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RESEARCH ARTICLES

MOLECULAR CLONING, PHYLOGENETIC ANALYSIS AND STRUCTURE PREDICTION OF AMT1 FROM *AZOLLA-ANABAENA AZOLLAE* SYMBIOTIC SYSTEM

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ABSTRACT

Azolla anabaena azollae is a symbiotic association between the aquatic fern *Azolla* and nitrogen fixing cyanobacteria *anabaena azollae*, by the exchange of carbon and nitrogen between them. The nitrogen fixed by the cyanobacterium is converted into ammonium and is transported via ammonium transporters. AMT1family of ammonium transporters are high affinity transporters hence are quick to respond under very low levels of nitrogen supply.AMT1 will be the best AMT to be studied under nitrogen limiting growth conditions. Putative AMT1 sequence was cloned from the symbiotic *Azolla-Anabaena* using reverse transcription PCR and phylogenetic analysis was conducted with the nearly similar sequences using BLAST. The amplified putativeAMT1sequence from the cDNA of *Azolla anabaena azollae* was sequenced and the sequence was submitted to NCBI. Multiple sequence alignment was performed using ClustalW and the Maximum Likelihood and Neighbour Joining tree was developed using Tamura-Nei model of MEGA 6.0.The reliability of each branch was tested by bootstrap analysis with 1,000 replications. In the phylogenetic analysis, AMT1of *Azolla anabaena azollae* showed a close similarity with *Azolla caroliniana* AMT1.The transmembrane AMT1 protein structure was predicted with Raptorx structure prediction program.

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INTRODUCTION

Ammonium and nitrate are considered to be the principal sources of nitrogen for plant growth. Ammonium is generally preferred over nitrate when provided to plants at similar concentrations (Fried *et al*. 1965; Clarkson *et al*., 1986, Macduff, and Jackson, 1991). Ammonium and nitrate are rarely available in equal quantities and their concentrations in the soil can vary over several orders of magnitude, from micro molar to hundreds of milli molar (Marschner, 1995). Plants have evolved a collection of transporters that efficiently import ammonium and nitrate over a wide range of concentrations. There are structural and functional parallels between ammonium transporters, and nitrate transporters in plants, animals and bacteria. There exist regulatory parallels, although plants have almost certainly evolved novel mechanisms of regulation in order to integrate the transport of these compounds with their complex and unique multicellular metabolism. Ammonium is imported from the external environment via ammonium transporters in the plasma membrane of root cells (Forde and Clarkson, 1999) and leaf cells (Yin, 1996).

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Ammonium absorbed to the cell is assimilated either in the cytoplasm via glutamine synthetase (GS; EC 6.3.1.2), or in plastids and possibly also mitochondria, following transport to these organelles. Ammonium may also enter the vacuole where it is `stored' temporarily. Ammonium can also be generated de novo from N_2 by nitrogen-fixing bacteria in some plant cells, such as rhizobia in legume root nodule cells. Most of this ammonium is transferred to the plant cytoplasm where it is assimilated by GS(Lea and Ireland, 1999).Previous studies on ammonium transport have indicated that there are two distinct transport systems for ammonium i) HATS- High-affinity transport system and ii) LATS-Low-affinity transport system (Howitt and Udvardi, 2000).The HATS is regulated by the nitrogen status of plants i.e. the activity of HATS is inversely proportional to the concentration of ammonium (Gazzarrini, *et al* 1999).There are both cytoplasmic and plastid forms of GS and they have high affinity for ammonium (Stewart *et al.,* 1980). The two intracellular locations of GS in plant cells have evident implications for ammonium transport: ammonium transport activity can be expected at both the plasma membrane and the plastid inner membrane.GS catalyzes the amidation of glutamate to yield glutamine in presence of ATP (Purich, 1998).GOGAT catalyses the reductive transfer of the amide group from glutamine to 2-oxoglutarate in order to yield two molecules of glutamate (Van den Heuvel *et al*., 2004. The

