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Identification of hypo-osmotically induced genes in *Kappaphycus alvarezii* (Solieriaceae, Rhodophyta) through expressed sequence tag analysis

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Abstract

Hypo-osmotic conditions are among the most stressful environmental factors that affect seaweed physiology and productivity. To improve understanding of hypo-osmotic adaptation and to find genes involved in low salinity stress, we conducted an analysis of expressed sequence tags (ESTs) on the κ-carrageenan-producing red algae, *Kappaphycus alvarezii*. Of 454 tentatively unique genes (TUGs), 281 matched protein sequences by BlastX analysis (*e* value≤10^{-3}), 112 TUGs matched ESTs from other agar- or carrageenan-producing red algae, and 98 TUGs were annotated. The transcriptional profiles of six functional genes from *K. alvarezii* ESTs were examined. An ATP-binding cassette transporter gene and a sodium phosphate symporter gene were significantly up-regulated, indicating the importance of membrane transport proteins in coping with hypo-osmotic stress conditions.

Keywords: ATP-binding cassette transporter; expressed sequence tag; hypo-osmotic stress; *Kappaphycus alvarezii*; sodium phosphate symporter.

Introduction

Kappaphycus alvarezii (Doty) Doty ex Silva is an economically important red tropical seaweed that is in high demand because of its cell wall polysaccharide – the most important source of κ-carrageenan in the world (Bixler 1996). The market for carrageenan continues to grow, and current sources of cultivated eucheumoids seem incapable of meeting industrial demand in terms of quality, price and volume (Ask et al. 2003).

Rainfall in equatorial monsoon climates causes prolonged low-salinity stress for seaweeds, and results in significant loss in both biomass and gel strength (Phang et al. 1996). *Kappaphycus* experiences rapid decreases in salinity because of tropical downpours when cultured on shallow, floating platforms that are exposed at low tide (Ask and Azanza 2002). Liu et al. (unpublished data) demonstrated that some strains of *K. alvarezii* soaked in water at a salinity of 28 for 2 h are injured by induced "ice-ice" disease; more serious "ice-ice" disease, or even death, occurs in seaweeds soaked in water at a salinity of 22 for 2 h. In seaweeds, hypo-osmotic stress caused by decreased salinity increases external water potential and may trigger excessive uptake of water with concomitant increase of cell volume and turgor pressure, loss of ions and organic solutes, and osmotic shock (Teo et al. 2009). In red algae, the molecular mechanisms of hypo-osmotic tolerance of *Chondrus crispus* Stackh and *Gracilaria changii* (B.M. Xia *et* I.A. Abbott) I.A. Abbott, J. Zhang *et* B.M. Xia have been explained based on genomic methods. However, there are some contradictory results for these two species (Collén et al. 2007, Teo et al. 2009), such as the up-regulated expression in *G. changii* of the vanadium-dependent bromoperoxidase gene, which is not differentially expressed in *C. crispus* in response to hypo-osmotic conditions.

Expressed sequence tag (EST) analysis is one of the most effective means for gene discovery; it is specifically relevant to the transcriptome of an organism under different experimental conditions. The advance of gene sequence knowledge (e.g., gene annotation) is of crucial importance in revealing underlying mechanisms of physiological phenomena. To date, >35,000 EST sequences of economically important red algae have been published, including *Porphyra*, *Gracilaria*, and *Chondrus*. Many genes involved in stress responses of red algae have been identified (Nikaido et al. 2000, Asamizu et al. 2003, Collén et al. 2006, Teo et al. 2007). A small-scale EST analysis of 311 clones of *Eucheuma denticulatum* (N.L.Burman) F.S.Collins *et* Hervey, the closest relative species to *K. alvarezii*, has been conducted. More than 60% of ESTs of *E. denticulatum* do not occur in other red algal EST libraries (Aspilla et al. 2010).

