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The heat shock protein 70a from *Pyropia seriata* increases heat tolerance in *Chlamydomonas*

San Choi[1], Sun-Hee Kim[1], Sung Oh Im[1], Won-Joong Jeong[2], Mi Sook Hwang[3], Eun-Jeong Park[3], Dong-Woog Choi[1,*]

[1]Department of Biology Education and Kumho Life Science Laboratory, Chonnam National University, Kwangju, 500-757, Korea
[2]Plant Systems Engineering Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon, 305-806, Korea
[3]Marine Algae Research Institute, National Fisheries Research and Development Institute, Mokpo, Korea

***Corresponding author: dwchoi63@jnu.ac.kr**

Abstract

The heat shock protein 70 (HSP70) molecular chaperones constitute a large family of highly conserved proteins, which are key survival components under stress and normal physiological conditions. *Pyropia seriata* grows on intertidal rocks, where it is exposed to environmental changes including desiccation and temperature variations. We identified five *HSP70* cDNAs from *P. seriata* transcriptome. An amino acid sequence analysis suggested that *PsHSP70a* and *PsHSP70d* are cytosolic *HSP70s*, whereas *PsHSP70b* and *PsHSP70c* are in the mitochondria and *PsHSP70e* may be transported into the endoplasmic reticulum. Most of the *PsHSP70* genes were detected under normal growth and heat stress conditions, except *PsHSP70c*. Among them, *PsHSP70a* displayed the strongest response to heat stress. *PsHSP70a*-transformed *Chlamydomonas* showed much higher survival and growth rates than those of wild-type under high temperature conditions. These results indicate that the *Pyropia* genome contains at least five *HSP70* genes, and that cytosolic *PsHSP70a* is involved in high temperature stress tolerance.

Keywords: Red algae, *Pyropia seriata*, Heat shock protein 70, Heat tolerance, Transgene.
Abbreviations: HSP_heat shock protein; HSF_heat shock factor; HSE_heat shock element; qRT-PCR_ quantitative reverse-transcription-polymerase chain reaction; ORF_open reading frame; GFP_green florescent protein; ER_endoplasmic reticulum; CBF_C-repeat-binding factor; DRE_drought response element; ABRE_abscisic acid responsive element; ABF_ABRE binding factors.

Introduction

Pyropia species are important marine macro red algae and have been proposed as model plants to study the mechanisms underlying abiotic stress tolerance (Blouin et al., 2011). *Pyropia* grow naturally in the intertidal zone, where they are frequently exposed to a variety of potentially stressful environmental conditions with the turning tides (Sahoo et al., 2002). These changes include variations in humidity, from drying to partial or total immersion; changes in temperature, from heat stress to freezing during hibernal low tides; osmotic shock, and different light intensities (Hwang et al., 1997).
Heat shock proteins (HSPs) are a group of proteins induced by harmful environmental conditions, including abiotic and biotic stressors. HSPs have been found in virtually all living organisms, from bacteria to animals and plants, and are named according to their molecular weight: HSP100, HSP90, HSP70, HSP60, and small HSP. The presence of HSPs and their induction by environmental stressors are a central tolerance mechanism in many organisms (Feder and Hofmann 1999; Ireland et al., 2004). Among all HSPs, the HSP70 family is large and highly conserved in prokaryotes and eukaryotes (Karlin and Brocchieri 1998; Feder and Hofmann 1999; Renner and Waters 2007). HSP70 proteins perform essential roles in folding of newly translated proteins, repair of misfolded proteins, and transport of nascent proteins across membranes into organelles, as well as help target damaged proteins for degradation (Nelson et al., 1992; Hartl 1996; Fink 1999). Much of the structural and functional information about how HSP70s work comes from studies of *Escherichia coli* DnaK, which is an HSP70 considered to be the archetype for all HSP70s. HSP70s have two major functional domains, such as an ATPase domain in the N-terminal region and a peptide-binding domain in the C-terminal portion of the protein. ATP binding causes a conformational change in the ATPase domain, which is then transduced to the peptide-binding domain, causing opening of the binding cleft through lifting of the α-helical trap door lid. In this open structural arrangement, peptide binding and release occur rapidly. In addition, a C-terminal subdomain is necessary in many HSP70s for certain co-chaperone interactions. An N-terminal transit peptide in the variable sequence is present in the precursor form of those members destined for organelles. Plant HSP70s have been found in the cytosol, endoplasmic reticulum (ER), mitochondria, chloroplast, and peroxisome (Lin et al., 2001). Another feature of plant HSP70s is the presence of identifiable C-terminus motifs that can be used to distinguish the protein's subcellular location. The motif for the cytosolic group is EEVD, whereas that for the ER is HDEL (Lin et al., 2001; Sung et al., 2001a). Plant *HSP70* genes are encoded by a highly conserved multi-gene family. Fourteen genes encode HSP70 proteins in *Arabidopsis*, and at least 12 are present in spinach (Guy and Li 1998; Lin et al., 2001; Sung et al., 2001a). Of the 14 genes in Arabidopsis, 12 encode full-length proteins. Sequence analyses of the *HSP70*

