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

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11

12

13 **Abstract**

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

28

29 **Keywords**

30 ammonium transporter, full-length cDNA library, potassium deficiency, *Pyropia*

31 *yezoensis*, salt tolerance

32 **Introduction**

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

40 It has been long known that the marine red algae Bangiales (Rhodophyta) which
41 include *Pyropia* and *Porphyra* (Sutherland et al., 2011) accumulate K^+ in the cytoplasm
42 and either exclude or contain Na^+ preferentially in the vacuoles (Eppley, 1958; Wiencke
43 et al., 1983). In order to maintain the appropriate cytosolic K^+/Na^+ ratios, active
44 K^+ uptake mechanisms are considered essential. Unlike land plants and green algae
45 (Chan et al., 2012; Pedersen et al., 2012), red algae such as *Pyropia yezoensis* and
46 *Porphyridium purpureum* have been reported to possess animal-type Na^+/K^+ -ATPases
47 which extrude three ions of Na^+ while taking up two ions of K^+ into the cell and they are
48 predicted to provide the driving force for Na^+ -driven solute transporters (Barrero-Gil et
49 al., 2005; Bhattacharya et al., 2013). There seems a tendency that freshwater algae and

50 land plants utilise H^+ gradient generated by H^+ -ATPases to energise secondary
51 transporters whereas marine algae make use of Na^+ gradient albeit with some exceptions
52 (Chan et al., 2012) and this notion is evolutionarily quite interesting.

53 Recently, the 43 Mb genome of *P. yezoensis* was sequenced, with more than 10,000
54 gene models predicted (Nakamura et al., 2013). In this alga, a gene encoding K^+ P-type
55 ATPase, *PyKPA1*, was found to be phylogenetically related to the animal H^+/K^+ - and
56 Na^+/K^+ -ATPases. Heterologous expression of *PyKPA1* in the *Escherichia coli* strain
57 deficient in K^+ uptake demonstrated that *PyKPA1* had a growth promoting effect in the
58 K^+ -limited condition and that addition of Na^+ further enhanced the effect of *PyKPA1*
59 (Barrero-Gil et al., 2005), suggesting *PyKPA1* to be a Na^+/K^+ -ATPase. Although gene
60 expression of *PyKPA1* was not altered in response to salt stress in *P. yezoensis* (Uji et al.,
61 2012a), ectopic overexpression of *PyKPA1* in rice plants increased salt tolerance by
62 restoring growth (Kishimoto et al., 2013). Another P-type ATPase, *PyKPA2*, which
63 shares a 65% sequence identity with *PyKPA1* and Na^+/H^+ antiporters, *PySOS1* and
64 *PyNhaD*, have also been isolated from the genome of *P. yezoensis* (Barrero-Gil et al.,
65 2005; Uji et al., 2012a; Uji et al., 2012b). These membrane proteins could potentially be
66 the major machineries in ion homeostasis and Na^+ extrusion in *P. yezoensis*, however,
67 ATPases may be too energetically costly to be the major K^+ uptake mechanism and

68 additional K^+ transporters/channels are expected to exist. Although K^+ uptake in land
69 plants is commonly mediated by K^+ channels and transporters such as AKT1, HAK5
70 and KUPs in a model land plant *Arabidopsis thaliana* (Adams and Shin, 2014), proteins
71 with similar sequences and/or function have not been reported in *P. yezoensis*. In order
72 to resolve the molecular mechanisms underlying the ability of red seaweeds to survive
73 in the marine environment, investigation of regulatory components involved in
74 K^+/Na^+ homeostasis in *P. yezoensis* needs to be performed.

75 *P. yezoensis* spends the winter in the form of gametophytes, the leafy structure
76 commonly harvested as seaweed, and it turns into sporophytes, the filamentous structure
77 during the summer. In autumn, sporophytes form conchosporangia from which
78 conchospores are emitted to produce a new generation of gametophytes. There are
79 several reports describing that different sets of genes are expressed in the extremely
80 diverse structures observed throughout the life cycle of *P. yezoensis*: for instance, only
81 22.5% of ESTs and one out of 14 microRNAs are common among gametophytes and
82 sporophytes (Asamizu et al., 2003; Shen et al., 2011; He et al., 2012). Indeed,
83 phase-specific gene expression include genes encoding urea transporters (*PyDUR3s*), an
84 alginate lyase (*PyAly*) and a bromoperoxidase (*PyBPO1*) (Inoue et al., 2015; Matsuda et
85 al., 2015; Kakinuma et al., 2016b). Interestingly, *PyKPA1* has been reported to be

86 predominantly expressed in sporophytes while *PyKPA2* is expressed specifically in
87 gametophytes (Uji et al., 2012a) These findings strongly suggest the existence of
88 distinct regulatory mechanisms upon K^+/Na^+ homeostasis in each life stage.

