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Research Article

Overexpression of the Small Heat Shock Protein, PtsHSP19.3 from Marine Red Algae, *Pyropia tenera* (Bangiales, Rhodophyta) Enhances Abiotic Stress Tolerance in *Chlamydomonas*

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Abstract Water temperature is one of the major factors that impacts the growth and life cycle of *Pyropia tenera*, one of the most valuable and cultivated marine red algae belonging to Bangiales (Rhodophytes). We analyzed transcriptome from gametophyte of *P. tenera* under normal and high temperature conditions, and identified four small heat shock proteins (*sHSPs*). They have no significant amino acid sequence homology with known proteins in public databases except PhsHSP22 from *Pyropia haitanensis*. *PtsHSP19.3* gene responded to high temperature but slightly or not to desiccation, freezing or high salt condition. When the *PtsHSP19.3* gene was overexpressed in *Chlamydomonas reinhardtii*, transformed *Chlamydomonas* lines revealed much higher growth rate than that of control cells under heat stress condition. Transformed cells also grew well in those of the control cell onto the medium containing high salt or H_2O_2. When the *PtsHSP19.3* was fused to *GFP* and introduced into tobacco protoplast, fluorescence was detected at several spots. Results indicate that PtsHSP19.3 may form super-molecular assembles and be involved in tolerance to heat stress.

Keywords Red algae, *Pyropia tenera*, small heat shock protein, Heat tolerance, Abiotic stress tolerance

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Introduction

Temperature is a major environmental factor that limit the growth and yield of plant. Under high temperatures or heat stress, plants alter gene expression patterns to adapt to a given environment. Heat shock protein (HSP) genes are key components turned on under heat stress condition and contributing to cellular homeostasis (Scharf et al. 2001; Wang et al. 2004; Schroda and Vallon, 2009; Basha et al. 2012). They are responsible for protein folding, assembly, translocation and degradation in a broad array of normal cellular processes; they also function in the stabilization of proteins and membranes, and can assist in protein refolding under stress conditions. All organism response to high temperature and turning on the HSPs which are a number of conserved protein families such as the HSP100s, HSP90s, HSP70s, HSP60s and sHSP (Wang et al. 2004).

The small heat shock protein (sHSP) is a family of heat shock protein that range in size from approximately 12 ~ 42 kDa. Most sHSPs are in the range of 15 ~ 22 kDa. This protein is characterized by having a conserved C-terminal domain of approximately 90 amino acids referred to as the a-crystallin domain (ACD) (Basha et al. 2012; Scharf et al. 2001; Waters, 2013). Except ACD domain, sHSP are variable in both length and sequence. The sHSPs that function in specific cellular organelles or compartments have N-terminal transit or signal sequences needed to get the sHSP to the proper cellular compartment (Waters, 2013).

The high resolution crystal structures studies show that sHSP are composed of a β-sandwich of two antiparallel sheets and form a hollow ball (van Montfort et al. 2002; Waters, 2013; Zhang et al. 2015). The oligomers are of different sizes. The plant Hsp16.9 from wheat (*Triticum aestivum*) (van Montfort et al. 2001; 2002) forms a dodecamer, and the oligomer of Hsp16.5 from an archaea, *Methano*

caldococcus (*Methanococcus*) *jannaschii* (Kim et al. 1998) has 24 subunits. (van Montfort et al. 2001). A model for the structure of Hsp21 from *Arabidopsis* indicates that this plant sHSP is also a dodecamer with two hexameric disks (Lambert et al. 2011). Stengel et al. (2010) showed that the dodecamer form of Hsp18.1CI from *Pisum sativum* is in equilibrium with Hsp18.1 dimers, monomer, and higher order oligomers.

The sHSPs are ubiquitously present in all organisms. The number of genes encoding sHSPs in different organisms varies greatly. Investigations of sHSPs from mammals, yeast, plants and bacteria have unveiled their role in thermotolerance such that the overexpression of eukaryotic recombinant sHSPs could increase the thermotolerance of *E. coli* cells. The plant sHSPs are particularly diverse and are a crucial component of the plant heat shock response.

