Characterization and expression profiles of small heat shock proteins in the marine red alga *Pyropia yezoensis*

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Abstract

Small heat shock proteins (sHSPs) are found in all three domains of life (Bacteria, Archaea, and Eukarya) and play a critical role in protecting organisms from a range of environmental stresses. However, little is known about their physiological functions in red algae. Therefore, we characterized the sHSPs (PysHSPs) in the red macroalga *Pyropia yezoensis*, which inhabits the upper intertidal zone where it experiences fluctuating stressful environmental conditions on a daily and seasonal basis, and examined their expression profiles at different developmental stages and under varying environmental conditions. We identified five PysHSPs (PysHSP18.8, 19.1, 19.2, 19.5, and 25.8). Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis showed that expression of the genes *PysHSP18.8*, *PysHSP19.5*, and *PysHSP25.8* was repressed at all the developmental stages under normal conditions, whereas *PysHSP19.1* and *PysHSP19.2* were overexpressed in mature gametophytes and sporophytes. Exposure of the gametophytes to high temperature, oxidative stress, or copper significantly increased the mRNA transcript levels of all the five genes, while exogenous application of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) significantly increased the expression levels of *PysHSP19.2*, *PysHSP19.5*, and *PysHSP25.8*. These findings will help to further our understanding of the role of PysHSP genes and provide clues about how *Pyropia* species can adapt to the stressful conditions encountered in the upper intertidal zone during their life cycle.

Keywords *Pyropia yezoensis* · Small heat shock proteins · Red algae · Abiotic stress · Plant growth hormone

Introduction

Molecular chaperones perform important functions in protein–protein interactions, including assisting with proper protein folding, stabilizing partially unfolded proteins, and preventing irreversible protein aggregation (Hasanuzzaman et al. 2013). Heat shock proteins (HSPs), which can function as molecular chaperones, are induced by heat and other related stresses, and are found in all the three domains of life, from

bacteria to humans (Jacob et al. 2017). Five families of HSPs have been defined in plants and other organisms according to their molecular sizes: Hsp100, Hsp90, Hsp70, Hsp60, and small HSPs (sHSPs/HSP20). Among these HSP families, the low-molecular-weight sHSPs (12–43 kDa) have the greatest expression during heat stress, reaching > 1% of the total cell protein concentration in some cells (DeRocher et al. 1991).

sHSPs have the ability to construct dynamic oligomers, the disassembly of which is usually required for effective chaperoning. sHSP monomers are composed of a conserved α -crystalline domain (ACD) and enriched β -strands organized in a β -sheet sandwich that are responsible for dimer formation (Waters 2013). sHSPs can bind to denatured proteins as chaperones in an ATP-independent pattern, protecting them from aggregation to maintain cellular homeostasis, and also play a role in various physiological processes, such as cell proliferation and differentiation, development, and cytoskeletal organization (Carra et al. 2017). They can be induced in plants and animals under various environmental stress conditions, such as heat or cold, oxidative stress, and heavy metal exposure (Sun et al. 2002; de Thonel et al. 2012), and it has

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also been shown that, in higher plants, plant growth regulators (PGRs) such as abscisic acid (ABA) and salicylic acid (SA) regulate sHSP genes (Ham et al. 2013; Yang et al. 2014).

