Research Article

Algae 2021, 36(3): 207-217
https://doi.org/10.4490/algae.2021.36.8.26
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Downregulation of *PyHRG1*, encoding a novel secretory protein in the red alga *Pyropia yezoensis*, enhances heat tolerance

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An increase in seawater temperature owing to global warming is expected to substantially limit the growth of marine algae, including *Pyropia yezoensis*, a commercially valuable red alga. To improve our knowledge of the genes involved in the acquisition of heat tolerance in *P. yezoensis*, transcriptomes sequences were obtained from both the wild-type SG104 *P. yezoensis* and heat-tolerant mutant Gy500. We selected 1,251 differentially expressed genes that were up- or downregulated in response to the heat stress condition and in the heat-tolerant mutant Gy500, based on fragment per million reads expression values. Among them, *PyHRG1* was downregulated under heat stress in SG104 and expressed at a low level in Gy500. *PyHRG1* encodes a secretory protein of 26.5 kDa. *PyHRG1* shows no significant sequence homology with any known genes deposited in public databases to date. However, *PyHRG1* homologs were found in other red algae, including other *Pyropia* species. When *PyHRG1* was introduced into the single-cell green alga *Chlamydomonas reinhardtii*, transformed cells overexpressing *PyHRG1* showed severely retarded growth. These results demonstrate that *PyHRG1* encodes a novel red algae-specific protein and plays a role in heat tolerance in algae. The transcriptome sequences obtained in this study, which include *PyHRG1*, will facilitate future studies to understand the molecular mechanisms involved in heat tolerance in red algae.

Key Words: heat stress tolerance; *PyHRG1*; *Pyropia yezoensis*; red algae; transcriptome

Abbreviations: DEG, differentially expressed gene; FPKM, fragments per kilobase per million reads; HSP, heat shock protein; ORF, open reading frame; qRT-PCR, quantitative reverse transcription polymerase chain reaction; ROS, reactive oxygen species

INTRODUCTION

Pyropia yezoensis (Bangiales, Rhodophyta) is a commercially valuable and most cultivated marine red alga. It has a heteromorphic life cycle, alternating between the foliose thallus gametophyte and filamentous sporophyte generations (Blouin et al. 2011). The gametophyte thalli, which are used as an important food resource, grow in cold water during winter and transitions to the sporophytic conchocelis phase in summer. Temperature is one

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of the major abiotic stresses affecting *P. yezoensis* gametophyte growth rate (Avila et al. 1986, Hwang et al. 1997, Luo et al. 2014, Hwang and Park 2020). Therefore, the rise in seawater temperature owing to global warming is expected to significantly limit *Pyropia* growth. To mitigate the effects of increasing seawater temperatures, it may be necessary to develop high-temperature-resistant varieties of *Pyropia*.

Understanding the molecular genetic circuits of plants exposed to high temperatures will be key to successful breeding of heat-resistant crop varieties. Plant responses to heat stress include a reduction in the expression level of genes involved in photosynthesis, the biosynthesis of storage compounds, such as starch, and an increase in the expression level of the genes that are important for cellular homeostasis (Shinozaki et al. 2015). Heat shock proteins (HSPs) are typically genes that are upregulated under high-temperature conditions and play an important role in the cellular response to heat stress (Wang et al. 2004). HSPs act as molecular chaperones that prevent irreversible aggregations and re-solubilize already aggregated proteins under heat stress conditions (Wang et al. 2004). Heat stress results in the formation of reactive oxygen species (ROS), which at low concentrations play a role as signaling molecules but at high concentrations may lead to oxidative damage. To protect against this damage, the levels of ROS scavengers such as superoxide dismutases, catalases, and peroxidases in the cells are increased (Mittler 2002, Mittler et al. 2004). In addition, compatible solutes, such as proline, glycine betaine, and sugar alcohols, are accumulated in the cells, similar to those observed in the response to osmotic stress (Mittler 2002, Livingston et al. 2009, Van den Ende and Valluru 2009, Dai et al. 2020). Furthermore, previous studies have demonstrated that heat stress affects the structure of the cell wall (Moore et al. 2008, Sasidharan et al. 2011, Le Gall et al. 2015, Chen et al. 2020); the plant cell wall determines the size and shape of the cell through the mechanical control of cell expansion (Chen et al. 2020). Some cell wall-related genes may play a role in the acquisition of thermotolerance (Yang et al. 2006, Ha et al. 2007, Rienth et al. 2013, Le Gall et al. 2015, Chen et al. 2020). However, additional studies are needed to fully understand the role of the plant cell wall in heat tolerance at the physiological, genetic, and biochemical levels. Our knowledge of the heat response in plants is based on green plants including *Arabidopsis*, but research on red algae including *Pyropia* is currently limited.

To find the genes involved in the acquisition of heat tolerance in *P. yezoensis*, we compared transcriptomes from gametophytes of wild-type and heat-tolerant mutants *P. yezoensis* under control and heat-stress conditions and identified differentially expressed genes (DEGs). We selected a DEG, *PyHRG1*, which was downregulated in the gametophytes of wild-type *P. yezoensis* under high-temperature conditions and in the heat-tolerant mutant, and characterize its physiological function. This study would be valuable by providing the gene resources for heat tolerance in *P. yezoensis*.

