

CLONING AND EXPRESSION ANALYSIS OF A NOVEL AMMONIUM TRANSPORTER GENE FROM *EICHHORNIA CRASSIPES*

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Abstract

In order to explore the molecular mechanism for *Eichhornia crassipes* to transport ammonium from outside, we cloned a novel ammonium transporter (EcAMT) gene from *E. crassipes* and identified its function by using yeast complementation experiment. The full-length cDNA of EcAMT contains a 1506 nucleotide-long open reading frame which encodes a protein of 501 amino acids. Bioinformatics analysis predicted that EcAMT had 8 transmembrane regions. The expressions of EcAMT gene under three different nitrogen conditions were evaluated by quantitative reverse transcriptase PCR (qRT-PCR) and the results showed that the expression of EcAMT gene was up-regulated under nitrogen starvation. Our study results revealed some molecular mechanism of *E. crassipes* to absorb the ammonium in eutrophic water.

Key words: *Eichhornia crassipes*; Ammonium transporter; Clone; Expression.

Introduction

Nitrogen is a fundamental nutrient for all kinds of life. It exists in protein, nucleic acid, growth factor and so on, and engages in many biochemical reactions in plants. So it is a major limiting factor in plant production (Hammad *et al.*, 2011). Previous study indicated that crop production was positively correlated to nitrogen absorption and utilization (Yin *et al.*, 2010; Jan *et al.*, 2011) significantly. Although the average NH_4^+ concentrations of most soils are often 10~1000 times lower than that of NO_3^- (von Wirén *et al.*, 2000a), plants usually prefer ammonium to nitrate as their nitrogen source. Perhaps ammonium-absorption process is more energy-saved because nitrate must be transformed into ammonium before incorporated into compounds.

Usually, ammonium can be absorbed from outside. It also can be produced by plant cells during metabolism. Ammonium is generally not used for long-distance transport in plants, instead, most of ammonium transported into plant cells is assimilated locally via glutamine synthetase in cytoplasm and plastid. So Different kinds of ammonium transporters (AMTs) play different roles in plant to fulfill various needs, such as moving ammonium from intracellular sites of production to sites of consumption, retrieving ammonium leaked out during metabolism (Pérez-Tienda *et al.*, 2011) or taking up ammonium from outside (Howitt & Udvardi, 2000) and so on. AMT which absorb ammonium from outside was demonstrated to be a membrane protein so far. Lots of AMTs were cloned and characterized from different plants, such as rice, *Lotus japonicas* (Simon-Rosin *et al.*, 2003), *Phaseolus vulgaris* (Ortiz-Ramirez *et al.*, 2011) and so on. Among those plants, rice has the most AMTs, including 3 AMT1s and 9 AMT2s, which may be attributed to its roots submerged in water where nitrogen exists as reduced state ammonium. As a result, plant evolves an enhanced mechanism to adapt various external environments. Biphasic kinetics for ammonium uptake in green alga and higher plant roots and leaves indicates that there are at least two distinct transport systems for

ammonium (Howitt & Udvardi, 2000): high-affinity transport system (HATs) and low-affinity transport system (LATs). HATs is more sensitive to ammonium. It can be detected at low (sub-millimolar) external ammonium concentrations where LATs can't be detected. AMT1 generally belongs to HATs while AMT2 usually belongs to LATs. Previous studies showed that the expression of AMT could be affected by many kinds of factors, such as nitrogen, phosphorus, potassium (Li *et al.*, 2012), sunshine, CO_2 and so on. When it appears low level of external nitrogen, most of AMTs' expressions exhibit up-regulation. For example, yeast MEP and many plant AMT1s.

Eichhornia crassipes, a native of South America, is now distributed almost all over the world. Due to its high reproductive capability and strong ability to adapt, *E. crassipes* can live in almost all water bodies and keep growing indeterminately in suitable conditions. Moreover, it can quickly become the dominant population in water from 25°C to 35°C (Wang *et al.*, 2011) which resulted in damage on aquatic ecology and brought about channel jam. However, *E. crassipes* is able to absorb large amount of nitrogen and phosphorus in eutrophic water and accumulate heavy metal ions. Its rate of nitrogen utilization is significantly higher than main crops. Biochemical characteristic analysis shows that *E. crassipes* is the best choice in both dry matter production yield and nitrogen absorption in phytoremediation (Zheng *et al.*, 2008).

In order to gain better understanding of the molecular basis and expression of *E. crassipes* ammonium transporter, we've cloned the full-length cDNA of EcAMT and analyzed its expression under different nitrogen status through quantitative real-time PCR.

