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Metabolic Phenotypes Associated with High-Temperature Tolerance of *Porphyra haitanensis* Strains

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ABSTRACT: Colored mutants of *Porphyra haitanensis* have superior production and quality characteristics, with two mutants, Shengfu 1 (SF-1) and Shengfu 2 (SF-2), having good high-temperature tolerances. To understand the molecular aspects of high-temperature tolerance, this study comprehensively investigated the metabolic differences between the high-temperature tolerant strains and wild type. Nuclear magnetic resonance (NMR) methods identified 35 algal metabolites, including sugars, amino acids, carboxylic acids, aldehydes, amines, and nucleotides. The results indicated that the high-temperature tolerant strains had significantly different metabolic phenotypes from the wild type. The high-temperature tolerant mutants had significantly higher levels in a set of osmolytes consisting of betaine, taurine, laminitol, and isofloridoside than the wild type, indicating the particular importance of efficient osmoregulation for high-temperature resistance. These findings provided essential metabolic information about high-temperature adaptation for *P. haitanensis* and demonstrated NMR-based metabolomics as a useful tool for understanding the metabolic features related to resistance to stressors.

KEYWORDS: *high temperature, metabolic profiling, multivariate data analysis, nuclear magnetic resonance (NMR), metabolic phenotype, Porphyra haitanensis*

INTRODUCTION

Endemic seaweeds in the genus *Porphyra* are widely cultivated on a commercial scale worldwide. Among them, *Porphyra haitanensis* (Note: The taxonomic name for *Porphyra haitanensis* has been changed to be *Pyropia haitanensis* recently) has long been used as a sea vegetable because of its high protein, mineral, and vitamin contents but low fat content.[1] Because *P. haitanensis* can tolerate an inherently stressful intertidal environment, its capability of adapting and acclimating to environmental stresses, such as intense sunlight, rapid temperature fluctuations, osmotic stress and desiccation, is particularly important for its growth. However, the original cultivar (i.e., wild-type strain) of *P. haitanensis* has dominated the cultivation industry for the time being.[2] Serious germplasm degeneration and frequent disease problems have become adverse effects on the quality and output of seaweed production. [3] For example, sustained high-temperature exposures weaken the resistance of *P. haitanensis* to diseases caused by bacteria or fungi, leading to thallus decay.[4,5]

A number of breeding studies have been conducted by selecting the protoplast mutants[6] and pigmentation mutants[7−10] to improve the temperature resistance of *P. haitanensis*. Subsequently, two pigmentation mutants of *P. haitanensis* strain Chang et Zheng, namely, Shengfu 1 (SF-1) and Shengfu 2 (SF-2), have been generated with high-temperature tolerance.[9,11] These strains further showed good properties including more rapid growth and higher productivity than the wild-type strain.[11] Therefore, the physiological processes have been subjected to extensive studies with particular interests in the high-temperature tolerance of these strains.[2,4] However, little information is available so far on the biochemical mechanisms for the high-temperature tolerance of *P. haitanensis* strains.

It has been well-documented that metabolites have important functions in the resistance to high-temperature stress. For example, choline provides thermoprotection by preventing protein denaturation and disaggregation in bacteria,[12] whereas aspartate can function as a compatible solute in the thermoadaptation of organisms.[13] It is therefore likely that metabolites produced by *P. haitanensis* also have important functions to play in adaptation and tolerance to the high temperatures and other stresses. Some studies have already been reported for metabolites in *P. haitanensis*. Sulfated galactan and polysaccharides in *P. haitanensis* have shown antioxidant and antiaging activities[14−16] due to their free radical scavenging properties.[17] The primary metabolites of *P. haitanensis* have also been investigated with the nutritional value of the seaweed as the primary focus.[18] The question of whether these metabolites of *P. haitanensis* are associated with high-temperature stress tolerance remains to be further investigated.

The combination of ^{1}H nuclear magnetic resonance (NMR) spectroscopy with multivariate statistical analysis has proven to be a powerful tool in revealing the stress-induced metabolomic changes. Such approaches have found widespread application in understanding the stress-induced metabolomic responses in animal models.[19−22] These methods were also successfully employed in defining the metabolic phenotypes of ecotypic plant subspecies[23] and plant metabolic reprogramming against

