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Variations in the Expression of Photosynthesis–Related Proteins in Field Chattonella Marina Cells During a Harmful Algal Bloom

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Time series variations in protein expression profiles in field *Chattonella marina* cells were investigated during a HAB occurred in the inner part of Ariake Sea, Japan (5–14 September, 2012). This study aimed to gather information on the molecular mechanisms underlying the physiological responses in field C. marina population during HAB. Proteomic analysis showed that the abundance of ~37% protein spots (40 out of 108 detected from 2–DE gel images) significantly varied with the sampling date. Significant decreases in the abundances of proteins involved in photosystem II (LHCP 4), electron transfer chain (Cyt c553), Calvin cycle (GAPDH), and chloroplast antioxidant system (2–Cys Prx) were observed as the bloom progressed, suggesting the efficiencies of those photosynthetic pathways declined during the bloom. In addition, the abundances of the above proteins showed significant positive correlations with the F_v/F_m ratio and growth rate of *C. marina* and with DIN concentrations (except LHCP 4). Our findings suggested that declined expressions of those photosynthesis–related proteins presented some molecular foundation of the decreases in F_v/F_m ratio and growth rate of *C. marina* during the bloom, and also provided insight into mechanistic links between the external/internal factors and physiological responses of *C. marina* that may ultimately dictate the ecology of the bloom.

Key words: *Chattonella marina*; F_v/F_m ratio; Growth rate; Harmful algal bloom; Proteomics Two–dimensional gel electrophoresis

Abbreviations: 2–Cys Prx, 2–cysteine peroxiredoxin; 2–DE, two–dimensional gel electrophoresis; AtpB, ATP synthase CF 1 beta subunit; Cyt c553, Cytochrome c553; DIN, dissolved inorganic nitrogen (NO_2^-, NO_3^-, and NH_4^+); DIP, dissolved inorganic phosphorus (PO_4^{3-}); GAPDH, glyceraldehyde–3–phosphate dehydrogenase; ISP: rieske (2Fe–2S) region protein; LHCP 4, chloroplast light harvesting protein isoform 4; OEE 1, oxygen evolving enhancer 1; RPL12, 50S ribosomal protein L12

INTRODUCTION

The harmful algal blooms (HABs) of species belonging to the genus *Chattonella* (Raphidophyceae) have been reported to cause significant economic and ecological damages in various regions of the world (Hallegraeff *et al*., 1998; Tiffany *et al*., 2001; Imai and Yamaguchi, 2012). Recent years, HABs of *Chattonella* spp. continually cause serious damages to aquaculture and fishery production in the coastal waters of western Japan (Yamatogi *et al*., 2006; Imai and Yamaguchi, 2012; Katano *et al*., 2012). Thus, numerous studies have been performed to reveal their life history, growth physiology, and bloom ecology (Imai and Yamaguchi, 2012). However, only a few studies have focused on physiological responses in *Chattonella* to various stresses via defined laboratory culture experiments (Warner and Madden, 2007; Portune *et al*., 2010; Qiu *et al*., 2013), while studies on mechanisms involved in cellular responses in field populations are completely lacking.

Proteomics, a large–scale study of the structure and function of proteins in complex biological samples, has been shown to have powerful potential with regard to revealing the physiological and metabolic characteristics of HAB species (Anderson *et al*., 2012; Poulson–Ellestad *et al*., 2014). Proteomics have been applied to the adaptive responses of marine dinoflagellates and diatoms under various environmental stresses (Wang *et al*., 2014; Yang *et al*., 2014; Muhseen *et al*., 2015). Although most proteomic studies on HAB species were conducted via laboratory culture experiments, obtained results have provided clues to elucidate the relationship between algae and environmental variables, and promoted our understanding of the biochemical processes that contribute to algal ecological success. Furthermore, a study combining field research and omics–related technologies has been considered as an essential and powerful way to ensure a comprehensive understanding of the factors that promote the HABs within specific environments (McLean,

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2013; Poulson–Ellestad *et al*., 2014; Wang *et al*., 2014).

Our previous proteomic analysis on batch cultured *Chattonella marina* var. *antiqua* (Hada) Demura & Kawachi demonstrated that some phase–dependent expressed proteins have potential to serve as indicators for rapid assessment of cellular responses, and monitoring their abundance may provide useful information on the molecular mechanisms involved in the initiation and decline of algal blooms, by correlating those changes with physiological and ecological parameters (Qiu *et al*., 2013). In the present study, we investigated variations in protein profiles of field *Chattonella* cells at different stages of a HAB dominated by *C. marina*. Subsequently, we focused on photosynthesis related proteins and correlated their abundances with the measured physiological parameters (F_v/F_m ratio and growth rate of *C. marina*) and observed environmental variables (DIN, DIP, temperature, and salinity). This study aimed to gather information on the molecular mechanisms underlying the physiological responses in field *C. marina* population during HAB.