Although it is commercially valuable, fewer studies have been made on genomic functions of *Kappaphycus alvarezii* than on many other red algae. The present study primarily focused on building a cDNA library for low salinity stress-induced *K. alvarezii* and conducting EST analysis. An attempt was made to identify functional genes and to examine expression patterns of those genes in *K. alvarezii* under hypo-osmotic stress.

Materials and methods

RNA extraction and cDNA library construction

Seaweed samples of *Kappaphycus alvarezii* were collected from an aquaculture farm off Lingshui, Hainan province in

China in August 2008. After cleaning and soaking in seawater at salinity 28 (seawater diluted with distilled water and salinity calculated with a salinity calibrator) for 2 h, samples were stored at -80°C. Two RNA extraction methods were compared: the first using TRI reagent (Molecular Research Centre, Cincinnati, OH, USA) performed according to the manufacturer's recommendations and the second using the CTAB-LiCl protocol performed with minor modifications recommended by Zeng and Yang (2002).

Briefly, seaweed was ground entirely with liquid nitrogen and transferred into a 1.5 ml tube containing 600 µl of CTAB extraction buffer [2% (w/v) CTAB (Sigma, St Louis, MO, USA), 2% (w/v) PVP (Sigma), 100 mmol Tris-HCl (pH 8.0), 25 mmol EDTA, 2.0 mol NaCl, and 10% β-mercaptoethanol added just before use]. The mixture was blended thoroughly and incubated at 65°C for 10 min with occasional agitation. The solution was extracted twice using an equal volume of chloroform: isoamyl (24:1, v/v), and centrifuged at 10,000×*g* for 10 min at 4°C. The upper aqueous phase was transferred into a new tube and then one third of the volume of 8.0 mol LiCl was added. Extracts were mixed well and stored at -80°C for 1 h. After centrifuging and washing with 80% ethanol, the RNA pellet was dissolved in Diethylpyrocarbonate (DEPC)-treated water, and mRNA was purified from total RNA using an mRNA isolation kit (Qiagen, Hilden, Germany). cDNA was synthesized by using 5 µg of the Poly (A)+RNA and employing a ZAP-cDNA Synthesis Kit (Stratagene, La Jolla, CA, USA). After sizing the fraction, cDNA was ligated into a Uni-zap XR vector and packaged in a Lambda phage with a ZAP-cDNA Gigapack III Gold Cloning Kit (Stratagene, Agilent Technologies, Santa Clara, CA, USA). The resulting primary cDNA library containing 1.2×10^6 independent phages was amplified to 2.3×10^8 pfu ml^{-1}.

EST aligner and function annotation

Around 523 clones were randomly selected and sequenced on an ABI 3730 Sequence Analyzer (Applied Biosystems, Foster City, CA, USA). After sequencing, the vector part was trimmed and all 519 cDNA sequences were assembled into contigs using a CodonCode Aligner (http://www.codoncode.com/aligner/). Tentatively unique genes (TUGs) were compared to both the National Center for Biotechnology Information (NCBI) nr (non-redundant) protein database and the nucleotide database by using the BlastX algorithm and the BlastN algorithm, respectively. TUGs were also compared with the EST database. Sequences of the ESTs were batch aligned with Blastcl3 programs (www.ncbi.nlm.nih.gov/BLAST/), and function annotation was classified with the EGENES database platform (Kanehisa et al. 2006; Masoudi-Nejad et al. 2007).

Real time PCR

Transcript profiles of six functional genes: elongation factor EF-3, sodium phosphatesymporter, vanadium-dependent bromoperoxidase, pyruvate dehydrogenase, sulfate adenylyltransferase, and ATP-binding cassette were analyzed by real-time PCR. Both actin and glyceraldehyde-3-phosphate dehydrogenase gene were chosen as endogenous controls.

