Molecular mechanism underlying *Pyropia haitanensis* *PhHsp22*-mediated increase in the high-temperature tolerance of *Chlamydomonas reinhardtii*

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Abstract

Global warming is one of the key limiting factors affecting the cultivation of *Pyropia haitanensis* which is an economically important macroalgae species grown in southern China. However, the mechanism underlying the high-temperature tolerance of *P. haitanensis* remains largely unknown. In a previous study, we showed that the expression of the small heat shock protein 22 gene (*Hsp22*) is upregulated in *P. haitanensis* in response to high-temperature stress, but the associated regulatory mechanism was not fully elucidated. In this study, a transgenic *Chlamydomonas reinhardtii* expression system was used to functionally characterize *P. haitanensis* *Hsp22*. Our analyses indicated that the C-terminal of *PhHsp22* is highly conserved and contains an A-crystal structure domain. A phylogenetic analysis revealed *PhHsp22* is not closely related to small heat shock protein genes in other species. Additionally, *PhHsp22* expression significantly increased at 3 and 6 h after initiating 33 °C treatment, which improved the survival rate of transgenic *C. reinhardtii* during the early stage of high-temperature treatment. The further transcriptome analysis revealed that *PhHsp22* expression can promote pathways related to energy metabolism, metabolites metabolism, and protein homeostasis in transgenic *C. reinhardtii* cells exposed to high temperatures. Therefore, *PhHsp22* may be crucial for the response of *Pyropia* species to high-temperature stress. Furthermore, this gene may be useful for breeding new high-temperature algal strains.

Keywords *PhHsp22* · *Pyropia haitanensis* · *Chlamydomonas* · *Rhodophyta* · High-temperature stress · Homeostasis

Introduction

Pyropia species are economically important marine red algae used to produce food, fertilizer, and medicine (Blouin et al. 2011; Brawley et al. 2017; Cao et al. 2020). With the expansion of artificial seeding and the development of a floating culture method, the farming and processing of *Pyropia* species

is now a major part of the largest seaweed industries in East Asian countries, including China, Japan, and South Korea (Sahoo et al. 2002; Hwang et al. 2019). However, because of global warming, seawater temperatures have gradually increased in recent years which has adversely affected the cultivation and yield of *Pyropia* species (Shi et al. 2017; Wang et al. 2018a; Hwang et al. 2019). To adapt to global climate change and to continue to develop improved methods for cultivating *Pyropia* species, several high-temperature (HT) tolerant *Pyropia haitanensis* strains (Z-26, Z-61, and ZS-1) have been selected and are now widely cultivated in southern China (Chen et al. 2008). Although the mechanisms underlying this high-temperature tolerance have been investigated (Shi et al. 2017; Wang et al. 2018a, b), they remain largely uncharacterized. Therefore, deciphering the molecular basis for the heat stress tolerance of *P. haitanensis* is important for increasing the commercial value and productivity of red algal species.

The development and application of high-throughput sequencing technology and molecular biology research tools

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have resulted in some recent progress regarding the elucidation of the molecular mechanisms behind *P. haitanensis* heat stress tolerance. For example, Wang et al. (2018b) compared the transcriptomes between Z-61 and wild-type *P. haitanensis* strains, revealing that Z-61 is more thermotolerant than the wild type because of an increase in the transcriptional regulation of genes mainly related to photosynthesis, antioxidant systems, and energy metabolism. In another investigation, 151 heat stress-responsive *P. haitanensis* proteins associated with various processes, mainly including photosynthesis, protein synthesis and degradation, defense response, and energy and carbohydrate metabolism, were identified based on the iTRAQ technique (Shi et al. 2017). On the basis of a lipidomics analysis, Chen et al. (2016b) detected a number of lipid biomarkers for high-temperature stress. Additionally, some candidate genes related to high-temperature responses have been cloned, including genes encoding manganese superoxide dismutase (Yang et al. 2013) and carbonic anhydrases (Chen et al. 2016a). In order to cope with HTs and other abiotic stresses, *Pyropia* species initiate various defense mechanisms to carry out the appropriate physiological and biochemical changes (Qian et al. 2015; Im et al. 2015; Wang et al. 2018a, 2019, 2020; Zheng et al. 2020). Notably, regardless of the pathway that is activated by HT stress, non-native proteins aggregate and partially unfolded proteins are produced and accumulate in *Pyropia* species, with detrimental effects on the thalli cells.