gene family have revealed four major subgroups (Guy and Li 1998; Sung et al., 2001a), each localized to one of the major subcellular compartments of cytosol, ER, plastids, and mitochondria. The conservation of *HSP70* genes in plants is astonishing in that the least conserved plant cytosolic member among 23 full-length sequences shares no less than 75% amino acid identity with all other plant HSP70 sequences. In addition, the mitochondrial and plastid members show higher sequence relatedness to the bacterial HSP70 homolog DnaK, whereas the ER luminal member binding protein (BiP) and HSP70s targeted to other compartments are more closely related to cytosolic HSP70s (Wimmer et al., 1997). A unique feature of the plant *HSP70* gene family is the presence of multiple ER luminal members. Five genes encoding BiP have been found in tobacco, four in soybean, three in Arabidopsis, and two in maize (Denecke et al., 1991; Cascardo et al., 2000; Sung et al., 2001a). A hallmark of many *HSP70* genes is their strong and universal induction in response to heat shock. Plant *HSP70s* show strong and rapid induction at 30 min to 2 h after exposure to 37–45℃ (Sung et al., 2001a). At least one Arabidopsis *HSP70* gene shows as much as 20-fold induction in response to heat shock at 40℃. Several plant *HSP70s* are also induced by cold shock (Li et al., 1999). Induction of *HSP70s* in response to heat is mediated by heat shock transcription factors (HSFs). Active trimeric HSFs bind to heat shock elements (HSEs), which are present in the promoters of many heat-inducible genes, including *HSP70s*. Expression of *HSP70s* and other heat-shock genes in response to heat shock is autoregulated by HSF/HSP70 interactions (Schoffl et al., 1998). HSP70 either remains free or binds to and inhibits HSF under non-heat shock conditions. The inhibition of HSF involves not only HSP70, but also HSP90 (Zou et al., 1998). The recent transcriptome sequencing of the marine red algae, *Pyropia,* has allowed for the identification of abiotic stress tolerance genes in the Rhodophyta genome. Herein, we describe the isolation and characterization of the cDNAs encoding HSP70 from *Pyropia seriata* (*PsHSP70a*). Five *HSP70* cDNAs from the *P. seriata* transcriptome were isolated and characterized, and cellular localization and physiological functions of the *PsHSP70a* were analyzed.

Results and Discussion

Identification of the P. seriata HSP70 gene family

The transcriptome sequences from *P. seriata* gametophyte thalli have been generated under normal growth and heat stress conditions (Kim et al., 2011; Im et al., 2015), and attempts were made to identify the differentially expressed genes (DEG) under heat stress conditions. Among the identified DEGs, the most popular and well-known genes were heat shock proteins (HSPs), which constitute a large and highly conserved family in eukaryotes and prokaryotes and are highly involved in the heat shock response (Nelson et al., 1992; Hartl 1996; Karlin and Brocchieri 1998; Feder and Hofmann 1999; Fink 1999; Renner and Waters 2007). The transcriptome sequences were screened for HSP70 members, and 42 contigs encoding putative HSP70s were obtained. The open reading frame (ORF) findings and results from homolog searches showed that many of the contig sequences were truncated at the 5′- or 3′-regions. Finally, five contig sequences covering full ORF were selected (Table 1). These results indicate that there are more than five *HSP70* genes in the *P. seriata* genome. The cDNAs corresponding to the selected contigs were cloned by polymerase chain reaction (PCR) with gene specific primers, sequenced, and named *PsHSP70a, PsHSP70b, PsHSP70c, PsHSP70d*, and *PsHSP70e* (Genebank accession no. KP241940 for *PsHSP70a*, KP241941 for *PsHSP70b*, KP241942 for *PsHSP70c*, and KP241943 for *PsHSP70e*). Plant *HSP70* genes are encoded by a highly conserved multi-gene family. There are 14 genes encoding HSP70 proteins in *Arabidopsis* and nine in *Chlamydomonas*, which is a single cell green algae (Lin et al., 2001; Schroda and Vallon 2009). The full genome sequences of several red algae have been reported. Gene identification results from the KEGG database (http://www.genome.jp/kegg/catalog/org_list.html) showed at least one *HSP70* gene in *Cyanidioschyzon merolae*, a unicellular extremophile red algae, and three in *C. crispus*, a multicellular marine red algae.