Collected *Kappaphycus alvarezii* samples were cultured for two days at 25°C, salinity 33 and 12:12 L:D daily photoperiod. Branches (4–5 cm) were cut from thalli for low-salinity treatment and RNA extraction. The experiment was repeated three times, and *K. alvarezii* samples for each test group were taken from a single plant to ensure the homogeny of experimental material. Samples were treated with salinity 28 seawater for 0 h, 1 h, 3 h, 6 h, 12 h, and 24 h. RNA was extracted from the samples and treated with RNase free-DNase (Invitrogen, Carlsbad, CA, USA) for 30 min at 37°C. Primers for real-time PCR (Table 1) were designed using Primer Premier 5.0 (Premierbiosoft, Palo Alto, CA, USA). Real time PCR was performed in an Mx3005P real time PCR machine (Stratagene) using a SYBR® PrimeScript™ RT-PCR Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. Forty cycle reactions were conducted (95°C for 10 s; annealing for 10 s; 72°C for 20 s). To evaluate amplification of primers, standard curves of each pair of primers were constructed and PCR efficiencies were calculated from the slopes of the curves. Real time PCR efficiencies for endogenous controls and genes examined ranged from 95% to 105%. Reactions were performed in triplicate and relative gene expression determinations were made with the comparative Δ-Δ C_T method ($2^{-\Delta\Delta CT}$) described by Livak and Schmittgen (2001).

Results

RNA extraction and cDNA library construction

Construction of the cDNA library for an organism rich in polysaccharides and proteins is frequently problematic because of the low yield of RNA extraction. RNA extraction from *Kappaphycus alvarezii* was performed using two different extraction methods. Higher quality RNA was obtained by using the CTAB-LiCl method compared with low yield and poor quality of RNA obtained by using TRI reagent; with TRI reagent, considerable amounts of polysaccharide still remained in the RNA sample. After CTAB-LiCl extraction, electrophoresis on agarose gels indicated that little RNA degradation had occurred, and the OD 260/280 ratios of 1.8–2.0 showed that very little protein or polysaccharide contaminated the *K. alvarezii* RNA product.

Forty-eight colonies were randomly selected to test the fragment length of cDNA in the cDNA library constructed. Twelve clones were longer than 2000 bps and only one clone was shorter than 500 bps, indicating the integrity of mRNA for *Kappaphycus alvarezii* cDNA library construction.

General characteristics of *Kappaphycus* ESTs

Single pass sequencing of the amplified cDNA library of *K. alvarezii* generated 523 ESTs. Five sequences contaminated by cloning vectors or low-quality sequences were not analyzed. The 518 readable sequences consisted of 454 tentatively unique genes (TUGs) that were submitted to GenBank with accession numbers from HS416812 to HS417265. Of

Table 1 *Kappaphycus alvarezii*: primers used in real-time PCR assays.

Genes	Primers 5′-3′	Annealing temperature (°C)
Genes examined		
Elongation factor EF-3 (HS416858)	F: GTGACATACCCAATGACCTG R: TCGCTGCTTCTGCCCTC	50
Sodium phosphate symporter (HS416898)	F: AGACTACGGTCAACTGGAAGC R: CGTGATGAGCAGGAACAAGAT	55
Vanadium-dependent bromoperoxidase (vBPO, HS417223)	F: AGTTGCCGTTTGCGTATGTC R: CAGAGTTTGTTCAGCTCTCCCT	45
Pyruvate dehydrogenase (PdhA, HS417161)	F: TCAAAGGCAGAGTCTAACAA R: AATCGTCGTAGGAAATCG	50
Sulfate adenylyltransferase (HS416997)	F: CAGGGCTGACGACTTTG R: CCTCCTACCGCATTACTTT	50
ATP-binding cassette (ABCD3, HS417124)	F: GGTCGTATTCACTTACCCTG R: CGTTTGCGTGCTTCCA	50
Endogenous controls		
Actin	F: TGGATTGGTGGGTCTATT R: AAGCACTTGCGATGGA	45
Glyceraldehyde-3-phosphate dehydrogenase	F: ATGCCACCACTGCTACAC R: CATACCCGTCAACTTTCC	45

the 454 TUGs, there were 403 tentatively unique singletons (TUSs) and 51 tentatively unique contigs (TUCs) that were assembled by 109 ESTs. Two hundred and eighty one of the 454 TUGs (62%) matched significantly with sequences in the non-redundant protein database (*e* value $\leq 0^{-3}$), which contains genes encoding both "known proteins" and "predicted/unknown proteins" based on BlastX results.