Molecular Evolutionary Genetics Analysis (MEGA) software is developed for the comparative analyses of DNA and protein sequences that are aimed at inferring the molecular evolutionary patterns of genes, genomes, and species over time (Kumar *et al.*, 1994; Tamura *et al.,* 2011).The phylogenetic analysis of the cloned AMT1 will be helpful in finding its relationship with other genes of AMT1 family. A protein structure can be predicted from the amino acid sequence of cloned AMT1 by structure prediction tools. The 3D structure of an unknown protein can be predicted using experimentally determined protein template having better homology with the target protein. Comparative modelling is the most reliable and accurate protein structure prediction method (Baker and Sali, 2001). Protein structure prediction helps to provide the biological function and mechanism of action of an unknown protein (Khan *et al*., 2016).RaptorX structure prediction server (Källberg *et al.,* 2012,Peng, and Xu, 2011) helps in predicting the 3D structure for protein sequences when homologs are lacking in the Protein Data Bank (PDB). When an input sequence is given, RaptorX predicts its secondary and tertiary structures, contacts, solvent accessibility, disordered regions and binding sites. RaptorX also assigns some confidence scores to indicate the quality of a predicted 3D model: P-value for the relative global quality, GDT (global distance test) and uGDT (un-normalized GDT) for the absolute global quality and modeling error for each residue.

MATERIALS AND METHODS

Plant material

Azolla-Anabaena azollae plants were collected from the Botanical garden, University of Calicut. The association was washed vigorously in large quantities of water for several times, followed by surface sterilization with 0.12% (v/v) sodium hypochlorite and few drops of Tween 20 for 10 to 20 mins. The fronds were then washed with sterile distilled water 6 times and transferred to sterile nutrient media in Erlenmeyer flasks and excess water from the fronds was blotted. Modified Hoaglands Nutrient solution E with or without combined nitrogen was used as the culture medium. The cultures were maintained in a culture room provided with 16-hr light, 8-hr dark cycle and temperature of 25±2ºC.Illumination was provided by cool white fluorescent lamps.

Total RNA Isolation

Total RNA was isolated from *Azolla anabaena azollae* by modified CTAB method (Zeng, and Yang, 2002).100mg of fresh fronds were ground into fine powder using liquid nitrogen in presence of 2mg fresh PVPP powder. The powdered sample was transferred to eppendorf tube containing 700µl pre-warmed (65ºC) RNA extraction buffer containing 100mM Tris HCl (pH 8.0), 25mM Na-EDTA (pH 8.0), 2M NaCl and 2% (w/v) Cetyl Trimethyl Ammonium Bromide (CTAB).β-mercaptoethanol (2.0µl) was added to the sample and vortex vigorously. The sample was incubated at 65ºC for 5min. The homogenate was extracted with an equal volume of chloroform: isoamyl alcohol (24:1) and mixed by inversion to emulsify. The sample was centrifuged at 10,000 rpm for 10min to separate the phases. The upper aqueous phase was transferred to another sterile eppendorf tube. The sample was extracted twice with equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). The phases were separated by centrifuging at 10,000 rpm for 10min in a cooling centrifuge. The upper aqueous phase was collected in a sterile eppendorf tube.0.25vol of 10M LiCl was added to the aqueous phase and mixed well. RNA was precipitated by incubating overnight at 4 ºC. Incubated sample was centrifuged at 10,000rpm for 20min at 4 ºC to pellet the RNA.The pellet was dissolved in 50µl sterile DDW. 0.1vol of 3M sodium acetate and 2.5vol of 96 % (v/v) ethanol was added and incubated at -80 ºC for 30min to precipitate RNA completely.RNA was pelleted by centrifuging at 10,000rpm at 4 ºC for 20min. RNA pellet was rehydrated by 70% (v/v) ethanol and air dried for 10min.RNA pellet was dissolved in 20 µl DDW and stored at -80 ºC until use.

cDNA synthesis by RT-PCR

cDNA was prepared using Takara Prime Script^{TM} RT Reagent Kit (Cat.#: RR037Q). All steps were performed according to the manufacturer's protocols.

