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# *Vigna subterranea* ammonium transporter gene (*VsAMT1*): Some bioinformatics insights

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ABSTRACT

Ammonium transporters (AMTs) play a role in the uptake of ammonium, the form in which nitrogen is preferentially absorbed by plants. *Vigna subterranea* (VsAMT1) and *Solanum tuberosum* (StAMT1) AMT1s were characterized using molecular biology and bioinformatics methods. AMT1-specific primers were designed and used to amplify the AMT1 internal regions. Nucleotide sequencing, alignment and phylogenetic analysis assigned *VsAMT1* and *StAMT1* to the AMT1 family. The deduced amino acid sequences showed that VsAMT1 is 92% and 89% similar to *Phaseolus vulgaris* PvAMT1.1 and *Glycine max* AMT1 respectively, while StAMT1 is 92% similar to *Solanum lycopersicum* LeAMT1.1, and correspond to the 5th–10th trans-membrane domains. Residues VsAMT1 D23 and StAMT1 D15 are predicted to be essential for ammonium transport, while mutations of VsAMT1 W1A-L and S87A and StAMT1 S76A may further enhance ammonium transport. In addition to nitrogen uptake from the roots, VsAMT1 may also contribute to interactions with rhizobia.



## 1. Introduction

*Vigna subterranea* (common name: 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 [3]. 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 [3]. *V. subterranea* 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 [3,26]. It has a crucial impact in sustainable agriculture due to its ability to fix atmospheric $N_2$. *V. subterranea* however remains one of the most neglected crops by science. Hence, this under-utilized crop with great potential is not well funded.

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 [10] and agricultural [11] environments. The N composition of plant tissues also has important nutritional effects, since plants constitute a significant source of protein in the diet of humans and animals.

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 nitrate ($NO_3^-$) and ammonium ($NH_4^+$) in the soil [4]. The first step that occurs in nitrogen assimilation is the uptake of $NO_3^-$ and $NH_4^+$ into root cells from the soil solution [6]. Roots sense and respond to changes in internal and external N status, which include the regulation of gene expression, metabolism and further N uptake and assimilation [13]. The uptake and assimilation of $NH_4^+$ requires less energy than that of $NO_3^-$, because $NO_3^-$ has to be reduced before assimilation [5]. Hence, $NH_4^+$ is the preferential form of N uptake when plants are subjected to N deficiency [6,12].

In legume crops, $NH_4^+$ is the principal product of symbiotic N fixing bacteria [32]. Symbiotic relationship occurs between rhizobium bacteria and its legume host plant. The rhizobia present within nodules of the root systems fixes nitrogen from atmospheric nitrogen ($N_2$) into $NH_3/NH_4^+$ which is then transferred to the plant cytoplasm where it is assimilated mainly through the glutamine synthetase/glutamine GS/GOGAT pathway. Hence, $NH_4^+$ plays a key role in nodule metabolism and in plant N nutrition as a whole [8,29].

Ammonium transporters (AMTs) of the Ammonium Transporter/Methylammonium Permease/mammalian Rhesus (AMT/MEP/

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Rh) protein family are present in all domains of life [22,37]. 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 [7,16,41]. 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 [38]. Plant AMT encodes a high-affinity transport system and a low-affinity transport system and constitutes multi-gene family [6,35,36]. All plant AMT proteins investigated so far are located in the plasma membrane, indicating that their role is in $NH_4^+$ acquisition by plant cells [20,21,23,30,31,39]. Furthermore, AMT1 in leguminous plants may function in different steps of the rhizobium infection and colonization N-fixation [8,29].

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 such as *V. subterranea*. *Solanum tuberosum* and *Lotus japonicus* can be used as a model crop from which results can be extrapolated to *V. subterranea*. In the long term, this would result in more rapid development and wider utilization of neglected African crops. This paper reports the isolation and characterization of the ammonium transporter genes (AMT1) of *S. tuberosum* and *V. subterranea* using molecular biology and bioinformatics methods.