MATERIAL AND METHODS

Field survey

Seawater samples were collected at those stations located in the inner part of the Ariake Sea, Japan (Fig. 1). About 2 L seawater was collected from depths of 0, 2, and 5 m (depth permitting) using a Niskin water sampler (Model 1080, Rigo, Saitama, Japan), and were brought to the laboratory within 4 h from the first sampling time and used for subsequent experiments. The vertical profiles of water temperature and salinity were measured using a Compact–CTD recorder (Model ASTD687, ALEC Electronics Co., Ltd., Kobe, Japan). For phytoplankton counting, each bottle was gently turned upside down five times before subsamples were taken. Vegetative cells were counted under a microscope in triplicate 0.1 ml subsamples. Subsequently, 50 ml subsamples were gravity–filtered through glass microfiber filters (GF/C, Whatman International Ltd., Maidstone, UK), and then were passed through 0.22–μm syringe filters. Those water samples were frozen at −80°C for macronutrient analysis, The DIN and DIP concentrations were measured with an Autoanalyzer (TRACCS 2000; Bran + Luebbe, Hamburg, Germany).

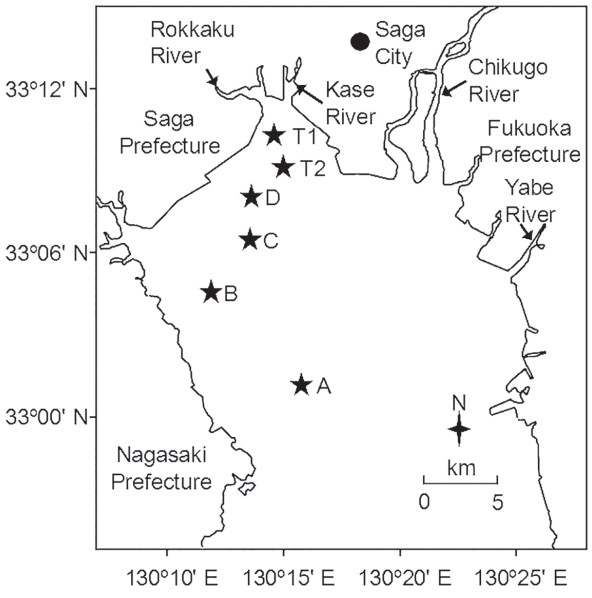

Fig. 1. Field sites for sampling from 5 to 18 September, 2012, in the inner part of Ariake Sea, Japan. Stars indicate the sampling stations: A (33°01.165′ N, 130°15.971′ E), B (33°04.715′ N, 130°10.885′ E), C (33°04.813′ N, 130°11.737′ E), D (33°07.157′ N, 130°12.886′ E), T1 (33°07.963′ N, 130°13.436′ E), T2 (33°10.571′ N, 130°14.218′ E).

Determination of the Fv/Fm ratio and growth rate of *C. marina*

The F_v/F_m ratio was used as the photosynthetic activity indicator and determined on the sampling day. To avoid the potential impact of elevated irradiance on the F_v/F_m ratio, seawater samples were kept under weak natural light (<10 μmol m^{-2} s^{-1}) during transport. For F_v/F_m ratio measurement, the bottle was gently turned upside down 5 times before subsamples (1.5 ml) were taken. Subsequently, the subsamples were kept in darkness for at least 30 min and then assayed with a Xe–PAM fluorometer (H. Walz, Effeltrich, Germany), as described by Qiu *et al*. (Qiu *et al*., 2013). To determine the growth rate of *C. marina*, triplicate 25–ml subsamples were transferred into 70–ml sterile flasks (Nunc, Thermo Fisher Scientific Inc., Suwanee, GA, USA) on the sampling day (defined as day 1), and were grown in an incubator at 27.5°C under cool–white fluorescent illumination (110 ± 10 μmol m^{-2} s^{-1}) at a 14:10 h light:dark cycle for 3 days. Phytoplankton cells were counted under a microscope in triplicate 0.1–ml subsamples on the day 3, and the growth rate (GR, divisions d^{-1}) of *C. marina* was determined as GR = $LN(N_3/N_1)$ / [2LN(2)], where N_1 and N_3 are cell densities of *C. marina* on the days 1 and 3 (Guillard, 1973).