Amplification and Sequencing of AMT1 gene

AMT1 genes were cloned by PCR using *Azolla anabaena azollae* cDNA as template. PCR was first performed using two primers (Forward: 5'-CATCATGCTTACCAACGTCCTTG-3'; and Reverse: 5'-AGTTTTCCTCCACCGCCCATAAA-3') based on the nucleotide sequences encoding AMT1s in other plant species such as *Azolla caroliniana*(AMT1) *Brassica napus* (AMT1;2), *Solanum lycopersicum* (AMT1;1),*Citrus trifoliata* (AMT1;2),*Lotus japonicus* putative (AMT1.1)and *Lotus japonicas* (AMT1;2). The consensus sequence was derived using MultAlin, software. The PCR reactions were carried out using Emerald Amp^{R} GT PCR Master Mix (Cat. # RR310A) by Takara. Thermocycling was performed using an initial denaturation at 95ºC for 5min followed by 36 cycles of 95ºC for 30 s, annealing temperature of 48ºC for45 s and 72ºC for 1min with extension at 72ºC for 5min.The PCR products were sequenced from SciGenom Labs Private Ltd, India using AMT1 forward and reverse primers. Homology of the cloned sequences was carried out using BLAST. The sequences obtained using the forward and reverse primers were merged using BioEdit program.

Phylogenetic Analysis

Phylogenetic analysis of AMT1 of *Azolla-Anabaena azollae* with AMT1 from BLAST analysis which showed a similarity above 72% and AMT1 of few randomly selected nitrogen fixing plants was performed (Table.1). Sequence homology searches were performed on the BLAST server (http:www.ncbi.nlm.nih.govblast) and the nucleotide sequences were obtained from gene-bank. For complete Phylogenetic analysis MEGA6.0 software (Tamura *et al.,* 2013) was employed. Prior to the tree construction, sequences were aligned by multiple sequence alignment method using ClustalW (http:www2.ebi.ac.ukclustalw) (Thompson *et al.,* 1994) Maximum likelihood tree was constructed using Tamura-Nei model. The reliability of each branch was tested by bootstrap analysis with 1000 replications.

Sequence analysis and structure prediction of Az-AMT1 protein

ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/) was employed to find the open reading frame of the putative Az-AMT1 sequence.

The sequence was translated to the corresponding protein sequence. The 3D structure of the transmembrane Az-AMT1protein was predicted using RaptorX server and validated by ERRAT and Ramachandran plot.

RESULTS

Total RNA isolation

Good quality RNA was isolated from *Azolla-Anabaena azollae* and run on 1.2% EtBr Formaldehyde Agarose (FA) gel. 28S rRNA and 18S rRNA bands were clearly visible without any degradation.

Table 1. Members of AMT1 involved in phylogenetic analysis

GenBank ID	Organism	AMT Family
KX881762.1	*Azolla pinnata*	Amt1
EF030058.2	*Azolla caroliniana*	Amt1
XM_009383922.2	*Musa acuminata subsp. malaccensis*	Amt1.2
XM_008803050.2	*Phoenix dactylifera*	Amt1.2
XM_011464127.1	*Fragaria vesca subsp. vesca*	Amt 1.1
DQ887678.2	*Citrus sinensis x Poncirus trifoliata*	Amt 1
JX049223.1	*Citrus trifoliata*	Amt1.1
XM_008225790.2	*Prunus mume*	Amt1.1
XM_010026627.2	*Eucalyptus grandis*	Amt1.2
AY135020.1	*Lotus japonicus*	Amt1.2
XM_020245985.1	*Ananas comosus*	Amt 1.3
AF182188.1	*Lotus japonicus putative*	Amt1.1
XM_012979220.1	*Erythranthe guttatus*	Amt1.2
XM_010468526.1	*Camelina sativa putative*	Amt1.5
XM_016327959.1	*Arachis ipaensis*	Amt1.1
XM_010103892.1	*Morus notabilis*	Amt1.2
XM_014663561.1	*Vigna radiata var. radiata*	Amt1.3
XM_014659367.1	*Vigna radiata var. radiata*	Amt1.1
XM_014658248.1	*Vigna radiata var. radiata*	Amt1.2
XM_014639498.1	*Vigna radiata var. radiata*	Amt1.4
XM_004497742.2	*Cicer arietinum*	Amt1.1
XM_004500192.2	*Cicer arietinum*	Amt1.3
XM_004493825.1	*Cicer arietinum*	Amt1.4
XM_020353542.1	*Cajanus cajan*	Amt1.1
XM_020382298.1	*Cajanus cajan*	Amt1.2
XM_020366444.1	*Cajanus cajan*	Amt1.3
KR024012.1	*Vigna subterranea*	Amt1
XM_019604524.1	*Lupinus angustifolius*	Amt1.4
XM_019577026.1	*Lupinus angustifolius*	Amt1.1
XM_019589707.1	*Lupinus angustifolius*	Amt1.3

