

Proteomic profile analysis of *Pyropia haitanensis* in response to high-temperature stress

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Abstract High-temperature stress is a major abiotic stress that affects the yield and quality of *Pyropia*, which is produced for use as a nutrient. *Pyropia haitanensis* strain Z-61 is tolerant to high temperature and is widely cultivated in South China. Here, we conducted a comparative proteomic analysis of the blades of Z-61 strain at 29 °C. Changes in the proteome of the blades were analyzed every other day for 8 days at 29 °C. Approximately 1,000 protein species were reproducibly detected at all time points, whereas 87 protein spots were differentially expressed at least at one time point. Fifty-nine protein spots were successfully identified using liquid chromatography–tandem mass spectroscopy and database searching. The proteins were classified into nine functional categories: photosynthesis (27.12 %), energy metabolism (22.03 %), other metabolic pathways (11.86 %), redox homeostasis (11.86 %), response to stimuli (8.47 %), proteins involved in transcription and translation (6.78 %), cytoskeleton-related proteins (5.08 %), proteins that mediate signal transduction (1.69 %), and unknown proteins (5.08 %). These findings indicated that the blades of Z-61 resist high-temperature stress by inhibiting photosynthesis and other nonessential metabolic processes. In contrast, to increase energy metabolism, the expression of proteins related to redox homeostasis and response to stimuli were upregulated, thereby maintaining physiological balance during stress.

Keywords *Pyropia haitanensis* · Rhodophyta · High-temperature stress · Proteomic analysis · Differentially expressed proteins

Introduction

Pyropia, a genus of marine red algae, is one of the most economically important mariculture crops, with an annual harvest of more than 120,000 t (dry weight) and a value of over US\$ 2 billion per year (Blouin et al. 2010). With the expansion of artificial seeding and development of the floating culture method, farming and processing of *Pyropia* have generated the largest seaweed industries in East Asian countries, including China, Japan, and South Korea (Sahoo et al. 2002). In China, two major cultivars, *Pyropia yezoensis* (Ueda) Hwang et Choi and *Pyropia haitanensis* (Chang et Zheng) Kikuchi et Miyata, are distributed in North and South China, respectively. *P. haitanensis*, a typical warm temperate zone species originally found in Fujian Province, is widely cultivated along the coasts of South China (Zhang et al. 2005). The production of *P. haitanensis* now represents 75–80 % of the total production of cultivated *Pyropia* in China (Xie et al. 2010).

Under natural conditions, the optimum temperature for conchospore release and net seeding by *P. haitanensis* is 25 to 27 °C. However, in early seeding period (autumn in South China), *Pyropia* farms often suffer from sustained high temperatures or temperature increases following a dramatic drop to temperatures suitable for conchospore release and germination, which is known as “temperature rebound” (Zhang et al. 2011a, b). The persistence of high temperatures can negatively affect *Pyropia* blades by inhibiting the survival and division of the conchospores. In addition, the germlings would be prone to disease, premature senility, and eventual decay, leading to a substantial reduction in yield (Yan et al. 2010). In recent years, high temperatures associated with global warming have markedly affected the cultivation of *P. haitanensis* and reduced its yield along the coasts of Fujian and Zhejiang (China), which comprise one of the two primary areas used for cultivation. Therefore, in addition to screening for cultivars with higher yield and better quality,

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breeding cultivars for tolerance to high temperature is a major objective.

Chen et al. (2008) and Yan et al. (2010) selected several strains (*Z-26*, *Z-61*, and *ZS-1*) of *P. haitanensis* exhibiting high-temperature tolerance and high growth rates. These strains are widely cultivated in South China, and their traits of high-temperature tolerance have been validated by field cultivation. To understand the physiological mechanism of high-temperature tolerance, Zhang et al. (2011a, b) compared the physiological response of a high-temperature tolerance strain (*Z-61*) to that of a wild-type strain and found that *Z-61* can induce its antioxidant and osmoregulation systems, whereas the wild-type strain only induced its osmoregulation system. Further, the baseline activity of the antioxidant system differed significantly between the two strains. However, little is known about the molecular changes that occur in the tolerant strains that affect the biochemical pathways involved in their response to high-temperature stress. Although much evidence exists detailing the molecular mechanisms of high-temperature stress tolerance in vascular plants, and many genes related involved in the high-temperature response have been identified (Wahid et al. 2007), much less is known about the molecular mechanism of high-temperature tolerance in algae.

Discovering and understanding the functions of genes whose expression responds to high-temperature stress is essential. To this end, a global transcriptomic study of the response to high temperature identified more than 1,000 differentially expressed genes (Xie et al., unpublished). It is important, however, to confirm studies of transcription with those that address protein expression. In fact, several studies have shown that changes in transcription often do not correspond with changes in protein expression, and only direct measurements of proteins will reveal authentic functional changes (Gygi et al. 1999; Hack 2004; Ghazalpour et al. 2011). Proteins are the direct effectors of the response of plants to stress. They not only include enzymes that mediate changes in the levels of metabolites but also serve as components of the transcription and translation machinery. Proteomic studies can thus lead to identification of proteins involved in the stress response and contribute significantly to our understanding of the molecular mechanisms underlying tolerance to stress. It is imperative to determine the pattern of proteins expressed by *P. haitanensis* in response to high-temperature stress to gain a better understanding of the molecular basis of high-temperature stress, as well as identifying proteins that mediate this process.