After searching the EST database, 89 ESTs from *Kappaphycus alvarezii* first hit ESTs from *Eucheuma denticulatum*, and 23 ESTs first hit those from *Chondrus crispus*, *Gracilaria lemaneiformis* (Bory de Saint-Vincent) Greville, and *Gracilaria changii*. Nucleotide sequence identity ranged from 81% to 89% for *K. alvarezii* and other red algae.

Gene function annotation of *Kappaphycus* ESTs

Of the 454 TUGs from *K.alvarezii*, 98 (21.6%) were annotated and assigned into 8 KEGG orthology (KO) groups analyzed by the EGENE database platform (Figure 1). Proportions of the 98 annotated ESTs that fell into "metabolism", "translation", "folding, sorting and degradation", "transcription", "cellular process", "replication and repair", "organismal system", and "environmental information processing" are shown in Figure 1. Transcripts involved in metabolic pathways of *K. alvarezii* are listed in Table 2.

Genes differentially expressed under hypo-osmotic stress identified by real time PCR

To investigate expression patterns under hypo-osmotic stress conditions, we examined transcript profiles of six functional genes isolated from *Kappaphycus alvarezii* ESTs (Figure 2). Samples were treated separately in seawater at a salinity of 28 for 1 h, 3 h, 6 h, 12 h, and 24 h. The expressions of several genes were up-regulated. The most significant changes occurred in the ATP-binding cassette gene, with an approximately 8-fold increase in salinity 28 treatment for 6 h compared with 0 h. The transcription of the ATP-binding cassette gene stayed high during the course of treatment from 1 h to12 h, reaching maximum at 6 h and decreasing thereafter. Another significantly changed transcription was for the gene encoding sodium phosphate symporter, which increased about 5-fold when treated at salinity 28 for 3 h compared with 0 h. The sulfate adenyltransferase gene had an expression pattern similar to that of the sodium phosphate symporter gene, with <4-fold up-regulation after treatment in salinity 28 water for 3 h compared with 0 h. Pyruvate dehydrogenase gene expression was consistently up-regulated by about 3-fold from 1 h to 12 h compared with 0 h.

The up-regulation of vanadium-dependent bromoperoxidase was <2-fold at 1 h, but expression was down-regulated at other

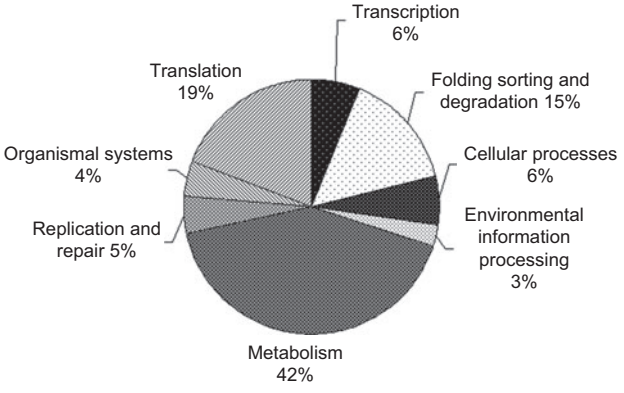

Figure 1 *Kappaphycus alvarezii*: EGG orthology group percentages representation of ESTs.

Table 2 *Kappaphycus alvarezii*: ESTs in primary and other biological processes shown by KEGG orthology annotation.

