the T-DNA knockout and wild type lines. Cellular localisation of AtAMT1;2 and observed changes in expression in response to external nitrogen provision was analysed using a promotor::GFP::AtAMT1;2 construct which was under the activity of an endogenous AMT1;2 promoter.

4.3 Results

Growth analysis of *amt1;2* and its related parental line

Two lines containing independent T-DNA insertions in the *AtAMT1;2* gene were obtained. A T-DNA insertion line in the Col 0 background was obtained from DuPont-Pioneer (R.Gupta, unpublished result) which contains an insertion event 890 bp downstream of the start codon of AtAMT1;2 (Figure 1a). The other *amt1;2* line (Figure 1b) was obtained from the GARLIC population developed by Syngenta with a T-DNA insertion 13 bp downstream of the first methionine of the open reading frame of *AtAMT1;2* (Carter, 2004). In order to check the function of the gene at the mRNA level, total RNA was extracted from individual root and shoot tissues from both lines and corresponding parental lines. A pair of primers was designed to amplify 100 bp of open reading frame of *AtAMT1;2*. Semi-quantitative reverse transcriptase PCR analysis revealed a loss of *AMT1;2* transcript in both T-DNA lines, while amplified product was amplified in the corresponding wild types (Figure 1c).

To detect whether the loss of *AtAMT1;2* results in any detectable growth phenotype in response to different nitrogen sources, we measured growth of the DuPont T-DNA line relative to a wild-type parent. Plants were grown hydroponically in nutrient solution containing 1 mM NH_4NO_3 or 2mM KNO_3. In the presence of ammonium nitrate there was no statistically significant difference in growth (shoot and root dry weight) between the T-DNA and wild type lines (Figure 3a and b). Interestingly, when plants were grown on 2 mM KNO_3, a statistically significant ($P<0.05$) growth advantage (root and shoots) was observed in the *amt1;2* line between day 40 and 43 (Figure 2a and b). The shoot and root growth rate of the mutant was approximately 4.2 and 3 fold greater than that of the WT line (Figure 2c, d). However, as plants continued to grow, the WT began to

grow quicker than the *amt1;2* KO (Figure 2a and b) resulting in a 3.1 and 1.6 fold increase in shoot and root dry weight over that of the *amt1;2* (days 43-47) (Figure 2c and d). One interesting feature of the *amt1;2* growth pattern was its linearity relative to the wild-type. It would appear the loss of *AtAMT1;2* disrupts the rapid growth rate observed once plants develop sufficient roots within the hydroponic growing conditions.

AtAMT1;2 does not alter net ammonium or nitrate uptake

Nitrate and ammonium uptake was measured in the *amt1;2* and wild type lines to investigate any links between nitrogen uptake/transport and AMT1;2 activity. In media containing 2mM $^{15}NO_3$, there was no significant difference in root nitrate uptake over the time period tested for both sets of lines (Figure 4a and b). Nitrate uptake accumulated in a linear fashion over the first hour and then increased significantly (approximately 2 fold) in the final 30 minutes of the assay (Figure 4a and b).

Interestingly, loss of *AMT1;2* did not appear to disrupt net $^{15}NH_4^+$ uptake (1 mM NH_4^+) over the time period for both of the T-DNA alleles (Figure 5a and b). Instead, ammonium uptake increased equally over 60 minutes in both the T-DNA and corresponding wild type lines. Although net ammonium uptake in the WT Syngenta line exceeded the *amt1;2* T-DNA line over the period from 60 to 90 minutes, (Figure 5a), this difference was found not to be statistically significant ($P<0.05$).

AMT1;2 does not contribute to high affinity ammonium uptake in sufficient or nitrogen starved plants

In order to understand the contribution of AtAMT1;2 to high affinity ammonium uptake, we performed a flux experiment in the high affinity range of 100 μM NH_4^+ (Figure 6). HATS activity was measured in both N grown and N starved plants. For the nitrogen starvation

treatment, plants were transferred to a nitrogen free medium 4 days prior to the uptake experiments. Unlike other members of the AMT1 family (AMT1;1 and AMT1;3), loss of AMT1;2 did not disrupt high-affinity ammonium uptake in either nitrogen starved or nitrogen sufficient grown plants (Figure 6). A slight enhancement in ammonium uptake was measured in the DuPont T-DNA line over the wild type control; however the differences were not statistically significant.

Tissue specific localization of AtAMT1;2

Cellular localisation of AMT1;2 was investigated using GFP fusion proteins and confocal microscopy to help define its role in the plant and location within tissues where ammonium transport is believed to be important. We were able to source an Arabidopsis line which contained an AMT1;2::GFP fusion construct that was transcriptionally activated by the endogenous AMT1;2 promoter (R. Gupta, DuPont-Pioneer). Plants were grown in nitrogen free (Figure 7 a), 1 mM NH_4NO_3 (Figure 7 b) or normal 0.5 X MS media which contains high amount of N (concentration is ~20 mM N) (Figure 7 c). In order to avoid N/C imbalance no sucrose was added to media. In the root tip, GFP signal was barely detectable (Figure 7G,J and M). Behind the root tip where root hairs begin to emerge, AMT1;2::GFP signals was mainly detected in the endodermis (Figure 7 B,H,K and N). Further up the root, the GFP signal was mostly found in the root cortex (Figure 7 C, I, L,). Interestingly when plants were grown with high nitrogen concentrations, AMT1;2/GFP was specifically localised to the endodermis in most roots examined (Figure 7,N and O).

4.4 Discussion

Plants need multiple strategies in order to accumulate and transport ammonium across various cell types and tissues. For example, in many plant species there is a need for long distance transport from root to shoot where assimilation of root accumulated ammonium is primarily in the cytosol or chloroplasts of leaf tissues (Schjoerring *et al.*, 2002). Furthermore, ammonium is routinely generated in mitochondria during photorespiration in C3 plants (Coschigano *et al.*, 1998). This ammonium is recaptured in the chloroplast necessitating the existence of an intercellular organelle transport mechanism (Howitt *et al.*, 2000).

Our results are indicative of a marginal improved growth of *amt1;2* over its corresponding wild type line in media containing 2mM KNO_3 as a sole N source. In an attempt to understand why the loss of *AMT1;2* could transiently improve the growth on nitrate, we examined the net nitrate (Figure 4a and b) and ammonium uptake (Figure 5a and b) capacity of the *amt1;2* plants (six weeks old) relative to the parental wild-types. We found no difference in the capacity of the *amt1;2* mutant to accumulate nitrate or ammonium over an extended incubation period (100 minutes). Under the conditions tested, this result suggests the growth phenotype may be related to the plants capacity to assimilate nitrate rather than accumulate more. The data also suggests the role of amt1;2 in ammonium uptake is minor and can be easily covered (compensated for) by other AMT's as before has been seen for other AMTs (Kaiser et al., 2002; Yuan et al., 2007a). Furthermore, our analysis of the ammonium HATS in the *amt1;2* mutant (Figure 6) revealed no significant differences in the capacity of NH_4^+ uptake relative to the controls. A previous estimation by Yuan et al., (2007a) that AMT1;2 was responsible for up to 18-26% of root ammonium absorption was not confirmed in this study. Based on these results, it would appear AMT1;2 has a minor contribution to overall net NH_4^+ uptake but instead could be more suited/responsible for intercellular ammonium transport.