## 2. Materials and methods

### 2.1. PCR amplification of VsAMT1 and StAMT1

Nucleotide sequences of thirty one AMT1.1, 1.2 and 1.3 genes from various plant species were aligned using BLAST in the NCBI in order to identify conserved regions. AMT1-specific primers were designed from regions of similarity between the aligned sequences. The primers were evaluated in-silico, and the optimum annealing temperature and optimum $MgCl_2$ concentration determined experimentally.

DNA was extracted from *V. subterranea* and *S. tuberosum* variety Up To Date leaves using cetyl trimethylammonium bromide (CTAB) method [9]. The DNA was re-suspended in 60 μL sterile distilled water. The quality and concentration of DNA samples were determined using Thermo Scientific NanoDrop 2000 spectrophotometer, Lithuania and agarose gel electrophoresis. Amplification of target gene (AMT1) by PCR was performed using a Bio-Rad T100 Thermal Cycler. The reaction volume was 25 μL consist of 500 ng total DNA template, 1.25 mM $MgCl_2$, 0.1 mM dNTP mix, 0.4 mM forward primer, 0.4 mM reverse primer, 2.5 units of Taq DNA pol and 1× PCR buffer (Thermo scientific, Lithuania). The following gene-specific primers were used: forward primer 5′-GCCATCGCCGCCGCCGG-3′, and reverse primer 5′-GGGTCAGATC-CATACCCGC-3′, which targeted a 976 bp internal region of the AMT1 gene. The amplification processes included an initial denaturation step at 95 °C for 2 min, followed by 35 cycles of 95 °C for 1 min, 60 °C for 30 s and 72 °C for 1 min. The final cycle included an extension for 5 min at 72 °C, before storage at 4 °C. The presence and quality of PCR products were visually inspected using electrophoresis through a 1% molecular grade agarose gel that was stained with SYBR Green. The nucleotide sequence of the amplicons was determined using the ABI Prism dye Terminator Cycle Sequencing method (Inqaba Biotech, SA).

### 2.2. Amino acid sequence alignment

Nucleotide sequences of the internal-regions of *VsAMT1* and *StAMT1* were aligned using Mafft version 7, BLAST (using Basic Local Alignment Search Tool software) against the NCBI GenBank data base and compared with known nucleotide sequences. Expasy translate tool was used to translate nucleotide sequences to give the corresponding amino acid sequences. *V. subterranea* VsAMT1 and *S. tuberosum* StAMT1 deduced amino acid sequences were aligned with *Phaseolus vulgaris* PvAMT1.1, *Glycine max* GlycineAMT1, *Lotus japonicus* LjAMT1.1, *Solanum lycopersicum* LeAMT1.1 and *S. lycopersicum* LeAMT1.2 using ClustalX 2.1. The GeneBank accession numbers of these genes are shown in Table 1.

### 2.3. Phylogenetic analyses

Amino acid sequences of AMT1 and AMT2 subfamily members from various plant species (including putative VsAMT1 and StAMT1 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 neighbor joining (NJ) method. Table 1 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. Results

### 3.1. PCR amplification of VsAMT1 and StAMT1

Literature and database searches for plant AMT1 genes revealed several reports on the gene. Accession numbers for 31 of the sequences reported could be found in public databases and are listed in Table 1. Four of these; 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 *S. tuberosum* and *V. subterranea*. The multiple

**Table 1**
Accession numbers of AMT1 genes that was 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 |
| CusAMT1 | AY642427.1 |
| MhAMT1.1 | JQ072026.1 |
| MhAMT1.2 | JQ072027.1 |
| PtrAMT1.1 | XM_002314482.1 |

sequence alignment was used to design AMT1 specific-primers that were used in this experiment: forward primer 5′-GCCATCGCCGCCGCCGG-3′, and reverse primer 5′-GGGTCAGATC-CATACCCGC-3′. Amplicons whose sizes approximate the expected AMT1 internal region of 976 bp were obtained. The nucleotide sequences and phylogenetic tree based on the sequences are shown in Ref. [1].

Nucleotide sequence analysis of the *V. subterranea* amplicon revealed high percentage similarity to *P. vulgaris PvAMT1.1* and *G. max GlycineAMT1* (Table 2). Nucleotide sequence analysis of the *S. tuberosum* amplicon also revealed high percentage similarity to *S. lycopersicum LeAMT1.1* and *S. lycopersicum LeAMT1.2* (Table 2). These results suggest that the amplicons are indeed the internal regions of AMT1 genes. The nucleotide sequences were deposited into the NCBI database and assigned the following accession numbers: KR024012 for VsAMT1 and KR024013 for StAMT1.