With the development and improvement of protein separation and identification techniques, comparative proteomics has evolved into a powerful tool for identifying previously unrecognized proteins and those expressed differentially in response to abiotic stress in higher plants (Neilson et al. 2010; Kosová et al. 2011). However, to the best of our knowledge,

few proteomic studies have focused on macroalgae. Examples include reports describing the extraction and identification of proteins obtained from *Gracilaria changii* (Wong et al. 2006), *Ecklonia kurome* (Nagai et al. 2008), and *Saccharina japonica* (Kim et al. 2011); the analysis of seasonal variation in protein expression in the sporophyte of *S. japonica* (Yotsukura et al. 2010); and the identification of 19 proteins involved in copper tolerance in *Scytosiphon gracilis* (Contreras et al. 2010). One reason for this is that extracting proteins from macroalgae is difficult because of the presence of secondary metabolites, mainly polysaccharides and polyphenols, which cause proteins to precipitate or streak or generate artifactual spots in two-dimensional electrophoresis (2-DE) gels (Yotsukura et al. 2010; Kim et al. 2011). We have developed an effective method for protein isolation from *P. haitanensis* and the separation these proteins by 2-DE to facilitate their identification (Hang et al. 2011). The aim of the present study was to analyze the proteomic profiles of *P. haitanensis Z-61* in response to high-temperature stress and to identify proteins that are differentially expressed using a combination of 2-D PAGE gels and mass spectrometry analysis, coupled with database searching.

Materials and methods

Plants and stress treatment We used *P. haitanensis* strain *Z-61*, which is tolerant to high temperatures and grows with a high yield. It was selected and purified by researchers working in the Laboratory of Germplasm Improvements and Applications of *Pyropia* in Jimei University, Fujian, China. Fifty 20±2-cm long blades of *Z-61* were randomly selected and cultured in ten aerated flasks (2,000 mL) with five blades each, at 21 °C (control temperature) and 29 °C (high temperature) and illuminated by 50–60 μmol photons m⁻² s⁻¹ (10:14, L/D) for 0, 2, 4, 6, and 8 days. The culture medium was natural seawater enriched with MES medium and was refreshed every 2 days.

Chlorophyll fluorescence measurements To monitor the intensity of high-temperature stress on *Z-61* blades of *P. haitanensis*, the optimum quantum yield, a chlorophyll fluorescence marker for photosynthetic efficiency, was measured with a portable pulse amplitude-modulated fluorometer (DIVING-PAM, Walz, Germany). Intrinsic fluorescence (F_0) was determined after maintaining the tissue in darkness for 30 min. A saturating actinic light pulse (5,000 μmol photons m⁻² s⁻¹, 800 ms) was applied to obtain maximum fluorescence (F_m) in the dark-adapted samples. Variable fluorescence (F_v) was determined as the difference between F_m and F_0 , and optimum quantum yield (F_v/F_m) was calculated (Schreiber et al. 1994).

Protein extraction and purification The blade proteins of *Z-61* were extracted according to a previously published

method (Hang et al. 2011). Briefly, blades were ground in a precooled mortar in the presence of liquid nitrogen. Approximately 0.2 g of the homogenate was precipitated for 2 h with 10 mL acetone, 10 % trichloroacetic acid, 0.07 % β -mercaptoethanol (BME), and 0.2 % polyvinylpyrrolidone at -20°C . After centrifugation at $15,000\times g$ for 20 min at 4°C , the supernatant was removed and the pellet was rinsed three times in ice-cold acetone (including 0.07 % BME). Between the rinsing steps, the sample was incubated for 30 min at 25°C . The pellet was air-dried, resuspended in 5 mL lysis buffer (7 M urea, 2 M thiourea, 40 mM Tris base, 4 % 3-[(3-cholamidopropyl)-dimethylammonio]-propanesulfonate, 2 % immobilized pH gradient (IPG) buffer (pH 3~10), 65 mM dithiothreitol (DTT)), and vortexed for 1 h at room temperature. The homogenate was centrifuged at $15,000\times g$ for 20 min at 4°C , and the supernatant was collected for 2-DE analysis.

2-DE and data analysis For 2-DE, a 250- μg protein sample was loaded onto immobilized IPG strips (GE Healthcare; pH 4–7, 24 cm linear) and rehydrated passively with 450 μL protein solution for 12 h at 20°C using an Ettan IPGphor system (GE Healthcare). Isoelectric focusing was conducted in six steps as follows: 100 V for 1 h, 200 V for 1 h, 500 V for 1 h, 1,000 V for 1 h, 8,000 V for 2.5 h, and 10 h at 8,000 V (total ~ 80 kV h). The focused strips were then equilibrated twice in equilibration solution for 15 min. The first equilibration was performed in 75 mM Tris-HCl (pH 8.8), 6 M urea, 30 % glycerol, 2 % sodium dodecyl sulfate (SDS), 0.01 % bromophenol blue, and 1 % DTT. The second equilibration was performed in 75 mM Tris-HCl (pH 8.8), 6 M urea, 30 % glycerol, 2 % SDS, 0.01 % bromophenol blue, and 4 % iodoacetamide. For the second dimension, the proteins were separated on 12 % SDS-polyacrylamide gels using an Ettan DALTsix electrophoresis system (GE Healthcare). The proteins in the gels were visualized by silver staining (Yan et al. 2000) and scanned at 300 dpi using a GE image scanner. Image analysis was performed using ImageMaster 2D Platinum software version 6.0 (GE Healthcare). The relative abundance of each protein spot was estimated by the volume percentage (vol%) of total spot densities in the gel. The levels of protein expression were calculated with three biological replicates of each sample, and the results were analyzed using ANOVA. Proteins were considered differentially expressed only if they exhibited reproducible twofold changes in vol% that were statistically significant ($p < 0.05$).

Protein excision and digestion Protein spots of interest were manually excised from 2-D gels with a scalpel and washed two times with 70 μL deionized water. Gel slices were destained twice with 100 mM NH_4HCO_3 in 30 % acetonitrile (ACN) and lyophilized overnight before digestion (16 h)

with trypsin (Promega, USA) in 25 mM NH_4HCO_3 containing 10 % ACN at 37°C . The peptides were extracted three times with 0.1 % trifluoroacetic acid (TFA) in 60 % ACN, and the extracts were pooled and lyophilized for mass spectrometry (MS) analysis.