Genome sequence analyses have revealed that sHSPs are a diverse and complex family of proteins in various eukaryotic organisms. For example, 10 sHSPs have been identified and characterized in the human genome and 18 have been found in the nematode *Caenorhabditis elegans* (Kappé et al. 2003; Aebermann and Waters 2008). Furthermore, 19, 23, and 36 sHSP genes have been identified in the terrestrial plant genera *Arabidopsis*, *Oryza*, and *Populus*, respectively, and it has been shown that plant sHSPs are encoded by six nuclear multigene families that occur in distinct cellular compartments (i.e., the cytosol, chloroplast, endoplasmic reticulum, and mitochondrion) (Waters 2013). In addition, six sHSP genes have been found in the genome of the green alga *Chlamydomonas reinhardtii* (Waters and Rioflorida 2007). However, the sHSP family appears to be less diverse in red algae than in other eukaryotic organisms. For example, two sHSP genes that are expressed at the survival threshold temperature have been reported in the unicellular red alga *Cyanidioschyzon merolae*, which inhabits sulfate-rich hot springs (Kobayashi et al. 2014), and five and six sHSPs have been found in the genomes of multicellular red algae *Porphyra umbilicalis* and *Chondrus crispus*, respectively, which inhabit the intertidal zone (Collén et al. 2013; Brawley et al. 2017).

Red algae are evolutionarily distant from green plants, which evolved from green algae and consist of terrestrial plants, and have a fossil record that dates back 1.2 billion years (Butterfield 2000), making the studies on the mechanisms that regulate their stress response of critical importance for the understanding of eukaryotic evolution. Red algae are important components of many marine ecosystems, including rocky intertidal shores, with the red alga *Pyropia* (formerly *Porphyra*) being one of the most important marine crops worldwide, with a value of over US\$1.3 billion per year (Sahoo et al. 2002; Blouin et al. 2011). In addition to its economic importance, *Pyropia* appears to be a suitable species for elucidating the molecular mechanisms that regulate environmental stress responses in marine red algae because it can inhabit the stressful upper intertidal zone with its rapidly changing physical conditions caused by the turning tides and, therefore, has high levels of tolerance to various abiotic stresses, such as temperature, desiccation, and osmotic stress (Blouin et al. 2011).

Transcriptome analysis has revealed the upregulation of HSPs, including sHSPs, under high-temperature conditions in *Pyropia* species (Park et al. 2012; Sun et al. 2015), and it has also been shown that the overexpression of sHSPs from *Pyropia tenera* enhances heat stress tolerance in *C. reinhardtii* (Jin et al. 2017). Furthermore, proteome analysis has shown that HSP22 is upregulated under desiccation stress in *Pyropia orbicularis* (López-Cristoffanini et al. 2015). However,

information about the physiological function of sHSPs in *Pyropia* is currently lacking.

The aim of the present study was to identify and characterize the sHSPs (hereafter PysHSPs) in *Pyropia yezoensis*, cultivated in Japan as the food “nori.” Then, we examined the transcriptional profiles of these PysHSP genes at different developmental stages and in response to abiotic stresses such as temperature, oxidative stress, and copper, and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC). The findings of this study will help to further our understanding of the important contributions sHSPs make toward the adaptation of *Pyropia* species to intertidal zone conditions.

Materials and methods

Algal materials and stress treatments

The leafy gametophytes and filamentous sporophytes of *P. yezoensis* strain TU-1 were cultured at 15 °C in enriched sea life (ESL) medium under a 10 h light/14 h dark photoperiod provided by cool-white fluorescent lamps with a light intensity of 60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, as described previously (Uji et al. 2016).

Since the gametophytes of *P. yezoensis* inhabit the intertidal zone, where they are exposed to harsh environmental stresses, immature gametophytes that had been grown to a blade length of 20–50 mm were used for the transcriptional analysis of the sHSP genes following heat, cold, copper, oxidative stress, and ACC treatments. For the heat stress and cold stress treatments, thalli that had been grown at 15 °C were placed in cultivation chambers set at 25 and 5 °C, respectively. For the oxidative stress, copper stress, and ACC treatments, cultured algae were treated with ESL medium containing 10 μM copper(II) chloride (Kanto Chemical Co., Inc., Tokyo, Japan), 1.0 mM hydrogen peroxide (H_2O_2) (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), or 500 μM ACC (Tokyo Chemical Industry, Tokyo, Japan), respectively. For the combination of heat stress and cold stress treatments, thalli grown at 15 °C were placed in cultivation chambers set at 25 °C for 8 h, followed by 15 °C, and then cultured in chambers set at 5 °C for 8 h. Following treatment, the algal materials were harvested and were immediately frozen with liquid nitrogen and stored at -80 °C until the extraction of RNA for quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis.