Characterization of PsHSP70

The characteristics of the five PsHSP70 polypeptides are summarized in Table 1. All PsHSP70 polypeptides had molecular weights of about 70 kDa and isoelectric points of 4.98–7.51. The PsHSP70 polypeptides also harbored the ATPase domain and substrate binding domain found in HSP70s. Amino acid sequence alignment of the PsHSP70 polypeptides showed sequence variations, including deletions and substitutions, particularly in the N- and C-termini (Fig. 1). Prediction of the signal peptides using the PrediSi program (www.pridisi.de) demonstrated that PsHSP70b harbors a putative signal peptide in the N-terminal region for mitochondria, as reported previously (Park et al., 2012). However, a signal peptide sequence was not detected in PsHSP70e, which has a long N-terminal region upstream of the ATPase domain, nor was one detected in PsHSP70a, PsHSP70c, or PsHSP70d. PsHSP70a and PsHSP70d harbored an EDID motif at the C-terminus, which was similar to the EEVD motif found in *Arabidopsis* cytosolic HSP70 (Lin et al., 2001; Sung et al., 2001a). PsHSP70e harbored a KEEL motif at the C-terminus, which was similar to the HDEL motif found in *Arabidopsis* ER HSP70 (Lin et al., 2001). All HSP70 polypeptides from *Arabidopsis* and *Chlamydomonas,* as well as three HSP70s from *Pyropia yezoensis* were used to construct a phylogenetic tree using the Clustal X program to predict the subgroup of each PsHSP70 identified. The phylogenetic tree grouped PsHSP70a and PsHSP70d with the cytosolic HSP70s from *Arabidopsis* and *Chlamydomonas*, whereas PsHSP70b and PsHSP70c were clustered with mitochondrial HSP70, and PsHSP70e was grouped with the ER HSP70, respectively (Fig. 2). These results indicate that PsHSP70a and PsHSP70d are cytosolic HSP70s, PsHSP70b and PsHSP70c are mitochondrial, and PsHSP70e is based in the ER. However, no mitochondria or ER signal peptide sequence was detected in PsHSP70c or PsHSP70e (Fig. 1). Therefore, the cellular localization of these PsHSP70s should be confirmed using a reporter gene system, such as the green fluorescent protein (GFP). HSP70s have long been recognized as one of the most conserved protein families. They can be divided into DnaK- and HSP110-type HSP70s, and are found in the cytosol, ER, mitochondria, chloroplast, and peroxisomes. The higher plant *Arabidopsis thaliana* harbors 18 HSP70 genes, 14 of which are in the DnaK-subfamily and the remaining four are in the HSP110-subfamily (Fig. 2). Among the 14 DnaK-type HSP70s, six are located in the cytosol, three in the ER, two in the mitochondria, and three in plastids (Lin et al., 2001). The phylogenetic tree of HSP70s showed that all of the *Pyropia* HSP70s belonged to the DnaK subfamily, whereas none were grouped with plastid HSPs. These results demonstrate that there are additional HSP70 genes, including those in the plastid HSP70 and HSP110-type subfamilies, in the *Pyropia* genome that were not identified

Table 1. Summary of the *Pyropia seriata* heat shock protein 70 *(HSP70)* gene family.

PsHSP70	No. of amino acid residues	Molecular weight (kDa)	Isoelectric point (IP)	Target description*
PsHSP70a	664	71.8	4.93	Putative heat shock protein 70-2 [*Pyropia yezoensis*]
PsHSP70b	736	76.9	5.28	Heat shock protein 70 [*Ostreococcus tauri*]
PsHSP70c	662	72.4	6.29	MitochondrialHSP70 [*Trypanosoma congolense*]
PsHSP70d	673	71.6	5.17	Putative heat shock protein 70 [*Pyropia yezoensis*]
PsHSP70e	720	78.6	7.51	Heat shock protein 70 [*Suberites domuncula*]

