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Isolation and functional characterization of an ammonium transporter gene, *PyAMT1*, related to nitrogen assimilation in the marine macroalga *Pyropia yezoensis* (Rhodophyta)

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ABSTRACT

Ammonium and nitrate are the primary nitrogen sources in natural environments, and are essential for growth and development in photosynthetic eukaryotes. In this study, we report on the isolation and characterization of an ammonium transporter gene (*PyAMT1*) which performs a key function in nitrogen (N) metabolism of *Pyropia yezoensis* thalli. The predicted length of PyAMT1 was 483 amino acids (AAs). The AA sequence included 11 putative transmembrane domains and showed approximately 33–44% identity to algal and plant AMT1 AA sequences. Functional complementation in an *AMT*-defective yeast mutant indicated that PyAMT1 mediated ammonium transport across the plasma membrane. Expression analysis showed that the *PyAMT1* mRNA level was strongly induced by N-deficiency, and was more highly suppressed by resupply of inorganic-N than organic-N. These results suggest that *PyAMT1* plays important roles in the ammonium transport system, and is highly regulated in response to external/internal N-status.

1. Introduction

Some species of the rhodophyte genus *Pyropia* are economically important as edible "*nori*" in many places of the world, and have been cultivated in coastal areas of Japan, Korea, and China for generations. In Japan, *P. yezoensis* is one of the most important algal species used as food, and approximately 300,000–350,000 tons (wet weight) of the thalli are produced in *Pyropia* cultivation farms every year (e-Stat, Portal Site of Official Statistics of Japan: http://www.e-stat.go.jp/SG1/estat/eStatTopPortalE.do "accessed 20 Nov 2015"). Production and quality of the cultivated *P. yezoensis* thalli are frequently influenced by environmental factors. Sufficient dissolved inorganic nitrogen (DIN) content in seawater, which consists of nitrate-N (NO_3-N), nitrite-N (NO_2-N), and ammonium-N (NH_4-N), is a key factor for growth, development, and quality of *P. yezoensis* thalli (Sano, 1955; Sakaguchi et al., 2003; Oyama et al., 2008). When the DIN in the cultivation area decreases below 50 μg/L due to low inflow from rivers (low rainfall) or diatom blooms, severe thallus discoloration ("*iroochi*") occurs in the *Pyropia* cultivation farms, resulting in lower quality *Pyropia* thalli (Sano, 1955; Nishikawa et al., 2007; Oyama et al., 2008). Although phycobiliproteins, the predominant photosynthetic pigments in red algae, decrease 20–50% in the discolored *Pyropia* thalli, recovery of discolored *Pyropia* thalli has been obtained by chemical fertilization using not only inorganic-N but also organic-N such as urea-N and amino acid-N (AA-N) (Sano, 1955; Nozawa, 1959; Ito et al., 1960; Amano and Noda, 1987; Sakaguchi et al., 2003). Thus, it is necessary for effective management of *P. yezoensis* cultivation to understand the molecular mechanisms and characteristics for inorganic/organic-N uptake and assimilation in *Pyropia* thalli. To date, a nitrate transporter gene (*PyNRT2*) and three urea transporter genes (*PyDUR3.1/3.2/3.3*), which may be involved in uptake and transport of nitrate and urea, respectively, have been identified and characterized in *P. yezoensis* (Kakinuma et al., 2008, 2016). In natural seawater, NO_3-N and NH_4-N are major N sources utilized by *P. yezoensis* thalli. Nevertheless, no *P. yezoensis* ammonium transporter genes (*AMTs*) related to NH_4-N uptake have been isolated and characterized.