Transcriptional analysis of PysHSP genes

RNA extraction and qRT-PCR analysis were performed as described by Uji et al. (2016). Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The extracted RNA

was purified with the TURBO DNA-free kit (Invitrogen/Life Technologies, Carlsbad, CA) to obtain DNA-free RNA. First-strand cDNA was synthesized from 0.5 µg of total RNA using the PrimeScript II 1st strand cDNA Synthesis Kit (TaKaRa Bio, Shiga, Japan). For qRT-PCR analysis, the cDNA was diluted 10-fold and 3.0 µl of the diluted cDNA was used as a template in a 20-µl reaction volume following the manufacturer's instructions. SYBR® Premix Ex Taq™ GC (TaKaRa Bio) Real-time PCR was then performed with a LightCycler® 480 System (Roche Diagnostics, Basel, Switzerland) under the following conditions, 30 s at 95 °C followed by 40 cycles of 5 s at 95 °C and 31 s at 60 °C. Table 1 lists the primers that were used in this study.

The mRNA levels for each of the PysHSP genes were calculated based on a standard curve and were normalized to levels of the transcription elongation factor 1 (*PyElf1*) gene (Uji et al. 2010) and the 18S ribosomal RNA (*Py18SrRNA*) gene (Uji et al. 2016) for the developmental stage and stress treatment experiments, respectively. The standard curve for each primer set was prepared by plotting serial cDNA dilutions (1:10 to 1:10⁵) against the threshold cycle (CT). The relative expression level was calculated as a ratio of the mRNA level to the transcription level at 0 h after the stress or ACC treatment. All the experiments were performed in triplicate.

Identification and analysis of the PysHSP genes

Sequences of the PysHSP genes were retrieved from *P. yezoensis* genome sequence data (Nakamura et al. 2013). The isoelectric point (pI) and molecular weight (Mw) of PysHSP20 were calculated using the ExPASy program (http://web.expasy.org/cgi-bin/compute_pi/). The conserved domains of the PysHSP proteins (ACDs) were confirmed by Pfam (<https://pfam.xfam.org/>) and the PROMALS3D structural alignment program (<http://prodata.swmed.edu/promals3d/promals3d.php>) was used to align the deduced amino acids sequences. To explore the possible regulation mechanism of PysHSP genes in *P. yezoensis*, the upstream regions (> 1.0 kb from the translated start site) were derived from *P. yezoensis* genome sequence data and were searched for regulatory elements, including HSE in PLACE (<http://www.dna.affrc.go.jp/PLACE/>) (Higo et al. 1999). Finally, the programs Predotar (<https://urgi.versailles.inra.fr/predotar/>), TargetP (<http://www.cbs.dtu.dk/services/TargetP/>) (Emanuelsson et al. 2007), and WoLF PSORT (<https://wolfpsort.hgc.jp/>) were used to predict the subcellular localization of the PysHSPs. For phylogenetic analysis, amino acid sequences for sHSP homologs from eukaryotes were obtained from the GenBank database. Only the conserved C-terminal regions were used for phylogenetic analysis. A phylogenetic tree was by neighbor-joining (NJ)

implemented by MEGA version 7.0 (<http://www.megasoftware.net>).

Statistical analysis

The data obtained from the transcriptional analyses were analyzed using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test for the developmental stage experiment and Student's *t* test for the stress and ACC treatment experiments (control at 0 h vs. stress/ACC treatment at 2–8 h). In all the analyses, *p* < 0.05 was considered statistically significant.