Materials and Methods

Plant preculture and RNA extraction: *E. crassipes* was obtained from the west pool in Guangdong university of technology and precultured in complete nutrient solution (59mg/L $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 25.5mg/L KNO_3 , 24.5mg/L

MgSO₄·7H₂O, 7mg/L KH₂PO₄) for two weeks at 28°C, under a 16/8-hour light (2000lx)/dark cycle. The solution was replaced every 2 days. After that, it was cultured in distilled water for about one week, and then fresh roots of *E. crassipes* were collected and grinded into powder as liquid nitrogen existed. Then total RNA was extracted according to the RNAiso plus (from TAKARA, Dalian) manual. Integrity of total RNA was detected by agarose gel electrophoresis.

RT-PCR with degenerate primers: Total RNA was reverse transcribed into cDNA by using a prime-scriptTM RT-PCR kit (from TAKARA, Dalian). PCR reaction was performed with root cDNA as template and P1 and P2 (Table 1) as degenerate primers. In this PCR program, an initial denaturation step for 3min at 94°C was followed by 30 cycles of 30s at 94°C, 30s at 53°C and 1min at 72°C, with a final elongation step at 72°C for 10min. PCR production was purified using agarose DNA purification kit (from TAKARA, Dalian), and a 408bp DNA sequence was obtained. This target gene fragment was put into pMD19-T vector, and then transformed into competent *E.coli* cell and sequenced (Invitrogen, Guangzhou).

3' and 5' rapid-amplification of cDNA ends (RACE): Using RACE technology, we can get the unknown 3' end and 5' end based on a known gene fragment, and then obtain the complete gene sequence. RNA was extracted as mentioned before. It was used as template to prepare 3' adaptor cDNA and 5' ligated cDNA as described in the 3' full RACE core set ver.2.0 and 5' full RACE kit (from TAKARA, Dalian). This cDNA was used as template in nested-PCR and touch-down PCR reactions. Three pairs of primers P3-5 were designed in RACE according to the above conserved sequence (Table 1).

3' RACE: In the first program, an initial denaturation step for 3min at 94°C was followed by 20 cycles of 30s at 94°C, 30s at 60°C with a -1°C down at each cycle, and 90s at 72°C. No bands were seen in electrophoretogram, and a second touch-down PCR program was performed, an initial denaturation step for 3min at 94°C was followed by 16 cycles of 30s at 94°C, 30s at 59°C with a -0.5°C down at each cycle, and 90s at 72°C, followed by 19 cycles of 30s at 94°C, 30s at 51°C, 90s at 72°C.

5' RACE: In this program, an initial denaturation step for 3min at 94°C was followed by 15 cycles of 30s at 94°C, 30s at 60°C with a -0.6°C down at each cycle, and 1min at 72°C, followed by 15cycles of 30s at 94°C, 30s at 51°C, and 2min at 72°C, with a final elongation step at 72°C for 10min.

Full-length cDNA clone: The template cDNA was prepared as described before. A pair of primers P6-7 with restriction sites was designed (Table 1) and synthesized by the company of Invitrogen. PrimeSTAR HS DNA Polymerase from TAKARA was used in PCR process. In this PCR program, 11 cycles of 10s at 98°C, 10s at 49°C with +0.5°C up at each cycle, 2min at 72°C, followed by 24 cycles of 10s at 98°C, 10s at 55°C, 2min at 72°C, with a final elongation step at 72°C for 10 min. The full-length cDNA about 1500bp was obtained and sequenced (Invitrogen, Guangzhou).

Yeast growth and function complementation: The *Saccharomyces cerevisiae* strain 31019b (*mep1 mep2 LEU2 mep3 KanMX2 ura3*) can't grow when the external concentration of ammonium below 5mM as all of its three ammonium transporter genes are missed (Ortiz-Ramirez *et al.*, 2011). Competent yeast cells (31019b) were prepared to be the acceptor of vector pYES2 according to kits (FunGenome, Beijing) manuals. The full-length cDNA of EcAMT was linked into yeast vector pYES2, and then transformed into competent yeast cell to form trans-formant. Yeast trans-formants were firstly selected in *ura* minus media and followed by transferred to yeast nitrogen base (YNB) without amino acids and supplemented with 0.5mM (NH₄)₂SO₄ as its sole nitrogen source. They were incubated at 30°C for three days.