The sHSP are known to act as ATP-independent molecular chaperones work with other chaperones to prevent irreversible aggregations and to re-solubilize proteins that have already aggregated (Waters, 2013; Zhang et al. 2015). They do not require ATP to bind substrate proteins. The sHSP bind to the non-native substrate proteins and release them for refolding with the help of other ATP-dependent chaperones (Waters, 2013). In the absence of such stresses, however, sHSPs can also be produced specifically in reproductive organs at certain developmental stages, including seed maturation and germination, pollen development, and fruit maturation (Neta-sharir et al. 2005; Volkov et al. 2005).

Pyropia (Bangiales, Rhodophyta) are one of the most commercially valuable marine red algae; it is used as a source of food, fertilizer, medicine, and chemicals (Sahoo et al. 2002). Among them, several species have been cultured in aquaculture industry in East Asia including Korea (Hwang et al. 2005). *Pyropia* grow naturally in the intertidal zone, where they are frequently exposed to a variety of potentially stressful environmental including variations in humidity, changes in temperature, osmotic shock and different light intensities (Hwang et al. 1997; Blouin et al. 2011). Therefore, *Pyopia* have developed a variety of strategies and mechanisms to respond to and survive through those environmental stresses (Choi et al. 2013; Im et al. 2015). *Pyropia* exhibits a exhibits a heteromorphic life cycle with an alternation of generation between foliose thallus gametophyte and filamentous sporophyte; this is referred to as conchocelis phase (Sahoo et al. 2002, Blouin et al. 2011). The thallus that we see and use as a food source is the haploid generation, and it can reproduce asexually by forming spores that grow to replicate the original thallus. Gametophyte thalli are grown in cold water during the winter season and changed into conchocelis phase for summer. Temperature is one of the major factors that affect growth rate and life cycle of the *Pyropia* (Hwang et al. 1997). The increase of seawater temperature due to global warming affect the growth of *Pyropia*. However, information currently known regarding genes affecting the regulatory and biochemical pathways of high temperature responses in *Pyropia* is limited. Identifying genes and studying their expression patterns and physiological function in response to high temperature will provide a molecular basis for life cycle as well as heat tolerance of the *Pyropia*.

We identified four small heat shock genes (*sHSP*) based on transcriptome generated from *P. tenera* thalli under normal and high-temperature condition. In this study, we report that expression pattern of *PtsHPS19.3* and its physiological function in heat and abiotic stress tolerance in heterologous expressed *Chlamydomonas* cells.

Material and Methods

Plant materials and growth condition

Pyropia tenera (LB) was provided from Seaweed research center at Mokpo, Korea. Leafy gametophytes of *P. tenera* were cultured in modified Grund medium (McLachlan, 1973) at 12°C under irradiation with 80 μmol photon m^{-2} s^{-1}, provided by cool-white fluorescent lamps on a 10:14 (light: dark) photoperiod in a growth room. Growth bottles containing *P. tenera* were transferred to a 20°C growth chamber with the same light intensity and photoperiod for the heat treatment. Desiccation and freezing stress were applied as described by Im et al. (2015).

Chlamydomonas reinhardtii strain mut-11 (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}).

PtsHSP 19.3 cDNA isolation and sequence analysis

Transcriptome sequence reads generated from *P. tenera* gametophyte thalli were analyzed for the s*HSP* gene family (Choi et al. 2013). The contigs encoding putative s*HSPs* were analyzed to determine if they covered the full open reading frame (ORF). Finally, four contigs encoding putative full s*HSP* ORFs were selected for further analysis. Gene-specific primer sets were designed for each contig to clone the s*HSP* 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 contigs 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). Phylogenic trees for sHSPs from green plants and PtsHSP19.3 were built with the neighbor-joining method using the Geneious program (Geneious, Auckland, New Zealand). 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 *PtsHSP19.3* 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 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 10, 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, and 500 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 60 sec at 72°C, followed by 5 min of termination at 72°C. The gene-specific primers were as follows: 5′-CTCTCTCCCATCACACGACTC-3′ and 5′-GAACCCAGGTGTCTCGAACC-3′ for *PtsHSP19.3*. The PCR products were separated on 1% agarose gels and stained with ethidium bromide for imaging.

