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Cloning and quantitative analysis of five heat shock protein 70 genes from *Pyropia haitanensis*

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Abstract Heat shock protein 70s (Hsp70s) play particularly important roles in a variety of stress responses and have been widely studied in many higher plants and algae. *Pyropia haitanensis* blades inhabit the upper intertidal zones of rocky shores and are exposed to dynamic environmental changes associated with the turning tides, including desiccation and high-temperature stress. In this study, based on unigene sequences of *P. haitanensis*, five full-length *PhHsp70* genes were obtained by rapid amplification of complementary DNA (cDNA) ends or direct PCR and named *PhHsp70-1*, *PhHsp70-2*, *PhHsp70-3*, *PhHsp70-4*, and *PhHsp70-5*. The full-length cDNA of the five *PhHsp70* genes comprised 2,249, 2,380, 1,945, 2,177, and 2,327 nucleotides, respectively. The cDNAs encoded proteins of 664, 668, 621, 654, and 686 amino acids, with isoelectric points of 4.93, 5.17, 4.87, 5.09, and 5.04, respectively. On the basis of conserved motifs and phylogenetic tree analysis, the five PhHsp70s could be divided into four subfamilies, two in the cytoplasmic subfamily and one for each in the chloroplast, mitochondria, and endoplasmic reticulum subfamilies. The expressions of the five *PhHsp70* genes, as measured by real-time quantitative PCR, were significantly induced by high-temperature stress but had different expression patterns under desiccation stress. These results suggested that PhHsp70s play important roles in the response to high-temperature stress but have no obvious functions in adaptation to desiccation stress, except under extreme desiccation conditions.

Keywords Desiccation stress · Heat shock protein 70 · High-temperature stress · qRT-PCR · *Pyropia haitanensis*

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Introduction

Pyropia, a genus of marine red algae, is among the most common algae in the intertidal zones of temperate rocky shores (Sutherland et al. 2011). It is considered a commercially valuable source of foods, fertilizers, medicines, and chemicals; farming and processing of *Pyropia* represent the largest aquaculture industry in East Asian countries, including China, Japan, and South Korea (Sahoo et al. 2002). Among the species of *Pyropia*, *Pyropia haitanensis* is endemic to China and has been commercially cultivated for more than 50 years. Currently, its output accounts for about 75 % of the total production of *Pyropia* in China (Ma and Xu 2005).

Pyropia species are not only economically important but also are research models for studying physiological ecology of intertidal seaweed communities (Blouin et al. 2011). The intertidal zones where the *Pyropia* blade grows are subjected to particularly severe conditions, with turning tides caused by diurnal and seasonal variations. The stressful environmental conditions include extreme temperatures, rapid nutrient and salinity changes, desiccation, intense sunlight, and various forms of pollution (Blouin et al. 2011). Therefore, adaptation and acclimation to these stressful environmental conditions are essential to the survival of *Pyropia* blades.

As sessile organisms that are not able to avoid exposure to adverse effects, plants have evolved intricate defense systems at multiple levels for adapting to environmental stressors. One important and universal behavior is the ability to synthesize and accumulate a small set of proteins called heat shock proteins (Hsps) that participate in the refolding and relocalization of proteins damaged by stresses (Al-Whaibi 2011). The Hsps represent a super gene family. On the basis of approximate molecular weight (MW), amino acid (AA) sequence homology and functions, Hsps can be divided into several families, including Hsp100, Hsp90, Hsp70, and

Hsp60, and small Hsps (sHsps; with MWs in the range of 12–43 kDa) (Gupta et al. 2010).

Among the Hsp families, the Hsp70 family is a major class that has been widely studied in many higher plants and algae. Hsp70s are encoded by highly conserved multigene families in plants, and different members of the *Hsp70* gene family encode proteins targeted to different cellular compartments, including the cytoplasm, chloroplast, mitochondria, and endoplasmic reticulum (ER) (Sung et al. 2001a). Recently, the completion of the genome sequences of some model plants and unicellular algae demonstrated that *Arabidopsis thaliana* harbors 14 *Hsp70* genes, comprising six cytoplasmic, three ER, two mitochondrial, and three chloroplast members (Lin et al. 2001). *Chlamydomonas reinhardtii* has nine *Hsp70* genes (two cytoplasmic and four mitochondrial or chloroplast members and three ER members) (Schroda and Vallon 2009). *Cyandioschyzon merolae* has four *Hsp70* genes (one each for cytoplasmic, ER, mitochondrial, or chloroplast members, and *Thalassiosira pseudonana* has five *Hsp70* genes (one each for cytoplasmic, ER, mitochondrial members, and two unknown, based on cDNA or genomic DNA annotation) (Renner and Waters 2007).

Hsp70s are involved in various cellular processes, including correct folding of de novo-synthesized proteins, protein transport across membranes, modulation of protein activity, regulation of protein degradation, and prevention of irreversible protein aggregation (Al-Whaibi 2011). The presence of Hsp70s and their induction by environmental stresses are key elements of the stress response system at the cellular level in divergent organisms, from prokaryotes to eukaryotes (Morris et al. 2013). Therefore, it is of great interest to evaluate the roles of Hsp70s in the adverse stress resistance mechanisms of *Pyropia*.

High temperature is one of the most important factors determining the yield and quality of *Pyropia*. High temperatures render *Pyropia* blades prone to disease, premature senility, and eventual decay, leading to a substantial reduction in yield (Yan et al. 2010; Xu et al. 2014). Desiccation is a daily stress factor of *Pyropia* blades in the turning tides. *Pyropia* blades demonstrate extreme tolerance to desiccation; they can survive a loss up to 85–95 % of their water during daytime desiccation when the tide is low (Blouin et al. 2011). To determine the role of Hsp70s in adaptation and tolerance mechanisms to high-temperature and desiccation stresses in *Pyropia* at the molecular level, cloning and sequencing the *Hsp70* genes from *Pyropia* genomic DNA or cDNA are useful. Three *Hsp70* genes, one *Hsp70* gene, and three *Hsp70* genes have already been cloned from cDNA of *P. yezoensis*, *P. haitanensis*, and *P. seriata*, respectively (Zhou et al. 2011; Liu et al. 2012; Park et al. 2012). The recent sequencing of the whole transcriptome of *P. haitanensis* allowed us to identify further *Hsp70* genes (Xie et al. 2013). Thus, the present study aimed to clone and sequence the five *Hsp70* cDNAs from *P. haitanensis* blades. In addition, to understand the relationship between physiological and molecular characteristics in response to temperature and desiccation stresses in the blades of *P. haitanensis*, the expression level of each *Hsp70* gene was investigated.