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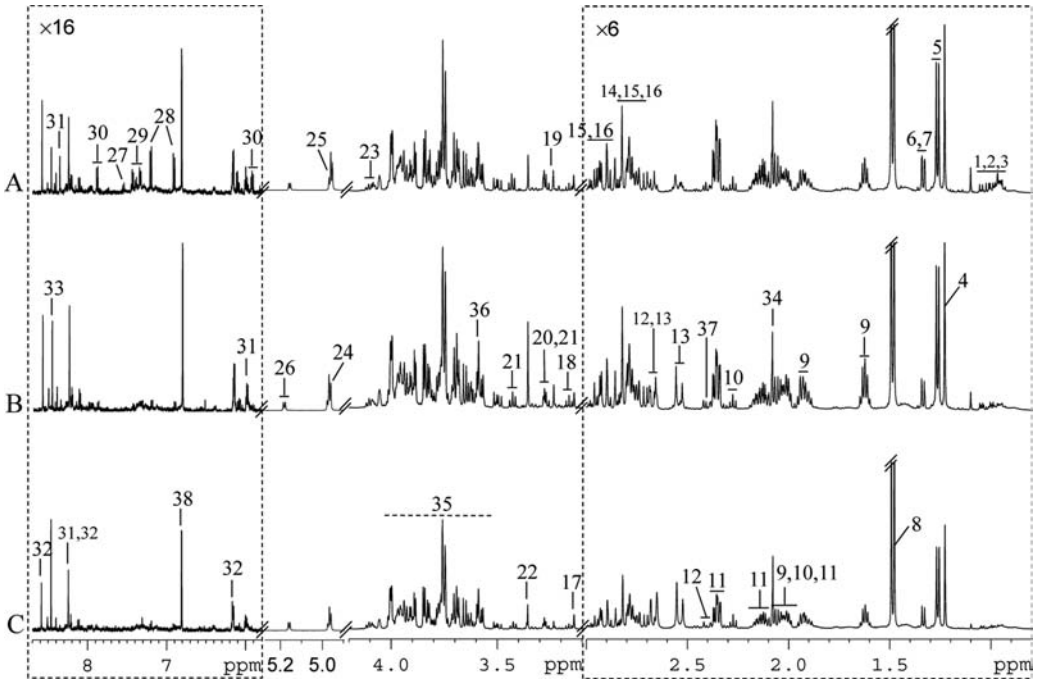

Figure 1. Three typical 500 MHz ^{1}H NMR spectra of *P. haitanensis* strains SF-1 (A), SF-2 (B), and WT (C) extracts. Compared to the chemical shift range at δ 3.1−5.2, the spectra in the regions δ 0.8−3.0 and 5.8−8.7 are displayed at 6- and 16-fold magnification, respectively. Keys for metabolites are in Table 1.

biotic[24] and abiotic stresses.[25−27] It was particularly interesting to note that the metabolomics approaches were particularly powerful in understanding the systems metabolic reprogramming of microorganisms in responding to various stresses[28,29] and adaptations of fungi toward gene dysfunctions.[30] The feasibility of ^{1}H NMR in metabolic phenotyping has also been confirmed in studies of marine unicellular algae.[31]

In this work, we comprehensively analyzed the metabolomic differences of three *P. haitanensis* strains including wild type and two high-temperature tolerant strains using NMR in conjunction with multivariate statistical analysis. Our objectives are to define the metabolic phenotypes (metabotypes) of these three *P. haitanensis* strains and to search for the metabotypic features associated with high-temperature tolerance.

MATERIALS AND METHODS

Chemicals. Analytical grade acetonitrile, $K_2HPO_4{\cdot}3H_2O$, and $NaH_2PO_4{\cdot}2H_2O$ were all purchased from Sinopharm Chemical Co., Ltd. (Shanghai, China). Deuterated water (D_2O, 99.9%) and sodium 3-trimethylsilyl (2,2,3,3,-d_4) propionate (TSP) were bought from Sigma-Aldrich, Inc. (St. Louis, MO, USA).

Sample Collection and Extraction. Dried samples of the two modified strains (SF-1 and SF-2, respectively) and wild type (designated WT) of *P. haitanensis*[11] were kindly provided by Dr. Xinghong Yan of Shanghai Ocean University. High-temperature tolerant strains SF-1 and SF-2 are pigmentation mutants of a wild-type strain of *P. haitanensis* Chang et Zheng generated by ^{60}Co γ-ray treatments.[9,11] In brief, the blades (n) of the wild-type strain (about 5 cm long) were exposed to the ^{60}Co γ-ray with 700 Gy of irradiation dose. The irradiated thalli were cultured in dark for 24h, and then cultured in light/dark (12h in each step). The thalli with color mutants were collected after 3 weeks. Then the single cell of the mutant was cultured to form the conchosporagia. Once conchosporagia formed, the conchocelis was transferred into a 500 mL Erlenmeyer flask containing 150 mL of culture medium and cultured with aeration at 25 °C under 80 μmol photons m^{-2} s^{-1} to develop into gemetophytic blades. Samples were transported in sealed plastic bags and stored at −20 °C for later analysis.

The dried seaweed material (50 mg) was extracted sequentially twice with 600 μL of ice-cold acetonitrile and phosphate buffer (1:1, v/v) using a tissue lyser (Qiagen TissueLyser, Retsch GmBH, Germany) at 20 Hz for 90 s. K_2HPO_4 and NaH_2PO_4 were used to prepare the phosphate buffer (0.1 M, pH 7.4), which contained 10% D_2O (v/v) and 0.005% TSP (w/v), with good solubility and low-temperature storage stability.[32] After centrifugation for 10 min (12000 rpm and 4 °C), the resultant two supernatants were combined and lyophilized following removal of acetonitrile *in vacuo*. The extracts from each sample were respectively dissolved in 600 μL of phosphate buffer. Following centrifugation, 550 μL of the supernatant from each extract was pipetted into a 5 mm NMR tube for NMR analysis.