The tissue specific expression pattern of AtAMT1;2 does not change significantly when plants are exposed to different nutrient solutions containing either no nitrogen (Figure 7a), 1mM NH_4NO_3 (Figure 7b) or normal high N 0.5 x MS media (Figure 7 c). However, it was previously reported that provision of exogenous sucrose to roots significantly increased transcript levels of OsAMT1,3 and AtAMT1;1 and increased the uptake of ^{13}N-NH_4^+ in the dark (Rawat *et al.*, 1999; Glass *et al.*, 2003). The mechanism for this carbon induced phenotype is unknown, however it has been suggested that it could be involved in maintaining C/N balance in plants and that AMT's could be a component of the signalling cascade influenced by rich carbon provisions (Kaiser *et al.*, 2002). Our profile of GFP tagged AMT1;2 did not result in any significant difference in GFP localization in the presence or absence of exogenous sucrose in the growth media (sucrose data not shown). In plates where no sucrose was present GFP tagged AMT1;2 was mainly in endodermis (Figure 7 B,H,K and N) and cortex of root cells (Figure 7 C,F,I,L,), with higher levels in elongation zone behind the root tip and in more matured basal regions. These results are consistent with the studies of both Yuan *et al.* (2007a) and Neuhauser *et al*. (2007) that used similar approaches in Arabidopsis. However rarely the endodermal expression is detectable in the distal regions of the root (Figure 7 D and O) or cortex protein expression in root hair emerging zone were detectable (Figure 7O). The substrate affinities of AMT transporters have been measured *in planta* (Yuan *et al.*, 2007a). Based on these studies it was shown that AtAMT1;2 has a K_M for ammonium of ~ 234 μM compared to that of AtAMT1;1 (50 μM) and AtAMT1;3 (60.5 μM). If AMT1;2 is involved in ammonium transport in the endodermis and cortex its apparent lower affinity for ammonium suits this location where higher ammonium concentrations are predicted to be (Yuan *et al.*, 2007a). Therefore, it has been suggested that AtAMT1;2 is responsible for transfer of captured ammonium via AtAMT1;1 and AtAMT1;3

(located primarily in the endodermis and root hairs) to vascular tissues via the symplastic pathway (Neuhäuser *et al.*, 2007).

Nitrate absorbed by roots can either be directly assimilated in the root or translocated into the shoot and subsequently be stored or assimilated (Schobert and Komor, 1990). The first step in the utilization of nitrate as a N source is its reduction to nitrite by the cytosolic enzyme nitrate reductase (NR) (Galván *et al.*, 2002). Nitrite is highly reactive in the cytosol and rapidly transferred to plastids (chloroplast or root plastids) either via simple diffusion through a saturable plastidic nitrite transporter or chloride channel located on the inner envelope membrane of chloroplasts (Rexach et al., 2000; Galván et al., 2002). In the chloroplast / root plastid, nitrite is reduced to ammonia via nitrite reductase (NiR). Ammonium is then assimilated into glutamine which is used for various transamination reactions to produce amino acids (Bothe et al., 2007). Ammonium assimilation occurs via the GS/GOGAT (glutamine synthetase/glutamate-oxoglutarat amminotransferase) pathway which operates in both the cytosol and chloroplast/plastid (Weber and Flügge, 2002). Ammonium transport within the root plastid as well as the chloroplast may involve AMT1;2. AMT1;2 is the only AMT with a predicted N-terminal plastidic signaling motif. Assimilation of nitrate in both root and shoots will involve the generation of ammonium pools within plastids. Maintenance of these pools may involve the activity of AMT1;2 either in efflux pathway to encourage cytosolic NH_4^+ assimilation via GS. Alternatively, NH_4^+ in the cytosol which is released from mitochondria during photorespiration (in shoots) needs to be transported into the chloroplasts for re-assimilation. When amt1;2 plants are grown on nitrate, the movement of ammonium into (influx) or out (efflux) of plastids may be perturbed. This could alter the ammonium assimilation pathway where plastidic GS/GOGAT activity dominates that of the cytosolic pathway or alternatively reduce xylem delivery of nitrate derived NH_4^+-N to the shoot. This may be particularly important in root nitrate assimilation where efficiencies in amino

acid synthesis in the root may modify amino acid levels between roots and shoot tissues. Both ammonium and nitrate transport and assimilatory systems are negatively regulated by direct or indirect phloem mobile N feed-back mechanisms respectively (Rawat et al.,1999). A reduction of shoot amino acid pools through improved assimilation of nitrate in the roots could disrupt the N feed-back mechanism and trick the plant that it is still N deprived encouraging further N uptake and assimilation. However, as we observed in this study the improved growth on nitrate was eventually lost, where wild type plants were able to catch up and grow better than the *amt1;2* line. We also didn't see any improvement to either nitrate or ammonium uptake with the loss of *amt1;2*. Thus to conclude the plants may have improved uptake capacity through a modification of N-feedback is purely speculative at the moment.

At this stage, it is still too premature to offer a definitive explanation for the growth response, however it is clear AMT1;2 does seem to participate in the assimilaton of NO_3^- and offers a useful tool to further explore this mechanism in future experiments. In particular there needs to be continued work in defining the intercellular localization of AMT1;2 using either GFP or immunogold labelleing and to confirm whether or not it is localized within a plastid.

4.5 Experimental procedure

Plant growth

Arabidopsis thaliana seeds were hydrated in water and vernalised at 4°C for 3 days prior to germination and then grown hydroponically. The seeds were germinated on end cut rockwool filled microfuge tubes placed in racks above double distilled water. The water was replaced with nutrient solution after 10-14 days. Nutrient solutions were replaced weekly. The nutrient solutions contained: 0.5 mM $MgSO_4.7\ H_2O$; 0.5 mM KH_2PO_4; 0.0250 mM H_3BO_3; 0.002 mM $MnSO_4.H_2O$; 0.002 mM $ZnSO4.7H_2O$; 0.5 μM $CuSO_4$; 0.5 μM $Na_2MoO_4.2H_2O$; 0.05 mM KCl; 0.02 mM Fe-EDTA; 0.25 mM K_2SO_4; 0.25 mM $CaSO_4.2H_2O$. PH was adjusted to 6 with approximately 200 mg $CaSO_4.2H_2O$ per litre of solution. 1mM NH_4NO_3 or 2mM KNO_3 was supplied depending on the experiment. Plants were grown for 6 weeks in temperature and light controlled growth room at 23°C, short day condition (8-h light /16h dark) with fluorescent lights with a light Intensity at plant level of 150 μmole $m^{-2}\ s^{-1}$. Plate grown plants were planted in ½ MS media with ± 2% (w/v) sucrose. For plant growth analysis the plants were harvested and fresh weight of roots and shoots were measured separately. Plants were oven dried at 42°C for at least 15 days before measuring the dry weight.

Flux experiments and ^{15}N uptake analysis

Net uptake over time

Plants were grown vegetatively (8 hour days) at a canopy light level of ~ 150 μmole $m^{-2}\ s^{-1}$ in nutrient solution containing 1mM NH_4NO_3 for 4-5 weeks. Plants of suitable size and age were transferred to new media containing 1 mM NH_4NO_3 or 2 mM KNO_3 4 days prior to the flux experiment. The nitrogen flux experiment consisted of placing the plants in nutrient solution

containing either 1 mM ($^{14}NH_4^+$) or 2 mM ($^{14}NO_3^-$) for 10 min (preincubation). The plants were then transferred to an identical media containing 1 mM ($^{15}NH_4^+$) or 2 mM ($^{15}NO_3^-$) for the designed time period (influx) and then transferred (wash step) to a new nutrient solution containing 1 mM ($^{14}NH_4NO_3$) or 2 mM ($K^{14}NO3$) for 10 min. For nitrate flux experiments, a second wash in 2 mM $CaSO_4$ was also performed. The pH of all solutions was adjusted to 6. The fresh weight of roots and shoots were measured separately (data not shown). Tissues were dried 42˚C for a minimum of 15 days. Each dried sample was ground and 1-3 mg of powder used for analysis in an isotope ratio mass-spectrometer (Plant Research Centre, Adelaide, South Australia). ^{15}N samples were determined as described above.

Short term flux

Short-term flux experiments were performed on 6 week old plants (See figure 2 for development profile). Prior to the flux experiment, plants were transferred to nutrient solutions with or without N and grown for 4 days. The flux protocol was similar to that explained previously but with the following modifications: 1) preincubation with 100 μM $^{14}NH^+_4$ for 10 minutes; 2) 10 minute ^{15}N uptake with 100 μM ^{15}N-NH_4^+; 3) Wash with 100 μM $^{14}NH^+_4$ for 10 minutes.

Reverse transcript PCR

Six week-old hydroponically grown plants (nutrient solution contained either 1mM NH4NO3 or 2mM KNO3) were harvested and root and shoot tissues separated and snap frozen in liquid nitrogen. The RNA was extracted using RNeasy plant mini kit (Qiagen) from 100 mg of frozen tissue. Total RNA quality was tested by running on the 2% w/v agarose gel. One μg of total RNA was used for reverse transcript cDNA synthesis using the iScript cDNA synthesis kit (Biorad).

