J Appl Phycol (2017) 29:2617–2626
DOI 10.1007/s10811-017-1196-1

Selection and functional analysis of a *Pyropia yezoensis* ammonium transporter PyAMT1 in potassium deficiency

Eri Adams[1] **· Koji Mikami**[2] **· Ryoung Shin**[1]

Received: 31 October 2016 / Revised and accepted: 4 June 2017 / Published online: 19 June 2017
© Springer Science+Business Media B.V. 2017

Abstract Seaweeds are believed to have developed unique mechanisms to maintain optimal cellular potassium and sodium concentrations in order to survive in the saline marine environment. To gain a molecular understanding of underlying potassium/sodium homeostasis in seaweeds, full-length cDNA libraries from the multiple stages in the life cycle, including gametophytes, conchosporangia and sporophytes of a marine red alga, *Pyropia yezoensis*, were constructed. A large portion of genes from each library through the life cycle was revealed to be functionally unknown reconfirming the uniqueness of *P. yezoensis* genes in terms of evolutionary lineage. Genes that could potentially contribute to potassium deficiency tolerance were selected from the potassium uptake defective *Escherichia coli* strain expressing gametophytes and conchosporangia libraries under the low potassium conditions. Of those, an ammonium transporter gene, *PyAMT1*, was demonstrated to enhance potassium deficiency tolerance effectively when expressed in the *E. coli* strain. Potential roles of PyAMT1 and other candidate components in this context are discussed.

Keywords Ammonium transporter · Full-length cDNA library · Potassium deficiency · *Pyropia yezoensis* · Salt tolerance

✉ Ryoung Shin
ryoung.shin@riken.jp

[1] RIKEN Center for Sustainable Resource Science, 1-7-22 Suehirocho, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan

[2] Faculty of Fisheries Sciences, Hokkaido University, 3-1-1 Minato-cho, Hakodate, Hokkaido 041-8611, Japan

Introduction

Seaweeds have adapted to the extremely high salt environment in the ocean, an environment that most of land plants never encounter. High levels of sodium (Na^+) in the cell cause osmotic and ionic stress and disturb potassium (K^+) uptake and functions due to their similar physicochemical properties, often resulting in a K^+ deficiency response (Adams and Shin 2014). Despite the high concentrations of Na^+ in seawater, cytosolic concentrations of Na^+ are generally maintained at low levels in marine algae, suggesting the existence of Na^+ extrusion mechanisms (Kirst 1990; Karsten 2012).

It has been long known that the marine red algae Bangiales (Rhodophyta) which include *Pyropia* and *Porphyra* (Sutherland et al. 2011) accumulate K^+ in the cytoplasm and either exclude or contain Na^+ preferentially in the vacuoles (Eppley 1958; Wiencke et al. 1983). In order to maintain the appropriate cytosolic K^+/Na^+ ratios, active K^+ uptake mechanisms are considered essential. Unlike land plants and green algae (Chan et al. 2012; Pedersen et al. 2012), red algae such as *Pyropia yezoensis* and *Porphyridium purpureum* have been reported to possess animal-type Na^+/K^+-ATPases which extrude three ions of Na^+ while taking up two ions of K^+ into the cell and they are predicted to provide the driving force for Na^+-driven solute transporters (Barrero-Gil et al. 2005; Bhattacharya et al. 2013). There seems a tendency that freshwater algae and land plants utilise H^+ gradient generated by H^+-ATPases to energise secondary transporters, whereas marine algae make use of Na^+ gradient albeit with some exceptions (Chan et al. 2012) and this notion is evolutionarily quite interesting.

Recently, the 43 Mb genome of *P. yezoensis* was sequenced, with more than 10,000 gene models predicted (Nakamura et al. 2013). In this alga, a gene encoding K^+ P-type ATPase, *PyKPA1*, was found to be phylogenetically

related to the animal H^+/K^+- and Na^+/K^+-ATPases. Heterologous expression of *PyKPA1* in the *Escherichia coli* strain deficient in K^+ uptake demonstrated that PyKPA1 had a growth promoting effect in the K^+-limited condition and that addition of Na^+ further enhanced the effect of PyKPA1 (Barrero-Gil et al. 2005), suggesting PyKPA1 to be a Na^+/K^+-ATPase. Although gene expression of *PyKPA1* was not altered in response to salt stress in *P. yezoensis* (Uji et al. 2012a), ectopic overexpression of *PyKPA1* in rice plants increased salt tolerance by restoring growth (Kishimoto et al. 2013). Another P-type ATPase, PyKPA2, which shares a 65% sequence identity with PyKPA1 and Na^+/H^+ antiporters, PySOS1 and PyNhaD, have also been isolated from the genome of *P. yezoensis* (Barrero-Gil et al. 2005; Uji et al. 2012a, b). These membrane proteins could potentially be the major machineries in ion homeostasis and Na^+ extrusion in *P. yezoensis*; however, ATPases may be too energetically costly to be the major K^+ uptake mechanism and additional K^+ transporters/channels are expected to exist. Although K^+ uptake in land plants is commonly mediated by K^+ channels and transporters such as AKT1, HAK5 and KUPs in a model land plant *Arabidopsis thaliana* (Adams and Shin 2014), proteins with similar sequences and/or function have not been reported in *P. yezoensis*. In order to resolve the molecular mechanisms underlying the ability of red seaweeds to survive in the marine environment, investigation of regulatory components involved in K^+/Na^+ homeostasis in *P. yezoensis* needs to be performed.

Pyropia yezoensis spends the winter in the form of gametophytes, the leafy structure commonly harvested as seaweed, and it turns into sporophytes, the filamentous structure during the summer. In autumn, sporophytes form conchosporangia from which conchospores are emitted to produce a new generation of gametophytes. There are several reports describing that different sets of genes are expressed in the extremely diverse structures observed throughout the life cycle of *P. yezoensis*: for instance, only 22.5% of ESTs and 1 out of 14 microRNAs are common among gametophytes and sporophytes (Asamizu et al. 2003; Shen et al. 2011; He et al. 2012). Indeed, phase-specific gene expression includes genes encoding urea transporters (*PyDUR3s*), an alginate lyase (*PyAly*) and a bromoperoxidase (*PyBPO1*) (Inoue et al. 2015; Matsuda et al. 2015; Kakinuma et al. 2016). Interestingly, *PyKPA1* has been reported to be predominantly expressed in sporophytes while *PyKPA2* is expressed specifically in gametophytes (Uji et al. 2012a) These findings strongly suggest the existence of distinct regulatory mechanisms upon K^+/Na^+ homeostasis in each life stage.

To identify the genes playing roles in K^+ deficiency tolerance throughout the life cycle of *P. yezoensis*, we here constructed full-length complementary DNA (cDNA) libraries using three different stages, gametophytes, conchosporangia and sporophytes, and these libraries were then transferred into the *E. coli* expression vector system to isolate the genes involved in K^+ deficiency response. Candidate genes and possible mechanisms by which *P. yezoensis* tolerates K^+ deficiency are discussed.