Results

Characterization of *Pyropia* cDNAs encoding sHSPs

Five unigenes with complete open reading frames (ORFs) were found in the draft *P. yezoensis* genome sequence that showed significant similarity to other eukaryote sHSP sequences in the GeneBank database. These were named according to their molecular weights (Table 1). The lengths of the PysHSP proteins ranged from 171 (PysHSP19.1/19.2) to 245 (PysHSP25.8) amino acid residues, and the predicted pI values ranged from 5.42 (PysHSP19.2) to 9.29 (PysHSP25.8). Blast analysis showed that the protein sequences of PysHSP19.1, 19.2, and 19.5 exhibited 65–84% identities with HSP22 from *Pyropia haitanensis*, while PysHSP18.8 and PysHSP25.8 exhibited 41% identity with sHSPs from the extremophilic unicellular red alga *Galdieria sulphuraria*.

Secondary structure analysis revealed that all the five PysHSPs shared a conserved ACD that consisted of eight β-strands (Fig. 1). Multiple sequence alignment showed that all of the PysHSPs conserved the “GVL” motif, thought to play a very important role in maintaining not only the structural stability of sHSPs but also their chaperone activity (Mao and Chang 2001). In addition, the PysHSPs contained a “I/V-x-I/

Table 1 The list of the identified *PysHSP* gene family members in *P. yezoensis*

Name	Conting ID	Size (aa)	pI	Mw(KDa)
PyHSP18.8	conting_13836_g3328	175	8.75	18.84
PyHSP19.1	conting_22184_g5485	171	5.64	19.09
PyHSP19.2	conting_17765_g4357	171	5.42	19.18
PyHSP19.5	conting_9957_g2378	178	7.07	19.50
PyHSP25.8	conting_32316_g7859	245	9.29	25.75

aa-amino acid; pI-isoelectric point; Mw-Molecular weight

Fig. 1 Structural characteristics of the small heat shock proteins (PysHSPs) identified in *Pyropia yezoensis*. The PROMALS3D structural alignment program was used to align the deduced amino acid sequences of five PysHSPs. Shading indicates the percentage of conserved amino acid residues (black, 100%; dark gray, 80%; light gray, 80%), while bars

represent gaps. Eight β -strands within the α -crystallin domain are indicated by black arrows. The characteristic motifs "GVL" and "I/V-x-I/V" are indicated by a solid box and a dashed box, respectively. The numbers correspond to the amino acid positions from the first methionine residue

V" motif, highly conserved across many sHSPs and affects the oligomerization (Pasta et al. 2004). These two conserved motifs have also been found in sHSPs from the red algae *P. tenera* (Jin et al. 2017) and *C. merolae* (Kobayashi et al. 2014).

Phylogenetic analysis revealed that All PysHSPs were classified with the clusters of sHSPs from the red algae that were distinct from those of land plants and green algae (Fig. 2). PysHSP19.1, 19.2, and 19.5 had a close relationship with sHSPs from *P. haitanensis* and *P. umbilicalis*, while PysHSP18.8 and PysHSP25.8 had a relatively close relationship with sHSPs from *C. merolae* as well as *P. umbilicalis*.

Expression profiles of PysHSP genes at different developmental stages

P. yezoensis has a heteromorphic life cycle that includes a macroscopic leafy gametophyte and a microscopic filamentous sporophyte. The immature gametophytes and sporophytes form vegetative cells only, while the mature gametophytes produce both male gametes (spermatia) and female

gametes (carpogonia), and the mature sporophytes produce conchospores that are released from conchosporangia and settle on the substratum, where they germinate to form new gametophytes. Therefore, the expression patterns of PysHSP genes were examined in both immature and mature gametophytes and sporophytes under normal conditions. The mRNA transcripts of all the five PysHSP genes were variously expressed in the different developmental stages of *P. yezoensis* (Fig. 3). The expression of *PysHSP18.8*, *PysHSP19.5*, and *PysHSP25.8* was repressed at all the developmental stages, whereas *PysHSP19.1* and *PysHSP19.2* were overexpressed in the mature gametophytes and sporophytes. Only *PysHSP19.2* was found to have high expression in the immature gametophytes. *PysHSP18.8* and *PysHSP19.5* were most highly expressed in the mature gametophytes.