cDNA synthesis by RT-PCR

DNase I treated total RNA (1µg) was transcribed into high quality cDNA using Prime Script RT enzyme, Oligo dT primers and random hexamers. cDNA was observed as a smear in 1%TAE-EtBr agarose gel. (Fig: 1).

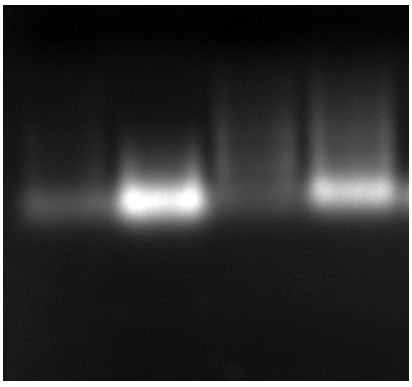

Fig. 1. Electrophoretic profile of total cDNA on 1% TAE-EtBr agarose gel

Amplification of AMT1 gene by PCR

Polymerase chain reaction using AMT1 primers (Forward: 5'-CATCATGCTTACCAACGTCCTTG-3'; and Reverse: 5'-AGTTTTCCTCCACCGCCCATAAA-3') developed a band of approximately 1100bp in 1% TAE-EtBr agarose gel (Fig: 2). The PCR product with a clear band was selected for sequencing.

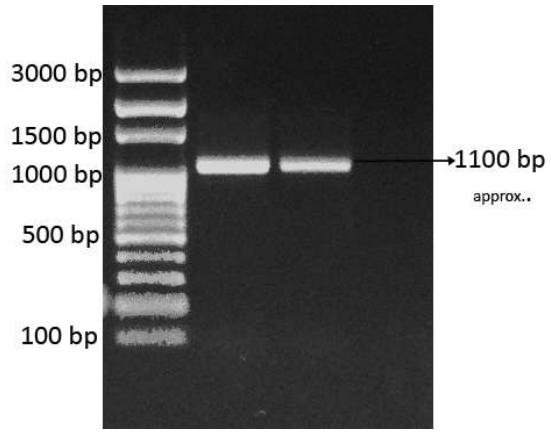

Fig. 2. Electrophoretic profile of PCR product on 1%TAE-EtBr Agarose gel

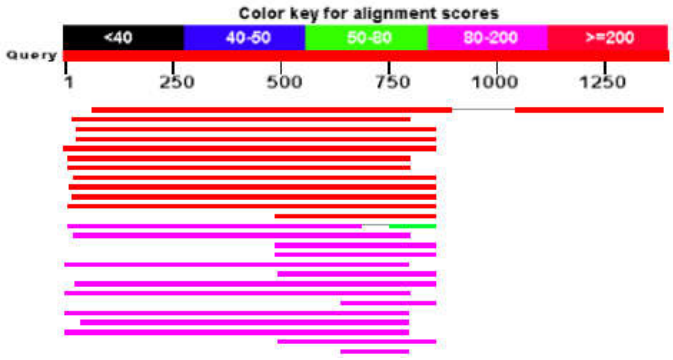

Fig. 3. BLAST analysis of AMT1 gene sequence showing homology with other AMT1 gene sequences

Gene sequencing and sequence analysis

The PCR product was sequenced using Sanger dideoxy sequencing method. BLAST analysis of the sequence obtained confirmed homology with already existing AMT1 gene of *Azolla caroliniana* (Acc. No.EF030058.2) with 87% identity, 84% Query coverage, and an E-value of 0.0 (Table.3, Fig. 3).The sequence was submitted in NCBI and an accession number (KX881762.1) was provided and designated as Az-AMT1.