Forward and reverse primers AtAMT1;2 (amt1;2 F:TGCTTTGTGCTGGATCAGTC, R:AGGTGTACCAAAGGCGAATG) and AtAMT1;1 (for control) were designed to amplify 100 bp of cDNA. The PCR cycling conditions were (cycle 1) 95°C for 3 min, (cycle 2, 35times) 95°C for 15 seconds; 60°C for 30 seconds; 72°C for 30 seconds, (cycle 3) 72 for 5 min. The PCR product was size separated on a 1.5% (w/v) agarose gel in 1 X TAE buffer.

Microscopy

For tissue specific localization of AtAMT1;2, plants were grown on plates containing 0.5 x MS media with altered ammonium and nitrate concentrations with or without 2% (w/v) sucrose as indicated. The plates were put vertically in a temperature and light controlled growth chamber [24°C (day) and 21°C (night), 70 μmole $m^{-2}s^{-1}$ at plate surface level]. Plants at different developmental periods were harvested when required. The seedlings and different parts of the roots were placed in a solution of 10 μg/ml of propidium iodide (Sigma) for 5-10 minutes. The imaging was conducted using a ZEISS laser scanning LSM5 microscope (Australian Centre for Plant Functional Genomics, Adelaide, South Australia). 488 nm filter set for laser excitation and GFP filter (510 nm) for emission were used.

Statistical Analysis

Where statistically significant differences were mentioned in the text, two-way ANOVA and Bonferroni posttests were used on whole data sets (based on mean). The data sets were then compared to detect the strength of difference at each point via unpaired t-test with 95% confidence interval. The Graph Pad Prism 5 software was used for data analysis.

4.6 Acknowledgements

We would like to thank Nenah Mackenzie for skilful technical support and Alex Johnson for helping in confocal microscopy. This Study was supported by a grant from Australia Research Council to B.N.K.

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4.8 Figure legends

Figure 1 Characterization of AtAMT1;2 T-DNA insertion lines used in this study.

(a) T-DNA insertion site in the DuPont/Pioneer AtAMT1;2 line (Rajeev Gupta, unpublished results). The T-DNA is inserted within the open-reading frame of AtAMT1;2 (b) Transposon insertion site in the Syngenta line (Garlic population) obtained from Brent N Kaiser (unpublished results). A modified T-DNA which consists of a spliced variant of the original T-DNA is inserted 13 bp downstream of the first ATG of AtAMT1;2 open reading frame (Carter,2004). (c) Semi-quantitative reverse transcriptase PCR analysis of *AtAMT1;2* expression. Total RNA was extracted from both root and shoot tissues from both *amt1;2* and WT lines grown for 6 weeks in hydroponics

Figure 2. Effect of nitrate on root and shoot growth in *amt1;2* and WT plants.

Dry weight of roots (a) and shoots (b) over time. Plants were grown hydroponically with 2 mM KNO_3 and harvested at the indicated times. The fresh weights were measured (data not shown) and the dry weight measured after the samples were dried at 42°C for 15 days. The growth rate (c, d) was calculated based on mean dry weights achieved across the respective sampling periods. Symbols indicate means ± SEM (n= 7-8 plants per harvest). Where indicated * data between lines are significantly different at $p < 0.05$ while *** shows significant difference at $p<0.001$.

Figure 3. Effect of ammonium nitrate on root and shoot growth in amt1;2 and WT plants.

Dry weight of roots (a) and shoots (b) over time. Plants were grown in hydroponics with 1 mM NH_4NO_3 and harvested at the indicated times. The fresh weights were measured (data not shown) and the dry weight measured after the samples were dried at 40°C for 10-15 days. Symbols indicate means ± SEM (n= 7-8 plants per harvest).

Figure 4. Characterisation of nitrate uptake into roots of amt1;2 and WT plants.

Net influx over time of ^{15}N –KNO_3^- was measured in WT and *amt1;2* mutant lines. Plants were grown in hydroponics for 6 weeks in nutrient solution containing 1 mM NH_4NO_3. Plants were transferred to 2mM KNO_3 for 4 days and then transferred to nutrient solutions containing 2 mM ^{15}N –KNO_3^- for the indicated periods of time. Symbols indicate means ± SEM (n= 5 plants per harvest).

Figure 5. Characterisation of ammonium uptake into roots of *amt1;2* and WT plants..

Net influx over time of ^{15}N –NH_4^+ was measured in WT and amt1;2 mutant lines. Plants were grown in hydroponics for 6 weeks in nutrient solution containing 1 mM NH_4NO_3. Plants were then transferred to nutrient solutions containing 0.5 mM ^{15}N–$(NH_4)_2SO_4^-$ for the indicated periods of time. Symbols indicate means ± SEM (n= 5 plants per harvest).

Figure 6. Comparative analysis of high-affinity (HATS) ammonium uptake in two T-DNA *amt1;2* lines in response to N provision and starvation.

Plants were grown in hydroponics for 6 weeks in nutrient solution containing 1 mM NH_4NO_3. Plants were then transferred to nutrient solutions containing either 1 mM NH_4NO_3 or –N and

grown for a further 4 days. Plants were then precultured in a nutrient solution containing 100 μM NH_4^+ before transfer to an identical solution containing 50 μM ^{15}N–$(NH_4)_2SO_4^-$ for 10 minutes. Symbols indicate means ± SEM (n= 7 plants).

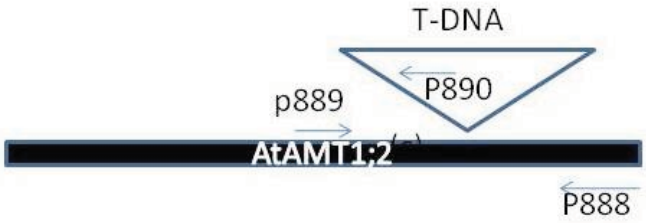
Figure 7. Cellular localization of AtAMT1;2 in roots

Col O plants containing an AMT1;2 promoter:AMT1;2/GFP construct (R. Gupta, unpublished result, DuPont-Pioneer) were examined for GFP localization in roots. Plants were cultured directly on a modified MS media lacking sucrose and containing either no nitrogen (a), 1mM NH_4NO_3 (b) or 30 mM N (c) which consisted of a combination of 10 mM NH_4NO_3 and 9.3 5mM KNO_3 in normal 0.5 x MS media. Representative images of AMT1;2/GFP fusion lines. Root tip zone (A, G, J and M). Elongation zone (25-40 mm) behind root-tip (B, H, K). Matured root zone (40-70mm from root tip) (C, E, I, F). Bars=50 μm. Plants were 12 - 21 days old.

4.9 Figures

Figure 1

(a)



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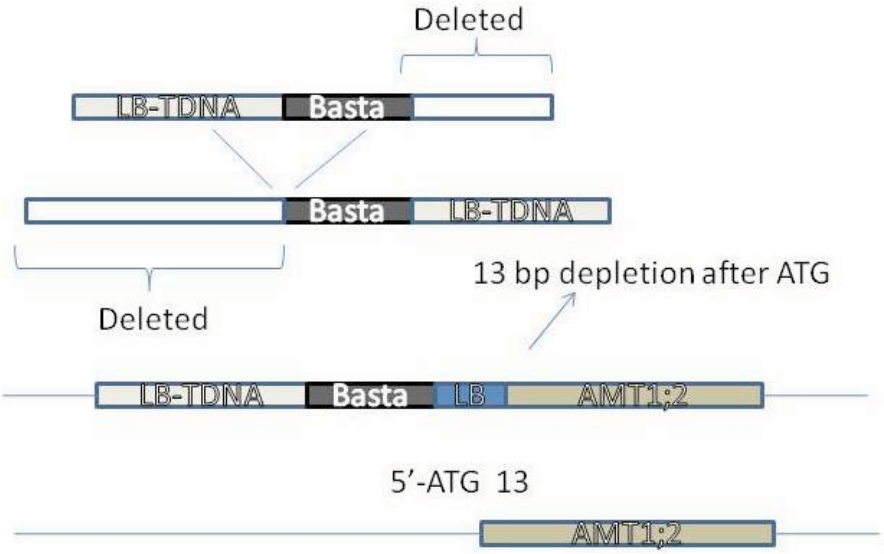

