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

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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*

31 *yezoensis*, salt tolerance

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

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^+ antiproters, 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

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 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 the *P. 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^oC in a 10 h light/14 h dark photocycle with a light intensity of 60 μmol 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, MA) and 75 μg of total RNA was used to isolate 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. Titer was determined by spreading 1:10 serial dilutions (10^{-2}, 10^{-3}, 10^{-4}) of each library onto LB plates containing kanamycin. Titer was calculated as colony forming unit (cfu mL^{-1}) = colonies on plates × dilution factor / volume plated (mL) and total CFU (cfu) = average titer (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 manufacture's instruction (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 five-fold 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

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

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, 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., 2016a). 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 (Table 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 titer. 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 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.

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

248 (Zhang et al., 2010). Although PyβCA1 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* βCAs (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 PyβCA1 in K^+ deficiency response. Furthermore, this
254 particular βCA might be important in this response as only one gene was repeatedly
255 isolated though multiple *βCAs* 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 PyβCA1 (Fig. 3), it is predicted as a functional βCA. 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 βCA 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_3^- requirement for amino acid, nucleotide and fatty acid synthesis (Merlin et al.,
262 2003), indicating that expression of *PyβCA1*, but not the innate CA activity of *E. coli*,
263 could contribute to K^+ deficiency tolerance (Fig. 5). Although a direct interaction
264 between βCA and K^+ has yet to be reported in seaweeds (Escassi et al., 2002), we
265 speculate that increased carbon source and photosynthesis by PyβCA1 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,

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.

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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

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	

Figure legends

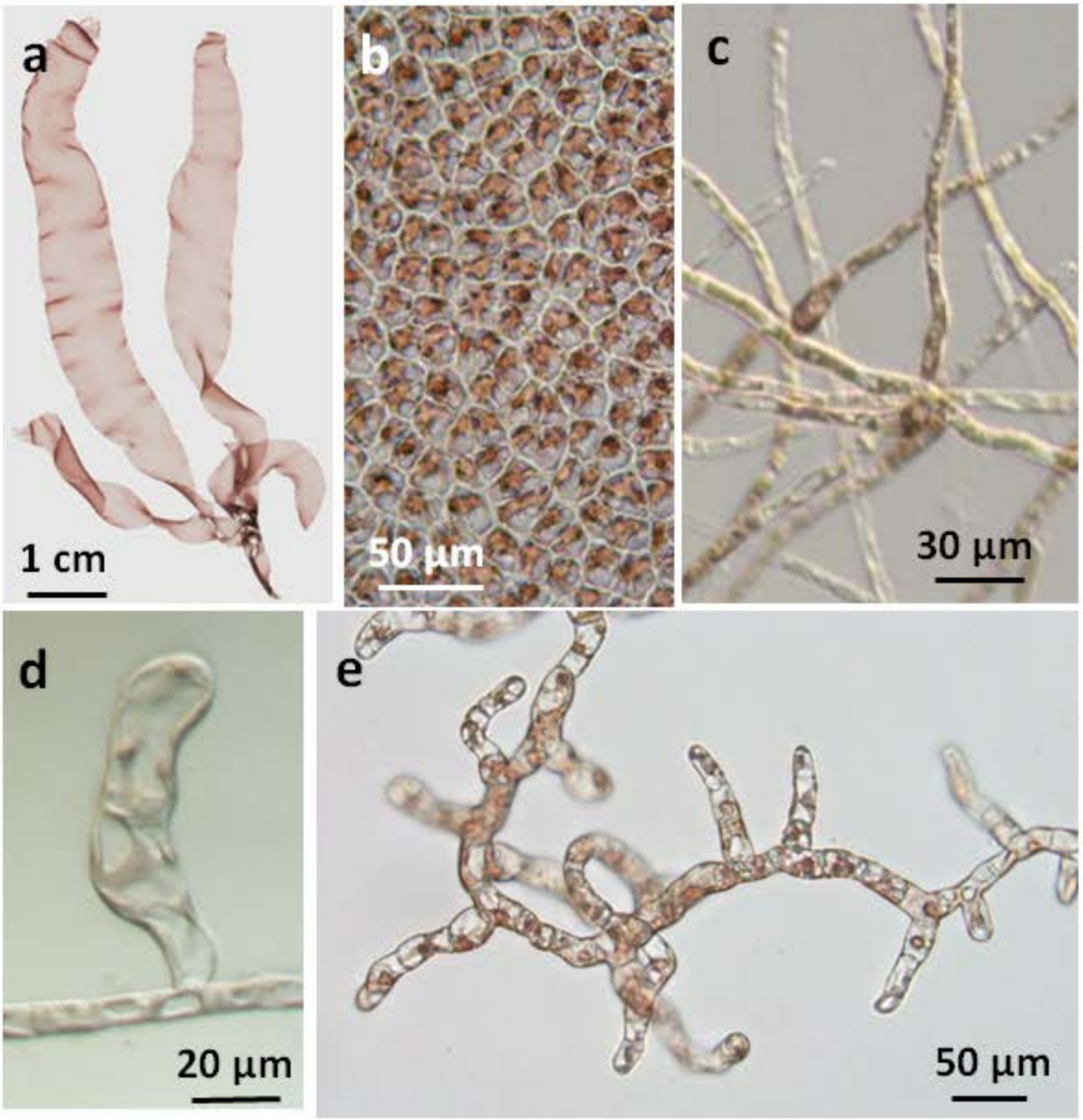
Fig. 1 Images of the multiple stages in the life cycle of *Pyropia yezoensis*. **a** Gametophytes. **b** Vegetative cells of gametophytes. **c** Sporophytes. **d** Generation of a conchosporangium from a sporophyte. **e** Conchosporangia. Full-length cDNA libraries were constructed with RNA extracted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