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

95

96 **Materials and methods**

97 **Plant material and growth conditions**

98 The cultivation of the *P. yezoensis* strain U51 was performed as previously reported (Li
99 et al., 2008) with a slight modification. Briefly, free-living sporophytes, free-living
100 conchosporangia and gametophytes attached to polyvinyl alcohol (PVA) monofilaments
101 were suspended in ESL (enriched SEALIFE) media, continuously aerated with
102 filter-sterilised air and grown at 15°C in a 10 h light/14 h dark photocycle with a light
103 intensity of $60 \mu\text{mol m}^{-2} \text{s}^{-1}$. The sterile ESL medium was made by

104 dissolving commercially available SEALIFE powder (Marintech Co. Ltd., Tokyo,
105 Japan) in distilled water with added ESS₂ solution (Kitade et al., 2002) and this was
106 exchanged weekly.

107

108 **RNA extraction and cDNA library construction**

109 An excess amount of sporophyte, gametophyte and conchosporangium samples were
110 flash frozen in liquid N₂ and ground into fine powder using a mortar and a pestle. Total
111 RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, MA) and 75 µg of
112 total RNA was used to isolate mRNA using Ambion Dynabeads mRNA Purification Kit
113 (Thermo Fisher Scientific). Purified mRNA was concentrated by glycogen precipitation
114 with 1 µL of glycogen, 0.5 volumes of NH₄OAc and 2.5 volumes of 100% ethanol.
115 0.88~3.31 µg of mRNA was used to create full-length cDNA libraries using CloneMiner
116 II cDNA Library Construction Kit according to the manufacture's instruction (Thermo
117 Fisher Scientific). In short, hybridisation of Biotin-*attB2*-Oligo(dT) Primer to the
118 mRNA poly(A) tail and the first strand cDNA synthesis by SuperScript III Reverse
119 Transcriptase were followed by the second strand cDNA synthesis by *E. coli*
120 Polymerase I and ligation of *attB1* Adapter to the 5' end of the cDNA. The resultant
121 double-stranded cDNA was size fractionated by a column to remove truncated cDNA

122 shorter than 500 bp and cloned into a Gateway entry vector pDONR222 through BP
123 recombination reaction. The cDNA construct was then transformed into ElectroMAX
124 DH10B T1 Phage Resistant Cells to create the final cDNA library. Titer was determined
125 by spreading 1:10 serial dilutions (10^{-2} , 10^{-3} , 10^{-4}) of each library onto LB plates
126 containing kanamycin. Titer was calculated as colony forming unit (cfu mL⁻¹) =
127 colonies on plates × dilution factor / volume plated (mL) and total CFU (cfu) =
128 average titer (cfu mL⁻¹) × total volume of cDNA library (mL). Single colonies were
129 picked and plasmid DNAs (pDNAs) were prepared. Each pDNA was digested by *Bsr*G
130 I to determine the insert size and sequenced using M13 forward and reverse universal
131 primers and the Sanger sequencing technique (HITACHI gene analysis system with ABI
132 PRISM 3100-21 genetic analyser).

133

134 **Selection of K⁺ deficiency tolerance-related genes**

135 The pDONR222 entry libraries from gametophyte and conchosporangium samples were
136 transferred into the pBAD-DEST49 Gateway destination vector according to the
137 manufacture's instruction (Thermo Fisher Scientific). Plasmid DNA was prepared from
138 the entry library culture grown till an OD₆₀₀ to be approximately 1.0. Polyethylene
139 glycol (PEG) precipitation was performed to purify pDNA using 0.4 volumes of 30%

140 PEG/Mg solution. The entry library was transferred into the destination vector using
141 Gateway LR Clonase II enzyme and transformed into ElectroMAX DH10B T1 Phage
142 Resistant Cells. Plasmid DNA of the pBAD-DEST49 library prepared from the *E. coli*
143 culture with an OD₆₀₀ of approximately 1.0 was transformed into an *E. coli* strain
144 defective in K⁺ uptake, TK2463 (Epstein et al., 1993), and selected on minimal media
145 (Ahn et al., 2004) containing 1-3 mM KCl, 0.1% arabinose and ampicillin. For
146 functional analysis, overnight culture of TK2463 expressing pBAD-PyAMT1, PyβCA1
147 or PyHSP70 grown in KML media (10 g Bacto Tryptone, 10 g KCl, 5 g Bacto Yeast
148 Extract in 1 L MilliQ water) containing ampicillin were pelleted, washed three times
149 with autoclaved MilliQ water, resuspended in autoclaved MilliQ water and dropped
150 onto minimal media containing 30, 1.5 or 1.25 mM KCl, 0.1% arabinose and ampicillin
151 as five-fold serial dilutions.