* Best deduced amino acid sequence Blastp results

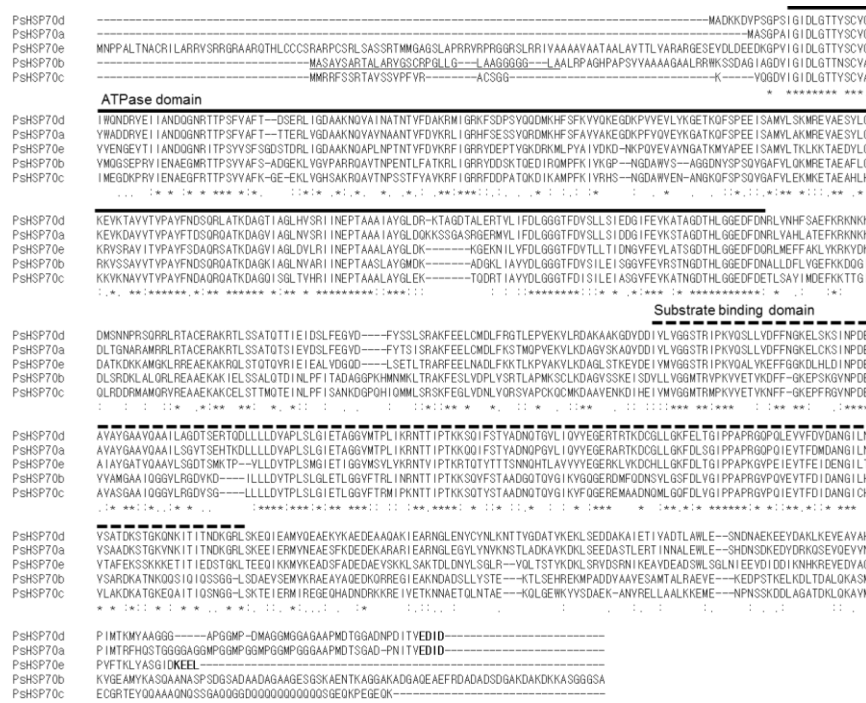

Fig 1. Amino acid sequence alignment of the *Pyropia seriata* heat shock protein (HSP) 70s. Amino acid residues are marked with single letters. The putative signal peptide is underlined, and the ATPase and substrate binding domains are marked above with linear and dotted lines, respectively. The conserved amino acids motif, HDEL, for the endoplasmic reticulum (ER) group and the EEVD or EEID motifs for the cytosolic group are marked in bold. Asterisk (*) and : indicate identical and similar amino acid residues, respectively. The deduced amino acid sequences were aligned using the ClustalX program.

here. Some of the *Pyropia* contigs sequences containing a partial ATPase domain or substrate binding domain may have the truncated sequence of the other HSP70 members, including plastid HSP70 or HSP110-type HSP70.

Expression of PsHSP70 genes

A hallmark of many *HSP70* genes is their strong and universal induction in response to heat stress. Several plant *HSP70s* are also induced by other biotic and abiotic stressors, such as drought and cold (Li et al., 1999). Total RNA was isolated after *P. seriata* was subjected to heat, desiccation, and freezing, and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analyses were conducted to evaluate the expression of each *PsHSP70* gene. Figure 3A shows the expression of each *PsHSP* gene under heat, desiccation and freezing stress conditions. *PsHSP70a*, *PsHSP70b*, and *PsHSP70d* responded to heat stress (Fig. 3A). Interestingly, *PsHSP70d* was also expressed in response to desiccation and freezing stress, as well as heat. However, *PsHSP70a* and *PsHSP70b* did not respond to desiccation stress. *PsHSP70a* demonstrated the strongest response to heat stress. *PsHSP70a* transcripts responded quickly and increased more than 250-fold 4 hr after heat treatment (Fig. 3B). The *PsHSP70c* transcript was not detected in the *P. seriata* gametophyte after exposure to any of the stressors; however, it was detected in the genomic DNA template. These results indicate that *PsHSP70c* is expressed at relatively low levels or at different life stages, either in a tissue-specific manner or under stimuli not tested herein. In addition, *PsHSP70e* was detected at low levels and demonstrated a downregulated response to heat, desiccation and freezing stress. *HSP70* genes are generally encoded by multiple genes; some *HSP70* family members are constitutively expressed, whereas others are expressed only in response to heat and/or other abiotic stressors (Fu et al., 2009; Tominaga et al., 2010). The constitutively expressed *HSP70s* frequently assist in the folding *of de novo* synthesized polypeptides and import or translocation of precursor proteins (Frydman 2001; Wang et al., 2004). The other family members that respond to environmental fluctuations may facilitate refolding or proteolytic degradation of non-native proteins (Hartl 1996; Miernyk 1997). The *P. seriata* heat response *PsHSP70a, PsHSP70b*, and *PsHSP70c*

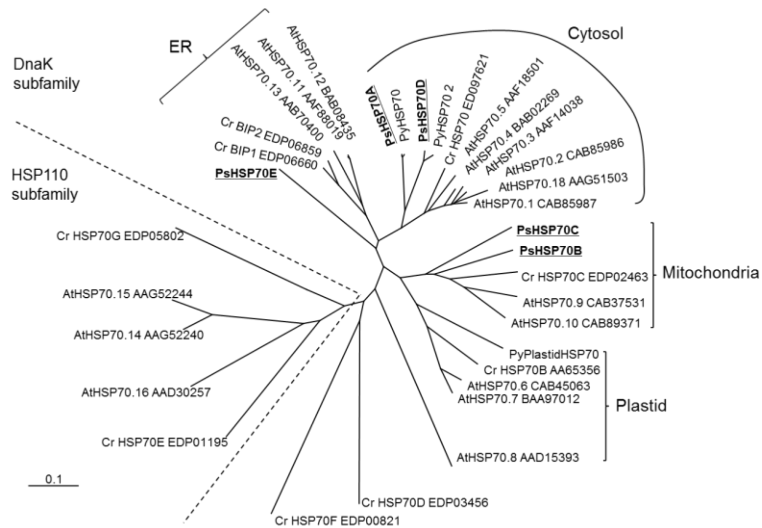

Fig 2. Phylogenetic tree of the *P. seriata* heat shock protein (HSP) 70s with HSP70s from *Arabidopsis thaliana*, *Chlamydomonas reinhardtii*, and *P. yezoensis*. The phylogenetic tree of the HSP70 family members was constructed via the unrooted neighbor-joining method using the ClustalX program. Accession numbers of the HSP70s from *Arabidopsis* and *Chlamydomonas* follow the name of each HSP.