Sequence analysis: Nucleotide sequences were blasted against non-redundant nucleotide database (Altschul, 1997). Their translation was performed by translation tool from EXPASY (<http://web.expasy.org/translate/>). The program ProtParam (Gasteiger *et al.*, 2005) was employed to analyze the physico-chemical properties of EcAMT. Transmembrane domains were predicted by TMHMM (Krogh *et al.*, 2001). Multiple sequence alignments of translated gene sequences were carried out with the program ClustalX-1.83 (Thompson *et al.*, 1997). The phylogenetic tree was constructed by MEGA 5.1 program (Tamura *et al.*, 2011) using Neighbour-joining method.

Table 1. Primers used in this study.

Primer name	Sequence(5'-3')
P1(forward degenerate primer)	GACTTCTTCCTGTACCAGTGGGCNTTYGC
P2(reverse degenerate primer)	GGGTTGAAGCCGTACCANCCRAACCA
P3(3' outer special primer)	CGGCATCTTCTACTACCTGTTTGG
P4(3' inner special primer)	CACTCAGCCTCCCTCGTGGTCCTT
P5(5' inner special primer)	GGGTTGAAGCCGTACCATCCGAACCA
P6(forward full-length primer)	<u>AAGCTT</u> ATGTGCGACGTGCAGCGCGG
P7(reverse full-length primer)	<u>GAATTC</u> GCTAGCTGACGACGTGCGCCG
P8(forward EcAMT1 primer)	GATACCCCGACCATGTGTA
P9(reverse EcAMT1 primer)	CACAGTTCTCCGCCTACCTC
P10(forward 18s rRNA primer)	CTTCGGGATCGGAGTAATGA
P11(reverse 18s rRNA primer)	AACCCCAACTTTCGTTCTT

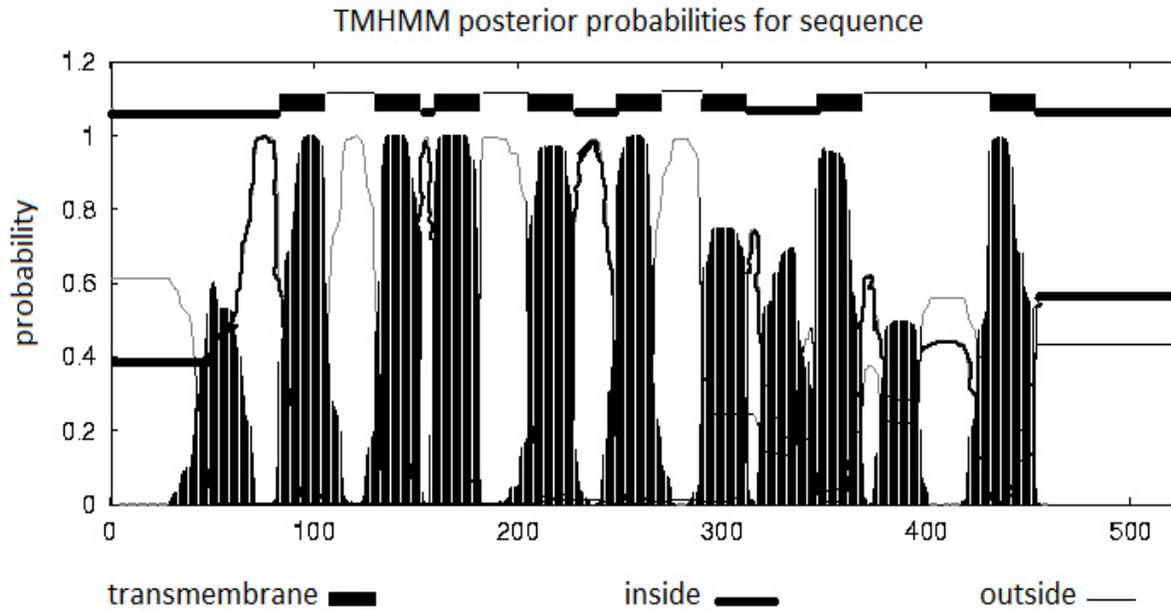


Fig. 2. Transmembrane domains of EcAMT were predicted by TMHMM.