Materials and methods

Pyropia haitanensis strain Z-61, the seaweed material in this study, is tolerant to high temperatures and produces a high yield (Chen et al. 2008). It was selected and purified by the Laboratory of Germplasm Improvements and Applications of *Pyropia* in Jimei University, Fujian Province, China.

Under normal conditions, the blades of Z-61 were cultured in natural seawater with Provasoli's enriched seawater (PES) medium at 21 °C and illuminated by 50–60 μmol photons m^{-2} s^{-1} (10:14, L/D). The cultured medium was refreshed every 2 days.

Under high-temperature experiments, thirty 15±2 cm long blades of Z-61 were randomly selected and cultured in six aerated flasks (3,000 mL) containing five blades each at 29 °C (high temperature) and illuminated by 50–60 μmol photons m^{-2} s^{-1} (10:14, L/D) for 0, 3, 6, 12, 24, and 48 h.

In the desiccation experiments, twenty-five 15±2 cm long blades of Z-61 were randomly selected and separated into five groups, each with five blades. The blades of each group were desiccated in an incubator at 50–60 μmol photons m^{-2} s^{-1} and 21 °C. The desired levels of desiccation (0, 10, 40, 60, and 90 %) were obtained by varying the duration of exposure. Degrees of desiccation were expressed as percentage of water loss from the samples. The water loss (W_L, %) was estimated using the equation: $W_L=(W_0–W_t)/(W_0–W_d)\times 100$ % where W_0 is the initial wet weight measured after removing surface water drops by lightly blotting with tissue paper; W_t is the desiccated weight after a known time interval; and W_d is the dry weight (60 °C, 6 h).

In the rehydration experiment, five 15±2 cm long blades of Z-61 were first desiccated to a water loss of 90 %, and then recultured in culture medium for 30 min. Each treatment was carried out in three biological replicates.

RNA extraction and cDNA synthesis

Total RNA was isolated from each sample listed above. The collected samples were first cleaned with sterilized water. After drying with hygroscopic filter paper, the samples were ground into powder with liquid nitrogen. RNA was extracted and purified by an E.Z.N.A.™ Plant RNA Kit (Omega, Germany). The quality and quantity of the purified RNA were determined by measuring the absorbance at 260 nm/280 nm (A_{260}/A_{280}) and 260 nm/230 nm (A_{260}/A_{230}) using a Nanodrop ND-1000 spectrophotometer (LabTech, USA); only RNA

samples with an A_{260}/A_{280} ratio between 1.9 and 2.1 and an A_{260}/A_{230} ratio greater than 2.0 were used for subsequent analyses. The integrity of RNA sample was also assessed by 1.2 % agarose gel electrophoresis.

The cDNA used for 5′ rapid amplification of cDNA ends (RACE) and 3′ RACE were synthesized using a SMART RACE cDNA kit (Clontech Lab. Inc., USA). The first strand of cDNA used for quantitative real-time PCR (qRT-PCR) was synthesized with total RNA (1 μg) in each reaction using a PrimeScript RT reagent kit (TaKaRa, China) with 6-mer Random Primers, according to the manufacturer's protocol. All cDNAs were diluted with nuclease free water to 5 ng μL^{-1} to be used as templates in qRT-PCR.

Cloning of the full-length cDNA of *PhHsp70* genes

Among the unigene database of *P. haitanensis*, which was obtained from whole transcriptome sequencing, five sequences (Unigene6746, CL285.Contig2, Unigene3117, Unigene851, and Unigene11112) were identified as homologous to the conserved domains of *Hsp70* genes. These five sequences were used to amplify the full-length cDNA of the *PhHsp70* genes. Four head-to-toe primers, H702F, H702R, H703F, and H703R were used to amplify the full-length *PhHsp70-2* and *PhHsp70-3* cDNAs via PCR, respectively. Six gene-specific primers, GSP701-5′, GSP701-3′, GSP704-5′, GSP704-3′, GSP705-5′, and GSP705-3′ were used to amplify the 5′ and 3′ ends of the *PhHsp70-1*, *PhHsp70-4*, and *PhHsp70-5* genes via RACE using a SMART RACE cDNA kit (Clontech Lab. Inc.) (Table 1). All the processes were performed according to the manufacturer's protocol. The obtained PCR products were cloned into pMD19-T simple vector (TaKaRa) and transformed into competent *Escherichia coli* JM109 cells. The recombinants were identified through blue-white selection on ampicillin-containing Luria-Bertani plates and white colonies were verified via PCR with primers RV-M and M13-20. Positive clones were sequenced using an ABI Prism 3730 automated DNA sequencer. The sequence of each primer used in this study is shown in Table 1.

Sequence analysis

The obtained 5′ and 3′ end sequences were first assembled into the full-length cDNA of each *PhHsp70* gene using DNAMAN 5.2.2 software (Lynnon BioSoft). The obtained sequences were examined for identity with other known sequences using the BLAST program, available at the National Center for Biotechnology Information (NCBI) Website (http://www.ncbi.nlm.nih.gov/blast) and the deduced AA sequence was analyzed using the Conserved Domain of NCBI and the Expert Protein Analysis System (http://www.expasy.org/). Multiple sequence alignments were generated using Clustal X (Thompson et al. 1997) and DNAMAN. The neighbor-joining algorithm of the MEGA5.10 program (Tamura et al. 2011) was used to construct a phylogenetic tree with Poisson correction and pair-wise deletion parameters; all other parameters were set to default. A total of 1,000 bootstrap replicates were performed. The internet program PrediSi (http://www.pridisi.de/) was used to predict the signal peptide.

qRT-PCR analysis of *PhHsp70* genes expression

The expressions of *PhHsp70* genes under different stress treatments were measured by qRT-PCR. qRT-PCR reactions were performed in 96-well plates using an ABI 7300 Real-time PCR Detection system. No-template control (NTC) and no-reverse transcriptase (NRC) reactions were included in the same 96-well plates to confirm no target contamination and negligible genomic DNA contamination. Reactions were performed in 25-μL volumes containing 12.5 μL of 2× SYBR green Master Mix (ToYoBo, Japan), 0.25 μL (20 mM) of each sense and antisense primers, 2 μL of the diluted template, and 10 μL of RNA-free water. The ubiquitin conjugating enzyme (*UBC*) gene was used as an internal control gene. The sequence of each primer is shown in Table 1. The thermal profile for qRT-PCR was 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Dissociation curve analysis of the Amplicons was performed at the end of each PCR reaction to confirm that only one specific PCR product was amplified and detected. qRT-PCR was performed in triplicate for each sample. After the PCR program, the data were analyzed with the ABI optical system software. To maintain consistency, the baseline was set automatically by the software. All data are presented as means ± SE in terms of relative mRNA expression. The results were analyzed with the Student's *t* test, and $P<0.05$ was set as the level of statistical significance.