Materials and methods

Plant material and growth conditions

The cultivation of *Pyropia yezoensis* strain U51 was performed as previously reported (Li et al. 2008) with a slight modification. Briefly, free-living sporophytes, free-living conchosporangia and gametophytes attached to polyvinyl alcohol (PVA) monofilaments were suspended in ESL (enriched SEALIFE) media, continuously aerated with filter-sterilised air and grown at 15 °C in a 10 h light/14 h dark photocycle with a light intensity of 60 μmol photons m^{-2} s^{-1}. The sterile ESL medium was made by dissolving commercially available SEALIFE powder (Marintech Co. Ltd., Tokyo, Japan) in distilled water with added ESS_2 solution (Kitade et al. 2002) and this was exchanged weekly.

RNA extraction and cDNA library construction

An excess amount of sporophyte, gametophyte and conchosporangium samples were flash frozen in liquid N_2 and ground into fine powder using a mortar and a pestle. Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, USA) and 75 μg of total RNA was used to isolate messenger RNA (mRNA) using Ambion Dynabeads mRNA purification kit (Thermo Fisher Scientific). Purified mRNA was concentrated by glycogen precipitation with 1 μL of glycogen, 0.5 volumes of NH_4OAc and 2.5 volumes of 100% ethanol; 0.88 ~ 3.31 μg of mRNA was used to create full-length cDNA libraries using CloneMiner II cDNA Library Construction Kit according to the manufacture's instruction (Thermo Fisher Scientific). In short, hybridisation of Biotin-*att*B2-Oligo(dT) Primer to the mRNA poly(A) tail and the first strand cDNA synthesis by SuperScript III Reverse Transcriptase were followed by the second strand cDNA synthesis by *E. coli* Polymerase I and ligation of *att*B1 Adapter to the 5′ end of the cDNA. The resultant double-stranded cDNA was size fractionated by a column to remove truncated cDNA shorter than 500 bp and cloned into a Gateway entry vector pDONR222 through BP recombination reaction. The cDNA construct was then transformed into ElectroMAX DH10B T1 Phage Resistant Cells to create the final cDNA library. Titre was determined by spreading 1:10 serial dilutions (10^{-2}, 10^{-3}, 10^{-4}) of each library onto LB plates containing kanamycin. Titre was calculated as colony forming unit (cfu mL^{-1}) = colonies on plates × dilution factor / volume plated (mL) and total CFU (cfu) = average titre (cfu mL^{-1}) × total

volume of cDNA library (mL). Single colonies were picked and plasmid DNAs (pDNAs) were prepared. Each pDNA was digested by *Bsr*G I to determine the insert size and sequenced using M13 forward and reverse universal primers and the Sanger sequencing technique (HITACHI gene analysis system with ABI PRISM 3100-21 genetic analyser).

Selection of K^+ deficiency tolerance-related genes

The pDONR222 entry libraries from gametophyte and conchosporangium samples were transferred into the pBAD-DEST49 Gateway destination vector according to the manufacturer's instructions (Thermo Fisher Scientific). Plasmid DNA was prepared from the entry library culture grown till an OD_{600} to be approximately 1.0. Polyethylene glycol (PEG) precipitation was performed to purify pDNA using 0.4 volumes of 30% PEG/Mg solution. The entry library was transferred into the destination vector using Gateway LR Clonase II enzyme and transformed into ElectroMAX DH10B T1 Phage Resistant Cells. Plasmid DNA of the pBAD-DEST49 library prepared from the *E. coli* culture with an OD_{600} of approximately 1.0 was transformed into an *E. coli* strain defective in K^+ uptake, TK2463 (Epstein et al. 1993), and selected on minimal media (Ahn et al. 2004) containing 1–3 mM KCl, 0.1% arabinose and ampicillin. For functional analysis, overnight culture of TK2463 expressing pBAD-PyAMT1, PyβCA1 or PyHSP70 grown in KML media (10 g Bacto Tryptone, 10 g KCl, 5 g Bacto Yeast Extract in 1 L MilliQ water) containing ampicillin were pelleted, washed three times with autoclaved MilliQ water, resuspended in autoclaved MilliQ water and dropped onto minimal media containing 30, 1.5 or 1.25 mM KCl, 0.1% arabinose and ampicillin as fivefold serial dilutions.

Sequence analysis

Contig numbers were retrieved from the obtained sequences using the public *Pyropia* database (Nakamura et al. 2013). *Pyropia* genes were annotated using blastx function against the non-redundant protein sequences database at the NCBI search engine (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Amino acid sequences were aligned using Vector NTI (Thermo Fisher Scientific).

Results

Construction and validation of full-length cDNA libraries of *Pyropia yezoensis*

In order to gain molecular information on each stage in the life of *P. yezoensis*, cDNA libraries derived from gametophyte, sporophyte and conchosporangium samples, whose diverse structures are shown in Fig. 1 (a, b for gametophytes, c for sporophytes and d, e for conchosporangia), were constructed. Total CFU was greater than 10^7 for all the libraries with the greatest being >10^8 for the sporophytes library (Table 1). Twenty-four single colonies from each library were randomly picked to validate the diversity of the cDNA libraries and pDNA was digested with restriction enzyme *Bsr*G I to determine the insert size. The recombination efficiency was 100% for all libraries and the average size of inserts was approximately 1 kb (Table 1). Inserted cDNA from each colony was also sequenced and annotated against the public protein sequences database (Table 2). For the gametophytes library, one third of genes were either not annotated or annotated to encode proteins of unknown function and most of the other genes were predicted to encode rather ubiquitous proteins involved in the general biological processes such as protein synthesis, regulation and degradation (Table 2 and Fig. 2). By contrast, more than half of the genes were annotated as unknown in the conchosporangia and sporophytes libraries. The annotated genes were for general functions such as protein synthesis and degradation.