MATERIALS AND METHODS

Plant material and stress treatment

P. yezoensis var. Sugwawon 104 (SG104) and Gy500 were obtained from the Fisheries Seed and Breeding Research Institute, Korea. Gy500 is a heat-tolerant mutant developed from *P. yezoensis* SG104 (Park et al. unpublished data). Heat-tolerant mutant Gy500 was developed by radiation breeding as described by Shin et al (2018). Conchocelis line from selected heat tolerant gametophyte was established after three rounds of the asexual cycle from single monospore to thallus. Gametophyte thalli of Gy500 grew better than those of SG104 under both high-temperature and normal growth conditions. *P. yezoensis* was cultured in modified Grund medium (McLachlan 1973) in a growth chamber under the following conditions: temperature, 12°C; irradiation, 80 µmol photon m^{-2} s^{-1} provided by cool-white fluorescent lamps; photoperiod, 10 : 14 (light : dark). For heat treatment, growth bottles containing *P. yezoensis* were transferred to a growth chamber at 20°C with the same light intensity and photoperiod. All gametophyte thalli were snap frozen using liquid nitrogen and maintained at -80°C until RNA extraction.

Transcriptome sequencing and identification of DEGs

Transcriptome sequence reads were obtained in triplicate from gametophyte thalli of wild-type (SG104) and mutant (Gy500) *P. yezoensis*, under control (12°C) and high-temperature (20°C) conditions. Library construction and RNA sequencing was performed using the Illumina Hi-Seq 2500 platform at G&C Bio Company (Daejeon, Korea). Briefly, sequencing reads were evaluated for quality using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and preprocessed to remove adapter sequences using Cutadapt software (ver.

1.9.1; https://cutadapt.readthedocs.io/en/stable/). Finally, high-quality, clean reads were subjected to *de novo* assembly using the Trinity (https://github.com/trinityrnaseq/trinityrnaseq) software with the default parameters.

Fragments per kilobase per million reads (FPKM) values were applied to measure gene expression levels. Sequencing reads from each cDNA library were mapped to the *de novo* assembled contigs using Bowtie2 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml), and DEGs were detected using edgeR (https://bioconductor.org/packages/release/bioc/html/edgeR.html), with criteria of at least 2-fold ratio changes and q-values < 1.0×10^{-3}. The heatmap of the sample correlation matrix was generated across all replicates using the Trinity-bundled Differential Expression analysis script PtR with default parameter. For gene annotation, DEGs were compared to the sequences in the NCBI non-redundant database (updated Jul 30, 2021) and Uniprot / Swissprot (updated Jul 31, 2021) using BLASTX program 2.12.0+ with default parameter. Contigs with an E-value of >1.0E-10 were considered not available.

Identification and characterization of *PyHRG1*

Among the selected DEGs, the contig Py97124 had no sequence homology to any known gene and was highly downregulated under heat stress conditions and in the heat-tolerant mutant. The downregulated expression pattern of contig Py97124 was validated using quantitative reverse transcription polymerase chain reaction (qRT-PCR). Therefore, we selected Py97124 for further study and named it "*Heat Response Gene 1* of *P. yezoensis* (*PyHRG1*)."

The cDNA covering the full open reading frame (ORF) of *PyHRG1* was amplified from wild-type (SG104) and mutant (Gy500) *P. yezoensis* and cloned to create a pGEM T-easy vector (Promega, Madison, WI, USA). The putative molecular weight and isoelectric point (pI) of *PyHRG1* were predicted using Geneious R8 software (Biomatters Limited, Auckland, New Zealand). To identify *PyHRG1* homologs in the *Pyropia* genome, amino acid sequences deduced from *PyHRG1* were used to search the draft-genome sequence of *P. yezoensis* (Kim et al. 2021). Multiple sequence alignments of amino acid sequences were performed using ClustalX software (http://www.clustal.org/clustal2). A phylogenic analysis was conducted using the neighbor-joining method using CLC viewer 8.0 (CLC Bio, Aartus, Denmark).

Expression and subcellular localization of *PyHRG1*

Total RNA was obtained from the gametophyte thalli using an RNeasy plant mini kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. qRT-PCR was performed as described by Choi et al. (2013) using *PyHRG1*-specific primer sets (5′-GTCAAAACGCCGACCAAGAC-3′ and 5′-CACGATCCATCGTCCTGCTT-3′). *PyUBQ* (5′-TTTCCAAGGTGCTCCTCTCCATC-3′ and 5′-CGTCTCTTCATAGCGACTGCGGTT-3′) was used as an internal control. All the samples were run in duplicate, and the n-fold differential expression was calculated using the comparative Ct method ($2^{-\Delta\Delta Ct}$) with three replicates. Differences between the samples were compared using t-test in Microsoft Excel software.