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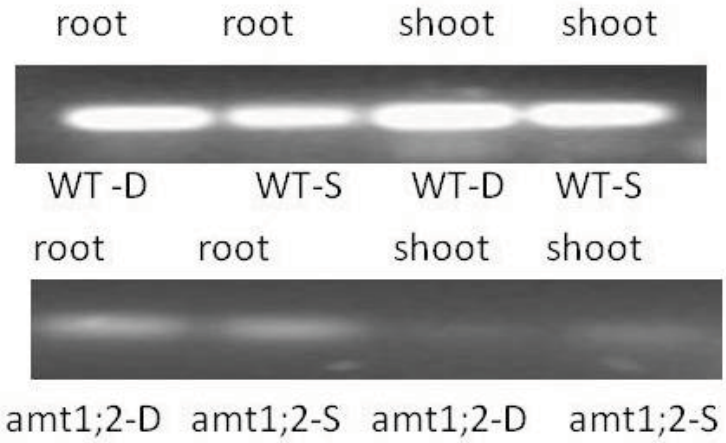


Figure 2

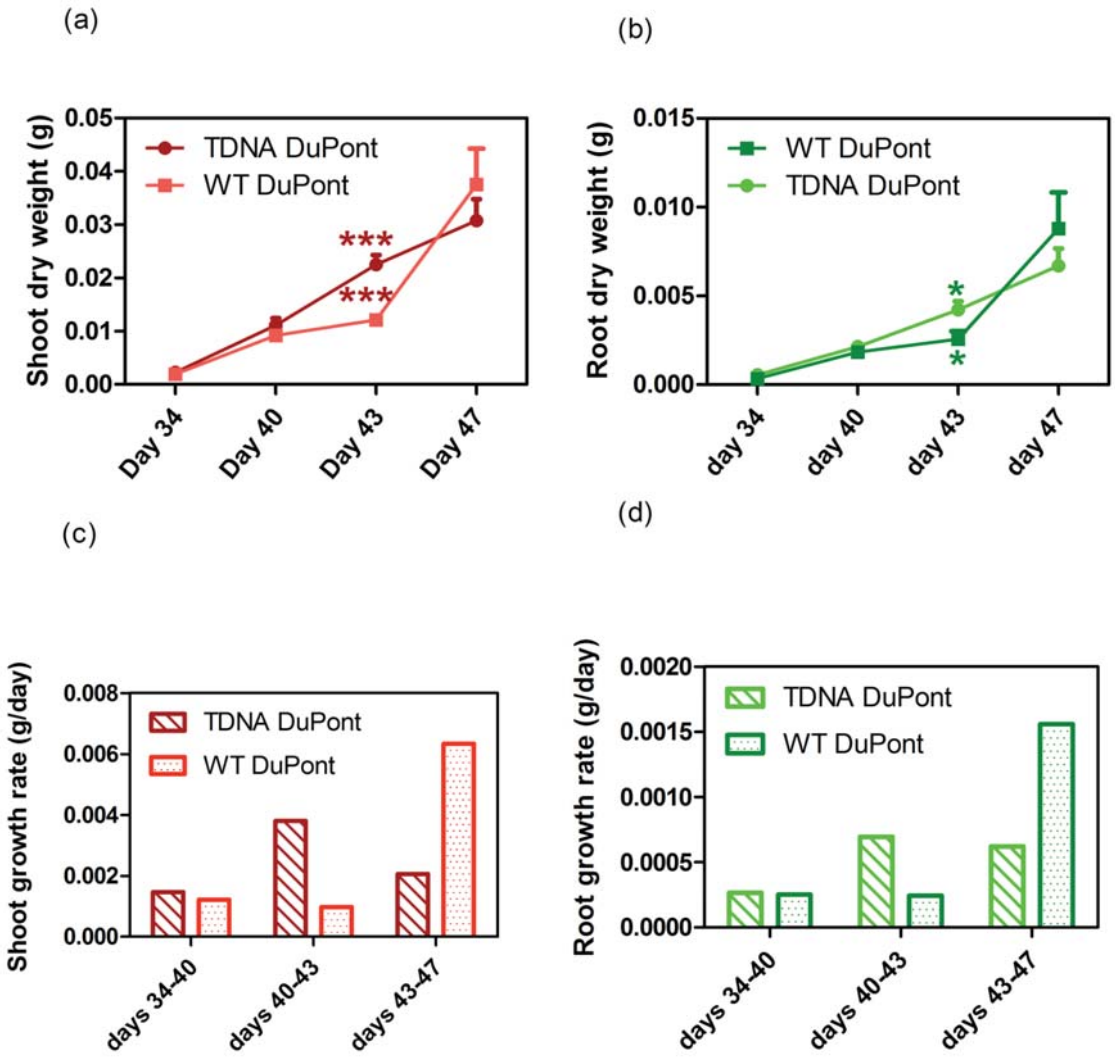

(a)
Shoot dry weight (g)
0.05
0.04
0.03
0.02
0.01
0.00
TDNA DuPont
WT DuPont
***
***
Day 34
Day 40
Day 43
Day 47
(b)
Root dry weight (g)
0.015
0.010
0.005
0.000
WT DuPont
TDNA DuPont
*
*
day 34
day 40
day 43
day 47
(c)
Shoot growth rate (g/day)
0.008
0.006
0.004
0.002
0.000
TDNA DuPont
WT DuPont
days 34-40
days 40-43
days 43-47
(d)
Root growth rate (g/day)
0.0020
0.0015
0.0010
0.0005
0.0000
TDNA DuPont
WT DuPont
days 34-40
days 40-43
days 43-47

Figure 3

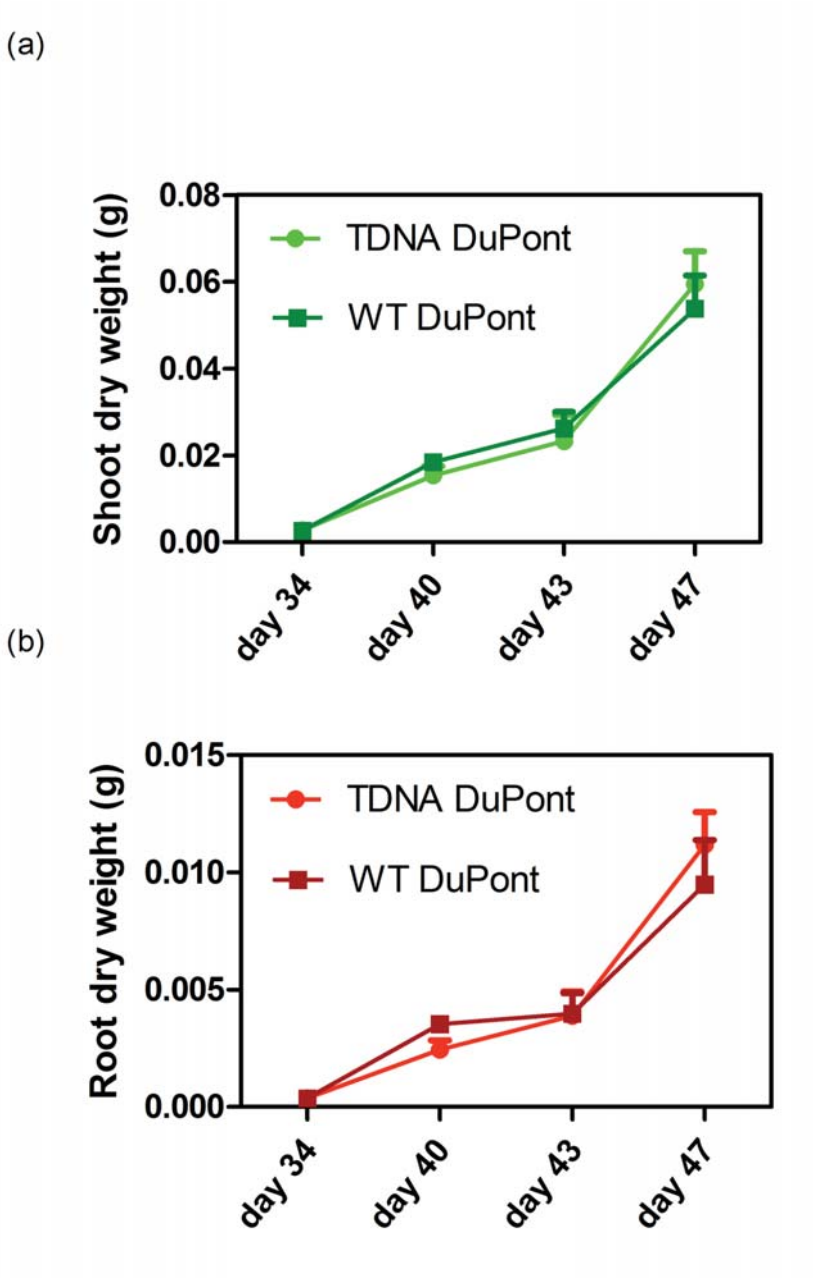

(a)
Shoot dry weight (g)
0.08
0.06
0.04
0.02
0.00
TDNA DuPont
WT DuPont
day 34
day 40
day 43
day 47
(b)
Root dry weight (g)
0.015
0.010
0.005
0.000
TDNA DuPont
WT DuPont
day 34
day 40
day 43
day 47

