



**Potato and Bambara groundnut ammonium transporter (AMT1)
structure and variation in expression level in potato leaf tissue
in response to nitrogen form and availability**

by

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DECLARATION

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ABSTRACT

Plants require nitrogen (N) to support desired production levels. Nitrogen fertilization strategy is a major consideration in field management with regard to achieving both economic and environmental objectives. For instance, in potato, insufficient N supply reduces tuber size and overall yield while excessive N supply can reduce tuber quality and increase environmental risk through nitrate (NO_3^-) leaching and nitrous oxide emission. Selection of an adequate N fertilizer application rate for crops is difficult, due to marked variations in soil N supply and crop N demand in both the field and over time. This research was conducted to characterise the ammonium transporter gene (AMT1) of Bambara groundnut and potato using molecular biology and bioinformatics methods. Nucleotide database sequences were used to design AMT1-specific primers which were used to amplify and sequence the core-region of the gene from Bambara groundnut and potato. Bioinformatics techniques were used to predict the structure and infer properties of the proteins. Nucleotide sequence alignment and phylogenetic analysis indicate that BgAMT1 and PoAMT1 are indeed from the AMT1 family, due to the clade and high similarity they respectively shared with other plant AMT1 genes. Amino acid sequence alignment showed that BgAMT1 is 92%, 89% and 87% similar to PvAMT1.1, GlycineAMT1 and LjAMT1.1 respectively, while PoAMT1 is 92%, 83% and 76% similar to LeAMT1.1, LjAMT1.1 and LeAMT1.2 respectively. BgAMT1 and PoAMT1 fragments were shown to correspond to the 5th - 10th transmembrane spanning-domains. Mutation of Bg W1A-L and S28A (for BgAMT1) and Po S70A (for PoAMT1) is predicted to enhance ammonium (NH_4^+) transport activity. Residues Bg D23 (for BgAMT1) and Po D16 (for PoAMT1) must be preserved otherwise NH_4^+ transport activity is inhibited. In all, BgAMT1 and PoAMT1 play a role in N uptake from the root while BgAMT1 may contribute more in different steps of rhizobia interaction.

In an investigation of the correlation between AMT1 gene expression levels and leaf chlorophyll content index (CCI) with plant N status, potato plants were grown in a hydroponic greenhouse with 0.75 or 7.5 mM NO_3^- and 0.75 or 7.5 mM NH_4^+ as forms of N supply in a completely randomized design. Leaf CCI as measured by chlorophyll content meter, showed that an increase in N supply results in increased leaf CCI in response to both forms of N. Total RNA was isolated from leaf sampled at 28 days after treatment and expression level of the AMT1 gene was determined by reverse transcription-qPCR using a second set of primers designed for qPCR. The results showed that expression levels of AMT1 increased from 8.731 ± 2.606 when NO_3^- supply was high to 24.655 ± 2.93 when NO_3^- supply was low. However, there was no significant response in AMT1 expression levels to changes in NH_4^+ .

This result suggested that AMT1 transports NO_3^- less efficiently than NH_4^+ , and thus more transport channels are required in the cell membrane when NO_3^- levels are low. Such variation in AMT1 expression levels are not necessary for NH_4^+ transport since the transport mechanism for NH_4^+ is efficient even at low NH_4^+ levels.

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DEDICATION

I dedicate this work to God Almighty the source of my strength.

RESEARCH OUTPUTS

The following research outputs represent contributions by the candidate to scientific knowledge and development during his master's candidacy (2013-2014):

➤ **Publication**

Adetunji, A.T., Lewu, F.B. & Mundembe, R. *Vigna subterranea* ammonium transporter gene: some bioinformatics insights. Submitted to *Biotechnology Reports* (2015)

➤ **Presentations**

Adetunji, A.T., Lewu, F.B. & Mundembe, R., 24th The South African Society of Biochemistry and molecular biology conference 6 – 9 July 2014, Rawsonville, South Africa, Paper: Development of a reverse transcription polymerase chain reaction protocol for the expression profiling of the ammonium transporter gene.

Adetunji, A.T., Lewu, F.B. & Mundembe, R., 2nd U6 Consortium international conference 8-10 September 2014, Cape Town, South Africa, Paper: Expression profiling of the ammonium transporter gene using a reverse transcription-quantitative polymerase chain reaction.

LAYOUT OF THESIS

The overall aim of this study is to amplify and sequence the core-region of AMT1 gene from potato and Bambara groundnut, and quantitatively examine the expression of potato AMT1 gene in response to nitrogen rate and form. The references are listed at the end as a separate chapter in accordance with the Harvard method of referencing.

The thesis is subdivided into the following chapters:

- **Chapter 1**, the introduction, provides the background information on nitrogen, ammonium transporter and reverse transcription-quantitative polymerase chain reaction. It also presents the problem statement, hypothesis, research objectives and the significance of the study.
- **Chapter 2**, the literature review, provides information on nitrogen, nitrogen fertilizer and nitrogen assimilation from root. It includes information about ammonium transporter gene and their homologs in various plant species. This section reports information on the use of reverse transcription-quantitative PCR for mRNA quantification.
- **Chapter 3**, the research methodology. It summarises the materials and methods used in this study to design ammonium transporter1-specific primers, amplify and sequence Bambara groundnut and potato ammonium transporter1 genes. It involves growing potatoes hydroponically, isolate total RNA and measure potato ammonium transporter1 expression levels.
- **Chapter 4**, entails the results and discussion chapter.
- **Chapter 5**, overall conclusion and recommendations, presents the answers to the research questions in chapter 1 while also listing recommendations for future research.

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GLOSSARY

Abbreviation	Description
AMT	Ammonium transporter
Bg	Bambara groundnut
BLAST	Basic local alignment search tool
bp	Base pair
CCI	Chlorophyll content index
cDNA	Complementary deoxyribonucleic acid
GS	Glutamine synthase
GS/GOAT	Glutamate synthase/Glutamine oxoglutarate aminotransferase
HAT	High affinity transporter
HGT	Horizontal gene transfer
K_m	Michaelis constant
LAT	Low affinity transporter
MEP	Methyl ammonium permease
MPSS	Massive parallel signature sequencing
mRNA	Messenger ribonucleic acid
N	Nitrogen
NCBI	National center for biotechnology information
NH_3	Ammonia
NH_4^+	Ammonium
NO_3^-	Nitrate
PCR	Polymerase chain reaction
Rh	Mammalian rhesus
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SAGE	Serial analysis of gene expression
UTD	Up to date

Clarification of Terms

Ammonium transporter: Proteins which transport ammonium ions across the cell membrane.

Polymerase chain reaction: A sensitive technique used to amplify DNA.

Reverse transcription-qPCR: A sensitive technique used to quantitatively detect RNA expression levels.

CHAPTER ONE

INTRODUCTION

1.1 Background

Nitrogen (N) is an essential macronutrient required by all plants to thrive. It is the mineral nutrient needed in the highest amount and its availability is a major factor restricting plant growth in natural (Epstein and Bloom, 2005) and agricultural (Galloway and Cowling, 2002) environments. The N composition of plant tissues also has important nutritional effects, since plants constitute a significant source of protein in the diet of human and animals. Although plants can absorb small amount of N from the atmosphere through their foliage, a greater part of N is acquired in specific forms in the soil (Below, 2001). Plant roots mostly obtain N from the rhizosphere as inorganic ammonium (NH_4^+) or nitrate (NO_3^-), and later absorb intracellular NH_4^+ into amino acids (Lea and Azevedo, 2007). Roots sense and respond to changes in internal and external N status, which includes the regulation of gene expression, metabolism, and further N uptake and assimilation (Glass *et al.*, 2002). Most soils however do not have sufficient N in the available form to support desired production levels. Therefore, addition of N from fertilizer is typically needed to maximize crop yield and quality.

Management of nitrogen fertilizer is important in attaining both economic and environmental objectives in crop production. It was reported in potato that deficient supply of N leads to economic losses owing to decrease in tuber size and overall yield (Millard and Marshall, 1986, Zebarth and Rosen, 2007). Excessive supply of N can reduce tuber quality through decreased tuber specific gravity (Long *et al.*, 2004), increased tuber nitrate concentration (Zebarth *et al.*, 2004) and increased accumulation of asparagine which can contribute to acrylamide formation during cooking (Lea *et al.*, 2007). However, corresponding effects of excessive N supply have not been studied in Bambara groundnut. Increased N fertilizer rate above the optimum rate may lead to rapid increase in N loss from the environment through nitrate leaching (Van Es *et al.*, 2002) and nitrous oxide emissions (Zebarth *et al.*, 2008).

Correlating the supply of N to the crop N demand is one of the most efficient approaches to meet both economic and environmental objectives (Zebarth *et al.*, 2009). Although the idea is simple, selection of an adequate fertilizer N rate is difficult owing to marked variation in both soil N supply and crop N demand in both the field and over the time (Olfs *et al.*, 2005, Scharf *et al.*, 2005). As a result of this, efficient approaches have been put in place where the in-season management of N fertilizer is optimised by measuring crop N sufficiency (Goffart and Olivier, 2004, Goffart *et al.*, 2008).

Olfs *et al.* (2005) evaluated a few chemical and optical methods of estimating crop N sufficiency. The most frequently used chemical and optical measures of crop N sufficiency in potato production are petiole nitrate concentration (Westcott *et al.*, 1991) and relative leaf chlorophyll concentration as measured by light transmittance using a SPAD-502 meter (Goffart *et al.*, 2008, Minotti *et al.*, 1994). A recent study by Tremblay *et al.* (2007) has also reported the potential to identify plant N sufficiency using a Dualex instrument, which quantifies plant polyphenolics optically. Although, these methods are generally effective, yet they provide indirect measures of crop N sufficiency. Another drawback to using these approaches is that they may be influenced by the availability of water or other nutrients in soil, environmental conditions (Olfs *et al.*, 2005) and the stage of crop development (Millard and Mackerron, 1986).

Ammonium transporters (AMTs) of the Ammonium Transporter/Methylammonium Permease/mammalian Rhesus (AMT/MEP/Rh) protein family have been revealed in all domains of life, particularly plants, bacteria, archaea, yeast, and animals (Ludewig *et al.*, 2001). All plant ammonium transporter (AMT) proteins investigated so far are located in the plasma membrane, indicating that their role is in ammonium acquisition by plant cells (Loque *et al.*, 2006, Ludewig *et al.*, 2002, Ludewig *et al.*, 2003, Simon-Rosin *et al.*, 2003, Sohlenkamp *et al.*, 2002, Yuan *et al.*, 2007b). The expression levels of AMT1 mostly increase when N supply is low and decrease in response to high N supply, consistent with an optimized ammonium uptake function required in N-starvation conditions (Couturier *et al.*, 2007, Gazzarini *et al.*, 1999, Pearson and Stewart, 1993, Rawat *et al.*, 1999, Von Wiren *et al.*, 2000a). Recent evidence indicates that the expression of AMT1 in potato is less influenced by N form, developmental stage and environmental factors, than currently used chemical (petiole nitrate concentration) or optical (SPAD meter) measures of plant N status (Zebarth *et al.*, 2012). Hence, quantification of AMT1 gene expression in leaf tissue of plants could be better than petiole nitrate concentration or SPAD-502 meter readings as measures of accessing plant N status (Zebarth *et al.*, 2012). However, while the method used for gene expression analysis by Zebarth *et al.* (2012) is ideal for research, it is too expensive for routine diagnosis; a simple reverse transcription-quantitative polymerase chain reaction (RT-qPCR) method may be more appropriate.

Gene expression profiling provides new insights into regulatory gene networks and offers more in-depth understanding of various biological processes (Wise *et al.*, 2007). Reverse transcription-PCR (RT-PCR) is a technique widely used among other robust methods to study changes in gene expression (Cruz *et al.*, 2009). Messenger ribonucleic acid (mRNA)

quantification method avoids the need to use suitable antibodies and has the ability to detect very small amounts of target molecule, depending on the technique used (Thellin *et al.*, 2009). In order to measure messenger RNA (mRNA), reverse transcriptase is used to convert mRNA into complementary DNA (cDNA), which will then be amplified by PCR (Bustin, 2005). Thus, RT-PCR may be used for qualitative, semi-quantitative or quantitative detection. Real time-PCR is one of the most powerful techniques for analysing mRNA expression because of its sensitivity, specificity, wide dynamic range and low template concentration requirements (Shi and Chiang, 2005). Apart from giving quantitative results, real-time PCR is also easy and convenient to use (Radonic *et al.*, 2004).

This work proposes to amplify and sequence AMT1 gene fragments from potato and Bambara groundnut, and quantitatively examine the expression of potato AMT1 gene in response to nitrogen (ammonium and nitrate) supply.

1.2 Statement of research problem

Nitrogen is available to plants in NH_4^+ or NO_3^- form (Barker and Mills, 1980). Most soils however do not have sufficient N in the available form to support desired production levels. Therefore, addition of N from fertilizer is typically needed to maximize crop yield and quality. Selection of the adequate N fertilizer application rate for crops is difficult, due to marked variation in both soil N supply and crop N demand in the field and over time (Zebarth *et al.*, 2009). There is need for greater understanding of AMT and N transport mechanism. Thus, it is proposed that the in-season management of N fertilizer is improved by measuring crop N sufficiency through determination of ammonium transporter expression levels in crop species.

1.3 Hypothesis

It is hypothesized that the similarity between Bambara, potato and other plant AMT genes is sufficient to enable design of primers that amplify a significant portion of the gene. In addition, the amplified gene should allow assignment of function to some sections of the amplified gene. It is further hypothesized that the primers similarly designed may be used in real-time RT-PCR to determine levels of AMT1 expression, which in turn, may be used to give an insight into the nitrogen status of the plant.

1.4 Objectives of the research

The objectives of the research are to:

- I. Amplify and sequence the core-regions of the AMT1 gene from potato UTD and Bambara groundnut.
- II. Examine the expression of AMT1 in relation to nitrogen supplied.
- III. Examine the response of leaf chlorophyll content index to nitrogen supplied.

1.5 Significance of the research

This research guides fertilizer management on potato cultivars in South Africa. This study will contribute to the following:

- I. Contribute to better understanding of the structure and function of AMT1 gene.
- II. Assist farmers in applying the optimal N fertilizer for maximum crop yield and quality.
- III. Minimise nitrogen fertilizer wastage.
- IV. Minimise environmental pollution.

CHAPTER TWO LITERATURE REVIEW

2.1 Bambara groundnut

Bambara groundnut (*Vigna subterranean*) is a member of the family *Fabaceae*. Bambara groundnut is indigenous to Africa and it is the third most important grain legume after groundnut (*Arachis hypogaea* L.) and cowpea (*Vigna unguiculata*) (Baryeh, 2001). It adapts well to hot, dry temperature, yet it also tolerates high rainfall (Dakora and Muofhe, 1996). Furthermore, Bambara grows in soils 5.0 - 6.5 pH with 600 - 1200 mm annual rainfall (Baryeh, 2001). It is easy to cultivate and makes very little or no demand on the soil (Swanevelder, 1998). Bambara groundnut matures in 4 - 5 months with average yield of 650 - 850 kg/ha (Baryeh, 2001). This legume crop is largely produced and consumed in semi-regions of Africa where rainfall is erratic and low, and losses to run-off, drainage and evaporation may leave only a small portion of precipitation available for crop growth (Mwale *et al.*, 2007). Hence, it is an important crop owing to the fact that it is drought resistant and has the ability to produce reasonable yield in poor soils (Mwale *et al.*, 2007, Swanevelder, 1998). In fact, studies have shown that it performs more than groundnut in dry environments (Collinson *et al.*, 1996, Mwale *et al.*, 2007). It serves as a key source of protein in the diets of a large percentage of the population in Africa, especially to poorer people who cannot afford expensive animal protein (Baryeh, 2001). Indeed, the protein content of Bambara is comparable or superior to other legumes (Linnemann and Azam Ali, 1993), making it a good complement for cereal-based diets (Mwale *et al.*, 2007). Bambara groundnut seed consist of 49%–63.5% carbohydrate, 15% – 25% protein, 4.5% – 7.4% fat, 5.2% – 6.4% fibre, 3.2% – 4.4% ash, 0.098% calcium, 0.007% iron, 1.2% potassium, 0.003% sodium and 2% mineral (Baryeh, 2001, Mwale *et al.*, 2007). It has a crucial impact in sustainable agriculture due to its ability to fix atmospheric N₂. Despite the fact that Bambara groundnut has high nutritional values, functional properties, antioxidant potential, and a drought resistant ability, it remains one of the most neglected crops by science. Hence, the under-utilised crop with great potential is not well funded.

2.2 Potato

Potato (*Solanum tuberosum* L.) is a member of the family *Solanaceae*. It is one of the world's major staple food crops that was first cultivated 8000 years ago in the Andean highlands of southern Peru (Lutaladio and Castaldi, 2009), which later spread to Europe and other parts of the world from the beginning of 16th century (Bradeen and Haynes, 2011).

There are about 5000 varieties of potato (Burlingame *et al.*, 2009, Litaladio and Castaldi, 2009) and its global production has been estimated at 368 million tonnes in 2013 (<http://faostat3.fao.org/>). Potato is the fourth most essential food crop in the world, behind rice, wheat and maize. It is however, the only major food crop that is a tuber (Ezekiel *et al.*, 2013, Spooner and Bamberg, 1994). With respect to fresh matter, potato is the third highest yielding crop following sugarcane and sugar beet (Abdel-Aal *et al.*, 1977, Ayyub *et al.*, 2011). Potatoes are herbaceous perennial plants usually about 60cm height depending on variety. A large number of potato cultivars are autotetraploid ($2n=4x=48$ chromosomes), highly heterozygous, suffer acute inbreeding depression and, although it produces true botanical seed, is usually propagated asexually (Bradeen and Haynes, 2011, Consortium, 2011).

In 1845, a large number of peasant populations in Ireland and across Europe were so dependent on potato as a primary food that at least one million people died through famine when the potato farm was infested by late-blight pathogen (*phytophthora infestans*) (Bradeen and Haynes, 2011, Litaladio and Castaldi, 2009). Since potatoes are cheaply grown and reach maturity within a short period of time, it relieved the entire population from hunger, nourished the emerging urban working class and released rural people for work in 19th-century factories (Litaladio and Castaldi, 2009). Potato is increasingly cultivated because it produces more dry matter, protein and minerals (potassium) per hectare than cereal crops. In addition, it is a major food source of inexpensive energy, iron, vitamin B (B1, B3, B6) and C as well as carbohydrate (Buono *et al.*, 2009, Burton, 1989, Ezekiel *et al.*, 2013). Potatoes produce 130 kcal of energy per person per day in the developed countries where it is known as a staple food, against 41 kcal in the developing countries where it is still considered as a vegetable (Burlingame *et al.*, 2009, Ezekiel *et al.*, 2013, Friedman, 1997). The potato tuber is free from fat and cholesterol and relatively low in calories consequently promoting healthy living (Buono *et al.*, 2009). Apart from its rich nutritional content, potato contains good quantity of antioxidants like ascorbic acid (8 - 54 mg/100g), polyphenols (123 – 441 mg/100g), carotenoids (up to 0.4 mg/100g) and tocopherols (up to 0.3 mg/100g) in the human diet, whose beneficial effects on health has also been reported (Burlingame *et al.*, 2009, Delaplace *et al.*, 2008, Leo *et al.*, 2008). Potato tuber can be boiled for consumption or processed into products including chips, French fries, starch, alcohol production as well as a wide range of frozen and chilled products (Abdel-Aal *et al.*, 1977, Buono *et al.*, 2009, Mcgregor, 2011). However, proper management of nutrient input especially nitrogen is crucial for maximizing potato tuber quality and yield (Guler, 2009, Rowe, 1993).

2.3 Nitrogen

Nitrogen is a key macronutrient and primary mineral constituent limiting agricultural productivity (Galloway and Cowling, 2002). It is required by all organisms for the basic processes of life to make nucleic acids, proteins, chlorophyll molecules, which are crucial for photosynthesis and further growth, and to reproduce (McCallister *et al.*, 2012, Smil, 2002). In the biosphere, N is available for plants in molecular nitrogen (N_2), volatile ammonia or nitrogen oxides, mineral N (NO_3^- and NH_4^+) and organic N forms such as amino acids and peptides (Von Wiren *et al.*, 1997). Nitrogen application is necessary to maintain high crop yields. Most soils however do not have sufficient N in the available form to support desired production levels.

2.3.1 Nitrogen fertilizer

The addition of N from fertilizer has become one of the main yield-enhancing techniques in modern crop production (Scheromm *et al.*, 1992). In fact, adequate N fertilization is crucial for optimizing potato yield and quality (Lauer, 1986, Westermann and Kleinkopf, 1985). As a result of this, about 85 - 90 million metric tonnes of nitrogenous fertilizers are added to the soil worldwide annually (Good *et al.*, 2004). The application of N fertilizer is mostly the single highest input cost for many crops and, since its production is energy intensive, this cost is dependent on the price of energy (Rothstein, 2007). Much of the N from fertilizer added to the soil is lost to the environment, with an average of only 30% - 50% being taken up by the plant depending on the species and cultivar (Garnett *et al.*, 2009). However, the predominant part is usually lost to surface run-off, leaching of nitrates, ammonia volatilization or bacterial competition (Garnett *et al.*, 2009).

Proper management of nitrogen fertilizer plays a crucial role in achieving both economic and environmental objectives in crop production. For instance, in potato, inadequate N supply leads to economic losses owing to reduction in tuber yield and quality (Millard and Mackerron, 1986, Rowe, 1993, Zebarth and Rosen, 2007). These may be as a result of reduced growth (Harris, 1992), reduced light interception (Chase *et al.*, 1990), delayed tuber set (Harris, 1992), reduced dry matter content (Love *et al.*, 2005, Westermann *et al.*, 1994) and an increase in disease which include early die, late blight, and verticillium wilt (Davis *et al.*, 1990, Rowe, 1993). On the other hand, excessive N supply can reduce tuber quality through decreased tuber specific gravity (Long *et al.*, 2004), increased tuber nitrate concentration (Zebarth *et al.*, 2004) and increased accumulation of asparagine that can

contribute to acrylamide formation during cooking (Lea *et al.*, 2007). In addition, excess N may result in increased vegetative growth rather than in tuber production and delayed maturity (Ojala *et al.*, 1990). It may also result in a humid environment that promotes diseases associated with moisture such as aerial stem rot, *Sclerotinia* stem rot, pink rot, and other foliar as well as tuber diseases (Rowe, 1993), and an influence on storability (Long *et al.*, 2004). Environmental losses of N through nitrate leaching to ground water (Houles *et al.*, 2004, Van Es *et al.*, 2002) and nitrous oxide emissions (Chantigny *et al.*, 1998, Zebarth *et al.*, 2008) increase rapidly as fertilizer N rate is increased above the optimum rate. The continuous leaching of nitrate into drainage water as well as the release of nitrous oxide and reactive N gases into the troposphere accelerate the eutrophication of waterways and acidify soils (Guo *et al.*, 2010, Robertson and Vitousek, 2009). Consequently, the risk of environmental pollution is increased.

2.3.2 Correlation of soil N supply to crop N demand

Since N fertilization strategy is a major consideration in field management, synchronization between N availability in the soil and N uptake by plant roots has been reported as a means to meet both economic and environmental objectives (Ladha *et al.*, 2005, Zebarth *et al.*, 2009). Indeed, Zebarth and Rosen (2007) stated that, “*One of the most effective means of improving the efficiency of N use in agricultural crop production is to match the supply of N to the crop N demand in both space and time*”. This can be attained by targeting the rate and form of fertilizer N that meet but do not exceed crop N requirements (Hong *et al.*, 2007). Although the idea is simple, selection of an adequate fertilizer N rate is difficult owing to marked variation in both soil N supply and crop N demand in the field and over time (Olfs *et al.*, 2005, Scharf *et al.*, 2005, Tremblay *et al.*, 2007, Zebarth *et al.*, 2009).