Cellular localization of PyHRG1 was analyzed using the web-accessible software WoLF PSORT (https://wolfpsort.hgc.jp) and DeepLoc (http://www.cbs.dtu.dk). Bioinformatic analysis predicted that PyHRG1 could be a secretory protein (Supplementary Table S1). To examine the cellular localization of PyHRG1, the *PyHRG1* coding region was amplified using the primers 5′-TCTAGAATGGTCGGTACCGC-3′ and 5′-GGATCCAGCACTTCTGCCA-3′, containing an *Xba*I site upstream and a *Bam*HI site downstream. PCR products were introduced into the *Xba*I and *Bam*HI sites of the 326-GFP-3G vector. The constructed *PyHRG1-GFP* vector was introduced into tobacco (*Nicotina benthamiana*) protoplasts. The transformed tobacco protoplasts were then incubated at 25°C for 12–24 h under dark conditions. The recombinant DNA was also introduced into onion (*Allium cepa*) epidermis cells via particle bombardment, as described by Ha et al. (2007). Subsequently, the fluorescence signals of PyHRG1-GFP fusion protein were evaluated under a fluorescence microscope (Leica, Wetzlar, Germany).

Transformation and abiotic stress tolerance assay of *Chlamydomonas*

The physiological function of *PyHRG1* in the single-cell green alga *Chlamydomonas reinhardtii* was assayed as described by Im et al. (2017). The ORF of *PyHRG1* was amplified using primers 5′-CGCCATATGGTCGGTACCGCCGC-3′ and 5′-CTGCAGGCACTTCTGCCACGATCCA-3′ and then subcloned into the *NdeI* and *PstI* sites of the *PsaD* promoter in pCr112, a *Chlamydomonas* expression vector. This pCr112-*PyHRG1* plasmid was then introduced into *C. reinhardtii* Mut11. The introduction of *PyHRG1* into transgenic *Chlamydomonas* cells and its expression were confirmed using qRT-PCR. The *Chlam-*

ydomonas actin gene (*CrActin*) was used as a control (5′-TGTGCATACGTGGATAGCTTG-3′ and 5′-ATGACCCG-CTCCTCATATCTT-3′).

To assay *C. reinhardtii* cell growth, cells were cultured to an absorbance of 0.75 at 750 nm. *C. reinhardtii* cells were diluted (10^{-1} to 10^{-3}) with TAP fresh medium, inoculated onto agar plates, and cultured at 23°C in a growth chamber under a 14 : 10 (light : dark)-photoperiod and cool-white fluorescent light (50 μmol photon m^{-2} s^{-1}). For the heat treatment, cells were incubated in a growth chamber at 35°C, under the same light intensity and photoperiod. Four days after heat treatment, cells were transferred to a growth chamber kept at 23°C and cultured for 1 week. *C. reinhardtii* Mut11 harboring an empty pCr112 vector was used as a control.

RESULTS AND DISCUSSION

Transcriptome sequencing and identification of DEGs

To identify genes that respond to heat stress in *P. yezoensis*, cDNA libraries were constructed from the gametophytes of wild-type SG104 and heat-tolerant mutant Gy500 under normal culture conditions (control) or heat stress (20°C) conditions. Three cDNA libraries were generated from three replicates for each sample. A total of 113.2 Gb of transcriptome sequences were generated from 12 libraries, and a total of 69.4 Gb of high-quality sequences were obtained after preprocessing (Table 1). *De novo* assembly processing generated a total of 242,470 contigs, with an N50 length of 961 bp and covering a total of 161.8 Mb (Table 1). Clustering analysis of the sample correlation matrix showed that each replicate was strongly correlated within samples and was reliable (Fig. 1). Transcriptomes of the heat-tolerant mutant Gy500, in both control conditions and under heat stress, were correlated significantly higher with those generated from gametophytes of wild-type SG104 under heat stress than with those generated under control condition (Fig. 1).

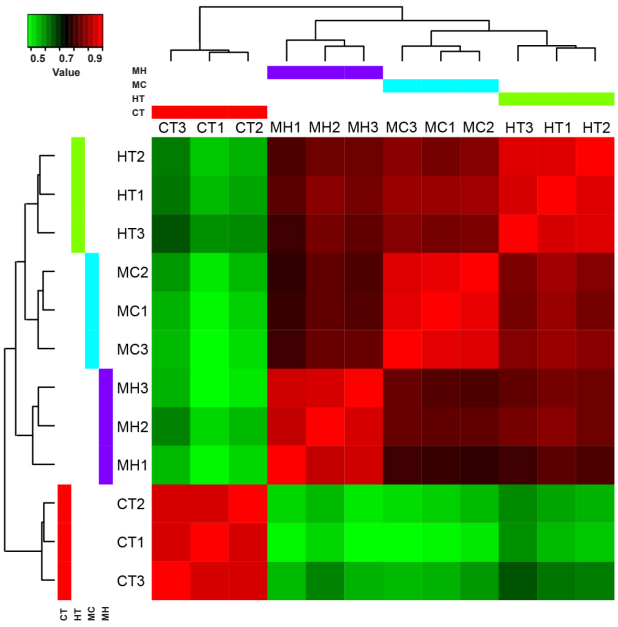

Fig. 1. Heatmap of the sample correlation matrix. Three cDNA libraries were generated from three replicates for each sample. A total of 113.2 Gb of transcriptome sequences were obtained from 12 libraries (see Table 1): CT1, CT2, and CT3 from wild-type *Pyropia yezoensis* SG104 under the control condition; HT1, HT2, and HT3 from SG104 under the heat stress condition; MC1, MC2, and MC3 from the heat-tolerant mutant Gy500 under the control condition; and MT1, MT2, and MT3 from Gy500 under the heat stress condition.

