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

3

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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 ( $\text{Na}^+$ ) in  
35 the cell cause osmotic and ionic stress and disturb potassium ( $\text{K}^+$ ) uptake and functions  
36 due to their similar physicochemical properties, often resulting in a  $\text{K}^+$  deficiency  
37 response (Adams and Shin, 2014). Despite the high concentrations of  $\text{Na}^+$  in seawater,  
38 cytosolic concentrations of  $\text{Na}^+$  are generally maintained at low levels in marine algae,  
39 suggesting the existence of  $\text{Na}^+$  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  $\text{K}^+$  in the cytoplasm  
42 and either exclude or contain  $\text{Na}^+$  preferentially in the vacuoles (Eppley, 1958; Wiencke  
43 et al., 1983). In order to maintain the appropriate cytosolic  $\text{K}^+/\text{Na}^+$  ratios, active  
44  $\text{K}^+$  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  $\text{Na}^+/\text{K}^+$ -ATPases  
47 which extrude three ions of  $\text{Na}^+$  while taking up two ions of  $\text{K}^+$  into the cell and they are  
48 predicted to provide the driving force for  $\text{Na}^+$ -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 ESS<sub>2</sub> 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 N<sub>2</sub> 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 NH<sub>4</sub>OAc 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<sup>-1</sup>) =  
127 colonies on plates × dilution factor / volume plated (mL) and total CFU (cfu) =  
128 average titer (cfu mL<sup>-1</sup>) × 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<sup>+</sup> 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 OD<sub>600</sub> 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 OD<sub>600</sub> of approximately 1.0 was transformed into an *E. coli* strain  
144 defective in K<sup>+</sup> 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<sup>+</sup> deficiency tolerance**

181 In order to isolate genes responsible for efficient K<sup>+</sup> utilisation and K<sup>+</sup>/Na<sup>+</sup> 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<sup>+</sup> uptake. Under the less stringent K<sup>+</sup> 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  $\beta$ -carbonic anhydrase ( $\beta$ 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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:  $\beta$ -carbonic anhydrase  
241 (Py $\beta$ 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  $\beta$ 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 $\beta$ 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*  $\beta$ 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<sup>+</sup> assays, it seems  
253 to point to the significance of Py $\beta$ CA1 in K<sup>+</sup> deficiency response. Furthermore, this  
254 particular  $\beta$ CA might be important in this response as only one gene was repeatedly  
255 isolated though multiple  $\beta$ 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 $\beta$ CA1 (Fig. 3), it is predicted as a functional  $\beta$ 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  $\beta$ 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 HCO<sub>3</sub><sup>-</sup> requirement for amino acid, nucleotide and fatty acid synthesis (Merlin et al.,  
262 2003), indicating that expression of *Py $\beta$ CA1*, but not the innate CA activity of *E. coli*,  
263 could contribute to K<sup>+</sup> deficiency tolerance (Fig. 5). Although a direct interaction  
264 between  $\beta$ CA and K<sup>+</sup> has yet to be reported in seaweeds (Escassi et al., 2002), we  
265 speculate that increased carbon source and photosynthesis by Py $\beta$ CA1 might

266 compensate the loss of K<sup>+</sup>.

267 Two independent transformants from the stringent K<sup>+</sup> 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<sup>+</sup> uptake, to  
282 survive in K<sup>+</sup> 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

## 324 **References**

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

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

329 Alvarez-Pizarro JC, Gomes E, Prisco JT, Grossi-De-Sa MF, Neto OBO (2011)  
330 NH<sub>4</sub><sup>+</sup>-stimulated low-K<sup>+</sup> uptake is associated with the induction of H<sup>+</sup> extrusion  
331 by the plasma membrane H<sup>+</sup>-ATPase in sorghum roots under K<sup>+</sup> deficiency. *J*  
332 *Plant Physiol* 168: 1617-1626.

333 Asamizu E, Nakajima M, Kitade Y, Saga N, Nakamura Y, Tabata S (2003) Comparison  
334 of RNA expression profiles between the two generations of *Porphyra yezoensis*  
335 (Rhodophyta), based on expressed sequence tag frequency analysis. *J Phycol* 39:  
336 923-930.

337 Barrero-Gil J, Garcíadeblas B, Benito B (2005) Sodium, potassium-ATPases in algae

338 and oomycetes. *J Bioenerg Biomembr* 37: 269-278.