Heat shock proteins (HSPs) are molecular chaperones that can prevent irreversible protein aggregations (Liberek et al. 2008; Tyedmers et al. 2010). Based on their molecular weight, homology and functions, HSPs can be classified into several families. Small heat shock proteins (sHSPs) are the most ubiquitous HSPs subgroup, with molecular weights ranging from 12 to 42 kDa (Zhang et al. 2016). They are usually undetectable in vegetative tissues under normal growth conditions but can be induced by environmental stresses and developmental stimulation. In cells exposed to abiotic stresses, sHSPs function as the first line of defense by binding and stabilizing proteins at intermediate stages of folding, assembly, degradation, and translocation across membranes (Waters 2012). Compared to sHSP family in plants, those in red macroalgae seem to be less diverse. To date, only a few sHSPs have been identified in *Chondrus crispus* (Collén et al. 2013), *Porphyra umbilicalis* (Brawley et al. 2017), *Pyropia yezoensis* (Uji et al. 2019), and *P. haitanensis* (Chen et al. 2015). Identifying and clarifying the physiological functions of sHSPs in response to HT will provide a basis for molecular analyses and the selective breeding of *Pyropia* species. We previously cloned the *P. haitanensis* *Hsp22* gene and found its expression was up-regulated more than 1000-folds under HT stress (Chen et al. 2015). The stable genetic transformation system of hygromycin resistance gene by codon optimization has only been reported in *P. yezoensis* (Uji et al. 2014). Although

successful genetic transformation system in *P. yezoensis* has been reported, transgenic strains of *P. yezoensis* can only be propagated through monospore germination, which is a very convenient and fast way of propagation. However, *P. haitanensis* has no such asexual reproduction, resulting in it being difficult to obtain stable transformed cells for *P. haitanensis*. Therefore, sHSP functions remain largely unknown in these species.

Unlike *Pyropia* species, *Chlamydomonas reinhardtii* is a unicellular green alga broadly used for elucidating fundamental biological processes. There are several genetic resources and analytical tools available for *C. reinhardtii*, including nuclear, chloroplast, mitochondrial transformation systems, RNAi knockdown methods, a sequenced genome, and hundreds of genetically characterized mutants (Kindle 1990; Shimogawara et al. 1998; Neupert et al. 2012). Moreover, many heterologous genes have been expressed in *C. reinhardtii*, including genes from microalgae (Cordero et al. 2011), macroalgae (Kim et al. 2011), higher plants (Siripornadulsil et al. 2002), and humans (Rasala and Mayfield 2011). Specifically, Jin et al. (2017) inserted the *Pyropia tenera* *HSP19.3* gene into *C. reinhardtii* and confirmed that the physiological function of the encoded protein may influence HT tolerance. Accordingly, *C. reinhardtii* may be suitable for expressing *P. haitanensis* genes. However, the molecular mechanism underlying the HSPs function related to *C. reinhardtii* HT tolerance is unclear. The objectives of the present study were to establish a stable genetic transformation system of *P. haitanensis* gene (*PhHsp22*) in *C. reinhardtii* and investigate the *PhHsp22* expression pattern. Additionally, the regulation and expression of genes in response to HT stress were examined via the genome-wide high-throughput transcriptomic analysis of *C. reinhardtii* overexpressing *PhHsp22*.