Expression profiles of PysHSP genes with environmental stress and ACC treatments

The mRNA transcripts of all the five PysHSP genes increased significantly in the gametophytes after 2 h of heat stress

Fig. 2 Phylogenetic tree of small heat shock proteins (PysHSPs) in *Pyropia yezoensis*. The phylogenetic tree was constructed by neighbor-joining (NJ) implemented by the MEGA 7.0 software. Numbers at each branch show the percentage of times a node was supported in 1000 bootstrap pseudoreplications. PysHSPs are marked by the black triangle. The numbers next to each species indicate molecular weight (Mw) of sHSPs. GenBank accession numbers for amino acid sequences of sHSPs are shown in parentheses

treatment and, then, gradually decreased after 4 h (Fig. 4a). *PysHSP19.1* exhibited the most remarkable increase of 2378.9-fold after 2 h, while the expressions of *PysHSP19.2*, *PysHSP19.5*, and *PysHSP25.8* increased by 442.8- to 1159.3-

Fig. 3 Relative expression levels of small heat shock protein (PysHSP) genes in *Pyropia yezoensis* at different developmental stages. RNA samples were prepared from immature gametophytes (Im GA), mature gametophytes (Ma GA), immature sporophytes (Im SP), and mature sporophytes (Ma SP), and expression levels were assessed using the *PyElf1* gene for normalization. Different letters above each set of columns indicate significant differences in the expression levels (ANOVA, $p < 0.05$). The data are presented as means $\pm$ standard deviations ($n = 3$)

fold, and *PysHSP18.8* was overexpressed by 30.8-fold. With the exception of *PysHSP19.5*, cold stress treatment did not significantly affect the expression of any of the PysHSP genes, after 8 h of treatment (Fig. 4b). Next, to investigate the expression of PysHSP genes under oxidative stress, we firstly analyzed the expression profiles of antioxidation-related genes such as superoxide dismutase (SOD) and glutathione S-transferase (GST) after H_2O_2 treatment to confirm the occurrence of oxidative stress by the treatment of 1 mM H_2O_2 . H_2O_2 treatment activated the expression of PySOD and PyGST within 2 h of the treatment (Table. S2). Thus, the expression analysis of PysHSPs genes under oxidative stress was performed by the treatment of 1 mM H_2O_2 . Oxidative stress induced by H_2O_2 promoted significant upregulation of all the five PysHSP genes, with *PysHSP18.8* exhibiting the greatest increase in expression (946.6-fold at 4 h after treatment) (Fig. 4c). The expression levels of *PysHSP18.8*, *PysHSP19.1*, and *PysHSP25.8* increased within 2 h of the H_2O_2 treatment and peaked at 4 h, following which they decreased, whereas those of *PysHSP19.2* and *PysHSP19.5* peaked at 2 h and, then, declined in a time-dependent manner. Exposure to copper also enhanced the expression of all the five PysHSP genes at 2 h after treatment and their high expression levels were maintained until 8 h, with *PysHSP18.8*, *19.1*, *19.2*, *19.5*, and *25.8* being increased 3738.8-, 12,486.2-, 5839.3-, 5349.5-, and 659.3-fold, respectively (Fig. 4d). The