### 3.2. Amino acid sequence alignment

Sequence translation of VsAMT1 and StAMT1 gene fragments showed a peptide of 246 aa and 220 aa long, respectively (Fig. 1). The amino acid sequence alignment of *V. subterranea* VsAMT1 with *P. vulgaris* PvAMT1.1, *G. max* GlycineAMT1 and *L. japonicus* LjAMT1.1 revealed high percentage similarity (Table 2). Furthermore, amino acid sequence alignment showed that StAMT1 is highly similar to LeAMT1.1, LjAMT1.1 and LeAMT1.2, respectively (Table 2).

The amino acid sequence alignment also showed that the amplified fragments of VsAMT1 and StAMT1 corresponded to 5th–10th AMT1 transmembrane spanning regions (Fig. 1). Tryptophan (Vs W1), Serine (Vs S87) and Aspartic acid (Vs D23) represent selected residues that are constitutive of the pore in VsAMT1 (Fig. 1). Aspartic acid (St D15) and Serine (St S76) represent the selected residues that are constitutive of the pore in StAMT1 (Fig. 1).

### 3.3. Phylogenetic analysis

Fig. 2 shows that the VsAMT1 belongs to the AMT1 subfamily, and that it is most closely related to PvAMT1.1 followed by GlycineAMT1 and LjAMT1.1, respectively. Furthermore, Fig. 2 shows that StAMT1 belongs to the AMT1 subfamily and that it is most closely related to LeAMT1.1.

## 4. Discussion

The analysis of the nucleotide sequences amplified in this work confirms that VsAMT1 and StAMT1 are indeed AMT1 genes. While reports of *S. tuberosum* AMT1 could be found in literature [40], the nucleotide and amino acid sequences could not be found in the database.

In legume crops, $NH_4^+$ is the principal product of symbiotic N fixing bacteria, hence contribute greatly to N nutrition [32]. Apart from the fact that AMT1 assimilates N from roots, they probably play a crucial role in the efficiency of the symbiosis [29], indicating that AMT1 in leguminous plants may function in the regulation of nodule formation [8]. 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 [8].

Amino acid sequence alignment indicated that VsAMT1 is most closely related to PvAMT1.1, followed by GlycineAMT1 and LjAMT1.1 respectively. It is not surprising that the aforementioned closely related homologues of VsAMT1 are all from the legume family (Fabaceae). *P. vulgaris* PvAMT1.1 mediates the high affinity and electrogenic transport of $NH_4^+$ and it is pH dependent in that $NH_4^+$ transport increased at low pH (5.5) [27]. The high affinity transport property of PvAMT1.1 for $NH_4^+$ is evidence that VsAMT1 is a member of AMT1 family and may be responsible for $NH_4^+$ transport. *G. max* GlycineAMT1 is a putative AMT1, but this transporter has not yet been further analyzed. Apart from the fact that *L. japonicus* LjAMT1.1 is the first $NH_4^+$ transporter gene with an intron (1009 bp) and no open reading frame of any marked length, it is also a high affinity AMT gene (transport affinity: 1.7 μM) which is highly expressed in roots, leaves and nodules in low N conditions [8,29]. LjAMT1.1 has been reported to likely function in recovering $NH_4^+$ escaped from nodule cells in the different tissues during normal metabolism apart from transporting $NH_4^+$ across the symbiosome membrane. This is a further indication that the internal region of VsAMT1 that we isolated is a high affinity ammonium transporter and may be predicted to play a role in *Rhizobium* infection.