Materials and methods

Algae and growth conditions

The *Pyropia haitanensis* Z-61 strain used in this study is tolerant to HT and produces a high yield. It was selected and purified by researchers in the Laboratory of Germplasm Improvements and Applications of *Pyropia* in Jimei University, Fujian Province, China. Five randomly selected blades (15 ± 2 cm long) were cultured at 21 °C with a 10-h light:14-h dark cycle ($50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ irradiance). Additionally, *Chlamydomonas reinhardtii* strain “CC-400 cw15 mt+” was used to examine the physiological function of *PhHsp22*. The *C. reinhardtii* cells were grown in Tris-acetate-phosphate (TAP) medium at 25 °C with shaking (100 rpm) and a 14:10 (light/dark) cycle with cool fluorescent light ($50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$).

Sequence and phylogenetic tree analyses of *PhHsp22*

The *PhHsp22* nucleotide sequence, deduced amino acid sequence, and open reading frame were analyzed with tools available on the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>) (Chen et al. 2015). Conserved domains were identified with SMART (<http://smart.embl-heidelberg.de/>). MEGA6 program was used to construct a phylogenetic tree according to the maximum likelihood method.

RNA extraction and cDNA synthesis

Total RNA was extracted from *P. haitanensis* and *C. reinhardtii* and purified with the E.Z.N.A. Plant RNA Kit (Omega, Germany). The quality and quantity of the purified RNA were determined by measuring the absorbance at 260 nm/280 nm (A_{260}/A_{280}) and 260 nm/230 nm (A_{260}/A_{230}) with a NanoDrop ND-1000 spectrophotometer (LabTech, USA). The integrity of the purified RNA was assessed by 1.2% agarose gel electrophoresis. The total RNA (1 µg) was used as the template for the first-strand cDNA synthesis with the PrimeScript RT reagent kit (TaKaRa, Japan) and random primers. The cDNA samples were diluted with nuclease-free water to 5 ng µL⁻¹ and then used as the templates for the quantitative real-time (qRT)-PCR analysis.

Isolation of *PhHsp22* and construction of a transformation vector

The complete *PhHsp22* cDNA was cloned with *PhHsp22* specific primers (Table S1). The pMD19-T (TaKaRa, Japan) and pChlamy_3 (Invitrogen, USA) vectors were used for constructing the transformation vector. The amplified *PhHsp22* sequence was first inserted into the pMD19-T vector. The resulting recombinant plasmid and pChlamy_3 were digested with KpnI and PstI (TaKaRa, Japan), after which the *PhHsp22* and linearized pChlamy_3 vectors were ligated to form the transformation vector.

Chlamydomonas reinhardtii nuclear transformation

The nuclear transformation of *C. reinhardtii* was completed according to a modified version of the electroporation method described by Yamano et al. (2013). Briefly, *C. reinhardtii* cells were cultured until the cell density reached 1–2 × 10⁶ cells mL⁻¹, after which a 10 mL aliquot of the cultured cells was centrifuged at 600×g for 5 min. The pelleted cells were re-suspended in 200 µL TAP medium containing 40 mM sucrose for a final density of 1 × 10⁸ cells mL⁻¹. The pChlamy-*PhHsp22* plasmids (2 µg) were linearized with PstI and added to the cell suspension. The cell suspension was added to an electroporation cuvette (ECM830, USA). Parameters were

optimized as three Pps of 300 V with 6 ms pulse length, 50 ms pulse interval. After electroporation, the cell suspension from the cuvette was transferred into 10 mL TAP medium containing 40 mM sucrose and incubated at dim light (1–2 µmol photons m⁻² s⁻¹) for 24 h. Transformed colonies were selected on TAP agar medium containing 10 µg mL⁻¹ hygromycin after 7–14 days of growth.