Ammonium transport across membranes is mediated by the ammonium transporter/methylammonium permease/mammalian

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rhesus protein (AMT/MEP/Rh) family (von Wirén and Merrick, 2004; McDonald et al., 2012). In higher plants, AMTs can be subdivided into AMT1 (AMT1 cluster) and AMT2 (AMT2/3/4 cluster) subfamilies at the molecular level (Loqué and von Wirén, 2004; Ludewig et al., 2007; Koegel et al., 2013). Although the physiological roles of AMT2s are still unclear, it is well known that the AMT1s are channel-like proteins which function as NH_4^+ uniporters or NH_3/H^+ cotransporters, and are responsible for high-affinity NH_4^+ transport system (HATS) (von Wirén and Merrick, 2004; Yuan et al., 2007). Plant *AMT1s* were first characterized in the model land plant *Arabidopsis thaliana* (Ninnemann et al., 1994). Recent genome sequence analysis indicates that plant *AMT1s* are encoded by multigene families, e.g., three genes (*OsAMT1;1–1;3*) for *Oryza sativa*, five genes (*AtAMT1;1–1;5*) for *A. thaliana*, and seven genes (*PtrAMT1;1–1;7*) for *Populus trichocarpa*, which specify isoforms with organ-specific distributions and different functions (Loqué and von Wirén, 2004; Li et al., 2012; von Wittgenstein et al., 2014; Wu et al., 2015). In *A. thaliana*, three *AMT1s* (*AtAMT1;1/1;2/1;3*), which are responsible for majority of the total high-affinity NH_4^+ uptake in roots, are differentially affected by external N status (i.e., *AtAMT1;1/1;3* are N-deficient inducible, while *AtAMT1;2* is insensitive to N nutrition changes) (Shelden et al., 2001; Loqué et al., 2006; Yuan et al., 2007), whereas *AtAMT1;4* is related to NH_4^+ uptake across the plasma membrane of pollen (Yuan et al., 2009). In unicellular algae, more than two *AMT1s* have been identified in the green algae *Clamydomonas reinhardtii*, *Dunaliella viridis*, and *Volvox carteri* (González-Ballester et al., 2004; Merchant et al., 2007; Prochnik et al., 2010; Song et al., 2011), red alga *Galdieria sulphuraria* (Schönknecht et al., 2013), diatom *Cylindrotheca fusiformis* (Hildebrand, 2005), and coccolithophore *Emiliania huxleyi* (Read et al., 2013), and exhibited different functions or efficiencies in NH_4^+ uptake in some species. The molecular characteristics of *AMT1s* in multicellular algal species remain unclear, although some putative *AMT1s* have been identified in the model alga *Ectocarpus siliculosus*, whose genome sequencing project has been completed and publicly released (Cock et al., 2010).

A publicly-available *P. yezoensis* expressed sequence tag (EST) database exists which includes more than 20,000 ESTs from both the gametophytic and sporophytic generations (Nikaido et al., 2000; Asamizu et al., 2003). A database search of these ESTs using the BLAST algorithm indicated that some sequences have similarity to *AMT1s* identified in land plants and algae, two of which may include a putative 5′-untranslated region (UTR) and translational start codon. The present study was undertaken to clone and sequence the *P. yezoensis AMT1* (*PyAMT1*) cDNA and genomic DNA (gDNA). In addition, in order to understand the physiological and molecular characteristics of *PyAMT1* in NH_4-N uptake, functional complementation analysis of *PyAMT1* using the fission yeast expression system was performed, and transcriptional regulation of the *PyAMT1* in *P. yezoensis* thalli grown under different N-conditions was investigated.

2. Materials and methods

2.1. Algal strain and cultivation conditions

Pyropia yezoensis strain FA-89, which was originally isolated by selective breeding and maintained by the Fukuoka Fisheries and Marine Technology Research Center, Japan (Fukunaga and Iwabuchi, 2004), was used in this study. Leafy gametophytes of *P. yezoensis* were maintained using one-fifth strength Provasoli's enriched seawater (1/5 PES) medium (Provasoli, 1968) in the laboratory. The culture medium was changed every 3 days throughout the experiments. The culture was aerated with air filtered through a FP30/0.2PTFE-S filter (Whatman, Germany), irradiated with 50 μmol photons $m^{-2}s^{-1}$ light on a 10:14 h (light/dark) cycle (10 L/14D photoperiod), and maintained at 10 °C. Thalli were inoculated into the experimental seawater media upon reaching an average length of 3 cm.