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 predenaturation 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 *actin* (5′-GGTGCAGAAGGAGG TGGAGA-3′ and 5′-GCAACGCAACCCATAGAGAA-3′) gene was used as an internal control. All samples were run in duplicate, and n-fold differential expression was calculated using the comparative Ct method. N-fold differential expression was calculated by $2^{-\Delta\Delta Ct}$.

Cellular localization of *PtsHSP19.3*

The *PtsHSP19.3* coding region was amplified via PCR with ExTaq DNA polymerase (Takara, Shiga, Japan) using forward (5′-TCTAGAATGGACCTTTTCGCACTTGA-3′) and reverse (5′-GGATCCCTACTCAGTCACCTGCGGTC-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 tobacco (*Nicotina benthamiana*) protoplasts. The tobacco protoplasts were examined under a fluorescence microscope (Leica, Wetzlar, Germany) to evaluate GFP expression.

Stress tolerance assay for the *PtsHSP19.3* gene in Chlamydomonas

The *PtsHSP19.3* gene ORF was amplified via PCR and subcloned under the *PtsHSP19.3* pCr112 vector using the NdeI and EcoRV sites. The pCr112-*PtsHSP19.3* plasmid was introduced into *Chlamydomonas* strain mut-11 via the glass bead method for transformation. Transformants were selected on TAP agar medium containing 20 μg mL^{-1} hygromycin after 14 ~ 16 days of growth. *Chlamydomonas* genomic DNA was purified from 100-ml liquid cultures and used for PCR with the *PtsHSP19.3*-specific primer set to confirm insertion of the *PtsHSP19.3* gene into the *Chlamydomonas* genome. Total RNA was isolated from control and transformed cells and applied to RT-PCR to assess *PtsHSP19.3* gene expression in transgenic *Chlamydomonas* cells. The *Cr_actin* (5'-TGTGCATACGTGGATAGCTTG-3' and 5'-ATGACCCGCTCCTCATATCTT-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 $2 \sim 4 \times 10^6$ cells mL^{-1} and concentrated to 10^7 cells mL^{-1} before dilution to $10^0 \sim 10^{-2}$ in fresh medium. The diluted cells (8 μL) were inoculated onto agar plates. To evaluate high temperature tolerance, the cells were exposed to 38°C for 1 ~ 3 days, and transferred to a 25°C growth chamber. *C. reinhardtii* strain mut-11 and the transformed cells with the pCr112

vector, Hyg5414, were used as controls. To check osmotic, oxidative or salt tolerance, cells were inoculated onto agar medium with 200 mM Mannitol, 0.03% H_2O_2 or 50 mM NaCl.

Results and Discussion

Identification and characterization of the *P. tenera sHSP 19.3* gene

The transcriptome sequences from *P. tenera* gametophyte thalli have been generated under normal growth and heat stress conditions (Choi et al. 2013), 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 small heat shock proteins (sHSPs), which constitute a small and highly conserved family in eukaryotes and prokaryotes and are highly involved in the heat shock response (Choi et al. 2013; Waters, 2013). The transcriptome sequences were screened for sHSP members, and 19 contigs encoding putative alpha crystalline domain (α-crystalline, ACD) 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, four unique sequences covering full ORF were selected (Table 1). These results indicate that there are more than four *sHSP* genes in the *P. tenera* genome. The characteristics of the four PtsHSP polypeptides are summarized in Table 1. All PtsHSP polypeptides had molecular weights of 16.4 ~ 20.2 kDa and isoelectric points of 4.7 ~ 6.42. Amino acid sequence alignment of the PtsHSP polypeptides showed sequence variations, including deletions and substitutions (Fig. 1A). An ACD domain was detected in all identified PtsHSPs. Amino acid homology searching by Blast program result show that four PtsHSPs have significant homology with previously deposited PhsHSP22 from *P. haitanensis*, red algae (Table 1). PtsHSP19.3 have the highest sequence homology with PhsHSP22 from *P. haitanensis* (Table 1). Therefore we chose the *PtsHSP19.3* for further analysis of its expression and physiological function.