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.

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 gray and identical amino acids between two or three among four are highlighted as light gray. The amino acids which form the conserved zinc binding site are marked in blue.

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 gray and identical amino acids between two of three AMTs are highlighted as light gray. 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).

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.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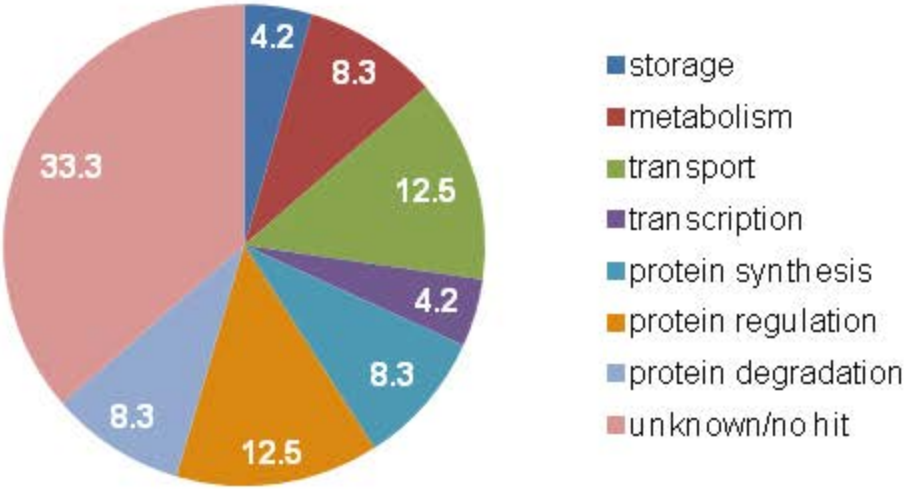
Gametophytes