Results

Identification of the *PhHsp70* unigenes

Previously, high-throughput sequencing was used to identify 24,575 unigenes from one cDNA library constructed with mixed samples, including thallus (gametophytes) and free-living conchocelis (sporophytes) of *P. haitanensis* cultured under normal or different stress conditions (Xie et al. 2013). Functional annotation of each unigene allowed us to identify unigenes encoding putative Hsp70s. Among the putative *Hsp70* unigenes, five unigenes (Unigene6746, CL285.Contig2, Unigene3117, Unigene851, and Unigene11112) that had the longest sequences and the largest query scores were selected and used as core sequences to clone the full sequences of *PhHsp70* cDNAs. Homology

Table 1 Primers used in the present study

Purpose	Gene	Primer name	Sequences(5′–3′)	Annealing temperature (°C)	Size of product length (bp)
qRT-PCR	*PhHsp70-1*	Q701F Q701R	GATTGAGATTGACTCGCTGTTT TCAGGGTTGATGCTCTTCG	60	118
	PhHsp70-2	Q702F Q702R	GACGATGGCATCTTTGAGGT CCTTCTTGTTCTTCCGCTTG	60	255
	PhHsp70-3	Q703F Q703R	CAAGAAACACTACTATTCCGACAA GGTGCAGGCATAATACCATCTA	60	98
	PhHsp70-4	Q704F Q704R	TCAACTTGCCGTTCATCACT CCTTCAGGCACGACTTCATT	60	135
	PhHsp70-5	Q705F Q705R	GGGATTGAGACGGTTGGG TTGTCCTGGTAGGTCGTGAAG	60	160
	PhUBC	UBCF UBCR	TCACAACGAGGATTTACCACC GAGGAGCACCTTGGAAACG	60	107
Head-to-toe	*PhHsp70-2*	H702F H702R	CCACCGCTTCACACATACC GACCGACACAACGTGCTATG	60	2,380
	PhHsp70-3	H703F H703R	TAATGGGTAAAGTTGTTGGA CGGGTATAGCTCAGTGGTAG	60	1,945
RACE	*PhHsp70-1*	GSP701-5′	TCAGGGTTGATGCTCTTCG	60	1,259
		GSP701-3′	GATTGAGATTGACTCGCTGTTT	60	1,255
	PhHsp70-4	GSP704-5′	CCTTCAGGCACGACTTCATT	60	991
		GSP704-3′	GGTGCCTAAGGTCGTGGAG	60	1,127
	PhHsp70-5	GSP705-5′	CTCAACAGCGACCTCTTCCG	60	1,263
		GSP705-3′	ATGAGGCTGTGGCGTTTGGC	60	900
Validation of positive clones		RV-M M13-20	GAGCGGATAACAATTTCACACAGG CGACGTTGTAAAACGACGGCCAGT	58	–

searching using the BLASTX program showed that the five putative *PhHsp70* cDNAs encoded different PhHsp70s. The *PhHsp70* genes that were generated based on these unigenes were named *PhHsp70-1*, *PhHsp70-2*, *PhHsp70-3*, *PhHsp70-4*, and *PhHsp70-5*, respectively (Table 2).

Cloning and sequence analysis of PhHsp70 cDNAs

PhHsp70-1

On the basis of the sequence of Unigene6746, two gene-specific primers (GSP701-5′ and GSP701-3′) were designed to clone the partial cDNA of *PhHsp70-1*. Using SMART RACE technology, a fragment comprising 1,245 bps at the 5′ end and a fragment comprising 1,259 bps at the 3′ end (Fig. 1a) of the *PhHsp70-1* cDNA were obtained. The three fragments were then assembled into the full-length cDNA of *PhHsp70-1*, which comprised 2,249 bp (Additional file 1). ORF finder analysis showed that the *PhHsp70-1* cDNA comprised a 5′ untranslated region (UTR) of 115 bp, a 3′ UTR of 139 bp, and an open reading frame (ORF) of 1,995 bp. The ORF encoded a polypeptide of 664 AA with a predicted molecular mass (MM) of 71.7 kDa and a theoretical PI of 4.93 (Table 2).

PhHsp70-2 and PhHsp70-3

CL285.Contig2 and Unigene3117 included the full-length cDNA of *PhHsp70-2* and *PhHsp70-3*, respectively; therefore, four head-to-toe primers (H702F, H702R, H703F, and H703R) were used to amplify the full-length cDNAs of the two genes, respectively. The nucleotide sequence of *PhHsp70-2* was 2,380 bp (Additional file 1; Fig. 1b), comprising a 2,007-bp ORF encoding a protein of 668 AA with a predicted MM of approximately 72.0 kDa and a p*I* of 5.17 (Table 2). The sequence also contained a 224-bp 5′ UTR and a 149-bp 3′ UTR. The nucleotide sequence for *PhHsp70-3* was 1,945 bp (Additional file 1; Fig. 1c), comprising a 1,866-bp ORF encoding a protein of 621 AA with a predicted MM of approximately 67.7 kDa and a p*I* of 4.87 (Table 2). The sequence also contained a 2-bp 5′ UTR and a 67-bp 3′ UTR.

PhHsp70-4

Using the sequence of Unigene851, two gene-specific primers (GSP704-5′ and GSP704-3′) were designed to clone the partial cDNA of PhHsp70-4. Using SMART RACE technology, a 991-bp fragment from the 5′ end and a 1,127-bp fragment from the 3′ end (Fig. 1d) of the *PhHsp70-4* cDNA were

Table 2 Characterization of *PhHsp70* genes isolated from *Pyropia haitanensis*

Gene	Unigene ID	AA no.	MM (kDa)	p*I*	*E* value[a]	Description
PhHsp70-1	Unigene6746	664	71.7	4.93	0	Putative heat shock protein 70 (*Pyropia yezoensis*)
PhHsp70-2	CL285.Contig2	668	72.0	5.17	0	Putative heat shock protein 70-2 (*P. yezoensis*)
PhHsp70-3	Unigene3117	621	67.7	4.87	0	Heat shock protein 70 (*P. haitanensis*)
PhHsp70-4	Unigene851	654	69.1	5.09	0	*dnaK* gene product (*Sinorhizobium medicae*)
PhHsp70-5	Unigene11112	686	73.9	5.04	0	Heat shock protein 70 (*Cucumis sativus*)

AA amino acid
[a] BLASTP results using a deduced AA sequence

obtained. The three fragments were then assembled into the full-length cDNA of *PhHsp70-1*, which comprised 2,177 bp (Additional file 1). ORF finder analysis showed that the complete sequence of the *PhHsp70-4* cDNA comprised a 5′ UTR of 84 bp, a 3′ UTR of 128 bp, and an ORF of 1965 bp. The ORF encoded a polypeptide of 654 AA with a predicted MM of 69.1 kDa and a theoretical PI of 5.09 (Table 2).