2.3.3 Methods of estimating crop N status

Studies have been done to assess a number of chemical and optical methods of estimating crop N sufficiency (Olfs *et al.*, 2005, Wu *et al.*, 2007). The most frequently used chemical and optical measures of crop N sufficiency in potato production are petiole nitrate concentration (Westcott *et al.*, 1991) and relative leaf chlorophyll concentration that is estimated by light transmittance using a SPAD-502 meter (Goffart *et al.*, 2008, Minotti *et al.*, 1994). Tremblay *et al.* (2007) also reported the potential to identify plant N sufficiency using a Dualex instrument, which quantifies plant polyphenolics optically.

2.3.3.1 Chemical tests of crop N sufficiency

Measurement of petiole nitrate concentration is an efficient approach to assessing plant N status (Alva, 2007, Westcott *et al.*, 1993). In potatoes, there exists a quantitative research technique for petiole nitrate concentration for several production regions (Porter and Sisson, 1991, Westcott *et al.*, 1991). Indeed, it is considered to be an effective means of measuring current crop N status owing to a strong correlation between petiole nitrate, N availability in the plant and N uptake (Alva, 2007, Errebhi *et al.*, 1998b, Westcott *et al.*, 1993, Westcott *et al.*, 1991). This method has also been used to assess the N status for other crop species such as sugar beet (Sexton and Carroll, 2002), broccoli (Villeneuve *et al.*, 2002), grapes (Nagarajah, 1999) and tomatoes (Rhodes *et al.*, 1996). Petiole nitrate concentration may be measured on either a dry plant tissue basis, or on freshly expressed petiole sap (Errebhi *et al.*, 1998a). However, a major problem with this kind of approach is that, critical petiole nitrate values decrease over time as the crop develops and the season progresses. Furthermore, petiole nitrate value varies with potato cultivar (Porter and Sisson, 1991) not to mention the sampling, preservation, extraction, dilution and measurement effort required (Tremblay *et al.*, 2011).

2.3.3.2 Optical tests of crop N sufficiency

There is a close correlation between leaf chlorophyll concentration and N status of a plant (Mackerron, 2000, Piekielek and Fox, 1992). This has further been reported in wheat (Singh *et al.*, 2002, Vidal *et al.*, 1999, Vouillot *et al.*, 1998), rice (Esfahani *et al.*, 2008, Turner and Jund, 1991), Maize (Blackmer and Schepers, 1995, Ercoli *et al.*, 1993, Piekielek *et al.*, 1995, Varvel *et al.*, 1997, Ziadi *et al.*, 2008) and potato (Gianquinto *et al.*, 2004, Majic *et al.*, 2008, Vos and Bom, 1993, Zebarth *et al.*, 2012). Thus, optical tests are effective in estimating leaf chlorophyll concentration for the purpose of measuring plant N status (Botha *et al.*, 2006). Furthermore, Guler (2009) reported significant correlation between leaf chlorophyll content and total yield in potato plants. The SPAD-502 chlorophyll metre is the most commonly used optical method of assessing plant N sufficiency using light reflectance or transmittance (Goffart *et al.*, 2008, Minotti *et al.*, 1994). It is a small, light-weight, hand-held device that measures light transmittance in the red (650 nm, chlorophyll) and near-infrared (960 nm) spectrum without damaging the leaf (Yadawa, 1986, Zebarth *et al.*, 2009). In potato, the chlorophyll content index is usually estimated in the terminal leaflet of the fourth completely expanded leaf on the stalk because the upper leaves that are more exposed to solar radiation tend to have higher N content than those that are below (Giletto *et*

al., 2010, Westermann, 2005). Indeed, SPAD meter readings have been used to guide in-season fertilizer N management with the aim of reducing N losses without loss of potato yield. However, there are a number of factors that influences leaf chlorophyll content index such as; varieties (Hoel, 2002, Minotti *et al.*, 1994), growth stage (Ramesh *et al.*, 2002), leaf position (Chapman and Barreto, 1997, Hoel, 1998), nutritional deficiencies apart from nitrogen (sulphur, magnesium or iron) (Turner and Jund, 1991), soil type, radiation, foliar diseases (Giletto *et al.*, 2010) and environmental conditions (Schepers *et al.*, 1992).

2.3.3.3 Dualex

The dualex is a portable leaf-clip instrument that can be used to monitor plant N status. It functions by in situ measurement of the polyphenolic content of leaves in the field (Cartelat *et al.*, 2005, Goulas *et al.*, 2004). Polyphenolic are carbon-based compounds playing multiple roles in plants, such as chemical defence against herbivores as well as protection from ultraviolet radiation, free radicals, stress, and pathogens (Cartelat *et al.*, 2005). Estiarte *et al.* (1994) reported a significant reverse relationship between the leaf polyphenol concentration and the level of N fertilization in pepper. Cartelat *et al.* (2005) further revealed that with increasing N fertilization in wheat, leaf chlorophyll content increased and the leaf polyphenolic content decreased. Therefore, the presence of high polyphenolic concentrations denotes N deficiency in a crop.

2.3.4 Nitrogen assimilation

In spite of the fact that plants can absorb small amount of N from the atmosphere through their foliage, the greater part of N is by far acquired in specific forms of NO_3^- and NH_4^+ in the soil (Below, 2001). The first step that occurs in nitrogen assimilation is the uptake of nitrate (NO_3^-) and ammonium (NH_4^+) into root cells from the soil solution (Bu *et al.*, 2011). Roots sense and respond to changes in internal and external N status, which includes the regulation of gene expression, metabolism and further N uptake and assimilation (Glass *et al.*, 2002).

In plants, the nitrate that is taken up by the root can be transported in the root cell vacuoles or through the xylem to the shoot, where it is first reduced to nitrite by nitrate reductase, then enter the plastid and reduced to ammonia ion by nitrite reductase. It is then rapidly assimilated into amide residue of glutamine by the coupled reactions catalysed by glutamine synthetase (GS) and glutamate synthase (GOGAT) (Bu *et al.*, 2011, Lam *et al.*, 2003) (Fig

2.1). Ammonium, on the other hand, is essential from the external environment, as well as both the rhizosphere and atmosphere through NH_4^+ transporters in the plasma membrane of the root cells and leaf cells (Bu *et al.*, 2011). Assimilation of NH_4^+ into the cell either takes place in the cytoplasm via glutamine synthetase or in the plastids and possibly mitochondria following transport into these organelles, and NH_4^+ may as well move into the vacuole where it is briefly stored (Bu *et al.*, 2011). In addition, NH_4^+ can be produced from N_2 by root nodule cells. The majority of this NH_4^+ is transferred to the plant cytoplasm where it is assimilated into glutamine by GS/glutamine-2-oxoglutarate aminotransferase (GS/GOGAT) cycle for plant growth (Bu *et al.*, 2011, Xu *et al.*, 2012). Hence, N is transported to other tissues for utilization in the form of glutamate, aspartate, glutamine, and asparagine through amino acid permeases (Masclaux-Daubresse *et al.*, 2010).

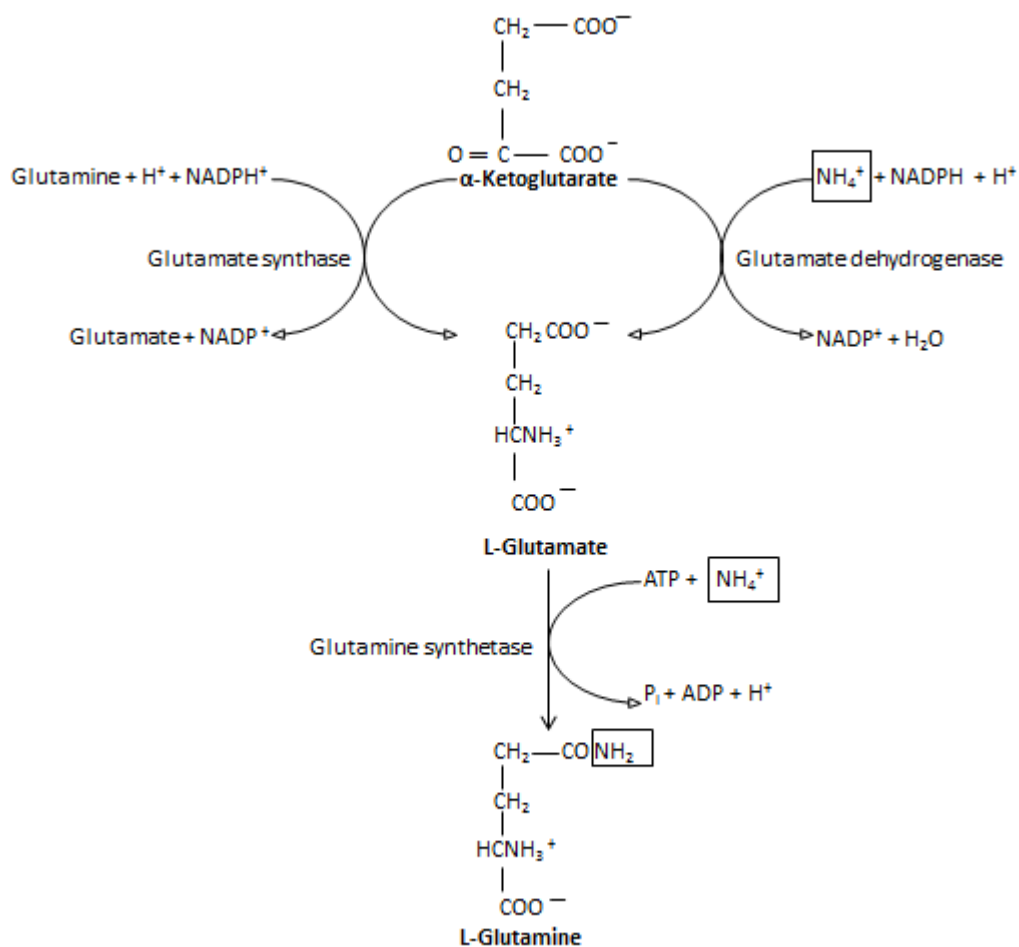


Figure 2. 1: Assimilation of NH_4^+ into glutamine

2.3.4.1 Ammonium; a preferential form of nitrogen uptake

Studies have shown that the average NH_4^+ concentrations of soils are usually 10 to 1000 times lower than those of NO_3^- (Marschner, 1995). However, the difference in concentration of this N nutrients in the soil do not necessarily determine the uptake ratio of each N source (Von Wiren *et al.*, 2000a). The role of NH_4^+ in plant nutrition cannot be over emphasised. Although NH_4^+ concentration is lower than NO_3^- in the soil, most plants preferentially take up NH_4^+ when both forms are present. In addition, NH_4^+ uptake and assimilation requires less energy than that of nitrate probably because NO_3^- has to be reduced before assimilation (Bloom *et al.*, 1992). Hence, NH_4^+ is the preferential form of nitrogen uptake when plants are subjected to nitrogen deficiency (Bu *et al.*, 2011, Gazzarini *et al.*, 1999). However, crop yield is usually maximized when N is supplied in both forms (Bloom *et al.*, 1993).

The difference between the associated inward and outward movement of NH_4^+ in root systems explains the net uptake of NH_4^+ by plant roots (Morgan and Jackson, 1988). Short-term labelling (with the aid of either $^{13}\text{NH}_4^+$ or $^{15}\text{NH}_4^+$) can be used to estimate the inflow of NH_4^+ in view of the fact that outflow of the tracer increases with time of exposure. Furthermore, NH_4^+ transport into plant roots shows biphasic kinetics that can be separated into not less than two different components. The inflow of external NH_4^+ approaches Michaelis-Menten kinetics when the concentration is less than 1mM, although uptake rates seem to increase linearly at higher concentrations (Kronzucker *et al.*, 1996, Ullrich *et al.*, 1984, Wang *et al.*, 1993b).

Several studies have revealed that plants possess two distinct NH_4^+ transport systems in roots. The first type is a low affinity channel-like transporter (Wang *et al.*, 1994, Wang *et al.*, 1993a, Wang *et al.*, 1993b) which some have suggested not to be an exclusive NH_4^+ transporter but more likely a shared transporter, for example a potassium (K^+) channel (Marini *et al.*, 1997). The second type is the high-affinity transporters (Wang *et al.*, 1994, Wang *et al.*, 1993a, Wang *et al.*, 1993b), which may be primarily responsible for the transport of NH_4^+ for N assimilation (Marini *et al.*, 1997). High-affinity and low-affinity transport systems are differentiated by their obvious Michaelis-Menten constant (K_m) values (Wang *et al.*, 1993b), with the substrate affinity often described as the most relevant parameter in describing transport systems. High affinity transporters are known for drawing out nutrients at low external concentrations, yet low affinity usually corresponds with high capacity, which is a key parameter for the maintenance of large inflows at high external availability (Von Wiren *et al.*, 2000a). In fact, the high affinity system is saturable and energy-

dependent with a K_m in the submillimolar concentration range while the low affinity system is non-saturable with a K_m in the millimolar concentration range (D'apuzzo *et al.*, 2004). Therefore, a difference between high affinity/ low capacity and low affinity/high-capacity NH_4^+ transport systems reflects their physiological role more precisely than does a distinction based on affinity alone (Von Wiren *et al.*, 2000a).

It has been documented that plants starved of N for a few days results in an increased capacity for NH_4^+ uptake, which correlates to a specific stimulation of inflow (Gazzarini *et al.*, 1999, Glass and Siddiqi, 1995, Morgan and Jackson, 1988). Also, the high-affinity ammonium uptake capacity of *Arabidopsis* increased under N limitation, and rapidly declined when external N sources were resupplied to the root (Gazzarini *et al.*, 1999, Lanquar *et al.*, 2009, Rawat *et al.*, 1999, Yuan *et al.*, 2007b). This was explained by the concomitant changes in cytoplasmic glutamine levels that are thought to regulate *Arabidopsis* ammonium transporter (AtAMT) gene expression by a negative feedback mechanism (Gazzarini *et al.*, 1999, Rawat *et al.*, 1999). In a similar way, Wang *et al.* (1993b) reported a decrease in the K_m and an increase in the maximum inflow (V_{max}) of the high-affinity transport system in rice during pre-culture stage, when it was subjected to a decrease in external NH_4^+ from 1 mM to 2 μM . This may be a way by which the uptake system adaptively responds to the N demand of the plant, and shows the involvement of feedback regulation that entails changes in both the affinity and capacity of the uptake system (Von Wiren *et al.*, 2000a).

It is a known fact that NH_4^+ reduces growth when it is solely supplied as the N source for plant nutrition, yet the NH_4^+ ion is the preferred source of N over NO_3^- when NH_4NO_3 is available, (Gazzarini *et al.*, 1999, Gessler *et al.*, 1998, Gojon *et al.*, 1986). Furthermore, NH_4^+ is the principal form of N obtained from symbiotic N_2 -fixing bacteria (Udvardi and Day, 1997). Hence, NH_4^+ is a crucial component of N metabolism which accumulates in millimolar (mM) concentrations in the cytosol, the vacuole, and even in the apoplast (Nielsen and Schjoerring, 1998, Wells and Miller, 2000). Therefore, plant cells need transport systems at the plasma membrane that bring about both the retrieval of $\text{NH}_3/\text{NH}_4^+$ and the initial uptake of external NH_4^+ (Von Wiren *et al.*, 2000a).

2.4 Ammonium transporter gene

Ammonium is transported across cellular membranes by a family of integral membrane proteins that is conserved in all domains of life. The family consist of the Ammonium Transporter/Methylammonium Permease/mammalian Rhesus protein (AMT/MEP/Rh)

(Winkler, 2006, Wiren and Merrick, 2004b). AMT/MEP/Rh have been reported to possess a number of features in common which include, high affinity (K_m in the micromolar range) and high selectivity for ammonium and saturation at ammonium concentrations less than or equal to 1 mM (Boeckstaens *et al.*, 2008, Ortiz-Ramirez *et al.*, 2011, Pantoja, 2012).

The evolutionary history of AMT/MEP/Rh genes are complex and are tagged by replication and larger gene family expansions (Couturier *et al.*, 2007), loss and horizontal gene transfer (HGT) (McDonald *et al.*, 2010). For instance, the genome of higher plants not only consist of AMT1 family which was identified in 1994 but now includes AMT2, a second plant family of AMTs that has been shown to be more closely related to bacteria than to other plant AMTs (Sohlenkamp *et al.*, 2002). Fungal AMTs are also more closely related to prokaryotic AMTs than to most other eukaryotic AMTs (Monahan *et al.*, 2002). However, a number of eukaryotes including apicomplexans, microsporidia, and diplomonads such as *Giardia* have no AMTs at all in their genomes. In all, a large sampling of diverse groups of organisms is crucial to reconstruct the evolutionary history of the AMT/MEP/Rh family (McDonald *et al.*, 2012).

The AMT proteins have been characterized genetically and biochemically from a variety of organisms with the most detailed information coming from studies of the *Escherichia coli* ammonia channel, AmtB (Fig. 1) (Conroy *et al.*, 2007, Khademi *et al.*, 2004, Zheng *et al.*, 2004). These studies have shown that the ammonium transporters are conformed as homotrimers, with a substrate conduction channel within each monomer (Conroy *et al.*, 2007, Zheng *et al.*, 2004). Despite their original designation, studies in bacteria suggest that they function as channels through which ammonia (NH_3) is transported (Javelle *et al.*, 2007). However, studies of plant AMTs have argued that they transport charged (NH_4^+) or co-transport NH_3 with a proton (Ludewig *et al.*, 2002).

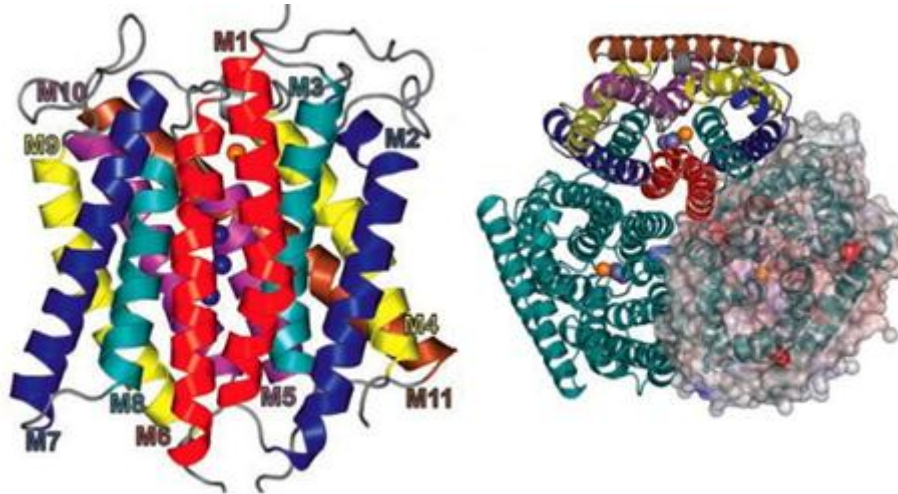


Figure 2.2: The structure of AmtB ammonia channel of *Escherichia coli* with 11 transmembrane helices (M1-M11) adopted from Khademi *et al.* (2004)

In microorganisms and plants, the expression of AMT proteins is subject to nitrogen repression, in such a way that expression is essentially only instigated at low external ammonium concentration (Wiren and Merrick, 2004a). Under this trophic condition, the high NH_4^+ affinity of AMTs may be important for efficient substrate capture. Furthermore, the specific features of the binding site may play a role in discriminating against other cations (Luzhkov *et al.*, 2006, Winkler, 2006). Analysis of the predicted amino acid sequences of AMT family members combined with X-ray structures of *E. coli* AmtB, *Archaeoglobus fulgidus* Amt1 and *Nitrosomonas europaea* Rh50 suggest that all ammonia channel proteins are likely to have either 11 or 12 transmembrane helices (Andrade *et al.*, 2005, Loque *et al.*, 2007, Zheng *et al.*, 2004). All three structures elucidated to date have 11 transmembrane helices with an N-out, C-in topology. Analyses of the human rhesus proteins (the animal homologues of the AMT proteins) however suggest that in some cases, there may be a 12th N-terminal helix giving an N-in, C-in topology.

2.4.1 Isolation and characterisation of plant AMTs

Ammonium uptake from the soil solution is mainly mediated by ammonium transport systems at the root plasma membrane. AMTs encode a high-affinity transport system (HATS) and a low affinity transport system (LATS) and constitute a multi-gene family (Bu *et al.*, 2011, Wang *et al.*, 1993a, Wang *et al.*, 1993b). They have been isolated in several plant species including *Arabidopsis thaliana* (AtAMT1.1 - AtAMT1.5, AtAMT2) (Gazzarini *et al.*, 1999, Kaiser *et al.*, 2002, Ninnemann *et al.*, 1994, Sohlenkamp *et al.*, 2002), *Oryza sativa*

(OsAMT1.1 - OsAMT1.4, OsAMT2.1 - OsAMT2.3, OsAMT3.1 - OsAMT3.3, OsAMT4 and OsAMT5) (Deng *et al.*, 2007, Li *et al.*, 2009, Sonoda *et al.*, 2003a, Suenaga *et al.*, 2003), *Solanum lycopersicum* (LeAMT1.1 - LeAMT1.3, LeAMT2) (Lauter *et al.*, 1996, Von Wiren *et al.*, 2000b), *Triticum aestivum* (TaAMT1.1 - TaAMT1.3, TaAMT2.1) (Jahn *et al.*, 2004), *Lotus japonicus* (LjAMT1.1 - 1.3, LjAMT2.1) (D'apuzzo *et al.*, 2004, Salvemini *et al.*, 2001, Simon-Rosin *et al.*, 2003), *Brassica napus* (BnAMT1.2) (Pearson *et al.*, 2002), *Phaseolus vulgaris* (PvAMT1.1) (Ortiz-Ramirez *et al.*, 2011), *Zea mays* (ZmAMT1.1a, ZmAMT1.3) (Gu *et al.*, 2013), *Populus trichocarpa* (Ptr1.1 - Ptr1.6, Ptr2.1 - Ptr2.2, Ptr3.1, Ptr4.1 - Ptr4.5) (Couturier *et al.*, 2007), *Citrus sinensis x Poncirus trifoliata* (CitAMT1) (Camanes *et al.*, 2009), *Chlamydomonas* (Gonzalez-Ballester *et al.*, 2004), *Pyrus betulifolia* (PbAMT1.2) (Cong *et al.*, 2011), *Puccinellia tenuiflora* (PutAMT1.1) (Bu *et al.*, 2013) and *Solanum tuberosum* AT1 (Zebarth *et al.*, 2012). Furthermore, putative *Cucumis sativus*, *Malus hupehensis* var. *mengshanensis*, *Citrus trifoliata*, *Pyrus ussuriensis*, *Camellia sinensis*, and *Medicago truncatula*'s AMT genes have also been identified (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

All plant's AMT proteins investigated so far are located in the plasma membrane suggesting that their role is in ammonium acquisition by plant cells (Loque *et al.*, 2006, Ludewig *et al.*, 2002, Ludewig *et al.*, 2003, Simon-Rosin *et al.*, 2003, Sohlenkamp *et al.*, 2002, Yuan *et al.*, 2007b). Moreover, the diverse nature of AMT homologs probably allows a greater regulatory flexibility and organelle, cell, tissue, or organ specialization, in addition to enabling plant cells to take up NH_4^+ over a wide range of concentrations (Sonoda *et al.*, 2003a). Ammonium transporters are expressed with a different pattern of regulation in several plant organs such as roots, leaves, stems and buds (Gazzarini *et al.*, 1999, Ninnemann *et al.*, 1994, Von Wiren *et al.*, 2000a), although preferentially expressed in the root tissue. On the other hand, the biochemical characterization in yeast of three *A. thaliana* AMT1 genes revealed different substrate affinities for both NH_4^+ and methylammonium (MA) used as an NH_4^+ analog (Gazzarini *et al.*, 1999).