Protein extraction and 2–DE

To collect sufficient cells for 2–DE, about 500 ml seawater samples were transferred into 1–l clean plastic jars, and those jars were let stand for 30–60 min until the *C. marina* had concentrated in the surface of water. Subsequently, 120 ml supernatant suspensions were transferred into four 50–ml centrifuge tube and *C. marina* cells were obtained by centrifugation at 670 × g at 25°C for 5 min. After triplicate re–suspensions with sterile seawater and centrifugations, *Chattonella* cell pellets were stored at −80°C until protein extraction.

With an Analog sonifier (Model 250, Branson Ultrasonics Co., Danbury, CT, USA) cell pellets were lysed in 3 ml of extraction buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 5 mM magnesium acetate) on ice, and the resulting homogenate was centrifuged at 10,000 × g at 4°C for 10 min. Subsequently, the supernatant was transferred into a new centrifuge tube containing 12 ml of cold acetone (−20°C), and the mixture was maintained at −20°C for at least 2 h, after which the mixture was centrifuged at 10,000 × g at 4°C for 10 min. Repeated re–

suspensions with cold acetone and centrifugations were conducted for 3 times to remove substances that might interfere with the first dimension (isoelectric focusing) of the electrophoresis. The resulting protein pellet was dried at room temperature and dissolved in 0.5 ml of urea buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 2% w/v DTT, trace amount of bromophenol blue) by vortex mixing and sonication.

Protein concentration of each sample was measured with a BCA protein assay kit (Thermo Scientific, Pierce, IL, USA). Exactly 0.5 mg of protein (350 μl) with 1.75 μl of IPG (immobilized pH gradient) buffer (pH 4–7, GE Healthcare, Uppsala, Sweden) was loaded onto each IPG strip (pH 4–7, 18 cm, GE Healthcare). After rehydration for 10 h at 20°C, isoelectric focusing (IEF) was performed with an Ettan IPGphor 3 system (GE Healthcare) using the following program at 20°C: 500 V for 4 h, a linear gradient to 1,000 V over 1 h, a linear gradient to 8,000 V over 2 h, and 8,000 V for 7 h. The focused IPG strips were equilibrated in equilibration buffer (6 M urea, 30% w/v glycerol, 2% w/v SDS, 50 mM Tris–HCl, and a trace of bromophenol blue, pH 8.8) supplemented with 130 mM DTT for 10 min and subsequently equilibrated with the same buffer containing 135 mM iodoacetamide (instead of DTT) for 10 min. For the second dimension electrophoresis, the equilibrated strips were loaded onto 12% SDS–polyacrylamide gels (20 × 20 cm). The gels were run in a Protean Ⅱ xi 2–D system (Bio–Rad, Hercules, CA, USA) at 25 mA for 1.5 h and 35 mA per gel until the front dye reached the bottom of the gel at 10°C. To consider technical variation, protein extract from each sample was analyzed 3 times. This resulted in a total of 33 gels used for subsequent correlation analysis.

Image capture, abundance analysis, and protein identification

The 2–DE gels were fixed in 10% (w/w) acetic acid and 40% (w/w) ethanol for 6 h and stained with Flamingo gel stain solution (Bio–Rad) for 3 h. The stained gels were scanned at a resolution of 100 dots cm^{-1} by an FX molecular imager (Bio–Rad). The gel images were analyzed by Proteomweaver 2–D analysis software version 4.0 (Bio–Rad). The relative protein abundance in each spot was analyzed with automated routines from the software combined with manual editing to remove artifacts.

For protein identification, protein spots in 2–DE gels were electrotransferred onto PVDF membranes (Bio–Rad) with a Semi–dry transblot apparatus (ATTO, Tokyo, Japan). The PVDF membrane–bound proteins were visualized by staining with 0.1% w/v amido black in 45% v/v methanol for 10 min and destained in distilled water. Selected protein spots were excised and analysis of *N*–terminal amino acid sequences was determined by a PSQ–1 protein sequencer (Shimadzu, Kyoto, Japan). Obtained amino acid sequences were firstly searched against NCBI–BLAST using protein–protein BLAST algorithm with a non–redundant (nr) protein database (http://www.ncbi.nlm.nih.gov/protein/). Those sequences that failed to find a match were then searched against the available sequences of Chattonellaceae (taxid: 658124) and Heterokonts (taxid: 33634) to find homologous proteins with default parameters.