To screen for DEGs responsive to heat stress, contigs were analyzed by FPKM value fold change comparisons. A

Table 1. Summary of the generation and *de novo* assembly of transcriptome sequence reads from the gametophyte thalli of wild-type SG104 and heat-tolerant mutant Gy500 *Pyropia yezoensis* under control and heat stress conditions

		Wild-type (SG104)						Heat-tolerant mutant (Gy500)						Total
		Control (CT)			Heat (HT)			Control (MC)			Heat (MT)			
		1	2	3	1	2	3	1	2	3	1	2	3	
Raw	Total No. (10^6 pairs)	43.9	25.3	33.5	29.5	32.2	27.2	25.2	41.6	32	30.5	30.5	23.4	374.7
	Total length (Gb)	13.3	7.6	10.1	8.9	9.7	8.2	7.6	12.6	9.7	9.2	9.2	7	113.2
Clean	Total No. (10^6 pairs)	40.9	14.6	18.9	17.9	19.4	16.7	17.4	19.4	14	15.1	23.9	19.8	238.1
	Total length (Gb)	11.9	4.3	5.5	5.2	5.7	4.9	5	5.6	4.1	4.5	6.9	5.8	69.4
Assembly	No. of contigs													242,470
	Total length (Mb)													161.8
	N50 (bp)													961
	No. of contigs >1 kb													40,906
	Average contig length (bp)													667.3

RNA sequences were generated from three replicates for each sample.

total of 1,251 contigs were identified as DEGs in response to heat stress (Supplementary Tables S1 & S2). A total of 409 contigs among the identified DEGs were downregulated in heat stressed wild-type SG104 and showed low levels even under the control condition in heat-tolerant mutant Gy500 (Supplementary Table S2). Transcriptome analysis is a rapid and efficient method being used to identify genes involved in specific metabolic or stress tolerance processes (Rodriguez et al. 2010, Song et al. 2016, Chen and Li 2017, Wang et al. 2017). RNA sequencing projects have been applied to the phylum Rhodophyta to identify genes involved in the development and abiotic stress responses (Chan et al. 2012, Choi et al. 2013, Im et al. 2015, 2017). Previous studies in Rhodophyta, however, observed transcriptional changes in gametophyte of wild type plants under control and stress condition. Transcriptome sequences from heat-tolerant mutant Gy500 of *P. yezoensis* will facilitate future studies for identification and understanding of the molecular mechanisms involved in heat stress tolerance in *P. yezoensis*.

Identification and characterization of *PyHRG1*

In this study, we focused on DEGs, which were significantly downregulated in the gametophytes of wild-type SG104 under heat stress and also detected at low levels in the heat-tolerant mutant Gy500 under both control and heat stress conditions. DEGs with FPKM values greater than 20 in SG104 under control conditions and SG104 : Gy500 ratios greater than 32 under control conditions were selected and summarized in Table 2. Among them, we selected the DEG *Py97124*, which was strongly downregulated in the gametophytes of wild-type SG104 under heat stress and detected at low levels in the heat-tolerant mutant Gy500 (Table 2). Downregulation of the DEG *Py97124* in gametophyte under heat stress condition and in heat-tolerant mutant were confirmed using qRT-PCR (Supplementary Fig. S1).

The cDNA of *PyHRG1* (accession No. MT122996) encodes a polypeptide of 241 amino acids with a molecular weight of 26.5 kDa and a pI of 9.11. Glycine (14.2%) was the most abundant amino acid in the *PyHRG1* polypeptide (Fig. 2A). *PyHRG1* showed no sequence homology with any known genes currently deposited in public databases, except with those of other red algae; *PyHRG1* homologs were also identified from other *Pyropia* species, *P. tenera* and *P. seriata* (Fig. 2A). *PyHRG1* shared 97.9% sequence identity with the *PtHRG1* of *P. tenera*, the closest relative of *P. yezoensis*. *PyHRG1* homologs were also identified in another *Porphyra* species, *Porphyra umbilicalis* (Fig. 2B). Although red algae and green plants share abiotic stress tolerance mechanisms, not all stress response genes identified in green plants are found in red algae; some stress genes are specific to red algae (Choi et al. 2013, Lu and Xu 2015, Im et al. 2017, Na et al. 2018). Data from this study suggest that *PyHRG1* is a novel red algae-specific gene or its homologs in green plants have significantly lower sequence similarity for identification.

Based on the analysis of the draft-genome sequence of *P. yezoensis*, we identified three *PyHRG1* homologs, which shared approximately 31.1–41.2% amino acid sequence identity with *PyHRG1* (Supplementary Table S3 & Fig. S2). *PyHRG1* homolog 4 had the highest sequence homology with *PtDEG5*, which was previously reported to be a *P. tenera* desiccation response gene (Im et al. 2017). These results suggest that *PyHRG1* may be involved in responses to desiccation as well as to heat stress. Although its expression patterns were different for SG104 and Gy500, cDNA sequences of *PyHRG1* were identical for both *P. yezoensis* variants (Supplementary Fig. S3). These results suggest that the high-temperature tolerance phenotype of Gy500 is not a result of *PyHRG1* mutation but rather a result of downregulation of *PyHRG1* expression in Gy500.