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

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

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

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

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

355 Derelle E, Ferraz C, Rombauts S, Rouze P, Worden AZ, Robbens S, Partensky F,

356 Degroeve S, Echeynie S, Cooke R, Saeys Y, Wuyts J, Jabbari K, Bowler C,  
357 Panaud O, Piegu B, Ball SG, Ral JP, Bouget FY, Piganeau G, De Baets B, Picard  
358 A, Delseny M, Demaille J, Van De Peer Y, Moreau H (2006) Genome analysis of  
359 the smallest free-living eukaryote *Ostreococcus tauri* unveils many unique  
360 features. Proc Natl Acad Sci USA 103: 11647-11652.

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

363 Epstein W, Buurman E, Mclaggan D, Naprstek J (1993) Multiple mechanisms, roles and  
364 controls of K<sup>+</sup> transport in *Escherichia coli*. Biochem Soc T 21: 1006-1010.

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

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

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

374 Kakinuma M, Nakamoto C, Kishi K, Coury DA, Amano H (2016a) Isolation and  
375 functional characterization of an ammonium transporter gene, *PyAMT1*, related  
376 to nitrogen assimilation in the marine macroalga *Pyropia yezoensis*  
377 (Rhodophyta). Mar Environ Res, In press.

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

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

383 Kimber MS, Pai EF (2000) The active site architecture of *Pisum sativum* b-carbonic  
384 anhydrase is a mirror image of that of a-carbonic anhydrases. Embo J 19:  
385 1407-1418.

386 Kirst GO (1990) Salinity tolerance of eukaryotic marine algae. Annu Rev Plant Phys 41:  
387 21-53.

388 Kishimoto M, Shimajiri Y, Oshima A, Hase A, Mikami K, Akama K (2013) Functional  
389 expression of an animal type- $\text{Na}^+$ -ATPase gene from a marine red seaweed  
390 *Porphyra yezoensis* increases salinity tolerance in rice plants. Plant Biotechnol  
391 30: 417-422.



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

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

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

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

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

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

408 Nakamura Y, Sasaki N, Kobayashi M, Ojima N, Yasuike M, Shigenobu Y, Satomi M,  
409 Fukuma Y, Shiwaku K, Tsujimoto A, Kobayashi T, Nakayama I, Ito F, Nakajima

410 K, Sano M, Wada T, Kuhara S, Inouye K, Gojobori T, Ikeo K (2013) The first  
411 symbiont-free genome sequence of marine red alga, Susabi-nori (*Pyropia*  
412 *yezoensis*). PLoS One 8: e57122.

413 Ortiz-Ramirez C, Mora SI, Trejo J, Pantoja O (2011) PvAMT1;1, a highly selective  
414 ammonium transporter that functions as H<sup>+</sup>/NH<sub>4</sub><sup>+</sup> Symporter. J Biol Chem 286:  
415 31113-31122.

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

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

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

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

427 Rubio F, Nieves-Cordones M, Aleman F, Martinez V (2008) Relative contribution of

428 AtHAK5 and AtAKT1 to K<sup>+</sup> uptake in the high-affinity range of concentrations.  
429 Physiol Plantarum 134: 598-608.

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

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

437 Szczerba MW, Britto DT, Ali SA, Balkos KD, Kronzucker HJ (2008) NH<sub>4</sub><sup>+</sup>-stimulated  
438 and -inhibited components of K<sup>+</sup> transport in rice (*Oryza sativa* L.). J Exp Bot  
439 59: 3415-3423.

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

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

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

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

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

454

455

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 <sup>-1</sup> )	Total CFU	Recombination (%)	Average insert (kb)
Gametophytes	$1.93 \times 10^6$	$1.93 \times 10^7$	100	1.22
Conchosporangia	$1.30 \times 10^6$	$1.56 \times 10^7$	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
<b>Gametophytes</b>		
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 $\beta$ subunit	protein degradation
8	unknown/no hit	
<b>Conchosporangia</b>		
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	
<b>Sporophytes</b>		
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	

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465

466

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

470

Number	Name	Involved in
<b>Gametophytes</b>		
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<sup>+</sup> tolerance screening of the gametophytes and  
 474 conchosporangia libraries expressed in the *E. coli* strain defective in K<sup>+</sup> uptake under  
 475 severe K<sup>+</sup> deficiency (1 mM KCl)