Stress treatment and quantitative real-time PCR analysis

Chlamydomonas was treated at 33 °C for 3, 6, and 12 h to observe the biomass. Cell growth was monitored based on the cell counts determined by measuring the optical density at 750 nm (Kumar et al. 2013). To assess the effects of HT on gene expression, cDNA was prepared for HT treatment samples for a qRT-PCR analysis with *PhHsp22*-specific primers (*Hsp22QF* and *Hsp22QR*). A β-tubulin gene was used as an internal control. The primers for qRT-PCR are listed in Table S1. The qRT-PCR was performed with 96-well plates and the ABI 7300 real-time PCR Detection system. The 20 µL reaction volumes comprised 10 µL 2 × SYBR Green Master Mix (Takara, China), 0.4 µL sense and antisense primers (20 mM), 2 µL diluted template, 0.4 µL ROX Reference Dye (50×), and 6.8 µL RNA-free water. The qRT-PCR program was as follows: 95 °C for 30 s and 40 cycles of 95 °C for 5 s and 60 °C for 31 s. A dissociation curve analysis of the amplification was completed after the PCR cycles to confirm that only one PCR product was amplified and detected.

Transcriptome assembly, annotation, and differential gene expression analysis

Only RNA samples with an A_{260}/A_{280} ratio of 1.9–2.1 and an $A_{260}/A_{230} > 2.0$ were used in the subsequent analyses. The de novo transcriptome assembly and annotation were completed by GENE DENOVO Biotech, Ltd. (Guangzhou, China). Gene expression levels were calculated based on the RPKM (reads per kilobase per million reads) method (Mortazavi et al. 2008). The gene expression data were then used to analyze differentially expressed genes (DEGs) with the DESeq2 software (Love et al. 2014).

Statistical analysis

All experiments were repeated three times. The standard deviation of the mean was used to determine the precision of the mean. The significance of any differences between the treated and control samples was assessed by a one-way ANOVA and post hoc testing with the LSD test of SPSS 12.0 (SPSS Inc, USA). The threshold for significance was set at $p < 0.05$.

Results

Identification of *PhHsp22* cDNA

In a previous work, we cloned *P. haitanensis* *Hsp22* from a cDNA library based on high-throughput sequencing. *PhHsp22* encodes a 19.1 kDa polypeptide with 172 amino acids (pI of 5.24) (Chen et al. 2015). The prediction of domain structures in *PhHsp22* by SMART revealed the presence of *PhHsp22* contained A-crystal structure domain from amino acids 52 to 149, as well as the HSP20 domain at amino acids 60–164 (Fig. 1a). A phylogenetic analysis supported the existence of a sister-group relationship between *P. haitanensis* and *Chondrus crispus* but implied that *Pyropia* species diverged from Cyanophyta, Chlorophyta, Phaeophyta, and land plant species (Fig. 1b).

Confirmation of *C. reinhardtii* transformation and *PhHsp22* expression under high-temperature stress

To investigate whether the constitutive expression of *PhHsp22* can improve the HT tolerance of transgenic *C. reinhardtii*, we transformed *C. reinhardtii* with the *PhHsp22* coding sequence under the control of a tandem promoter comprising the *Hsp70A* and *Rbc S2* promoters (Fig. S1). Many independently regenerated hygromycin-resistant lines were obtained. The transformation was then confirmed

by PCR analyses using *PhHsp22*-specific primers (Table S1). The expected amplicons were produced for all randomly selected hygromycin-resistant lines, but not for the wild-type control, indicating *PhHsp22* was integrated into the genome of the hygromycin-resistant lines (Fig. 2a). The 5th transgenic lines (T5) were selected for the subsequent HT tolerance analyses at the transcriptional and morphological levels. The *PhHSP22* expression levels in T5 were determined in a qRT-PCR assay. As shown in Fig. 2c, at least a fifteenfold increase of *PhHsp22* expression in transgenic *C. reinhardtii* was induced by HT stress, with peak expression levels at 33 °C (Fig. 2b). Thus, we evaluated the effects of this temperature on the transgenic *C. reinhardtii* over time (Fig. 2c). As shown in Fig. 2c, the maximal expression of the *PhHsp22* gene is detected after 3 h of induction at 33 °C. The *PhHsp22* expression level decreased gradually after 6 h and would gradually decline to the normal level after 12 h.