Dissolved inorganic nitrogen (DIN) content in natural seawater for preparation of each experimental seawater medium was analyzed using a TRAACS 2000 (Bran + Luebbe, Germany). The average DIN concentration of the natural seawater was 138.3 μg/L (NO_3-N = 125.8 μg/L, NO_2-N = 2.1 μg/L, and NH_4-N = 10.4 μg/L).

2.2. Total RNA and genomic DNA extraction

Fresh *P. yezoensis* thalli collected from experimental treatments were immediately immersed in RNA*later* Stabilization Solution (Applied Biosystems, USA) and stored at −20 °C until total RNA extraction. The thalli used for genomic DNA (gDNA) extraction were immediately frozen with liquid nitrogen and stored at −80 °C. Total RNA extraction from RNA-stabilized *P. yezoensis* thalli was performed using the RNeasy Plant Mini Kit (Qiagen, Germany). After DNase treatment using the MessageClean Kit (GenHunter, USA), the purified total RNA was used for single-stranded (ss) cDNA synthesis for 3′ rapid amplification of cDNA ends (RACE) and for quantitative-polymerase chain reaction (qPCR) analysis. Genomic DNA (gDNA) extraction from frozen *P. yezoensis* thalli was performed according to the method of Liu et al. (1995). The extracted gDNA was further purified using the Genomic-tip 100/G Kit (Qiagen). This purified gDNA was used for isolation of partial gDNA clones.

2.3. cDNA and gDNA cloning

Five micrograms of total RNA were used for ss cDNA synthesis using the 3′ RACE System for Rapid Amplification of cDNA Ends (Invitrogen, USA). The gene-specific primers PyAMT-GSP1 and PyAMT-GSP2 (Table 1) were designed based on the nucleotide (nt) sequences of ESTs AV430486 (495 bp) and AU193810 (311 bp) in the *P. yezoensis* EST Index of the Kazusa DNA Research Institute, Japan (http://est.kazusa.or.jp/en/plant/porphyra/EST/) (Nikaido et al., 2000; Asamizu et al., 2003). The primary PCR amplification of *PyAMT1* cDNA was performed using primers PyAMT-GSP1 and the adapter primer AUAP from the 3′ RACE kit. The secondary PCR amplification was performed using primers PyAMT-GSP2 and AUAP. The cDNA fragments amplified by PCR were subcloned into a pT7Blue T-vector (Novagen, USA) and sequenced.

A genomic copy of *PyAMT1* was amplified using Long and Accurate-PCR (LA-PCR, Takara Bio, Japan) according to the

Table 1
Nucleotide sequences of primers for PCR amplifications.

Primer name	Sequence[a]
3′ RACE	
PyAMT-GSP1	5′-dCACAATCGCTGTTCCCTCCCGCTCCCA-3′
PyAMT-GSP2	5′-dTAACTACGCAGACCGCCGGCAACG-3′
LA-PCR	
PyAMT-GF	5′-dTAACTACGCAGACCGCCGGCAACGCCT-3′
PyAMT-GR	5′-dGCCGCAGAGATCGGAGATGACGATAGA-3′
PCR for probe preparation	
PyAMT-PF	5′-dGGTCGACTCTGGGTATCTCGAACA-3′
PyAMT-PR	5′-dTGCTTGGAGACGTCGTTACCAATC-3′
PCR for vector construction	
PyAMT-SAC	5′-dTGAGCTCATGATTGCTACCGACATGACCGCT-3′
PyAMT-PST	5′-dTCTGCAGTTAGACCATCGTCGGGTCGTC-3′
Quantitative-PCR	
PyAMT-QF	5′-dGCACATTCATTCTGTGGTTTGG-3′
PyAMT-QR	5′-dCCGTGTTCGAGATACCCAGAGT-3′
PyAMT-QP	5′-dTGGTACGGCTTCAAC-3′

[a] Recognition sites of *Sac*I (GAGCTC) and *Pst*I (CTGCAG) are underlined.

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