Figure 4

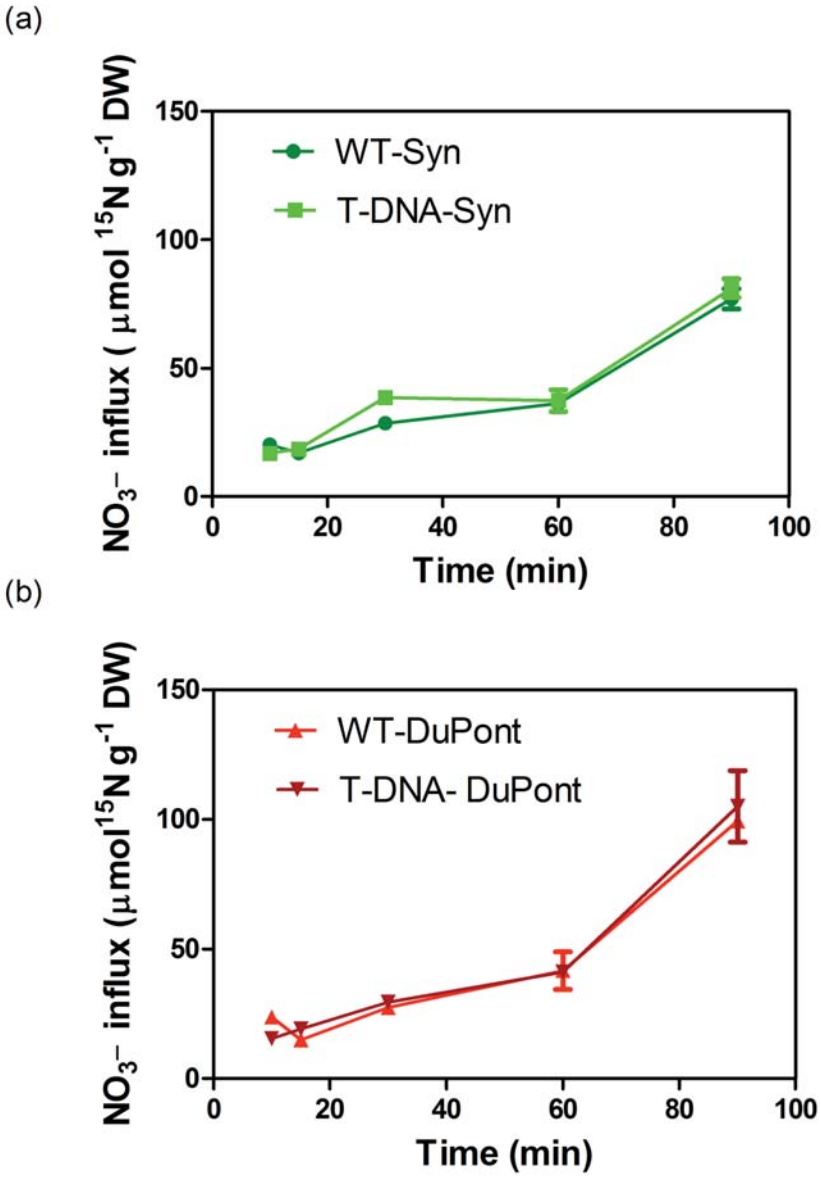
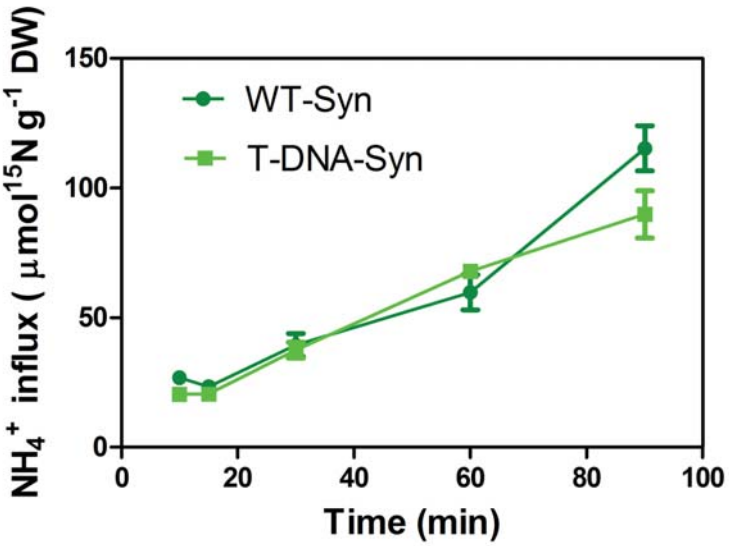

(a)
NO_3^- influx (µmol ^{15}N g^{-1} DW)
150
100
50
0
0
20
40
60
80
100
Time (min)
WT-Syn
T-DNA-Syn
(b)
NO_3^- influx (µmol^{15}N g^{-1} DW)
150
100
50
0
0
20
40
60
80
100
Time (min)
WT-DuPont
T-DNA- DuPont

Figure 5

(a)


WT-Syn
T-DNA-Syn
NH_4^+ influx (μmol^{15}N g^{-1} DW)
Time (min)
0
50
100
150
0
20
40
60
80
100

(b)

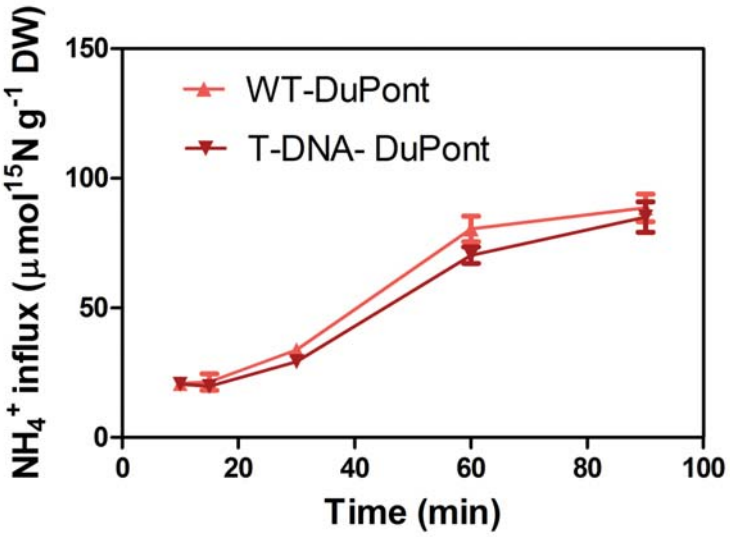

WT-DuPont
T-DNA- DuPont
NH_4^+ influx (μmol^{15}N g^{-1} DW)
Time (min)
0
50
100
150
0
20
40
60
80
100