Tolerance of transgenic *C. reinhardtii* to high-temperature stress

The transgenic *C. reinhardtii* was clearly more tolerant to HT treatment (33 °C) than the WT control (Fig. 3). The cultures for the T5 were obviously greener than WT cultures at 33 °C. The differences between the WT and transgenic cultures exhibited a time-dependent profile.

Fig. 1 Domain and phylogenetic analyses of *PhHsp22*. **a** Protein domain analysis. **b** Phylogenetic tree comprising *PhHsp22* and genes encoding small heat shock proteins in other species. The tree was constructed based on amino acid sequences, and the number at each node indicates the percentage of bootstrapping after 1000 replications

Fig. 2 Transgenic verification of *PhHsp22*. **a** Confirmation that the putative transformants carried *PhHsp22* based on PCR analyses with gene-specific primers. **b** Expression of *PhHsp22* under different temperatures. **c** Expression of *PhHsp22* at 33 °C over time

Verification of differentially expressed genes

To further verify the reliability of the RNA-seq data, 8 unigene sequences were randomly selected from the DEGs of the transcriptome for qPCR analysis. There was a good correlation between the transcript abundances based on the qRT-PCR analysis and the expression profiles determined by RNA-Seq (Figure S2).

Functional analyses of differentially expressed genes

Based on the abovementioned results, we selected the *PhHsp22*-overexpressing transgenic *C. reinhardtii* incubated at 33 °C for 3 h for a transcriptome analysis, with the WT *C. reinhardtii* used as the control. The mRNA samples,

including different strains of *C. reinhardtii*, were subjected to paired-end sequencing using the Illumina HiSeq2500 platform. Details regarding the transcriptome assembly are provided in supplementary table S2. The high-temperature stress affected the WT control more than the transgenic *C. reinhardtii*. Specifically, compared with the WT-unstressed (CK) expression levels, the 3-h heat treatment of the WT *C. reinhardtii* (WT-3 h) upregulated and downregulated the expression of 3666 and 3487 genes, respectively. In contrast, the 3-h heat treatment of the transgenic *C. reinhardtii* (HSP-3 h) upregulated and downregulated the expression of 363 and 556 genes, respectively, relative to the HSP-CK expression levels. Additionally, there were 6958 DEGs between WT-CK and HSP-CK, whereas only 89 DEGs were detected between WT-3 h and HSP-3 h (Fig. 4a). Furthermore, the top

Fig. 3 Effects of *PhHsp22* on transgenic *Chlamydomonas reinhardtii*. **a** Biomass of *C. reinhardtii* under heat stress for 3 days. **b** Concentration of *C. reinhardtii* under high-temperature stress

Fig. 4 Functional analyses of differentially expressed genes. **a** Global overview of the differentially expressed genes (DEGs) among samples (WT-CK, wild-type unstressed control; WT-3 h, wild type exposed to high-temperature stress for 3 h; HSP-CK, transgenic *C. reinhardtii*

unstressed control; HSP-3 h, transgenic *C. reinhardtii* exposed to high-temperature stress for 3 h). **b** Top 20 enriched KEGG pathways among the DEGs between WT-CK and HSP-CK. **c** Number of up- and downregulation genes related to the top 20 enriched KEGG pathways

20 enriched KEGG pathways among the DEGs between WT-CK and HSP-CK included proteasome, photosynthesis-antenna proteins, citrate cycle, metabolic pathway, and ABC transporters (Fig. 4b). The expression levels of most of the DEGs related to these pathways were significantly upregulated in HSP-CK (Figs. 4c and 5).

To reveal the functional relationships among the DEGs involved in proteasome, ubiquitin-mediated proteolysis, protein processing in the endoplasmic reticulum, and citrate cycle, a protein-protein interaction network was generated with the STRING program. This network showed that the sub-genes encoded diverse proteins, including protein disulfide isomerase (prtp), heat shock protein 70 (HSP70), 20S proteasome alpha subunit E (PAE1), and malate dehydrogenase (MMDH1) (Fig. 6). Moreover, the expression levels of the DEGs associated with the top 20 enriched KEGG pathways were mainly downregulated and upregulated in HSP-3 h and CK-3 h samples, respectively (Fig. 7).