	Genbank ID		Genbank ID
Carbohydrate metabolism		Folding, sorting and degradation	
Pyruvate dehydrogenase E1 component subunit α	HS417161	Thioredoxin 1	HS417154
Glyceraldehyde 3-phosphate dehydrogenase	HS417175	Heat shock 70 kDa protein	HS417158
Glucose-6-phosphate isomerase	HS417179	Ubiquitin-conjugating enzyme E2 G1	HS417187
Glucose-1,6-bisphosphate synthase	HS417242	20S Proteasome subunit β 4	HS416985
Pyruvate, orthophosphate dikinase	HS416816	Disulfide isomerase family A, member 6	HS416991
Mannose-6-phosphate isomerase	HS417200	Ubiquitin-conjugating enzyme E2 D/E	HS417024
Pyruvate, water dikinase	HS416844	Syntaxin	HS416829
Glucosamine-6-phosphate deaminase	HS416886	Molecular chaperone GrpE	HS416831
6-Phosphogluconate dehydrogenase	HS416892	20S Proteasome subunit α 6	HS416835
Starch phosphorylase	HS416943	Elongation factor EF-3	HS416858
UTP – glucose-1-phosphate uridylyltransferase	HS417120	Cell division protease FtsH	HS416863
Energy metabolism		Cullin 3	HS416904
Sulfate adenylyltransferase	HS416997	Ubiquitin C	HS416921
Phycobilisome core-membrane linker protein	HS417005	T-complex protein 1 subunit epsilon	HS416934
Pyruvate, orthophosphate dikinase	HS416816	26S Proteasome regulatory subunit N6	HS417230
Glutamate synthase (NADPH/NADH) large chain	HS416834	RNA-binding protein PNO1	HS417232
Phycocyanin α chain	HS417204	COP9 signalosome complex subunit 2	HS417235
Peroxiredoxin 6, 1-Cys peroxiredoxin	HS417216	Molecular chaperone DnaK	HS417084
Phycobilisome core component	HS416894	Replication and repair	
Cytochrome b6	HS416927	Ribonuclease HI	HS417197
Catalase/peroxidase	HS417227	Ataxia telangectasia mutated family protein	HS417064
Inorganic pyrophosphatase	HS417060	Histone H2A	HS417194
Glutamate dehydrogenase	HS417095	Eukaryotic translation initiation factor 2C	HS416912
Lipid metabolism		Environmental information processing	
Stearoyl-CoA desaturase (Δ-9 desaturase)	HS417199	ATP-binding cassette, subfamily D (ALD), member 2	HS417124
3-Oxoacyl-[acyl-carrier-protein] synthase II	HS416969	Outer membrane usher protein	HS417220
Acyl-CoA oxidase	HS417093	Heat shock 70 kDa protein	HS417158
3-Oxoacyl-[acyl-carrier-protein] synthase III	HS417256	Zinc finger protein ZIC 2	HS416871
Cell growth and death		Glycerol uptake facilitator protein	HS417229
Ataxia telangectasia mutated family protein	HS417064	Glycan biosynthesis and metabolism	
Cell cycle arrest protein BUB2	HS417173	Arylsulfatase B	HS417031
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	HS417068		

times compared with 0 h. The translation elongation factor 3 gene was the only locus that that was always down-regulated.

Discussion

We analyzed the profile of gene expression in *K. alvarezii* by EST after low salinity treatment, and determined expressions of six genes under hypo-osmotic stress by real-time PCR. As ion concentration is relatively low in hypo-osmotic medium, the first effect of reduced salinity treatment would be on ion transport pressure. The expression of a Na/Pi symporter gene was significantly up-regulated by low salinity stress, which may compensate for the loss of Na^+ (*via* membrane leakiness under low salt conditions) through uptake of more sodium ions. Meanwhile, the reduced phosphate concentration in seawater at a salinity of 28 may be another inducer that triggers expression of the Na/Pi symporter gene. A deduced amino acid sequence of the Na/Pi symporter gene identified in this study demonstrated 58% identity to the phosphate transporter (TcPHO) gene of *Tetraselmis chui* Butcher, whose expression is triggered by low-phosphate treatment and repressed by high-phosphate treatment (Chung et al. 2003).