152

153 **Sequence analysis**

154 Contig numbers were retrieved from the obtained sequences using the public *Pyropia*
155 database (Nakamura et al., 2013). *Pyropia* genes were annotated using blastx function
156 against the non-redundant protein sequences database at the NCBI search engine
157 (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Amino acid sequences were aligned using

158 Vector NTI (Thermo Fisher Scientific).

159

160 **Results**

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

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

176 than half of the genes were annotated as unknown in the conchosporangia and
177 sporophytes libraries. The annotated genes were for general functions such as protein
178 synthesis and degradation.

179

180 **Selection of genes potentially involved in K⁺ deficiency tolerance**

181 In order to isolate genes responsible for efficient K⁺ utilisation and K⁺/Na⁺ balance in *P.*
182 *yezoensis*, the entire entry clone libraries from gametophytes and conchosporangia were
183 transferred into the *E. coli* expression vector system and transformed into TK2463, an *E.*
184 *coli* strain defective in K⁺ uptake. Under the less stringent K⁺ deficiency conditions (2 or
185 3 mM KCl), 48 colonies were recovered and 45 genes were successfully sequenced
186 from the gametophytes library (Table 3). Under the stringent condition (1 mM KCl), 23
187 genes from the gametophytes library and 16 genes from the conchosporangia library
188 were revealed (Table 4). Although a large portion of genes could not be annotated for
189 function as in the entry libraries (34.8% for gametophytes and 62.5% for
190 conchosporangia), a higher number of the annotated genes was associated with specific
191 functions in biological processes such as metabolism and signalling rather than general
192 functions. Of these, three genes were annotated as β -carbonic anhydrase (β CA, two
193 from 2 or 3 mM KCl, one from 1 mM KCl screening). All three were predicted to

194 represent the same gene model (*PyβCA1*, contig_16545_g4020). Upon sequence
195 alignment with known βCAs from *Chlamydomonas reinhardtii* (CrCAH4),
196 *Ostreococcus tauri* (OtβCA) and *Arabidopsis* (AtβCA5.1), *PyβCA1* was shown to
197 possess all three conserved zinc binding sites, two cysteine residues (C) and histidine
198 (H) (marked in blue in Fig. 3) (Provart et al., 1993; Bracey et al., 1994; Kimber and Pai,
199 2000), however, the rest of the sequence was fairly diverse among the species (20.1%,
200 22.0%, 23.1% identity with AtβCA5.1, CrCAH4, OtβCA, respectively). Two genes
201 recovered from stringent K⁺ deficiency screening were predicted to be a single
202 ammonium transporter (*PyAMT1*, contig_16335_g3953) (Kakinuma et al., 2016a).
203 Sequence alignment of *PyAMT1* with well-studied AtAMT1;2 (Yuan et al., 2007) and
204 algal OtAMT (Derelle et al., 2006) indicated that many conserved amino acids such as
205 those which form the ammonium binding site, tryptophan (W) and serine (S) (marked in
206 red in Fig. 4), phenylalanine (F) and aspartate (D) (marked in blue) were identical
207 among three whereas others, such as F and threonine (T) (marked in green) highlighted
208 the difference between algae and land plants (Pantoja, 2012). The TK2463 *E. coli*
209 strains expressing *PyβCA1* and *PyAMT1* were further analysed in the K⁺ deficient
210 conditions (1.25 and 1.5 mM KCl) and compared with the strain expressing *PyHSP70*
211 as a negative control. The strains expressing *PyAMT1* and, to a lesser extent, *PyβCA1*

212 grew well in K⁺ deficiency while the strain expressing *PyHSP70* could not survive (Fig.
213 5). Multiple ribosomal proteins of various sizes were also selected from the
214 gametophyte library (Table 3 and 4).