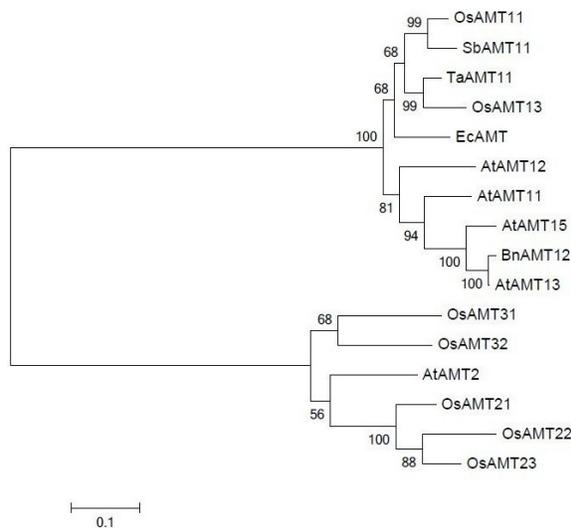


Fig. 3. Neighbor-joining phylogenetic tree of plant AMTs protein sequences. Sequences were obtained from NCBI with the following accession numbers, *Arabidopsis thaliana*: AtAMT1;1 (AEE83287), AtAMT1;2 (NP_176658), AtAMT1;3 (NP_189073), AtAMT1;5 (NP_189072), AtAMT2 (XP_002881576); *Brassica napus*: BnAMT1;2 (AAG28780); *Oryza sativa*: OsAMT1;1 (AAL05612), OsAMT1;3 (AAL05614), OsAMT2;1 (Q84KJ7), OsAMT2;2 (Q8S230), OsAMT2;3 (Q8S233), OsAMT3;1 (Q84KJ6), OsAMT3;2 (Q851M9); *Sorghum bicolor*: SbAMT1;1 (XP_002446777); *Triticum aestivum*: TaAMT1;1 (AAS19466)

E. crassipes is one of the most productive plants, accordingly, one of the top ten world's weeds, caused extremely serious ecological, economical and social problems. Most studies are focused on how to make full use of it (Munavalli & Saler, 2009; Aboul-Enein *et al.*, 2011) and how to repress its production (Harley, 1990). But there was little study about the molecular mechanism of its

nitrogen absorption. *E. crassipes* is very efficient in taking up nitrogen. AMT is an important protein in its nitrogen absorption. So it will be meaningful to study its AMTs in order to reveal the mechanism of its nitrogen utilization.

Since AMT genes from *Saccharomyces cerevisiae* (Marini *et al.*, 1994) and *Arabidopsis thaliana* (Ninnemann *et al.*, 1994) were isolated in 1994, many AMTs in rice, tomato, *Brassica napus* and other plants have been identified. In this study, we first cloned a novel AMT gene from *E. crassipes* root and studied its expressions under different nitrogen conditions.

Functional identification: To further identify the ammonium transporting capacity of this protein, we transferred EcAMT gene into *Saccharomyces cerevisiae* 31019b. This mutant yeast can't grow in a medium containing less than 5mM NH_4^+ as the sole nitrogen source as it is defective in the two high-affinity NH_4^+ transporters (*mep1*, *mep2*) and one low-affinity NH_4^+ transporter (*mep3*). Wide-type yeast and EcAMT-carrying cells were able to grow on 1mM NH_4^+ as the sole nitrogen source while triple *mep* mutants can't grow (Fig. 4), revealed that EcAMT expressed a functional protein which acted as ammonium transporter and enabled the mutants take up NH_4^+ from outside.

Expression analysis of EcAMT under different nitrogen conditions: *E. crassipes* was precultured in complete nutrient solution for two weeks, and then transferred to nutrient solutions containing different nitrogen sources. EcAMT was up-regulated markedly under nitrogen-starved status shown in Fig. 5. EcAMT transcripts level was repressed by several folds in the presence of nitrogen, to an even larger extent by nitrate than ammonium, which may be partially resulted from nitrate transporters (NRT), which is ubiquitous in plants and may take part of role in nitrogen uptake from external environment.

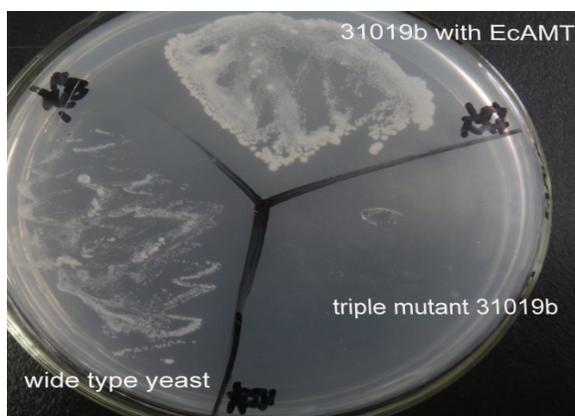


Fig. 4. Yeast complementation of triple mep mutant by EcAMT1. Yeast strain 31019b was transformed with the yeast expression vector pYES2 harboring coding sequence of EcAMT. Growth test on YNB medium containing 0.5mM $(\text{NH}_4)_2\text{SO}_4$ as a sole nitrogen source.