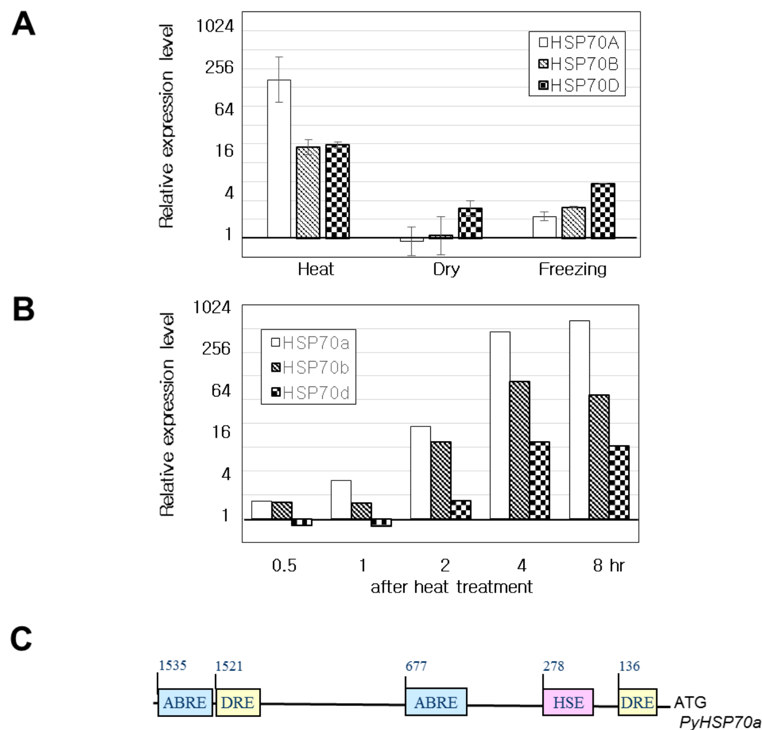

Fig 3. Expression pattern of the *PsHSP70s*. A, Total RNA was purified from gametophyte thalli under heat, desiccation, or freezing stress conditions and used for qRT-PCR. B, Gametophyte thalli were transferred to a 25°C incubator and harvested at 0.5, 1, 2, 4, and 8 hr intervals to determine the *PsHSP70* gene expression patterns under heat stress condition. C, Putative cis-elements in the *PyHSP70a* promoter region. The approximately 1,500 bp upstream from the start codon of *PyHSP70a* was analyzed for putative regulatory sequence elements. The consensus sequences identified were CCAATT for the heat shock element (HSE), ACGTGT/GC for the ABA responsive element (ABRE), and CCGAC for the drought response element (DRE). Numbers on the boxes indicate the nucleotides relative to the site of the start codon.

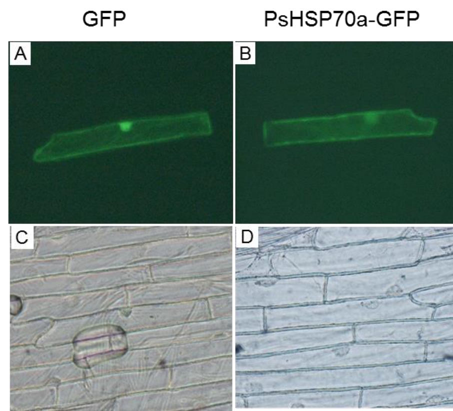

Fig 4. Cellular localization of PsHSP70a in *Allium cepa*. A reporter gene encoding the green fluorescent protein GFP was fused to *PsHSP70a* under control of the CaMV 35S promoter to identify the cellular location of the PsHSP70a proteins. The constructs were introduced into *A. cepa* epidermal cells. The 326-GFP vector served as a control (A, C). After overnight induction, individual cells were observed for the location of PsHSP70a by GFP fluorescence (B, D). A and B, cell image with green filter. C and D, contrast interference imagery for the whole-cell structure.