215

216 **Discussion**

217 Full-length cDNA libraries from various life stages, including gametophytes,
218 conchosporangia and sporophytes, were created for a model marine alga *P. yezoensis*
219 with excellent recombination percentages and titer. The average insert size of
220 approximately 1 kb corresponds with the predicted average coding sequence length in *P.*
221 *yezoensis* (Nakamura et al., 2013). Analysis of the whole genome sequence of *P.*
222 *yezoensis* has revealed that the function of 35% of the genes is unknown (Nakamura et
223 al., 2013) and our results obtained from the gametophytes library was consistent with
224 their report. It is intriguing to postulate why the conchosporangia and sporophytes
225 libraries contain more than 50% of genes that are functionally unknown. Many of these
226 genes do not even have any previously characterised conserved amino acid motif or
227 domain, underlining the uniqueness of *P. yezoensis* genes, especially in the
228 conchosporangia and sporophytes stages.

229 In the search of contributory factors in K⁺ deficiency tolerance in *P. yezoensis*, we

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

240 There were two types of proteins selected multiple times: β -carbonic anhydrase
241 (Py β CA1) and ammonium transporter (PyAMT1). CA catalyses the reversible reaction
242 between CO_2 and $HCO_3^- + H^+$ and is crucial for aquatic photosynthetic organisms
243 which suffer in the low- CO_2 environment to concentrate CO_2 in the vicinity of ribulose
244 1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Moroney et al., 2001). The
245 existence of CA activity in marine macroalgae has been known for a long while (Bowes,
246 1969). A β CA has previously been cloned in *P. yezoensis* and its expression was
247 reported to be the lowest in gametophytes, followed by sporophytes and conchospores

248 (Zhang et al., 2010). Although Py β CA1 selected in this screen is different from the one
249 identified in the previous study, its expression is predicted to be low in gametophytes
250 since the same expression pattern was also observed for *P. haitanensis* β CAs (Chen et
251 al., 2016). Considering its lower abundance in the original gametophytes library and the
252 fact that three independent transformants were recovered in the low K⁺ assays, it seems
253 to point to the significance of Py β CA1 in K⁺ deficiency response. Furthermore, this
254 particular β CA might be important in this response as only one gene was repeatedly
255 isolated though multiple β CAs were expected to exist in the genome. As all three
256 conserved amino acids which contribute to binding of the catalytic zinc ion are present
257 in Py β CA1 (Fig. 3), it is predicted as a functional β CA. By contrast, overall sequence
258 identity is not high among the species tested (approximately 20%) and it is possible that
259 regulation and function of each β CA are distinct. This notion is also supported by the
260 fact that CA is essential for *E. coli* growth under aerobic conditions probably due to
261 HCO₃⁻ requirement for amino acid, nucleotide and fatty acid synthesis (Merlin et al.,
262 2003), indicating that expression of *Py β CA1*, but not the innate CA activity of *E. coli*,
263 could contribute to K⁺ deficiency tolerance (Fig. 5). Although a direct interaction
264 between β CA and K⁺ has yet to be reported in seaweeds (Escassi et al., 2002), we
265 speculate that increased carbon source and photosynthesis by Py β CA1 might

266 compensate the loss of K⁺.

267 Two independent transformants from the stringent K⁺ deficiency screen were found
268 to carry a single *AMT* gene (*PyAMT1*). During the review process of the current paper,
269 another group reported isolation of PyAMT1 as a functional ammonium transporter
270 whose gene expression is dramatically induced in response to nitrogen deficiency
271 (Kakinuma et al., 2016a). It is known that ammonium is preferentially taken up over
272 nitrate by many algae and multiple *AMT* genes are present and expressed in the
273 *Porphyra* species (Chan et al., 2012). Amino acid sequence alignment of PyAMT1 with
274 *Arabidopsis* AtAMT1;2 and green alga *O. tauri* OtAMT indicated that many of the
275 functional residues were conserved but some of the amino acids were the same among
276 algae but this was not the case with *Arabidopsis*. Interestingly, substitution of the H125
277 residue identified from bean (*Phaseolus vulgaris*), which is generally replaced by
278 proline (P) in other plant homologues (marked in orange in Fig. 4), for arginine (R)
279 renders the transporter more active (Ortiz-Ramirez et al., 2011). Since the H125 position
280 is R in PyAMT1, this might suggest it to be an active form. Expression of *PyAMT1*
281 dramatically improved the ability of TK2463, an *E. coli* strain defective in K⁺ uptake, to
282 survive in K⁺ deficiency compared to the negative control line expressing *PyHSP70* (Fig.
283 5). Although the negative control line showed somewhat compromised growth in the