Figure 6

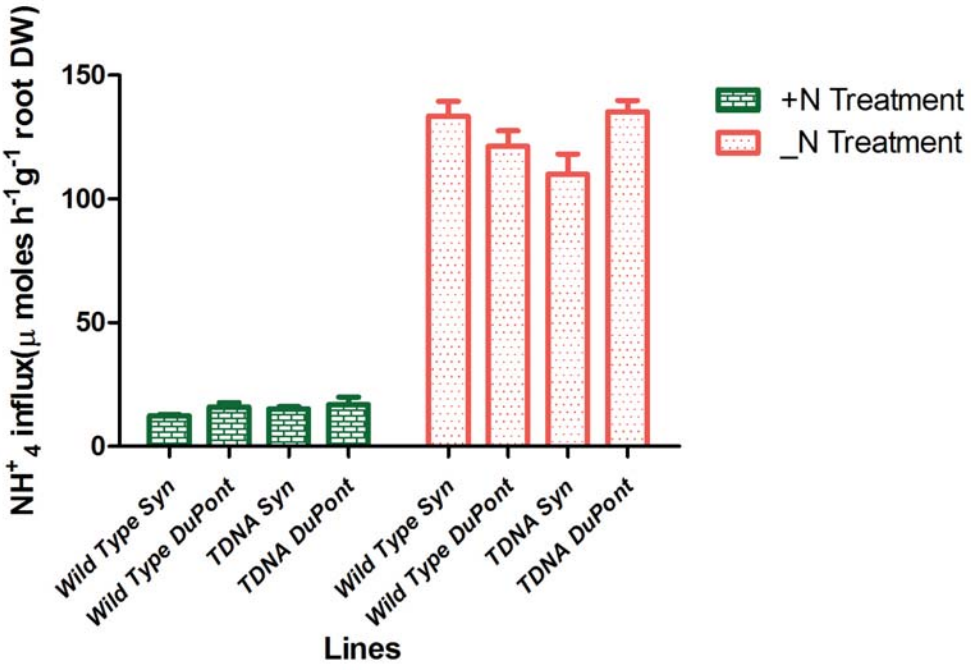
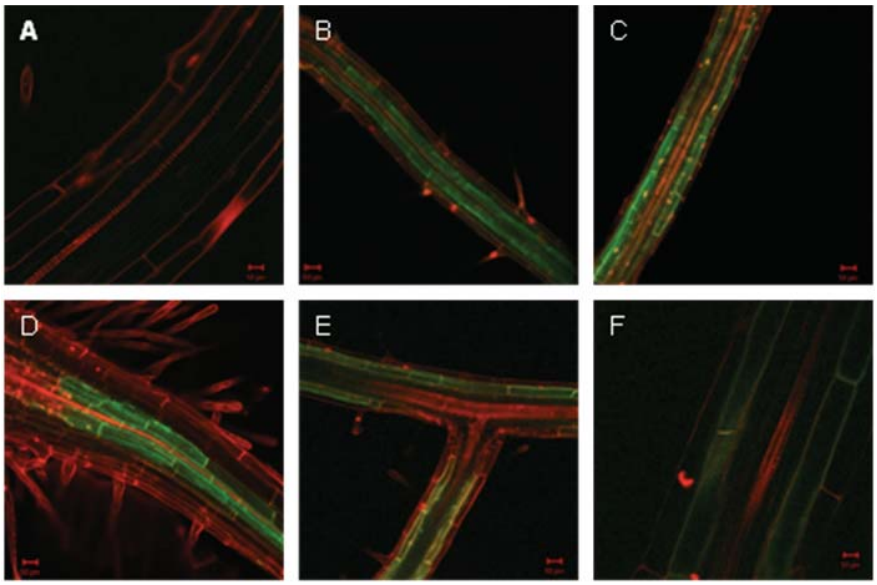

NH^+_4 influx(μ moles $h^{-1}g^{-1}$ root DW)
150
100
50
0
Wild Type Syn
Wild Type DuPont
TDNA Syn
TDNA DuPont
Wild Type Syn
Wild Type DuPont
TDNA Syn
TDNA DuPont
Lines
+N Treatment
_N Treatment

Figure 7

(a)



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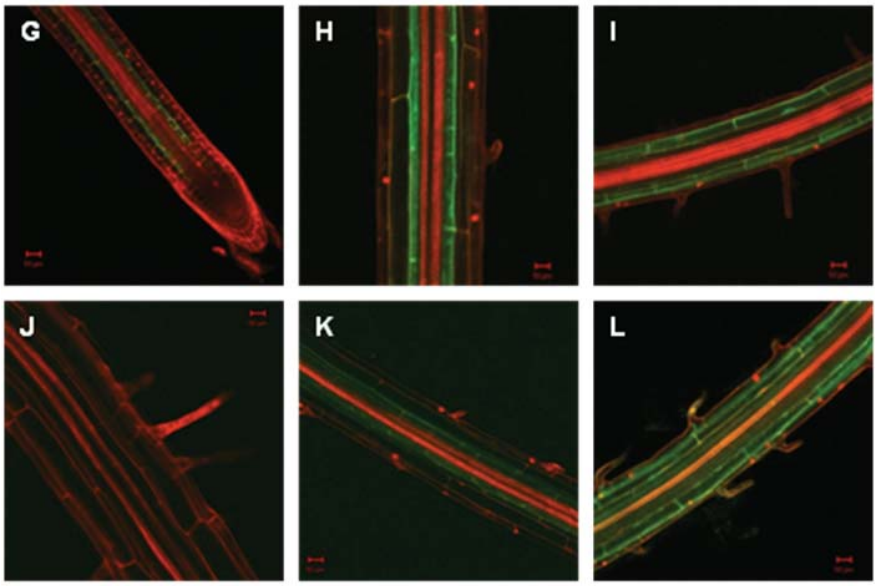
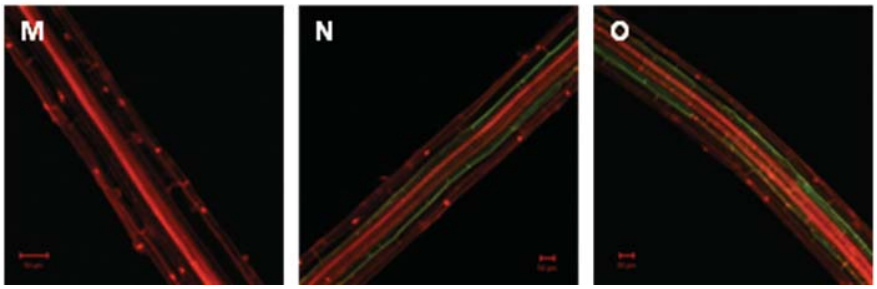


(c)



Chapter 5. Appendices

5.1 Characterisation of an AtAMT1;2 antibody

5.1.1 Microsomal preparation

Arabidopsis plants were grown hydroponically on nutrient solution containing 1mM NH_4NO_3 for six weeks. Four days prior to harvest the plants were moved to fresh nutrient solutions containing 1 mM NH_4NO_3, 2mM KNO_3 or a N free media. Shoot and root tissues were harvested and snap frozen in liquid N_2 and stored at -80°C for later use. Root and shoot tissues were eventually ground in liquid N_2 in a mortar and pestle. Approximately 500-700 mg of frozen ground tissue was transferred to an ice-cold mortar and ground in 2.5 ml of extraction buffer [25mM MES-KOH (pH 7), 350 mM Mannitol, 3 mM $MgSO_4$, 3 µL Sigma Plant Protease Inhibitor Cocktail per 100 mg frozen tissue]. Homogenised tissues were passed through 4 layers of miracloth (Calbiochem) and centrifuged at 20,000 x *g* for 20 minutes. The supernatant was collected and centrifuged further at 125, 000 x *g* for 1 hour at 4°C. The pellet (microsomal fraction) was resuspended in 100-300 µl of solubilisation buffer [50 mM KPO_4 (pH 7.5), 20% v/v Glycerol, 1mM $MgCl_2$, 3 µL Sigma Plant Protease Inhibitor Cocktail].

5.1.2 Protein quantification and SDS-PAGE analysis

Proteins in both the soluble and microsomal fractions were quantified using a modified Lowry assay (BioRad DC). All protein fractions were examined by SDS-PAGE and stained in Coomasie brilliant blue R250 (Sigma) to verify both quality and concentrations. In a typical SDS-PAGE experiment, 15 µg of protein was loaded per lane in either a 2x [42 mM Tris-HCl, 56 mM Tris-Base, 2 mM SDS, 5 mM EDTA and 40 mM Glycerol] or 5x [15% (v/v) β-mercaptoethanol, 15%

(w/v) SDS, 50% (v/v) Glycerol and 1.5% (w/v) Bromophenol blue] loading buffer. The samples were heated at 90° C for 5 min before loading. The gels were electrophoresed in Tris-Glycine running buffer [0.019 M Tris-HCl , 4% (w/v) SDS and 0.19 M glycine]. Polyacrylamide gels (12% v/v) were prepared using the BioRad minigel system according to the manufacturers instructions (BioRAD).