The expression of an ABC transporter protein gene in *Kappaphycus alvarezii* was significantly up-regulated under hypo-osmotic conditions, more so than other transcripts. This protein is a member of the ATP-binding cassette family D (ABCD3) located on the peroxisomal membrane. ABC subfamily D is present in all eukaryotic taxa and plays multiple metabolic and developmental functions in different organisms. One of the common functions is importation of substrates for β-oxidation, although many functions remain to be determined for substrates and transport mechanisms, which appear to differ among phyla (Theodoulou et al. 2006). The response of ABCD3 to various abiotic stresses has not been previously reported. However, an ABC transporter protein (putatively subfamily G, member 3) gene from *Gracilaria changii* is also significantly up-regulated (about 6-fold) under hypo-osmotic treatment (Teo et al. 2009).

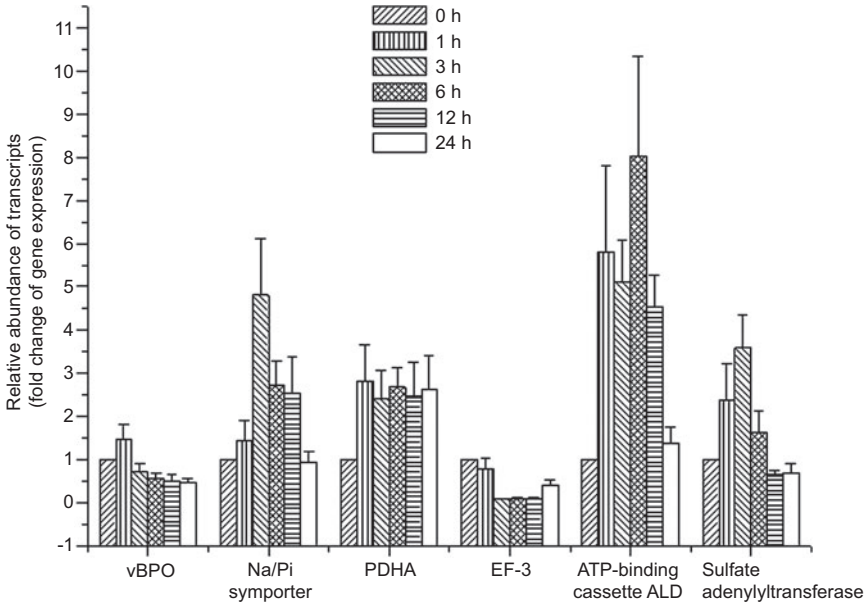

Figure 2 *Kappaphycus alvarezii*: Relative abundance of transcripts (fold-change of gene expression) of selected candidate loci after treatment in seawater at salinity 28 for 0 h, 1 h, 3 h, 6 h, 12 h, and 24 h. Values are means±SE (n=3).

A pyruvate dehydrogenase E1 component subunit α (PdhA) gene was also up-regulated by low salinity stress in this study. It encodes a mitochondrial PdhA protein. Mitochondrial Pdh protein is the site of carbon entry into the tricarboxylic acid cycle (Tovar-Méndez et al. 2003). The up-regulation of PdhA gene expression in low salinity water indicates that energy metabolism is important under this stress. PdhA and PdhD proteins of *Listeria monocytogenes* (E.G.D. Murray, R.E. Webb, M.B.R. Swann) P.J.H Pirie that are highly induced by salt stress may be vital enzymes that are rather salt sensitive and whose production is necessary to keep up a minimal level of catabolic metabolism (Duché et al. 2002). Thus, we suggest that elevation of carbon assimilation by over-expression of PdhA may be important in seaweed acclimation to hypo-osmotic stress.