Except PhsHSP22, there are no known genes showing significant amino acid sequence homology with PtsHSPs in public database. These results indicate that red algae sHSPs are much different from the known sHSPs from green plants including single cell algae and higher land plants (Fig 1B). Or PtsHSP19.3 may have no chaperon activity although ACD domain was found.

Expression of *PtsHSP 19.3* genes

A characteristic of many s*HSP* genes is their strong and universal induction in response to heat stress. Several s*HSPs* are also induced by other biotic and abiotic stressors, such as drought and cold (Wehmeyer and Vierling, 2000; Waters, 2013). Total RNA was isolated after *P. tenera* was subjected to heat, low temperature, desiccation, and high salt condition, and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analyses were conducted to evaluate the expression of each *PtsHSP 19.3* gene. Figure 2 shows the expression of each *PtsHSP 19.3* gene under heat, desiccation and freezing stress conditions. *PtsHSP19.3* responded to heat stress (Fig. 2a). But *PtsHSP* 19.3 response slightly or not to desiccation, freezing or high salt condition. Expression of the *PtsHSP 19.3* was increased until 8 hours after heat treatment and then decreased (Fig. 2b). The most common expression pattern of *sHSP* gene in model plant *Arabidopsis thaliana* is no expression in unstressed tissue and high level of expression during heat stress (Waters, 2013). There is no single pattern of gene expression for *sHSP* genes. Most of the *Arabidopsis sHSP* genes response to various abiotic stress including osmotic, oxidative, salt and wounding stress as well as heat (Malik et al. 1999; Lee et al. 2000; Sun et al. 2001; Waters et al. 2008). Example, class I *sHSPs* of *Arabidopsis* are highly expressed during heat stress, but they are also expressed in response to a wide range of other stressors including osmotic stress, oxidative stress and UV. But expression level of the each *Arabidopsis sHSP* gene

Table 1 Summary of small heat shock proteins (PtsHSPs) isolated from *P. tenera*

Gene	contig name	No. amino acid residues	MW (kDa)	pI	e-value	Description
PtsHSP19.3	*Ptcontig714*	173	19.3	5.44	6e-96	HSP22 of *Pyropia haitanensis*
PtsHSP19.6	*Ptcontig6407*	179	19.6	6.05	3e-72	HSP22 of *Pyropia haitanensis*
PtsHSP20.2	*Ptcontig6022*	200	20.2	6.42	7e-21	HSP22 of *Pyropia haitanensis*
PtsHSP16.4	*Ptcontig3221*	154	16.4	4.70	1e-19	HSP of *Galdieria sulphuraria*