The higher plants have two families of ammonium transporters (AMT1 and AMT2) in their genome (Couturier *et al.*, 2007, Simon-Rosin *et al.*, 2003). These two transporters both show a high affinity for NH_4^+ , yet those from the AMT2 family do not transport the ammonium analog, methylammonium; has lower transport capacity. Furthermore, ammonium transport by AtAMT2 has been shown to be electro-neutral, suggesting that the transported species corresponds to the uncharged NH_3 and not NH_4^+ (Neuhauser *et al.*, 2009, Simon-Rosin *et al.*, 2003, Sohlenkamp *et al.*, 2002).

The activity of plant AMT1 family members is pH-independent (Ludewig *et al.*, 2002, Ludewig *et al.*, 2003, Wood *et al.*, 2006) with the exception of *Phaseolus vulgaris* PvAMT1.1 and *Triticum aestivum* TaAMT1.1 (Ortiz-Ramirez *et al.*, 2011, Sogaard *et al.*, 2009). AMT1 transcript levels mostly increase during N deprivation and decrease rapidly in response to high N supply, consistent with an optimized ammonium uptake function required in N-starvation conditions (Couturier *et al.*, 2007, Gazzarini *et al.*, 1999, Pearson and Stewart, 1993, Rawat *et al.*, 1999, Von Wiren *et al.*, 2000a).

2.4.1.1 Characterization of *Arabidopsis* AtAMT

Ninnemann *et al.* (1994) isolated the first plant NH_4^+ transporter (AtAMT1.1) through heterologous complementation of the double mutant yeast strain 26972c (mep1-1; mep2-1) with cDNAs from an *Arabidopsis thaliana* library. Other members of the AMT1 gene family in *A. thaliana*, including AtAMT1.2, AtAMT1.3, AtAMT1.4, AtAMT1.5 and AtAMT2.1 were further isolated by screening of DNA libraries and PCR-based approaches (Gazzarini *et al.*, 1999, Sohlenkamp *et al.*, 2000, Von Wiren *et al.*, 2000b).

The AtAMTs have an open reading frame of 500-530 amino acids and encode highly hydrophobic proteins with 11 putative membrane-spanning regions (Von Wiren *et al.*, 2000a) as shown in Fig. 2. The K_m of the transporters for NH_4^+ was reported to be less than 0.5-40 μM (Gazzarini *et al.*, 1999, Ninnemann *et al.*, 1994). Amino acid sequence shows that AtAMT1.3 and AtAMT1.5 are the most closely related with greater than 90% similarity, yet, AtAMT1.1 is 80% similar to AtAMT1.3 and AtAMT1.5. AtAMT1.2 and AtAMT1.4 are more distantly related with 70% similarity to the other AtAMTs (Von Wiren *et al.*, 2000a).

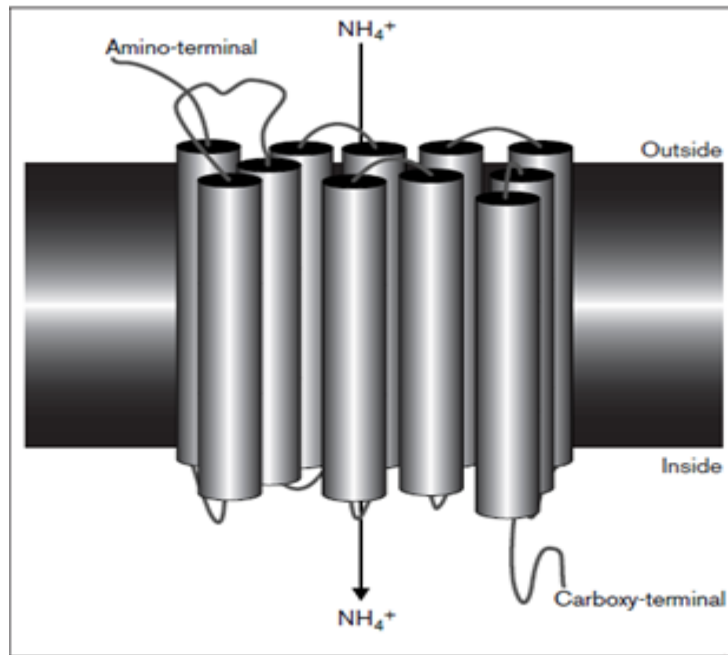


Figure 2. 3: Structural model of AtAMT1.1 with 11 transmembrane-spanning domains adopted from Von Wiren *et al.* (2000a)

Transcriptome and RNA gel blot analyses have shown that four of the six AMT/MEP homologs in *Arabidopsis* are expressed in roots and up-regulated under nitrogen deficiency (Birnbaum *et al.*, 2003, Gazzarini *et al.*, 1999, Schmid *et al.*, 2005, Sohlenkamp *et al.*, 2002). The AtAMT1.1, AtAMT1.2, AtAMT1.5 and AtAMT2.1 are mainly expressed in roots and shoots, AtAMT1.3 specifically in roots and AtAMT1.4 in pollen (Ludewig *et al.*, 2007, Sohlenkamp *et al.*, 2000, Yuan *et al.*, 2009). Their expression in shoots may play roles in ammonium recycling, especially after photorespiration (Sohlenkamp *et al.*, 2000).

In roots, the point of location of AtAMT1.1 is the plasma membrane (rhizodermis, cortex, and pericycle) (Mayer and Ludewig, 2006), which is responsible for about 30% of the NH_4^+ uptake in *Arabidopsis* roots (Kaiser *et al.*, 2002). AtAMT1.3 partially shares expression site with AtAMT1.1 (rhizodermis and cortex) and contributes another 30% of NH_4^+ acquisition in roots (Loque *et al.*, 2006). AtAMT1.2 may consequently transport the remaining 40% NH_4^+ inflow in roots (Neuhauser *et al.*, 2007, Sohlenkamp *et al.*, 2000). Location of AtAMT1.2 in the plant root endodermis and cortex, facilitates the uptake of NH_4^+ from external environments as well as the translocation of NH_4^+ from roots to shoots (Li *et al.*, 2009). Owing to the fact that the additive role of AtAMT1.1 and AtAMT1.3 in taking up NH_4^+ in nitrogen-deficient roots accounts for 65 to 70% of the total uptake capacity (Loque *et al.*,

2006), AtAMT1.1, AtAMT1.2, and AtAMT1.3 might jointly explain 90% of the overall ammonium uptake capacity in *Arabidopsis* roots.

AtAMT1.5, a second, saturable transport system from nitrogen-deficient quadruple insertion line (qko) plants has a low K_m (4.5 μM) and a very low capacity that tallied only from 5 to 10% of the wild-type transport capacity (Yuan *et al.*, 2007a). Engineer and Kranz (2007) as well as Yuan *et al.* (2007a) in their studies, agreed that only AtAMT1.5 could potentially represent this second saturable transport system, among the *Arabidopsis* AMT proteins. This is because AtAMT1.5 transcripts were accumulated under nitrogen deficiency, yet nitrogen deficiency did not have any effect on AtAMT1.4 expression and was at least a thousand-fold lower under either nitrogen-deficient or-sufficient condition. In addition, AtAMT1.5 may be localized at the plasma membrane in *Arabidopsis* due to the fact that it encodes a functional ammonium transporter (Yuan *et al.*, 2007a). It has also been reported that AtAMT1.5 may play a role in accessing external ammonium by uptake across the plasma membrane of rhizodermis cells (Yuan *et al.*, 2007a).

High-affinity NH_4^+ inflow is strongly correlated with AMT1 transcript abundance, which appears to be regulated by root glutamine levels (but not NH_4^+) concentration (Gansel *et al.*, 2001, Gazzarini *et al.*, 1999, Rawat *et al.*, 1999). Moreover, the transcript levels of AtAMTs are regulated by the available N supply, photosynthetic products and diurnal change (Gazzarini *et al.*, 1999).

Ammonium can be directly taken up from the soil solution by AtAMT1.1, AtAMT1.3 and AtAMT1.5 at the surface and root hairs of the rhizodermis (Yuan *et al.*, 2007a). AtAMT1.2 faces higher ammonium concentrations than the AtAMT1.1 and AtAMT1.3 rhizodermis-located proteins. The lower K_m of AtAMT1.2 is hence suited to this environment, enabling efficient substrate transport close to saturation of the transporter (Yuan *et al.*, 2007a). This shows that the biochemical transport properties and specific location of AtAMT1.2 represent an adaptation to retrieval of ammonium that is released from the cortex and that enters the root tissue via the apoplastic transport route (Yuan *et al.*, 2007a).

The substrate affinities of *Arabidopsis* ammonium transporter proteins have been calculated to be 50.0, 233.9, 60.5 and 4.5 μM for AtAMT1.1, AtAMT1.2, AtAMT1.3 and AtAMT1.5, respectively (Yuan *et al.*, 2007a). AtAMT1.2 expressed in yeast mutant displays biphasic kinetics (K_m values of 36 μM and 3.0 mM) for methylammonium uptake (Shelden *et al.*, 2001). AtAMT1.1 and AtAMT1.3 are involved in the high affinity loading of NH_4^+ at the

rhizodermis (Loque *et al.*, 2006, Mayer and Ludewig, 2006, Wood *et al.*, 2006), yet AtAMT1.2 has a lower affinity to NH_4^+ and is preferentially localized in the root endodermis. The significantly lower substrate affinity shown by AtAMT1.2, suggests adaptation to higher substrate concentrations (Yuan *et al.*, 2007a). From a biological perspective, differing substrate affinities of individual transporters might then represent one factor for the existence of multiple AMT1 homologues (Gazzarini *et al.*, 1999).

In wild-type plants, the order in which the AMT genes were expressed in roots after N starvation from most to least abundant was AtAMT1.2 > AtAMT1.1 > AtAMT1.3 > AtAMT2.1 (Kaiser *et al.*, 2002). Yuan *et al.* (2007a) in the same way, reported the capacities of the four ammonium transporters in *Arabidopsis* roots in their decreasing order: AtAMT1.1= AtAMT1.3> AtAMT1.2>AtAMT1.5. Nitrogen deficiency led to increase in ammonium transport capacities of all the four AtAMT1 proteins, although individual ammonium transport capacities (more importantly those of AtAMT1.1 and AtAMT1.3) might depend on the presence of other transporters (Loque *et al.*, 2006, Yuan *et al.*, 2007a). Moreover, the steep increase in AtAMT1.1 mRNA suggested that the gene product makes a major contribution to the overall ammonium uptake capacity in the roots (Loque and Von Wiren, 2004). To meet the plant's nitrogen demand, its nutritional status determines the overall ammonium transport capacity, which in turn is broken down into individually modulated AtAMT1 capacities (Yuan *et al.*, 2007b).

Isolation and partial characterization of the ammonium transporter AtAMT2 from *Arabidopsis* was later reported. The study shows that AtAMT2 is only distantly related to plant AMT1 proteins (Howitt and Udvardi, 2000, Sohlenkamp *et al.*, 2000). When sequenced, AtAMT2 was more closely related to the ammonium transporters Mep1, 2 and 3 from *Saccharomyces cerevisiae* and with AmtB from *E.coli*, forming, together with other prokaryotic homologues, the MEP subfamily (Ludewig *et al.*, 2001). Hence, plant AMT2 family is more closely related to ammonium transporters from prokaryotes than it is to plant AMT1 transporters (Sohlenkamp *et al.*, 2002). As a result of this, AtAMT2.1 is the only member of the MEP subfamily, while the five homologs, AtAMT1.1 to AtAMT1.5, constitute the AMT clade (Ludewig *et al.*, 2001).

2.4.1.2 Characterisation of AMT1 in legume crops

2.4.1.2.1 *Lotus japonicus* LjAMT1

Three homologs of ammonium transporter gene (LjAMT1.1, LjAMT1.2 and LjAMT1.3) were cloned and characterized from *Lotus japonicus* which is a legume plant (D'apuzzo *et al.*, 2004, Salvemini *et al.*, 2001). They were identified through the functional complementation of the yeast triple mutant strain 31019b and homology to other members of the AMT1 family (D'apuzzo *et al.*, 2004, Salvemini *et al.*, 2001).

They have high affinity for ammonium with transport affinity of 1.7, 3 and 15 μM for LjAMT1.1, LjAMT1.2 and LjAMT1.3 respectively (D'apuzzo *et al.*, 2004, Salvemini *et al.*, 2001). Amino acid sequence comparison shows that LjAMT1.1 and LjAMT1.2 are more closely related with 76% similarity while LjAMT1.3 is more distantly related with 73% and 71% similarity to LjAMT1.1 and LjAMT1.2 respectively (D'apuzzo *et al.*, 2004). The LjAMT1 homologs are expressed in the roots, leaves, nodule and flowers of *Lotus japonicus* (D'apuzzo *et al.*, 2004, Salvemini *et al.*, 2001). They are highly expressed in leaves than in roots and nodule with LjAMT1.1 having the highest expression level followed by LjAMT1.2 then LjAMT1.3. The expression level of LjAMT1.3 was reported to be higher in flower than in leaves while LjAMT1.1 showed the least expression in flower (D'apuzzo *et al.*, 2004, Rogato *et al.*, 2010, Salvemini *et al.*, 2001). In roots, the expression of LjAMT1.1 and LjAMT1.2 was induced when subjected to N deficiency while the expression of LjAMT1.3 remained low regardless N supply (D'apuzzo *et al.*, 2004, Rogato *et al.*, 2010). However, there was high expression level of LjAMT1 homologs in leaves when *Lotus japonicus* was grown in hydroponic condition with both high and low N supply (D'apuzzo *et al.*, 2004). This strange pattern of expression was also reported for the LeAMT1.3 and AtAMT2 in non-legume plants (Sohlenkamp *et al.*, 2002, Von Wiren *et al.*, 2000b). The localization of LjAMT1 homologs in different tissues as well as their varying expression pattern indicates differential role in both the N metabolism of the organs and the NH_4^+ translocation in sink and source tissues (D'apuzzo *et al.*, 2004). LjAMT1.1 is the first ammonium transporter gene to be reported to have an intron (1009 base pairs) and no open reading frames of significant length have been found in this unusually long intron sequence (Salvemini *et al.*, 2001). Yet, LjAMT1.2 and LjAMT1.3 has complete open reading frames of 519 and 507 respectively (D'apuzzo *et al.*, 2004).

The principal form of N transported from symbiotic N_2 -fixing bacteria to their host plant is likely NH_4^+ (Howitt and Udvardi, 2000, Tate *et al.*, 1999, Tyerman *et al.*, 1995, Von Wiren *et*

al., 2000a). It is crucial to isolate and study the ammonium transporters from leguminous plants since these genes may play a strategic role in different steps of the *Rhizobium* infection, colonization N-fixation and N export (Salvemini *et al.*, 2001). LjAMT1.1 and LjAMT1.2 could be involved in recovering NH_4^+ lost from nodule cells in the different tissues during normal metabolism (D'apuzzo *et al.*, 2004, Salvemini *et al.*, 2001).

2.4.1.2.2 *Phaseolus vulgaris* PvAMT1

The ammonium transporter of *Phaseolus vulgaris* (PvAMT1.1) which is a legume and common bean was characterized in a study by Ortiz-Ramirez *et al.* (2011). PvAMT1.1 mediates the high affinity (in micromolar range) and electrogenic transport of NH_4^+ (Ortiz-Ramirez *et al.*, 2011). PvAMT1.1 encodes for a highly selective ammonium transporter and its activity relies on pH. PvAMT1.1 was subjected to different external pH values in which the analysis showed that NH_4^+ transport increased as the medium became more acidic at an optimum pH close to 5.5 involving the transport of H^+ . This indicates that PvAMT1.1 functions as an H^+/NH_4^+ (1:1 ratio) symporter (Ortiz-Ramirez *et al.*, 2011).

2.4.1.3 Characterisation of AMT1 in non-legume crops

2.4.1.3.1 *Solanum lycopersicum* LeAMT1

Three *Solanum lycopersicum* (tomato) AMT1 homologs (LeAMT1.1-1.3) were isolated by screening a cDNA library of different tissues, and of the tomato genome using a PCR-based approach (Lauter *et al.*, 1996, Von Wiren *et al.*, 2000b) and a single putative orthologue of AtAMT2 and LeAMT2.

Transcript of LeAMT1.1 and LeAMT1.2 were detected both in roots and shoots, yet LeAMT1.1 and LeAMT1.2 were preferentially expressed in root hairs of tomato roots, indicating that they act by acquiring NH_4^+ from the rhizosphere (Von Wiren *et al.*, 2000a). On the other hand, LeAMT1.3 is mainly expressed in shoots (Lauter *et al.*, 1996) which makes it the only member of the plant AMT1 family that is not preferentially expressed in roots (Von Wiren *et al.*, 2000b). The expression patterns of LeAMT1.2 and LeAMT1.3 varies in the sense that the highest LeAMT1.3 transcript levels occur during the dark period while the highest LeAMT1.2 transcript levels occur after the onset of light (Von Wiren *et al.*, 2000b). Consequently, LeAMT1.2 plays a role in the uptake of xylem-derived NH_4^+ or in the retrieval of photorespiratory NH_3 . However, LeAMT1.3 may function by transporting or retrieving NH_4^+ during the dark period. NH_4^+ could be released from reactions involving other light-repressed

enzymes, such as asparagine synthetase and glutamate dehydrogenase (Lam *et al.*, 1995, Lam *et al.*, 1998). Deficient N in roots increases the expression level of LeAMT1.1 whereas the expression level of LeAMT1.2 is mainly induced by the supply of NH_4^+ or NO_3^- (Lauter *et al.*, 1996, Von Wiren *et al.*, 2000b). The contrary responses of LeAMT1.1 and LeAMT1.2 to N supply indicates that different NH_4^+ transporters are required to meet the plant's demand for N when growing in N-deficient and N-abundance soil (Von Wiren *et al.*, 2000b).

2.4.1.3.2 *Brassica napus* BnAMT1

Pearson *et al.* (2002) isolated two homologues of *Brassica napus* AMT (BnAMT1.1 and BnAMT1.2) from the shoot mRNA using a PCR based approach. The study revealed that both BnAMTs are high-affinity transport system in the Kinetic analysis of NH_4^+ entry into leaf protoplasts of *Brassica napus*.

The high expression of BnAMT1.2 under most conditions in the leaves has led to the suggestion that, the primary function of the leaf BnAMT1.2 protein is the transport of NH_4^+ from the apoplast and may play an important role in the recovery of photorespiratory NH_4^+ (Pearson *et al.*, 2002). A RT-PCR analysis indicates that the long-term exposure of BnAMT1.2 to 1.0 mM NH_4^+ results in an 80% decrease in mRNA expression (Pearson *et al.*, 2002).

2.4.1.3.3 *Populus trichocarpa* PtrAMT

The *Populus trichocarpa* genome contains 14 putative AMT genes (Ptr1.1-Ptr1.6, Ptr2.1-Ptr2.2, Ptr3.1, Ptr4.1-Ptr4.5), comprising of AMT1 subfamily (six genes) and AMT2 subfamily (eight genes) (Couturier *et al.*, 2007). Indeed, *Populus trichocarpa* possesses much more AMT2 homologs than *Arabidopsis* while it also possesses much more AMT1 homologs than rice (Couturier *et al.*, 2007).

The expression of PtrAMT1.2 is high and specific to roots. In a like manner, PtrAMT1.1, PtrAMT1.5, PtrAMT2.2 and PtrAMT4.5 are expressed in roots, an indication that they respond to N demand through uptake of soil NH_4^+ in various N regime (Couturier *et al.*, 2007). On the other hand, their expression was also indicated in other poplar tissues including petiole (PtrAMT1.1 and PtrAMT2.2) or stamen (PtrAMT1.5). Consequently, their role may not be specific to root uptake (Couturier *et al.*, 2007). PtrAMT1.6, PtrAMT2.1 and PtrAMT3.1 are exclusively expressed in shoot tissues (Couturier *et al.*, 2007). The expression of PtrAMT1.5, PtrAMT1.6 and PtrAMT3.1 increased as the leaves matures

(Couturier *et al.*, 2007) but also highly corresponds with the expression of the marker gene, cysteine protease whose involvement in senescence has been reported (Bhalerao *et al.*, 2003). The expression of PtrAMT3.1 was however, only discovered in senescing leaves which is in contrast to PtrAMT1.5 and PtrAMT1.6 (Couturier *et al.*, 2007). However, PtrAMT1.5 and PtrAMT1.6 also differs in their expression patterns in which both were fully expressed in senescing leaves, yet PtrAMT1.5 expression in flowers was specific to stamens and that of PtrAMT1.6 specific to female flowers. PtrAMT1.5 has a similar high expression pattern in the stamen with its closest ortholog AtAMT1.4 (Zimmermann *et al.*, 2004). Indeed PtrAMT1.5 and PtrAMT1.6, might have important and specific roles in reproductive organ development in the dioecious plant, poplar (Couturier *et al.*, 2007).

2.4.1.3.4 *Zea mays* ZaAMT1

A recent publication by Gu *et al.* (2013) reported the isolation of two ammonium transporter genes (ZmAMT1.1a and ZmAMT1.3) from a maize root-specific cDNA library by functional complementation of an ammonium uptake-defective yeast mutant (Gu *et al.*, 2013). The ectopic expression of ZmAMT1.1a and ZmAMT1.3 in an ammonium uptake defective *Arabidopsis* mutant, supported ammonium transport by both genes (Gu *et al.*, 2013). Moreover, a short-term inflow study on ZmAMT1.1a and ZmAMT1.3 proteins revealed that they function as high-affinity ammonium transporters with substrate affinities close to AtAMT1.1 and AtAMT1.3 (Gu *et al.*, 2013, Yuan *et al.*, 2007a). The two proteins have K_m values of between 30 and 60 μM (48 and 33 μM for ZmAMT1.1a and ZmAMT1.3, respectively) and consequently appear highly adapted to the ammonium concentrations found in most soils (Barber, 1995, Marschner and Marschner, 2012, Miller *et al.*, 2007). Just like the regulation of OsAMT1.1 and OsAMT1.2, the up-regulation of the ZmAMT1.1a and ZmAMT1.3 are brought about by NH_4^+ but not NO_3^- or N deficiency (Gu *et al.*, 2013). As a consequence, ammonium inducible expression of ZmAMT1.1a and ZmAMT1.3 is controlled specifically by a local NH_4^+ signal (Gu *et al.*, 2013)

2.4.1.3.5 *Oryza sativa* OsAMT

Twelve AMT homologs have been reported so far in rice and allocated to five sub-families (OsAMT1.1-OsAMT1.4, OsAMT2.1-OsAMT2.3, OsAMT3.1-OsAMT3.3, OsAMT4 and OsAMT5.1- OsAMT5.2) (Bu *et al.*, 2011, Deng *et al.*, 2007, Li *et al.*, 2009, Sonoda *et al.*, 2003a, Suenaga *et al.*, 2003).