Selection of genes potentially involved in K^+ deficiency tolerance

In order to isolate genes responsible for efficient K^+ utilisation and K^+/Na^+ balance in *P. yezoensis*, the entire entry clone libraries from gametophytes and conchosporangia were transferred into the *E. coli* expression vector system and transformed into TK2463, an *E. coli* strain defective in K^+ uptake. Under the less stringent K^+ deficiency conditions (2 or 3 mM KCl), 48 colonies were recovered and 45 genes were successfully sequenced from the gametophytes library (Table 3). Under the stringent condition (1 mM KCl), 23 genes from the gametophytes library and 16 genes from the conchosporangia library were revealed (Table 4). Although a large portion of genes could not be annotated for function as in the entry libraries (34.8% for gametophytes and 62.5% for conchosporangia), a higher number of the annotated genes was associated with specific functions in biological processes such as metabolism and signalling rather than general functions. Of these, three genes were annotated as β-carbonic anhydrase (βCA; two from 2 or 3 mM KCl, one from 1 mM KCl screening). All three were predicted to represent the same gene model (*PyβCA1*, contig_16545_g4020). Upon sequence alignment with known βCAs from *Chlamydomonas reinhardtii* (CrCAH4), *Ostreococcus tauri* (OtβCA) and *Arabidopsis* (AtβCA5.1), PyβCA1 was shown to possess all three conserved zinc binding sites, two cysteine residues (C) and histidine (H) (marked in blue in Fig. 3) (Provart et al. 1993; Bracey et al. 1994; Kimber and Pai 2000); however, the rest of the sequence was fairly diverse among the species (20.1, 22.0, 23.1% identity with AtβCA5.1, CrCAH4,

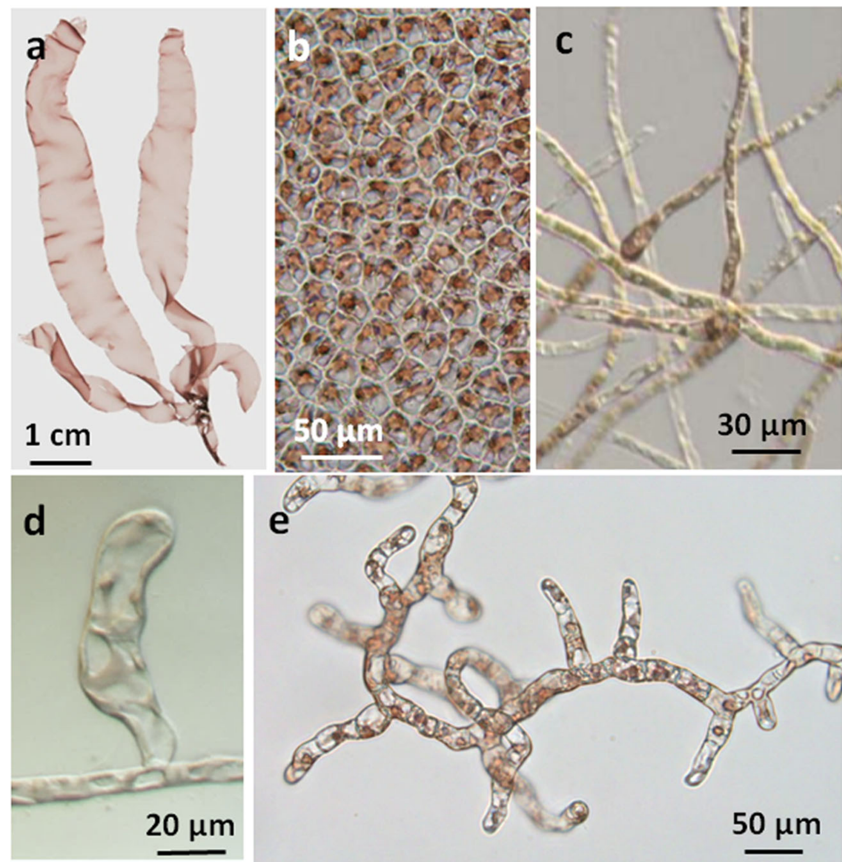

Fig. 1 Images of the multiple stages in the life cycle of *Pyropia yezoensis*. **a** Gametophytes. **b** Vegetative cells of gametophytes. **c** Sporophytes. **d** Generation of a conchosporangium from a sporophyte. **e** Conchosporangia. Full-length cDNA libraries were constructed with RNA extracted from gametophytes, sporophytes and conchosporangia

OtβCA, respectively). Two genes recovered from stringent K^+ deficiency screening were predicted to be a single ammonium transporter (PyAMT1, contig_16335_g3953) (Kakinuma et al. 2017). Sequence alignment of PyAMT1 with well-studied AtAMT1;2 (Yuan et al. 2007) and algal OtAMT (Derelle et al. 2006) indicated that many conserved amino acids such as those which form the ammonium binding site, tryptophan (W) and serine (S) (marked in red in Fig. 4), phenylalanine (F) and aspartate (D) (marked in blue) were identical among three, whereas others, such as F and threonine (T) (marked in green), highlighted the difference between algae and land plants (Pantoja 2012). The TK2463 *E. coli* strains expressing *PyβCA1* and *PyAMT1* were further analysed in the K^+ deficient conditions (1.25 and 1.5 mM KCl) and compared with the strain expressing *PyHSP70* as a negative control. The strains expressing *PyAMT1* and, to a lesser extent, *PyβCA1* grew well in K^+ deficiency while the strain expressing *PyHSP70* could not survive (Fig. 5). Multiple ribosomal proteins of various sizes were also selected from the gametophyte library (Tables 3 and 4).

Discussion

Full-length cDNA libraries from various life stages, including gametophytes, conchosporangia and sporophytes, were created for a model marine alga *P. yezoensis* with excellent recombination percentages and titre. The average insert size of approximately 1 kb corresponds with the predicted average coding sequence length in *P. yezoensis* (Nakamura et al. 2013). Analysis of the whole genome sequence of *P. yezoensis* has revealed that the function of 35% of the genes is unknown (Nakamura et al. 2013), and our results obtained from the gametophytes library was consistent with their report. It is intriguing to postulate why the conchosporangia and sporophytes libraries contain more than 50% of genes that are

Table 1 Titre, recombination % and average insert size of cDNA libraries for three life stages of the marine red alga *P. yezoensis*

cDNA library	Titre (cfu mL^{-1})	Total CFU	Recombination (%)	Average insert (kb)
Gametophytes	1.93×10^6	1.93×10^7	100	1.22
Conchosporangia	1.30×10^6	1.56×10^7	100	1.00
Sporophytes	$>10^7$	$>10^8$	100	1.20

Table 2 Annotation of genes from cDNA libraries for three life stages of the marine red alga *P. yezoensis*

Number	Name	Involved in
Gametophytes		
1	Ferritin	Storage
1	Nitrate reductase	Metabolism
1	5-Formyltetrahydrofolate cycloligase	Metabolism
1	Transmembrane 9 protein	Transport
1	Phosphate transporter	Transport
1	Mitochondrial substrate carrier family protein	Transport
1	Transcription initiation factor	Transcription
1	Ribosomal protein	Protein synthesis
2	Ribosomal RNA/hypothetical protein	Protein synthesis
1	Ser/Thr protein phosphatase	Protein regulation
1	Ser/Thr protein kinase	Protein regulation
1	Protein kinase	Protein regulation
1	F-box protein	Protein degradation
1	Proteasome β subunit	Protein degradation
8	Unknown/no hit	
Conchosporangia		
1	Thioredoxin	Redox reaction
1	Phosphotransferase	Metabolism
1	Actin	Structure
1	bHLH DNA-binding superfamily protein	Transcription
1	Zinc finger transcription factor	Transcription
1	Ribosomal protein	Protein synthesis
1	Disulphide isomerase (thioredoxin superfamily)	Protein regulation
1	Transducin family protein/WD-40 repeat family protein	Protein regulation
1	F-box protein	Protein degradation
1	RING/U-box superfamily protein/E3 ubiquitin-protein ligase	Protein degradation
14	Unknown/no hit	
Sporophytes		
1	Catalase	Redox reaction
1	Alanine:glyoxylate aminotransferase	Metabolism
1	Kinesin	Transport
1	ER membrane protein	Transport
3	Ribosomal protein	Protein synthesis
1	GTPase	Protein synthesis
1	FKBP-type peptidyl-prolyl cis-transisomerase	Protein regulation
1	Peptidase	Protein degradation
1	Proteasome activator protein	Protein degradation
2	YGGT family protein	Unknown function
11	Unknown/no hit	