Phylogenetic analysis

The phylogenetic analysis (Fig.5) revealed two major clades; cladeI and cladeII, clade I consists of AMT1.3 gene family. Clade II is divided into 3 clusters AMT1.2, AMT1.1 and AMT1.5/1.4. *Azolla pinnata* AMT1 and *Azolla caroliniana* AMT1 together formed a separate cluster with the support of 100% bootstrap value in clade2, which is placed very close toAMT1.2 cluster. Thus it can be assumed that Az-AMT1 (KX881762.1) is in close relation with AMT1.2 phylogenetically.

Table 2. BLAST analysis of Az-AMT1 gene sequence showing homology with other AMT1 gene sequences

Plant Species	Max. Score	Query coverage	E-value	Identity	GenBank accession No.
A.caroliniana AMT 1	963	84%	0.0	87%	EF030058.1
Musa acuminate AMT1.2	337	75%	4e-88	75%	XM009383922.1
Phoenix dactylifera AMT1.2	318	80%	1e-82	74%	XM008803050.2
Fragaria vesca AMT1.1	307	80%	3e-79	74%	XM011464127.1
Citrus trifoliate AMT1.1	296	76%	7e-76	74%	JX049223.1
Eucalyptus grandis AMT1.2	274	81%	3e-69	73%	XM010026627.1
Prunus mume AMT1.1	265	81%	2e-66	73%	XM008225790.2
Lotus japonicas AMT1.2	206	35%	1e-48	77%	AY135020.1

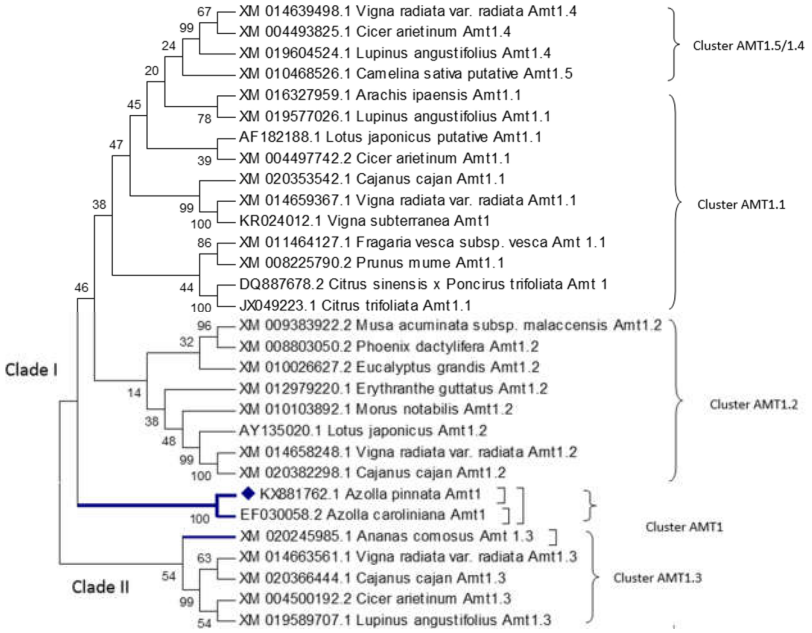

Fig. 4. A phylogenetic tree for AMT1of *Azolla pinnata* with members showing above72% homology in BLAST analysis and selected N_2 fixing plants, generated using MEGA6.0 (Tamura *et al*., 2013)