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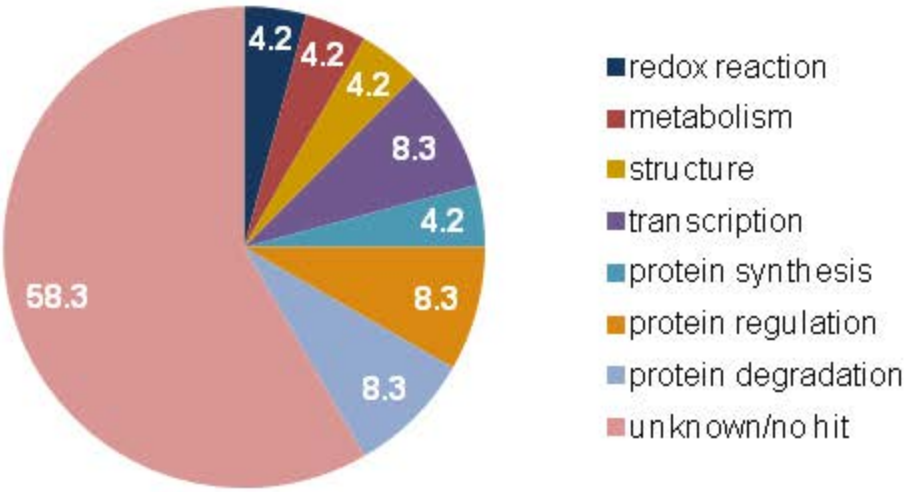

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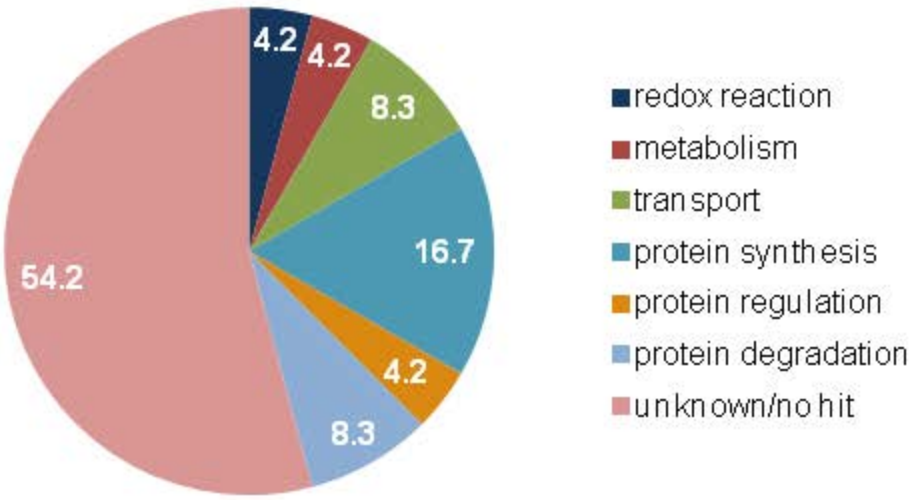