NMR Measurements. All ^{1}H NMR spectra were acquired at 298 K on a Bruker Avance II 500 MHz spectrometer equipped with a BBI probe (Bruker, Biospin, Germany). A standard one-dimensional NMR spectrum was recorded using the first increment of NOESY pulse sequence (recycle delay−90°−t_1−90°−t_m−90°−acquisition). Water signal was suppressed by irradiation during the recycle delay (2 s) and mixing time (t_m, 100 ms). Typically, the 90° pulse was adjusted to approximately 10 μs and t_1 was set to 6 μs. Thirty-two transients were collected into 32K data points for each spectrum with a spectral width of 20 ppm. Prior to Fourier transformation (FT), an exponential window function with a line-broadening factor of 0.5 Hz was applied to all free induction decays. All spectra were referenced to TSP (δ 0.0). To facilitate NMR signal assignments, a series of 2D NMR spectra were acquired and processed as previously described[25,26] for selected samples, including the ^{1}H−^{1}H COSY, ^{1}H−^{1}H TOCSY, ^{1}H−^{13}C HSQC, and ^{1}H−^{13}C HMBC spectra.

For quantitative analysis, completely relaxed ^{1}H NMR spectra were further acquired for a randomly selected 18 samples from three *P. haitanensis* strains (6 samples per strain). The NMR experiments were carried out at 298 K on a Bruker Avance III 800 MHz spectrometer equipped with an inverse detection cryogenic probe (Bruker, Biospin, Germany). A recycle delay of 15 s was used prior to a standard NOESYPR1D pulse sequence with t_1 of 6.5 μs and 90° pulse length of approximately 10 μs. Water suppression was achieved with a weak irradiation during 2 s of the recycle delay and mixing time (t_m, 100

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Table 1. NMR Data for Low-Molecular-Weight Metabolites Detected in *P. haitanensis*

	metabolite[a]	group	δ ^{1}H (multiplicity)[b]	δ ^{13}C
1	isoleucine	βCH, γCH, γCH′, $\gamma'CH_3$, δCH_3	1.98(#), 1.27(#), 1.46(#), 1.01(d), 0.94(t)	#
2	leucine	βCH_2, δCH_3, $\delta' CH_3$	1.71(#), 0.97(d), 0.96(d)	27.1
3	valine	βCH, γCH_3, $\gamma' CH_3$	2.28(#), 0.99(d), 1.04(d)	32.1
4	laminitol	CH_3, 1-C, 2-CH, 3-CH, 4-CH	1.23(s), 3.33(d), 3.58(#), 4.09(#)	18.8, 79.5, 74.5, 74.3, 76.2
5	6-DA	6-CH_3, 5-CHO, 4-CHO, CO, COOH	1.26(d), 4.32(dd), 4.08(#)	22.2, 71.0, 67.1, 161.5, 178.2
6	lactate	αCH, βCH_3	4.11(q), 1.33(d)	#
7	threonine	αCH, βCH, γCH_3	3.59(d), 4.26(#), 1.33(d)	63.4, 69.3, 22.3
8	alanine	αCH, βCH_3, COOH	3.79(q), 1.49(d)	53.4, 19.1, 178.8
9	2H5AV	αCH, βCH, βCH′, γCH_2, δCH_2 δCH_2 COOH	4.02(m), 1.93(m), 2.02(m), 1.62(m), 3.99(m)	71.9, 59.3, 28.3, 22.3, 176.7
10	2O5AV	αCH_2, βCH_2, γCH_2, COOH	2.27(t), 2.03(m), 3.32(#)	36.3, 22.4, 68.3, 183.6
11	glutamate	αCH, βCH_2, γCH_2, δCO, COOH	3.77(m), 2.06(m), 2.1(m), 2.36(dt)	57.6, 29.8, 36.4, 184.3, 177.5
12	malate	αCH, βCH, βCH′	4.35(dd), 2.40(dd), 2.67(dd)	74.9, 45.9, 188.6
13	citrate	αCH_2, βCOH, γCOOH, δCH_2, COOH	2.54(d), 2.67(d)	48.5, 78.1, 182.1, 48.5, 184.7
14	aspartate	αCH, βCH_2, γCOOH	3.91(dd), 2.68(dd), 2.82(dd)	54.9, 39.4, 180.3
15	DMSP	αCH_2, βCH_2, S-CH_3, COOH	2.74(t), 3.47(t), 2.93(s)	33.9, 43.5, 27.8, 179.8
16	asparagine	αCH, βCH_2, $\gamma CONH_2$, COOH	4.00(dd), 2.84(dd), 2.94(dd)	54.2, 36.2, 180.3, 177.4
17	COS	αCH_2, βCH_2, N−CH_3	4.33(#), 3.69(#), 3.14(s)	69.0, 63.2, 55.6
18	isethionate	CH_2, OCH_2	3.16(t), 3.96(t)	55.7, 59.7
19	betaine	CH_2, N−CH_3	3.93(3), 3.23(s)	68.8, 56.9
20	betaine aldehyde	N−CH_3, CH_2	3.27(s), 3.43 (d)	55.0, 79.9
21	taurine	N−CH_2, S−CH_2	3.27(t), 3.42(t)	50.8, 37.9
22	scyllitol	CHO	3.36(s)	76.6
23	fructose	C_1, $C_{2,3}$H, C_4H, C_6H	4.11(m), 3.94(#), 3.75(d)	101.1, 73.9, 73.5, 63.9
24	L-isofloridoside	C_1H, C_2H, C_3H, C_4H, C_5H, C_6H, $C_{1'}$H, $C_{2'}$H, $C_{3'}$H	4.96(d), 3.85(#), 3.89(#), 4.00(#), 3.91(#), 3.75(d), 3.82(#), 3.50(dd)	101.4, 71.4, 72.1, 72.2, 73.8, 63.9, 71.2, 71.6, 65.4
25	D-isofloridoside	C_1H	4.97(d)	#
26	floridoside	C_1H, C_2H, C_3H, C_4H, C_5H, C_6H, $C_{1'}$H, $C_{2'}$H	5.17(d), 3.85(#), 3.90(#), 4.01(#), 4.10(#), 3.75(d), 3.83(#)	100.8, 71.2, 72.1, 72.2, 73.8, 63.9, 64.1, 81.4
27	uracil	C3H, C4H	7.54(d), 5.81(d)	#
28	tyrosine	C2,6H, C3,5H	7.19(d), 6.90(d)	#
29	phenylalanine	C2,6H, C3,5H, C4H	7.33(dd), 7.43(t), 7.38(t)	#
30	uridine	C4H, C5H	7.88(d), 5.91(d)	#
31	adenosine	C2H, C8H, C1′H, C2′H	8.24(s), 8.36(s), 6.10(d), 4.78(#)	#
32	AMP	C2H, C8H, C1′H	8.24(s), 8.58(s), 6.15(d)	#
33	formate	CH	8.46(s)	#
34	acetonitrile	CH_3	2.08(s)	#
35	sugars, amino acids (α-CH)		3.46−4.03	#
36	glycine	αCH_2, COOH	3.58(s)	175.8
37	succinate	CH_2	2.41(s)	36.4
38	U1		6.81(dd), 3.25(#)	117.9