Statistical analysis

Spearman's rank correlation coefficient (rs) was used to examine the correlation of abundance of each protein spot detected by 2–DE with sampling date, F_v/F_m ratio and growth rate of *C. marina*, and observed environmental variables (DIN, DIP, temperature, and salinity). Considering Chattonella cells have diel vertical migration behavior (Watanabe *et al*., 1991) and can migrate to a depth of about 5 m for taking up nutrient in the Ariake Sea (Katano *et al*., 2012), the average values of environmental variables (Table 1) of seawater samples collected from depths of 0, 2, and 5 m (depth permitting) in the same station were used for the above correlation analy-

Table 1. Cell abundance, F_v/F_m ratio, and growth rate of *Chattonella marina*, as well as environmental variables in seawater samples used for proteomic analysis

Sampling date	Site	Abundance (cells · mL^{-1})	F_v/F_m ratio	Growth rate (division · d^{-1})	Environmental variables[a]			
					DIN (μM)	DIP (μM)	Temperature (°C)	Salinity
5–Sep	Station A	1030	0.710	0.380	1.38	0.62	27.55	27.77
	Station B	2350	0.716	0.221	3.12	1.19	27.66	27.37
7–Sep	Station C	9444	0.711	0.278	1.39	0.76	29.43	24.45
	Station D	950	0.712	0.788	5.48	1.52	29.28	23.97
10–Sep	Station C	2340	0.665	0.498	1.37	0.65	28.17	22.75
	Station B	6100	0.661	0.276	0.48	0.77	28.05	23.12
12–Sep	Station T1	8667	0.690	0.097	0.79	0.72	27.37	25.01
	Station D	3217	0.651	0.196	0.43	1.34	27.14	24.61
	Station T2	6817	0.672	0.205	0.48	1.98	27.38	26.35
14–Sep	Station C	5948	0.648	–0.109	0.45	1.90	28.15	26.60
	Station B	4383	0.637	–0.027	0.39	1.35	28.20	26.70

[a] The average values of seawater samples collected from the depth of 0, 2, and 5 m (depth permitting) in the same station.

sis. All statistical analyses were performed using the Statistical Package for the Social Sciences software (SPSS 11.0; SPSS, Inc., Chicago, IL, USA).

RESULTS

On the basis of the dominance (became monospecific or almost dominated the phytoplankton community) and