Several studies have revealed that bacteria such as *E. coli* *Ec*AmtB and plant AMTs possess 11 transmembrane regions. The internal region of VsAMT1 that was isolated and sequenced in this study corresponds to 5th–10th TMDs of other plant AMT transmembrane (Fig. 1). A report on *Ec*AmtB 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 [17]. The similarity of plant AMTs with that of *E. coli* and *Archaeoglobus fulgidus* AMT was reported to be 20–25% lower [27]. However, plant proteins tend to maintain similar tertiary and quaternary structures (transmembrane domains, cytoplasmic loops and the carboxyl terminal), as revealed by molecular modelling [19,25,27]. In *Ec*AmtB, W148 and S219 among other conserved amino acid residues, were indicated to play a role in structuring the $NH_4^+$ binding site [2,16,41]. These residues (*Ec*AmtB: W148 and S219) which have been reported to increase transport activity at mutation between 2 and 10 fold [28], correspond to W1 and S87 of the predicted VsAMT1 amino acid residues (Fig. 1). The residues are well conserved with other selected proteins as shown in the alignment (Fig. 1). Therefore, mutations in Vs W1A-L and S28A may be predicted to function in aiding $NH_4^+$ transport activity. A Vs D23 residue was identified to be similar to the residues in *E. coli* *Ec*AmtB (D160) [28],*Saccharomyces cerevisae*

**Table 2**
Nucleotide similarity and amino acid identity for *V. subterranea* and *S. tuberosum* AMT1 gene.

| | AMT1 | Nucleotide sequence | Amino acid sequence |
|---|---|---|---|
| *V. subterranean* | *Phaseolus vulgaris* PvAMT1.1 | 92% | 92% |
| | *Glycine max* GlycineAMT1 | 82% | 89% |
| | *Lotus japonicus* LjAMT1.1 | N/A | 87% |
| *S. tuberosum* | *Solanum lycopersicum* LeAMT1.1 | 93% | 92% |
| | *Solanum lycopersicum* LeAMT1.2 | 92% | 76% |
| | *Lotus japonicus* LjAMT1.1 | N/A | 83% |