Reports from various studies showed that the expression levels of AMTs are generally regulated by N starvation, N form and N level. However, reports on AMTs in rice differ from different varieties, to growth conditions and experimental setups (Kumar *et al.*, 2003, Sonoda *et al.*, 2003b). OsAMT1:1 is constitutively expressed in shoots and stimulated by NH_4^+ in roots of rice (Bu *et al.*, 2011, Li and Shi, 2006, Sonoda *et al.*, 2003a). Although the expression of OsAMT1.2 and OsAMT1.3 are specific to root (Li *et al.*, 2012, Sonoda *et al.*, 2003a), they are as well expressed in shoots (Li and Shi, 2006, Zhao *et al.*, 2008). In addition, OsAMT1.2 is NH_4^+ inducible while OsAMT1.3 is NH_4^+ depressible (Sonoda *et al.*, 2003a). Li and Shi (2006) indeed reported cortical cells and vascular cylinder of rice roots as the site where OsAMT1.2 mRNA expression was detected. This consequently showed that OsAMT1.2 may play a role in NH_4^+ uptake and retrieval in the vascular system in addition to NH_4^+ uptake from soil solution (Bu *et al.*, 2011). The supply and resupply of NH_4^+ up-regulates the expression level of OsAMT1.1 (Li and Shi, 2006, Sonoda *et al.*, 2003a), yet OsAMT1.3 was strongly up-regulated when NH_4^+ , NO_3^- or both were resupplied for 2 h and also showed faint expression under N deprivation (Li and Shi, 2006, Sun *et al.*, 2006). However, several other studies pointed out that the expression pattern of OsAMT1.3 was repressed when N was supplied making it similar to AtAMT1.1 and LeAMT1.1 (Gazzarini *et al.*, 1999, Rawat *et al.*, 1999, Von Wiren *et al.*, 2000b). In a like manner, Tabuchi *et al.* (2007) suggested that the role of OsAMT1.3 in rice may be to support OsAMT1.1 in taking up NH_4^+ ion when soil NH_4^+ is low. Either OsAMT1.1 or OsAMT1.2 demands lesser ammonium concentration compared to OsAMT2.1 which needs higher NH_4^+ concentration in order to rescue the growth of a yeast *amt* deletion strain (5 mmol/L rather than 1 mmol/L) (Li *et al.*, 2009). This is an indication that OsAMT2.1 may have a low affinity for NH_4^+ under these circumstances (Li *et al.*, 2009).

OsAMT1.1, OsAMT1.2 and OsAMT1.3 showed 74.2%, 73.7% and 70.4% sequence-identity to AtAMT1.1 (Ninnemann *et al.*, 1994) and 74.4%, 75.4% and 70.9% to LeAMT1.1 respectively (Lauter *et al.*, 1996, Sonoda *et al.*, 2003a). Sequence analysis of two expressed sequence tags and one DNA clone in a study by Sonoda *et al.* (2003a), showed that the open reading frames of OsAMT1.1, OsAMT1.2 and OsAMT1.3 encode 499, 497 and 498 amino acid residues, with a predicted molecular mass of 52.6, 52.2 and 53.1 kDa, respectively.

2.4.1.3.6 *Triticum aestivum* TaAMT1

Sogaard *et al.* (2009) cloned, sequenced and investigated TaAMT1.1 from wheat (*Triticum aestivum*) expressed in *Xenopus oocytes* by electrode voltage clamp and radio-labelled uptakes. The study reported increase in the NH_4^+ - induced currents when the external pH was decreased from 7.4 to 5.5. This showed that TaAMT1.1 is pH dependent, a characteristic similar to PvAMT1.1 (Ortiz-Ramirez *et al.*, 2011). Indeed, it is a strong indication of NH_4^+ or MeA^+ transport. Hence, TaAMT1.1 expressed in *Xenopus oocytes* transport ammonia in the form of NH_4^+ (Sogaard *et al.*, 2009).

2.4.1.3.7 *Puccinellia tenuiflora* PutAMT1

Puccinellia tenuiflora is a monocotyledonous plant that thrives well under high pH (> 9) and extreme low N in the soil. PutAMT1.1, the first member of the AMT1 family was identified and characterized in *Puccinellia tenuiflora*. Its open reading frame encodes 499 amino acids with a predicted molecular mass of 55.47 kDa (Bu *et al.*, 2013).

A semi quantitative PCR carried out on various *P tenuiflora* tissues showed the expression of PutAMT1.1 in all the plant organs but the expression was higher in the panicle stem and the anther (Bu *et al.*, 2013). PutAMT1.1 is pH independent and it functionally complements a yeast mutant 31019b deficient in ammonium uptake. PutAMT1.1 protein is located in the plasma membrane and around the nuclear periphery in yeast cells and *P tenuiflora* suspension cells. Immunoelectron microscopy analysis further showed that PutAMT1.1 is located in the endomembrane (Bu *et al.*, 2013).

2.5 Expression profiling

Gene expression profiling provides new insights into regulatory gene networks and offers more in-depth understanding of various biological processes (Wise *et al.*, 2007). Two types of transcribed genes can be found in the genome. They can either be transcribed into messenger ribonucleic acids (mRNAs) that will be translated into proteins, or be transcribed into non-coding RNAs (Backofen *et al.*, 2007). Hence, mRNA gene expression profiling is crucial for investigating gene function in plant biology (Shi and Chiang, 2005).

Busch and Lohmann (2007) classified the different methods for gene expression profiling into three categories: (1) PCR-based methods, such as quantitative real-time reverse-transcription PCR; (2) sequencing based methods, such as serial analysis of gene

expression (SAGE), and massive parallel signature sequencing (MPSS); and (3) hybridization-based methods, such as microarrays and northern blotting. These state-of-the-art technologies are more frequently applied on model plants such as *Arabidopsis* and high value commercial crops such as potato, rice and maize. However, it has not been used on African crops such as Bambara groundnut. There are advantages and disadvantages associated with each group of methods. The method recommended also depends on the objective of the investigation and budget. Hence, this literature will be focusing on the PCR-based methods.

2.5.1 PCR-based approach

Quantitative real-time reverse transcription PCR is the best known technique for small and medium scale expression profiling (Heid *et al.*, 1996). It is one of the reliable technologies of the genomic age that is well adopted for the detection of mRNA (Bustin, 2000) and it also offers quantitative information about transcript levels (Bengtsson *et al.*, 2005). In fact, the simplest and impartial approach to analyse a RNA population is the sequencing of complementary DNA (cDNA) libraries and quantitative analysis of the resulting expressed sequence tags (Busch and Lohmann, 2007). mRNA quantification method avoids the need to use suitable antibodies and has the ability to detect very small amounts of target molecule, depending on the technique used (Thellin *et al.*, 2009).

2.5.1.1 Polymerase chain reaction

The polymerase chain reaction (PCR) is a technology in molecular biology that enables exponential amplification of short DNA sequence within a longer double stranded DNA molecule, generating thousands to millions of copies of a particular DNA sequence (Hunt, 2010). The process is carried out by thermal cycling with cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA (Hunt, 2010, Kubista *et al.*, 2006). PCR depends on the use of a pair of primers (short DNA fragment) that are complementary to a target sequence on each of the two strands of the DNA (Dieffenbach *et al.*, 1993, Hunt, 2010). These primers are extended by a DNA polymerase to allow selective and repeated amplification (Hunt, 2010). During the course of the reaction, the DNA generated is itself used as a template for replication, putting in place a chain reaction in which the DNA template is exponentially amplified. A heat-stable DNA polymerase, such as Taq polymerase (an enzyme originally isolated from the bacterium *Thermus aquaticus* that grows in hot pools) is a key component in PCR since these

technology requires the application of a high temperature to separate the two strands of the double helical DNA in each round of the amplification processes (Hunt, 2010, Kebelmann-Betzing *et al.*, 1998).

The PCR temperature cycles consist of three steps; the denaturation, annealing and elongation step. At denaturation step, the reaction is heated as high as 94 – 98 °C for 20-30 seconds to separate the two strands of the DNA double helix. This process is called DNA melting where the hydrogen bonds of the DNA template is disrupted between complementary bases, yielding single stranded DNA molecule (Hunt, 2010). The temperature is then lowered to 50 – 60 °C for 20 - 40 seconds at the annealing step so as to enable specific primers anneal to the single stranded DNA (Hunt, 2010, Kubista *et al.*, 2006). However, the choice of the annealing temperature depends on the primers. It is usually a few degrees lower than the melting temperature of the two primers so that they can form stable complexes with the targeted sequences but not with any other sequences (Kubista *et al.*, 2006). The temperature at the elongation step is raised to 72 °C, which is optimum for Taq DNA polymerase that extends the primers by incorporating the deoxynucleoside triphosphates (dNTPs) (Hunt, 2010, Kubista *et al.*, 2006, Lawyer *et al.*, 1993). In the absence of any limitation as a result of limiting substrates or reagents, at each extension step, the amount of DNA target is doubled, leading to geometric amplification of the specific DNA fragment. The reaction usually comes to an end after 30 - 40 rounds of amplification, with the PCR product being analysed by agarose gel electrophoresis (Hunt, 2010).

The PCR process can be divided into three phases; the exponential, linear and plateau phase. At the exponential phase, PCR reaches its optimal reaction efficiency in which the PCR product doubles at every cycle. Exponential amplification occurs at this phase due to the fact that all the reagents are fresh, available and at their optimum capacity. The reaction is very sensitive and precise at this stage (Reece, 2011). During linear phase, some of the reagents (like dNTPs and primers) are being used-up due to amplification, then the reaction begins to slow down and the PCR product no longer doubles at each stage. At the plateau phase, no more products are being made and the reaction eventually stops.

Agarose gel electrophoresis separates and analyses the size of the PCR product to examine whether the PCR generated the anticipated DNA fragment (Kryndushkin *et al.*, 2003). An agarose gel is stained with ethidium bromide (a dye that binds to double stranded DNA) which illuminates under ultraviolet light and causes the intercalated stain to fluorescent. The

size of PCR product is determined by comparison with a molecular weight marker that contains DNA fragment of known size, run on the gel along the PCR product.

2.5.1.2 Reverse transcription

Reverse transcription polymerase chain reaction is a qualitative or semi-quantitative method most commonly used to characterise or confirm gene expression as well as compare mRNA levels in different samples (Bustin *et al.*, 2005, Freeman *et al.*, 1999, Orlando *et al.*, 1998). In order to estimate the expression of a gene, reverse transcriptase is used to convert mRNA into complementary DNA (cDNA), which will then be amplified by PCR (Bustin, 2000, Hunt, 2010). The two reverse transcriptase that are often used are avian myeloblastosis virus reverse transcriptase (AMV-RT) and moloney murine leukaemia virus reverse transcriptase (MMLV-RT) (Bustin, 2000). Complementary DNA reaction can be primed from the RNA template using random primers, oligo-dT and target gene-specific primers (Bustin *et al.*, 2005, Bustin, 2005). Random primers yield the most cDNA, yet it initiates transcript from multiple origins along the RNA template and consequently produce more than one cDNA target per original mRNA target. Additionally, most of the cDNA synthesised from total RNA is ribosomal RNA (rRNA)-derived (Bustin *et al.*, 2005). Oligo-dT is more specific than random priming. It works best when the aim of the assay is to get a faithful cDNA representation of the mRNA pool. Indeed, it is the most suitable method when the assay is directed at amplifying several targets mRNAs from a limited RNA sample. However, it does not work well with poor quality RNA from formalin-fixed archival material (Bustin, 2005). Target-specific priming is the most specific method of synthesizing cDNA and the best system of quantification, due to its incomparable sensitivity. However, it demands separate priming reactions for each target (Bustin, 2005, Lekanne Deprez *et al.*, 2002).

2.5.1.3 Real time PCR

Real time PCR otherwise called quantitative PCR (Mackay, 2007), quantitative real-time PCR (Radonic *et al.*, 2004) and real time quantitative PCR (Livak and Schmittgen, 2001), proffers a simple and suitable method for determining the amount of a target sequence or gene that is present in a sample. It is one of the most powerful and widely used techniques for analysing mRNA expression because of its high sensitivity, sequence-specificity, wide dynamic range, low template concentration requirements and little to no post-amplification processing (Shi and Chiang, 2005, Wong and Medrano, 2005). Apart from giving quantitative results, real-time PCR is easy and convenient to use (Radonic *et al.*, 2004).

Indeed, real time PCR is a system that works by collecting data throughout the PCR process as it occurs, thereby combining amplification, detection and quantification into a single step (Higuchi *et al.*, 1993, Wong and Medrano, 2005). This is attained by using a fluorescent reporter that binds to the amplicons and reports its presence by fluorescence. The quantity of PCR product formed is monitored as the reaction is proceeding, by noting the fluorescence of dyes or probes introduced into the reaction that is correlative to the amount of product formed, and the number of amplification cycles required to obtain a particular amount of DNA molecules is registered (Jungebloud *et al.*, 2007, Kubista *et al.*, 2006). Hence, the correlation between fluorescence and amount of amplicons allows precise quantification of target molecules over a wide and dynamic range in the presence of suitable standards (Bustin, 2008). The fluorescent DNA probes that is often used in real time RT-PCR are SYBR Green, Taqman, Molecular Beacons, and Scorpions (Holden and Wang, 2008). The SYBR Green dye produces its fluorescent signal by binding to the double-stranded DNA in solution. However, the Taqman, Molecular Beacons and Scorpions emit their fluorescent based on Forster Resonance Energy Transfer (FRET) coupling of the dye molecule and a quencher moiety of the oligonucleotide substrates (Holden and Wang, 2008). However, SYBR Green is the easiest and cheapest probe to use (Schmittgen *et al.*, 2000).

The amplification plot contains valuable information for the quantitative measurement of DNA or RNA. The threshold line is the point at which a reaction reaches a fluorescent intensity above background. The PCR cycle at which the target amplification is first detected in a reaction is called the threshold cycle (C_t). Indeed, threshold cycle is the number of PCR cycles at which fluorescence produced from the amplification product first exceeds a baseline level (Bustin, 2005, Heid *et al.*, 1996, Wong and Medrano, 2005).

However, the quality or accuracy of the results got in real-time quantitative PCR assay greatly depends on the integrity of the RNA template, the choice of cDNA priming strategy and reverse transcriptase, the features of the primers and the validity of the normalisation method (Bustin, 2008, Dheda *et al.*, 2005, Dheda *et al.*, 2004, Huggett *et al.*, 2005, Tricarico *et al.*, 2002).

2.5.1.3.1 One-step and Two-step reverse transcription-quantitative PCR

In an experiment to quantify mRNA, reverse transcription-qPCR can be performed as either a one-step reaction or a two-step reaction (Battaglia *et al.*, 1998, Vandesompele *et al.*,

2002). One-step reaction permits the use of a single buffer, where the entire reaction from cDNA synthesis to PCR amplification is performed in a single tube (Aatsinki *et al.*, 1994, Wong and Medrano, 2005). In contrast, the two-step reaction entails that the reverse transcription and PCR amplification be performed in separate tubes (Vandesompele *et al.*, 2002, Wong and Medrano, 2005). Experimental variation is reduced in one-step real-time PCR owing to the fact that both enzymatic reactions occur in single tube. Furthermore, it is more convenient and reduces the risk of cross-contamination (Aatsinki *et al.*, 1994, Mallet *et al.*, 1995). One-step reaction may however, not be suitable in circumstances where the same sample is analysed severally over a period of time, because it uses an RNA starting template which is susceptible to rapid degradation if not handled properly (Wong and Medrano, 2005). On the other hand, two-step real-time PCR is more reproducible and more sensitive while it is more prone to DNA contamination compared to one-step real-time PCR (Vandesompele *et al.*, 2002, Wong and Medrano, 2005).

2.5.1.4 Relative quantification

In relative quantification, expression level of mRNA is measured based on a reference sample or internal control (Fink *et al.*, 1998, Livak and Schmittgen, 2001) . It involves the co-amplification of an internal control simultaneously with the mRNA of interest (Fink *et al.*, 1998, Minton, 1995). In this assay, there is a direct comparison between the target mRNA cycle threshold and the internal control cycle threshold values, and the results are expressed as ratios of target mRNA signal to internal control signal (Bustin, 2005). The values obtained here can then be used to compare between the samples in order to estimate the relative expression level of target mRNA (Bustin, 2005, Gause and Adamovicz, 1994). However, for relative quantification results to be accurate and relevant, it is important that the expression level of the internal control is constant across all samples and with the target mRNA. Moreover, amplification efficiency of a target gene and internal control must be similar due to the fact that they directly influence the precision of any calculated expression result (Liu and Saint, 2002, Pfaffl, 2001).

2.5.1.5 Absolute quantification

Absolute quantification entails the use of serially diluted standards with a known concentration of initial amount of total RNA or cDNA to generate a standard curve (Bustin, 2005, Wong and Medrano, 2005). The standard curve is generated by plotting the C_t values against the logarithm of the initial copy numbers there by enabling the calculation of the

concentration of unknown samples based on their C_t values (Heid *et al.*, 1996, Ke *et al.*, 2000). Absolute quantification presumes that all standards and samples have roughly equal amplification efficiency (Souaze *et al.*, 1996). The PCR standard curve can be constructed from fragments of double-stranded DNA, single-stranded DNA or in vitro T7-transcribed RNA (cRNA) bearing the target sequence (Fronhoffs *et al.*, 2002, Pfaffl and Hageleit, 2001). The standards used have to be a pure species. Furthermore, the accuracy of the standards is crucial for the accuracy of absolute quantification (Bustin, 2005, Wong and Medrano, 2005).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials and methods

This chapter presents the description of the materials, experimental protocols and analytical procedures used in this study. The rationale for the use of particular materials and methods is also discussed in this chapter.

3.2 Bioinformatics

Several plant AMT genes have been studied and reported, yet the information is not available in a consolidated manner. In fact no report has been made on AMTs from indigenous African crops. Potato can be used as a model crop from which results can be extrapolated to African crops such as Bambara groundnut. But even for potato, standard tools such as universal AMT primers for PCR as well as qPCR protocols are not available. This then necessitated a change in focus of the project, to initiate work on developing tools for AMT molecular biology studies.

3.2.1 Primer design

3.2.1.1 Multiple sequence alignment

Nucleotide sequences of thirty AMTs from various plant species, ranging from AMT1.1-1.3 were aligned (using BLAST in the NCBI) in order to identify conserved regions. Table 3.1 shows their accession numbers.

3.2.1.2 Primer design and BLAST

For PCR assay, ammonium transporter (AMT1) specific primers were designed from region of similarities between the aligned sequences. The primers were then evaluated by BLASTing (Primer-BLAST) against *Solanum lycopersicum* and *Solanum tuberosum* genomic sequence present in the NCBI database, to ensure that the primers amplified the right cDNA segment.

3.2.1.3 Primer optimization and validation

The optimum annealing temperature for the primers was selected by reducing as well as varying the melting temperature (T_m) of the primers. Furthermore, to allow the primers bind at their optimum capacity, final concentration of $MgCl_2$ was varied (0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, and 2.5 mM) for a 50 μ L reaction with the purpose of choosing the most suitable concentration. The primers were validated on genomic DNA isolated from both potato and Bambara groundnut.

Table 3. 1: Names and accession numbers of aligned AMT1 genes that were used to design primers

Species	Homologs	Accession number
<i>Arabidopsis thaliana</i>	AtAMT1	X75879.1
<i>Brassica napus</i>	BnAMT1.1	AF188744.1
	BnAMT1.2	AF306518.1
<i>Camellia sinensis</i>	CsAMT1.1 gene	AB597261.1
	CsAMT1.1 RNA	AB117640.1
	CsAMT1.2	AB114913.1
<i>Citrus sinensis</i> x <i>Poncirus trifoliata</i>	CitAMT1	DQ887678.2
<i>Citrus trifoliata</i>	AMT1.1	JX049223.1
	AMT1.2	JX049224.1
<i>Cucumis sativus</i>	CusAMT1	AY642427.1
<i>Lotus japonicas</i>	LjAMT1.1	AF182188.1
	LjAMT1.2	AY135020.1
	LjAMT1.3	AJ575588.1
<i>Malus hupehensis</i> var. <i>mengshanensis</i>	MhAMT1.1	JQ072026.1
	MhAMT1.2	JQ072027.1
<i>Medicago truncatula</i>	AMT1	XM_003590129.1
<i>Oryza sativa</i>	OsAMT1.1	AF289477.1
	OsAMT1.2	AF289478.1
	OsAMT1.3	AF289479.1
<i>Populus trichocarpa</i>	PtrAMT1.1	XM_002314482.1
	PtrAMT1.3	XM_002311667.1
	PtrAMT1.4	XM_002303068.1
<i>Puccinellia tenuiflora</i>	PutAMT1.1	JQ279059.1
<i>Pyrus betulifolia</i>	PbAMT1.2	JN650604.1
<i>Pyrus ussuriensis</i>	AMT1.2	JN650608.1
<i>Solanum lycopersicum</i>	LeAMT1.1	X92854.1
	LeAMT1.2	X95098.1
	LeAMT1.3	AF118858.1
<i>Triticum aestivum</i>	TaAMT1.1	AY525637.2
	TaAMT1	AY390355.1

3.3 Sequence analyses

3.3.1 DNA extraction

DNA was extracted from Bambara groundnut (Bg) (*Vigna subterranean*) and potato (*Solanum tuberosum*) leaves using Cetyl trimethylammonium bromide (CTAB) method. Leaf tissue (0.1g - 0.2g) was ground thoroughly in liquid nitrogen with a mortar and pestle. The tissue powder was then transferred into a 1.5 mL micro centrifuge tube containing 500 μ L CTAB (cetyl trimethyl ammonium bromide) buffer and 7 μ L β -mercaptoethanol. The CTAB and plant extract mixture was incubated for 15 minutes at 55°C in a recirculating water bath and then centrifuged at 12000g for 5 minutes. The supernatant was transferred into clean micro centrifuge tubes where 250 μ L of chloroform was added to each tube and mixed by inversion. After mixing, the tubes were centrifuged at 13000 rpm for 1 minute before the upper aqueous phase was transferred to a clean micro centrifuge tube. 50 μ L of 7.5 M ammonium acetate was added to each tube followed by 500 μ L of ice cold absolute ethanol. The tubes were inverted slowly several times (to precipitate the DNA) and then stored in -20°C for 1 hour. The precipitate was isolated by centrifuging the tubes at 13000 rpm for 1 minute to form a pellet. The supernatant was removed and the DNA pellet was washed by adding two changes of 500 μ L ice cold 70% ethanol. After the wash, the DNA was spun into a pellet by centrifuging at 13000 rpm for 1 minute. All the supernatant was removed and the DNA pellet was allowed to dry for about 15 minutes. The DNA was re-suspended in 60 μ L sterile autoclaved distilled water and then incubated at 65°C for 20 minutes.

3.3.1.1 DNA concentration

The concentration of DNA samples were determined using Thermo Scientific NanoDrop 2000 spectrophotometer. To establish a blank, 1 μ L of the blanking buffer (sterile distilled H₂O) was pipetted onto the bottom pedestal. The blank was then wiped away and 1 μ L of each sample was measured to determine their concentration.

3.3.1.2 DNA quality assessment

A 0.8% agarose gel was prepared by melting 0.8g of agarose (WhiteSci) in 100 ml of 1x Tris-acetate (TAE) electrophoresis buffer in a microwave for about 2 minutes. The agarose was left to cool to about 50°C before 1.5 μ L SYBR green was added (for stain) and mixed gently without introducing bubbles. The gel was poured into the casting apparatus and allowed to set for 30 minutes at room temperature on a flat surface. Two μ L 6x loading dye

of 2 μL was added to each 2 μL DNA samples before 8 μL sterile distilled water was added to make the volume 12 μL (2 μL DNA + 2 μL loading dye + 8 μL sterile distilled water). Ten μL GeneRuler 1kb DNA ladder was loaded in the first well while the samples were loaded separately in the following wells. The lid was closed and subjected to gel electrophoresis at 100V for 1 hour. The gel was viewed and photographed under UV light to determine the presence and quality of DNA.