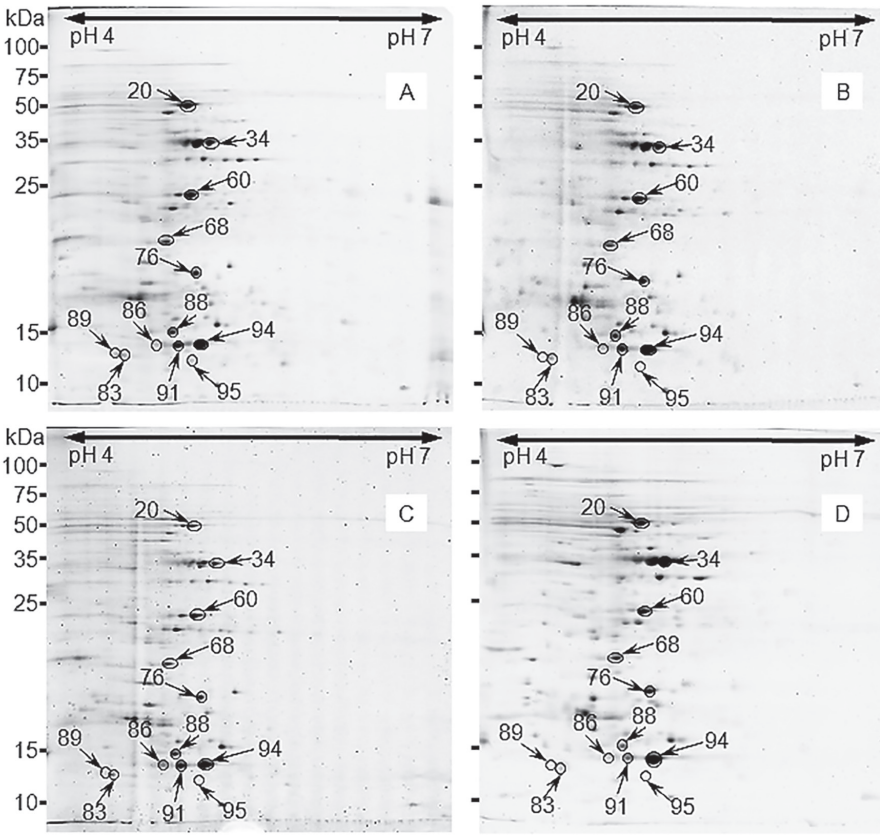

Fig. 2. Representative 2–DE maps of field *Chattonella marina* cells in seawater samples collected in the station B on 5 (A), 7 (B), 14 (C) September, 2012, as well as that of *C. marina* var. *antiqua* at early stationary phase (NIES–1 strain, 16,000 cells ml^{-1}) of a sterile batch culture (D). The numeric labeling of the spots in corresponds to the numbers assigned in Table 2.

Table 2. Proteins identified by *N*–terminal amino acid sequencing

Spot No. [a]	*N*–terminal amino acid sequencing	Matched proteins [marched species] [b]	Accession No.	Identity	E–value	MW(kDa) / *p*I	
						Observed	Calculated
20	METTNESLGYTDQIIGPVLD	AtpB [*H. akashiwo*]	YP_001936399.1	17/20 (85%)	2.0E–08	51.0 / 5.30	51.0 / 5.07
34	EGDVIATGINGFGRIG	GAPDH [*D. dichotoma*]	ABU54834.1	11/13 (85%)	6.0E–03	36.0 / 5.65	36.4 / 5.42
60	ITKEELRSLDYLQVKGVGLA	OEE1 [*H. akashiwo*]	AAN11311.1	16/20 (80%)	6.0E–06	29.5 / 5.40	31.9 / 5.52
68	SIRVGQKAPDFTATAVFDQE	2–Cys Prx [*H. akashiwo*]	YP_001936352.1	13/19 (68%)	8.0E–03	23.0 / 4.90	22.0/ 4.89
76	KLGSSAQGLIGSDLXXPXF	LHCP 4 [*N. gaditana*]	EWM23957.1	14/19 (74%)	1.0E–04	21.0 / 5.50	23.4 / 5.44
83	ADIENGEDIFTANAS	Cyt c553 [*H. akashiwo*]	YP_001936409.1	12/13 (92%)	7.6E–07	12.0 / 4.80	12.1/ 5.02
86	MRLTQGAFLXLP	RUBISCO SSU [*C. marina*]	BAS54019.1	10/12 (83%)	2.2E–02	16.5 / 4.90	16.1 / 5.01
88	AGVKAKDEWVXXLL	ISP [*N. gaditana*]	EWM24009.1	10/12(83%)	4.0E–03	18.0 / 5.00	20.6/5.66
89	SKVENIVDELKTLTL	RPL12 [*H. akashiwo*]	YP_001936329.1	12/15 (80%)	1.0E–05	13.0 / 4.60	13.5/ 4.55
91	MRLTQGAFSYLXDXH	RUBISCO SSU *[C. marina*]	BAS54019.1	12/13 (92%)	1.0E–05	16.5 / 5.30	16.1 / 5.01
94	MRLTQGAFSYLPDLTDAQII	RUBISCO SSU [*C. marina*]	BAS54019.1	20/20 (100%)	2.0E–14	16.5 / 5.50	16.1 / 5.01
95	MRLTQGAFSYLPDLXDAQXI	RUBISCO SSU [*C. marina*]	BAS54019.1	18/20 (90%)	6.0E–12	14.0 / 5.40	16.1 / 5.01

[a] The protein spot numbers are given in Fig. 2.

[b] *C. marina: Chattonella marina; D. dichotoma: Dictyota dichotoma; H. akashiwo: Heterosigma akashiwo; N. gaditana: Nannochloropsis gaditana*

abundance (peak densities of each sampling day), *C. marina* cells in a total of 11 samples (2 or 3 samples per sampling date) were selected for proteomic analysis, as shown in Table 1. As the bloom progressed, both F_v/F_m ratio (0.64–0.72) and growth rate (–0.1–0.29 div. d^{-1}) of *C. marina* cells showed a tendency to decrease throughout the sampling period. Relative small variations in the average temperature (27.1–29.4 °C), salinity (22.8–27.8), and DIP c oncentration (0.62–1.98 μM) were observed in the above sample. Relative low DIN (0.39–5.48 μM) were recorded during the bloom, especially after 10 September, 2012 (<0.8 μM).