Fig. 4 Relative expression levels of small heat shock protein (PysHSP) genes in *Pyropia yezoensis* gametophytes in response to environmental stress and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC). RNA samples were prepared from gametophytes that had been exposed to a high temperature (a), low temperature (b), H_2O_2 (c), copper (d), and ACC (e) for 2, 4, or 8 h, and expression levels were assessed using the *Py18srRNA* gene for normalization. The results are presented as the relative expression compared with that in non-stressed gametophytes (0 h). Significant differences between the control and treatment are indicated by asterisks (Student's *t* test, $p < 0.05$). The data are presented as means $\pm$ standard deviations ($n = 3$)

exogenous application of ACC significantly increased the expression levels of *PysHSP19.2* by 9.75- to 70.6-fold (2–8 h), *PysHSP19.5* by 17.4- to 211.9-fold (2–8 h), and *PysHSP25.8* by 12.8-fold (8 h) (Fig. 4e). Finally, we examined the expression of PysHSP genes under cold stress in the thalli which had experienced heat stress. There was no significant difference in the expression of all PysHSP genes observed, after 8 h of cold treatment, in the thalli exposed to heat stress for 8 h before cold stress treatment (Fig. 5). The expression levels of *PysHSP19.2*, and *PysHSP19.5* which were induced by cold stress in the thalli without heat shock treatment (Fig. 4b) repressed under cold stress in the thalli with heat stress. These

results indicate that *P. yezoensis* has the mechanism to repress the synthesis of the transcripts of PysHSP genes under stress condition in the thalli which have overexpressed PysHSP genes once.

Analysis of cis-acting elements in the PysHSP gene promoters

The upstream regions of *PysHSP19.1* (1.3 kbp from the translated start site) and *PysHSP19.5* (1.4 kbp from the translated start site) were retrieved from *P. yezoensis* draft genome sequence data and the PLACE database was used to scan them.

Fig. 5 Relative expression levels of small heat shock protein (PysHSP) genes in *Pyropia yezoensis* gametophytes exposed to high temperature stress in response to low temperature. RNA samples were prepared from gametophytes that had been exposed to high temperature (25 °C) for 8 h, followed by 16 h at 15 °C, and then to low temperature (5 °C) for 8 h. The expression levels were assessed using the *Py18srRNA* gene for normalization. The results are presented as the relative expression compared with that in non-cold stressed gametophytes (0 h). The data are presented as means $\pm$ standard deviations ($n = 3$)

A TATA box was detected in the *PysHSP19.1* (−211 to −218 from the translated start site) and *PysHSP19.5* (−202 to −194) promoters, while a CCAAT box was found in the *PysHSP19.1* (−497 to −492) and *PysHSP19.5* (−497 to −493) promoters (Fig. 6). Although none of the PysHSP gene promoters were found to contain a perfect HSE module (nGAAnnTTCnnGAAn or nTTCnnGAAnnTTCn), an imperfect HSE module (nnGAAnnTTC) was present in the upstream regions of *PysHSP19.1* (−1192 to −1184) and *PysHSP19.5* (−771 to −762) (Fig. 6).

Prediction of cellular localization of PysHSPs

Table S3 shows the subcellular localization predictions for the five PysHSPs. PysHSP18.8 was predicted to be targeted to the cytosol. However, no consistent predictions were made for the other PysHSPs, with the programs WoLF PSORT and TargetP predicting that PysHSP19.1 and PysHSP19.2 were localized to either the nucleus or plastid, respectively, and all the three programs variously predicting that PysHSP19.5 was targeted to the cytosol or the plastid with a low reliability score and PysHSP25.8 was targeted to the plastid or mitochondria.

Discussion

In this study, we investigated the structure of five sHSP genes from *P. yezoensis* and their expression patterns in the different developmental stages and under stress and ACC treatments as a basis for understanding the sHSP gene family in *Pyropia*. *Pyropia* species that inhabit the intertidal zone are periodically exposed to adverse environmental conditions such as high