3.3.2 PCR assay

Amplification of target gene (AMT1) by PCR was performed using a Bio Rad T100 Thermal Cycler for potato and Bambara groundnut. It was performed in a reaction volume of 25 μL containing 17 μL of sterile distilled water, 2.5 μL 10x PCR mix, 1.25 μL 25 mM MgCl_2 , 1 μL 2.5 mM dNTP mix, 1 μL 10 mM forward primer, 1 μL 10 mM reverse primer, 1 μL DNA sample (534.0 ng/ μL and 1836.3 ng/ μL for potato and Bg respectively) and 0.25 μL Taq polymerase (Thermo scientific, Lithuania). Table 4.1 shows the AMT1-specific primer sequence (976 bp) that was used. The amplification processes included an initial denaturation step at 95°C for 2 minutes, followed by 34 cycles of 95°C for 1 minute, 60°C for 30 s and 72°C for 1 min. The reaction was brought to an end with a final extension period of 5 min at 72°C followed by cooling and storage at 12°C. The presence and quality of PCR products was visually inspected using electrophoresis. Approximately 10 μL of the PCR amplicons were analysed on a 1% molecular grade agarose gel that was stained with SYBR Green, using 1x TAE electrophoresis buffer at 100V for 1 hour. The presence of a single band of the expected size was used as a criterion for specificity.

3.3.3 DNA Sequencing

DNA nucleotide sequence analysis of core-regions of potato and Bambara groundnut AMT1 was performed using 16S rRNA sequential analysis.

3.3.3.1 Phylogenetic analysis of Bambara groundnut and potato AMT1

Core-regions of BgAMT1 and PoAMT1 nucleotide sequences were aligned by Mafft version 7 and blasted (using Basic Local Alignment Search Tool software) against the NCBI GenBank data base and compared with known nucleotide sequences. Nucleotide sequences of AMT1 and AMT2 subfamily members from various plant species (including putative BgAMT1 and PoAMT1 sequences) were aligned by Mafft version 7. The aligned sequences were imported into the Molecular Evolutionary Genetics Analysis (MEGA)

package version 6, where phylogenetic analyses were conducted using the neighbour joining (NJ) method. Table 3.2 presents the GeneBank accession numbers. The pairwise deletion option was used for handling alignment gaps. The evolutionary distances were computed using Kimura 2- parameter method while the strength of the branches was calculated with 1000 Bootstrap replicates.

3.3.3.2 Amino acid sequence alignment of Bambara groundnut AMT1

Bambara groundnut BgAMT1 amino acid sequence was aligned with *Lotus japonicus* LjAMT1.1, *Phaseolus vulgaris* PvAMT1.1 and *Glycine max* GlycineAMT1 using Mafft version 7. The GeneBank accession numbers of these genes are shown in Table 3.2.

3.3.3.3 Amino acid sequence alignment of Potato AMT1

Potato PoAMT1 amino acid sequence was aligned with *Solanum lycopersicum* LeAMT1.1, *Lotus japonicus* LjAMT1.1, and *Solanum lycopersicum* LeAMT1.2 using Mafft version 7. The GeneBank accession numbers of these genes are shown in Table 3.2.

Table 3. 2: Accession numbers of AMT1 genes that were used for multiple sequence alignment and phylogenetic analyses

AMT gene	Accession number
PvAMT1.1	GQ377869.1
GlycineAMT1	XM_003535933.2
AtAMT1.1	X75879.1
AtAMT1.2	AF083036.1
AtAMT1.3	AF083035.1
AtAMT1.4	NM_119012
AtAMT1.5	NM_113335.1
AtAMT2	NM_129385.4
TaAMT1.1	AY525637.2
TaAMT1.2	AY525638.1
LeAMT1.1	X92854
LeAMT1.2	X95098
LeAMT1.3	AF118858
LjAMT1.1	AJ279059.1
LjAMT1.2	AY135020.1
LjAMT1.3	AJ575588.1
LjAMT2	AF189762
BnAMT1.1	AF188744
BnAMT1.2	AF306518
OSAMT1.1	AF289477.1
OSAMT1.2	AF289478
OSAMT1.3	AF289479.1
OSAMT2.1	AB051864.1
OSAMT2.2	AP003252.4
OSAMT2.3	NM_190448
PutAMT1.1	JQ279059
CsAMT1.1	AB597261.1

3.4 Experimental conditions and treatments

3.4.1 Location

The hydroponic experiment was carried out from January to April in a temperature controlled research greenhouse at Cape Peninsula University of Technology, Cape Town. The controlled climatic condition included 12 hours day length, temperature of 34 ± 0.5 °C and 35% average humidity. Sterile water filtered from a Hager IP65 water filtration plant deionizer was used for irrigation.

3.4.2 Hardening process

Three week old Up To Date (UTD) tissue culture-grown potato plantlets were obtained from Ansabi Mass Tissue Culture Laboratory, Pyramid, South Africa. Algae was gently removed from plantlets in running sterile water without damaging their roots, and then planted in small pots containing a 1:1 ratio of coco peat and Styrofoam which served as the media. A mini tunnel was created to cover the plantlets with a transparent polythene sheet to maintain humidity, and then placed in the shade, to avoid direct sunlight. The polythene sheet was opened little by little each day till it was fully opened on the seventh day to acclimatize the plants (Chandra *et al.*, 2010, Mathur *et al.*, 2008). The plantlets were sprayed with organic liquid fertilizer two weeks after (Super Natural Fish Emulsion Plant Food: 93 g/kg N, 13 g/kg P, 35 g/kg K and 15 g/kg S) in order to relieve stress.

3.4.3 Hydroponic set up

One meter plastic gutters were evenly laid on a 1 meter galvanized steel table. The gutters were made of eight separate sections which were attached by means of irrigation pipes (20 mm) to water pump placed underneath the table. The water recirculating was aided by a submersible pump that pumps 1,350 litres per hour, which was used to supply nutrients to the plants using a nutrient film technique (NFT). Each reservoir (60 litres) had its individual pump. The drainage pipes drained water to the same reservoir that was drawn from. Drippers were connected to the spaghetti tube (4 mm) to control the rate of nutrient supply to the plant at 4 litres per hour. Marine silicon, a water proof sealant was used to seal leakages and external fittings. Leca hydroponic clay pellets were selected as a medium to grow the potato plantlets on. The leca clay pellets were selected for their good capillary and absorption properties, which easily carry the nutrient to the roots. Leca clay pellets were washed in running tap water, and then evenly distributed in each 12 cm square pot that has been allocated to individual gutters. The exposed part of the gutter was lined with black plastic sheathing to prevent algae growth. A photograph of the hydroponic system is shown in Appendix A.

Once the set up was complete, a solution of dodecyl dimethyl ammonium chloride of 120 g/l (spore kill) was used to clean and disinfect the whole system over a period of two days. Deionised water was then used to rinse off the spore kill for about 24 hours prior to planting.

3.4.4 Experimental setup

A completely randomized design was used with eight nutrient solution treatments for a potato growth stage. The eight nutrient solution treatments involved two forms of N: NO_3^- only (N0.75 and N7.5), NH_4^+ only (A0.75 and A7.5), a 1:1 ratio of $\text{NH}_4^+:\text{NO}_3^-$ (A0.75N0.75 and A7.5N7.5) or a 1:10 and 10:1 ratio of $\text{NH}_4^+:\text{NO}_3^-$ (A0.75N7.5 and A7.5N0.75) at two levels of N supply (0.75 or 7.5mM), to represent deficient or abundant N supply, respectively (Li *et al.*, 2010, Zebarth *et al.*, 2012). The plantlets were grown for a four-week period which represented early tuber bulking, to assess gene expression response under different source relationships. All plants were sampled on the 28th day for early tuber bulking stage.

3.4.5 Plant selection and pre-treatment

Thirty two plantlets of the same size and growth range were gently taken out of the pots and the media (coco peat and Styrofoam) was washed off their roots with distilled water. Each plantlet was then transplanted into a 12.5 cm square pot with a sterile leca-growing media in the hydroponic system. The nutrient solution (Hoagland's solution as described by Li *et al.* (2010)) containing 7.5 mM nitrogen with a 1:1 ratio of $\text{NO}_3^-:\text{NH}_4^+$ was then introduced to the plantlets for two weeks in preparation for the experimental treatments. The pH of the nutrient solution was adjusted to 5.8 with 0.1 M KOH or 32% HCl using the hand pH meter (PCTestr 35 Multi-parameter).

3.4.6 Treatments

The experimental treatments were initiated; four plantlets received one of eight nutrient solution treatments (Table 3.3) after their roots were first flushed with distilled water (for about seven hours) to remove residual nutrient solution. Treatments were modified Hoagland's nutrients solution (Hammer *et al.*, 1978, Li *et al.*, 2010, Zebarth *et al.*, 2012). The concentration of N in the nutrient solutions was balanced by varying the concentrations of sulphur from 1 to 8.5 mM (Zebarth *et al.*, 2012). The concentrations of salts used for macronutrients in the nutrient solutions are outlined in Table 3.4 while Table 3.5 shows the micronutrients that were included in the solutions.

Table 3. 3: The nutrient solution treatments

Number	Treatments (mM)
1	0.75 (NO ₃ ⁻)
2	7.5 (NO ₃ ⁻)
3	0.75 (NH ₄ ⁺)
4	7.5 (NH ₄ ⁺)
5	0.75 (NH ₄ ⁺):0.75 (NO ₃ ⁻)
6	7.5 (NH ₄ ⁺):7.5 (NO ₃ ⁻)
7	0.75 (NH ₄ ⁺):7.5 (NO ₃ ⁻)
8	7.5 (NH ₄ ⁺):0.75 (NO ₃ ⁻)

Table 3. 4: Concentration of salts used to supply macronutrients in the eight treatment nutrient solutions

Salt	Nutrient solution (mM)							
	NO ₃ ⁻		NH ₄ ⁺		NH ₄ ⁺ :NO ₃ ⁻		NH ₄ ⁺ : NO ₃ ⁻	NH ₄ ⁺ : NO ₃ ⁻
	0.75	7.5	0.75	7.5	0.75	7.5	0.75:7.5	7.5:0.75
KNO ₃	0.25	2.5	-	-	-	-	0.25	2.5
Ca(NO ₃) ₂ .4H ₂ O	0.25	2.5	-	-	-	-	0.25	2.5
(NH ₄) ₂ SO ₄	-	-	0.375	3.75	-	-	3.75	0.375
K ₂ SO ₄	1.125	-	1.25	1.25	1.25	1.25	1.125	1.25
CaCl ₂	1.75	-	2.0	2.0	2.0	2.0	1.75	2.0
NH ₄ NO ₃	-	-	-	-	0.375	3.75	-	-
KH ₂ PO ₄	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
MgSO ₄ .7H ₂ O	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

Table 3. 5: Salts and micronutrients supplied to the eight treatment nutrient solution

Salt	Micronutrient	Mg L⁻¹
Iron chelate	Fe	2.3
H ₃ BO ₃	B	0.25
MnCl ₂ .4H ₂ O	Mn	0.25
ZnSO ₄ .7H ₂ O	Zn	0.025
CuSO ₄ .5H ₂ O	Cu	0.01
Na ₂ MoO ₄ .2H ₂ O	Mo	0.01

The pH of the nutrient solution was frequently monitored (with PCTestr 35 Multi-parameter) and adjusted to 5.8 with 0.1 M KOH or 32% HCl when necessary. Each plantlet received approximately 96 litres nutrient solution treatments on each day for the 28 days duration. Plantlets were all staked a day after treatment initiation and the stakes were replaced with longer ones about every five days. This is as a result of the regular increase in the plant size.

3.4.7 Sampling and analysis

All plants for early tuber bulking growth stage were sampled at the end of the 28th day after treatment. The third leaf from the top of the plant was sampled for gene expression analyses (Zebarth *et al.*, 2012). Leaf samples from four plants per treatment were individually put in a transparent polythene bag then immediately stored in -80°C until use.

Leaf chlorophyll content index was determined on the same day (28th day) using a Chlorophyll Content Meter (CCM-200). Two readings were made on the third leaf from the top of each plant for all the treatments. The same leaf tissue from which leaf chlorophyll content index were measured was sampled for the 28th day gene expression analysis.

3.5 Quantification of gene expression

3.5.1 Primer design

The AMT nucleotide sequences that were used for the multiple sequence alignment in section 3.2.1.1 above were also used to design AMT1-specific primers, for gene expression analysis. The primers were designed from region of similarities between the aligned sequences and were evaluated as described in section 3.2.1.2 above.

3.5.2 RNA extraction

Total RNA from leaves was isolated using GeneJET Plant RNA Purification Mini Kit (Cat. No. K0801) following the manufacturer's instructions (Thermo scientific; Lithuania). Frozen leaf tissue (0.01g - 0.09g) was grounded thoroughly in liquid nitrogen with a mortar and pestle. The tissue powder was immediately transferred into a 1.5 mL micro centrifuge tube containing 500 μ L of plant RNA Lysis solution (supplemented with 10 μ L 2M DTT) and vortex for 20 seconds to mix thoroughly. The homogenate was incubated for 3 min at 56°C then centrifuged for 5 min at 14,000 rpm. Supernatant was collected and transferred to the clean micro centrifuge tube where 250 μ L of 96% ethanol was added and mixed by pipetting. The prepared mixture was transferred to a purification column inserted in a collection tube which was then centrifuged for 1 min at 11,000 rpm. The flow through solution was discarded and the column and collection tube was reassembled. 700 μ L of Wash Buffer WB 1 was added to the purification column (containing ethanol) and centrifuged for 1 min at 11,000 rpm. The flow through and collection tube was discarded while the purification column was placed into a clean 2 mL collection tube. 500 μ L of Wash Buffer 2 (containing ethanol) was added to the purification column and then centrifuged for 1 min at 11,000 rpm. The flow through solution was discarded while column and collection tube was reassembled. Wash Buffer 2 of 500 μ L (containing ethanol) was added to the purification column the second time and then centrifuged for 1 min at 11,000 rpm. The flow through solution was discarded while column and collection tube was reassembled. The column was re-spined for 1 min at maximum speed 14,000 rpm. The collection tube containing the flow through solution was discarded while the purification column was transferred to an RNase - free 1.5 mL collection tube. To elute the RNA, 50 μ L of nuclease free water was added to the centre of the purification column membrane and centrifuged for 1 min at 11,000 rpm.

The presence and quality of RNA was visualised using gel electrophoresis as described in section 3.3.1.2. The concentration of RNA was also determined using Thermo Scientific NanoDrop 2000 spectrophotometer as described above.

3.5.3 Preliminary experiment

3.5.3.1 Qualitative PCR assay

In an exploratory experiment to optimize the reference gene (β -tubulin) and AMT1-specific primers (160 bp) for RT-qPCR assay, expression level of potato AMT1 was determined by end point PCR. Total RNA was extracted from potato leaf samples of five selected N treatments (A7.5N7.5, N0.75, N7.5, A7.5 and A0.75 represented by C, P1, P2, P3, and P4 respectively) using Thermo Scientific GeneJET Plant RNA Purification Mini Kit (Cat. No. K0801) as described in section 3.5.2. The presence and quality of total RNA was visualised using gel electrophoresis also as described in section 3.3.1.1. Thermo Scientific NanoDrop 2000 spectrophotometer was used to determine RNA concentration as similarly described above. The reverse transcription-PCR for amplification of target gene (AMT1) was performed using Thermo scientific Verso 1-step QRT-PCR kit according to manufacturer's description in a Rotor-Gene 3000 (Corbett research). It was performed in a reaction volume of 25 μ L containing 0.25 μ L verso enzyme mix, 12.5 μ L 1-step qPCR mix, 1.25 μ L RT enhancer, 1 μ L 10 mM forward primer, 1 μ L 10mM reverse primer, 1 μ L 1 ng RNA sample, 5.5 μ L sterile distilled water and 2.5 μ L SYBR green. AMT1 primer of 160 bp was used to amplify target gene while β -tubulin was used to account for variation in total RNA. Table 4.1 shows the sequences of both primers. The amplification processes included 50°C for 15 minutes, 95°C for 15 minutes, 35 cycles of 95°C for 20 sec, 54°C for 20 sec and 72°C for 30 sec. The quality and sizes of end point PCR was visually inspected using electrophoresis as earlier described.

3.5.4 Reverse transcription-quantitative PCR assay

Absolute quantification approach was used to determine potato AMT1 expression level in a One-Step RT-qPCR, with SYBR Green 1 using a Rotor-Gene 3000 (Corbett research). The assay was performed over a range of RNA concentrations. A stock of 50 ng/ μ L was prepared for all the total RNA samples. Samples of 5 μ L each was pulled into a single 1.5 ml micro centrifuge tube where a 5-point 10 fold serial dilution was prepared from the pulled gene to generate a standard curve by Rotor-Gene 6.1.93 software as described above. KAPA SYBR FAST One-Step qRT-PCR kit was used in the assay following the manufacturer's instructions. Reactions contained 6.8 μ L nuclease-free water, 10 μ L 2X KAPA SYBR FAST qPCR Master Mix, 0.4 μ L of each 10 μ M AMT1-specific primer or *Solanum tuberosum* β -tubulin, 0.4 μ L 50X KAPA RT Mix and 2 μ L of 50 ng total RNA in a final volume of 20 μ L. The AMT1-specific primer (160 bp) and β -tubulin (101 bp: used to

account for variation in total RNA) that was used is shown in Table 4.1. Two separate master mixes were prepared prior to dispensing into individual PCR tubes, to reduce pipetting errors and to make sure that each reaction contains the correct and equal amount of primers as well as RNA samples. Both master mixes were prepared without RNA samples. However, the first master mix contains the reference primer while the second master mix contains AMT1-specific primer. Two μL of each dilution point was aliquoted in duplicate into the PCR tubes containing 18 μL master mix with the reference primer. Two μL of unknown RNA sample was also aliquoted into the PCR tubes containing 18 μL master mix with AMT1-specific primer. Two μL nuclease-free water was also pipetted in both PCR tube containing reference gene and specific primer to represent No Template Control (NTC). A No RT Control (NRTC) was included in the reaction whereby KAPA RT Mix was excluded in a reaction tube component. All the tubes were centrifuged briefly without introducing bubbles before they were placed into the appropriate wells of the Rotor-Gene. The following cycle protocol was used for all the One-step RT-qPCR reactions: 42°C for 5 minutes, 95°C for 5 minutes, 40 cycles of 95°C for 20 sec, 54°C for 20 sec and 72°C for 30 sec. Data were analysed using Rotor-Gene 6.1.93 software.

3.6 Statistical Analysis

The data generated were subjected to descriptive and inferential statistics using SPSS version 21.

CHAPTER FOUR RESULTS AND DISCUSSION

4.1 Bioinformatics; primer design, evaluation and optimization

4.1.1 Primer design

Literature and database searches for AMT1 gene revealed about 31 sequences as listed in section 2.4.1 and Table 3.1. Four of the homologs; MhAMT1.1, CusAMT1, CsAMT1.1 and LjAMT1.1 were selected for further analysis because of availability of full gene sequences in the database and the relationship of the species to both potato and Bambara groundnut. The multiple sequence alignment shown in Figure 4.1, was used to design AMT1 specific-primers. The non-degenerate primer sequence which was designed to amplify AMT1 gene in a PCR (target product, 976 bp) assay is shown in Table 4.1.



Figure 4. 1: Sequence alignment of several AMT1 plant species; the highlighted nucleotides with the region of similarities are used to design the 976 bp AMT1 large enough for bioinformatics analysis

Table 4. 1: Primer sequence for one reference gene and two AMT1-specific genes, the amplification length, nucleotide length and the melting temperature of the amplified product

Name	Accession number	Primer sequence 5'-3'	Primer sequence 5'-3'	Length (bp)	Tm (°C)
β -tubulin	609267	ATGTTTCAGGCGCAAGGCTT	TCTGCAACCGGGTCATTCAT	101	79
AMT1		ATGGGTGGTGGAGGGA	GGGTCAGATCCATACCCGC	160	63
AMT1		GCCATCGCCGCCGCCGG	GGGTCAGATCCATACCCGC	976	67

4.1.2 Primer evaluation and optimization

The primers were evaluated by BLASTing against *Solanum lycopersicum* and *Solanum tuberosum* genomic sequences present in NCBI database to ensure that the designed primers would specifically target the AMT1 gene. The bioinformatics evaluation of the primers revealed that they were AMT1-specific to *Solanum lycopersicum* and *Solanum tuberosum*. The optimum annealing temperature selected for the AMT1-specific primers was 60°C. In a preliminary experiment to optimize MgCl₂ for the 976 bp AMT1 primers, final concentration of MgCl₂ in a 50 μ L PCR reaction component was varied (0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, and 2.5 mM for 0, 0.5, 1, 1.5, 2.0, 2.5, 3.0 and 5.0 μ L, respectively). PCR product of the expected size was only observed for 0.75, 1, 1.25, 1.5 and 2.5 mM respectively out of all the MgCl₂ treatments. Figure 4.2 presents the agarose gel result which shows AMT1 amplicon/sizes (976 bp) for MgCl₂ optimization.

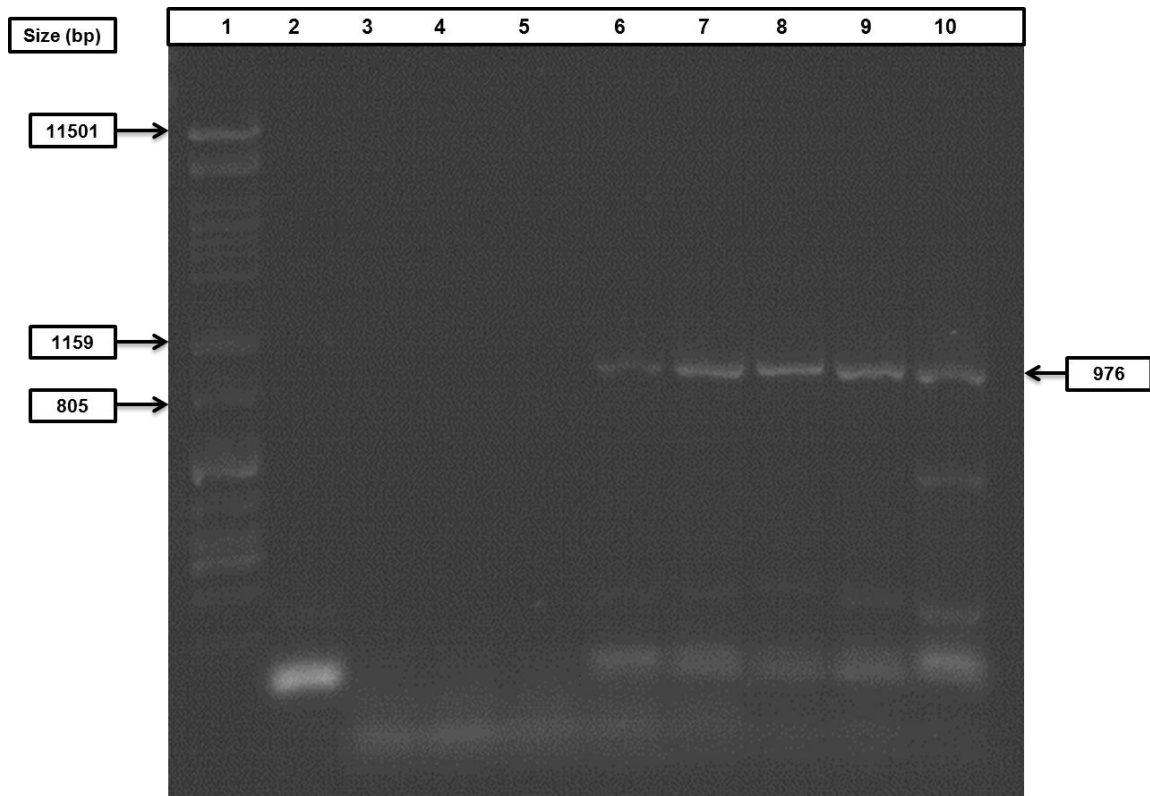


Figure 4. 2: Photograph of a SYBR Green stained agarose gel showing the results of a ($MgCl_2$) optimization reaction. The lanes represents: 1- Molecular weight marker, 2- No Template Control and 3-10- Varied $MgCl_2$ treatments: 0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, and 2.5 mM

No amplification was found on 0, 0.25 and 0.5 mM $MgCl_2$ lanes while amplification of PCR product was observed on 1.0, 1.25, 1.5 and 2.5 mM $MgCl_2$ lanes. The result shows that the optimum conditions are 1.0 and 1.25 mM $MgCl_2$. Higher $MgCl_2$ concentration resulted in un-specificity as shown by multiple amplification products while lower $MgCl_2$ concentration resulted in low or no amplification at all. The 1.25 mM $MgCl_2$ for a 50 μ L PCR reaction component was chosen.