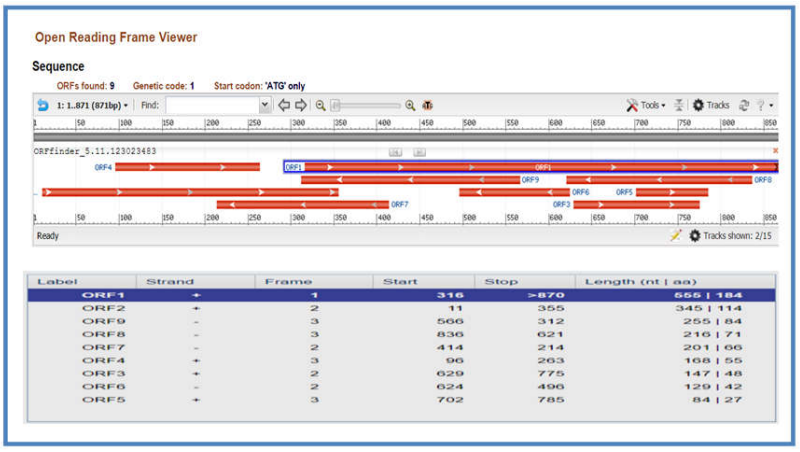

Fig 5. Open Reading Frame of Az-AMT1gene of *Azolla Anabaena azollae* (https://www.ncbi.nlm.nih.gov/orffinder/)

Sequence analysis of Az-AMT1 protein

The ORF Finder revealed a putative 555 bp open reading frame (ORF) for the Az-AMT1gene in +1 frame and that code a protein with 184 amino acid residues having an ATG initiation codon at 316th nucleotide position and the TAG stop codon at 870th position (Fig. 5). The sequence of the Az-AMT1 protein, KX881762.1, used for this study:

MVGGIAGLWGAFIEGPRMGRFDADGQPQRLKGHSATL
VVLGSFLLWFGWYGFNPGSFLVILASPYDAFKGNWSGV
GRTAVTTTIAGSTAALTTLFGKRIIGGHWNVLDVCNGL
LGGFAAITAGCSVVDPWASIICGFVSAWVLIGLNILAEK
LKFDDPLEAAQLHGGCGAWGLIFTGLFANENYV

Prediction of transmembrane protein of Az-AMT1 sequence: The 3D structure for Az-AMT1 transmembrane protein (Fig.6) was predicted using RaptorX structure prediction server (http://raptorx.uchicago.edu/joblog/).The unknown sequence aligned with template structure 5aex (Structure for Mep2 Ammonium Transporter Activation by Phosphorylation) A chain, about 290 residues were modelled and only 2% of the residues were predicted as disordered. A p-value 1.59e-09 was obtained for the predicted structure. Overall uGDT (GDT) which measures the absolute model quality was 234 (80). The uGDT (GDT) score satisfies the criteria for good quality model.

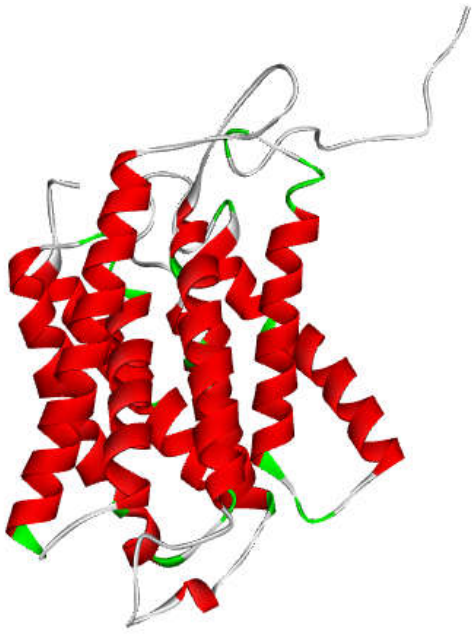

Fig. 6. Modeled structure for Az-AMT1 transmembrane protein of *Azolla –Anabaena azollae* obtained by RaptorX server

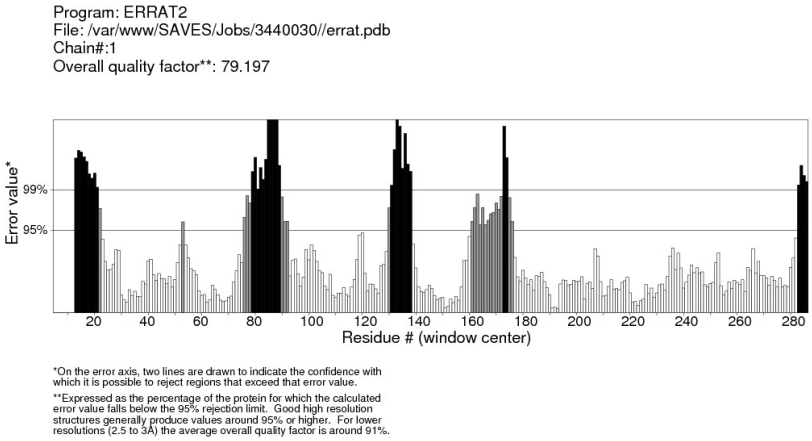

Fig.7. Graphical output of the predicted model of Az-AMT1 transmembrane protein of *A.Anabaena azollae* ERRAT program