284 sufficient K^+ condition, the degree of viability under K^+ deficiency between the lines
285 expressing *PyHSP70* and *PyAMT1* was fairly clear. The effect of *PyAMT1* was stronger
286 than that of *PyβCA1* (Fig. 5) and this point was consistent with the fact that *PyAMT1*
287 was recovered solely from the stringent screen while *PyβCA1* from the mild screen as
288 well. Interaction between K^+ and ammonium is known due to their chemical similarities
289 such as charge and size. Replacement of a nitrogen source as nitrate to ammonium in
290 tobacco (*Nicotiana tabacum*) was reported to cause growth retardation and a decrease in
291 K^+ uptake (Lu et al., 2005). In *Arabidopsis*, ammonium has been shown to inhibit
292 K^+ deficiency-induced expression of a high-affinity K^+ transporter gene, *AtHAK5* (Qi et
293 al., 2008; Rubio et al., 2008). By contrast, in ammonium-tolerant rice species,
294 ammonium inhibits high-affinity K^+ transport but promotes low-affinity K^+ uptake
295 (Szczerba et al., 2008). Tomato (*Solanum lycopersicum*) *LeHAK5* expression is induced
296 by ammonium although K^+ concentrations in roots are not altered, and K^+ uptake and
297 accumulation are stimulated by ammonium in sorghum (*Sorghum bicolor*)
298 (Alvarez-Pizarro et al., 2011). As shown in the examples from the previous reports,
299 whether ammonium prevents or activates K^+ uptake depends on the plant species. There
300 is no information available at present on the effect of ammonium on K^+ uptake in *P.*
301 *yezoensis*, but it is possible that increased concentrations of ammonium due to *PyAMT1*,

302 directly or indirectly, help accumulate K^+ under K^+ starvation. It would be interesting to
303 demonstrate the functions of PyAMT1 *in planta* and compare those with the functions
304 of AMTs from land plants in terms of K^+ deficiency tolerance.

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

311

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323

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454

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456

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

459

cDNA library	Titer (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

460

461

462 **Table 2** Annotation of genes from cDNA libraries for three life stages of the marine red
 463 alga *P. yezoensis*
 464

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	disulfide isomerase (thioredoxin superfamily)	protein regulation
1	transducin family protein/WD-40 repeat family protein	protein regulation
1	F-box family 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	

465

466

467 **Table 3** List of genes selected from the K⁺ tolerance screening of the gametophytes
 468 library expressed in the *E. coli* strain defective in K⁺ uptake under mild K⁺ deficiency (2
 469 or 3 mM KCl)
 470

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	

471

472

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

476

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 <i>c</i> 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 <i>c</i> 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	

477

478

479 **Figure legends**

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

485

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

490

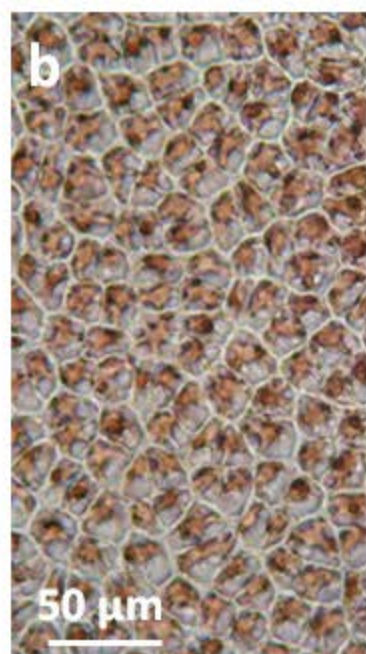
491 **Fig. 3** Amino acid sequence alignment of β CAs. *Pyropia yezoensis* Py β CA1
492 (contig_16545_g4020) was aligned with *Arabidopsis thaliana* At β CA5.1 (At4g33580),
493 *Chlamydomonas reinhardtii* CrCAH4 (GI: 159475801) and *Ostreococcus tauri* Ot β CA
494 (GI: 308799709). Identical amino acids among all four β CAs are highlighted as dark
495 gray and identical amino acids between two or three among four are highlighted as light
496 gray. The amino acids which form the conserved zinc binding site are marked in blue.