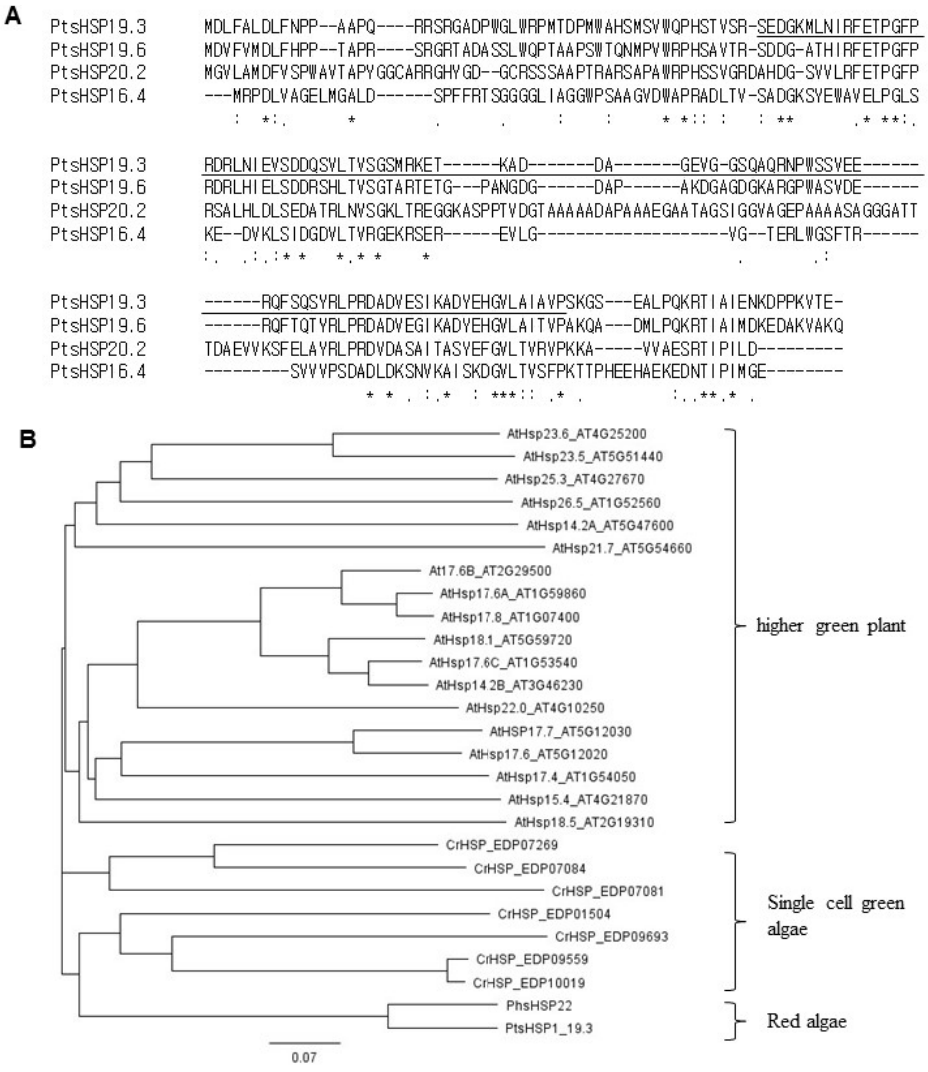

Fig. 1 Amino acid sequence alignment and phylogenic tree of PtsHSPs isolated from *P. tenera*. A, ACD domain identified in PtsHSP19.3 was underlined. Asterisk (*) and : indicate identical and similar amino acid residues, respectively. The alignment was conducted using the CLUSTAL W. B, Phylogenic tree of sHSPs. Two sHSP, PtsHSP19.3 and PhsHSP from *Pyropia* (red algae), sHSPs from *Chlamydomonas* (single cell green algae) and *Arabidopsis* (higher green plant) were selected for phylogenic analysis

differs depending on the type of abiotic stress. In addition to being heat induced, some *sHSPs* are expressed during embryogenesis and seed maturation (Wehmeyer and Vierling, 2000; Sun et al. 2002; Kotak et al. 2007).

Localization of PtsHSP 19.3

To determine the cellular location of the PtsHSP 19.3 protein, the *PtsHSP 19.3* ORF sequence was fused upstream of a reporter gene encoding GFP in the plant expression vector p326-35S-*GFP* (*GFP*). The recombinant vector, p326-35S-*PtsHSP19.3-GFP* (*PtsHSP19.3-GFP*), was then subjected to a transient assay using tobacco protoplast. The p326-*GFP* vector showed GFP signals in both the cytoplasm and cell organelles including nucleus (Fig. 3). The p326-*PtsHSP19.3-GFP* showed GFP signals in the several locations as spots or granules (Fig. 3). Prediction of the signal peptides using the PrediSi program (www.pridisi.de) demonstrated that PtsHSP19.3 do not harbor a putative signal peptide in the N-terminal region for subcellular organelle. These results suggest that PtsHSP19.3 may be in cytosol and not located in cell organelles such as nucleus, chloroplast or mitochondria.