Sulfate adenylyltransferase, the first enzyme in the plant sulfate assimilation pathway, catalyzes formation of adenosine phosphosulfate from ATP and sulfate. Hypo-osmotic stress may induce the over-expression of the sulfate adenylyltransferase gene in *Kappaphycus alvarezii*. A sulfate adenylyltransferase gene was chosen as a marker locus for hypo-osmotic stress in *Chondrus crispus* because its expression is up-regulated only in response to this stressor (Collén et al. 2007).

The vanadium-dependent bromoperoxidase (vBPO) gene, which is involved in reactive oxygen metabolism (ROM), is an important functional gene in red and brown algae (Dring 2005). BPO in red algae, such as *Eucheuma denticulatum* (Mtolera et al. 1996) and *Meristiella gelidium* (J.Agardh) D.P.Cheney *et* P.W.Gabrielson (Collén et al. 1994), produces volatile halogenated compounds when exposed to stress conditions that include elevated pH and high light intensities. The vBPO gene was shown (using the cDNA microarray approach) to be up-regulated in *Gracilaria changii* in response to both hyper-osmotic and hypo-osmotic conditions (Teo et al. 2009). The up-regulation of vBPO was not significant in this study; however, its expression in *Chondrus crispus* was found (using the cDNA microarray approach) to be down-regulated by both high and low salinity stresses (Collén et al. 2007), but real-time PCR methodology did not detect differential expression of vBPO in this species under hypo-osmotic conditions (Collén et al. 2007). A vBPO has been isolated from *K. alvarezii* and the enzyme activated is specific for Br^- and I^- ions (Kamenarska et al. 2007). We suggest that decreases in Br^- and I^- ion concentrations in hypo-osmotic conditions may repress vBPO gene transcription and that would reduce elimination of H_2O_2; the excessive H_2O_2 may then stress the algae.

There appears to be a coordinated action of translation initiation and elongation factors operating together with several accessory proteins that regulate stress-associated translation in controlling various cellular adaptations during salt stress (Sahi et al. 2006). The expression of translation elongation factor 3 was detected in *Kappaphycus alvarezii* and it was slightly down-regulated under hypo-osmotic conditions. The roles of transcriptional and translational machineries during low salinity adaptation require further study.

Other than genes responding to hypo-osmotic stress, we attempted to identify loci involved in carrageenan synthesis in *Kappaphycus alvarezii*. Arylsulfatase B was the sole transcript involved in glycan metabolism in this study. Arylsulfatase has been detected in *Chlamydomonas* under sulfur-deprived conditions; the enzyme cleaves sulfate from aromatic compounds and enables cells to use these molecules as a source of sulfur when free sulfate is not available (Davies et al. 1994). Blastx analysis indicated that the arylsulfatase gene from *K. alvarezii* resembles that of the bacterium *Pseudoalteromonas atlantica* (Akagawa-Matsushita, Matsuo, Koga *et* Yamasato) Gauthier,

Gauthier *et* Christen T6c (Score=391 bits, Expect=7e-107). Arylsulfatase isolated from the bacterium *Sphingomonas* sp. AS6330 hydrolyzes sulfate ester bonds in agar, and the sulfate ester hydrolytic enzyme activity on agar is higher than on other sulfated marine polysaccharides such as porphyran, fucoidan, and carrageenan (Kim et al. 2004). The role of arylsulfatase in the synthesis or hydrolysis of carrageenan in *K. alvarezii* should be clarified through detection of enzyme activities in future studies.

In conclusion, although the annotation rate of red algae ESTs was low, several genes controlling various cellular adaptations during hypo-osmotic stress on *K. alvarezii* were identified. Combining KO annotation with real time PCR analysis proved efficient in determining and characterizing gene functions during low-salinity stress.

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