PhHsp70-5

The sequence of Unigene11112 was used to design two gene-specific primers (GSP705-5′ and GSP705-3′) to clone the partial cDNA of PhHsp70-5. Using SMART RACE technology, a 1,263-bp fragment from the 5′ end (Fig. 1e) and a 900-bp fragment from the 3′ end (Fig. 1f) of the *PhHsp70-1* cDNA were obtained. The three fragments were then assembled into the full-length cDNA of *PhHsp70-5*, which contained 2,327 bp (Additional file 1). ORF finder analysis showed that the complete sequence of the *PhHsp70-1* cDNA comprised a 5′ UTR of 169 bp, a 3′ UTR of 97 bp, and an ORF of 2,061 bp. The ORF encoded a polypeptide of 686 AA with a predicted MM of 73.9 kDa and a theoretical PI of 5.04 (Table 2).

Multiple-sequence alignment and phylogenetic analysis of *PhHsp70* genes

Multiple-sequence alignments with the deduced AA sequence of the five PhHsp70s by Clustal X and DNAMAN program indicated that the sequence similarities (%) of the five PhHsp70s were between 38.2 and 77.3 %, with an average similarity of 48.3 % (Table 3). The C termini were more divergent, including deletions and substitutions; however, the N termini are relatively conserved (Fig. 2). Although the sequence similarities among of the five PhHsp70s are low, a BLASTP search of GenBank revealed that the five PhHsp70s were all highly similar to other members of the Hsp70 family. Analysis of the proteins using the conserved domain of NCBI and the ExPaSy Prosite Program showed that three conserved signature motifs of the Hsp70 family, IDLGTTYS, IFDLGGGTFDVSLL, and VLLVGGMTRVPKVVE, were present in all the PhHsp70 sequences. A conserved EDID motif, which is similar to the EEID motif found in cytoplasmic Hsps, was detected in the C termini of PhHsp70-1 and PhHsp70-2, and a conserved KEEL motif, the ER targeting sequence, was found at the C terminus of PhHsp70-5 (Fig. 2). The PrediSi program predicted that PhHsp70-5 harbors a

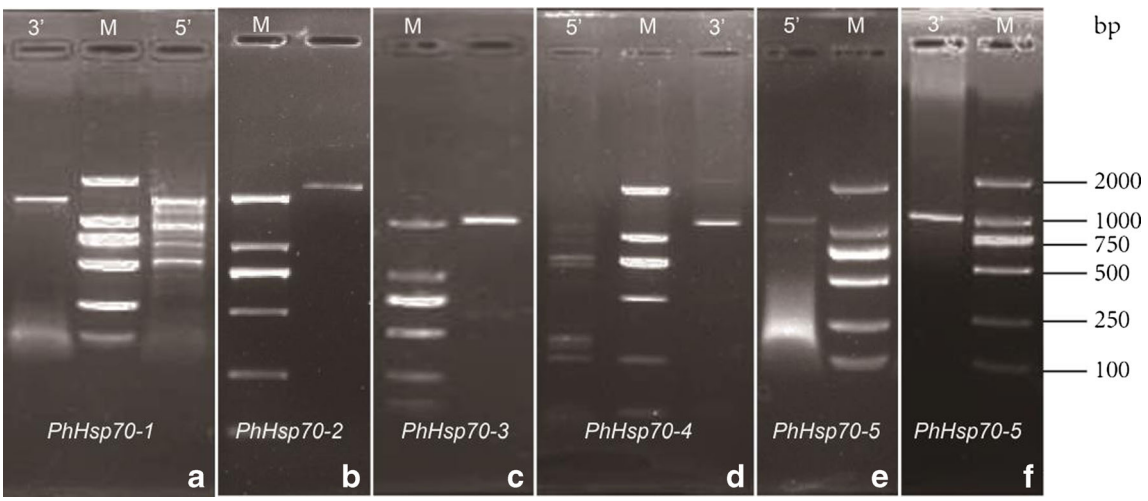


Fig. 1 Agarose gel electrophoresis of RACE or head-to-toe products of *PhHsp70* genes. M: DL2000 marker

Table 3 Sequence similarity (%) among PhHsp70s

	PhHsp70-1	PhHsp70-2	PhHsp70-3	PhHsp70-4
PhHsp70-2	77.3			
PhHsp70-3	43.7	44.7		
PhHsp70-4	42.8	43.3	48.4	
PhHsp70-5	51.9	52.3	40.6	38.2

putative signal peptide at its N terminus (Fig. 2). These results indicate that PhHsp70-1 and PhHsp70-2 may be located in the cytosol and PhHsp70-5 may be transported into subcellular organelles, perhaps the ER.

To further analyze the sequence homology and predict the subfamily of each PhHsp70, the full-length amino-acid sequences of the five PhHsp70s, together with other published Hsp70 sequences from higher plants and algae, were used to construct a phylogenetic tree. MEGA 5.10 was used to construct the neighbor-joining phylogenetic tree (Fig. 3). The phylogenetic tree consisted of four branches, with the Hsp70 family members of the different intracellular compartments divided into four different clusters (cytoplasm, ER, mitochondria, and chloroplast). As expected, PhHsp70-1 and PhHsp70-2 clustered together with cytoplasmic Hsp70s, and PhHsp70-3, PhHsp70-4, and PhHsp70-5 were grouped with the chloroplast, mitochondria, and ER Hsp70 families, respectively (Fig. 3). Thus, the phylogenetic tree and the analysis of conserved motifs in the PhHsp70s indicated that PhHsp70-1 and PhHsp70-2 are cytoplasmic Hsp70s, and PhHsp70-3, PhHsp70-4, and PhHsp70-5 belong to the chloroplast, mitochondria, and ER subfamilies, respectively.

Expression of *PhHsp70* genes under different levels of stress

Information on the expression of the *PhHsp70* genes would promote a better understanding of their physiological functions. Therefore, the relative expression of the *PhHsp70* genes under different levels of high-temperature stress and different levels of desiccation was measured by qRT-PCR. In the qRT-PCR, NTC and NRC reactions consistently demonstrated lack of target contamination and negligible genomic DNA contamination. The amplification specificity for each *PhHsp70* gene and *UBC* gene was determined by analyzing the dissociation curves of PCR products. There was only one peak in the dissociation curve for the *PhHsp70* and *UBC* genes (Additional file 2).