476

Number	Name	Involved in
<b>Gametophytes</b>		
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	
<b>Conchosporangia</b>		
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	

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

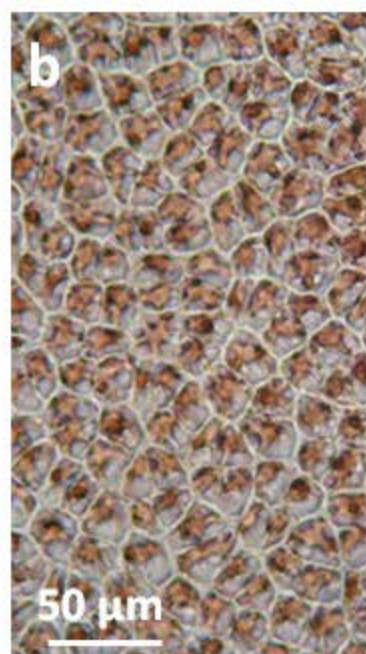
491 **Fig. 3** Amino acid sequence alignment of  $\beta$ CAs. *Pyropia yezoensis* Py $\beta$ CA1  
492 (contig\_16545\_g4020) was aligned with *Arabidopsis thaliana* At $\beta$ CA5.1 (At4g33580),  
493 *Chlamydomonas reinhardtii* CrCAH4 (GI: 159475801) and *Ostreococcus tauri* Ot $\beta$ CA  
494 (GI: 308799709). Identical amino acids among all four  $\beta$ 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

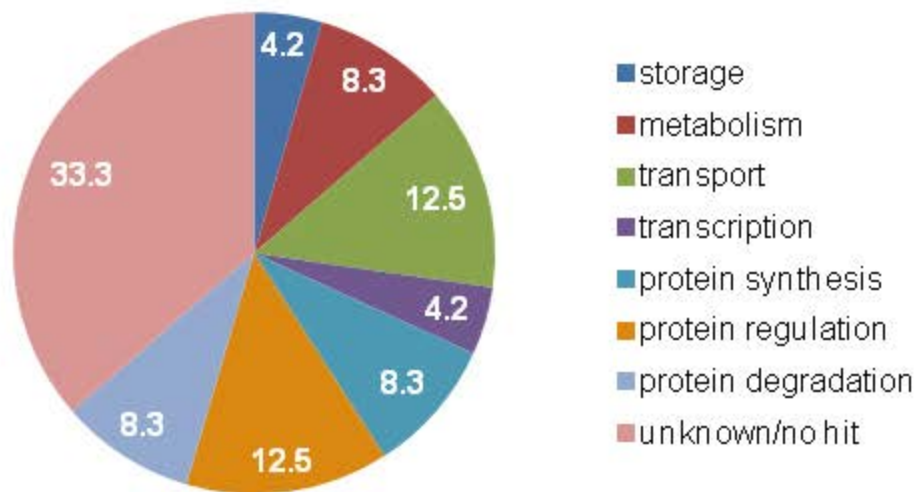
498 **Fig. 4** Amino acid sequence alignment of AMTs. *Pyropia yezoensis* PyAMT1  
499 (contig\_16335\_g3953) was aligned with *Arabidopsis thaliana* AtAMT1;2 (At1g64780)  
500 and *Ostreococcus tauri* OtAMT (GI:693496005). Identical amino acids among all three  
501 AMTs are highlighted as dark gray 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