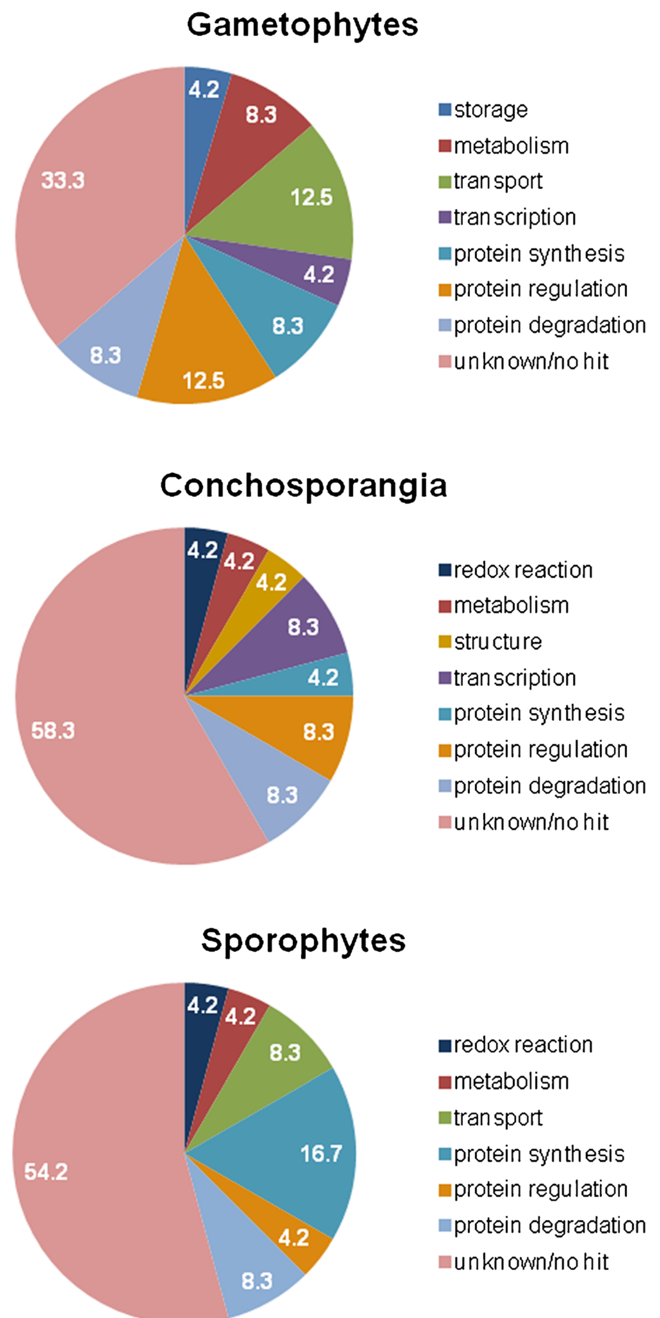

Fig. 2 Functional categories (%) of representative genes recovered from each cDNA library. Randomly selected 24 colonies from each of gametophytes, conchosporangia and sporophytes libraries were sequenced for the inserted genes and annotated based on the sequence similarities against the public protein sequences database

functionally unknown. Many of these genes do not even have any previously characterised conserved amino acid motif or domain, underlining the uniqueness of *P. yezoensis* genes, especially in the conchosporangia and sporophytes stages.

In the search of contributory factors in K^+ deficiency tolerance in *P. yezoensis*, we identified a series of genes that might be involved in such response as efficient uptake and use of K^+. Gametophytes and conchosporangia cDNA libraries were expressed in the *E. coli* system and selected in two different stringency conditions of K^+ deficiency. More colonies were found in the less stringent condition (45 transformants from gametophytes) than in the more stringent condition (23 transformants from gametophytes and 16 transformants from conchosporangia). The ratios of unannotated genes were similar to the pattern in the entry libraries. Unlike the genes annotated in the entry libraries, selected genes were annotated as proteins with specific biological functions rather than ubiquitous proteins, suggesting specific pathways at work in K^+ deficiency response.

Table 3 List of genes selected from the K^+ tolerance screening of the gametophytes library expressed in the *E. coli* strain defective in K^+ uptake under mild K^+ deficiency (2 or 3 mM KCl)

Number	Name	Involved in
Gametophytes		
1	Carrier superfamily protein	Transport
1	Glycyl-tRNA synthetase	Metabolism
1	5′ Adenylyl phosphosulfate reductase	Metabolism
1	GDP-D-mannose 3′,5′-epimerase	Metabolism
1	Serine hydroxymethyl transferase	Metabolism
1	Carbohydrate binding protein	Metabolism
1	Alanine:glyoxylate transaminase	Metabolism
1	Glutamate-5-semialdehyde dehydrogenase	Metabolism
2	β-Carbonic anhydrase	Metabolism
1	Fructose/ketose-bisphosphate aldolase	Metabolism
1	Nicotinic acetylcholine receptor-like protein	Signalling
1	Calmodulin/centrin	Signalling
1	Chromosome associated-like protein	Transcription
1	NAC transcription factor	Transcription
1	Histone superfamily protein	Transcription
1	RNA-binding protein	RNA regulation
1	Translational elongation factor EFG/EF2 protein	Protein synthesis
10	Ribosomal protein	Protein synthesis
1	Kinase-like protein	Protein regulation
1	Proteasome subunit	Protein degradation
15	Unknown	

Table 4 List of genes selected from the K^+ tolerance screening of the gametophytes and conchosporangia libraries expressed in the *E. coli* strain defective in K^+ uptake under severe K^+ deficiency (1 mM KCl)

Number	Name	Involved in
Gametophytes		
2	Ammonium transporter	Transport
1	Voltage-dependent anion channel	Transport
1	Valine-tRNA ligase/valyl trans synthase	Metabolism
1	β-Carbonic anhydrase	Metabolism
1	Phosphoglycerate mutase-like protein	Metabolism
1	Cytochrome *c* oxidase-like protein	Respiration
1	Calcium-binding EF-hand family protein/calcineurin	Signalling
1	Heat shock protein	Defence
1	RNA-binding protein	RNA regulation
3	Ribosomal protein	Protein synthesis
1	Ser/Thr kinase/phototropin	Protein regulation
1	F-box protein	Protein degradation
8	Unknown	
Conchosporangia		
1	Ubiquinol-cytochrome *c* reductase	Metabolism
1	Senescence-associated protein	Defence
1	DNA repair helicase	Defence
2	GTP-binding protein	Transcription
1	Ser protease-like protein	Protein degradation
10	Unknown	