First, the relative expressions of each *PhHsp70* gene at different time points under high-temperature stress (29 °C) were measured. During high-temperature stress, the expression levels of all five *PhHsp70* genes increased significantly after 3 h of stress, reaching their maximum levels at 6 h (*PhHsp70-1*, *PhHsp70-2*, *PhHsp70-4*, and *PhHsp70-5*) or 12 h (*PhHsp70-3*). Their expression levels then decreased

Fig. 2 Multiple alignment of AA sequences of five PhHsp70 using the Clustal X program. The three conserved signature motifs are *boxed*. The conserved AA motif, EDID, for the cytoplasmic group, is marked in *bold* in PhHsp70-1 and PhHsp70-2. The conserved AA motif, KEEL, for the ER targeting sequence, is marked in *bold* in PhHsp70-5. The putative signal peptide is *underlined* in PhHsp70-5. *Asterisks* and *colons* indicate identical and similar AA residues, respectively

significantly, reaching normal levels after being cultured at a high temperature for 24 h ($P>0.05$) (Fig. 4a–e). However, as the high-temperature stress continued to 48 h, the expression levels of all five *PhHsp70* genes increased again (Fig. 4a–e). The results also showed that the expression levels of *PhHsp70-2* were weak under normal conditions but were induced strongly by high-temperature stress, by up to 69-fold compared with the normal level after only 3 h of stress (Fig. 4b).

Second, the relative expressions levels of each *PhHsp70* gene under different levels of desiccation and in rehydration for 30 min were also measured. The expression level changes of the five *PhHsp70* genes were different. During desiccation and rehydration, the expression level of *PhHsp70-1* was not significantly different compared with the normal level ($P>0.05$) (Fig. 4f). For *PhHsp70-2* and *PhHsp70-3*, their expression levels were not significantly different from the normal level when the water loss was <60 %; however, when the water loss was >60 %, their expression levels increased significantly ($P<0.05$). In addition, the expression level of the two genes did not return to the normal level after rehydration in culture medium for 30 min (Fig. 4g, h). For *PhHsp70-4*, its expression level was significantly increased after 10 and 40 % water loss ($P<0.05$); however, as the water loss continued, the expression level return to the normal level ($P>0.05$) (Fig. 4i). The expression level of *PhHsp70-5* was significantly decreased after 10 % water loss ($P<0.05$). However, during the subsequent desiccation and rehydration process, the expression level did not change significantly ($P>0.05$) (Fig. 4j).

Discussion

Sessile organisms have adapted to various harsh habitats and developed complex defense mechanisms at multiple levels to withstand environmental stresses. The expressions of stress response genes are a key part in the cellular defense mechanism. Several studies have confirmed that the rapid expression of *Hsp70* genes and synthesis of their respective protein products can be induced and increased not only by high-temperature stimulation, but also by various environmental stressors, such as osmosis, heavy metals, and pathogens (Morris et al. 2013). This suggested that Hsp70s may represent the interaction point between multiple stress response pathways. Hsp70s have also received attention in marine organisms as a kind of biomarker of stress, because their