MS analysis and database searching The lyophilized peptide samples were dissolved in 0.1 % TFA, and MS analysis was conducted using a matrix-assisted laser desorption/ionization-time-of-flight mass spectrometer 4800 Plus proteomics analyzer (Applied Biosystems, USA). Peptide mass maps were generated in positive ion reflector mode (accelerating voltage 20 kV) with 1,000 laser shots per spectrum, with a signal-to-noise ratio minimum set to 10 and a local noise window width of m/z 250. Up to 10 of the most intense ions were selected as precursors for MS/MS data acquisition. Further processing and interpretation of the MS/MS spectra were performed using GPS Explorer (V3.6, ABI). MS/MS data from each individual spot were merged into a single file for database searching. The MS/MS data were searched for matches to the NCBI and Swiss-Prot databases using MASCOT V2.1 software (Matrix Science, UK). The search parameters were set as follows: trypsin was used to digest the proteins, up to two missed cleavages were allowed, carbamidomethyl (Cys) was the fixed modification and oxidation (Met) was the variable modification, maximum peptide mass tolerance was 70 ppm, and the general fragment mass tolerance was ± 0.25 Da. According to the search engine parameters, scores greater than 65 ($p < 0.05$) were considered positive.

Results

Effects of high-temperature stress on photosynthesis

Optimum quantum yield is a chlorophyll fluorescence marker for photosynthetic efficiency during abiotic stress (Schreiber et al. 1994). Figure 1 shows the change of optimum quantum

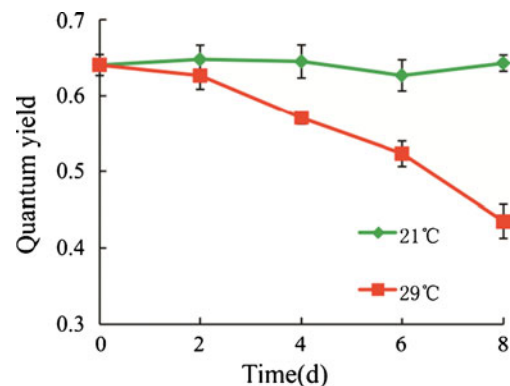


Fig. 1 Optimum quantum yield of *Z-61* blades under high temperature (29°C) and control temperature (21°C) for different days

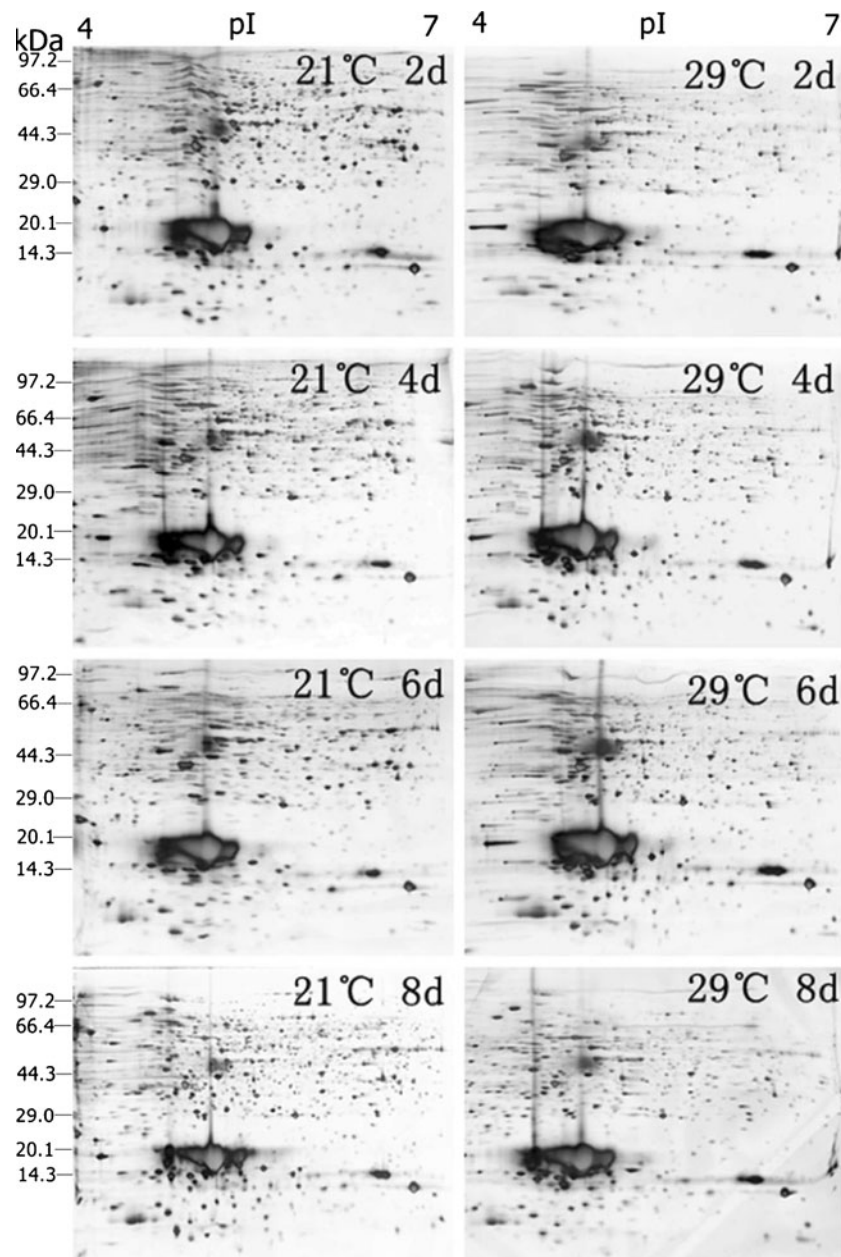


Fig. 2 2-DE maps of *Z-61* blades proteins under different temperatures and time gradients