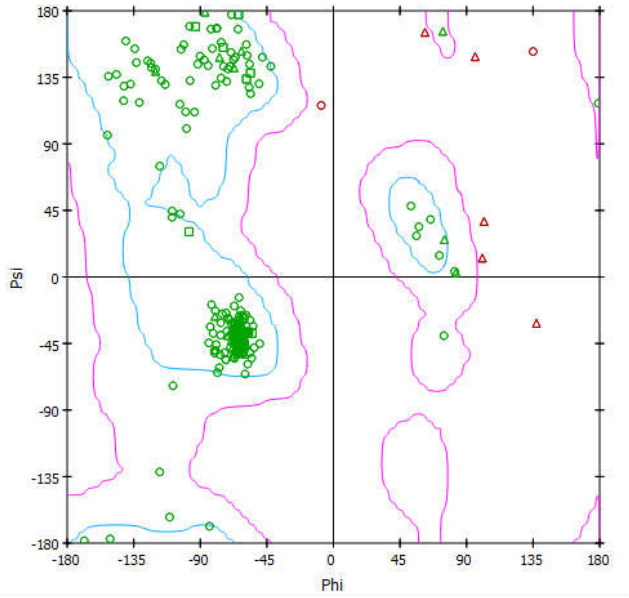

Fig.8. Ramachandran plot showing the external quality validation of the model with Number of residues in; favoured region (~98.0% expected): 272 (94.4%), allowed region (~2.0% expected): 14 (4.9%), outlier region: 2 (0.7%)

For 3-state secondary structure, 60%H, 0%E, 39%C indicates that 60% of the modeled residues are helical, no beta-sheet and 39% residues are arranged in a coil. This is in perfect agreement to transmembrane protein as most of the transmembrane proteins have limited beta sheets. The solvent accessibility is divided into three states by 2 cutoff values: Buried (<10%), Medium (between 10% and 42%) and Exposed (>42%) are abbreviated as B, M and E, respectively. The overall modelled structure shows 50 % buried surface and remaining 24% is exposed of larger and medium surface area of the protein. ERRAT revealed a quality factor of 79.197% (Fig.7).

Model validation by Ramachandran Plot

Ramachandran plot analysis showed 94.4% residues in the favoured region, 4.9% residues in allowed region and only 0.7% residues in outlier region (Fig. 8).Thus the overall result indicated that 99.3% of the amino acid residues of the AMT1 protein are in the allowed region of the Ramachandran plot.

DISCUSSION

Az-AMT1 (Azolla-Ammonium transporter1) gene was cloned by primer based gene cloning using cDNA as template, reverse transcribed from total RNA. Primer based gene cloning is reported in various species using RNA as template. cDNA band cloning was reported in the case of *Oryza sativa* AMT gene using specific primer (Sonoda *et al.,* 2003).The homology of the cloned sequence was established using BLAST program which indicated that the cloned cDNA has a high level of identity with the AMT1 proteins of *Azolla caroliniana*, *Musa acuminata*, *Phoenix dactylifera*, *Fragaria vesca*. In Dunaliella viridis, two ammonium transporter genes, DvAMT1;1 and DvAMT1;2, were cloned from the cDNA. The BLAST program was employed to analyze the sequences of cDNA fragments and the alignments were performed by ClustalW (Song *et al.,* 2011).Li *et al.,* (2009) reviewed the AMT1 family proteins and discussed the key role in high affinity ammonium uptake from soil. Studies revealed that in *Arabidopsis*, AtAMT1.1, AtAMT1.2 and AtAMT1.3 contribute 30%–40%, 18%–26% and 30% of ammonium influx into roots under low external N concentration.