PhHsp70-1 ----------MADKKDVPSGPS 12
PhHsp70-2 ----------MASGPA 6
PhHsp70-5 MMRAGSLAPRRVRPCGGRSLRRIVAAAAVAATVALAVTTLVARARGESEVDLDEEDKGPV 60
PhHsp70-3 ----------MGKV 4
PhHsp70-4
PhHsp70-1 IGIDLGTTYSCVGIWQNDRVEIIANDQGNRTTPSFVAFT--DSERLIGDAAKNQVAINAT 70
PhHsp70-2 IGIDLGTTYSCVGYWADDRVEIIANDQGNRTTPSFVAFT--TTERLVGDAAKNQVAVNAA 64
PhHsp70-5 IDIDLGTTYSCVGVVENGEVTIIANDQGNRITPSYVSFSGDSTDRLIGDAAKNQAPMNPS 120
PhHsp70-3 VGIDLGTTNSVIAVMEGGKPTVIPNAEGFRTTASVVAYTK-SGDKLVGQIARRQAVINPE 63
PhHsp70-4 ----------MQGSEPRVIENAEGMRTTPSVVAFSA-DGEKLVGVPARRQAVTNPE 45
PhHsp70-1 NTVFDAKRMIGRKFSDPSVQQDMKHFSFKVVQKEGDKPVVEVLYKGETKQFSPEEISAMV 130
PhHsp70-2 NTVFDVKRLIGRHFSESSVQRDMKHFSFAVVAKEGDKPFVQVEYKGATKQFSPEEISAMV 124
PhHsp70-5 NTVFDVKRLIGRKFSDPTVQRDKKLLPFSIVNKDG-KPMVEVSVNKAKKVMSPEEVSAMV 179
PhHsp70-3 NTFYSVKRFIGRKQ--NEISQEIRQTSYNVKTS---GSSIKIACPALDKDFAPEEISAQV 118
PhHsp70-4 NTLFATKRLIGRRYDDNKTQEDIRQMPFKIVK----GPNGDAWVSAGGDNYSPSQVGAFV 101
PhHsp70-1 LSKMREVAESYLGKEVKTAVVTVPAYFNDSQRLATKDAGTIAGLHVSRIINEPTAAAIAY 190
PhHsp70-2 LAKMREVAESYLGKEVKDAVVTVPAYFTDSQRQATKDAGVIAGLNVSRIINEPTAAAIAY 184
PhHsp70-5 LSKMKKTAEEYLGKEVTNAVVTVPAYFTDAQRQATKDAGQIAGLNVMRIINEPTAAAIAY 239
PhHsp70-3 LRKLVEDASTYLGETVTQAVITVPAYFNDSQRQATKDAGKIAGLDVLRIINEPTAAPLSY 178
PhHsp70-4 LQKMRETAEAFLGRKVSSAVVTVPAYFNDSQRQATKDAGKIAGLNVARIINEPTAASLAY 161
PhHsp70-1 GLDRKTAGDTAL-ERTVLIFDLGGGTFDVSLLSIEDGIFEVKATAGDTHLGGEDFDNRLV 249
PhHsp70-2 GLDQKKASGTGRGERMVLIFDLGGGTFDVSLLSIDDGIFEVKSTAGDTHLGGEDFDNRLV 244
PhHsp70-5 GLNKK-------GEKNILVFDLGGGTFDVSLLTIEDDVFEVLATNGDTHLGGEDFDQRVM 292
PhHsp70-3 GLDKQ-------NNETILVFDLGGGTFDVSVLEVGDGVFEVLSTSGDTHLGGDDFDQQIV 231
PhHsp70-4 GMDKA-------DGKLIAVYDLGGGTFDVSILEISGGVFEVKSTNGDTHLGGEDFDNALL 214
PhHsp70-1 NHFSAEFKRKNKKDMSNNPRSLRRLRTACERAKRTLSSATQTTIEIDSLFEGVD----FY 305
PhHsp70-2 AHLASEFKRKNKKDLTGNARAMRRLRTACERAKRTLSSATQTSIEVDSLFDGVD----FY 300
PhHsp70-5 DYFIKLFKRKYSKDMSGDKRAVGKLRREVEKAKRALSSQTQVRVEIESLFDGQD----LS 348
PhHsp70-3 EWLIKDFKQNEGIDLAKDRQALQRLTEAAEKAKIELSNLTQTEINLPFITATQDGPKHLE 291
PhHsp70-4 DFLVGEFKKDQGIDLSRDKLALQRSREAAEKAKIELSSALQTDINLPFITADAGGPKHMN 274
PhHsp70-1 SSLSRAKFEELCMDLFRGTLEPVEKVLRDAKAAKGDVDDIVLVGGSTRIPKVQSLLVDFF 365
PhHsp70-2 TSISRAKFEELCMDLFKSTMQPVEKVLKDAGVSKAQVDDIVLVGGSTRIPKVQSLLVDFF 360
PhHsp70-5 ETLTRARFEELNSDLFRKTLKPVEKVLKDAAMSKAKVDEIVMVGGSSRIPKVQELLRGYF 408
PhHsp70-3 KTVTRGKFEELCSNLIDKCSIPVNNALKDAKLEASSIDEVVLVGGSTRIPAIQQMVKRLI 351
PhHsp70-4 MKLTRAKFESLVDPLVSRTLAPMKSCLKDAGVSSKDISDVLLVGGMTRVPKVVETVKDFF 334
PhHsp70-1 NGKELSKSINPDEAVAYGAAVQAAILAGDTSERTQDLLLLDVAPLSLGIETAGGVMTPLI 425
PhHsp70-2 NGKELCKSINPDEAVAYGAAVQAAILSGVTSEHTKDLLLLDVAPLSLGIETAGGVMTPLI 420
PhHsp70-5 DGKELNKGVNPDEAVAFGAAVQGGILS--NEGGSAGVVLLDVAPLSLGIETVGGVMSKII 466
PhHsp70-3 -GKDPNQSVNPDEVVAIGAAVQAGVLAG----EVKDILLLDVTPLSLGVETLGGVMTKII 406
PhHsp70-4 -GKEPSKGVNPDEVVAMGAAIQGGVLRG----DVKDILLLDVTPLSLGLETLGGVFTRLI 389
PhHsp70-1 KRNTTIPTKKSQIFSTYADNQTGVLIQVYEGERTRTKDCGLLGKFELTGIPPAPRGQPQL 485
PhHsp70-2 KRNTTIPTKKQQIFSTYADNQPGVLIQVYEGERARTKDCGLLGKFDLSGIPPAPRGQPQI 480
PhHsp70-5 ERNTVIPTRKTQTFTTYQDKQTTVSIQVYEGERAMTKDNRQLGKFDLNGIPAAARGTPQI 526
PhHsp70-3 PRNTTIPTKKSEVFSTAVDNQPNVEIQVLQGERELTKDNKSLGTFRLDGIMPAPRGVPQI 466
PhHsp70-4 NRNTTIPTKKSQVFSTAADGQTQVGIKVGQGERDMFQDNSILGSFDLVGIPPAPRGVPQV 449
PhHsp70-1 EVVFDVDANGILNVSATDKSTGKQNKITITNDKGRLSKEQIEAMVQEAEKYKAEDEAAQA 545
PhHsp70-2 EVTFDMDANGILNVSAADKSTGKVNKITITNDKGRLSKEEIERMVNEAESFKDEDEKARA 540
PhHsp70-5 EVTFEVDSNGILAVSAEDKASSAKEAITITNDKGRLSKEEIERMVKEAEEFADADAEVKR 586
PhHsp70-3 EVTFDIDANGILSVKAKEKATGKEQSITISGA-STLPKDDVERMVKEAEENFDTDQKRRK 525
PhHsp70-4 EVTFDIDANGILHVSARDKATNKQQSIQIQSS-GGLSDAEVSEMVKRAEAYAQEDKQRRE 508
PhHsp70-1 KIEARNGLENYCYNLKNTTVGDATVKEKLSEDDAKAIETIVADTLAWLE-SNDNAEKEE- 603
PhHsp70-2 RIEARNGLEAYLYNVKNSTLSDKAVKDKLSEEDASTLERTINNALEWLE-SHDMSDKED- 598
PhHsp70-5 KVEGKNSLETAIFSAKAAVEEGGSLVDKASEEDKESLKAAVKEAQDWLDESGEDATADD- 645
PhHsp70-3 NIDTKNQAESLCYQAEKQIK---EFEDKISQDLKTKIEELITELRSSLEKEE--YENIES 580
PhHsp70-4 GIEAKNDADSLLYSTEKTLS---EHREKMPADDVAAVESAMTALRAEVEKEDPSTKELKD 565
PhHsp70-1 YDAKLKEVEAVAHPIMTKMYAAGGG-----APGGMP-----DMAGGMGGAGAAPMDTGGA 653
PhHsp70-2 YERKQSEVQEVVNPIMTRFHQSTGGGGAGGMPGGMPGGMPGGMPGGMPGGGAAPMDTSGS 658
PhHsp70-5 LEGKLKDLQEIIQPIMAKGGAAGGG----------SGAA---QGGE 678
PhHsp70-3 ISQRLQNSLMDIGKMAAQAESKNTNTKDDG----------TV 612
PhHsp70-4 LTDALQKASMKVGEAMYKASQAANASPSDGGSGAADAGAAGEGGSKADGAKAGGAKADGA 625
PhHsp70-1 DNPDITVEDID---------- 664
PhHsp70-2 D-PNITVEDID---------- 668
PhHsp70-5 E--DDSKEEL---------- 686
PhHsp70-3 IDTDFSEAK---------- 621
PhHsp70-4 QEAEFRDADADSEGVKDSKDKKASGGGSA- 654