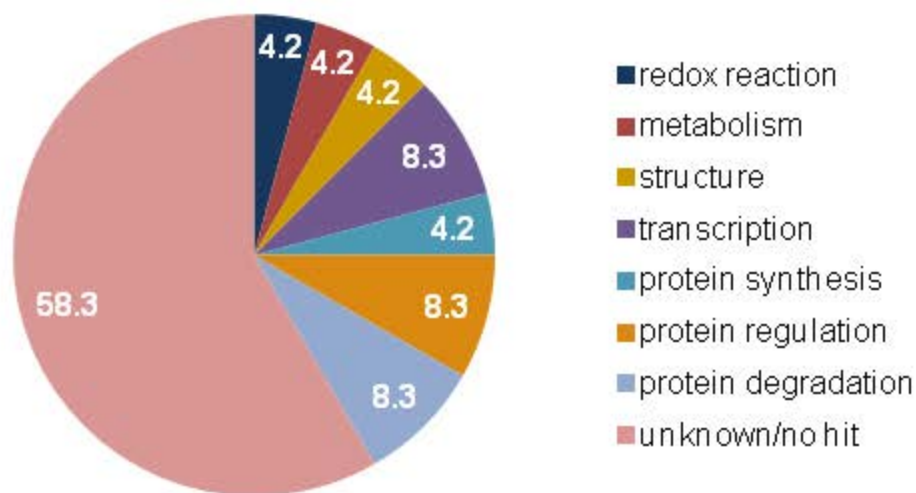
506 **Fig. 5** Functional analysis of PyAMT1 and Py $\beta$ CA1 in K<sup>+</sup> deficiency. The *E. coli* strain  
507 defective in K<sup>+</sup> uptake expressing *PyAMT1* and *Py $\beta$ CA1* were grown in the K<sup>+</sup> sufficient  
508 (30 mM KCl) and K<sup>+</sup> deficient (1.5 and 1.25 mM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



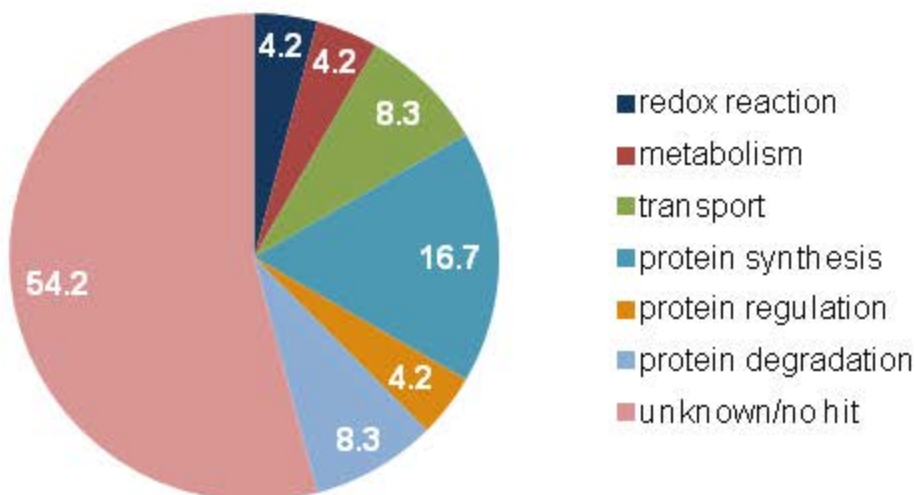
## Gametophytes



## Conchosporangia



## Sporophytes



PyβCA1 (1) -----MAAVASPTSVPATN  
CrCAH4 (1) -----MSSRNVATALRMFATLGRSQAGEASAMMGTGSALLAQRAAL  
OtβCA (1) -----  
AtβCA5 (1) MAATPTHFSVSHDPFSSTLLNLQTOAIFGNHSLKTTQLRIPASFRRKA

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

PyβCA1 (64) -----G-----GQSPSAVVVTCADSRLSPELLFARG  
CrCAH4 (93) KRFVNNKPHDSHPTRNLDRVKATAAGQKPFAPFLSCADSRVPVEIIFDQG  
OtβCA (43) -----GQRPRALVVACS DSRADPAIVFDTA  
AtβCA5 (100) -----DFEHYKNLDAQAPKFLVIACADSRVCP S AVLGFQ

PyβCA1 (90) LGELFVIRTAGNTTGDDS-----TVAS-VEYAVKNLSASLVVVLGHTK  
CrCAH4 (143) FGDVVFTRVAGNIVTNEIT-----ASL--EFGTAVLGSKVLMVLGHS  
OtβCA (68) PGDVFTIRNVGSLVPAYAGLDGGHHGTCAATEYATVHLEVPVILVMGHTQ  
AtβCA5 (135) PGDAFTVRNIANLVPPE---SGPTETKAALF S VNTLNVENILVIGHSR