Proteomics of vegetative cells of *C. marina* in seawater samples were performed to track variations in their protein expression patterns throughout the HAB (Table 1). The 2–DE protein profiles of field *C. marina* cells shared a majority of common protein spots (Fig. 2A–C), which were also found to be similar to those of *C. marina* var. *antiqua* cultured in laboratory (Fig. 2D). Among 108 protein spots detected from the Flamingo–stained 2–DE gels, 77 spots matched in at least 85% (≥ 28 gels) of all the 3 gels, which were used for further correlation analysis. Based on the Spearman's rank correlation between abundance of each protein spot and sampling date,

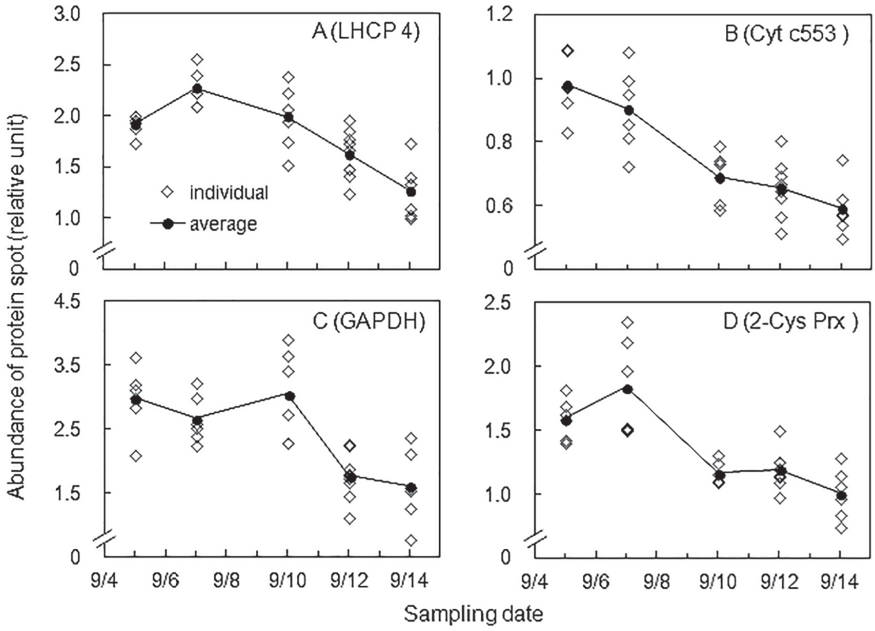

Fig. 3. Temporal variations in the expression levels of LHCP 4 (A), Cyt c553 (B), GAPDH (C), and 2–Cys Prx (D). Diamonds indicate relative abundance of protein spot in individual 2–DE gels and lines indicate the average values of all gels on the same sampling day. Note the different scales in vertical axis.

Table 3. Correlations of abundance of identified protein spots with physiological parameters and with environmental variables

Proteins (spot number) [a]	Spearman's coefficient [b]						
	Physiological parameters		Environmental variables				
	F_v/F_m ratio	Growth rate	Sampling date	DIN	DIP	Temperature	Salinity
AtpB (20)	0.102	0.214	–0.113	–0.154	–0.115	–0.059	–0.336
GAPDH (34)	0.523**	0.620**	–0.712**	0.457**	–0.487**	0.235	–0.183
OEE1 (60)	0.101	0.313	–0.161	–0.078	–0.091	0.116	–0.239
2–Cys Prx (68)	0.771**	0.494**	–0.762**	0.702**	–0.323	0.174	0.149
LHCP 4 (76)	0.602**	0.829**	–0.696**	0.176	–0.245	0.371*	–0.346*
Cyt c553 (83)	0.780**	0.574**	–0.812**	0.628**	–0.197	0.201	0.257
ISP (88)	0.043	0.107	–0.206	0.056	0.026	–0.046	–0.148
RPL12 (89)	0.284	–0.305	–0.094	0.192	0.112	0.006	0.509**
RUBISCO SSU (86)	0.043	0.158	–0.206	0.056	0.026	–0.148	–0.016
RUBISCO SSU (91)	–0.183	–0.284	0.208	–0.090	0.019	–0.307	–0.115
RUBISCO SSU (94)	0.018	0.189	–0.057	–0.160	–0.103	–0.142	–0.377*

[a] The protein spot's information is given in Fig. 2 and Table 2, respectively.

[b] Correlations were analyzed by Spearman's rank correlation (N = 33; *, $P < 0.05$; **, $P < 0.01$)

expression of 40 protein spots significantly decrease (33 spots; $P < 0.05$) or increase (7 spots; $P < 0.05$) as the bloom progressed.