5.1.3 Western Blotting

The size separation of proteins was performed using SDS/PAGE. Proteins were then transferred to nitrocellulose C extra membrane (Amersham-Biosciences) using the semi-dry method. Protein transfer was accomplished using 0.8 mA per square meter of membrane for 90-120 min with transfer buffer [0.037 M Glycine, 1.047 M Tris, 1.3 mM SDS and 20 % (v/v) MeOH]. The membrane was then blocked in 1% (w/v) BM Blocking reagent (Roche) overnight at 4°C or 1 hour at room temperature. The primary antibody was diluted in either TBS buffer [50 mM Tris, 150 mM NaCl] or 0.5% blocking reagent (Roche). The incubation was performed either overnight at 4°C or for 1 hour at room temperature. The membrane was then washed 3 times (10 minutes) in TBS buffer plus 0.2% (v/v) Tween-20 (Sigma). The membrane was incubated in a secondary horseradish peroxidase-conjugated anti-rabbit antibody (Roche) at a 1:10000 dilution of 0.5% (w/v) blocking solution (Roche) or 1 X TBS for 1 hour at room temperature. The blot was then washed three times (10 min each) with 1 X TBS-Tween buffer and then incubated in detection buffer (Roche) for 1 min at room temperature and luminescence captured on a BioRad Chemidoc XRS imager (Figure 1).

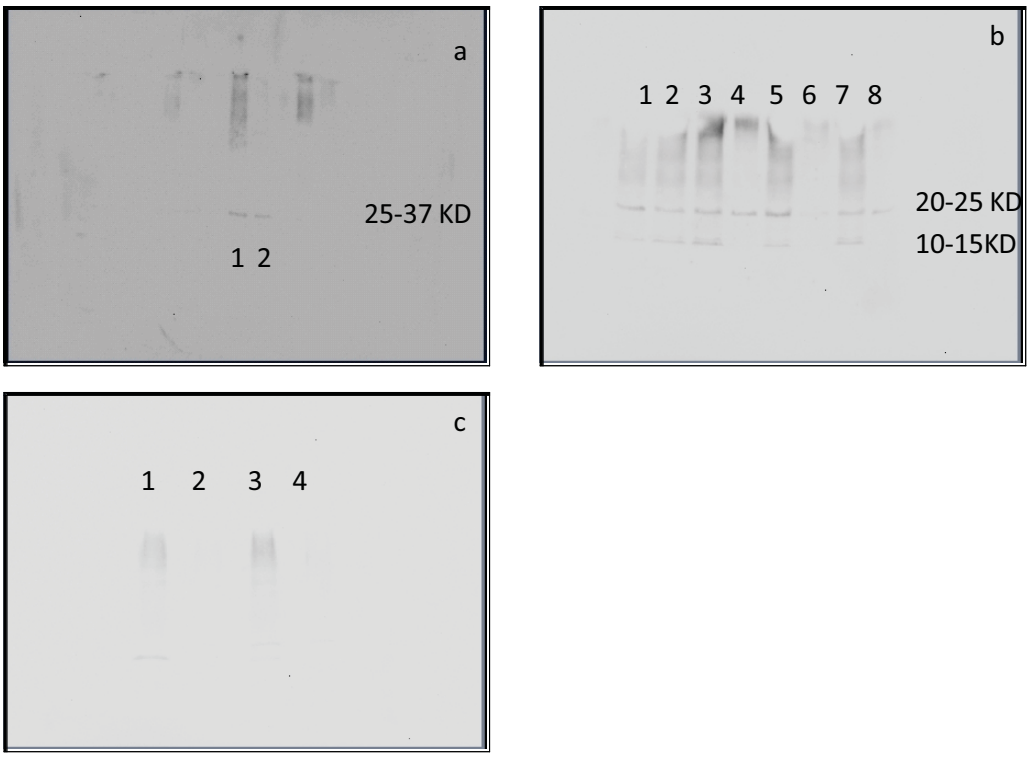


Figure 1.Western blot analysis of microsomal protein fractions of six-week-old Arabidopsis plants.

(a) Western blot analysis of the DuPont T-DNA and its corresponding parental line. Lanes 1, 2, 3 and 4 are wild type root, wild type shoot, T-DNA root and T-DNA shoot, respectively. The membrane was incubated in a dilution of 1:1000 primary and 1:10000 of secondary antibody. (b) Western blotting on DuPont T-DNA and WT lines. Lane 1 and 2 are the T-DNA root and shoot samples, while 3 and 4 correspond to wild type root and shoot, respectively. After blocking over night at 4°C, the membrane was incubated in a dilution of 1:1000 primary and 1:10000 of secondary antibody. (c) Western blot analysis against the Syngenta T-DNA insertion line (lane 3 and 4, shoot and root respectively) and wild type line (lane 1 and 2, shoot and root respectively). After blocking over night at 4°C, the membrane was incubated in a dilution of 1:1000 primary and 1:10000 of secondary antibody.

5.1.4 Protein detection of AMT1;2 expression in yeast

In order to determine the specificity of the antibody it was tested against a total yeast protein extract from the *Saccharomyces cerevisiae* strain 31019b which is deficient in all three AMT/MEP proteins. In these experiments 31019b was transformed with an empty yeast expression vector (pYES3) or one containing AtAMT1;2. Cells were grown to mid log phase in YNB (Yeast nitrogen base w/o amino acids) supplemented with 2% (w/v) galactose at 30° C with constant shaking (200 rpm). Cells were pelleted by centrifugation at 1500 x *g* for 3 min. Total yeast protein was extracted (PIERCE, protein extraction kit, USA) and mixed with equal volume of 2 x SDS sample buffer [25 mM Tris-HCL (pH 6.8), 9 M urea, 1mM EDTA, 1% (w/v) SDS, 0.7 M β-mercaptoethanol and 10% (v/v) glycerol] was added to extracted protein. The amount of protein was estimated using Commasie blue staining (Figure 2a). Unfortunately, the Western blot experiment failed to detect any band that could be indicative of AMT1;2 (Figure 2b).

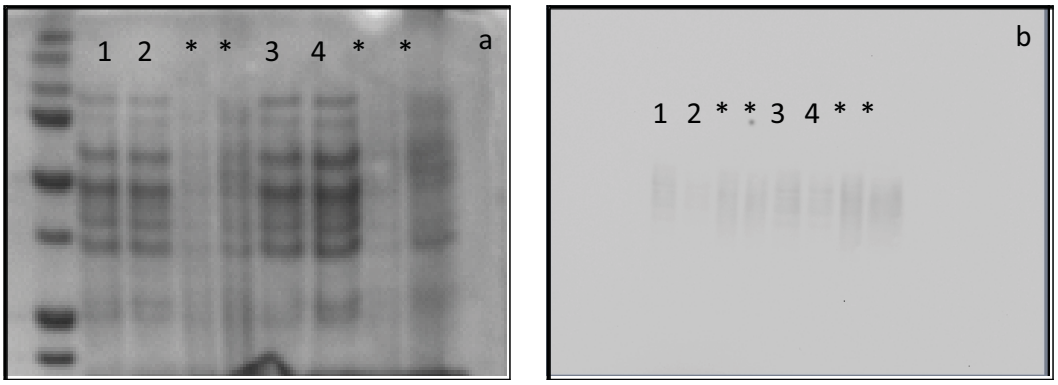


Figure 2. Western blot analysis of AMT1;2 expressed in yeast cells

Total yeast protein was extracted from yeast cells grown in YNB (yeast nitrogen base w/o amino acids) supplemented with 2% (w/v) galactose. (a) Coomassie stained SDS-PAGE gel of total protein extracts from yeast cells containing the empty pYES vector (lane 2 and 4) or pYES containing AtAMT1;2 (lane 1 and 3).Lane 1 and 2 contain 6 µL while 3 and 4 contain 9 µL of soluble protein fraction. The lanes which indicated with * contains insoluble pellet. (b) Western blot analysis of total yeast protein extracted from

yeast cells expressing AtAMT1;2 (lane 1 and 3) and empty control vector (lane 2 and 4). Lanes 1 and 2 contain 6 µL while 3 and 4 contain 9 µL of soluble protein fractions. The lanes which indicated with * contains insoluble pellet.

5.1.5 Western blot analysis of plant microsomal protein fractions using a purified primary anti-AtAMT1;2 antibody

The original AMT1;2 antibody was originally raised against a commercially synthesized AMT1;2 specific peptide (H-Cys-Pro-Trp-Gly-His-Phe-Ala-Gly-Arg-Val-Glu-Pro-Thr-Ser-Arg-Ser-OH) (Kaiser *et al.* unpublished results). In order to minimize non-specific binding of the AtAMT1;2 antibody to other proteins as well as other AMT family members in Arabidopsis, we purified the AtAMT1;2 antibody using the Microlink peptide coupling kit (PIERCE, USA), where the AMT1;2 peptide was used to affinity purify AMT1;2 specific antibodies within the crude rabbit serum. A 1:1000 dilution of the purified primary antibody was used on subsequent western blotting experiments (Figure 3).