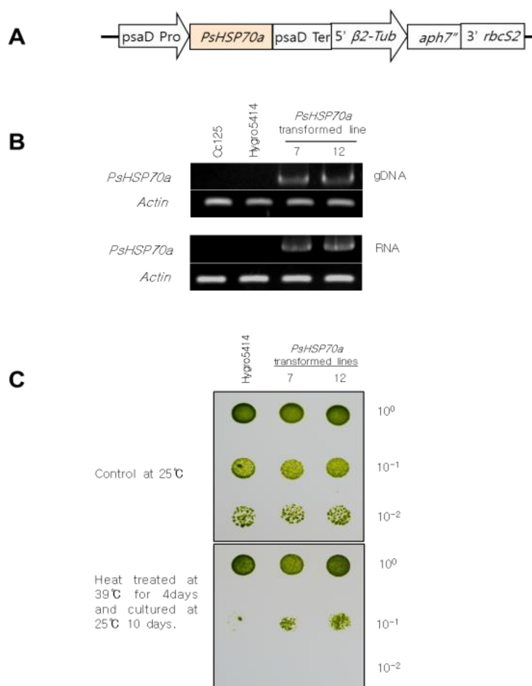

Fig 5. Effect of *PsHSP70a* on growth of *Chlamydomonas* under heat stress conditions. A. Vector map of *PsHSP70a* to express in *Chlamydomonas reinhardtii*. B, Introduction and expression of the *PsHSP70a* gene in transformed *Chlamydomonas.* The *PsHSP70a* were checked by PCR using genomic DNA (gDNA) and RNA from the transformed *Chlamydomonas*. The actin gene was used as an internal control. cc-125, wild-type; Hyg5414, vector control. C, Survival and growth assay of wild-type and transformed *Chlamydomonas* under heat stress conditions. *Chlamydomonas* was heat treated to 39°C for 4 days and transferred to a 25°C growth chamber and cultured for 10 days. Approximately 10^7 cells/ml were diluted 10^0 to 10^{-2} in fresh medium, and 8 µl of the diluted cells were used to inoculate agar plates.

genes may be involved in response to heat tolerance. It was previously reported that *Chlamydomonas* transformed with the *PsHSP70b* gene shows increased cell growth and survival under heat stress conditions (Park et al., 2012). In the present study, we determined whether *PsHSP70a* is involved in heat tolerance. The *PyHSP70a* promoter sequence was isolated from *Pyropia yezoensis*. A promoter analysis of the 5′-upstream region of > 1,500 bp to identify the *cis*-acting elements revealed a HSE 278 bp from the start codon (Fig. 3C). This result indicates that induction of the *PsHSP70a* gene via heat may be mediated by the heat shock factor (HSF), as in green plants and animals. HSF homologs from marine red algae, *Chondrus crispus*, support that HSF works as a heat-inducible transcription factor in Rhodophyta (Collen et al., 2013). However, *HSF* genes from the *Pyropia* genome have not been reported. It was interesting that abscisic acid (ABA) responsive elements (ABRE) and drought responsive elements (DRE) are found in the up-stream sequence of *PsHSP70a*. Stress-related gene expression is induced by the binding of the C-repeat-binding factor (CBF) to the *cis*-acting DRE in the promoters of relevant genes (Yamaguchi-Shinozki and Shinozaki 2005). ABRE act as binding sites for transcription factors ABF (a family of ABRE binding factors), which play an important role in the environmental stress and ABA gene responses in higher plants (Choi et al., 2000). ABA occurred in all red algae tested, including *Porphyra* (Yokoya et al., 2010). Despite detecting endogenous ABA and identifying the ABRE and DRE *cis*-acting elements in the upstream sequence of the genes, no information is available on ABF or CBF homologs in red algae.

Localization of PsHSP70a

The *PsHSP70a* ORF sequence was fused upstream of a reporter gene encoding GFP in the plant expression vector p326-35S-*GFP* (*GFP*) to determine the cellular location of the PsHSP70a protein. The recombinant vector, p326-35S-*PsHSP70a-GFP* (*PsHSP70a-GFP*), was then subjected to a transient assay using *Allium cepa* epidermal cells (Fig. 4). The p326-*GFP* vector showed GFP signals in both the cytoplasm and nucleus (Fig. 4A and C). The p326-*PsHSP70a-GFP* showed GFP signals in the cytosol (Fig. 4B and D), suggesting that PsHSP70a is located in the cytosol of *Pyropia* cells, and that the EDID motif at the C-terminal end is in the cytosol, similar to the EEVD motif in green plants. GFP signals were also detected in the nucleus as well as cytosol in onion epidermal cells harboring the *PsHSP70a-GFP* gene. The KRKNKK putative nuclear signal was detected between the ATPase and subtract-binding domains. These results suggest that PsHSP70a may be located in the cytosol and nucleus. Arabidopsis HSP70-1/HSC70-1 is located in both the cytosol and nucleus in Arabidopsis (Noel et al., 2007). PsHSP70a may interact with some other factor, resulting in co-localization in the cytosol and nucleus as observed for HSC70-1 in Arabidopsis (Noel et al., 2007). HSP70s localized in different subcellular compartments participate in different cellular processes. The specific roles of individual HSP70 proteins are likely to be determined by their locations in the different subcellular compartments, by differential expression in specific cells and at different developmental stages or via interactions with specific sets of HSP70-associated proteins (Michaud et al., 1997; May and Soll 2000; Sung et al., 2001b). PsHSP70a plays a role in heat tolerance in the cytosol and nucleus of *Pyropia*.