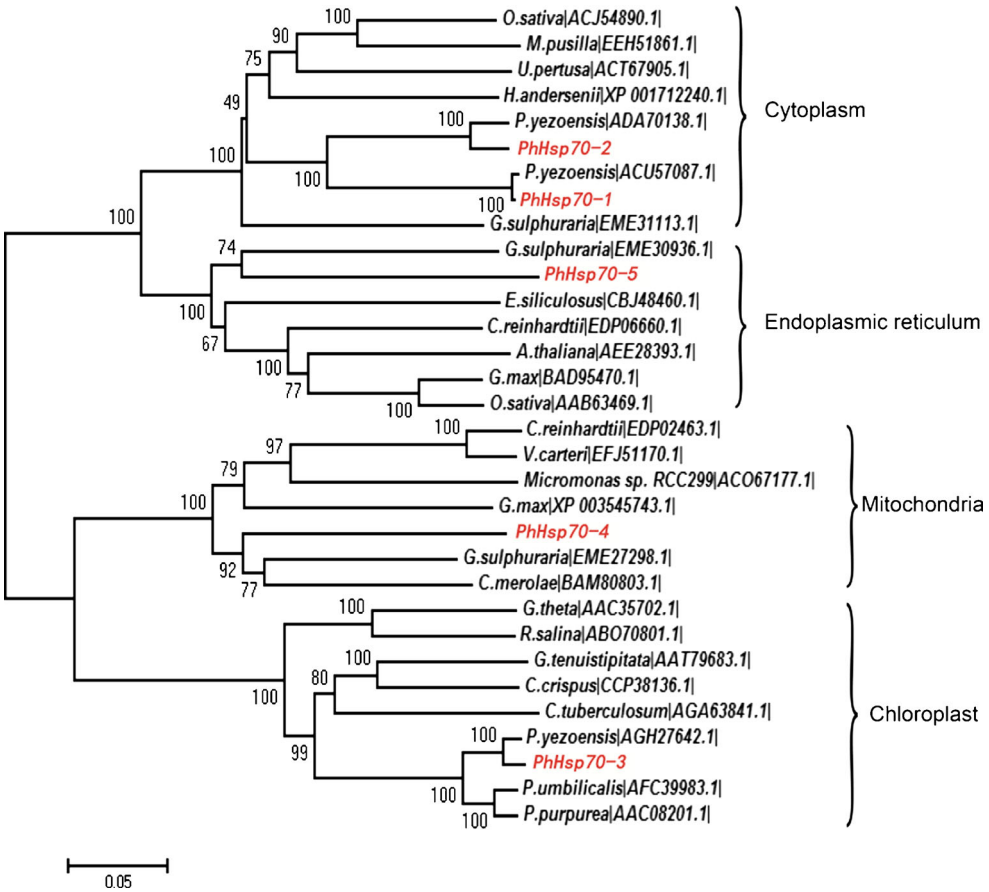


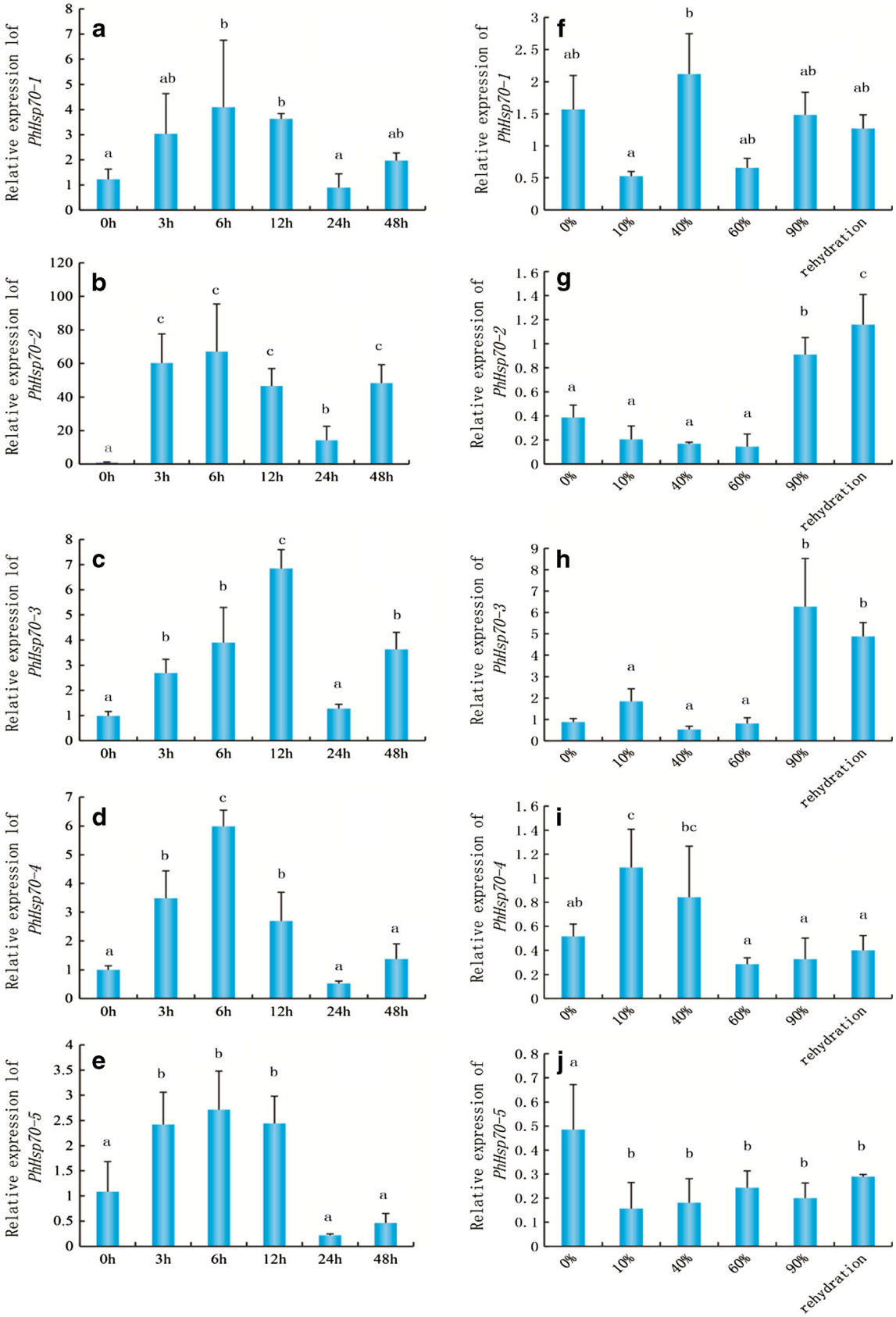
Fig. 3 Phylogenetic tree constructed using Hsp70 AA sequences. Putative Hsp70 sequences were aligned using Clustal X and analyzed with the MEGA 5.10 program by the neighbor-joining method