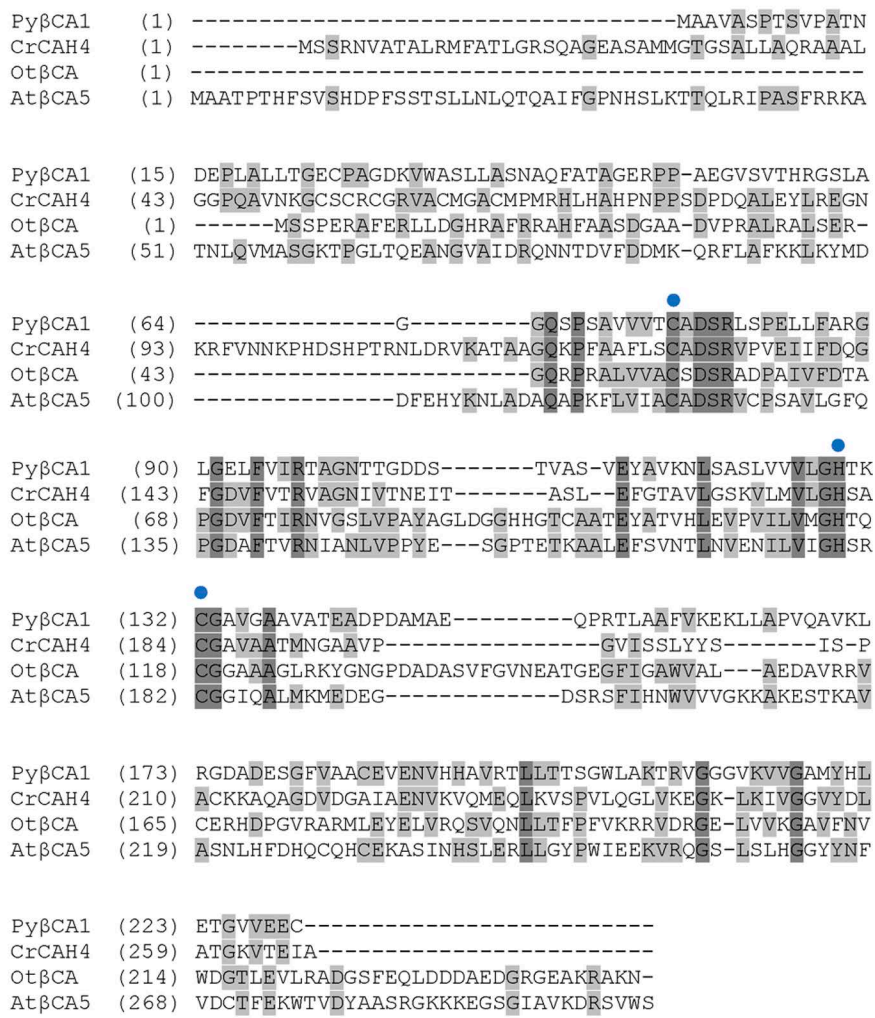
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OtβCA (1)
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PyβCA1 (15) DEPLALLTGECPAGDKVWASLLASNAQFATAGERPP-AEGVSVTHRGSLA
CrCAH4 (43) GGPQAVNKGCSCRCGRVACMGACMPMRHLHAHPNPPSDPDQALEYLREGN
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CrCAH4 (93) KRFVNNKPHDSHPTRNLDRVKATAAGQKPFAAFLSCADSRVPVEIIFDQG
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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