PyHRG1 inhibits cell growth in *Chlamydomonas reinhardtii*

PyHRG1 was downregulated in *P. yezoensis* SG104 gametophytes under heat stress condition. And in gametophytes of heat-tolerant mutant Gy500, the transcripts of *PyHRG1* was detected at a much lower level than that of SG104 (Table 2). To assess the physiological function of *PyHRG1*, the ORF of *PyHRG1* was subcloned into the *PsaD* promoter, a constitutive expression promoter of *Chlamydomonas*, in a pCr112 vector. *PyHRG1* was introduced into the single-cell green alga, *C. reinhardtii* Mut11. Introduction and expression of *PyHRG1* in transgenic *C. reinhardtii* were verified using reverse transcription polymerase chain reaction with *PyHRG1*-specific primers. *PyHRG1* transcripts were detected in all selected transformed *C. reinhardtii* cells, and no amplification bands were observed in control cells transformed with an empty pCR112 vector (Fig. 3A). Transgenic *C. reinhardtii* cells overexpressing *PyHRG1* had slower growth rates than wild-type cells under normal growth condition (23°C) (Fig. 3B). These results demonstrate that *PyHRG1* plays a role in the growth of *C. reinhardtii* cells. When transgenic *C. reinhardtii* cells overexpressing *PyHRG1* were exposed to heat stress, cell growth inhibition was further exacerbated. These results suggest that *PyHRG1* is not the only

Table 2. Summary of selected DEGs downregulated in the gametophyte of *Pyropia yezoensis* SG104 under heat stress condition and in heat-tolerant mutant *P. yezoensis* Gy500

DEG contig ID[a]	Expression level (FPKM[b])				Sequence homology			
	SG104		Gy500		NCBI NR		Swissprot	
	Control	Heat	Control	Heat	Description	E-value	Description	E-value
DN97124_c0_g3_i3[c]	290.09	2.03	0.73	0.81	Hypothetical protein BU14_0577s0005 [*Porphyra umbilicalis*]	1.00E-44	NA[d]	-
DN100909_c0_g3_i5	156.56	3.39	3.78	2.78	NA	-	NA	-
DN60776_c0_g1_i3	124.08	-	2.12	14.71	NA	-	NA	-
DN98375_c0_g2_i4	90.24	0.13	0.01	0.01	Beta-glucuronosyltransferase GlcAT14B [*Gracilariopsis chorda*]	2E-63	Beta-glucuronosyltransferase GlcAT14B	6E-20
DN103548_c0_g6_i4	89.98	-	1.51	0.08	NA	-	NA	-
DN104214_c1_g5_i3	86.05	0.98	2.60	0.27	Hypothetical protein KFL_013290010, partial [*Klebsormidium nitens*]	2E-97	Retrovirus-related Pol polyprotein from transposon TNT 1-94	1E-68
DN102638_c2_g4_i4	75.98	0.45	-	0.73	NA	-	NA	-
DN103283_c0_g1_i7	74.91	1.18	0.43	1.38	Hypothetical protein BU14_0055s0021 [*Porphyra umbilicalis*]	4E-89	Histone H3 [*Drosophila melanogaster*]	7E-88
DN105032_c1_g8_i4	65.85	0.99	1.84	0.17	Hypothetical protein AVDCRST_MAG94-1486, partial [uncultured *Leptolyngbya* sp.]	1E-117	Retrovirus-related Pol polyprotein from transposon TNT 1-94	3E-88
DN104263_c2_g4_i5	30.44	-	0.27	0.31	Hypothetical protein BU14_0207s0014 [*Porphyra umbilicalis*]	8E-106	NA	-
DN104187_c0_g3_i3	23.46	0.33	0.38	0.19	Hypothetical protein BU14_0728s0002 [*Porphyra umbilicalis*]	1E-75	NA	-
DN104436_c1_g2_i4	21.84	0.23	0.27	-	Hypothetical protein BWQ96_03366 [*Gracilariopsis chorda*]	8E-88	NA	-

DEG, differentially expressed gene; FPKM, fragments per kilobase per million reads.

[a]DEGs with FPKM values greater than 20 in SG104 under control conditions and SG104 : Gy500 ratios greater than 32 under control conditions were selected.

[b]Average of FPKM from three replicates.

[c]DN97124_c0_g3_i3 was selected for further research in this study and named PyHRG1.

[d]NA, not available. Contigs with an E-value of >1.0E-10 were considered not available.