[a]6-DA: 6-deoxy-ascobate; 2H5AV: 2-Hydroxy-5-aminovalerate; 2O5AV: 2-Oxo-5-aminovalerate; DMSP: Dimethylsulfoniopropionate; COS: Choline-O-sulfate; AMP: Adenosine monophosphate. [b]Multiplicity: s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublets; dt, doublet of triplets; m, multiplet; U, unidentified signal; #, signals or multiplicities were not determined.

ms). Sixty-four transients were collected into 32768 data points for each spectrum with a spectral width of 20 ppm. An exponential window function with a line-broadening factor of 0.5 Hz was applied to all free induction decays before FT.

Data Analysis. After manual phase and baseline corrections, the spectral region δ 0.8−9.4 was divided into bins with an equal width of 2 Hz using the AMIX software package (v 3.8, Bruker-Biospin). Regions δ 4.53−4.95 and 2.07−2.09 were discarded to remove residual water and acetonitrile signals, respectively. Each region was then normalized to the sum of total spectral integrals to compensate for the intersample differences in sample volume/concentration.

Multivariate data analysis was carried out on the normalized NMR data using SIMCA-P$^+$ software (v 11.0, Umetrics, Umeå, Sweden). Principal component analysis (PCA) was conducted on the mean-centered data to generate an overview and find outliners. Orthogonal projection to latent structure with discriminant analysis (OPLS-DA) was subsequently conducted using NMR data (scaled to unit variance) as the *X*-matrix and class information (i.e., strains in this study) as the *Y*-matrix to identify metabolites having statistically significant intergroup differences. All OPLS-DA models were calculated with a 10-fold cross-validation and further assessed with the CV-ANOVA method.[33] To facilitate easy interpretation of the results, the loadings that indicated metabolites having interstrain differences were back-transformed[34] and plotted with the correlation coefficients (r) color-coded for all variables (i.e., metabolites) using an in-house-developed Matlab script (v 7.1, MathWorks, Natick, MA, USA). The significance of a metabolite's contribution to the intergroup differentiation can be easily recognized with "hot" colored (e.g., red) ones being more significant than "cold" colored (e.g., blue) ones. In this study, a cutoff value of $|r| > 0.602$ (i.e., $r > 0.602$ and $r < 0.602$) was chosen for the correlation coefficients to determine the statistical significance ($p <$

0.05) for metabolite differentiations based on the discriminating significance of the Pearson's product-moment correlation coefficient.[34]