There were two types of proteins selected multiple times: β-carbonic anhydrase (PyβCA1) and ammonium transporter (PyAMT1). CA catalyses the reversible reaction between CO_2 and $HCO_3^- + H^+$ and is crucial for aquatic photosynthetic organisms which suffer in the low-CO_2 environment to concentrate CO_2 in the vicinity of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Moroney et al. 2001). The existence of CA activity in marine macroalgae has been known for a long while (Bowes 1969). A βCA has previously been cloned in *P. yezoensis* and its expression was reported to be the lowest in gametophytes, followed by sporophytes and conchospores (Zhang et al. 2010). Although PyβCA1 selected in this screen is different from the one identified in the previous study, its expression is predicted to be low in gametophytes since the same expression pattern was also observed for *P. haitanensis* βCAs (Chen et al. 2016). Considering its lower abundance in the original gametophytes library and the fact that three independent transformants were recovered in the low K^+ assays, it seems to point to the significance of PyβCA1 in K^+ deficiency response. Furthermore, this particular βCA might be important in this response as only one gene was repeatedly isolated though multiple *βCAs* were expected to exist in the genome. As all three conserved amino acids which contribute to binding of the catalytic zinc ion are present in PyβCA1 (Fig. 3), it is predicted as a functional βCA. By contrast, overall sequence identity is not high among the species tested (approximately 20%) and it is possible that regulation and function of each βCA are distinct. This notion is also supported by the fact that CA is essential for *E. coli* growth under aerobic conditions probably due to HCO_3^- requirement for amino acid, nucleotide and fatty acid synthesis (Merlin et al. 2003), indicating that expression of *PyβCA1*, but not the innate CA activity of *E. coli*, could contribute to K^+ deficiency tolerance (Fig. 5). Although a direct interaction between βCA and K^+ has yet to be reported in seaweeds (Escassi et al. 2002), we speculate that increased carbon source and photosynthesis by PyβCA1 might compensate the loss of K^+.

Two independent transformants from the stringent K^+ deficiency screen were found to carry a single *AMT* gene (*PyAMT1*). During the review process of the current paper, another group reported isolation of PyAMT1 as a functional ammonium transporter whose gene expression is dramatically

```
PyβCA1    (1) -------------------------------------MAAVASPTSVPATN
CrCAH4    (1) --------MSSRNVATALRMFATLGRSQAGEASAMMGTGSALLAQRAAAL
OtβCA     (1) --------------------------------------------------
AtβCA5    (1) MAATPTHFSVSHDPFSSTSLLNLQTQAIFGPNHSLKTTQLRIPASFRRKA

PyβCA1   (15) DEPLALLTGECPAGDKVWASLLASNAQFATAGERPP-AEGVSVTHRGSLA
CrCAH4   (43) GGPQAVNKGCSCRCGRVACMGACMPMRHLHAHPNPPSDPDQALEYLREGN
OtβCA     (1) ------MSSPERAFERLLDGHRAFRRAHFAASDGAA-DVPRALRALSER-
AtβCA5   (51) TNLQVMASGKTPGLTQEANGVAIDRQNNTDVFDDMK-QRFLAFKKLKYMD

PyβCA1   (64) ---------------G---------GQSPSAVVVTCADSRLSPELLFARG
CrCAH4   (93) KRFVNNKPHDSHPTRNLDRVKATAAGQKPFAAFLSCADSRVPVEIIFDQG
OtβCA    (43) -------------------------GQRPRALVVACSDSRADPAIVFDTA
AtβCA5  (100) ---------------DFEHYKNLADAQAPKFLVIACADSRVCPSAVLGFQ

PyβCA1   (90) LGELFVIRTAGNTTGDDS-------TVAS-VEYAVKNLSASLVVVLGHTK
CrCAH4  (143) FGDVFVTRVAGNIVTNEIT-------ASL--EFGTAVLGSKVLMVLGHSA
OtβCA    (68) PGDVFTIRNVGSLVPAYAGLDGGHHGTCAATEYATVHLEVPVILVMGHTQ
AtβCA5  (135) PGDAFTVRNIANLVPPYE---SGPTETKAALEFSVNTLNVENILVIGHSR

PyβCA1  (132) CGAVGAAVATEADPDAMAE---------QPRTLAAFVKEKLLAPVQAVKL
CrCAH4  (184) CGAVAATMNGAAVP----------------GVISSLYYS-------IS-P
OtβCA   (118) CGGAAAGLRKYGNGPDADASVFGVNEATGEGFIGAWVAL---AEDAVRRV
AtβCA5  (182) CGGIQALMKMEDEG-------------DSRSFIHNWVVVGKKAKESTKAV

PyβCA1  (173) RGDADESGFVAACEVENVHHAVRTLLTTSGWLAKTRVGGGVKVVGAMYHL
CrCAH4  (210) ACKKAQAGDVDGAIAENVKVQMEQLKVSPVLQGLVKEGK-LKIVGGVYDL
OtβCA   (165) CERHDPGVRARMLEYELVRQSVQNLLTFPFVKRRVDRGE-LVVKGAVFNV
AtβCA5  (219) ASNLHFDHQCQHCEKASINHSLERLLGYPWIEEKVRQGS-LSLHGGYYNF

PyβCA1  (223) ETGVVEEC--------------------------
CrCAH4  (259) ATGKVTEIA-------------------------
OtβCA   (214) WDGTLEVLRADGSFEQLDDDAEDGRGEAKRAKN-
AtβCA5  (268) VDCTFEKWTVDYAASRGKKKEGSGIAVKDRSVWS
```