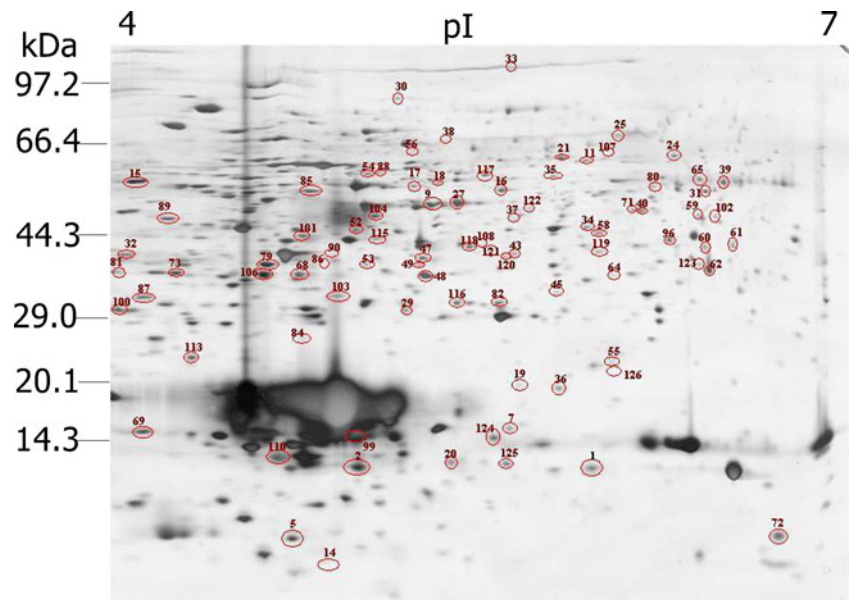
yield of *Z-61* blades under high temperature (29 °C) and control temperature (21 °C) over time. The quantum yield did not change significantly at the control temperature; however, it decreased significantly during high-temperature stress to 67.9 % of the control after 8 days. The result indicated that the high temperature inhibits photosynthesis in the *Z-61* blades significantly.

2-DE analysis of *P. haitanensis* proteins expressed under high-temperature stress

To investigate the dynamic changes in the response of proteins to high-temperature stress, the soluble proteins of *Z-61*

blades cultured at 21 °C (control group) and 29 °C (high-temperature stress group) for 0, 2, 4, 6, and 8 days were extracted and the proteomic dynamics were analyzed by high-resolution 2-DE. Protein spots displaying reproducible patterns were identified and their expression patterns were analyzed. Representative 2-DE gel maps of each sample are shown in Fig. 2. Between the two groups, 1,263 reproducible protein spots were detected, of which 937 coincided in all gels. Among the 937 coincident spots, the abundance (vol%) of 126 changed by more than twofold ($p < 0.05$) in the high-temperature stress groups. When they were compared with the control group, 39 were excluded because they also displayed significant changes in the control group. The remaining 87

Fig. 3 Distribution of differentially expressed proteins in a 2-DE map



protein spots were treated as differentially expressed proteins (DEPs) related to high-temperature stress (Fig. 3).

Identification of high temperature-responsive proteins

Among the 87 DEPs, 59 DEPs were successfully identified using LC-MS/MS and database searching (Table 1). Among them, four isoforms were identified, each with two spots located at a different position on the same gel. Spots 60 and 61 were identified as ferredoxin-NADP reductase, spots 2 and 110 as the beta subunit of allophycocyanin, spots 43 and 47 as enoyl-(acyl-carrier-protein) reductase (NADH), and spots 49 and 64 as membrane-associated 30-kDa protein. Further examination of the relative molecular weights (M_r) and isoelectric points (pI) showed that the observed and theoretical values of many spots are different (Table 1). This phenomenon might be caused by posttranslational modification or degradation. Posttranslational modifications such as glycosylation and phosphorylation can change the molecular weight, charge, or both.

Among the 59 identified DEPs, three main types of protein expression patterns were detected under high-temperature stress. The first group (group I) includes 24 DEPs whose abundances were consistently upregulated during high-temperature stress, such as spots 2, 16, and 100. The second group (group II) includes 19 DEPs whose abundances were consistently downregulated during high-temperature stress, such as spots 14, 64, and 122. The third group (group III) includes 15 DEPs whose abundances fluctuated, such as spots 11, 54, and 124. During the early stage of high-temperature stress, their abundance was upregulated and then diminished. The abundance of the remaining spot 65 was downregulated during the early stage of high-temperature stress and increased at later times. The dynamic changes in the level of each protein are shown in Table 1.

We were not able to identify 28 protein spots, perhaps because the entire genomic sequence of *P. haitanensis* has not been determined.

Functional classification of the stress-responsive proteins

The proteins were classified into nine categories according to their functions (Fig. 4). The proteins related to photosynthesis and energy metabolism form the largest two categories, which accounted for 27.12 and 22.03 % of the total. These two categories account for half of the proteins, indicating that high-temperature stress exerts a significant effect on the photosynthesis and energy metabolism. The other categories are as follows: other metabolic pathways (11.86 %), redox homeostasis (11.86 %), response to stimuli (8.47 %), proteins involved in transcription and translation (6.78 %), cytoskeleton-related proteins (5.08 %), proteins that mediate signal transduction (1.69 %), and unknowns (5.08 %).

Discussion

Proteomics and transcriptomics are becoming indispensable techniques for understanding how organisms adapt to abiotic stresses, and they have promoted the identification of numerous stress-related genes and proteins and the pathways in which they function (Hirayama and Shinozaki 2010; Kosova et al. 2011). Changes in protein accumulation under stress are directly related to the phenotypic response of plants to stress, and proteomic analysis can provide more direct information regarding abiotic stresses than transcriptomic analysis. High-temperature stress exerts a major impact on the yield and quality of *Pyropia* (Yan et al. 2010; Zhang et al. 2011a, b).