Expression of the PsHSP70a gene increased thermotolerance in Chlamydomonas

Among the *PsHSP70s* identified, *PsHSP70a* demonstrated the strongest heat stress response. The complete *PsHSP70a* coding sequence was introduced into *C. reinhardtii* strain cc-125 to assess the physiological functions of the *PsHSP70a* gene under heat stress conditions (Fig. 5A). Two *PsHSP70a*-transformed *Chlamydomonas* lines, line #7 and #12 were selected on media containing hygromycin, and introduction and expression of the *PsHSP70a* gene were confirmed via PCR and RT-PCR (Fig. 5B). In order to assess the thermotolerance conferred by *PsHSP70a*, Hyg5414 and transformed *Chlamydomonas* lines were subjected or not (control) to a 39°C for 4 days and then they were transferred to a 25°C incubator. All *Chlamydomonas* cells had grown to similar levels as those observed on control culture plates (Fig. 4C). However, the two transformed *Chlamydomonas* lines showed a much higher growth rate than that of the Hyg5414 line when heat stress was applied (Fig. 4C). These results demonstrate that *PsHSP70a* contributes to heat stress tolerance in *Chlamydomonas*. Overexpression of cytosolic *HSP70s* in plants is correlated with enhanced stress tolerance, including that from heat (Sugino et al., 1999; Ono et al., 2001; Cazale et al., 2009). Overexpression of DnaK from cyanobacteria (*Aphanothece halophytica*) enhances high temperature and salt stress tolerance in transgenic tobacco plants (Sugino et al., 1999; Ono et al., 2001). Constitutive overexpression of cytosolic HSP makes *Hsc70-1* plants more tolerant to heat shock but also appears to cause dwarfism in Arabidopsis (Sugino et al., 1999; Sung and Guy 2003; Cazale et al., 2009). Among all molecular chaperones, the HSP70s constitute a large highly conserved family in organisms that are essential for cell viability. Despite their physiological importance, information and functional studies on *HSP70* genes in Rhodophyta, including marine red algae, are very limited. Here, we present five *HSP70* genes from the marine red algae *Pyropia seriata*. Among them, *PsHSP70a* showed the strongest response to heat stress and was located in the cytosol and nucleus. Constitutive expression of the *PsHSP70a* gene in *Chlamydomonas* demonstrated that this *PsHSP70a* is involved in enhanced tolerance to high temperature.

Materials and Methods

Plant materials and growth condition

Pyropia seriata leafy gametophytes were cultured in modified Grund medium (McLachlan 1973) at 10°C under irradiation with 80 μmol photon m^{-2} s^{-1}, provided by cool-white fluorescent lamps on a 14:10 (light: dark) photoperiod in a growth room. Growth bottles containing *P. seriata* were transferred to a 25°C growth chamber with the same light intensity and photoperiod for the heat treatment. *Chlamydomonas reinhardtii* strain cc-125 (mt+) was grown in Tris-acetate-phosphate (TAP) medium at 25°C with shaking at 100 rpm under continuous cool fluorescent light (50 μmol photon m^{-2} s^{-1}). Desiccation and freezing stress were applied as described by Im et al. (2015).

PsHSP70 cDNA isolation and sequence analysis

Expressed sequence tags (ESTs) and RNA sequence reads generated from *P. seriata* gametophyte thalli were analyzed for the *HSP70* gene family (Kim et al., 2011; Im et al., 2015). The contigs encoding putative *HSP70s* were analyzed to determine if they covered the full open reading frame (ORF). Finally, five contigs encoding putative full *HSP70* ORFs were selected and cloned for further analysis. Gene-specific primer sets were designed for each contig to clone the *HSP70* cDNA, and the PCR products were cloned into the pGEM T-Easy vector (Promega, Madison, WI, USA). Plasmid DNA was purified using a Qiaquick Plasmid Extraction Kit (Qiagen, Hilden, Germany) and sequenced. Sequence editing and amino acid sequence prediction from the selected ESTs were conducted using the Sequencher program (Gene Code Corp., Ann Arbor, MI, USA). The putative molecular weights and PI values of the deduced polypeptides were predicted using the Compute pI/Mw program (http://web.expasy.org/compute_pi/). The deduced amino acid sequences were aligned using the ClustalX program (http://www.ebi.ac.uk/clustalw), which was also used to construct the phylogenetic tree. Conserved motifs or domains were predicted using the Prosite program (http://expasy.org/prosite).