Discussion

In the present study we determined that *PhHsp22* contained the theoretical domain of ACD and HSP20, which is consistent with the domains of *Arabidopsis thaliana* sHSPs (Scharf

et al. 2001). The N-terminal amino acid sequence of sHSPs is poorly conserved in other species, indicating that the N-terminal of *PhHsp22* has lower conservation (Chen et al. 2015). In contrast, the C-terminal is highly conserved and contains ACD (Scharf et al. 2001). A previous study confirmed that the C-terminal region of *PsHsp20* in *Porphyra* species is highly conserved (Park et al. 2012). Our findings suggest that the *PhHsp22* may play a similar function of response to stress at the C-terminal. sHSPs have a high capacity to bind to non-native proteins, but not able to refold, which maybe interact through hydrophobic. Phylogenetic analysis demonstrated that *PhHsp22* was poorly clustered with the sHSPs genes from other species (Fig. 1b), which reflect the diversity in the evolution of these genes.

HT stress has a complex effect on cell functions and can completely inactivate enzymes, resulting in protein denaturation (Kampinga et al. 1995). Accordingly, HT may disrupt the synthesis of native proteins in plants, while accelerating the production of HSPs and heat shock factors (Nover et al. 2001; Wang et al. 2004). Our earlier research involving transcriptome analyses and cloning revealed that the synthesis of sHSPs in *Pyropia* species might increase to mitigate the harmful effects of environmental stresses, including high temperatures (Chen et al. 2015), drought (Shi et al. 2019), and salinity (Wang et al. 2019). Because only *P. yezoensis* has a stable

Fig. 5 Heat map of the differentially expressed genes involved in the proteasome, ubiquitin-mediated proteolysis, protein processing in the endoplasmic reticulum (ER), and citrate cycle (TCA cycle) between

WT-CK (wild-type unstressed control) and HSP-CK (transgenic *C. reinhardtii* unstressed control)

transformation system, there is a lack of relevant research on *P. haitanensis* (Uji et al. 2014). We introduced *PhHsp22* into the *C. reinhardtii* genome and analyzed its heat-induced expression in the current study. Driven by the *Hsp70A* and *RbcS2* tandem promoter, *PhHsp22* in transgenic lines could respond positively to heat stress (Fig. 2b). The selection of an appropriate promoter is crucial for gene transformations and transgenes expression in plants (Potenza et al. 2004). Unfortunately, only a few promoters have been confirmed as suitable for red algae (Huddy et al. 2012; Son et al. 2012). There are some promoters in *P. yezoensis* that can be used normally, but they have not been proven to be suitable for *P. haitanensis* (Fukuda et al. 2008; Uji et al. 2010). The

observed upregulated expression of *PhHsp22* suggests that *Hsp70A* and *RbcS2* promoters may be effective for driving transgene expression in *C. reinhardtii*. In addition, the present result findings imply that *PhHsp22* contributes to HT stress response in *C. reinhardtii*. During the high-temperature (33 °C) treatment, the promoter of *hsp70a-rbcS2* induced high expression of *phhsp22* in transgenic *C. reinhardtii* from 3 to 6 h (Fig. 2c). Meanwhile, overexpression of *PhHsp22* contributes to heat stress tolerance in *Chlamydomonas* (Fig. 3). These results are similar to those of an earlier transgenic study (Jin et al. 2017). The expression of the *Pyropia tenera* *HSP19.3* gene inserted into the *C. reinhardtii* genome is reportedly upregulated soon after initiating a HT treatment and confers

Fig. 6 Protein-protein interaction network for differentially expressed genes related to proteasome, protein processing in endoplasmic reticulum, ubiquitin-mediated proteolysis, and citrate cycle (TCA cycle) in transgenic *C. reinhardtii* based on a STRING analysis. The proteins encoded by the sub-genes in the network are presented in different colors.