Table 1 Identification of differentially expressed proteins of *Z-6I* blades under high-temperature stress

Spot no.	Protein name	Species	Accession no.	Protein score	Number of peptides sequenced	Observed		Theoretical		Dynamic changes of expression abundances (vol.%)
						M_r	pI	M_r	pI	
Photosynthesis -related proteins										
124	Phycocyanin alpha subunit	<i>P. yezoensis</i>	gil90994569	99	2	14.4	5.59	17.5	7.71	
99	Allophycocyanin alpha subunit	<i>Cyanidium caldarium</i>	gil438529	179	4	14.5	5.07	17.5	5	
2	Allophycocyanin beta subunit	<i>P. purpurea</i>	gil11465727	202	4	12.4	5.04	17.5	5.17	
110	Allophycocyanin beta subunit	<i>P. purpurea</i>	gil11465727	247	5	13.0	4.69	17.5	5.17	
71	Phycobilisome linker polypeptide	<i>Paulinella chromatophora</i>	gil194477085	135	3	46.7	6.17	32.0	9.4	
1	RuBisCO small subunit	<i>P. haitanensis</i>	gil75226528	375	7	12.4	6.00	15.9	6.11	
68	Eukaryotic-type carbonic anhydrase family protein	<i>Oryza sativa japonica</i> group	gil115487388	87	6	33.6	4.78	30.8	6.36	
13	Fructose biphosphatase	<i>P. yezoensis</i>	gil168274233	220	5	40.1	5.21	39.2	5.16	
81	Fructose-1,6-bisphosphate aldolase	<i>Chlamydomonas reinhardtii</i>	gil159484548	75	5	36.3	4.04	42.1	8.76	
122	Fructose-bisphosphate aldolase	<i>Phytophthora infestans T30-4</i>	gil301119899	399	7	47.4	5.74	43.3	5.42	
60	Ferredoxin-NADP reductase	<i>Cyanophora paradoxa</i>	gil399485	164	5	39.0	6.47	40.5	8.36	
61	Ferredoxin-NADP reductase	<i>C. paradoxa</i>	gil399485	215	7	39.0	6.59	40.5	8.36	
27	Uroporphyrinogen decarboxylase, chloroplast precursor	<i>Ectocarpus siliculosus</i>	gil299115710	72	2	48.7	5.44	46.4	7.77	
14	Photosystem II protein H	<i>P. purpurea</i>	gil11465791	75	2	7.5	4.89	7.5	4.94	
86	Chloroplast-localized protein	<i>Nicotiana benthamiana</i>	gil157142955	90	2	35.1	4.84	33.8	8.79	
48	Oxygen evolving enhancer 1	<i>P. yezoensis</i>	gil57117322	473	6	33.4	5.31	34.4	5.74	
Energy metabolism-related proteins										
24	H ⁺ /Na ⁺ -translocating f-type, v-type, and A-type ATPase superfamily	<i>Micromonas</i> sp. <i>RCC299</i>	gil255088599	296	8	62.3	6.34	59.9	7.63	
73	Inorganic pyrophosphatase	<i>C. reinhardtii</i>	gil159489184	281	5	33.9	4.27	31.1	6.34	
30	Aconitate hydratase, mitochondrial	<i>Gracilaria gracilis</i>	gil1351858	121	5	83.7	5.19	83.7	5.2	
38	Glucose-6-phosphate 1-dehydrogenase	<i>P. infestans T30-4</i>	gil301122093	101	5	62.3	6.34	62.1	6.42	
52	Transaldolase	<i>Thalassiosira pseudonana</i>	gil223995561	114	4	42.3	5.02	34.9	4.79	
54	Enolase	<i>Arabidopsis lyrata</i>	gil297820024	310	7	56.6	5.07	47.8	5.57	
59	Cytosolic glyceraldehyde-3-phosphate dehydrogenase	<i>Gracilaria lemaneiformis</i>	gil60460510	99	2	45.7	6.44	33.7	5.74	
118	Pyruvate dehydrogenase E1 component beta subunit	<i>P. yezoensis</i>	gil90994457	72	2	38.8	5.49	36.4	5.57	
34	Trans-2-enoyl-CoA reductase, mitochondrial precursor	<i>E. siliculosus</i>	gil299471239	145	3	43.0	5.97	38.2	8.57	

Table 1 (continued)