Quantification of Metabolites. The completely relaxed NMR spectra were used to calculate the concentration of metabolites in three strains of seaweed. The concentration of a given metabolite (C_m) and that of TSP (C_{TSP}) in the same spectrum obeys the relationship described in eq 1 by taking the proton numbers related to NMR signals into consideration.

$$C_m = \frac{N_{TSP}C_{TSP}}{N_m} \times \frac{A_0^m}{A_0^{TSP}} \quad (1)$$

A_0^m and A_0^{TSP} denote the integral areas for the signals of a given proton (m) and internal reference TSP in a given spectrum, respectively. N_m and N_{TSP} denote proton numbers for the corresponding metabolite signal and the methyl groups of TSP (i.e., nine protons), respectively.[23−26]

RESULTS AND DISCUSSION

Analysis of ^{1}H NMR Spectra of *P. haitanensis* Extracts. Figure 1 shows some typical ^{1}H NMR spectra of *P. haitanensis* extracts obtained from strains SF-1, SF-2, and WT. Resonances were assigned to individual metabolites with both the ^{1}H and ^{13}C data (Table 1) according to the literature data,[35−38] the Human Metabolome Database (http://www.hmdb.ca/), and *de novo* structure determination from 2D NMR spectra. They were further confirmed by extensive 2D NMR analyses, including ^{1}H−^{1}H COSY, ^{1}H−^{1}H TOCSY, ^{1}H−^{13}C HSQC, and ^{1}H−^{13}C HMBC spectroscopy. To the best of our knowledge, this is the first comprehensive analysis of the low-molecular-weight metabolites in *P. haitanensis*. Apart from some amino acids previously detected in *P. haitanensis*,[18] a large number of sugars, carboxylic acids, aldehydes, amines, and nucleotides were also observed here in *P. haitanensis* with the metabolite concentration tabulated in Table 2. For example, three galactosides including floridoside and D- and L-isofloridoside were detected for the first time in *P. haitanensis*. The most abundant metabolites in *P. haitanensis* were sugars including isofloridoside (38.89 mg g^{-1} dried seaweed) and floridoside (13.88 mg g^{-1} dried seaweed). Amino acids were lower in concentration than these sugars, accounting for about 11.53 mg g^{-1} dried seaweed with alanine, aspartate, and glutamate being the most prevalent. Some metabolites were also relatively abundant (1−4 mg g^{-1} dried seaweed) including aspartate, glutamate, alanine, 2-hydroxy-5-aminovalerate, isethionate, and citrate in the WT strain. Some other metabolites were detectable but much less abundant (below 1 mg g^{-1} dried seaweed) in the WT strain including choline-*O*-sulfate, laminitol (i.e., 2-methyl-*myo*-inositol), 2-oxo-5-aminovalerate, betaine, 6-deoxyascobate, succinate, malate, formate, scyllitol, uracil, and uridine.

Previous colorimetric assay indicated that alanine and glutamate were the most abundant amino acids in *P. haitanensis* with glutamate level higher than alanine level.[18] However, we found that aspartate was also among the most abundant amino acids (Ala, Asp, Glu) with alanine level (3.95 mg g^{-1} dried seaweed) clearly higher than glutamate level (2.87 mg g^{-1} dried seaweed). The amounts of some other amino acids (e.g., leucine, isoleucine, valine, tyrosine, and phenylalanine) also differed from what were reported with colorimetric assay.[18] Such differences probably resulted from different culture areas, collection seasons, different drying methods, and, to a lesser extent, different techniques employed. Similar effects of these factors on metabotypic features were well-documented for plant leaves and roots.[25,26] Furthermore, serine, glycine, cysteine, methionine, lysine, histidine, and arginine were not detectable here, although they were detected with the colorimetric assay.[18] This is probably due to the limited NMR sensitivity in global analysis compared to the targeted analysis with colorimetric assay. Nevertheless, it is worth noting that our methods have much wider coverage of metabolites and enable the measurements of all abundant primary and secondary metabolites in a single spectrum.[25,26]