4.2 Isolation and sequencing of Bambara groundnut and Potato AMT1

4.2.1 DNA extraction and amplification of AMT1 gene

Bambara groundnut and potato DNA were successfully extracted and their concentration was determined; 534 and 1836.3 ng/ μ L for potato and Bg respectively. A260:A280 ratio indicates 2.05 and 2.04 for potato and Bg DNA respectively. DNA quality is presented in Figure 4.3. The quality of PCR products was visually checked using electrophoresis where the generation of a single band of 976 bp size was taken as the criterion for AMT1 specificity (Fig 4.4).

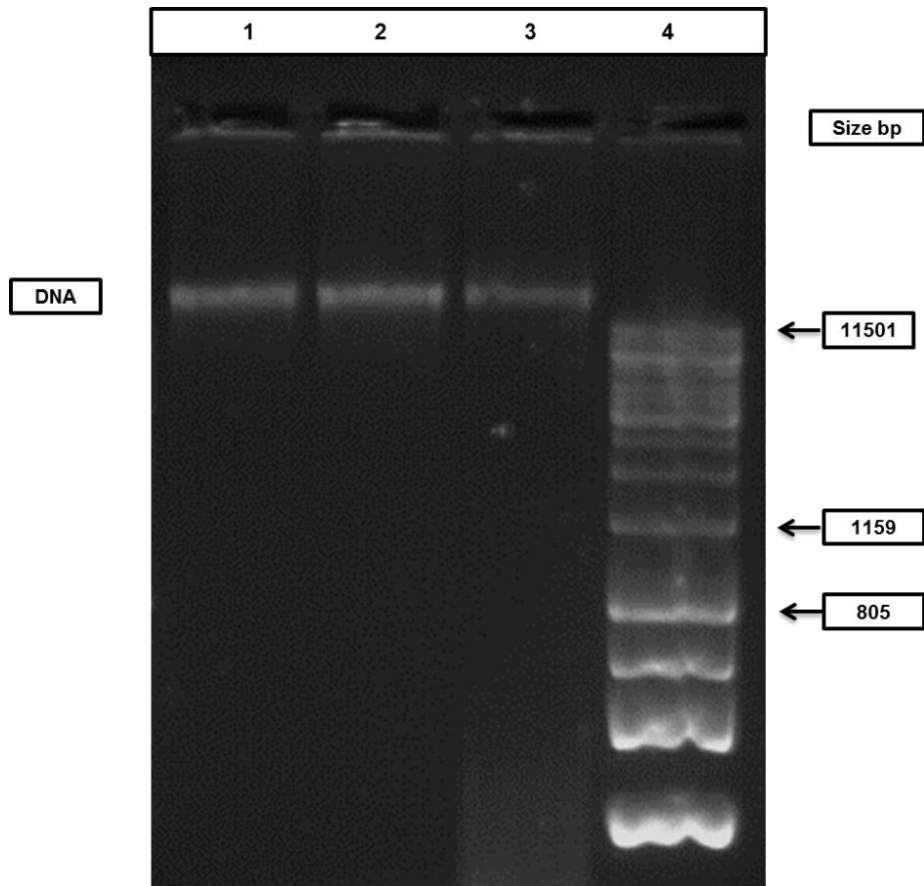


Figure 4. 3: Photograph of a SYBR Green stained agarose gel showing potato and Bambara DNA quality of different concentrations. The lanes are: 1- Potato DNA sample, 2- Potato DNA sample, 3- Bambara groundnut DNA sample and 4- Molecular weight marker

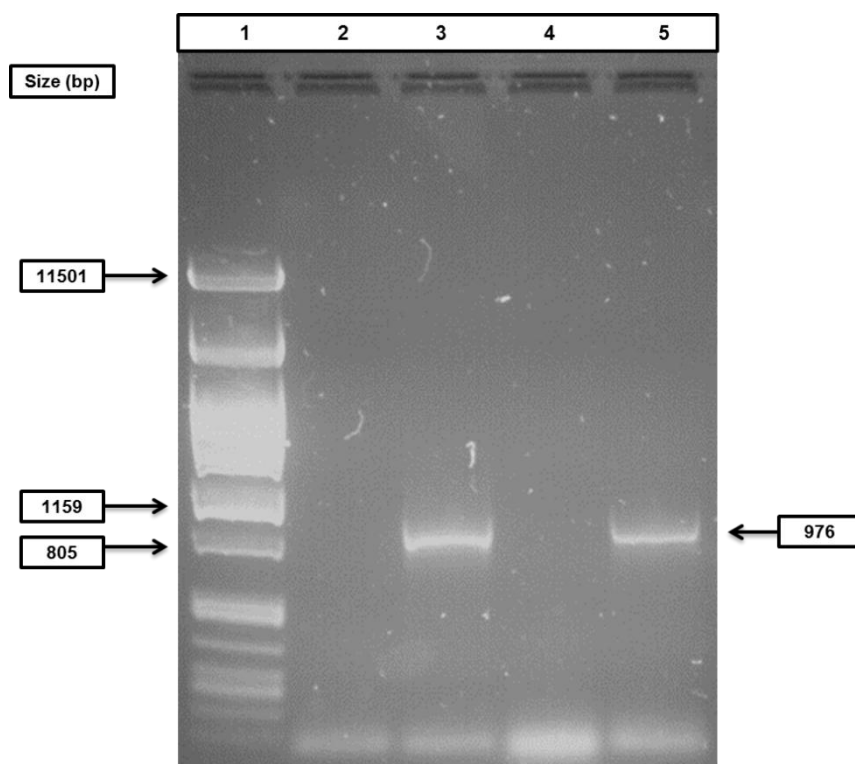


Figure 4. 4: Photograph of a SYBR Green stained agarose gel showing amplified products and sizes (976 bp) of potato and Bambara AMT1. The lanes are: 1- Molecular weight marker, 2- No Template Control (NTC), 3- Potato PCR product, 4- Potato PCR product and 5- Bambara groundnut PCR product

4.2.2 Bambara groundnut and potato AMT1 sequence identification

Core-regions of Bambara groundnut BgAMT1 and potato PoAMT1 genes of 976 bp long were amplified and sequenced. Their sequences are shown in Appendix B. This is the first sequence report on BgAMT1.

4.2.2.1 Phylogenetic analysis

4.2.2.1.1 Phylogenetic analysis of Bambara groundnut

Figure 4.5 shows that the predicted BgAMT1 belongs to the AMT1 subfamily, and that it is most closely related to PvAMT1.1 followed by GlycineAMT1 and LjAMT1.1, respectively.

Bambara groundnut is an important leguminous crop that is indigenous to Africa. It is known for its highly nutritional composition, functional properties, antioxidant potential and drought tolerance ability (Baryeh, 2001). It has a crucial impact in sustainable agriculture due to its ability to fix atmospheric N₂. It is however one of the neglected crops. In legume crops, NH₄⁺ is the principal product of symbiotic N fixing bacteria, hence contribute greatly to N nutrition

(Udvardi and Day, 1997). Apart from the fact that AMT1 assimilates N from roots, they probably play a crucial role in the efficiency of the symbiosis (Salvemini *et al.*, 2001), indicating that AMT1 in leguminous plants may function in the regulation of nodule formation (D'apuzzo *et al.*, 2004). Since NH_4^+ plays a key role in nodule metabolism and in plant N nutrition as a whole, it is worth studying the part that AMT1 plays in the development and functioning of nodules. Also, it is important to have knowledge of how they support the integration of the extra NH_4^+ supplied by the nodule organ in the general frame of partitioning of nitrogenous solutes to the whole plant (D'apuzzo *et al.*, 2004). This study reports the amplification and sequence analysis of the core-region of BgAMT1 for the first time, and opens up many possibilities for further studies on this subject.

The relationship of the predicted BgAMT1 and products of other AMTs reported in higher plants was analysed. When nucleotide sequences were compared, the phylogenetic tree showed that BgAMT1 forms a well-supported and monophyletic clade with other plant AMT1 genes that have been isolated so far (Fig 4.5). Plant AMT1 family have been reported to have high affinity and selectivity for NH_4^+ thereby playing a role in NH_4^+ uptake from the soil (Ortiz-Ramirez *et al.*, 2011, Pantoja, 2012). This result indicates that the predicted BgAMT1 not only belongs to plant AMT1 family but also has high affinity to assimilate NH_4^+ from the soil.

4.2.2.1.2 Phylogenetic analysis of potato AMT1

Figure 4.5 shows that PoAMT1 belongs to the AMT1 subfamily and that it is most closely related to LeAMT1.1 followed by LeAMT1.2 and LeAMT1.3 respectively.

The phylogenetic analysis showed that PoAMT1 forms a well-supported and monophyletic clade with other plant AMT1 genes that have been isolated so far (Fig 4.5). This shows that putative PoAMT1 belongs to plant AMT1 family and hence has high affinity and selectivity for NH_4^+ thereby playing a role in NH_4^+ uptake from the soil.

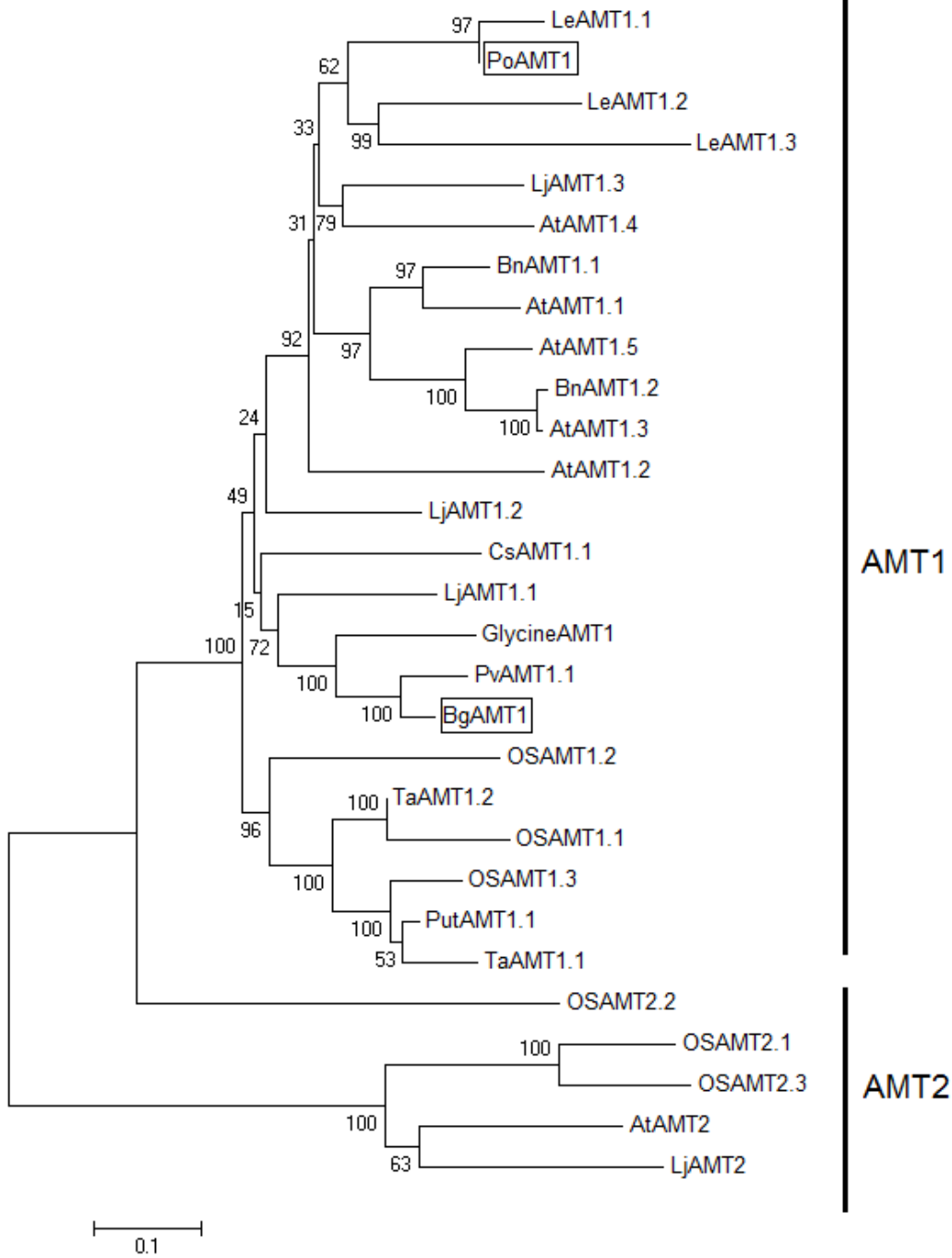


Figure 4. 5: Phylogenetic tree analysis of plant AMT families. The tree was constructed by multiple (nucleotides) sequences alignment of 29 members of plant AMT families using the Mega 6 software with the Neighbour joining method. The accession numbers are listed in Table 3.2

4.2.2.2 Amino acid sequence analysis of Bambara groundnut AMT1

Sequence translation of BgAMT1 gene fragment gives a peptide of 247 aa long (Fig 4.6). The amino acid sequence alignment of Bambara groundnut BgAMT1 with *Lotus japonicus* LjAMT1.1, *Phaseolus vulgaris* PvAMT1.1, and *Glycine max* GlycineAMT1 revealed that BgAMT1.1 is 92%, 89% and 87% similar to PvAMT1.1, GlycineAMT1 and LjAMT1.1 respectively. The amino acid sequence alignment also showed that the amplified fragment of BgAMT1 corresponds to 5th – 10th AMT1 transmembrane spanning regions (Fig 4.6). Tryptophan (Bg W1), Serine (Bg S87) and Aspartic acid (Bg D23) represent selected residues that are constitutive of the pore in BgAMT1 (Fig 4.6).

Amino acid sequence alignment indicated that BgAMT1 is most closely related to PvAMT1.1 (92% similarity) (Ortiz-Ramirez *et al.*, 2011), followed by GlycineAMT1 (89% similarity) (NCBI database) and LjAMT1.1 (87% similarity) (Salvemini *et al.*, 2001) respectively, suggesting that a portion of NH₄⁺ transporter gene was amplified. It is not surprising that the aforementioned closely related homologues of BgAMT1 are all from the legume family (*Fabaceae*). *Phaseolous vulgaris* PvAMT1.1 mediates the high affinity and electrogenic transport of NH₄⁺ and it is pH dependent in that NH₄⁺ transport increased at low pH (5.5) (Ortiz-Ramirez *et al.*, 2011). The high affinity transport property of PvAMT1.1 for NH₄⁺ is evidence that BgAMT1 is a member of AMT1 family and may be responsible for NH₄⁺ transport. *Glycine max* GlycineAMT1 is a putative AMT1, but this transporter has not yet been further analysed. Apart from the fact that *Lotus japonicus* LjAMT1.1 is the first NH₄⁺ transporter gene with an intron (1009 bp) and no open reading frame of any marked length, it is also a high affinity AMT1 gene (transport affinity: 1.7 μM) which is highly expressed in roots, leaves and nodules in low N conditions (D'apuzzo *et al.*, 2004, Salvemini *et al.*, 2001). LjAMT1.1 has been reported to likely function in recovering NH₄⁺ escaped from nodule cells in the different tissues during normal metabolism apart from transporting NH₄⁺ across the symbiosome membrane. This is a further indication that the core-region of BgAMT1 that we isolated is a high affinity ammonium transporter and may be predicted to play a role in different steps of *Rhizobium* infection.



Figure 4. 6: An alignment of the amino acid sequence of Bambara groundnut BgAMT1 with *Phaseolous vulgaris* PvAMT1.1, *Lotus japonicus* LjAMT1.1 and *Glycine max* GlycineAMT1. The accession numbers are listed in Table 3.2. The amino acid residues conserved in all sequences are designated by stars, and conservative substitutions are shown by dots. Predicted transmembrane-spanning domains are marked above the alignment (TMD 5-10). Some amino acids predicted as constitutive of the pore in BgAMT (Bg W1, Bg D23 and Bg S87) are marked at the top of the sequence in rectangles

Several studies have revealed that bacteria (*E.coli* EcAmtB) and plant AMTs possess 11 transmembrane regions. The core-region of BgAMT1 that was isolated and sequenced in this study corresponds to 5th – 10th TMDs of other plant AMT transmembrane (Fig 4.6). A report on EcAmtB showed that 1 – 10 transmembrane domain of the AMT family collectively

diverge outward from the central plane in a right-handed helical bundle to produce a vestibule on each side of the cell membrane (Khademi and Stroud, 2006).

The similarity of plant AMTs with that of *E.coli* and *A.fulgidus* has been reported to be 20 - 25% low. However, plant proteins tend to maintain similar tertiary and quaternary structures (transmembrane domains, cytoplasmic loops and the carboxyl terminal), as revealed by molecular modelling (Loque *et al.*, 2007, Mayer and Ludewig, 2006, Ortiz-Ramirez *et al.*, 2011). In *EcAmtB*, W148 and S219 among other conserved amino acid residues, were indicated to play a role in structuring the NH_4^+ binding site (Andrade *et al.*, 2005, Khademi *et al.*, 2004, Zheng *et al.*, 2004). These residues (*EcAmtB*: W148 and S219) which have been reported to increase transport activity at mutation by between 2 and 10 fold (Pantoja, 2012), correspond to W1 and S28 of the predicted BgAMT1 amino acid residues (Fig 4.6). The residues are well conserved with other selected proteins as shown in the alignment (Fig 4.6). Therefore, mutations in Bg W1A-L and S28A may be predicted to function in aiding NH_4^+ transport activity. The Bg D23 residue is equivalent to the residues in *E.coli EcAmtB* (D160) (Pantoja, 2012), *Saccharomyces cerevisiae* ScMep2 (D186N) (Marini *et al.*, 2006) and *Arabidopsis thaliana* AtAMT1.1 (D198N) (Loque *et al.*, 2007), which have all been stipulated to be inactive when substituted with Ala, hence inhibit NH_4^+ transport at mutation.

In all, it can be predicted that the properties of the predicted BgAMT1 will be high affinity and selectivity for NH_4^+ uptake, pH dependent, and may possibly contribute in different steps of rhizobia interaction. Cloning and further characterization of complete BgAMT1 gene will confirm this report. On the other hand, if for instance we propose to increase NH_4^+ transportation through genetic engineering, we can perform site directed mutagenesis of W1A-L or S87A which have been shown to increase NH_4^+ transport activity between 2 and 10 fold (Hall and Kustu, 2011, Javelle *et al.*, 2008).

4.2.2.3 Amino acid sequence analysis of potato AMT1

PoAMT1 gene fragment showed a peptide of 193 aa long at translation (Fig 4.7). Amino acid sequence alignment showed that PoAMT1 is 92%, 83% and 76% similar to LeAMT1.1, LjAMT1.1 and LeAMT1.2, respectively. The amino acid sequence alignment also showed that the amplified fragment of PoAMT1 corresponds to 5th – 10th AMT1 transmembrane spanning regions (Fig 4.7). Aspartic acid (Po D16) and Serine (Po S70) represent the selected residues that are constitutive of the pore in PoAMT1 (Fig 4.7).

```

PoAMT1 ----- 0
LeAMT1.1 -----MACSVDTLAPFLGPNTTNAVAASVYICNQFSGVSDRFVDTGYAIDSTYLLFS 42
LjAMT1.1 -----MAALPECSAANLAQLIGPNATDAAAVAGFICDQF TAVGQRFSDTAFAVDSTYLLFS 46
LeAMT1.2 AAQIMASAMTCSAAELFPHLGSSA-NATAAAEFICSRFSAVSEYLTNTTYAVDVTYLLFS 50

PoAMT1 ----- 0
LeAMT1.1 AYLVFMSQLGFAMLLAGSVR--NTMNIIMLTNVLDAAGGLFYLLFGFAFALGGPSNGFIG 90
LjAMT1.1 AYLVFMSQLGFAMLCAGSVRAKNTMNIIMLTNVLDAAGGLFYLLFGFAFAGAPSNGFIG 98
LeAMT1.2 AYLVFAMQLGFAMLCAGSVRAKNTMNIIMLTNVLDAAGGFSYLLFGFAFAGAPSNGFIG 100

PoAMT1 ----- 0
LeAMT1.1 RHFFGLKEIPSNFSDYMNFLYQWAFIAAAGITSGSIAERTQFVAYLIYSSFLTGFVYVPV 140
LjAMT1.1 RHFFGLKDVPTVAFDYSYFLYQWAFIAAAGITSGSIAERTQFVAYLIYSSFLTGFVYVPV 146
LeAMT1.2 KHFFGLKEFPSQAFDYSYFLYQWAFIAAAGITSGSIAERTQFVAYLIYSSFLTGFVYPI 150

PoAMT1 ----- 0
LeAMT1.1 -----ASP--GNSNXLFGSGVDPFAGSGVVVHVVGGIAGFYGALIEGPRIGRFDH 37
LjAMT1.1 VSHWFNTPDGWASP--TNSNLLFGSGVDPFAGSGVVHVVGGIAGFYGALIEGPRIGRYDH 188
LjAMT1.1 VSHWFNSGSGWASAT-NTGNLLFGTGVDPFAGSGVVHVVGGVAGLWGALIEGPRIGRFDH 195
LeAMT1.2 VSHWFNSGSGWASASKTDGNLLRFGVDPFAGSGVVHVVGGIAGLWGAFIEGPRIGRFDR 200
* * * * *
TMD 6 Po S70 TMD 7
TGRXVAXRGHSASLVVLTGTFLLWFGWYGFNPGSIFNKILIXYDGA-TGGYYGQWSAVGYXA 88
LjAMT1.1 TGRSVALRGHSASLVVLTGTFLLWFGWYGFNPGSIFNKILVTYG-A-SGGYYGQWSAVGRTA 236
LjAMT1.1 AGRVSLRGHSASLVVLTGTFMLWFGWYGFNPGSIFTKILSAYD--SGNYYGQWSAIGRTA 242
LeAMT1.2 SGRSVALRGHSASLVVLTGTFLLWFGWYGFNPGSIFLTIKSYDHTIRGTYGQWSAIGRTA 250
* * * * *
TMD 8 TMD 9
VTTTLAGCTAALTTFLGKRILSGHNVVTDVCGNLLGGFAAITAGCSXXEPWAAIICGXA 136
LjAMT1.1 VTTTLAGCTAALTTFLGKRILSGHNVVTDVCGNLLGGFAAITAGCSVVEPWAAIICGFVA 286
LjAMT1.1 VTTTLAGCTAALTTFLGKRILSGHNVVTDVCGNLLGGFAAITAGCSVVEPWAAIICGFVA 292
LeAMT1.2 VTTTLAGCTAALTTFLCKRLLVAHNVVTDVCGNLLGGFAAITSGCAVVEPWAAIICGFIA 300
* * * * *
TMD 10
ALVLIIGCXMLAEKFKYDDPLEAAQLHGGCGAWGIIIFTALFAKGEFVDQVYPGKPGRPHGL 186
LjAMT1.1 ALVLIIGFNMLAEKFKYDDPLEAAQLHGGCGAWGIIIFTGLFAKGEFVDQVYPGKPGRPHGL 336
LjAMT1.1 AVVLIACNMLAEKVRYDDPLEAAQLHGGCGAWGIIIFTALFAKEEYVNVQVYPGKPGRPHYGL 342
LeAMT1.2 AHVLIIGFNALAAKLYDDPLEAAQLHGGCGSGWIIIFTGLFAKKEEYVNEVYPGFPNRPYGL 350
* * * * *

PoAMT1 ----- 193
LjAMT1.1 FMGGGGKLLGAHQIQLVIIGWVSATMGPLFYLLHFKLLRISSEDEMAGMDLTRHGGA 386
LjAMT1.1 FMGGGGKLLGAHQIQLVIIGWVSATMGPLFFILNKMKLLRISTEDELAGMDLTRHGGA 392
LeAMT1.2 FMGGGGKLLGAHQIQLVIIGWVSATMGPLFYLLHFKLLRISRDETAGMDLTRHGGA 400
*****

PoAMT1 ----- 193
LjAMT1.1 YYH--EEDP--KLGQMRRIEPTTST----- 408
LjAMT1.1 YAY--EDDESHKPGIQLRKIEPNSSSTPSAES----- 422
LeAMT1.2 YIYHDEDEGSSMPGFKMTRVPTNTSTPDHQNRSVNVVVV*QINK*IKMLLYRMVTL*LRK 450

PoAMT1 ----- 193
LjAMT1.1 ----- 408
LjAMT1.1 ----- 422
LeAMT1.2 RQISKCFNNVILLVS**TIYIYAKYF*PKKKGLYLF*LYDFIVCVFLFVCLFI*SSYLVC 500

PoAMT1 ----- 193
LjAMT1.1 ----- 408
LjAMT1.1 ----- 422
LeAMT1.2 SKDVNK*IINYSGQKKKKKKX 500