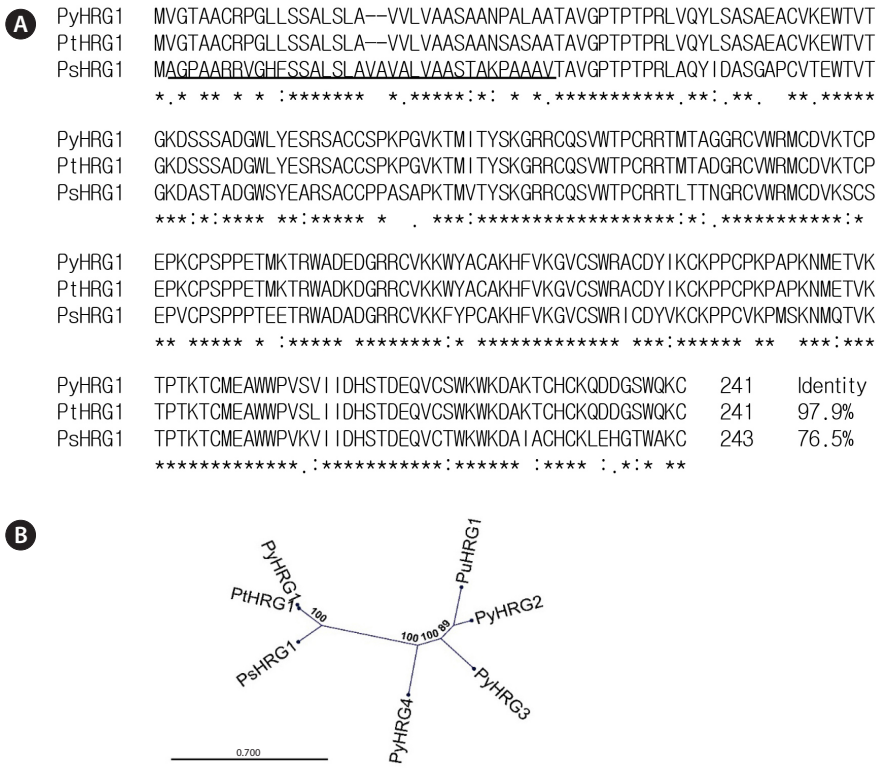

Fig. 2. Amino acid sequence alignment and phylogenic analysis of *PyHRG1*. (A) Amino acid sequence alignment of *PyHRG1* and its homolog from the *Pyropia* species—*P. yezoensis*, *P. tenera*, and *P. seriata*. The alignment was performed using CLUSTALW. The putative signal peptide is underlined. The asterisk (*) and colon (:) indicate identical and similar amino acid residues, respectively. (B) Phylogenic analysis of *PyHRG1* with its homologs. The three genes in (A), three *PyHRG1* homologs found in the *P. yezoensis* genome, and one gene from *Porphyra umbilicalis* were used for phylogenic tree analysis.

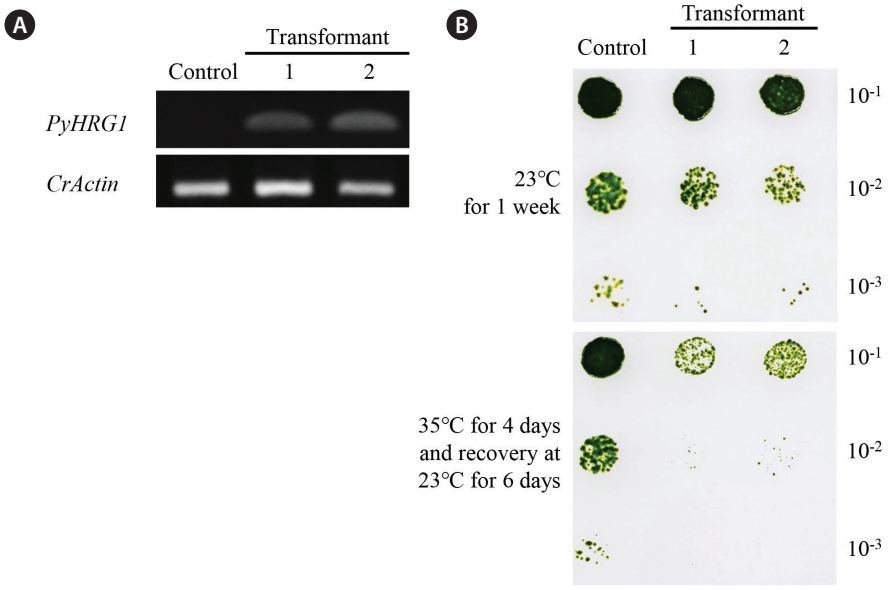

Fig. 3. Effects of *PyHRG1* on the growth of *Chlamydomonas reinhardtii.* (A) To verify the introduction and expression of *PyHRG1*, total RNA was purified from transgenic *C. reinhardtii* cells and used for reverse transcription polymerase chain reaction analysis with *PyHRG1*-specific primers. Transformed *C. reinhardtii* cells containing empty vectors were used as a control. *CrActin*, a *Chlamydomonas* actin gene, was used as an internal control. (B) To assay cell growth, *C. reinhardtii* cells were diluted to 10^{-1}, 10^{-2}, and 10^{-3} in fresh medium; 10 L of diluted cells was inoculated onto agar plates. Images were taken after 1 week of culture at 23°C. For the heat treatment, cells were kept at 35°C for 4 days and were subsequently transferred to a 23°C growth chamber for further growth. Images were also taken after 6 days of incubation at 23°C.