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

PyβCA1 (173) RGDADESGFVACEVENVHHAVRTLLTTSGLWLAKTRVGGGVKVVGAMYHL  
CrCAH4 (210) ACKKAQAGVDVGAIAENVKVQMEQLKVPVPLQGLVKEGK-LKIVGGVYDL  
OtβCA (165) CERHDPGVRARMLEYELVRQSVQNL LTFPFVKRRVDRGE-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**LAAATFLCSQISNISKLSDTTYA  
OtAMT (1) -----MSL**TESGAEIQSLYNN**

51

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

101

PyAMT1 (87) **YYLFGFAFAYGTEAN**----SFLGHSDFALSGDR----TDFHFFFQWTF**A**  
AtAMT1;2 (101) **YYLFGFAFAFGTPSNG**--FIGRHHSF**FALSSYPERPGSDFSF**FLYQW**AFA**  
OtAMT (67) **WFYFGYGFALGEASNGKLN**SFIGSGN**FAMKGVSGN**--TGIAMYL**FQWSEFS**

151

PyAMT1 (129) **ATAATIVSGSVAERTSFYAYLGYAFFLSGFVYPIVSHWVWGG**-GWLSTIF  
AtAMT1;2 (149) **IAAAGITSGSIAERTQFVAYLIYSTFLTGFVYPTVSHWFWS**SDGWASASR  
OtAMT (115) **AAATTIVSGSVAERTKFEAYLGYSFFLCAFVYVPV**VHWGW**SGQGWLGPWR**

201

PyAMT1 (178) TVGAK-----**DFAGDAVVHMGVGGFAGLAGATIVGPRLGRFDQ**  
AtAMT1;2 (199) SDNNLLFG-----SGAI**DFAGSGVVHMGVGGIAGLC**GALVEGPRI**GRFDR**  
OtAMT (165) CEGSSNGCGPLLAGSGML**DFAGSGIVHMTGGVAGLVGAI**IIVGPRT**GRFAP**

251

PyAMT1 (215) **DGRVVPMPGHSATLCTLGTFILWFGWYGFNPGSTLGI**SNTG----PDADY  
AtAMT1;2 (243) **SGRSVALRGHSASLVVLGTFLWFGWYGFNPGSFLT**ILKGYDKSR**PYYGQ**  
OtAMT (215) **DGRVNPMPGHSAPLVVLGTFILWLWFGWYGFNPGS**QLAIVAFGG---AAADN

301

PyAMT1 (261) **TVTAARCAVTTTIAAASAGVTTLIVIKLRDHIFDLLACLNG**ILAGLVAIT  
AtAMT1;2 (293) **WSAVGRTAVTTTTLTGCTAALTTLFSKRLLAGHWNVIDVCNGL**LGFAAIT  
OtAMT (262) **SRVIARTAVTTTTLAAGGGIMAMVLNYVLYHVWDLIAVCNGL**ILAGLVGIT

351

PyAMT1 (311) **ASCWVEVYAALVIGVIGALVYIGAAMLLLMFKI**DDPLEAFPIHGAVGVW  
AtAMT1;2 (343) **SGCAVVEPWAAIVCGFVASWVLIGFNLLAKKLKY**DDPLEAAQLHGCGGAW  
OtAMT (312) **AGCSTTEPWAAPICGALSALVIHASSKLLK**LKIDDPLEAAPMHGFCGAF

401

PyAMT1 (361) **GAFVGLFARIELLTLSGYGNDNGWE**--GVFYGGGG**RLLAANCVM**IASIA  
AtAMT1;2 (393) **GLIFTGLFARKEYVNEIYSGDR**----PYGLFMGGGG**KLLAAQIVQI**IVIV  
OtAMT (362) **GVLWVGFMAKQSYVAE**VFGTARNGYMPAGV**FYGGNGKLLGAQIAG**ICVIT

451

PyAMT1 (409) **GWTLVMIVPLFVVLNLVGVLRISPEMELIGN**DVSKHGG**AAYPDDVITTEE**  
AtAMT1;2 (439) **GWVTVTMGPLFYGLHKMNL**LRISAED**EMAGMDMTRHGGFAYAYN**DEDDVS  
OtAMT (412) **AWVGATLGAFELLMKKNL**LR**TSVEEETMGL**DESKHGG**SAYAMELVAPEP**

501 526

PyAMT1 (459) KQAGHTIDNLGVDD**SLSRADDPTMV**-  
AtAMT1;2 (489) TKPWGHFAGRVEPT**SRSSTPTPTLTV**  
OtAMT (462) A-----