Fig. 3 Amino acid sequence alignment of βCAs. *Pyropia yezoensis* PyβCA1 (contig_16545_g4020) was aligned with *Arabidopsis thaliana* AtβCA5.1 (At4g33580), *Chlamydomonas reinhardtii* CrCAH4 (GI: 159475801) and *Ostreococcus tauri* OtβCA (GI: 308799709). Identical amino acids among all four βCAs are *highlighted as dark grey* and identical amino acids between two or three among four are *highlighted as light grey*. The amino acids which form the conserved zinc binding site are *marked in blue*

induced in response to nitrogen deficiency (Kakinuma et al. 2017). It is known that ammonium is preferentially taken up over nitrate by many algae and multiple *AMT* genes are present and expressed in the *Porphyra* species (Chan et al. 2012). Amino acid sequence alignment of PyAMT1 with *Arabidopsis* AtAMT1;2 and green alga *O. tauri* OtAMT indicated that many of the functional residues were conserved but some of the amino acids were the same among algae but this was not the case with *Arabidopsis*. Interestingly, substitution of the H125 residue identified from bean (*Phaseolus vulgaris*), which is generally replaced by proline (P) in other plant homologues (marked in orange in Fig. 4), for arginine (R) renders the transporter more active (Ortiz-Ramirez et al. 2011). Since the H125 position is R in PyAMT1, this might suggest it to be an active form. Expression of *PyAMT1* dramatically improved the ability of TK2463, an *E. coli* strain defective in K^+ uptake, to survive in K^+ deficiency compared to the negative control line expressing *PyHSP70* (Fig. 5). Although the negative control line showed somewhat compromised growth in the sufficient K^+ condition, the degree of viability under K^+ deficiency between the lines expressing *PyHSP70* and *PyAMT1* was fairly clear. The effect of PyAMT1 was stronger than that of PyβCA1 (Fig. 5) and this point was consistent with the fact that PyAMT1 was recovered solely from the stringent screen while PyβCA1 from the mild screen as well. Interaction between K^+ and ammonium is known due to their chemical similarities such as charge and size. Replacement of a nitrogen source as nitrate to ammonium in tobacco (*Nicotiana tabacum*) was reported to cause growth retardation and a decrease in K^+ uptake (Lu et al. 2005). In *Arabidopsis*, ammonium has been shown to inhibit K^+ deficiency-induced expression of a high-affinity K^+

```
                1
   PyAMT1     (1) --------------MIATDMTAMAASPVGRQAVSEALAALTDQVSRNSDS
 AtAMT1;2     (1) MDTATTTCSAVDLSALLSSSSNSTSSLAAATFLCSQISNISNKLSDTTYA
    OtAMT     (1) ----------------------------------MSLTESGAEIQSLYNN
                51
   PyAMT1    (37) MDVFFILVSGYLVFLMQTGFAMLTAGSVRSKNTKNVLLKNVLDACVGAIA
 AtAMT1;2    (51) VDNTYLLFSAYLVFAMQLGFAMLCAGSVRAKNTMNIMLTNVLDAAAGAIS
    OtAMT    (17) LDANFLLSSAYLVFFMQAGFAMLCAGSVRSKNTKNILIKNVLDACVGALA
                101
   PyAMT1    (87) YYLFGFAFAYGTEAN----SFLGHSDFALSGDR----TDFHFFFFQWTFA
 AtAMT1;2   (101) YYLFGFAFAFGTPSNG--FIGRHHSFFALSSYPERPGSDFSFFLYQWAFA
    OtAMT    (67) WFYFGYGFALGEASNGKLNSFIGSGNFAMKGVSGN--TGIAMYLFQWSFS
                151
   PyAMT1   (129) ATAATIVSGSVAERTSFYAYLGYAFFLSGFVYPIVSHWVWGG-GWLSTIF
 AtAMT1;2   (149) IAAAGITSGSIAERTQFVAYLIYSTFLTGFVYPTVSHWFWSSDGWASASR
    OtAMT   (115) AAATTIVSGSVAERTKFEAYLGYSFFLCAFVYPVVVHWGWSGQGWLGPWR
                201
   PyAMT1   (178) TVGAK-------------DFAGDAVVHMVGGFAGLAGATIVGPRLGRFDQ
 AtAMT1;2   (199) SDNNLLFG------SGAIDFAGSGVVHMVGGIAGLCGALVEGPRIGRFDR
    OtAMT   (165) CEGSSNGCGPLLAGSGMLDFAGSGIVHMTGGVAGLVGAIIVGPRTGRFAP
                251
   PyAMT1   (215) DGRVVPMPGHSATLCTLGTFILWFGWYGFNPGSTLGISNTG----PDADY
 AtAMT1;2   (243) SGRSVALRGHSASLVVLGTFLLWFGWYGFNPGSFLTILKGYDKSRPYYGQ
    OtAMT   (215) DGRVNPMPGHSAPLVVLGTFILWLGWYGFNPGSQLAIVAFGG---AAADN
                301
   PyAMT1   (261) TVTAARCAVTTTIAAASAGVTTLIVIKLRDHIFDLLACLNGILAGLVAIT
 AtAMT1;2   (293) WSAVGRTAVTTTLSGCTAALTTLFSKRLLAGHWNVIDVCNGLLGGFAAIT
    OtAMT   (262) SRVIARTAVTTTLSAAGGGIMAMVLNYVLYHVWDLIAVCNGILAGLVGIT
                351
   PyAMT1   (311) ASCAWVEVYAALVIGVIGALVYIGAAMLLLMFKIDDPLEAFPIHGAVGVW
 AtAMT1;2   (343) SGCAVVEPWAAIVCGFVASWVLIGFNLLAKKLKYDDPLEAAQLHGGCGAW
    OtAMT   (312) AGCSTTEPWAAPICGALSALVIHASSKLLLKLKIDDPLEAAPMHGFCGAF
                401
   PyAMT1   (361) GAFAVGLFARIELLTLSGYGNDNGWE--GVFYGGGGRLLAANCVMIASIA
 AtAMT1;2   (393) GLIFTGLFARKEYVNEIYSGDR----PYGLFMGGGGKLLAAQIVQIIVIV
    OtAMT   (362) GVLWVGFMAKQSYVAEVFGTARNGYMPAGVFYGGNGKLLGAQIAGICVIT
                451
   PyAMT1   (409) GWTLVMIVPLFVVLNLVGVLRISPEMELIGNDVSKHGGAAYPDDVITTEE
 AtAMT1;2   (439) GWVTVTMGPLFYGLHKMNLLRISAEDEMAGMDMTRHGGFAYAYNDEDDVS
    OtAMT   (412) AWVGATLGAFFLLMKKLNLLRTSVEEETMGLDESKHGGSAYAMELVAPEP
                501                      526
   PyAMT1   (459) KQAGHTIDNLGVDDSLSRADDPTMV-
 AtAMT1;2   (489) TKPWGHFAGRVEPTSRSSTPTPTLTV
    OtAMT   (462) A-------------------------
```

Fig. 4 Amino acid sequence alignment of AMTs. *Pyropia yezoensis* PyAMT1 (contig_16335_g3953) was aligned with *Arabidopsis thaliana* AtAMT1;2 (At1g64780) and *Ostreococcus tauri* OtAMT (GI:693496005). Identical amino acids among all three AMTs are *highlighted as dark grey* and identical amino acids between two of three AMTs are *highlighted as light grey*. The amino acids which form the ammonium binding site are *marked in red* and other conserved amino acids reported are *marked in blue* (identical among three), *green* (identical among algae) and *orange* (not identical)

transporter gene, *AtHAK5* (Qi et al. 2008; Rubio et al. 2008). By contrast, in ammonium-tolerant rice species, ammonium inhibits high-affinity K^+ transport but promotes low-affinity K^+ uptake (Szczerba et al. 2008). Tomato (*Solanum lycopersicum*) *LeHAK5* expression is induced by ammonium although K^+ concentrations in roots are not altered, and K^+ uptake and accumulation are stimulated by ammonium in sorghum (*Sorghum bicolor*) (Alvarez-Pizarro et al. 2011). As