497

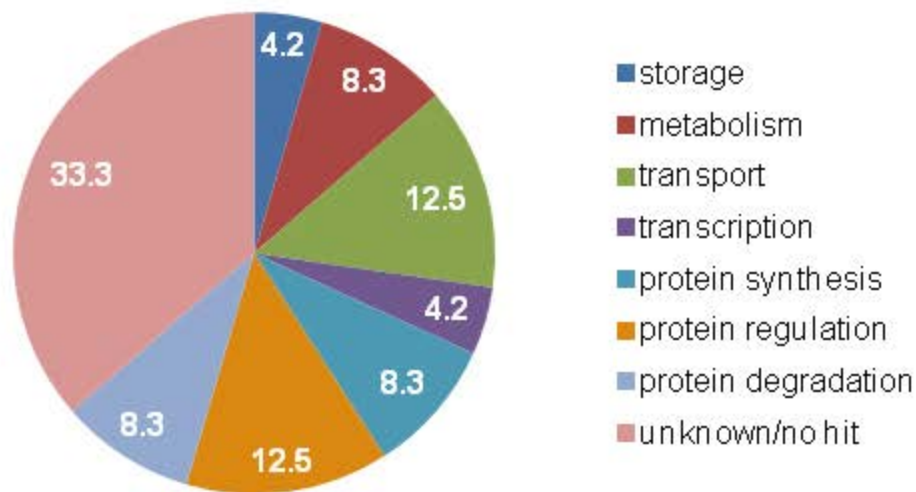
498 **Fig. 4** Amino acid sequence alignment of AMTs. *Pyropia yezoensis* PyAMT1
499 (contig_16335_g3953) was aligned with *Arabidopsis thaliana* AtAMT1;2 (At1g64780)
500 and *Ostreococcus tauri* OtAMT (GI:693496005). Identical amino acids among all three
501 AMTs are highlighted as dark gray and identical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

505

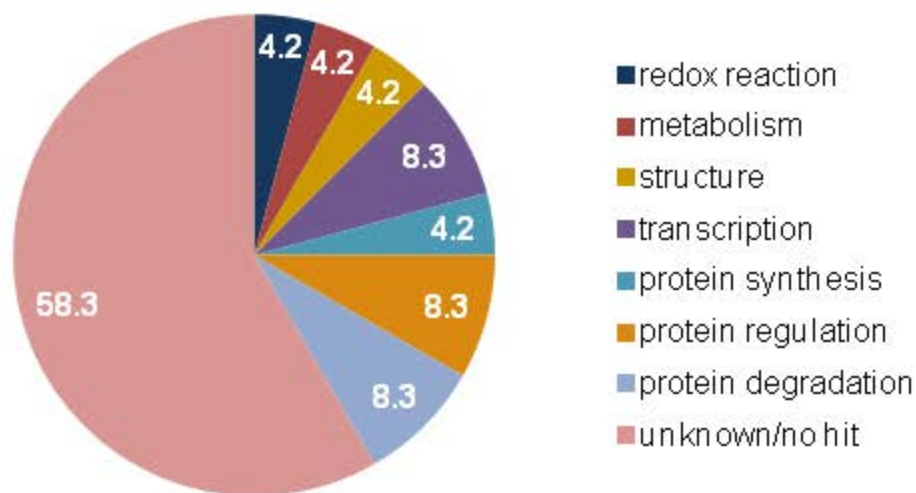
506 **Fig. 5** Functional analysis of PyAMT1 and Py β CA1 in K⁺ deficiency. The *E. coli* strain
507 defective in K⁺ uptake expressing *PyAMT1* and *Py β CA1* were grown in the K⁺ sufficient
508 (30 mM KCl) and K⁺ deficient (1.5 and 1.25 mM KCl) conditions. *PyHSP70* was used
509 as a negative control.