```

Figure 4. 7: An alignment of the amino acid sequence of potato PoAMT1 with *Solanum lycopersicum* LeAMT1.1, *Lotus japonicus* LjAMT1.1 and *Solanum lycopersicum* LeAMT1.2. The accession numbers are listed in Table 3.2. The amino acid residues conserved in all sequences are designated by stars, and conservative substitutions are shown by dots. Predicted transmembrane-spanning domains are marked above the alignment (TMD 5-10). Some amino acids predicted as constitutive of the pore in PoAMT (Po D16 and Po S70) are marked at the top of the sequence in rectangles

The amino acid sequence similarity which PoAMT1 shares with LeAMT1 (92%) (Lauter *et al.*, 1996), LjAMT1.1 (83%) (Salvemini *et al.*, 2001) and LeAMT1.2 (76%) (Von Wiren *et al.*, 2000b) showed that a portion of NH₄⁺ transporter gene was indeed amplified. *Solanum*

lycopersicum LeAMT1.1 and LeAMT1.2 are from the same family with potato PoAMT1 (*Solanaceae*). A substrate affinity study on tomato showed that LeAMT1.1 is a high affinity transporter with K_m value of about 10 μM (Von Wiren *et al.*, 2000a). The high transport activity of this homolog depends on concentration and voltage, but not affected by protons. Furthermore, LeAMT1.1 is preferentially expressed in root hairs indicating that it acquires NH_4^+ from the rhizosphere (Von Wiren *et al.*, 2000a). The affinity transport property of LeAMT1.1 is an indication that the putative PoAMT1.1 is from AMT1 family and has high affinity for NH_4^+ uptake from the rhizosphere. LjAMT1.1 has high affinity for NH_4^+ at 1.7 μM and is highly expressed in leaves, roots and nodules (Salvemini *et al.*, 2001). This further supports the stipulation that putative PoAMT1.1 is not only from AMT1 family but also a high affinity transporter of NH_4^+ . LeAMT1.2 is a close homologue of LeAMT1.1 with 76% amino acid similarity (Von Wiren *et al.*, 2000b) and it is preferentially expressed in root hairs indicating NH_4^+ uptake from the rhizosphere. In addition, the highest expression level of LeAMT1.2 occurs after the onset of light. It has been reported that LeAMT1.2 plays a role in the uptake of xylem-derived NH_4^+ or in the retrieval of photorespiratory NH_3 (Von Wiren *et al.*, 2000b).

Plant AMT1 family members have been shown to have 11 transmembrane spanning domain (Gazzarini *et al.*, 1999, Howitt and Udvardi, 2000). The core-region of putative PoAMT1 that was isolated and sequenced in this study corresponds to 5th – 10th TMDs of the plant AMT transmembrane (Fig 4.7). The amino acid sequence alignment showed that among other residues, the putative PoAMT1 possess Po S70 (equivalent to *EcAmtB* S219) which have been indicated to increase transport activity at mutation by between 2 and 10 fold (Pantoja, 2012). The residue (Po S70) is well conserved with other selected proteins as shown in the alignment (Fig 4.7). Residue Po D16 correlates with the residues in *E.coli EcAmtB* (D160) (Pantoja, 2012), *Saccharomyces cerevisiae* ScMep2 (D186N) (Marini *et al.*, 2006) and *Arabidopsis thaliana* AtAMt1.1 (D198N) (Loque *et al.*, 2007) and falls under the 5th transmembrane spanning region of PoAMT1 amino acid sequence. The implication of this is that PoAMT1 is a high affinity NH_4^+ transporter and mutation of Po S70A may play a role in enhancing NH_4^+ transport activity while residue Po D16 may inhibit NH_4^+ transport activity when substituted with Ala.

4.3 Effect of N rate and N form on leaf chlorophyll content index

In general good plant growth was recorded with all N treatments in the hydroponically grown potato over the 4-weeks period.

Leaf chlorophyll content index (CCI) measured using CCM-200 varied with different N treatments (Appendix C). Leaf chlorophyll content index showed a mean: 13.45 ± 1.36 in response to nitrate deficient (N0.75) nutrient while it showed a mean: 34.53 ± 2.21 for abundant nitrate (N7.5) nutrient. For ammonium deficient (A0.75) nutrient, leaf chlorophyll content index showed a mean: 15.0 ± 0.83 , while it showed a mean: 35.83 ± 5.11 when abundant ammonium (A7.5) nutrient was supplied. The leaf chlorophyll content index observed in plant treated with composite blend nutrient of both deficient ammonium and nitrate (A0.75N0.75) nutrient showed a mean: 15.70 ± 0.83 ; while index for abundant ammonium and nitrate (A7.5N7.5) nutrient treated plants showed a mean: 36.10 ± 3.68 . Plants treated with ammonium deficient/ abundant nitrate (A0.75N7.5) nutrient showed leaf chlorophyll index with mean: 36.53 ± 4.12 , while ammonium abundant/ deficient nitrate (for A7.5N0.75) nutrient treated plants showed a mean: 25.90 ± 1.06 (Appendix C).

There was significant difference ($P > 0.05$) between potato leaf CCI of treatments supplied with abundant N supply and treatments supplied with deficient N (Appendix E). Leaf CCI was increased with increased N supply whereas it decreased with decreased N supply. Leaf CCI was high for N7.5 treatment while N0.75 treatment resulted in low leaf CCI (Fig 4.8). In the same manner, A7.5 treatment showed high CCI yet A0.75 treatment showed low leaf CCI. Leaf CCI for N0.75 treatment was lower than A0.75 treatment while leaf CCI for A7.5 treatment was higher than that of N7.5 treatment. For A0.75N0.75 treatment, leaf CCI of potato was low while it was high for A7.5N7.5 treatment. High leaf CCI was observed for A0.75N7.5 treatment yet A7.5N0.75 treatment resulted in low leaf CCI. There was no significant difference ($P < 0.05$) between the leaf CCI of A0.75N0.75 treatment and N7.5 treatment (Appendix E). In all, A7.5N7.5 showed the highest leaf CCI followed by A0.75N7.5 treatment while N0.75 showed the least leaf CCI among other treatments (Fig 4.8).

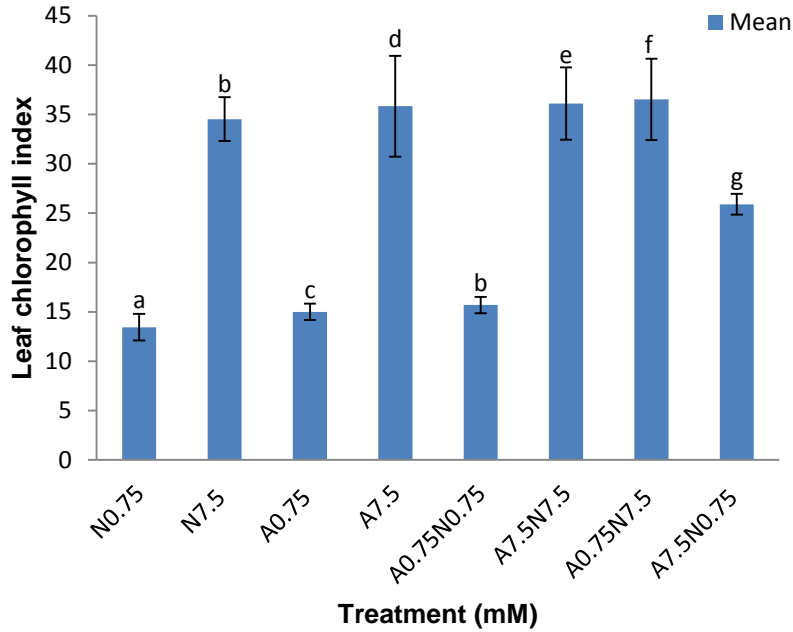


Figure 4. 8: Response of leaf chlorophyll content index to N rate and form. Values with the same letter are not significantly different

A number of studies have been done to assess potato N status by measuring leaf chlorophyll content (Gianquinto *et al.*, 2004, Li *et al.*, 2010, Vos and Bom, 1993, Zebarth *et al.*, 2012). The result of this study indicates that leaf chlorophyll content index (CCI) is sensitive to N form and rate. Leaf CCI increased as N supply was increased. This observation seems to be consistent with other research which reported that SPAD-502 chlorophyll metre value increases progressively with N supply (Guler, 2009, Majic *et al.*, 2008, Nemeth *et al.*, 2007, Turner and Jund, 1991, Zhu *et al.*, 2012). Leaf CCI was highest when a mixture of NO_3^- and NH_4^+ (A7.5N7.5) was supplied in abundance (Appendix C). This is an indication that leaf CCI may respond more to N supply when it is a mixture of NO_3^- and NH_4^+ . When N was supplied in abundance, leaf CCI for NO_3^- only was higher than that of NH_4^+ only. Furthermore, a comparison of a mixture of deficient NH_4^+ /abundant NO_3^- and a mixture of abundant NH_4^+ /deficient NO_3^- treatments showed higher leaf CCI for deficient NH_4^+ /abundant NO_3^- treatment (Fig 4.8). These results indicate that N supply in the form of NO_3^- only, has more effect on potato leaf CCI than NH_4^+ only. However, the findings of the current study do not support the previous research by Zebarth *et al.* (2012), who reported that leaf CCIs were mainly more responsive to N supply when NH_4^+ was the N form. There are several possible explanations for this difference. For NH_4^+ only, Zebarth *et al.* (2012) reported the lowest leaf CCI at tuber bulking stage while they recorded the highest leaf CCI at both tuber initiation stage and flowering stage among all the N treatments. The result of

the current study is only based on tuber bulking stage (four weeks after planting) which corroborates with the low leaf CCI recorded only at tuber bulking stage in Zebarth *et al.* (2012) study. Research under field and hydroponic conditions has however shown that potato leaf CCI differs with growth stages, growing season and variety (Giletto *et al.*, 2010, Guler, 2009, Li *et al.*, 2010, Minotti *et al.*, 1994, Wu *et al.*, 2007). Indeed, the timely fluctuation of leaf CCI varies between N treatments (Vos and Bom, 1993). In general, these findings suggest that leaf chlorophyll content indexes were more responsive to N supply when NO_3^- was the N form.

4.4 Gene expression analysis of AMT1 in potato leaf

4.4.1 Primer design

For a RT-qPCR experiment, it is recommended that the primer length should be between 100 - 200 bp. The 160 bp AMT1-specific primer sequence is presented in Table 4.1. The bioinformatics evaluation of the primers showed that they are AMT1-specific to *Solanum lycopersicum* and *Solanum tuberosum*. The optimum annealing temperature selected for the primers was 54°C.

	Query	1180	TGGTGCATGGGGGATAATTTTACCGGGTTATTCGCTAAAGGGGA-GTTTGTGGATCAAG	1238
<input type="checkbox"/>	JQ072026	1203	C..C..G.....C..G.C.C.G..T..G.-G..A..A..AC...A.CG.G.	1261
<input type="checkbox"/>	AB117640	1181	C..A..G.....G.G.....C..G.C.C.T..T..C.GC.A.A.-.AC...A.CG.G.	1239
<input type="checkbox"/>	AF182188	1215	C..C..G.....C..A..C.....C.C.....C..G.A.....AC...A.C..G.	1273
	Query	1239	TATA-CCCGGGTAAACCGGGTCGACCTCACGGGTTATTTGGGGTGGGTTGCTTAACTA	1297
<input type="checkbox"/>	JQ072026	1262	.G..-.....A...A...G..G..AT.....C.G.....C..C.....G..G	1320
<input type="checkbox"/>	AB117640	1240	.G..T.....A.GT...AC-..C..AT.T.....G..G.....C..A.....GGT.G	1298
<input type="checkbox"/>	AF182188	1274	.T..-.....G..AT.....G..C.....G..G	1332
	Query	1298	CTCGGGGCGCATATAATCCAGATACTCGTAATAATCGGGTGGGTCAGCGGACAATGGGT	1357
<input type="checkbox"/>	JQ072026	1321	..G.....C..CT.G..A.....G.G.....T..A.....G.....G.....G	1380
<input type="checkbox"/>	AB117640	1299	T.G.C.....G.G..T....TG.G..T..TG.T.....T..T.TT..T.....	1358
<input type="checkbox"/>	AF182188	1333A.....CG.G.....TT.G..G..T.....C.....	1392
	Query	1358	CCGCT-TT-TTTATATACTTCATAAATCAAGTTGCTTCGGATATCGTCCGAAGACGAAA	1415
<input type="checkbox"/>	JQ072026	1381	...T.-G.-.C.T.G.G..G..C..GC.G...C...GA.....G.G..G.....	1438
<input type="checkbox"/>	AB117640	1359	..AT.G..C...G.-.-.....C..GC.....C.T.....C..AG...G..T....	1416
<input type="checkbox"/>	AF182188	1393	..T..G..C..C..-.-.GA...GA.G.....C..AA.G..G..T...C	1450
	Query	1416	TGCGGGTATGGATCTCACCGGACATGGTGGGTTTGCTTAT	1456
<input type="checkbox"/>	JQ072026	1439G..C..G.....	1478
<input type="checkbox"/>	AB117640	1417	.A.....A.....A...	1457
<input type="checkbox"/>	AF182188	1451	.T.....A...	1491

Figure 4. 9: Sequence alignment of several AMT1 plant species; the highlighted nucleotides with the region of similarities are used to design the 160 bp AMT1 for use in qPCR

4.4.2 Total RNA isolation

In an experiment to investigate the response of the AMT1 expression level to N status, RNA was successfully isolated from hydroponically grown potato leaf collected at early tuber bulking growth stage, as described in section 3.4.7 above. Thermo Scientific NanoDrop 2000 spectrophotometer was used to determine concentration of each sample which varied from 36.4 - 1045 ng/μL. RNA quality was checked through SYBR Green stained gel electrophoresis (Fig. 4.10) where the quality of each treatment varied. Any variation observed at this stage is due to technical issues of RNA isolation that is why RNA quantity must be standardized when setting up amplification reactions.

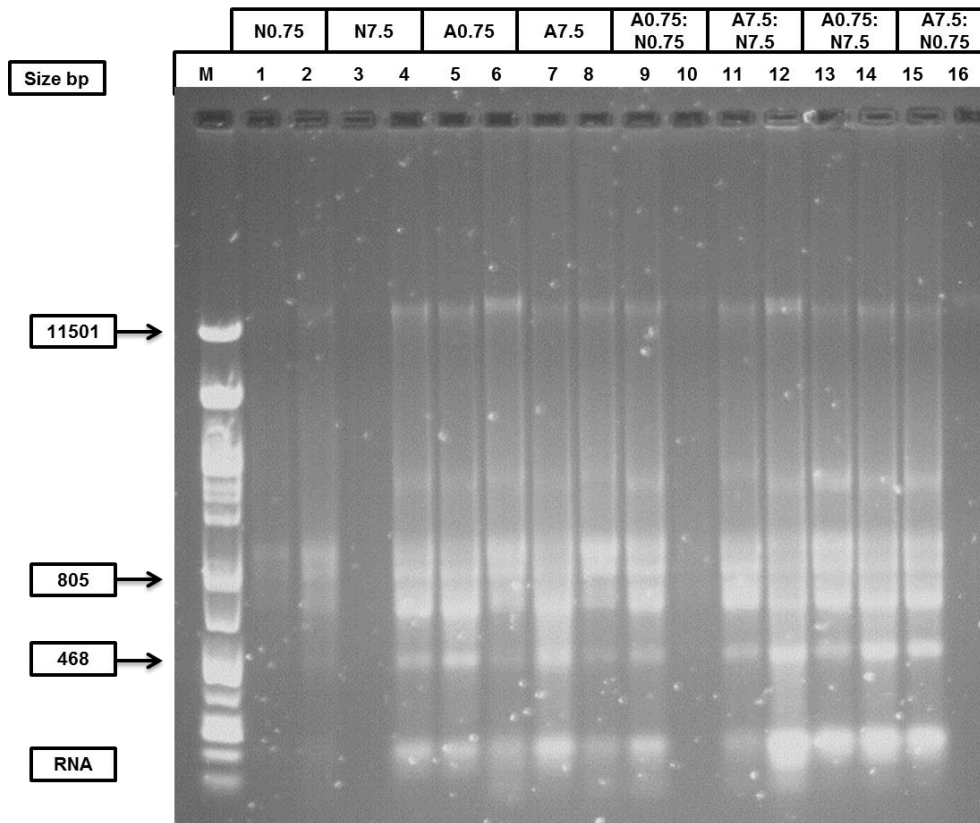


Figure 4. 10: Photograph of a SYBR Green stained agarose gel showing RNA quality of varying concentration. The lanes are: M (molecular weight marker), 1-2 (N0.75), 3-4 (N7.5), 5-6 (A0.75), 7-8 (A7.5), 9-10 (A0.75N0.75), 11-12 (A7.5N7.5), 13-14 (A0.75N7.5) and 15-16 (A7.5N0.75) respectively

4.4.3 Preliminary: Response of AMT1 expression level to N rate and form

4.4.3.1 Qualitative RT-PCR

Semi-quantitative RT-PCR analysis was performed on total RNA of potato leaf samples that were grown hydroponically with varying N rate and form. This was done to investigate the pattern of potato AMT1 expression under the aforementioned N conditions. The concentrations of total RNA that was extracted from selected leaf samples were 995, 1045, 1122.1, 1675.8 and 422.2 ng/ μ L, respectively for A7.5N7.5 (C), N0.75 (P1), N7.5 (P2), A7.5 (P3), and A0.75 (P4) treatments. One ng/ μ L was used for each sample. The amplified products and sizes as well as the qualitative expression of β -tubulin and AMT1 (160 bp) are shown in figure 4.11. The expression of potato AMT1 is given relative to β -tubulin mRNA levels. The expression of β -tubulin was relatively the same for all the treatments as expected apart from P3 whose expression was low and the size was 160 bp instead of the expected 101 bp size. Potato AMT1 expression was high for N0.75, N7.5 and A0.75 treatment with the

expression being lower for N7.54. Expression of AMT1 was low for A7.5N7.5 (C) and A7.5 (P3). Amplification was however found on 101 bp against the expected 160 bp size for P3 treatment.