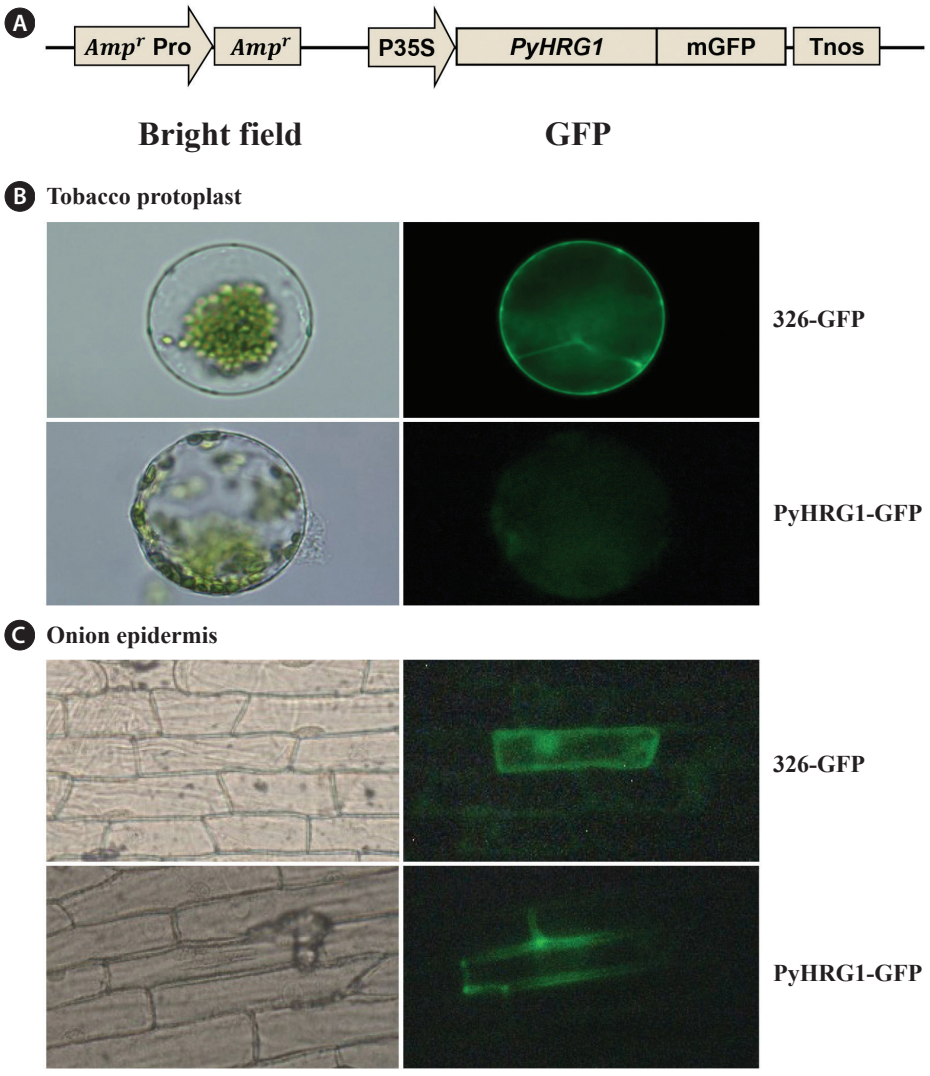

Fig. 4. Subcellular localization of PyHRG1. (A) Map of the recombinant vector for the PyHRG1-GFP fusion protein. *PyHRG1* was introduced into the 326-GFP vector and fused with GFP under the control of a CaMV 35S promoter. This construct was introduced into the protoplasts of tobacco (*Nicotina benthamiana*) (B) or the epidermis of onion (*Allium cepa*) (C). The 326-GFP empty vector was served as the control. The fluorescence signals of PyHRG1-GFP fusion protein were evaluated under a fluorescence microscope. Bright field contrast-interference images of the structure of the whole tobacco protoplast and onion epidermis cells are shown. GFP, cell images using a green filter for PyHRG1-GFP location with GFP fluorescence.

gene involved in cell growth inhibition under heat stress. It is not clear whether *PyHRG1* acts directly by inhibiting cell growth or indirectly by affecting other proteins that control cell growth. Studies in green plants have shown that under abiotic stressors, plants alter expression of genes to reduce growth and increase resistance to stress (Wahid et al. 2007, Shinozaki et al. 2015); likewise, in *P. yezoensis*, *PyHRG1* plays a role in cell growth regulation in response to heat stress.

Plants have evolved genetic systems to respond efficiently to adverse heat stress. The duration and severity of stress, susceptibility of cell types, and stage of development all influence the ability of a particular genotype to survive heat stress (Wahid et al. 2007, Shinozaki et al. 2015). HSPs plays critical role in heat tolerance process by prevent irreversible aggregations and re-solubilize proteins that have already aggregated by heat stress (Wang et al. 2004). A wide spectrums of heat response genes were identified by transcriptome analysis (Song et al. 2016, Chen and Li 2017, Wang et al. 2017, Xu and Hwang 2018). Besides HSPs, information of the molecular and physiological function of heat response genes from red algae is limited.