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PyβCA1 (223) ETGVVEEC----------
CrCAH4 (259) ATGKVTEIA----------
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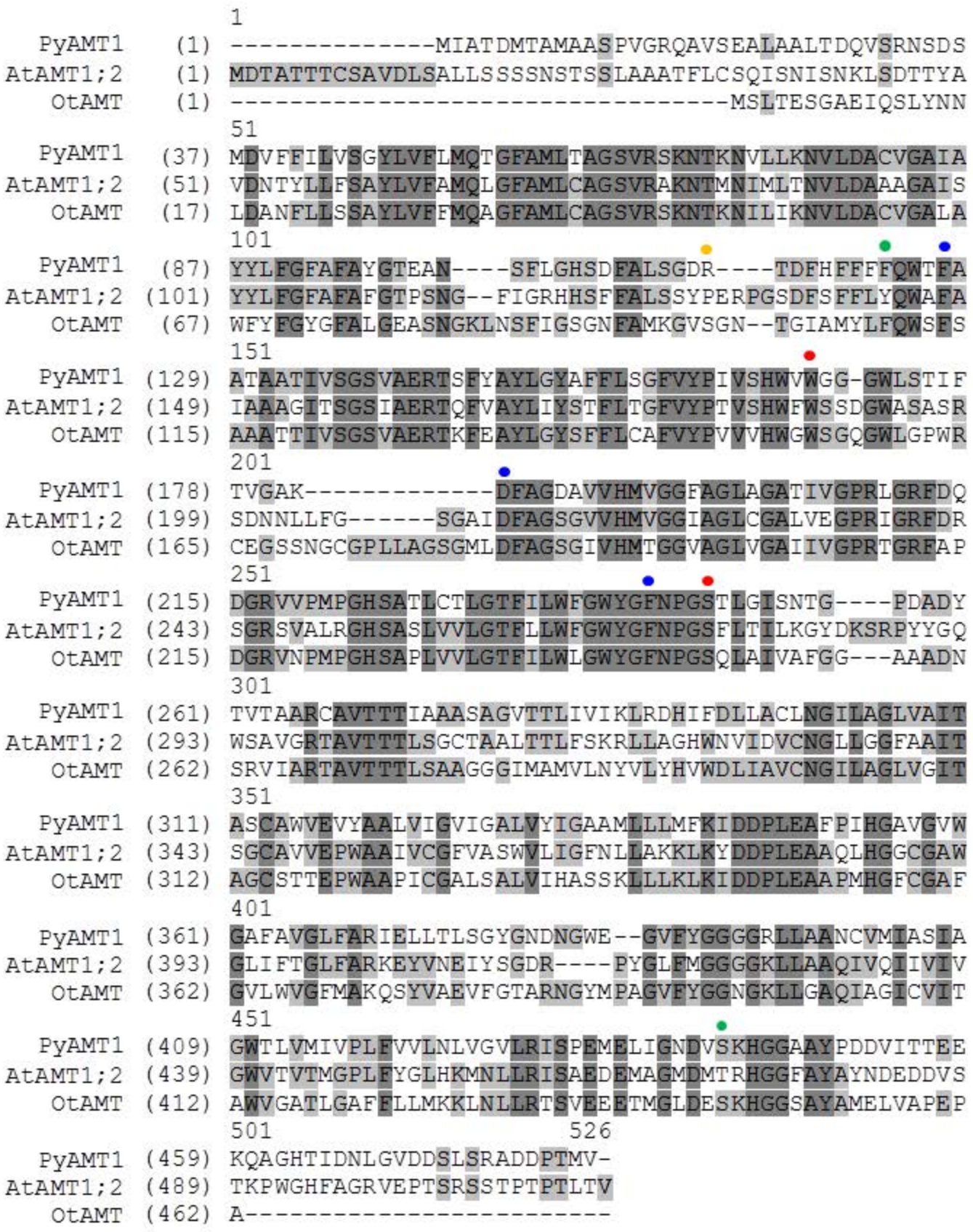
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OtAMT (1) ----------MSLTESGAEIQSLYNN
51
PyAMT1 (37) MDVFFILVSGYLVFLMQTGFAMLTAGSVRSKNTKNVLLKNVLDACVGAIA
AtAMT1;2 (51) VDNTYLLFSAYLVFAMQLGFAMLCAGSVRAKNTMNIMLTNVLDAAAGAIS
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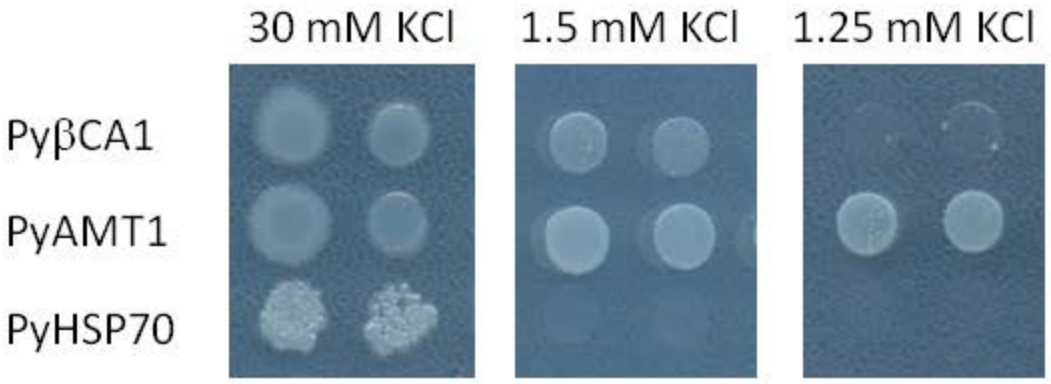
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