Table 2. Metabolite Content of Three Different *P. haitanensis* Strains

	mean ± SD (mg g^{-1})[a]		
metabolite	SF-1	SF-2	WT
isoleucine	0.146 ± 0.026	0.061 ± 0.009	0.060 ± 0.017
leucine	0.096 ± 0.013	0.045 ± 0.007	0.049 ± 0.015
valine	0.128 ± 0.021	0.143 ± 0.018	0.148 ± 0.046
laminitol	0.752 ± 0.246	0.643 ± 0.105	0.634 ± 0.072
threonine	0.773 ± 0.364	0.801 ± 0.181	0.803 ± 0.120
alanine	6.245 ± 2.099	4.105 ± 0.937	3.953 ± 0.539
2-hydroxy-5-aminovalerate	1.317 ± 0.567	1.720 ± 0.262	1.340 ± 0.419
4-oxo-5-aminovalerate	0.387 ± 0.127	0.358 ± 0.072	0.444 ± 0.079
glutamate	3.228 ± 1.553	2.148 ± 0.447	2.869 ± 0.708
succinate	0.053 ± 0.020	0.054 ± 0.009	0.090 ± 0.016
malate	0.414 ± 0.187	0.302 ± 0.068	0.423 ± 0.080
citrate	1.348 ± 0.777	1.402 ± 0.373	2.134 ± 0.481
aspartate	2.476 ± 1.858	2.287 ± 0.892	3.703 ± 0.874
choline-*O*-sulfate	0.513 ± 0.157	0.505 ± 0.094	0.609 ± 0.111
isethionate	2.495 ± 0.592	3.427 ± 0.445	2.976 ± 0.381
betaine	0.261 ± 0.103	0.271 ± 0.044	0.224 ± 0.036
scyllitol	0.641 ± 0.249	1.292 ± 0.171	0.681 ± 0.226
isofloridoside[b]	49.795 ± 4.474	44.010 ± 5.402	38.889 ± 3.931
floridoside	7.873 ± 2.367	9.486 ± 1.608	13.878 ± 1.910
uracil	0.040 ± 0.004	0.014 ± 0.003	0.012 ± 0.003
tyrosine	0.189 ± 0.022	0.036 ± 0.006	0.052 ± 0.010
phenylalanine	0.085 ± 0.009	0.035 ± 0.004	0.039 ± 0.014
uridine	0.133 ± 0.016	0.050 ± 0.008	0.045 ± 0.010
formate	0.035 ± 0.010	0.036 ± 0.004	0.032 ± 0.004

[a]The average concentration and standard deviation (mean ± SD, mg per gram dried *P. haitanensis*), were obtained from six parallel samples. [b]The sum of L-isofloridoside and D-isofloridoside concentrations.

Metabolic phenotypes of different *P. haitanensis* strains are expected to be highlighted in the variety of metabolites and their concentrations. Our results clearly showed obvious level differences for a number of metabolites between three different strains instead of metabolite types. Strain SF-1 had much higher levels for alanine, glutamate, tyrosine, and phenylalanine than the WT strain (Figure 1 and Table 2), whereas SF-2 had higher levels for choline and isofloridoside than the wild type. The detailed interstrain metabolomic differences for these three different strains were obtainable with multivariate data analysis.

Metabolomic Differences for *P. haitanensis* Strains Having Different High-Temperature Tolerances. The scores plot from PCA (Figure 2) showed obvious clustering of the samples from three different strains indicating that these three strains had differences in their metabolite compositions. The OPLS-DA results (Figure 3) further showed significant metabolomic differences between wild-type and both high-temperature tolerant mutants (SF-1 and SF-2), with the quality of all OPLS-DA models ensured with their Q^2 values and p values obtained from CV-ANOVA[33] (Table 3). Metabolites

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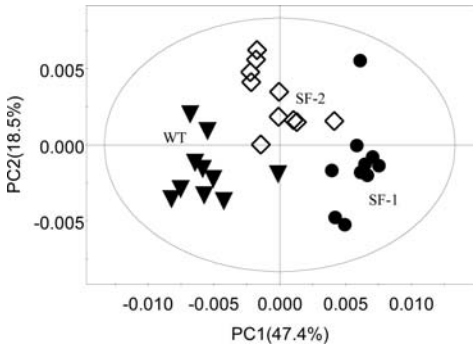

Figure 2. PCA scores plot for extracts from three *P. haitanensis* strains: SF-1 (●), SF-2 (◇), and WT (▼). PC1 and PC2 explained 47.7 and 18.5% of the total variance, respectively.

significantly contributed to the intergroup differentiations were extracted from the loadings plots (Figure 3D−F) and tabulated in Table 3 with their corresponding correlation coefficients.

Compared with the wild-type, high-temperature tolerant strain SF-1 contained significantly more betaine, betaine aldehyde, laminitol, isofloridoside, taurine, alanine, glutamate, tyrosine, phenylalanine, uridine, and adenosine but less malate, citrate, fructose, and floridoside. The other high-temperature tolerant strain, SF-2, contained higher levels of 6-deoxyascobate, laminitol, isethionate (i.e., 2-hydroxyethanesulfonate), betaine, taurine, and isofloridoside but lower levels of choline-*O*-sulfate, malate, citrate, fructose, and floridoside than the wild type. It is therefore obvious that both high-temperature tolerant *P. haitanensis* strains (SF-1 and SF-2) have some common metabolic features compared to the wild type. Both high-temperature tolerant mutants had higher levels of laminitol, betaine, isofloridoside, and taurine but lower levels of malate, citrate, fructose, and floridoside than the wild type. This implies that osmoprotectants are important for high-temperature tolerances because laminitol, betaine, isofloridoside, and taurine are all well-known compatible osmolytes. It is interesting to notice some metabolomic differences between these two pigmentation mutants as well. Strain SF-1 contained more alanine, glutamate, tyrosine, phenylalanine, betaine aldehyde, uridine, and uracil but less 6-deoxyascobate, 2-hydroxy-4-aminovalerate, isethionate, and floridoside than strain SF-2.