Plant have more sHSPs than other eukaryotes (Waters et al. 2008; Yan et al. 2017). Analysis of three angiosperm genome sequence including *Arabidopsis thaliana*, *Populus trichocarpa* and *Oryza sativa* show that there are 11 sHSP subfamilies (Waters et al. 2008). Among them, six subfamilies are localized in cytoplasm/nuclear and five sHSP subfamilies localized cellular organelles including endoplasmic reticulum (ER), chloroplast, mitochondria, and peroxisome. The sHSPs

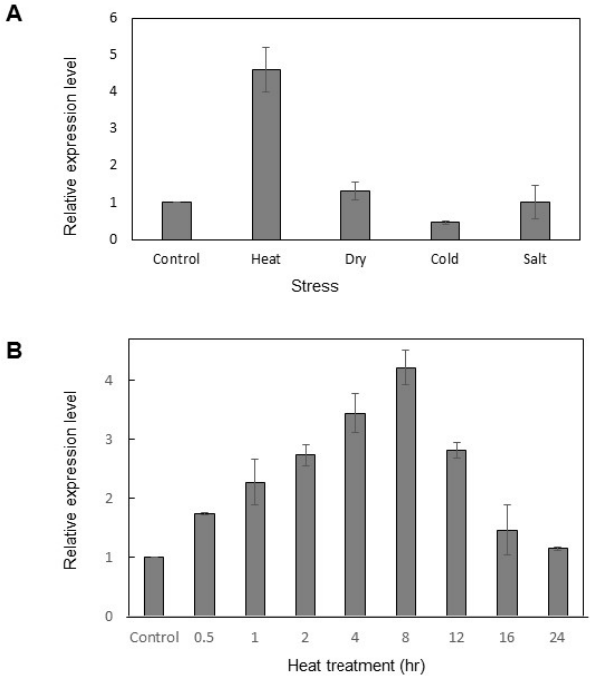

Fig. 2 Expression pattern of the *PtsHSP19.3* gene in *P. tenera*. A, Total RNAs were purified from the gametophyte thalli of *P. tenera* under heat, desiccation, cold or salt stress conditions and amplified by qRT-PCR. The *PtAct* gene was used as internal control. Bars are means ± standard deviation (SD). B, To check the expression pattern of the *PtsHSP19.3* gene during heat stress, total RNA was purified from the gametophyte thalli of *P. tenera* at indicated point after heat treatment

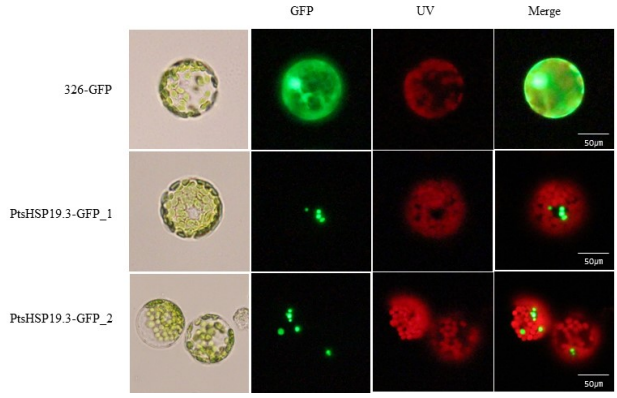

Fig. 3 Subcellular localizations of PtsHSP19.3
A reporter gene encoding green fluorescent protein (GFP) was fused to *PtHSP19.3* under the control of the CaMV 35S promoter in the 326-GFP vector. The constructs were introduced into the protoplasts of *Nicotiana benthamiana*. The 326-GFP empty vector served as the control. After overnight incubation, PtsHSP19.3-GFP localization in individual cells was observed by GFP fluorescence. Contrast interference images of the structure of the whole protoplast were presented at left. GFP, cell images using a green filter for PtsHSP19.3-GFP location with GFP fluorescence. Different protoplast (PtsHSP19.3-GFP_1 and PtsHSP19.3-GFP_2) expressing GFP are presented. UV, cell images using a UV filter for chloroplast location. Merge, merged cell image from GFP and UV filter

that function in specific cellular organelles or compartments have N-terminal transit, leader, or signal sequences needed to get the sHSP to the proper cellular compartment.

Arabidopsis study reported that some cytosolic sHSPs localize as multichaperone complex in cytosol (Siddique et al. 2008). The sHSPs of the cytoplasmic/nuclear subfamilies CI, CII and CIII are shown to be recruited to heat shock complex under heat stress condition (Siddique et al. 2008). Recently Zhang et al (2015) reported that small heat shock protein CeHSP17 from *Caenorhabditis elegans* exists as a 24 subunit spherical oligomer and forms large sheet like super molecular assemblies at the high temperature. These results suggest that PtsHSP 19.3 may form large super molecular assemblies in cytosol or located in heat stress granule.