light, extreme temperatures, desiccation, ultraviolet (UV) irradiation, and osmotic stress due to the turning tides. These environmental stresses enhance the concentrations of reactive oxygen species (ROS) such as H_2O_2 , superoxide radicals, or hydroxyl radicals in the plastids, mitochondria, cytosol, and apoplast due to an imbalance between ROS production and detoxification (Tripathy and Oelmüller 2012). ROS are capable of unrestricted oxidation of various cellular components and cause extensive damage to proteins, DNA, and lipids, thereby affecting normal cellular functioning (Das and Roychoudhury 2014). For example, the thalli of the red macroalga *Gracilaria corticata* showed enhanced ROS production and increased lipid peroxidation during desiccation (Kumar et al. 2011), while the green macroalga *Ulva fasciata* exhibited increased H_2O_2 contents and lipid peroxidation, as well as total peroxide contents following exposure to UV-B irradiance (Shiu and Lee 2005). Consequently, the ability to detoxify excess ROS under stress conditions will be crucial for intertidal seaweeds that are adapted to adverse environmental conditions—indeed, several studies have shown that upper-shore species display greater antioxidant responses and better recovery after oxidative stress than lower-shore species (Abe et al. 2001; Flores-Molina et al. 2014). In the present study, we observed a rapid elevation in the mRNA levels of PysHSP genes following heat stress and H_2O_2 treatments, suggesting that these proteins play a significant role in allowing *P. yezoensis* to survive in the upper intertidal zone.

Intertidal seaweeds are also exposed to pollutants, including heavy metals resulting from anthropogenic impacts. These pollutants have a major impact on marine ecosystems, affecting species distributions (Gledhill et al. 1997). Copper is one such heavy metal that is a vital micronutrient at low concentrations but becomes toxic when accumulated at high concentrations, with excess copper inducing an overproduction of ROS in macroalgae (Moenne et al. 2016). A number of sHSPs have been identified in intertidal invertebrates that are thought to be responsive to copper stress (Negri et al. 2013). Similarly, we demonstrated that the presence of Cu^{2+} causes the upregulation of PysHSP genes in *P. yezoensis*, suggesting that sHSPs also play an important role in the tolerance of intertidal seaweeds to copper stress. The ACDs of sHSPs selectively bind to Cu^{2+} over other divalent cations such as Zn^{2+} , Mg^{2+} , and Ca^{2+} , preventing the generation of ROS (Ahmad et al. 2008). We found that most of the PysHSP genes were more highly induced by the copper stress treatment than by any other abiotic stress treatment and that their high expression levels were maintained for up to 8 h. Thus, *P. yezoensis* appears to attenuate the accumulation of copper-mediated ROS by expressing PysHSPs to confer cytoprotection.

It has been demonstrated that PGRs play critical roles in regulating the protective responses of terrestrial plants against both biotic and abiotic stresses, and that crosstalk between different PGRs is important for generating a sophisticated

Fig. 6 The prediction of putative *cis*-elements in the promoter regions of *PysHSP* genes. The sequence of the upstream region of *PysHSP19.1* (a) and of *PysHSP19.5* (b). The number indicates the length of nucleotide

upstream from the translation start site which is shown in underline. All potential *cis*-elements are boxed

and efficient stress response through the regulation of gene expression (Verma et al. 2016). Several lines of evidence also indicate that PGRs are involved in the response of aquatic plants to environmental stress. For example, pretreatment with SA improved the thermotolerance of the brown alga *Saccharina japonica* by altering its antioxidant enzymatic activity (Zhou et al. 2010), and the exogenous application of methyl jasmonate (MeJA) or a combination of MeJA and SA resulted in the upregulation of stress-related genes, including sHSPs, in the red macroalgae *Chondrus crispus* and *Gracilariopsis lemaneiformis* (Collén et al. 2006; Wang et al. 2017). Furthermore, Uji et al. (2016) previously showed that *P. yezoensis* gametophytes that had been treated with the ethylene precursor ACC exhibited an increased tolerance to oxidative stress. In the present study, we found that the exogenous application of ACC significantly increased the expression levels of *PysHSP19.2*, *PysHSP19.5*, and *PysHSP25.8* in

P. yezoensis gametophytes, suggesting that their respective proteins are involved in an oxidative stress response via the ethylene signal transduction pathway.