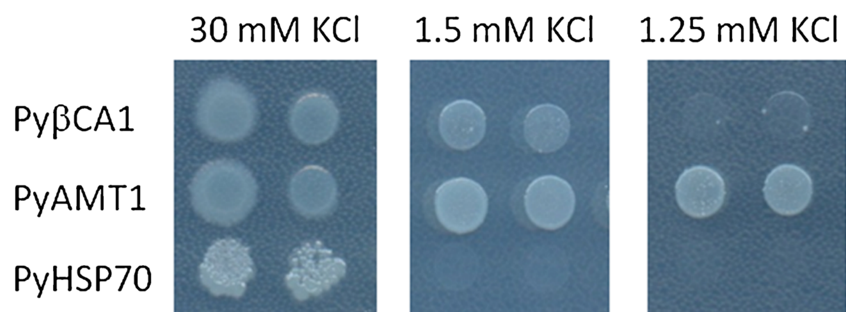

Fig. 5 Functional analysis of PyAMT1 and PyβCA1 in K^+ deficiency. The *E. coli* strain defective in K^+ uptake expressing *PyAMT1* and *PyβCA1* were grown in the K^+ sufficient (30 mM KCl) and K^+ deficient (1.5 and 1.25 mM KCl) conditions. *PyHSP70* was used as a negative control

shown in the examples from the previous reports, whether ammonium prevents or activates K^+ uptake depends on the plant species. There is no information available at present on the effect of ammonium on K^+ uptake in *P. yezoensis*, but it is possible that increased concentrations of ammonium due to PyAMT1, directly or indirectly, help accumulate K^+ under K^+ starvation. It would be interesting to demonstrate the functions of PyAMT1 *in planta* and compare those with the functions of AMTs from land plants in terms of K^+ deficiency tolerance.

Taken together, our findings provided insight into the potential pathways involved in K^+ uptake and response in *P. yezoensis*, PyAMT1 and probably ammonium being strong candidate components, although further investigation is required to clarify the roles of the selected genes in the K^+ deficiency response. The cDNA libraries created will serve as a useful tool to understand the molecular mechanisms underlying K^+/Na^+ homeostasis in seaweeds.

Acknowledgements We would like to thank Ms. Takae Miyazaki and Mr. Hajime Takiguchi for taking care of the seaweed cultures, Dr. Yuko Makita and Dr. Minami Matsui (RIKEN Center for Sustainable Resource Science) for creating the *P. yezoensis* BLAST search engine and Dr. Megumu Takahashi (Tokyo University of Agriculture) for providing a photo of conchosporangium generation. We appreciate the contribution of the Marine Resources Research Center, Aichi Fisheries Research Institute in kindly providing the *P. yezoensis* strain U51 and Dr. Nobuyuki Uozumi (Tohoku University) for providing the *E. coli* TK2463 strain. Many thanks also go to Dr. Michael Adams for comments and discussion on the paper. This work was supported by funding from the RIKEN Incentive Research Projects.

References

Adams E, Shin R (2014) Transport, signaling, and homeostasis of potassium and sodium in plants. J Integr Plant Biol 56:231–249

Ahn SJ, Shin R, Schachtman DP (2004) Expression of *KT/KUP* genes in *Arabidopsis* and the role of root hairs in K^+ uptake. Plant Physiol 134:1135–1145

Alvarez-Pizarro JC, Gomes E, Prisco JT, Grossi-De-Sa MF, Neto OBO (2011) NH_4^+-stimulated low-K^+ uptake is associated with the induction of H^+ extrusion by the plasma membrane H^+-ATPase in sorghum roots under K^+ deficiency. J Plant Physiol 168:1617–1626

Asamizu E, Nakajima M, Kitade Y, Saga N, Nakamura Y, Tabata S (2003) Comparison of RNA expression profiles between the two generations of *Porphyra yezoensis* (Rhodophyta), based on expressed sequence tag frequency analysis. J Phycol 39:923–930

Barrero-Gil J, Garciadeblas B, Benito B (2005) Sodium, potassium-ATPases in algae and oomycetes. J Bioenerg Biomembr 37:269–278

Bhattacharya D, Price DC, Chan CX, Qiu H, Rose N, Ball S, Weber APM, Arias MC, Henrissat B, Coutinho PM, Krishnan A, Zauner S, Morath S, Hilliou F, Egizi A, Perrineau MM, Yoon HS (2013) Genome of the red alga *Porphyridium purpureum*. Nat Commun 4: 1941

Bowes GW (1969) Carbonic anhydrase in marine algae. Plant Physiol 44: 726–732

Bracey MH, Christiansen J, Tovar P, Cramer SP, Bartlett SG (1994) Spinach carbonic anhydrase: investigation of the zinc-binding ligands by site-directed mutagenesis, elemental analysis, and EXAFS. Biochemistry 33:13126–13131

Chan CX, Zauner S, Wheeler G, Grossman AR, Prochnik SE, Blouin NA, Zhuang YY, Benning C, Berg GM, Yarish C, Eriksen RL, Klein AS, Lin SJ, Levine I, Brawley SH, Bhattacharya D (2012) Analysis of *Porphyra* membrane transporters demonstrates gene transfer among photosynthetic eukaryotes and numerous sodium-coupled transport systems. Plant Physiol 158:2001–2012

Chen CS, Dai ZZ, Xu Y, Ji DH, Xie CT (2016) Cloning, expression, and characterization of carbonic anhydrase genes from *Pyropia haitanensis* (Bangiales, Rhodophyta). J Appl Phycol 28:1403–1417

Derelle E, Ferraz C, Rombauts S, Rouze P, Worden AZ, Robbens S, Partensky F, Degroeve S, Echeynie S, Cooke R, Saeys Y, Wuyts J, Jabbari K, Bowler C, Panaud O, Piegu B, Ball SG, Ral JP, Bouget FY, Piganeau G, De Baets B, Picard A, Delseny M, Demaille J, Van De Peer Y, Moreau H (2006) Genome analysis of the smallest free-living eukaryote *Ostreococcus tauri* unveils many unique features. Proc Natl Acad Sci U S A 103:11647–11652

Eppley RW (1958) Sodium exclusion and potassium retention by the red marine alga, *Porphyra perforata*. J Gen Physiol 41:901–911

Epstein W, Buurman E, Mclaggan D, Naprstek J (1993) Multiple mechanisms, roles and controls of K^+ transport in *Escherichia coli*. Biochem Soc T 21:1006–1010

Escassi L, Aguilera J, Figueroa FL, Fernandez JA (2002) Potassium drives daily reversible thallus enlargement in the marine red alga *Porphyra leucosticta* (Rhodophyta). Planta 214:759–766