The *N*–terminal amino acid sequences of 15 protein spots were sequenced successfully, out of which 12 spots were identified as isoforms or enzymes involved in photosynthesis (Table 2). These identified proteins are involved in the PS Ⅱ (spot 60 and 76), electron transfer (spot 83 and 88), ATP synthesis (spot 20), Calvin cycle (spots 34, 86, 91, 94, and 95), chloroplastic antioxidant system (spot 68), and chloroplastic protein synthesis (spot 89). Correlations between abundance of identified protein spots and physiological parameters and environmental variables are listed in the Table 3. Significant decreases in the expression of LHCP 4 (spot 76), Cyt c553 (spot 83), GAPDH (spot 34), and 2–Cys Prx (spot 68) were observed as the bloom progressed (Fig. 3A–D), and their abundances were also parallel significantly correlated with the F_v/F_m ratio and growth rate of *C. marina* (Spearman's rank correlation, $P < 0.01$, Table 3).

DISCUSSION

This study represents the first attempt that using proteomics to investigate time series variations in protein expression profiles of field *C. marina* populations throughout a HAB when they became dominant. The dynamics of *Chattonella* cells during the HAB has been described in detail in our previous report (Qiu *et al*., 2016), and exhaustion of DIN concentration was likely responsible for controlling the photosynthetic activity and growth of algal cells. Assuming that the minimum cell quotas of nitrogen and phosphorus for *C. marina* var. *antiqua* are 7.7 and 0.60 pmol cell^{-1} (Nakamura, 1985), increase of 1000 cells in a milliliter water corresponds to consumptions of 7.7 and 0.6 μM, respectively. From this aspect, the DIN in samples used for proteomic analysis was likely a limited factor for the growth of *C. marina* cells, and become insufficient at the terminal stage of the HAB (Table 1). Indeed, the growth rate of *C. marina* decreased to < 0 divisions d^{-1} on 14 September, associated with the declined F_v/F_m ratios.

Proteomic study found that *C. marina* showed constancy of protein profiles at the different stages of the HAB (Fig. 2), suggested that the cells may maintain most of their protein functionality, even at the end of the bloom. Invariance of protein profiles was also observed in the *C. marina* var. *antiqua* (Qiu *et al*., 2013) and the dinoflagellate *Prorocentrum triestinum* (Chan *et al*., 2004) at different growth phases of batch culture. Given that equal amounts of proteins were loaded onto each gel, however, about 37% protein spots showed abundances significantly varied with sampling date, which are thought to play critical roles in mediating cellular responses and growth of *C. marina* during the HAB. Identification of proteins spots (Fig. 2 and Table 2) and subsequent correlation analysis (Table 3) suggested that variations in the expression levels of LHCP 4 (spot 76), Cyt c553 (spot 83), GAPDH (spot 34), and 2–Cys Prx (spot 68) present some molecular foundation of the physiological responses in *C. marina* cells during the bloom.

Pronounced decrease in abundance LHCP 4 (spot 76, Fig. 3A) may impair light absorption ability and thereby caused the decline of F_v/F_m ratio of *C. marina* (Table 1). The LHCP 4 is an isoform of light harvesting complex, which binds antenna chlorophyll and carotenoid pigments that augment light–capturing capacity of photosynthetic reaction centers of PS Ⅱ (Green and Durnford, 1996; Durnford *et al*., 1999). Since light absorption during photosynthesis is directly related to algal cell productivity, declined expression of LHCP 4 may also reduce the growth rate of *C. marina*. Those inferences were supported by correlation analysis, which showed that the abundance of LHCP 4 has significant positive correlations with both F_v/F_m ratio and growth rate of *C. marina* (Table 3).

Constantly decrease in the abundance of Cyt c553 (spot 83) suggested the electron transport ability of *C. marina* cells declined as the bloom progressed. Similar temporal decline in expression of Cyt c553 was also observed during the batch cultures of *C. marina* var. *antiqua* (Qiu *et al*., 2013), suggesting cellular senescence is one of the factors leading to declined expression of this protein. Since Cyt c553 is a mobile cytochrome that functions in the transfer of reducing equivalents from the cytochrome b_6f complex to PSI or ferredoxin (Merchant and Dreyfuss, 1998; Behrenfeld *et al*., 2006), declined expression of this protein may result in lower amount of electron available for the production of ATP and NADPH, which are used to promote the subsequent chemical processes of light–independent reactions.