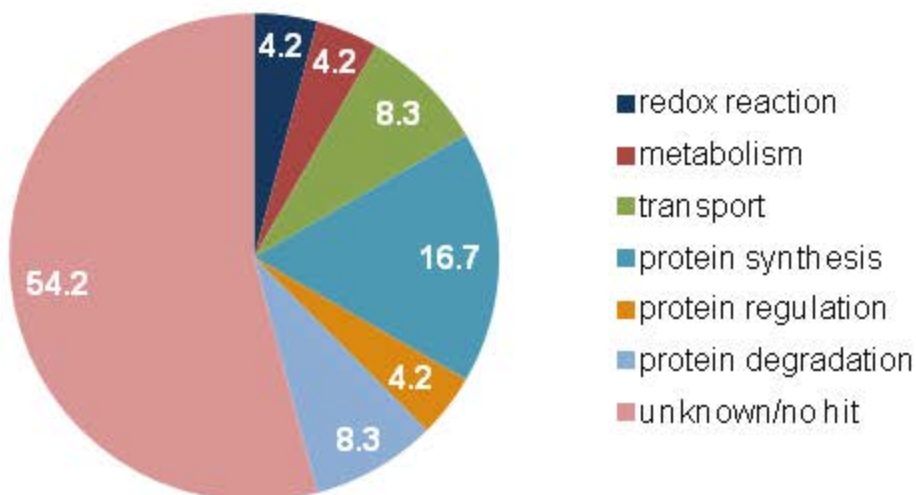
Gametophytes



Conchosporangia



Sporophytes



PyβCA1 (1) -----MAAVASPTSVPATN
CrCAH4 (1) -----MSSRNVATALRMFATLGRSQAGEASAMMGTGSALLAQRAAL
OtβCA (1) -----
AtβCA5 (1) MAATPTHFSVSHDPFSSTSLNLTQAI FGNHSLKTTQLRIPASFRRKA

PyβCA1 (15) DEPLALLTGECPAGDKVWASLLASNAQFATAGERPP-AEGVSVTHRGLA
CrCAH4 (43) GGPQAVNKGCSRCRCGRVACMGACMPMRHLHAHPNPPSDPDQALEYLREGN
OtβCA (1) -----MSSPERAFERLLDGHRAFRRRAHFAASDCAA-DVPRALRALSER-
AtβCA5 (51) TNLQVMASGKTFGLTQAEANGVAIDRQNNTDVFDMMK-QRFLAFKCLKYMD

PyβCA1 (64) -----G-----GQSPSAVVVTCADSRLSPELLFARG
CrCAH4 (93) KRFVNNKPHDSHPTRNLDRVKATAAGQKPF AAFSLSCADSRVPVEIIFDQG
OtβCA (43) -----GQRPRALVVACS DSRADPAIVFDTA
AtβCA5 (100) -----DFEHYKNLADAQAPKFLVIACADSRVCP S AVLGFQ

PyβCA1 (90) LGELFVIRTAGNTTGDDS-----TVAS-VEYAVKNLSASLVVVLGHTK
CrCAH4 (143) FGDVFTVRVAGNIVTNEIT-----ASL--EFGTAVLGSKVLMVLGHS
OtβCA (68) PGDVFTIRNVGSLVPAYAGLDGGHHGTCAATEYATVHLEVPVILVMGHTQ
AtβCA5 (135) PGDAFTVRNIANLVPPYE---SGPTETKAAL EFSVNTLNVENILVIGHSR

PyβCA1 (132) CGAVGAAVATEADPDAMAE-----QPRTLAAFFVKEKLLAPVQAVKL
CrCAH4 (184) CGAVAATMNGAAVP-----GVISSLYYS-----IS-P
OtβCA (118) CGGAAAGLRKYNGP DADASVFGVNEATGEGF IGAWVAL---AEDAVRRV
AtβCA5 (182) CGGIQALMKMEDEG-----DSRSFIHNWVVVGKKAKESTKAV

PyβCA1 (173) RGDADESGFVAACEVENVHHAVRTLLTTSGLWLA KTRVGGGVKVVGAMYHL
CrCAH4 (210) ACKKAQAGVDVGAIAENVKVQMEQLKVPV LQGLVKEGK-LKIVGGVYDL
OtβCA (165) CERHDPGVRARMLEYELVRQSVQNL LTFPFVKKRVD RGE-LVVKGA VFNV
AtβCA5 (219) ASNLFHFDHQCHCEKASINHSLERLLGYPWIEEKVRQGS-LSLHGGYYNF

PyβCA1 (223) ETGVVEEC-----
CrCAH4 (259) ATGKVTEIA-----
OtβCA (214) WDGTLVLRADGSFEQLDDDAEDGRGEAKRAKN-
AtβCA5 (268) VDCTFEKWTVDY AASRGKKKEGSGI AVKDRSVWS

1

PyAMT1 (1) -----MIATDMTAMAAS PVGRQAVSEALAAALTDQVSRNSDS
AtAMT1;2 (1) **MDTATTTCSAVDLSALLSSSSNSTSS**LAAATFLCSQISNINSLSDTTYA
OtAMT (1) -----MSLTESGAEIQSLYNN