Gene expression analysis

Gene-specific qRT-PCR was conducted to assay *PsHSP70* gene expression patterns. Total RNA was prepared from gametophyte thalli using a Plant RNeasy kit (Qiagen, Hilden, Germany). First-strand cDNA was constructed from about 2 μg of total RNA via reverse-transcription in 20 μL reaction volumes, using the oligo$(dT)_{17}$ primer, amfiRivertII cDNA Synthesis 2× Reaction Buffer with the oligo$(dT)_{17}$ primer, and the amfiRivertII cDNA Synthesis Enzyme Mix (GenDepot, Barker, TX, USA), according to the manufacturer's instructions. The reactions were conducted for 5 min at 25°C and 60 min at 50°C, followed by 15 min of heating at 70°C. The first-strand cDNA reaction was diluted by a factor of 5, and 2 μL of the dilution was applied for PCR-amplification in a reaction containing 5 μL of 10× PCR buffer (200 mM Tris-HCl, pH 8.4, and500 mM KCl), 1 μL 10 mM dNTPs, 1 μL of each gene-specific primer (10 μM), and 2.5 units of ExTaq DNA polymerase (Takara, Shiga, Japan). The PCR reactions were carried out for 35 cycles of 30 sec at 95°C, 30 sec at 58–64°C, depending on the primers, and 90 sec at 72°C, followed by 5 min of termination at 72°C. The *PsHSP70* gene-specific primers were as follows: 5′-GAGGTACAGGAGGTGGTCAAC-3′ and 5′-TCCTCCACCGTGATGTTGGGGTC-3′ for *PsHSP70a;* 5′-CTTTAATGATTCCCAGCGGCAG-3′ and 5′-TCTCCAGAATGCTCACGTCAAA-3′ for *PsHSP70b;* 5′-GTCCTCGAGAAGATGAAGGAGA-3′ and 5′-GGAGATATCGAAGGTACCACCA-3′ for *PsHSP70c;* 5′-GGTATCTTTGAGGTGAAGGCCA-3′ and 5′-AAAGAGCGAGTCAATCCAATCG-3′ for *PsHSP70d*; 5′-GCGCACTCGTCTCTATTTTTG-3′, and 5′-CCGTTCTCGTTTGTGTCTCC-3′ for *PsHSP70e.* The PCR products were separated on 1% agarose gels and stained with ethidium bromide for imaging.

The qRT-PCR was carried out on a Rotor-Gene RG-3000 cycler (Corbett, Sydney, Australia) using the QuantiTect SYBR Green PCR kit (Qiagen), according to the manufacturer's instructions. The qRT-PCR program consisted of a pre-denaturation step at 95°C for 10 min and 40 cycles of amplification at 95°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec. All samples were run in duplicate and n-fold differential expression was calculated using the comparative Ct method, $2^{-\Delta\Delta Ct}$. The *TubA1* (5′-ACAACGAGGCGATTTACGATA-3′ and 5′-ATAGGAGGAGAGCATGAAGTGG-3′) gene was used as an internal control.

Cellular localization of PsHSP70a

The *PsHSP70a* coding region was amplified via PCR with ExTaq DNA polymerase (Takara, Shiga, Japan) using forward (5′-TCTAGATGGCCTCGGGTCCGGCGATTG-3′) and reverse (5′-GGATCCAGTCAATATCCTCCACCGTGATGTT-GGGGT-3′) primers containing an *Xba*I site upstream and a *Bam*HI site downstream and was introduced into the *Xba*I /*Bam*HI site of the 326 GFP-3G vector. Recombinant DNA was introduced into onion epidermal cells via particle bombardment, according to the method of Ha et al., (2007). The onion cells were examined under a fluorescent microscope to evaluate GFP expression (Axioskop, Carl Zeiss, Jena, Germany).

Heat tolerance assay for the PsHSP70a gene in Chlamydomonas

The *PsHSP70* gene ORF was amplified via PCR and subcloned under the *PsHSP70a* pCr112 vector using the NdeI and EcoRV sites. The pCr112-*PsHSP70a* plasmid was introduced into *Chlamydomonas* strain cc-125 via the glass bead method for transformation (Kindle 1990). Transformants were selected on TAP agar medium containing 10 μg mL^{-1} hygromycin after 7–14 days of growth. *Chlamydomonas* genomic DNA was purified from 100-ml liquid cultures and used for PCR with the *PsHSP70a*-specific primer set to confirm insertion of the *PsHSP70a* gene into the *Chlamydomonas* genome. Total RNA was isolated from control and transformed cells and applied to RT-PCR to assess *PsHSP70a* gene expression in transgenic *Chlamydomonas* cells. The *actin* (5'-TGTGCATACGTGGATAGCTTG-3' and 5'-ATGACCCGCT-CCTCATATCTT-3') gene was used as an internal control. *Chlamydomonas* cells were grown in TAP medium containing hygromycin at 25°C under a 14 h d^{-1} photoperiod. The cells were first grown at a concentration of $2–4 \times 10^6$ cells mL^{-1} and concentrated to 10^7 cells mL^{-1} before dilution to 10^0– 10^{-2} in fresh medium to evaluate high temperature tolerance. The diluted cells (8 μL) were used to inoculate agar plates, the cells were exposed to heat stress during a 4-day incubation at 39°C, and transferred to a 25°C growth chamber under a 14 h d^{-1} photoperiod. *C. reinhardtii* strain cc-125 and the transformed cells with the pCr102 vector, Hyg5414, were used as controls.

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