Red color represents a degree between 634 and 425, whereas purple color represents a degree between 425 and 200. The green line indicates a combined score greater than 543, whereas the gray line indicates a combined score of less than 543. The circle size corresponds to the number of protein interactions. Protein annotations are listed in Table S2

the tolerance to heat (Jin et al. 2017). Our data suggest that *PhHsp22* from *P. haitanensis* might play a significant role in resisting HT stress.

As chaperones, sHSPs can bind to denatured proteins in an ATP-independent pattern to prevent them from aggregating thereby maintaining cellular homeostasis. *PhHsp22* is the most heat stress-responsive *P. haitanensis* sHSPs gene. Moreover, its positive effects on the HT tolerance of transformed *C. reinhardtii* have been confirmed. However, it remains unclear how much of this HT tolerance is due to this gene. Therefore, we compared the transcriptional profiles of WT and *PhHsp22*-overexpressing *C. reinhardtii* exposed to

the same HT stress. The results suggest that *PhHsp22* mainly affects the proteasome, photosynthesis-antenna proteins, citrate cycle, oxidative phosphorylation, biosynthesis of amino acid, protein processing in endoplasmic reticulum, ubiquitin-mediated proteolysis, and other pathways (Figs. 4 and 5). Thus, the transgenic *C. reinhardtii* had fewer DEGs than WT control following the same HT treatment (Fig. 4a), indicating that *PhHsp22* can maintain the cellular homeostasis of *C. reinhardtii* in response to HT stress.

Misfolded and denatured proteins inevitably accumulate in *Pyropia thalli* under HT stress conditions. The misfolded and unfolded proteins are perceived as endoplasmic reticulum

Fig. 7 Numbers of up- and downregulated genes among the top 20 enriched KEGG pathways between HSP-CK and HSP-3 h (a) as well as between WT-CK and WT-3 h. **b** WT-CK, wild-type unstressed control; WT-3 h, wild type exposed to heat stress for 3 h; HSP-CK, transgenic *C. reinhardtii* unstressed control; HSP-3 h, transgenic *C. reinhardtii* exposed to heat stress for 3 h

stress by specific sensor proteins in the endoplasmicreticulum membrane, ultimately resulting in the expression of genes encoding chaperones. In addition, misfolded and unfolded proteins are ubiquitinated by 26S proteasome for endoplasmic reticulum-associated degradation (ERAD) or inhibit protein translation to reduce the abundance of synthetic proteins and avoid endoplasmic reticulum stress (Helenius and Aebi 2004; Zhu 2016). The quality control system recognizes proteins in the endoplasmic reticulum based on interactions with calnexin and calreticulin (Helenius and Aebi 2004). Correctly folded proteins are transported to the Golgi apparatus through COP II, whereas misfolded and denaturation proteins are degraded by a proteasome or refolded via HSPs and other molecular chaperones (Wang et al. 2018a; Shi et al. 2019; Wen et al. 2020). In a recent study, all of the genes annotated by ubiquitin-mediated proteolysis, including those encoding the ubiquitin-activating enzyme, ubiquitin-conjugating enzyme, ubiquitin ligase, and most of the genes related to 26S proteasome pathway exhibited upregulated in *P. haitanensis* under severe HT conditions (Wang et al. 2018a). This HT-induced expression may be due to the fact that the ubiquitin-