Subcellular localization of PyHRG1

Bioinformatics analysis predicted that PyHRG1 could be a secretory protein (Supplementary Table S4). To examine the cellular localization of PyHRG1, we cloned *PyHRG1* between the 35S promoter and *GFP* in the plant expression vector 326-GFP (Fig. 4A). The recombinant *PyHRG1-GFP* construct was then introduced into tobacco (*N. benthamiana*) protoplasts and onion (*A. cepa*) epidermis cells. Fluorescence of the PyHRG1-GFP fusion protein was not detected in tobacco protoplasts (Fig. 4B). However, green fluorescence was predominantly observed in the cell wall region of the onion epidermis (Fig. 4C). These results demonstrate that *PyHRG1* encodes a secretory protein located in the cell wall or extracellular matrix. In plants, secreted proteins play major roles in cell wall assembly and modification, as well as in responses to biotic and abiotic stresses (Wang et al. 2004). The plant cell wall determines cell size and shape through the mechanical control of cell expansion. Common responses to heat stress observed in the cell wall architecture include thickening and reduction in plasticity (Le Gall et al. 2015). Cell wall proteins, which mediate cell enlargement and expansion, include xyloglucan endo-β-transglucosylases / hydrolases, endo-1,4-β-D-glucanase, and expansins (Eklöf and Brumer 2010). Pectin-modifying enzymes, such as pectin methylesterase, also play a major role in controlling cell wall plasticity (Sénéchal et al. 2014). Previous studies have shown that some cell wall proteins are required for abiotic stress responses in plants (Choi et al. 2011, Wu et al. 2018). During secondary cell wall formation, monolignols (precursors of lignin) as well as cellulose and hemicelluloses are secreted into the cell wall space (Vanholme et al. 2010, Le Gall et al. 2015). Expression of such cell wall proteins can be altered in response to heat stress (Xu and Hwang 2018). Other cell wall-related genes also play a role in the acquisition of thermotolerance (Yang et al. 2006, Ha et al. 2007, Choi et al. 2013, Rienth et al. 2013). Yang et al. (2006) reported that the cell wall-related genes might play a role in the acquisition of thermotolerance in Chinese cabbage (*Brassica rapa* L.). Ha et al. (2007) also found that the cell wall protein, GSAP3, from *Panax ginseng* plays a role in abiotic stress tolerance. However, cell wall composition and consequent interactions with the environment and abiotic stressors can vary between different plant groups (Popper et al. 2014, Chen et al. 2020). Overall, further research is needed to gain an improved understanding of the role of the cell wall in heat tolerance at the physiological, genetic, and biochemical levels in red algae, including *P. yezoensis*.

CONCLUSION

We generated transcriptomes sequences from both the wild-type SG104 *P. yezoensis* and heat-tolerant mutant Gy500, and selected DEGs that were up- or downregulated in response to the heat stress condition and in the heat-tolerant mutant Gy500. In this study, we characterized a high-temperature response gene *PyHRG1*, which was downregulated under heat stress in SG104 and expressed at a low level in the heat-tolerant mutant Gy500. *PyHRG1* encodes a novel red algae-specific secretory protein. Transformed *C. reinhardtii* cells overexpressing *PyHRG1* showed retarded cell growth. These results indicate that *PyHRG1* is involved in cell growth during heat stress. *PyHRG1* is a novel gene found only in red algae and further studies are needed to understand the molecular function of *PyHRG1*. The transcriptome sequences obtained in this study, which include *PyHRG1*, will facilitate future studies to understand the molecular mechanisms involved in heat tolerance in red algae.

ACKNOWLEDGEMENTS

This works was supported by the Korean Institute of Planning and Evaluation for Technology, Agriculture, Forestry and Fisheries (IPET) through a Golden Seed Project (Project number, 213008-05-4-SB830), funded by the Ministry of Oceans and Fisheries (MOF) and a grant (R2021013) from the National Institute of Fisheries Science, Republic of Korea.

CONFLICTS OF INTEREST

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

SUPPLEMENTARY MATERIALS

Supplementary Table S1. Summary of the differentially expressed genes (DEGs) upregulated in the gametophytes of *Pyropia yezoensis* SG104 under the heat stress condition and in the heat-tolerant mutant (Gy500) (https://www.e-algae.org).

Supplementary Table S2. Summary of the differentially expressed genes (DEGs) downregulated in the gametophytes of *Pyropia yezoensis* SG104 under the heat stress condition and in the heat-tolerant mutant (Gy500)

(https://www.e-algae.org).

Supplementary Table S3. Summary of the *PyHRG1* homologs found in the *Pyropia yezoensis* genome (https://www.e-algae.org).

Supplementary Table S4. Prediction of the cellular localization of PyHRG1 (https://www.e-algae.org).

Supplementary Fig. S1. *PyHRG1* expression in wild-type (SG104) and heat-tolerant (Gy500) *Pyropia yezoensis* (https://www.e-algae.org).

Supplementary Fig. S2. Amino acid sequence alignment of the three *PyHRG1* homologs identified from the *Pyropia yezoensis* genome (https://www.e-algae.org).

Supplementary Fig. S3. Alignment of the *PyHRG1* cDNA sequence from wild-type (SG104) and high-temperature tolerant mutant (Gy500) *Pyropia yezoensis* (https://www.e-algae.org).

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