expression is highly variable in the presence or absence of stimuli (Downs et al. 2000; Hamer et al. 2004; Ireland et al. 2004; Torres et al. 2008; Fu et al. 2009). The first step in the study of the function of Hsp70s under environmental stresses is to clone its members in various organisms. In the present study, using RACE or direct PCR with primer-based *PhHsp70* unigene sequences, five members of the *Hsp70* family were cloned in *P. haitanensis*. Based on conserved motifs and a phylogenetic tree analysis, the five PhHsp70s could be divided into four subfamilies (two cytoplasmic members and one each for the chloroplast, mitochondria, and ER subfamilies). These results are similar to those reported for Hsp70s in the genomes of *Ostreococcus lucimarinus*, *Ostreococcus tauri*, *T. pseudonana*, and *C. merolae*. Renner and Waters (2007) found that each species of these unicellular algae had four or five Hsp70s, with at least one belonging to the chloroplast, mitochondrial, ER, and cytoplasmic subfamilies.

There is accumulating evidence that the structural organization of Hsp70s is evolutionarily conserved. In general, all Hsp70s possess three signature motifs and a highly conserved motif in their C terminus. The motif in C terminus for the cytoplasmic subfamily is EEVD or EEID, and for the ER subfamily is HDEL or KEEL (Sung et al. 2001a). In this study, the AA sequence comparisons revealed that all typical signatures motifs were present in these PhHsp70s. In addition, PhHsp70-1 and PhHsp70-2 both have the cytoplasmic Hsp70 C-terminal motif EEID, which is involved in the cooperation with chaperone cofactors (Hartl 1996). PhHsp70-5 contains a highly conserved "KEEL" ER targeting sequence that is common for soluble ER-localized proteins and inhibits its exit from the ER (Munro and Pelham 1987).

Hsp70s have more in common with their homologs from different species than with other members of the Hsp70 protein family expressed in the same organism (Daugaard et al. 2007). This was true for the present study. The average sequence similarity among the five PhHsp70s was only 48.3 %. PhHsp70-1 and PhHsp70-2 cluster with known cytoplasmic Hsp70s, PhHsp70-3, PhHsp70-4, and PhHsp70-5 were grouped with chloroplast, mitochondrial, and ER Hsp70s from other species, respectively. Although the five *Hsp70* genes come from *P. haitanensis*, they belong to different branches in the phylogenetic tree in relation to their subcellular localization (Fig. 3).

Fig. 4 Relative expression levels of *PhHsp70* genes under different level ▶ of stress. **a**–**e** Relative expression levels of *PhHsp70* genes during different periods of high-temperature stress. **f**–**j** Relative expression levels of *PhHsp70* genes under different levels of desiccation and in rehydration for 30 min. *Bars of each column with different small letters* indicate significant differences



Although each member of the *PhHsp70* gene family shares a highly conserved structure, each member of the *PhHsp70* gene family may play distinct roles in response to environmental stresses. Accumulated evidence suggests that

regulation and expression levels of HSPs contribute to the thermotolerance of organisms (Grover et al. 2013). In this study, the five *PhHsp70* genes all exhibited similarity expression profiles during high-temperature stress; their expression levels all significantly increased under high-temperature stress, and the highest expression levels were all detected after 6 h (*PhHsp70-1*, *PhHsp70-2*, *PhHsp70-4*, and *PhHsp70-5*) or 12 h (*PhHsp70-3*) exposure and then decreased. Similar gene expression patterns were detected in *P. yezoensis* (Zhou et al. 2011) and *Laminaria japonica* (Fu et al. 2009). These data suggested that all the PhHsp70s serve general functions as Hsp70s under thermal stress. According to these results, we hypothesized that the expressions of *PhHsp70* genes are feedback regulated by high-temperature stress. During high-temperature stress, when protein unfolding increases and non-native proteins accumulate, the expressions of the *PhHsp70* genes are upregulated to prevent unfolding and to stabilize partially unfolded intermediates, thereby maintaining cell homeostasis. As the level of unfolding and non-native protein decreased, the expressions of *PhHsp70* genes are downregulated.

However, the expression patterns of the five *PhHsp70* genes under desiccation stress are different. During desiccation, the expression level of *PhHsp70-1* did not significantly change, the expression level of *PhHsp70-5* was significantly decreased, the expression levels of *PhHsp70-2* and *PhHsp70-3* were significantly increased only when the water loss was >60 %, and the expression level of *PhHsp70-4* was significantly increased after 10 and 40 % water loss, but as the water loss continued, the expression level returned to normal levels (Fig. 4f–j). These results indicated that the PhHsp70s have no obvious functions in adaptation to desiccation stress, except for PhHsp70-2 and PhHsp70-3 under extreme desiccation stress and PhHsp70-4 under low desiccation stress. Desiccation stress as a daily phenomenon that *Pyropia* blades should face, the *Pyropia* do not use the Hsp70 defense system to resolve it till the last extremity may be because the overexpression of Hsp70s can cause some deleterious effects in organisms (Krebs and Feder 1998; Sørensen et al. 2003; Morris et al. 2013). It is very important to keep the balance between benefits and costs for *Pyropia* blades, therefore the *Pyropia* blades must employ other more economic and effective mechanisms to resolve it, but the mechanisms are unclear.

Several studies showed that the cytoplasm of higher eukaryotes generally contains both stress-inducible *Hsp70s* and constitutively expressed *Hsp70s* (Lin et al. 2001; Sung et al. 2001b; Fu et al. 2009). The constitutively expressed *Hsp70s* are frequently involved in the folding of de novo synthesized polypeptides and the import or translocation of precursor proteins (Frydman 2001). Other members that respond to environmental stress may be involved in refolding stress-denatured proteins and proteolytic degradation of non-native proteins (Wang et al. 2004). The five cytoplasmic *Hsp70* genes in *A. thaliana* are well characterized, three (*AtHsc70-1*, *AtHsc70-2*, and *AtHsc70-3*) are expressed at a basal level in the vegetative organs, whereas two (*AtHsp70* and *AtHsp70b*) are highly heat-induced genes (Lin et al. 2001; Sung et al. 2001b). In this study, *PhHsp70-1* was constitutively expressed under normal, high-temperature, and desiccation stress conditions, indicating that it is a constitutively expressed member, although its expression upregulated during high-temperature stress (Fig. 4a, f). *PhHsp70-2* may be a stress-inducible member, because its expression level was strongly induced by high-temperature and extreme desiccation stress conditions (water loss, >60 %). The results suggest that *PhHsp70-2* is more sensitive to stress and may be more important for protection from stress than *PhHsp70-1*.

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