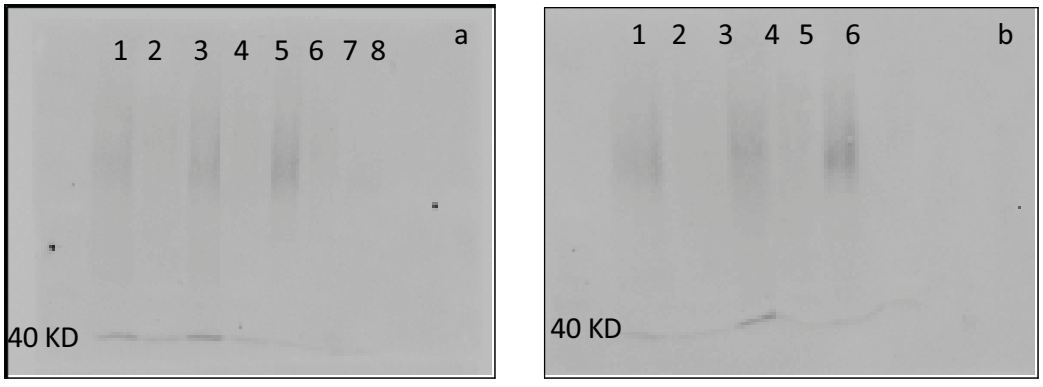


Figure 3.Western blot analysis of microsomal fractions of AtAMT1;2 T-DNA and WT lines using a purified AMT1;2 antibody.

Microsomal preparation was performed on plants grown on different N sources. SDS-PAGE and Western blotting was performed as described previously using a 1:1000 dilution of primary and 1:10000 of secondary antibody. (a) Lanes 1, 2, 5 and 6 are DuPont T-DNA root, DuPont T-DNA shoot, Syngenta T-DNA root and Syngenta T-DNA shoot, respectively. Microsomal fractions were extracted from tissues of plants grown on media containing 1mM NH_4NO_3. Lanes 3, 4, 7 and 8 are DuPont WT root, DuPont WT shoot, Syngenta WT root and Syngenta WT shoot, respectively. (b) Lane 1 and 2 are corresponding to root and shoot of 1mM NH_4NO_3 grown plants, respectively. Lanes 3 and 4 are root and shoot of WT plants grown in media containing minus N and 5 and 6 are root and shoot fractions from 4-day-treated plants containing 2mM KNO_3, respectively.

5.1.6 Conclusion:

Unfortunately, an antibody raised against AtAMT1;2 failed to detect native AMT1;2 in multiple western blot experiments of root or shoot microsomal fractions. In most cases there was considerable nonspecific binding by the crude AtAMT1;2 primary antibody (Figure 1B and C). The antibody also failed to pick up AtAMT1;2 when over expressed in yeast cells (Figure 2B). A third attempt using an affinity purified AMT1;2 antibody also failed to detect the correct sized protein (55 -80 kDa). At this stage it would appear the peptide derived AMT1;2 antibody is poor and not specific enough to detect AMT1;2 in either plant or yeast samples.

5.2 Confocal microscopy on leaves of the *amt1;2* T-DNA (DuPont) and its corresponding parental lines

We examined the expression of AtAMT1;2 using confocal microscopy on plants carrying an AMT1;2 promoter/AMT1;2/GFP (Rajeev Gupta, unpublished result) construct. Expression in roots was presented previously. In shoot tissues we were interested in identifying where AtAMT1;2 was localized and in particular if it was localized in the chloroplasts. In younger leaves (7 days post germination), there was an enhancement of GFP signal in chloroplasts of the GFP tagged line over the wild type. However this signal was quickly overcome by auto fluorescence as the plants increased in age. From 21 days it was not possible to detect any difference in signal intensity in wild type versus transgenic GFP lines (Figure 4).

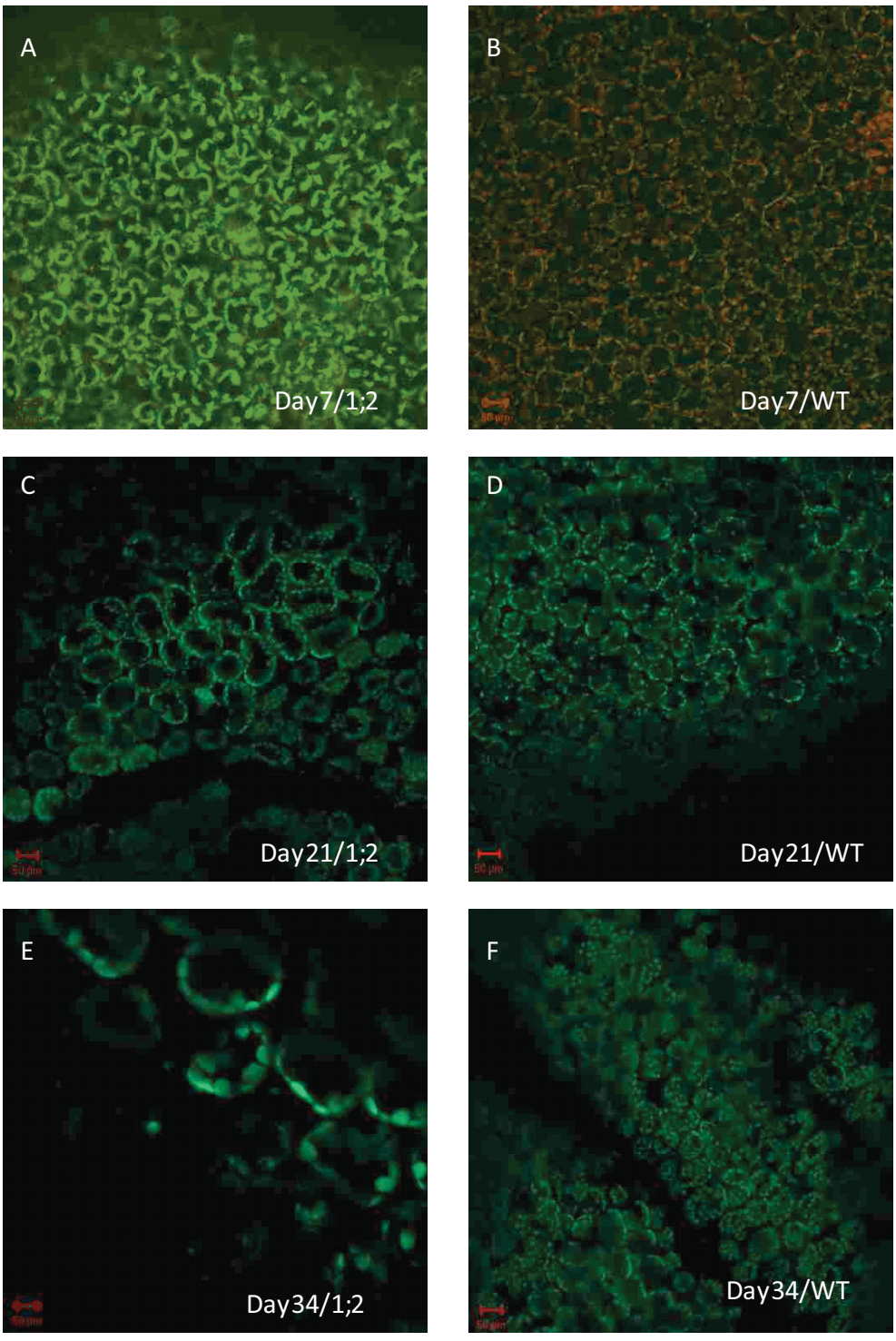


Figure 4. Cellular localization of AtAMT1;2 in leaves of Arabidopsis.

Col-0 plants containing a AMT1;2 promoter:AtAMT1;2/GFP construct were examined for GFP localization in shoot. Plants has been grown either on plates contained modified 0.5 x nitrogen free MS media (A, B, C and D) or in hydroponic solution containing 1mM NH_4NO_3 (E and F). No sucrose was added to media. Bars = 50µm. Each picture is a true representative from multiple plants (n=3).

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