The higher betaine levels observed in the high-temperature tolerant strains SF-1 and SF-2 are consistent with the fact that betaine (a choline metabolite) functions as an osmolyte in various organisms. The osmotic protection role of the betaine was reported for many marine organisms[39,40] already. In fact, this choline metabolite was accumulated in salt-stressed lactate-producing bacteria to balance the increased extracellular solute concentration.[41] The accumulation of choline metabolites was also observed in tobacco[27] and maize when exposed to osmotic stresses such as salinity.[42] Under osmotic stresses, the biosynthesis of choline can be facilitated through methylation of phosphatidylethanolamines.[42−44] Choline can then be oxidized to produce betaine aldehyde, which is further transformed to produce glycinebetaine functioning as a much more potent osmolyte in numerous bacterial, plant, and animal species.[29,42−44] It is interesting to note that betaine aldehyde also has an osmoprotective role in other microorganisms such as *Escherichia coli*.[45] However, betaine aldehyde was observed in one of the temperature-resistant *P. haitanensis* mutantd here

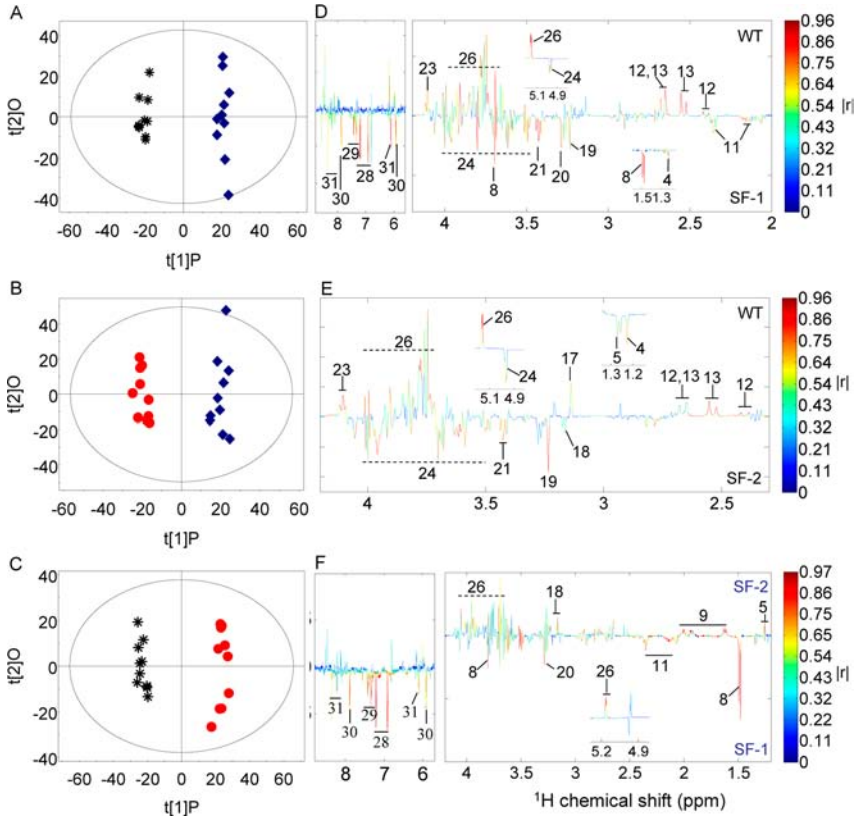

Figure 3. OPLS-DA scores (A, C) and coefficient-coded loading plots (D, E, F) for the models discriminating the SF-1 (black star), SF-2 (red dot), and WT (dark blue diamond) extracts (see Table 1 for metabolite keys).

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Table 3. OPLS-DA Coefficients of Metabolites Associated with *P. haitanensis* Strains

	coefficient (r)[a]		
	SF-1/WT	SF-2/WT	SF-1/SF-2
	$R^2X = 0.375$, $Q^2 = 0.948$	$R^2X = 0.403$, $Q^2 = 0.908$	$R^2X = 0.403$, $Q^2 = 0.934$
metabolite	$p = 1.84 \times 10^{-9}$	$p = 1.37 \times 10^{-7}$	$p = 1.16 \times 10^{-8}$
laminitol	−0.81	−0.68	–
6-deoxyascobate	–	−0.65	0.91
alanine	−0.80	–	−0.90
2-hydroxy-4-aminovalerate	–	–	0.89
glutamate	−0.83	–	−0.85
malate	0.81	0.84	–
citrate	0.90	0.88	–
choline-*O*-sulfate	–	0.61	–
isethionate	–	−0.73	0.96
betaine	−0.78	−0.85	–
betaine aldehyde	−0.81	–	−0.63
taurine	−0.89	−0.80	–
fructose	0.91	0.90	–
isofloridoside	−0.74	−0.61	–
floridoside	0.92	0.88	0.79
tyrosine	−0.93	–	−0.94
phenylalanine	−0.81	–	−0.82
uridine	−0.92	–	−0.90
adenosine	−0.62	–	−0.72

[a]Correlation coefficients with "+" and "−" indicate positive and negative correlation in the concentrations, respectively. "–" means that the correlation coefficient $|r|$ was less than the cutoff value.

and might act as a transient metabolite for the production of betaine rather than a major osmolyte.