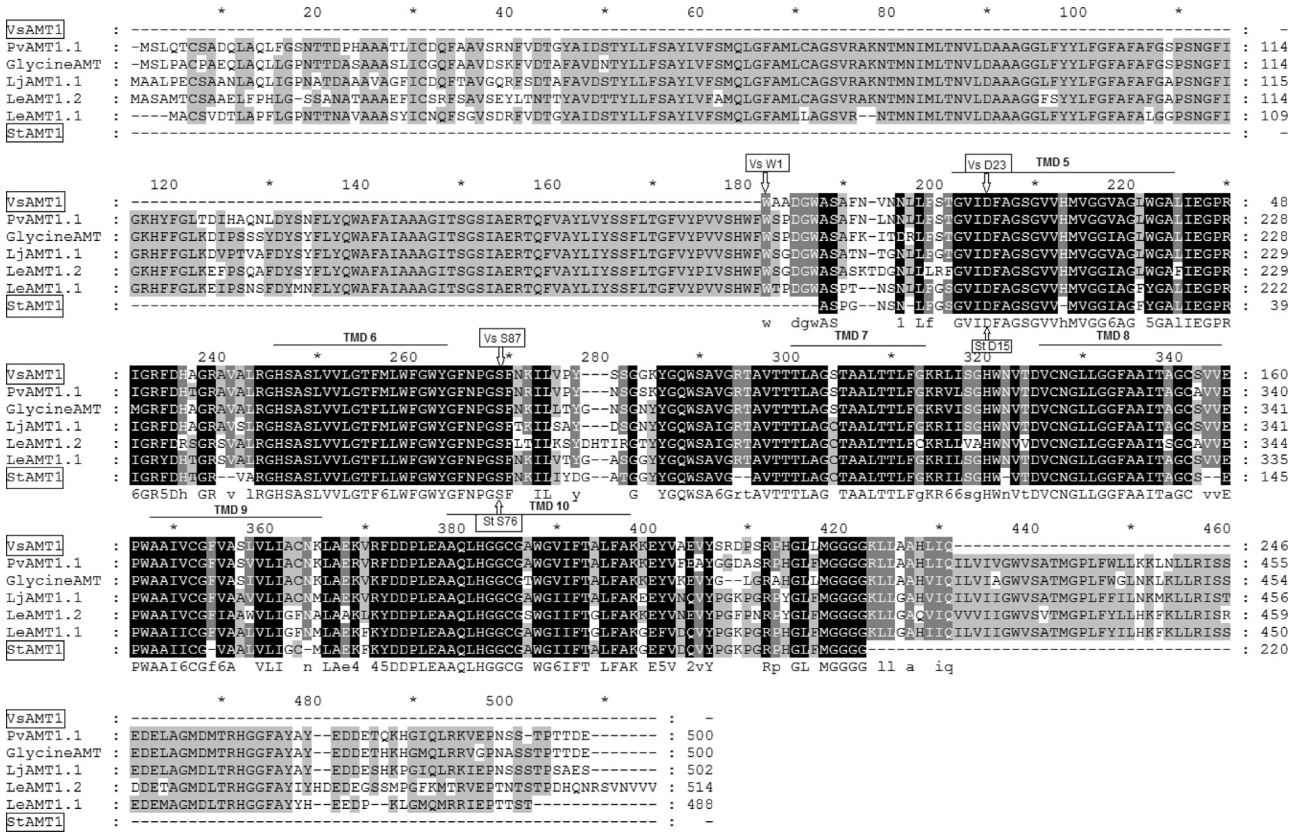


**Fig. 1.** An alignment of the amino acid sequence of VsAMT1 and StAMT1 with PvAMT1.1, GlycineAMT1, LjAMT1.1, LeAMT1.1 and LeAMT1.2. The accession numbers are listed in Table 1. The amino acid residues conserved in all sequences are written in white lettering inside black-filled rectangles. Conservative substitutions are written in black lettering inside grey-filled rectangles. Predicted transmembrane-spanning domains are marked above the alignment (TMD 5–10). Some amino acids predicted as constitutive of the pore are marked at the top and bottom of the sequence in rectangles for VsAMT1 (Vs W1, Vs D23 and Vs S87) and StAMT1 (St D15 and St S76), respectively.

ScMep2 (D186N) [24] and *Arabidopsis thaliana* AtAMT1.1 (D198N) [19], 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 VsAMT1 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 VsAMT1 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 [14,15].

StAMT1 has high amino acid sequence similarity with LeAMT1, LjAMT1.1 and LeAMT1.2. *S. lycopersicum* LeAMT1.1 and LeAMT1.2 are from the same family with *S. tuberosum* StAMT1 (*Solanaceae*). A substrate affinity study on tomato showed that LeAMT1.1 is a high affinity transporter with $K_m$ value of about 10 μM [34]. 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 [33]. The affinity transport property of LeAMT1.1 is an indication that the putative StAMT1.1 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 [29]. This further supports the stipulation that putative StAMT1.1 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 [34] 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 $NH_3$ [34].

The internal region of putative StAMT1 that was isolated and sequenced in this study corresponds to 5th – 10th TMDs of the plant AMT transmembrane (Fig. 1). The amino acid sequence alignment showed that among other residues, the putative StAMT1 possess St S76 (equivalent to *Ec*AmtB S219) which have been indicated to increase transport activity at mutation by between 2 and 10 fold [28]. The residue (St S76) is well conserved with other selected proteins as shown in the alignment (Fig. 1). Residue St D15 correlates with the residues in *E.coli Ec*AmtB (D160) [28], *S. cerevisae* ScMep2 (D186N) [24] and *A. thaliana* AtAMt1.1 (D198N) [19] and falls under the 5th transmembrane spanning region of StAMT1 amino acid sequence. The implication of this is that StAMT1 is a high affinity $NH_4^+$ transporter and mutation of St S76A may play a role in enhancing $NH_4^+$ transport activity while residue St D15 may inhibit $NH_4^+$ transport activity when substituted with Ala.

When amino acid sequences were compared, the phylogenetic tree showed that VsAMT1 and StAMT1 form a well-supported and monophyletic clade with other plant AMT1 genes that have been isolated so far (Fig. 2). This is an indication that the predicted VsAMT1 and StAMT1 belong to plant AMT1 family. 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 [27,28].