proteasome system mediates the removal of damaged and/or misfolded proteins and modulates regulatory protein contents. Additionally, Wen et al. (2020) pointed out that increases in the abundance of HSPs and proteins involved in ubiquitin-mediated proteasome activity stabilize the dynamic balance between protein folding and removal in *P. haitanensis* in response to hypersalinity. Here, the expression levels of 26 of 35 DEGs related to ubiquitin-mediated proteolysis as well as 25 of 38 DEGs related to protein processing in endoplasmic reticulum were upregulated in the *PhHsp22*-overexpressing transgenic *C. reinhardtii* (Fig. 5). Furthermore, *PhHsp22* up-regulated the expression of all but one of the 36 DEGs related to proteasomes which are large multi-subunit proteases in the cytosol (Bochtler et al. 1999). Proteasomes can recognize, unfold, and digest protein substrates that have been marked for degradation with an ubiquitin moiety (Bochtler et al. 1999). Thus, the *C. reinhardtii* cells expressing *PhHsp22* may be protected from fluctuations in environmental conditions via buffering proteasome and HSPs pools (Hanna et al. 2007). HSP expansions mediating protein homeostasis might reflect the adaptation of *Pyropia* species to the intertidal

environment (Wang et al. 2019, 2020). The protein-protein interaction network analysis also indicated that HSPs influence key regulatory activities (Fig. 6). Therefore, our results imply that *PhHsp22* contributes to the HT tolerance of *C. reinhardtii* by maintaining cellular homeostasis.

Additionally, we previously determined that severe HT stress substantially upregulates the expression of genes related to encoding energy metabolism in *P. haitanensis*, thereby enhancing carbohydrate metabolism to provide energy and the related carbon skeletons for key metabolic processes (Wang et al. 2018a) such as the citrate cycle is responsible for the oxidation of respiratory substrates that drive ATP synthesis (Sweetlove et al. 2010) and considerably influences the ability of plants to resist the effects of abiotic stresses (Mailloux et al. 2007). First, pyruvate dehydrogenase converts pyruvic acid to acetyl coenzyme A (acetyl-CoA). The formation of citric acid from acetyl-CoA and oxaloacetic acid via a reaction catalyzed by citric acid synthetase represents the beginning of the citrate cycle. Citric acid synthetase is the rate-limiting enzyme of the citrate cycle. We observed that compared with the WT levels, the expression levels of 28 of 29 DEGs related to pyruvate dehydrogenase, malate dehydrogenase, succinate dehydrogenase, citrate synthase, and other key enzymes were obviously upregulated in the transgenic *C. reinhardtii* cells (Fig. 5). This is consistent with previous studies, *C. reinhardtii* switching from regular metabolism to a specialized metabolism for stress protection (Schroda et al. 2015). Within the first 3 h of HS stress, several of TCA/glycolytic enzymes and the glyoxylate cycle were significantly downregulated, while the main metabolism metabolites are rapidly depleted (Hemme et al. 2014). At the same time, the quantitative results also verify this point (Figure S2). Therefore, *PhHsp22* may mediate energy metabolism in *P. haitanensis* exposed to high-temperature stress. The enhancement of the citrate cycle and other energy metabolism-related processes not only increase energy production but also provide sufficient amounts of carbon skeletons for amino acid biosynthesis, DNA replication, arginine biosynthesis, and protein processing (Fig. 4c) to protect thalli from HT stress.

Conclusion

In this study, the function of the *PhHsp22* gene from *P. haitanensis* was verified by transformed into *C. reinhardtii*. The early upregulation of *PhHsp22* expression following a HT treatment suggests this gene contributes to the early response to HT stress. Moreover, its HT-induced expression increases the survival of transgenic *C. reinhardtii* via its effects on cellular energy, secondary metabolites, HSPs, and proteasome pools. The results of this investigation may clarify the regulatory effects of HSPs on the HT tolerance of *P. haitanensis*. Furthermore, *PhHsp22* may be useful for the

breeding of new macroalgal strains adapted to global warming.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10811-020-02351-6>.

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Authors' contributions C.T.X., W.L.W., and J.Z.S. conceived and designed the experiment. J.C., J.Z.S., and W.L.W. performed experiments and data analysis. C.T.X. and C.S.C. contributed by planning, supervising, and financing the work. D.H.J. and Y.X. helped to prepare the materials and reagents. J.C., J.Z.S., and W.L.W. drafted and revised the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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