Expression of the *PtsHSP 19.3* gene increase heat and abiotic tolerance in *Chlamydomonas*

The complete *PtsHSP19.3* coding sequence was introduced into *C. reinhardtii* strain mut11 to assess the physiological functions of the *PtsHSP19.3* gene under heat, cold, high salt, and osmotic stress conditions. Three *PtsHSP19.3*-transformed *Chlamydomonas* lines were selected on media containing hygromycin, and introduction and expression of the *PtsHSP19.3* gene were confirmed via PCR (Fig. 4A) and RT-PCR (Fig. 4B). In order to assess the thermotolerance conferred by *PtsHSP19.3*, transformed *Chlamydomonas* lines were subjected or not (control) to a 38°C for 2 days and then they were transferred to a 25°C incubator and cultured for 10 days. *Chlamydomonas* strain Mut11 and Hyg5414 transformed by empty vector were used as control. All *Chlamydomonas* cells had grown to similar levels as those observed on control culture plates (Fig. 4C). However, the transformed *Chlamydomonas* lines showed a much higher growth rate than that of the Hyg5414, Mut 11 line when heat stress was applied. These results demonstrate that *PtsHSP19.3* contributes to heat stress tolerance in *Chlamydomonas*. However, when cold temperature was applied to *Chlamydomonas* lines, transformed *Chlamydomonas* grown similar level with control cells (data not shown). These results indicate that *PtsHSP19.3* does not involved in cold tolerance. Transformed *Chlamydomonas* also does not show tolerance to osmotic stress induced by mannitol (data not shown). These results are correlated with expression pattern of the *PtsHSP19.3* (Fig 2). qRT-PCR result show that *PtsHSP19.3* does not response to low temperature and desiccation stress. When the transformed *Chlamydomonas* were subjected into