Taken together, the evidence presented here indicates that the *V. subterranea* and *S. tuberosum* genes amplified are indeed AMT1

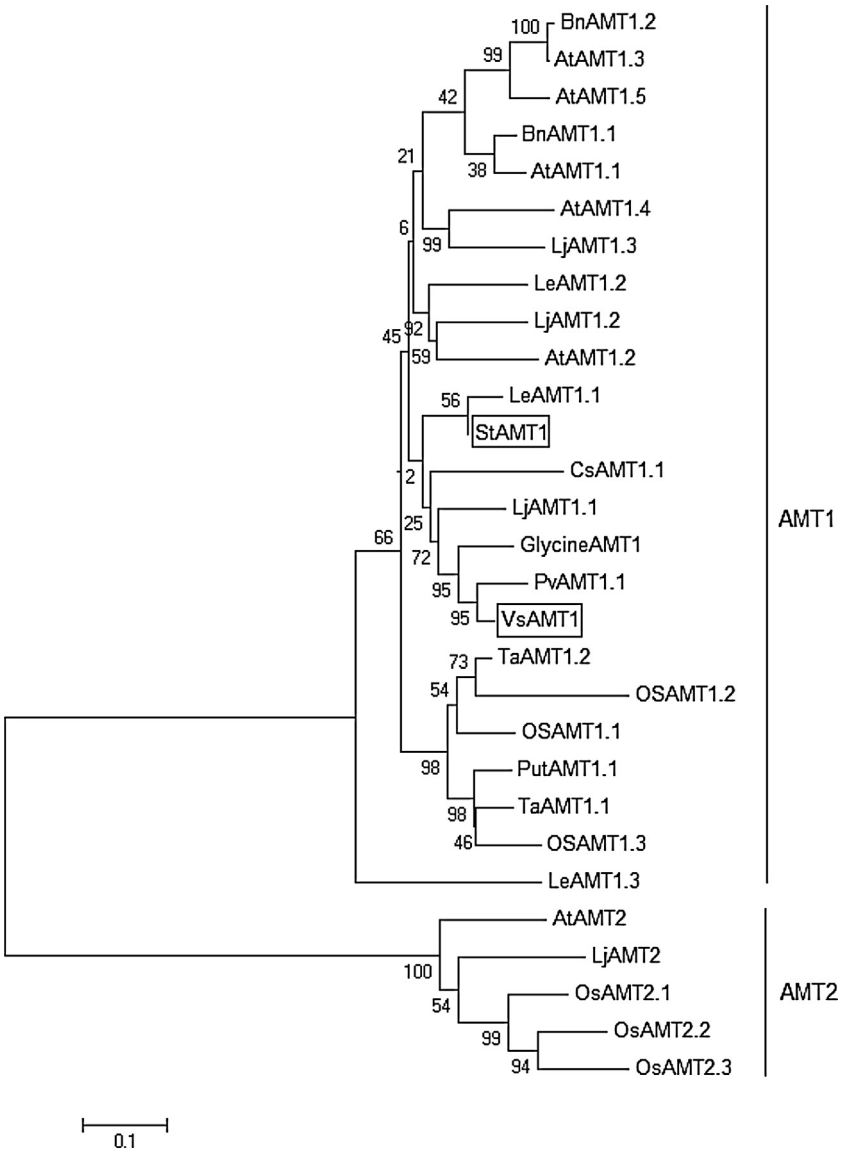


**Fig. 2.** Phylogenetic tree analysis of plant AMT families. The tree was constructed by multiple (amino acid) sequences alignment of 29 members of plant AMT families using the Mega 6 software with the neighbor joining method. The accession numbers are listed in Table 1.

genes. To the best of our knowledge, this is the first report of a *V. subterranea* AMT1, and we propose to abbreviate it as VsAMT1.

## 5. Conclusion

This work is to our knowledge the first report of nucleotide sequence of *V. subterranea* VsAMT1 internal-region, and its bioinformatics analysis. Amino acid sequence alignment as well as the phylogenetic analysis showed that VsAMT1 and StAMT1 are indeed from the plant AMT1 family and they have high affinity to assimilate $NH_4^+$ from the soil. VsAMT1 may contribute to different steps in rhizobia interaction. In *S. tuberosum*, site directed mutagenesis of St S76A can be performed to increase $NH_4^+$ transport activity. A corresponding mutation in *V. subterranea* would be Vs S87A.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.btre.2015.10.003.

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