Plants can evolve a comprehensive responsive mechanism to different living conditions. It was reported that nitrogen concentration of agricultural soil solution ranged across three to four orders of magnitude (Wolt, 1994). The situation was even more variable in natural soils, especially in flood fields, which resulted in an enhanced nitrogen absorption mechanism evolved by plants such as rice (Glass *et al.*, 2002) and so on. Many plants have more than one kind of ammonium transporter. Different ammonium transporters have different expression patterns, which may be the responsive mechanism of plants to different conditions. It was reported in *Arabidopsis thaliana* the transcript levels of AtAMT1;1 steeply increased with ammonium uptake in roots when nitrogen nutrition became limiting, whereas those of AtAMT1;3 increased slightly, with AtAMT1;2 being more constitutively expressed (Gazzarrini *et al.*, 1999). In rice, the expressions of OsAMT1;1, OsAMT1;2 and OsAMT1;3 were all upregulated under nitrogen limitation. However, Changes in transcript abundance of OsAMT1;2 were approximately 50% less than in OsAMT1;1, and changes of OsAMT1;3 expression were even less (Kumar *et al.*, 2003). Transcript levels of LeAMT1;2 in tomato increased after MH_4^+ or NO_3^- supply, while LeAMT1;1 was induced by nitrogen deficiency coinciding with low glutamine concentrations (von Wirén *et al.*, 2000b). D'Apuzzo *et al.*, (2004) also reported expression of LjAMT1;1 and 1;2 in *Lotus japonicus* roots was induced by nitrogen deprivation. In our study, the expression of EcAMT was enhanced by 7 to 8 folds under nitrogen starvation than in the presence of ammonium or nitrate, which was well consistent with the previous listed above. Molecular evidence suggests that glutamine in cells is an essential regulator. As we know, ammonium or nitrate should be transformed into glutamine firstly before it assimilated. Hirose *et al.*, (1997) demonstrated that the expression of NADH-dependent glutamate synthetase was induced within 3h of an NH_4Cl treatment in the roots of rice seedlings while the expression of cytosolic glutamine synthetase was constitutive throughout the treatment (Sakamoto *et al.*, 1989). It suggests that glutamine in cells is an essential regulator of the expression of OsAMT1 genes. The expression patterns of OsAMT1;3 and AtAMT1 were negatively correlated

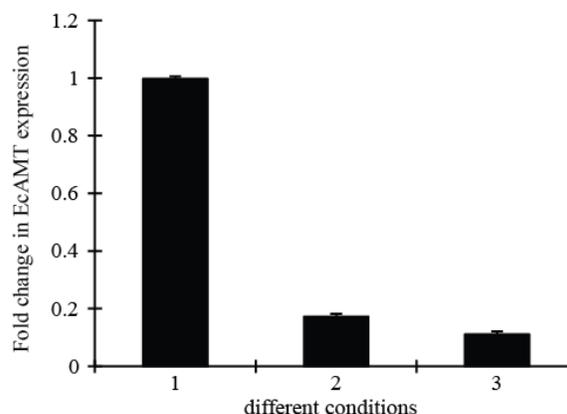


Fig. 5. EcAMT expressions under distilled water, 1.8mM NO_3^- or 1.8mM NH_4^+
1: cultured in distilled water; 2: cultured in 1.8mM NH_4Cl solution; 3: cultured in 1.8mM KNO_3 solution

with the glutamine concentrations in root (Rawat *et al.*, 1999; Li *et al.*, 2009). As for *E. crassipes*, there may be more than one kind of ammonium transporter, which may have different expression pattern to adapt different environmental conditions. So further studies on ammonium transporters of *E. crassipes* should be made, which can increase our understanding the molecular mechanism for *E. crassipes* to adapt eutrophic water and invade quickly to non-native environment, which can be used in improving crops nitrogen utilization, facilitating further development and utilization of *E. crassipes* as a water remediation plant, and controlling the growth of *E. crassipes* from the point of view of nutrient metabolism.

Conclusions

In this study we had cloned the first ammonium transporter gene in *E. crassipes*. EcAMT was able to functionally complement with triple mutant yeast defective in MEPs and acted as an ammonium transporter. The full-length cDNA of EcAMT contains 1506 nucleotides long open reading frame, which encodes 501 amino acids. Blast result showed that it shared above 70% identity with other plant AMTs, such as rice and *Triticum aestivum*. Besides, TMHMM algorithm predicted that it had 8 transmembrane regions, just like most other plant AMTs. qRT-PCR results indicated that EcAMT expression significantly increased under nitrogen starvation, while the expression kept relatively low in ammonium or nitrate solutions. The results revealed some molecular mechanism of *E. crassipes* to absorb the ammonium in eutrophic water.

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