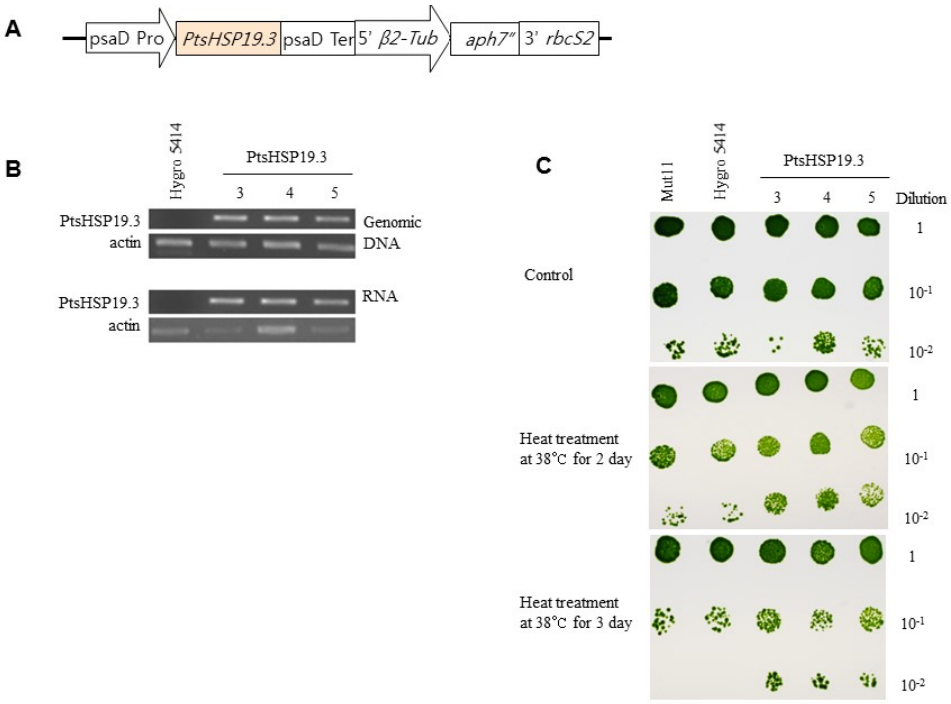

Fig. 4 Effects of *PtsHSP19.3* on growth of *Chlamydomonas* under heat stress conditions
A. Vector map for the expression of *PtsHSP19.3* in *Chlamydomonas*. B, To evaluate the introduction and expression of the *PtsHSP19.3* gene, genomic DNAs and RNA were purified from transgenic *Chlamydomonas* cells and amplified with a *PtsHSP19.3* specific primer. C, Survival and growth assay of the transgenic *Chlamydomonas* cells harboring *PtsHSP19.3* under heat stress. *Chlamydomonas* cells were diluted to $10^{-1} \sim 10^{-3}$ in fresh medium and 10 μl of diluted cells were inoculated onto agar plates. The *Chlamydomonas* cells were subjected or not (control) to a 38°C for 2 or 3 days and transferred to a 25°C incubator and cultured for 10 days. The *Chlamydomonas* strain Mut11 and Hyg5414 transformed by empty vector were used as control

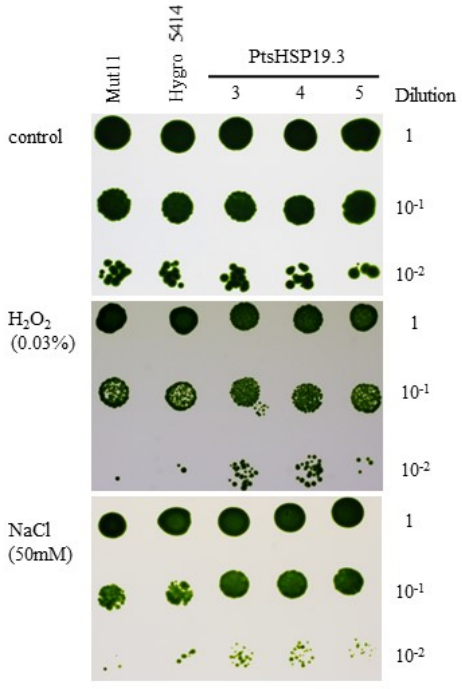

Fig. 5 Effects of *PtsHSP19.3* on growth of *Chlamydomonas* under abiotic stress condition. *Chlamydomonas* cells were diluted and inoculated onto agar plates containing 0.03% H_2O_2 or 50 mM salt as described in Fig 4

medium containing 50 mM salt or 0.03% H_2O_2, transformed cells showed a much higher growth rate than that of the control cells (Fig. 5). These results demonstrate that *PtsHSP19.3* contributes to oxidation stress and salt tolerance in *Chlamydomonas*. Previous studies in higher plants show that sHSPs are involve in salt and oxidation stress tolerance as well as high temperature (Malik et al. 1999; Lee et al. 2000; Sun et al. 2001; Mu at al. 2013; Ruibal et al. 2013). When a sHSP from lily, *LimHSP16.46*, was overexpressed in *Arabidopsis*, transgenic plant enhanced in viability under high temperature, high salt and oxidative stresss (Mu et al. 2013). Overexpression of a sHSP, *JrsHSP17.3* from walnut tree (*Juglans regia*) confer the tolerance to heat, freezing and salt stress in transgenic yeast (Zhai et al. 2016). These result demonstrate that *PtsHSP19.3* from red algae plays a role in abiotic stress tolerance including high temperature as *sHSPs* in higher plants, although, PtsHSP19.3 have low amino acid sequence homology to sHSP of higher plants.

Conclusion

Pyropia tenera is one of the most commercially valuable

marine red algae. We identified four putative small heat shock protein genes (*sHSP*) based on transcriptome generated from *P. tenera*. Although ACD domain was found, they have no significant amino acid sequence homology with known gene in public database. The *PtsHSP19.3* gene was highly upregulated by high temperature, and confer heat tolerance in *Chlamydomonas*. The PtsHSP 19.3 polypeptide may form large super molecular assemblies or located in stress granule in cytosol. Global warming affect increasing seawater temperature. Water temperature is one of the major factor affecting on the growth and life cycle in *Pyropia tenera.* This study provide information for sHSPs and its function in heat tolerance of *Pyropia*.

Acknowledgments

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