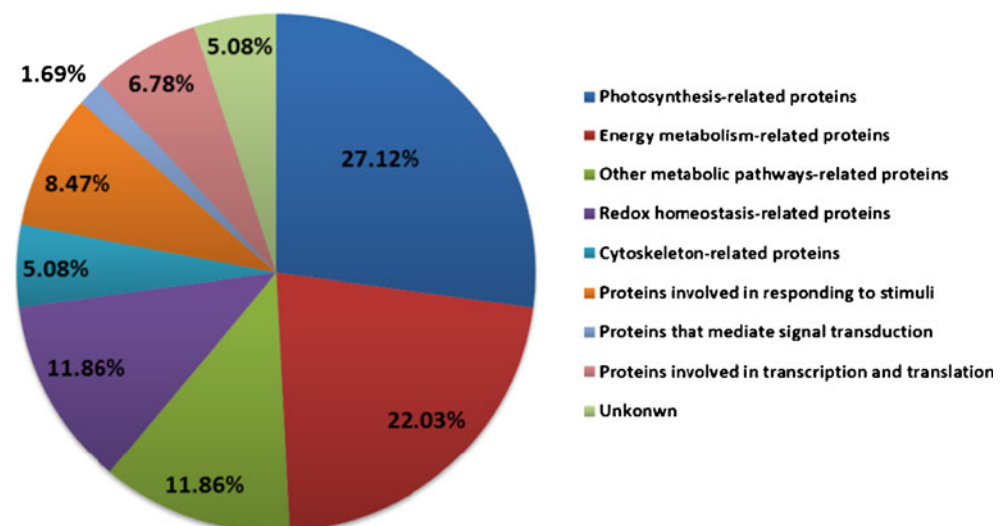
35	Beta-ketoacyl-acyl carrier protein synthase II	<i>O. sativa japonica</i> group	gil115466908	227	3	56.1	5.84	49.0	6.76	
43	Enoyl-(acyl-carrier-protein) reductase (NADH)	<i>P. chromatophora</i>	gil194476967	102	3	37.0	5.68	27.9	6.84	
47	Enoyl-(acyl-carrier-protein) reductase (NADH)	<i>P. chromatophora</i>	gil194476967	79	2	36.7	5.3	27.9	6.84	
82	Rossmann-fold NAD(P)(+)-binding protein	<i>O. sativa japonica</i> group	gil115470779	201	5	29.0	5.61	25.8	6.74	
Other metabolic pathways-related proteins										
17	Bifunctional 3-phosphoadenosine 5-phosphosulfate synthetase 2	<i>Zea mays</i>	gil226492878	273	8	53.2	5.26	52.4	8.3	
25	PAP2-like proteins	<i>E. siliculosus</i>	gil298714414	144	6	69.3	6.11	51.2	4.49	
65	Alanine-glyoxylate transaminase	<i>C. reinhardtii</i>	gil159487068	217	6	55.1	6.45	46.0	8.47	
80	Glycine cleavage system, T protein	<i>C. reinhardtii</i>	gil159469919	67	2	53.0	6.25	43.6	9.01	
90	Carbonyl reductase	<i>Jatropha curcas</i>	gil317106612	196	4	36.3	4.93	34.7	6.02	
102	Cinnamyl alcohol dehydrogenase-like protein	<i>C. reinhardtii</i>	gil159488737	198	7	33.2	7.83	34.7	8.79	
104	D-3-phosphoglycerate dehydrogenase	<i>Blastocystis hominis</i>	gil300121879	353	7	45.5	5.1	37.6	8.1	
Redox homeostasis-related proteins										
18	Pyridine nucleotide-disulphide oxidoreductase	<i>O. sativa japonica</i> group	gil115474811	345	8	54.2	5.36	52.8	6.84	
36	CBS domain-containing protein	<i>A. thaliana</i>	gil15238284	92	2	19.1	5.87	22.7	9.1	
45	Phosphomannomutase	<i>Solanum lycopersicum</i>	gil122194127	80	2	30.6	5.85	28.6	5.85	
85	Glutathione S-transferase	<i>O. sativa japonica</i> group	gil115477793	132	3	51.8	4.83	37.2	6.84	
100	Ferritin 3	<i>A. thaliana</i>	gil15228818	162	5	28.0	4.03	28.8	5.54	
101	Lactoylglutathione lyase	<i>O. sativa japonica</i> group	gil115462755	276	7	41.1	4.79	32.5	5.08	
116	Cytosolic ascorbate peroxidase	<i>P. haitanensis</i>	gil193792560	374	8	29.0	5.44	26.4	5.36	
Proteins involved in responding to stimuli										
11	Glutamate dehydrogenase	<i>P. yezoensis</i>	gil32480565	571	8	61.3	6.06	52.2	5.69	
19	Heat shock protein 22E	<i>C. reinhardtii</i>	gil159463182	130	4	18.9	5.7	25.2	9.09	
31	Serine type protease, similar to Protease Do-like 1, chloroplast precursor	<i>E. siliculosus</i>	gil298706275	135	3	51.8	6.47	41.3	5.07	
79	Pathogenesis-related protein 1-like protein	<i>Volvox carteri f. nagariensis</i>	gil302828408	122	3	35.5	4.65	74.8	9.93	
107	Stress-induced protein sti1-like protein (ISS)	<i>Ostreococcus tauri</i>	gil308804756	241	5	66.0	6.07	68.8	6.03	
Cytoskeleton-related proteins										
16	Actin	<i>P. yezoensis</i>	gil204022118	288	7	51.9	5.63	41.0	5.48	
103	PAP fibrillin	<i>E. siliculosus</i>	gil298705906	100	2	30.0	4.94	30.0	5.21	
125	Actin-depolymerizing factor 3	<i>Z. mays</i>	gil226500484	180	8	12.6	5.64	15.9	5.47	
Proteins involved in transcription and translation										

Table 1 (continued)

37	Elongation factor Tu	<i>O. tauri</i>	gil308804561	142	4	44.8	5.68	45.7	6	
87	Elongation factor 1β1-beta	<i>Z. mays</i>	gil195607814	105	3	30.4	4.16	23.4	4.55	
88	Elongation factor Tu	<i>P. purpurea</i>	gil11465753	357	4	57.1	5.17	44.7	5.17	
113	Transcription factor BTF3	<i>Z. mays</i>	gil195649741	99	2	21.8	4.33	17.8	6.62	
Proteins that mediate signal transduction										
69	ARF-GAP	<i>A. lyrata</i> subsp. <i>lyrata</i>	gil297813911	119	2	14.9	4.13	37.3	5.04	
Unknown										
49	Probable membrane-associated 30-kDa protein, chloroplast precursor	<i>E. siliculosus</i>	gil298708699	157	5	35.4	5.28	35.1	9.30	
64	Probable membrane-associated 30-kDa protein, chloroplast precursor	<i>E. siliculosus</i>	gil298708699	205	8	33.5	6.09	35.1	9.30	
121	38-kDa ribosome-associated protein precursor	<i>E. siliculosus</i>	gil299115197	209	5	37.9	5.58	42.3	6.43	

The mean seawater temperature is increasing because of global warming; therefore, the study of how *Pyropia* responds to high-temperature stress at the protein level is important for developing high temperature-tolerant strains. The proteomic responses to high-temperature stress are known for several higher plants, such as *Arabidopsis thaliana* (Palmlad et al. 2008), rice (Lee et al. 2007; Lin et al. 2005), wheat (Majoul et al. 2003, 2004; Skylas et al. 2002), barley (Sule et al. 2004), *Populus euphratica* (Ferreira et al. 2006), and *Carissa spinarum* (Zhang et al. 2010a, b).

In the present study, we analyzed the blade proteome of *P. haitanensis* under conditions of high-temperature stress for different times. Eighty-seven proteins were observed to be differentially expressed. Only limited genomic data are available for *P. haitanensis*; therefore, only 59 DEPs (67.82 %) could be identified (Table 1). These proteins were classified into nine categories based on function (Fig. 4, Table 1).

Fig. 4 Functional categories of 59 differentially expressed proteins

Photosynthesis-related proteins Photosynthesis is one of the systems that are most sensitive to high-temperature stress. Changes in environmental temperature are primarily reflected by photosynthesis, which triggers a response aimed at reaching the best possible performance under new conditions. For this, a balance is sought between the energy of absorbed light, carbon assimilation, and consumption in metabolic sinks. Several studies have shown that high-temperature stress can significantly inhibit the rate of photosynthesis (Salvucci and Crafts-Brandner 2004). In the present study, 16 proteins (27.12 %) related to photosynthesis were differentially expressed.