The Az-AMT1shared better sequence homology with other members of AMT1 family. The sequence obtained after annotation was of 871bp in length which was submitted to NCBI.Ding *et al*., (2011) reported that the coding region of OsAMT1.1 ranged from 860-1060bp in size. *Azolla caroliniana* AMT1-like mRNA, complete sequence submitted in NCBI (GenBank: EF030058.2) has a size of 891bp.*Arabidopsis thaliana* AMT1 (X75879.1) linear mRNA has 1700bp. *Musa acuminate* AMT1.2 (XM009383922.1) has a nucleotide size of 1820bp. The putative Az-AMT1 sequence from *Azolla-anabaena azollae* consisted of 871bp with an ORF of 555bp coding a protein of 184 amino acid residues. The evolutionary relationship of AMT1 gene of *Azolla – Anabaena azollae* was elucidated using few selected members of AMT1. Az-AMT1showed a separate cluster along with *Azolla caroliniana* AMT1in the phylogenetic tree. It was found that the cloned Az-AMT1was placed next to AMT1.2 cluster speculating that it is phylogenetically close to AMT1.2. Molecular phylogenies have become a vital part of biological research, pharmaceutical drug design, and bioinformatics techniques for protein structure prediction and multiple sequence alignment (Nakhleh *et al.,* 2005, Randal and Warnow, 2005). Genome-scale studies on gene content, conserved gene order, gene expression, regulatory networks, metabolic pathways, functional genome annotation can all be enriched by evolutionary studies based on phylogenetic statistical analyses (Thomas *et al*., 2006). For comparison of AMTs in other organisms, sequences were initially aligned using ClustalW (Thompson *et al.,* 1944) generating a sequence file comparing 600 residues including gaps, and then processed using MEGA (Kumar et al. 1993).

According to Chang *et al.,* (2004), proteins that are related by common descent are expected to display similar structures and functions to an extent proportional to the extent of their sequence similarity barring two independently evolving proteins. This principle provides the thrust to define protein phylogenetic relationships and interrelate families when possible. In some cases, available structural and functional data are evaluated in order to prove our claim that molecular phylogeny provides a reliable guide to protein structure and function. Comparative modelling has great importance in biological research due to its speed, simplicity, reliability, wide applicability and covering more than half of the residues in protein sequence space.

The 3D structure of Az-AMT1protein was predicted using RaptorX server and the best structure was selected using structure validation by Ramachandran plot analysis and ERRAT. Ramachandran plot was done to measure the accuracy of the protein model and ensures good stereo-chemical quality of the model. Model validation indicated that 99.3% of the amino acid residues of Az-AMT1protein were in the allowed region of the Ramachandran plot. According to Laskowski *et al.,* (1993), a typical model should have only a few residues in the disallowed region and many in the most favoured region. ERRAT algorithm interpreted the overall quality of the model with resulting score of 79.197%. This score denotes the percentage of the protein that falls below the rejection limit of 95% (Hasan *et al.,* 2015).Error values are plotted as a function of the position of a sliding 9-residue window. The error function is based on the statistics of non-bonded atom-atom interactions in the reported structure compared to a database of reliable high-resolution structures, (MacArthur *et al.,* 1994).

Conclusion

Differences in the affinity of different AMT1 proteins for ammonium suggest that they play balancing roles in ammonium uptake, which allow the plant to optimize the uptake of ammonium over a relatively wide range of soil ammonium concentrations (Gazzarrini *et al*., 1999). However, the absence of comprehensive data on gene expression at the cell and tissue levels, and of direct evidence for the intracellular location of the different AMT1 proteins limits our understanding of the real physiological roles of members of this family. For instance, it is not yet known if individual AMT1 proteins have unique, non-redundant roles in specific cell types. The Az-AMT1 gene sequence obtained can further be used in expression studies to understand its regulatory mechanisms. The behaviour of AMT1 proteins will be much different in case of symbiotic systems compared to non-symbiotic systems, hence molecular cloning, phylogenetic analysis and structure prediction of AMT1 from *Azolla-anabaena azollae* makes sense.

Acknowledgment

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