Expression analysis showed that transcripts of the *PysHSP19.1* and *PysHSP19.2* genes were accumulated in the mature gametophytes and sporophytes of *P. yezoensis* under normal conditions, and were also detected at low levels in the immature gametophytes. Uji et al. (2016) found previously that mature gametophytes exhibited an enhanced tolerance to oxidative stress compared with immature gametophytes, and it has been suggested that *PysHSP19.1* and *PysHSP19.2* play a role in enhancing the oxidative stress tolerance of mature gametophytes. *P. yezoensis* gametophytes usually grow in winter and produce male and female gametes at the beginning of spring, resulting in the sporophytes that germinate from the zygotes growing in summer when they are exposed to high temperatures. Therefore, the alteration of the *P. yezoensis* generation

from gametophyte to sporophyte through the maturation of the gametophyte may require the accumulation of PysHSPs to maintain metabolism under these high-temperature conditions.

There is growing evidence that plants use ROS as signaling molecules to acclimate against stressful conditions, although they are known to be harmful under stress conditions (Mittler 2017). For example, ROS that are generated in the chloroplast have been implicated as triggers of the signaling pathway that influences the expression of light-inducible genes in *P. yezoensis* (Uji et al. 2012), and a time-dependent increase in H₂O₂ release and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity was observed with an increasing duration of heat shock in *P. haitanensis* (Luo et al. 2014). Furthermore, a burst of ROS generated by the photosynthetic electron transport chain of the chloroplast thylakoids and plasma membrane-localized NADPH oxidases has been correlated with the induction of heat-responsive genes, including sHSPs, under the control of heat shock transcription factors (HSFs) that are assumed to be related to the direct sensing of ROS in animals and plants (Shigeoka and Maruta 2014).

Plant HSFs form homotrimers when they interact with ROS, which are transported to the nucleus to activate oxidative stress gene expression (Miller and Mittler 2006). HSFs bind to highly conserved elements in the promoter regions of genes that are known as heat shock elements (HSEs; palindromic motifs of nGAAn) (Scharf et al. 2012) and are a large multigene family in higher plants (for example, *Arabidopsis* has 21 HSFs) (Scharf et al. 2012). However, only one homolog of HSF (PyHSF) was detected in the draft genome sequence of *P. yezoensis*. In silico analysis of the promoter regions of the PysHSP genes detected imperfect HSE modules in *PysHSP19.1* and *PysHSP19.5*, known to be involved in the transcriptional regulation of plant sHSP genes under heat stress (Zhang et al. 2016). In addition, a CCAAT box was found in the promoter regions of these genes, shown to have a quantitative effect on the expression of sHSP genes by acting cooperatively with the HSE (Rieping and Schoffl 1992). These findings imply that PysHSP gene expression may be regulated by active PyHSF through ROS generation via the chloroplast and NADPH oxidase. In addition, it has been shown that multiprotein bridging factor 1c (MBF1c) from *Arabidopsis* controls the expression of two HSFs during heat stress and that the constitutive expression of MBF1c enhances the tolerance of transgenic plants to abiotic stresses (Suzuki et al. 2005, 2011). Uji et al. (2013) reported that during exposure to oxidative and heat stresses, transcripts of the MBF1 gene from *P. yezoensis* (*PyMBF1*) were upregulated in *P. yezoensis* gametophytes. Therefore, further research on the relationship between PyHSF and PyMBF1 in sHSP regulation is required to elucidate the regulatory mechanisms that occur in *Pyropia* in response to environmental stress.

In conclusion, five sHSPs were identified from *P. yezoensis* that showed different transcriptional expression profiles at

different developmental stages and in response to various abiotic stresses and ACC. This study provides insights that further our understanding of how *Pyropia* species can adapt to intertidal zone conditions through their life cycle.

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