He LW, Huang AY, Shen SD, Niu JF, Wang GC (2012) Comparative analysis of microRNAs between sporophyte and gametophyte of *Porphyra yezoensis*. Comp Funct Genom 2012:912843

Inoue A, Mashino C, Uji T, Saga N, Mikami K, Ojima T (2015) Characterization of an eukaryotic PL-7 alginate lyase in the marine red alga *Pyropia yezoensis*. Current Biotechnol 4:240–248

Kakinuma M, Suzuki K, Iwata S, Coury DA, Iwade S, Mikami K (2016) Isolation and characterization of a new *DUR3*-like gene, *PyDUR3.3*, from the marine macroalga *Pyropia yezoensis*. Fish Sci 82:171–184

Kakinuma M, Nakamoto C, Kishi K, Coury DA, Amano H (2017) Isolation and functional characterization of an ammonium transporter gene, *PyAMT1*, related to nitrogen assimilation in the marine macroalga *Pyropia yezoensis* (Rhodophyta). Mar Environ Res 128:76–87

Karsten U (2012) Seaweed acclimation to salinity and desiccation stress. In: Wiencke C, Bischof K (eds) Seaweed biology. Springer, Heidelberg, pp 87–107

Kimber MS, Pai EF (2000) The active site architecture of *Pisum sativum* β-carbonic anhydrase is a mirror image of that of α-carbonic anhydrases. EMBO J 19:1407–1418

Kirst GO (1990) Salinity tolerance of eukaryotic marine algae. Annu Rev Plant Physiol 41:21–53

Kishimoto M, Shimajiri Y, Oshima A, Hase A, Mikami K, Akama K (2013) Functional expression of an animal type-Na^+-ATPase gene from a marine red seaweed *Porphyra yezoensis* increases salinity tolerance in rice plants. Plant Biotechnol 30:417–422

Kitade Y, Fukuda S, Nakajima M, Watanabe T, Saga N (2002) Isolation of a cDNA encoding a homologue of actin from *Porphyra yezoensis* (Rhodophyta). J Appl Phycol 14:135–141

Li L, Saga N, Mikami K (2008) Phosphatidylinositol 3-kinase activity and asymmetrical accumulation of F-actin are necessary for establishment of cell polarity in the early development of monospores from the marine red alga *Porphyra yezoensis*. J Exp Bot 59:3575–3586

Lu YX, Li CJ, Zhang FS (2005) Transpiration, potassium uptake and flow in tobacco as affected by nitrogen forms and nutrient levels. Ann Bot 95:991–998

Matsuda R, Ozgur R, Higashi Y, Takechi K, Takano H, Takio S (2015) Preferential expression of a bromoperoxidase in sporophytes of a red alga, *Pyropia yezoensis*. Mar Biotechnol 17:199–210

Merlin C, Masters M, Mcateer S, Coulson A (2003) Why is carbonic anhydrase essential to *Escherichia coli*? J Bacteriol 185:6415–6424

Moroney JV, Bartlett SG, Samuelsson G (2001) Carbonic anhydrases in plants and algae. Plant Cell Environ 24:141–153

Nakamura Y, Sasaki N, Kobayashi M, Ojima N, Yasuike M, Shigenobu Y, Satomi M, Fukuma Y, Shiwaku K, Tsujimoto A, Kobayashi T, Nakayama I, Ito F, Nakajima K, Sano M, Wada T, Kuhara S, Inouye K, Gojobori T, Ikeo K (2013) The first symbiont-free genome sequence of marine red alga, Susabi-nori (*Pyropia yezoensis*). PLoS One 8:e57122

Ortiz-Ramirez C, Mora SI, Trejo J, Pantoja O (2011) *Pv*AMT1;1, a highly selective ammonium transporter that functions as H^+/NH_4^+ Symporter. J Biol Chem 286:31113–31122

Pantoja O (2012) High affinity ammonium transporters: molecular mechanism of action. Front Plant Sci 3:34

Pedersen CNS, Axelsen KB, Harper JF, Palmgren MG (2012) Evolution of plant P-type ATPases. Front Plant Sci 3:31

Provart NJ, Majeau N, Coleman JR (1993) Characterization of pea chloroplastic carbonic anhydrase. Expression in *Escherichia coli* and site-directed mutagenesis. Plant Mol Biol 22:937–943

Qi Z, Hampton CR, Shin R, Barkla BJ, White PJ, Schachtman DP (2008) The high affinity K^+ transporter AtHAK5 plays a physiological role *in planta* at very low K^+ concentrations and provides a caesium uptake pathway in *Arabidopsis*. J Exp Bot 59:595–607

Rubio F, Nieves-Cordones M, Aleman F, Martinez V (2008) Relative contribution of AtHAK5 and AtAKT1 to K^+ uptake in the high-affinity range of concentrations. Physiol Plantarum 134:598–608

Shen S, Zhang G, Li Y, Wang L, Xu P, Yi L (2011) Comparison of RNA expression profiles on generations of *Porphyra yezoensis* (Rhodophyta), based on suppression subtractive hybridization (SSH). BMC Res Notes 4:428

Sutherland JE, Lindstrom SC, Nelson WA, Brodie J, Lynch MDJ, Hwang MS, Choi HG, Miyata M, Kikuchi N, Oliveira MC, Farr T, Neefus C, Mols-Mortensen A, Milstein D, Muller KM (2011) A new look at an ancient order: generic revision of the Bangiales (Rhodophyta). J Phycol 47:1131–1151

Szczerba MW, Britto DT, Ali SA, Balkos KD, Kronzucker HJ (2008) NH_4^+-stimulated and -inhibited components of K^+ transport in rice (*Oryza sativa* L.) J Exp Bot 59:3415–3423

Uji T, Hirata R, Mikami K, Mizuta H, Saga N (2012a) Molecular characterization and expression analysis of sodium pump genes in the marine red alga *Porphyra yezoensis*. Mol Biol Rep 39:7973–7980

Uji T, Monma R, Mizuta H, Saga N (2012b) Molecular characterization and expression analysis of two Na^+/H^+ antiporter genes in the marine red alga *Porphyra yezoensis*. Fisheries Sci 78:985–991

Wiencke C, Stelzer R, Lauchli A (1983) Ion compartmentation in *Porphyra umbilicalis* determined by electron-probe X-ray microanalysis. Planta 159:336–341

Yuan LX, Loque D, Kojima S, Rauch S, Ishiyama K, Inoue E, Takahashi H, Von Wiren N (2007) The organization of high-affinity ammonium uptake in *Arabidopsis* roots depends on the spatial arrangement and biochemical properties of AMT1-type transporters. Plant Cell 19:2636–2652

Zhang BY, Yang F, Wang GC, Peng G (2010) Cloning and quantitative analysis of the carbonic anhydrase gene from *Porphyra yezoensis*. J Phycol 46:290–296