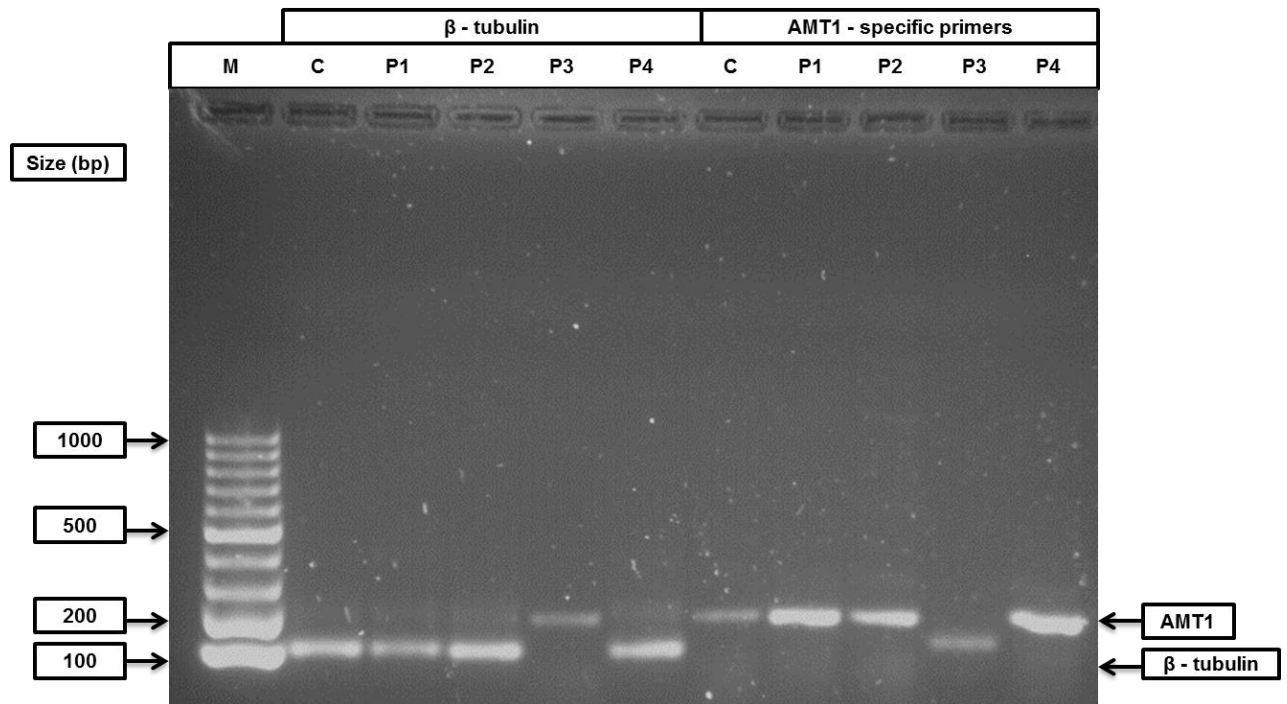


Figure 4. 11: Photograph of SYBR Green stained agarose gel showing amplicons of β-tubulin (101 bp) and AMT1 (160 bp) for each treatment

The expression of β-tubulin (Lopez-Pardo *et al.*, 2013, Nicot *et al.*, 2005) was used as a reference to determine AMT1 expression in this study. β-tubulin mRNA level for A7.5 (P3) treatment was not only low, but showed a different product size compared to other treatments (Fig. 4.11). The variation in β-tubulin mRNA level may indicate that P3 treatment had an influence on its expression or some form of contamination occurred. In this preliminary study, expression of AMT1 by semi-quantitative analysis showed that potato leaf AMT1 expression increased when N supply was decreased (P1 and P4) regardless of N form. This finding is consistent with a semi-quantitative analysis study on *Brassica napus* BnAMT1.2 in leaf (Pearson *et al.*, 2002). Furthermore, a study by Zebarth *et al.* (2012) indicated an increase in *Solanum tuberosum* AT1 expression when 0.75mM NH₄⁺ and 0.75 mM NO₃⁻ was supplied at tuber bulking stage. Increased NO₃⁻ (P2) supply however also increased the potato leaf AMT1 expression level along with the aforementioned expression for the N deficient treatment. This result corroborates Von Wiren *et al.* (2000b) study who

reports high expression of LeAMT1.2 in tomato leaf when NO_3^- was continuously supplied for 3 weeks. It is however contradictory to a quantitative analysis carried out on *Solanum tuberosum* AT1 (Zebarth *et al.*, 2012). The end-point PCR analysis in this study showed that AMT1 expression level decreased when N supply was increased (C and P3). This is in accord with Zebarth *et al.* (2012) results which showed that *Solanum tuberosum* AT1 expression decreased when 7.5 mM NH_4^+ , 7.5 mM NO_3^- as well as a mixture of 7.5mM NH_4^+ and 7.5 mM NO_3^- were applied. In addition, a semi-quantitative RT-PCR assay on *Brassica napus* BnAMT1.2 leaf also showed low expression when 5.0 mM NH_4^+ was continuously supplied (Pearson *et al.*, 2002). It is however not surprising that the low AMT1 expression level indicated for P3 treatment was observed on 160 bp instead of the expected 101 bp. This change in product size was also earlier observed in β -tubulin mRNA when treated with P3, possibly due to contamination. In general, the semi-quantitative RT-PCR assay in this preliminary study showed that AMT1 expression in potato leaf increased when N was deficient while it decreased when N supply was in abundance regardless of N form. However, these findings are qualitative and preliminary besides having a small sampling/treatment size.

4.5 Response of AMT1 expression level to N rate and form

RT-qPCR assay was performed on total RNA isolated from potato leaf which was subjected to varying N rate and form for four weeks.

The correlation coefficient (R^2) value from the standard curve analysis was 0.98433 (Fig. 4.12). AMT1 mRNA levels showed varied responses to different rates and forms of N (Appendix D). AMT1 expression level showed a mean: (24.65 ± 2.94 ng/ μL) when nitrate deficient (N0.75) nutrient was supplied, while the abundant nitrate (N7.5) nutrient results in AMT1 expression level which showed a mean: (8.73 ± 2.61 ng/ μL). For ammonium deficient (A0.75) nutrient, AMT1 expression level showed a mean: (9.83 ± 1.31 ng/ μL), while the abundant ammonium (A7.5) nutrient resulted in AMT1 expression level with a mean: (6.23 ± 0.46) ng/ μL . Furthermore, the AMT1 expression level in plants treated with ammonium/nitrate deficient (A0.75N0.75) N nutrient showed a mean: (18.76 ± 1.11 ng/ μL), while that for ammonium and nitrate abundant (A7.5N7) N nutrient treated plants showed a mean: (12.94 ± 0.32 ng/ μL). In plants treated with ammonium deficient/nitrate abundant (A0.75N7.5) N nutrient, the AMT1 expression level showed a mean: (11.96 ± 2.63 ng/ μL), while the AMT1 expression level observed in ammonium abundant/nitrate deficient (A7.5N0.75) N nutrient treated plants showed a mean: (22.69 ± 0.13 ng/ μL) (Appendix D).

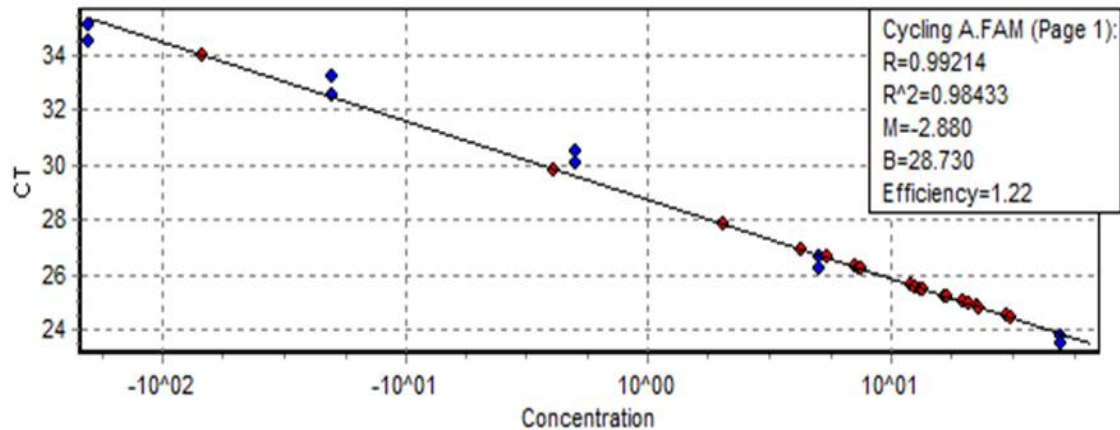


Figure 4. 12: Standard curve for potato AMT1 concentration

AMT1 mRNA levels showed varied responses to different rates and forms of N. Generally, expression level of potato AMT1 was increased when N supply was reduced, but not when N supply was in the form of deficient NH_4^+ only (A0.75) (Fig. 4.13). Increased N supply decreased the expression level of AMT1 regardless of N form. AMT1 expression level was high for N0.75 treatment while it was low for N7.5 treatment. However, the expression level of AMT1 decreased when A0.75 treatment and A7.5 treatment were supplied. AMT1 expression level was high for A0.75N0.75 treatment yet it was low for A7.5N7.5 treatment. For A0.75N7.5 treatment, expression level of AMT1 decreased while it increased for A7.5N0.75 treatment. When N deficient treatments were compared, it was observed that N0.75 had highest AMT1 expression level followed by A0.75N0.75 and A0.75 (Fig. 4.13). For abundant N supply, A7.5 had the lowest AMT1 expression level followed by N7.5 and A7.5N7.5. In all, the N0.75 and A7.5N0.75 treatments had the highest AMT1 expression level followed by the A0.75:N0.75 treatment.

In an attempt to study and quantitate AMT1 expression level for the purpose of correlating it to plant N status, two levels and forms of N supply were imposed on hydroponic grown potato plants. Ammonium transporters are expressed with a different pattern of regulation in several plant organs (roots, leaves, stems, shoots, buds, nodules) (D'apuzzo *et al.*, 2004, Gazzarini *et al.*, 1999, Ninnemann *et al.*, 1994, Von Wiren *et al.*, 2000a), although they are preferentially expressed in the root tissue. Expression of AMT1 in leaves have been reported in *Solanum lycopersicum* LeAMT1.2 and LeAMT1.3 (Von Wiren *et al.*, 2000b), *Brassica napus* BnAMT1.2 (Pearson *et al.*, 2002), *Lotus Japonicus* LjAMT1.1 - 1.3 (D'apuzzo *et al.*, 2004) and *Solanum tuberosum* AT1 (Zebarth *et al.*, 2012) and their primary function may be

NH_4^+ transport from the apoplast as well as the recovery of photorespiratory NH_4^+ (D'apuzzo *et al.*, 2004, Pearson *et al.*, 2002, Zebarth *et al.*, 2012).

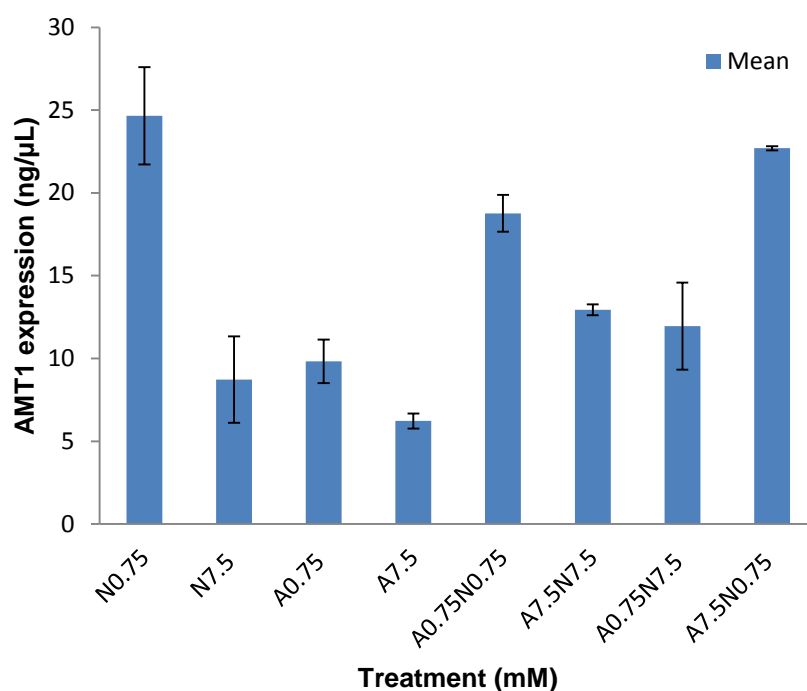


Figure 4. 13: RT-qPCR assay showing the effect of N form and N rate on expression of potato AMT1

The correlation coefficient (R^2) value from the standard curve analysis in this study was 0.984. This indicated the percentage of the data which matched the hypothesis that the given standards form a standard curve (Fig. 4.12). Increased N supply decreased the expression level of AMT1 in potato leaves regardless of N form. These findings further support previous research on leaves of *Brassica napus* BnAMT1 (Pearson *et al.*, 2002) and *Solanum tuberosum* AT1 (Zebarth *et al.*, 2012). AMT1 expression level was found to increase when N supply was reduced in the form of NO_3^- and a mixture of NH_4^+ and NO_3^- . The increase in AMT1 expression at low N supply is in agreement with other studies. Zebarth *et al.* (2012) reported an increase in AT1 expression when hydroponic grown potato was subjected to 0.75 mM NH_4^+ , 0.75 mM NO_3^- and a mixture of 0.75 mM NH_4^+ and NO_3^- respectively. In leaf, *Solanum tuberosum* AT1 expression was also high when N supply was reduced under field condition (Zebarth *et al.*, 2011). Contrary to expectations, this study showed low AMT1 expression level in potato leaf when N supply was 0.75 mM NH_4^+ only. This is in contrast with a number of studies (Pearson *et al.*, 2002, Wiren and Merrick, 2004a, Wiren and Merrick, 2004b, Zebarth *et al.*, 2012). This rather contradictory result may be due

to some factors. AMT1 expression level was low when N was supplied in a mixture of 0.75 mM NH_4^+ and 7.5mM NO_3^- while it was highly increased for a mixture of 7.5 mM NH_4^+ and 0.75 mM NO_3^- . Furthermore, 0.75 mM NO_3^- and a mixture of 7.5 mM NH_4^+ and 0.75 mM NO_3^- treatments showed the highest AMT1 expression level followed by a mixture of 0.75 mM NH_4^+ and 0.75 mM NO_3^- treatment. This result shows that NO_3^- has more effect on AMT1 in potato leaf. In addition, high AMT1 expression in potato leaf correlates with low NO_3^- regardless of presence or absence of NH_4^+ . It is however worth knowing that differential expression of AMT1 homologs (in roots and leaves) have been observed in several plant species which include; LjAMT1.1-3 (D'apuzzo *et al.*, 2004, Salvemini *et al.*, 2001), LeAMT1.1-3 (Von Wiren *et al.*, 2000b), AtAMT1.1-5 (Gazzarini *et al.*, 1999, Ninnemann *et al.*, 1994, Yuan *et al.*, 2009), PutAMT1.1 (Bu *et al.*, 2013). In addition, expression of rice OsAMT1 varies from different varieties, to growth conditions and experimental setup, whereas various studies have proven that expression of AMT1 in plants are generally regulated by nitrogen starvation (Kumar *et al.*, 2003, Sonoda *et al.*, 2003a, Wiren and Merrick, 2004a).

The findings of this study indicate that AMT1 probably transports NH_4^+ efficiently, but NO_3^- inefficiently. Low NO_3^- will therefore induce high levels of AMT1 expression to ensure N uptake. Monitoring of AMT1 expression levels in plants can therefore be used as a direct indicator of the plant's attempt to take up nitrogen in the form of nitrate from an environment where nitrate is limiting.

CHAPTER FIVE CONCLUSION AND RECOMMENDATION

5.1 Conclusion

This work is to our knowledge the first report of nucleotide sequence of Bambara groundnut BgAMT1 core-region, and its bioinformatics analysis. Amino acid sequence alignment as well as the phylogenetic analysis showed that BgAMT1 and PoAMT1 are indeed from the plant AMT1 family and they have high affinity to assimilate NH_4^+ from the soil. BgAMT1 may contribute to different steps in rhizobia interaction. In potato, site directed mutagenesis of Po S70A can be performed to increase NH_4^+ transport activity.

We also used RT-qPCR to study the response of potato PoAMT1 expression level to N rate and form and demonstrated the potential to correlate the expression level to potato N status. AMT1 expression in potato leaf was sensitive to N form and rate. AMT1 expression level in potato leaf increased when N supply was low and the expression was generally more responsive to NO_3^- form of nitrogen. We propose AMT1 as a candidate gene for determining the plant N status with NO_3^- .

5.2 Recommendation

Further work should be done to clone a complete BgAMT1 gene and report its uniqueness as AMT1 gene in a legume crop. It is important to study the role it plays in the development and functioning of nodules and how they bring about combination of additional NH_4^+ supplied by the nodule organ. The RT-qPCR study should be expanded to other crops to validate its utility in determining the plant N status.

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APPENDICES

Appendix A: Hydroponic system for potato N concentration studies



Appendix B: Forward (primer-1) and reverse (primer-2) sequences of BgAMT1 and PoAMT1

BgAMT1_Primer-1

TGGGCCGCAGACGGTTGGGCATCCGCGTTCAACGTAAACAATCTTTTATTCTCCACCG
GCGTAATCGACTTCGCCGGCTCCGGCGTTGTCCACATGGTCGGCGGTGTGGCGGGTC
TCTGGGGCGCGCTTATCGAGGGTCCCGAATCGGCCGATTGATCACGCGGGACGCG
CGGTGGCCTTACGTGGCCACAGCGCCTCGTTAGTCGTGCTTGAACCTTCATGCTGTG
GTTCCGGTTGGTACGGTTCAACCTGGTTCATTCAACAAAATCTTAGTCCCTTATAGCTC
AGGGGAAAAGTATGATCAATGGAGCGCGTTGGAGAACCGCGGTTACCAACAACACT
AGCAGGGTCAACGGCGGCGTTAACTACGCTGTTCCGGAAACGCCTGATATCTGGTCA
TGGAACGTCAACGATGTGTGCAACGTTTCTGGGTGGGTTCCGCCCATCACAGCAG
GTTGTTCTGTGGTGGAGCCGTGGGCGGCTATCGTGTGTGGTTTTGTGGCGTCCCTTGT
TCTGATCGGTGTAACAAACTGCGGGAGAAGGTGAGGT

BgAMT1_Primer-2

ATCTGAATCAGGTGCGCCGCCAACAACCTCCCTCCACCACCCATAAGCAACCCGTGCG
GCCTCGACGGATCTCTCGAATAAACCTCCGCCACGTACTCCTTCTTCGCGAACAGCGC
CGTGAAAATCACCCCCACGCGCCACACCTCCATGCAACTGCGCCGCTCAAAGGA
TCGTGCAACCTCACCTTCTCCGCCAGTTTGTACACGCGATCAGAACAAGGGACGCCA
CAAAACACACACGATAGCCGCCACGGCTCCACCACAGAACAACTGCTGTGATGGC
GGCGAACCCACCCAGCAAACCGTTGCACACATCGGTGACGTTCCAGTGACAGATATC
AGGCGTTTTCCGAACAGCGTAGTTAACGCCGCCGTTGACCCTGCTAGTGTTGTGGTAA
CCGCGGTTCTCCCAACCGCGCTCCATTGACCATCTTTCCCCCTGAGCTATAAGGGAC
TAAGATTTTGTGAATGAACAGGGTTGAAACCGTACCAACCGAACACAGCATGAAGG
TTCCAAGCACGACTAACGAGGCGCTGTGGCCACGTAAGGCCACCCGCGC

PoAMT1_Primer-1

GCTAGCCCGGTAATTCAAATCTKTTATTCCGGATCCGGTGTTATCGATTTCCCGGGTC
GGGTGTAGTTCAATGGTAGGTGGAATTGCCGGGTTTTATGGAGCTTTAATTGAAGGTC
CGAGAATCGGGCGGTTGATCATAACGGGTCGATCYGTTGCACTYCGTGGACATAGCGC
GTCACCTCGTGGTTCTAGGCACCTTTTTATTATGGTTCCGGATGGTACGGGTTTAAACCGG
GTTCAATTAATAAAAATCCTAATTACYTACGACGGTGCAACCGGAGGGTATTATGGTCAAT
GGAGCGCTGTGGGCCGWACYGCGGTTACAACCTAGCCGGTTGCACCGCTGCC
TAACAACCTTTTTCGTAAAAGGATCCTTTCCGGGTCATTGGAAAGTACCGATGTATGT
AACGGTTTACTAGGCGGATTTGCAGCAATCACGGCTGGATGCTCGGTYGTWAGCCAT
GGCCCGGATTATATGTGGGTTYGTGGCGGC

PoAMT1_Primer-2

TCCCTCCTCCACCCATGAATAACCCGTGAGGTCGACCCGGTTTACCCGGGTATACTTG
ATCCACAACCTCCCTTTAGCAAATAATGCAGTGAAAATTATCCCCATGCACCACAAC
CACCATGTAATTGTGCTGCTTCAAGTGGATCGTCTGATTTAAATTTCTCCGCTAACATRT
TACAACCGATTAAAATAAAGCCGCCACRAACCCACATATAATCGCGGCCATGGCTC
WACRACCGAGCATCCAGCCGTGATTGCTGCAAAATCCGCCTAGTAAACCGTTACATACA
TCGGTTAC

Appendix C: Descriptive statistics of leaf chlorophyll content index for potato N concentration studies

	N	Range	Minimum	Maximum	Sum	Mean	
	Statistic	Statistic	Statistic	Statistic	Statistic	Statistic	Std. Error
N0.75	4	6.20	9.80	16.00	53.80	13.4500	1.35554
N7.5	4	10.60	30.00	40.60	138.10	34.5250	2.20959
A0.75	4	3.90	12.70	16.60	60.00	15.0000	.83367
A7.5	4	21.70	20.70	42.40	143.30	35.8250	5.11263
A0.75N0.75	4	3.80	14.30	18.10	62.80	15.7000	.82865
A7.5N7.5	4	17.00	25.50	42.50	144.40	36.1000	3.67605
A0.75N7.5	4	18.80	24.80	43.60	146.10	36.5250	4.12096
A7.5N0.75	4	4.70	23.30	28.00	103.60	25.9000	1.06066
Valid N (listwise)	4						

Appendix D: Descriptive statistics of AMT1 expression level for potato N concentration studies

	N	Range	Minimum	Maximum	Sum	Mean	
	Statistic	Statistic	Statistic	Statistic	Statistic	Statistic	Std. Error
N0.75	4	10.17	19.57	29.74	98.62	24.6549	2.93668
N7.5	4	9.03	4.22	13.24	34.92	8.7312	2.60588
A0.75	4	4.53	7.56	12.09	39.31	9.8276	1.30872
A7.5	4	1.59	5.43	7.02	24.92	6.2291	.45856
A0.75N0.75	4	3.84	16.84	20.68	75.05	18.7624	1.10916
A7.5N7.5	4	1.13	12.38	13.50	51.76	12.9395	.32497
A0.75N7.5	4	9.12	7.40	16.52	47.83	11.9585	2.63350
A7.5N0.75	4	.46	22.46	22.92	90.78	22.6938	.13322
Valid N (listwise)	4						

Appendix E: Pearson Correlation Matrix for the measured chlorophyll content index across the different N rates and form

		Correlations				
		N0.75	N7.5	A0.75	A7.5	A0.75N0.75
N0.75	Pearson Correlation	1	-.326	.037	.947	-.283
	Sig. (2-tailed)		.674	.963	.053	.717
	N	4	4	4	4	4
N7.5	Pearson Correlation	-.326	1	-.060	-.019	.987 [*]
	Sig. (2-tailed)	.674		.940	.981	.013
	N	4	4	4	4	4
A0.75	Pearson Correlation	.037	-.060	1	-.075	.099
	Sig. (2-tailed)	.963	.940		.925	.901
	N	4	4	4	4	4
A7.5	Pearson Correlation	.947	-.019	-.075	1	.006
	Sig. (2-tailed)	.053	.981	.925		.994
	N	4	4	4	4	4
A0.75N0.75	Pearson Correlation	-.283	.987 [*]	.099	.006	1
	Sig. (2-tailed)	.717	.013	.901	.994	
	N	4	4	4	4	4
A7.5N7.5	Pearson Correlation	-.190	-.824	-.252	-.439	-.879
	Sig. (2-tailed)	.810	.176	.748	.561	.121
	N	4	4	4	4	4
A0.75N7.5	Pearson Correlation	-.481	.413	.800	-.452	.524
	Sig. (2-tailed)	.519	.587	.200	.548	.476
	N	4	4	4	4	4
A7.5N0.75	Pearson Correlation	.632	.341	.508	.727	.449
	Sig. (2-tailed)	.368	.659	.492	.273	.551
	N	4	4	4	4	4

		A7.5N7.5	A0.75N7.5	A7.5N0.75
N0.75	Pearson Correlation	-.190	-.481	.632
	Sig. (2-tailed)	.810	.519	.368
	N	4	4	4
N7.5	Pearson Correlation	-.824	.413	.341
	Sig. (2-tailed)	.176	.587	.659
	N	4	4	4
A0.75	Pearson Correlation	-.252	.800	.508
	Sig. (2-tailed)	.748	.200	.492
	N	4	4	4
A7.5	Pearson Correlation	-.439	-.452	.727
	Sig. (2-tailed)	.561	.548	.273
	N	4	4	4
A0.75N0.75	Pearson Correlation	-.879	.524	.449
	Sig. (2-tailed)	.121	.476	.551
	N	4	4	4
A7.5N7.5	Pearson Correlation	1	-.407	-.813
	Sig. (2-tailed)		.593	.187
	N	4	4	4
A0.75N7.5	Pearson Correlation	-.407	1	.282
	Sig. (2-tailed)	.593		.718
	N	4	4	4
A7.5N0.75	Pearson Correlation	-.813	.282	1
	Sig. (2-tailed)	.187	.718	
	N	4	4	4

*. Correlation is significant at the 0.05 level (2-tailed).