As a key enzymes of Calvin cycle, GAPDH (spot 34) catalyzes the reduction of 1,3–bisphosphoglyceric acid by NADPH to produce glyceraldehyde–3–phosphate (GAP) and inorganic phosphate. Because GAP is also an intermediate in the pathways of glycolysis and gluconeogenesis in plant and algae, GAPDH plays strategic roles in those cell metabolisms (Henze *et al*., 1995; Liaud *et al*., 2000; Trost *et al*., 2006; Mekhalfi *et al*., 2014). The notably declined expression of GAPDH in *C. marina* a few days prior to bloom termination (Fig. 3C) may impair those metabolic pathways and result in lower production of organic compounds. However, our previous study showed that GAPDH displayed a phase–dependent increased abundance during batch culture of *C. marina* var. *antiqua* (Qiu *et al*., 2013). Considering the difference in the nitrogen concentrations between the previous batch cultures (about 199 μM at late stationary phase) and the present field observation (<1 μM at the end of the bloom), we inferred that nitrogen limitation was one of the important factors that induced the decrease expression in GAPDH during the bloom. Consistent with this, significant positive correlations between abundance of GAPDH and DIN concentration was observed during the bloom (Table 3). Nitrogen deprivation induced declined expression of GAPDH at transcript and/or protein levels has also been demonstrated in several studies on diatoms (Hockin *et al*., 2012; Yang *et al*., 2014) and green algae (Juergens *et al*., 2015). In addition, carbon and nitrogen metabolism in plant and

algal cells are closely linked (Huppe and Turpin, 1994), and reduced nitrate assimilation causes metabolic imbalance leading to increased oxidative stress (Logan *et al*., 1999; Blaby *et al*., 2013).

Notable decrease in abundance of the antioxidant enzyme 2–Cys Prx (spot 68) was observed as the bloom progressed (Fig. 3D). Similarly, significant decrease in 2–Cys Prx prior to rapid cell death were also found in batch cultured *C. marina* var. *antiqua* (Qiu *et al*., 2013). It seems that cellular senescence is one of the critical factors leading to declined expression of this protein in *Chattonella* cells, as reported in various high plants (Baier and Dietz, 1996; Baier and Dietz, 1999; Horling *et al*., 2001). In various transgenic high plant or cyanobacteria, deficiency in 2–Cys Prx resulted in lower growth, inhibited photosynthesis, and higher stress sensitivity (Dietz, 2011). Those findings supported our results that abundance of 2–Cys Prx had significant positive correlations with both the growth rate and F_v/F_m ratio of *C. marina* during the HAB (Table 3). *Chattonella* is known to produce high levels of reactive oxygen species associated with photosynthesis and cell growth (Kim *et al*., 2004; Kim *et al*., 2006; Portune *et al*., 2010), and therefore large amount of antioxidant enzymes is necessary to protect themselves during active growth. As the bloom progressed, lower amount of 2–Cys Prx may become insufficient and result in peroxides accumulation within cells, which can impair the photosynthesis by damaging chloroplast structures (Baier and Dietz, 1999), and finally may induce the rapid termination of the bloom by triggering peroxide signaling events leading to cellular apoptosis (Wood *et al*., 2003).

In summary, the present proteomic study using field *C. marina* population demonstrates variations in abundances of some photosynthesis–related proteins can provided some insights into mechanistic links between external and/or internal factors (e.g., nitrogen limit and/or cellular senescence) and physiological responses (declined F_v/F_m ratio and growth rate) that may ultimately dictate the ecology of the bloom. Besides those indentified photosynthetic proteins, proteomic analysis of *C. marina* also detected many other differentially expressed proteins as the bloom progressed. Further study is necessary to investigate the synthesis and functions of those differentially expressed proteins of *C. marina* during a field HAB, which can ensure a more comprehensive understanding of their ecophysiology and bloom dynamics within specific environment conditions.

AUTHOR CONTRIBUTIONS

X. Qiu designed the study, performed the proteomic analysis and amino acid sequencing, analyzed the data and wrote the paper. K. Mukai performed the amino acid sequencing. Y. Shimasaki designed the study, performed the field sampling and amino acid sequencing, wrote the paper. M. Tsuyama analyzed the data. T. Matsubara performed the field sampling. T. Nakashima performed the amino acid sequencing. H. Ichinose and Y. Nakajima performed the proteomic analysis. T. Honjo supervised the work. Y. Oshima wrote the paper, supervised the work. All authors assisted in editing of the manuscript and approved the final version.

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