High levels of taurine and laminitol in both strains SF-1 and SF-2 further suggested the importance of osmoprotection in high-temperature tolerances for the seaweed cells. As a major free intracellular amino acid, taurine is well recognized as an osmoregulator under salinity,[27] temperature, and hypotonic stresses.[46,47] A higher level of isethionate in SF-2 is also related to taurine metabolism because it is common knowledge that isethionate is normally produced from sulfoacetaldehyde (under sulfoacetaldehyde reductase) resulting from taurine catalyzed by taurine-pyruvate aminotransferase. Laminitol is a methylated product of *myo*-inositol, which is a well-known osmolyte especially under osmotic stresses.[24−27]

Furthermore, significant isofloridoside accumulation accompanied by marked floridoside depletion was observed in both strains SF-1 and SF-2 (Figure 3). Extensive previous data indicate that floridoside is considered to be the main organic solute involved in osmotic adaptation in Bangiales.[48,49] However, it was rarely demonstrated that isofloridoside was the predominant heteroside associated with the osmotic acclimation unless its osmotic regulation role exceeded floridoside in *Porphyra columbina*.[50] In our study, three galactosides were simultaneously synthesized despite the different galactoside levels between wild-type *P. haitanensis* and the strains with improved temperature tolerance, which most likely reflected the conservation of essential physiological functions required for the survival of the seaweed in harsh intertidal zones. However, the presence of isofloridoside appeared to be correlated with the adaptation to high-temperature stress and might be considered as a biological marker in the selection of high-temperature tolerant mutants of *P. haitanensis*.

Moreover, it is particularly noteworthy that increased levels of metabolites, such as alanine, glutamate, and betaine aldehyde, but decreased levels of metabolites, such as floridoside, were observed in strain SF-1 compared with strain SF-2. Previous papers have suggested that alanine accumulation is a common phenomenon in plants and animals in response to their exposure to a variety of stress conditions, such as anoxia and extreme temperatures.[51,52] It has been proposed that alanine is a universal primary stress signal expressed by cells.[53] Elevated levels of glutamate were found during the response of organisms to environmental stresses, such as heavy metal and organic amines.[54] Therefore, alanine, glutamate, betaine aldehyde, and floridoside are possibly linked to osmoregulation, indicating that different osmoregulation strategies are probably adopted by strains SF-1 and SF-2.

Another prominent finding of our study is the marked depletion of malate, citrate, and fructose in the seaweed extracts from the two strains with improved temperature tolerance. Malate and citrate are the intermediates of the tricarboxylic acid cycle (TCA), and the depletion of these molecules suggests that the TCA cycle is inhibited in SF-1 and SF-2 compared with the WT. However, it remains unknown whether such TCA alterations have any direct contributions towards the high-temperature tolerance for these strains.

The above results indicated that a complex mixture of osmolytes, such as choline and taurine metabolites,[55,56] together with polyols (isofloridoside and laminitol) was functioning in *P. haitanensis*. Some organic osmolytes are functionally interchangeable.[57] This is not surprising because many organisms have developed a complex strategy for osmotic adjustment. The selective pressure for the evolution of osmolyte patterns and types to withstand high-temperature stress in organisms such as *P. haitanensis* remains to be fully understood. Nevertheless, understanding the metabolic differ-

ences between wild-type and high-temperature tolerant strains will help to elucidate the basic biochemical adaptations required for survival at high temperatures.

We found in this investigation that the metabolite composition of *P. haitanensis* is dominated by sugars, amino acids, carboxylic acids, aldehydes, amines, and nucleotides. The concentrations of some of these metabolites were significantly different between the extracts from the high-temperature tolerant strains and the wild-type strain. These metabolites, such as betaine, taurine, and isofloridoside, were good metabolic indicators of high-temperature tolerant *P. haitanensis* strains. It is therefore essential to consider metabolite constituents to aid in the selection of high-temperature tolerant mutants of *P. haitanensis*.

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Notes

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ABBREVIATIONS USED

NMR, nuclear magnetic resonance; TCA, tricarboxylic acid cycle; TSP, sodium 3-trimethylsilyl (2,2,3,3,-d_4) propionate; D_2O, deuterated water; PCA, principal component analysis; OPLS-DA, orthogonal projection to latent structure with discriminant analysis; COSY, correlated spectroscopy; TOCSY, total correlation spectroscopy; HSQC, heteronuclear single-quantum coherence; HMBC, heteronuclear multiple-bonding coherence; NOESY, nuclear overhauser effect spectroscopy

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