The process of photosynthesis is initiated by the absorption of light. *Pyropia* uses multimeric protein complexes, the phycobilisomes (PBSs), as their major light-harvesting complexes (Liu et al. 2005). PBSs comprise three basic types of biliprotein (phycoerythrin (PE), phycocyanin (PC), allophycocyanin (APC)) and linker polypeptides. Light energy that

is absorbed by PE migrates first to PC, then to APC, and finally to chlorophyll *a* (Samsonoff and MacColl 2001). Linker polypeptides function to stabilize the PBS structure and optimize absorbance characteristics and energy transfer (Liu et al. 2005). In the present study, under high-temperature stress, the downregulation of the phycobilisome linker polypeptide (spot 71) would significantly disturb the stability of PBSs and must, therefore, influence light absorbance and energy transfer of *P. haitanensis*. In such a case, to maintain the balance of light absorbance and energy transfer, the level of each biliprotein must be upregulated. Thus, the upregulations of the PC alpha subunit (spot 124), APC alpha subunit (spot 99), and APC beta subunit (spots 2 and 110) were understandable.

In seaweeds, carbonic anhydrase plays key roles in the CO₂-concentrating mechanism by accelerating the conversion of the external HCO₃⁻ pool into CO₂ or by mobilizing the internal pool of HCO₃⁻ to supply the carboxylation reaction of RuBisCo (Zhang et al. 2010a, b). RuBisCo catalyzes the first major step of carbon fixation in photosynthesis. Jordan and Ogren (1984) found that the affinity of RuBisCo for CO₂ decreased with increased temperature. In the present study, although the level of carbonic anhydrase (spot 68) was upregulated, the level of expression of the RuBisCo small subunit was downregulated. The diminished capacity of RuBisCo and its low affinity for CO₂ clearly indicate that carbon fixation or assimilation is highly inhibited when blades of *P. haitanensis* are exposed to high-temperature stress. Heat-induced changes of RuBisCo subunits have also been monitored in rice seedlings, and the data indicate that high temperature (over 40 °C), for prolonged periods (>2 h), significantly inhibited the activity and quantity of RuBisCo subunits (Lee et al. 2007).

Fructose bisphosphatase (spot 13), fructose-1,6-bisphosphate aldolase (spots 81 and 122), ferredoxin-NADP reductase (spots 60 and 61), photosystem II protein H (spot 14), and chloroplast-localized protein (spot 86) are the key enzymes of the Calvin cycle or participate in photosynthetic electron and energy transfer. Thus, the downregulated expression of these proteins further suggests that photosynthesis of *P. haitanensis* was inhibited by high-temperature stress. The speculation was validated by the measurement of optimum quantum yield of *Z-61* blades during high-temperature stress (Fig. 1).

Energy metabolism-related proteins Plants respond to stress conditions by triggering a network of events linked to energy metabolism (Hirayama and Shinozaki 2010; Cramer et al. 2011). As expected, 22.03 % of all proteins identified in the present study are related to energy metabolism. *trans*-2-Enoyl-CoA reductase (spot 34), beta-ketoacyl-acyl carrier protein synthase (spot 35), enoyl-(acyl-carrier-protein) reductase (NADH) (spots 43 and 47), and Rossmann-fold NAD(P)(+)-binding protein (spot 82) are involved in fatty acid biosynthesis. Aconitate hydratase (spot 30), glucose-6-phosphate 1-dehydrogenase (spot 38), transaldolase (spot 52), enolase

(spot 54), cytosolic glyceraldehyde-3-phosphate dehydrogenase (spot 59), and pyruvate dehydrogenase E1 component beta subunit (spot 118) are involved in the tricarboxylic acid cycle, the pentose phosphate pathway, or the glycolytic pathway. These differentially expressed proteins, which were all upregulated at least once under high-temperature stress, would lead to the accumulation of acetyl-CoA and cause a subsequent increase in adenosine triphosphate (ATP) production. The result suggests that the blades of *P. haitanensis* require high levels of energy to cope with stress and to repair damage under high-temperature stress. This speculation is consistent with the upregulation of two proteins known to be involved in the energy production pathway: protein belonging to the H⁺/Na⁺-translocating f-type, v-type, and A-type ATPase superfamily (spot 24) and an inorganic pyrophosphatase (spot 73). ATPases represent a class of enzymes that catalyze the decomposition of ATP into adenosine diphosphate (ADP) and a free phosphate ion. Inorganic pyrophosphatase catalyzes the conversion of one molecule of pyrophosphate to two phosphate ions. These two proteins play essential roles in energy conservation and provide the energy for many biosynthetic pathways. ATPase and inorganic pyrophosphatase are also upregulated in many higher plants when exposed to heat stress (Neilson et al. 2010).

Other metabolic pathways-related proteins In contrast to the upregulation of proteins related to energy metabolism, all of the differentially expressed proteins that function in other metabolism pathways were downregulated. Bifunctional 3'-phosphoadenosine 5'-phosphosulfate synthetase 2 (spot 17) mediates two steps in the sulfate activation pathway. PAP2-like proteins (spot 25), alanine-glyoxylate transaminase (spot 65), D-3-phosphoglycerate dehydrogenase (spot 104), and the glycine cleavage system T protein (spot 80) participate in amino acid metabolism. Carbonyl reductase (spot 90) participates in arachidonic acid metabolism. Cinnamyl alcohol dehydrogenase (spot 102) is a key enzyme in the biosynthesis of lignin. The downregulation of these proteins suggests that the synthesis of lignin and other metabolites was inhibited under high-temperature stress. This suggests that the blades of *P. haitanensis* can avoid cell damage under stress by reducing unnecessary consumption of metabolites. The same phenomenon occurs in many plants under stress (Kosová et al. 2011).