51

PyAMT1 (37) **MDVFFILVSGYLVFLMQTGFAMLTAGSVRSKNTKNVLLKNVLDACVGAIA**
AtAMT1;2 (51) **VDNTYLLFSAYLVFAMQLGFAMLCAGSVRAKNTMNIMLTNVLDAAAGAIS**
OtAMT (17) **LDANFLLSSAYLVFFMQAGFAMLCAGSVRSKNTKNILIKNVLDACVGALA**

101

PyAMT1 (87) **YYLFGFAFAYGTEAN**----SFLGHSDFALSGDR----TDFHFFFQWTF**A**
AtAMT1;2 (101) **YYLFGFAFAFGTPSNG**--FIGRHHSF**FALSSYPERPGSDFSF**FLYQ**WAF**A
OtAMT (67) **WFYFGYGFALGEASNGKLN**SFIGSGN**FAMKGVSGN**--TGIAMYL**FQWSE**S

151

PyAMT1 (129) **ATAATIVSGSVAERTS**FYAYLG**YAFFLSGFVYPIVSHWVWGG**-GWLSTIF
AtAMT1;2 (149) **IAAAGITSGSIAERTQ**FVAYLIY**STFLTGFVYPTVSHWFWS**SDGWASASR
OtAMT (115) **AAATTIVSGSVAERTK**FEAYLG**YSFFLCAFVYPVVHWGWSGQ**GWLG**PWR**

201

PyAMT1 (178) TVGAK-----**DFAGDAVVHMGVGGFAGLAGATIVGPRLGRFDQ**
AtAMT1;2 (199) SDNNLLFG-----**SGAIDFAGSGVVHMGVGGIAGLCGALVEGPRI**GRFDR
OtAMT (165) CEGSSNGCGPLLAGSGML**DFAGSGIVHMTGGVAGLVGAIIVGPRTGR**EAP

251

PyAMT1 (215) **DGRVVPMPGHSATLCTLGTFILWFGWYGFNPGSTLG**ISNTG----PDADY
AtAMT1;2 (243) **SGRSVALRGHSASLVVLGTFLWFGWYGFNPGSFLTIL**KGYDKSRP**YYGQ**
OtAMT (215) **DGRVNPMPGHSAPLVVLGTFILWLWFGWYGFNPGS**QLAIVAFGG---AAADN

301

PyAMT1 (261) **TVTAARCAVTTTIAAASAGVTTLIVIKLRDHIFDLLACLNGILAGLVAIT**
AtAMT1;2 (293) **WSAVGRTAVTTTTL**SGCTAAL**TTLFSKRLLAGHWNVIDVCNGLLGGFAAIT**
OtAMT (262) **SRVIARTAVTTTTL**SAAGGGIMAMVL**NYVLYHVWDLI**AVCN**GI**LAGLVGIT

351

PyAMT1 (311) **ASC**AWVEV**Y**AALVIGVIGALV**Y**IGAAMLL**LMFKI**DDPLEAF**PIHGAVGVW**
AtAMT1;2 (343) **SGCAVVEPWAAIVCGFVASWVLIGFNLLAKKLKY**DDPLEAA**QLHGGCGAW**
OtAMT (312) **AGCSTTEPWAA**PICGALSALV**I**HASSK**LLKLKI**DDPLEAA**PMHGF**CGAF

401

PyAMT1 (361) **GAF**AVGLFAR**I**ELLT**LSGYGNDNGWE**--GVFYGGGG**RLLAANCVM**IASIA
AtAMT1;2 (393) **GLIFTGLFARKEYVNEI**YSGDR----PYGL**FMGGGGKLLAAQIVQI**IVIV
OtAMT (362) **GVLWVGFM**AKQ**S**YVAE**V**FGTARNGYMPAG**V**FYGGNG**KLLGAQIAGIC**VIT

451

PyAMT1 (409) **GWT**LVMIV**PLFVVLN**LVGV**LRISPEMELIGN**DVSKHG**GAAYPDDVITTEE**
AtAMT1;2 (439) **GWVTVTMGPLF**YGLH**KMNLLRISAE**DEMAGMD**MRHGGFAYAYN**DEDDVS
OtAMT (412) **AWVGATLGA**F**ELLMK**KL**NLLR**TSVE**EETMGL**DESKHG**SAYAMELVAPEP**

501 526

PyAMT1 (459) KQAGHTIDNLGVDD**S**LSRADDPTMV-
AtAMT1;2 (489) TKPWGHFAGRVEPT**S**RSSTPTPTLV
OtAMT (462) A-----