Redox homeostasis-related proteins Abiotic stresses can induce the production of reactive oxygen species (ROS). ROS can act as signaling molecules for stress responses; however, they can also cause damage to cellular components. To protect against oxidative damage, cells have developed a wide range of antioxidant systems that scavenge or reduce excessive ROS or ROS-induced toxic substances. In the present study, a group of antioxidant enzymes were identified, including pyridine nucleotide-disulfide oxidoreductase (spot 18), phosphomannomutase (spot 45), glutathione S-transferase (spot 85), ferritin 3

(spot 100), lactoylglutathione lyase (spot 101), cytosolic ascorbate peroxidase (spot 116), and cystathionine β -synthase (spot 36) domain-containing protein. Several reports have shown that these antioxidant enzymes play important roles in the pathway for scavenging ROS and enhance tolerance to environmental and biotic stresses (Dixon et al. 2002; Seppänen et al. 2000; Sweetlove et al. 2002; Moons 2003; Asada 1992; Mittler et al. 2004; Hegedus et al. 2008; Qian et al. 2007; Yadav et al. 2005; Mustafiz et al. 2010; Kumari et al. 2008; Kushwaha et al. 2009). The consistently upregulated expression of these identified antioxidant enzymes in this study suggests that they may detoxify ROS-induced lipid peroxidation products and contribute to the survival of the blades of *P. haitanensis* under high-temperature stress.

Proteins involved in responding to stimuli High temperature leads to increased expression of several proteins that function as chaperones, particularly several members of the large family of heat shock proteins (HSPs), which are classified into five distinct subfamilies according to their molecular weights (HSP110, HSP90, HSP70, HSP60, and sHSPs) (Baniwal et al. 2004). In the transcriptome sequencing of gametophyte blades of *Pyropia tenera*, Choi et al. (2012) identified several high molecular weight HSPs whose expression was upregulated significantly under high-temperature stress for 1 h or 3 h, but no small HSPs (sHSPs) were identified. By contrast, the results of the present study show that high temperature increased the abundance of low molecular weight sHSPs (spot 19), but had little effect on the expression of high molecular weight HSPs. The difference may suggest that the changes in transcription do not often correspond with the changes of protein expression or that the expression of high molecular weight HSPs only increased significantly for a short time during high-temperature stress, but did not change as high-temperature stress continued for several days. Nevertheless, further studies should be conducted to clarify this point.

The chaperone activity of sHSPs, which function as a reservoir of intermediates of denatured proteins that prevent protein aggregation caused by stress, is usually undetectable in vegetative tissue under normal growth conditions. However, they can be induced by developmental stimuli or by environmental stresses (Sun et al. 2002). The upregulation of sHSPs in the present study suggests that they play an important role in counteracting high-temperature stress by reestablishing normal protein conformations and, thus, cellular homeostasis. Consistent with these findings, Lee et al. (2007) reported that several sHSPs are expressed in rice leaves during heat stress. Further, the overexpression of a tomato mitochondrial sHSP increased the thermotolerance of transgenic tobacco plants (Sanmiya et al. 2004).

Proteins involved in transcription and translation Transcriptional and translational control of the expression of stress-responsive

genes is a crucial part of the response of plants to abiotic stress. The elongation factor Tu (EF-Tu, spots 37 and 88) is a member of a highly conserved, nuclear-encoded multigene family (Momcilovic and Ristic 2007). During polypeptide synthesis, EF-Tu plays an important role in promoting the binding of aminoacyl-tRNA to the A site of the ribosome (Momcilovic and Ristic 2007). Moreover, EF-Tu acts as a molecular chaperone and protects chloroplast proteins from thermal aggregation and inactivation (Rao et al. 2004). In maize, Momcilovic and Ristic (2007) suggested that the expressions of EF-Tu genes correlate with the ability to tolerate stress. Elongation factor 1 β (EF-1 β , spot 87) is a member of the guanine nucleotide exchange factors family, which regulate the activity of G proteins and are critical for many cellular processes, including the stress response (Ejiri 2002). Transcription factor BTF3 (spot 113) is required for transcriptional initiation by RNA polymerase II. BTF3 is upregulated by drought in rice (Gorantla et al. 2005) and maize (Hu et al. 2011). In the present study, the upregulated expression of transcriptional and translational regulatory proteins suggested that they may play important roles in ensuring continued protein synthesis during high-temperature stress.

Cytoskeleton-related proteins Abiotic stress can also induce profound changes in the composition of the cytoskeleton (Abdrakhamanova et al. 2003). Here, the upregulation of the three cytoskeleton-related proteins (spot 16, 103, and 125) in response to high-temperature stress indicated that the dynamics of these proteins function in cellular homeostasis of *P. haitanensis* during high-temperature stress.

Proteins that mediate signal transduction Under stress, plants usually begin responding by sensing and transferring stress signals through signal transduction networks. The response to stress is likely mediated through signaling pathways that regulate the expression levels of a range of proteins (Hirayama and Shinozaki 2010). One protein (spot 69) involved in signal transduction was identified here. The ADP-ribosylation factor (ARF) GTPase-activating proteins (GAPs) represent a family of proteins that induce hydrolysis of GTP bound to ARF and act as a modulator or transducer in various transmembrane signaling systems (Randazzo and Hirsch 2004). The upregulation of ARF-GAP in response to high-temperature stress reflects the role of this protein in cell signaling under stress.

In conclusion, the comparative proteomic analysis presented identified a significant number of stress-responsive proteins from *P. haitanensis* blades placed under high-temperature stress. Based on the dynamic changes in the levels of expression of these proteins, we conclude that the blades of *Z-61* resist high-temperature stress by inhibiting photosynthesis and other unnecessary metabolic processes. Energy metabolism is increased, and upregulation of the expression of proteins related to redox homeostasis, as well as those that respond to stimuli,

serves to maintain the physiological balance during stress. Concurrently, the expression levels of proteins related to transcription and translation, the cytoskeleton, and signal transduction are also upregulated to promote survival under high-temperature stress.

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