

RESEARCH PAPER

Metabolite changes during the life history of *Porphyra haitanensis*

X. Wang¹, P. Zhao², Q. Luo³, X. Yan³, J. Xu³, J. Chen³ & H. Chen³¹ Zhejiang Provincial Key Laboratory of Plant Evolutionary Ecology and Conservation, Taizhou University, Taizhou, China² Jiangbei Centers for Disease Control and Prevention, Ningbo, China³ Marine Biotechnology Key Laboratory, Ningbo University, Ningbo, China**Keywords**

Growth phase; *Porphyra haitanensis*; ultra-performance liquid chromatography-quadrupole-time of flight-mass spectrometry.

Correspondence

X. Yan, Marine Biotechnology Key Laboratory, Ningbo University, 315211 Ningbo, China.

E-mail: yanxiaojun@nbu.edu.cn

Editor

H.-P. Mock

Received: 17 April 2014; Accepted: 26 September 2014

doi:10.1111/plb.12273

ABSTRACT

Plant metabolomics is essentially the comprehensive analysis of complex metabolites of plant extracts. Metabolic fingerprinting is an important part of plant metabolomics research. In this study, metabolic fingerprinting of different stages of the life history of the red alga *Porphyra haitanensis* was performed. The stages included conchocelis filaments, sporangial branchlets, conchosporangia, discharged conchospores and conchosporangial branchlets after conchospore discharge. Metabolite extracts were analysed with ultra-performance liquid chromatography coupled with electrospray ionisation quadrupole-time of flight mass spectrometry. Analyses profiles were subjected to principal components analysis and orthogonal projection to latent structures discriminant analysis using the SIMCA-P software for biomarker selection and identification. Based on the MS/MS spectra and data from the literature, potential biomarkers, mainly of phosphatidylcholine and lysophosphatidylcholine, were identified. Identification of these biomarkers suggested that plasma membrane phospholipids underwent major changes during the life history of *P. haitanensis*. The levels of phosphatidylcholine and lysophosphatidylcholine increased in sporangial branchlets and decreased in discharged conchospores. Moreover, levels of sphingaine (d18:0) decreased in sporangial branchlets and increased in discharged conchospores, which indicates that membrane lipids were increasingly synthesised as energy storage in sporangial branchlets, while energy was consumed in sporangial branchlets to discharged conchospores. A metabolomic study of different growth phases of *P. haitanensis* will enhance our understanding of its physiology and ecology.

INTRODUCTION

The species-rich and widely distributed red algal genus *Porphyra* (also known as *Pyropia*) belongs to Bangiophyceae (Brodie & Zuccarello 2007), which represents an ancient lineage, with fossil records that provide evidence for sexual reproduction at least 1.2 billion years ago (BYA; Butterfield 2000). *P. haitanensis* is widely cultivated along the coast of South China and is one of the most commercially important *Porphyra* species (Xie *et al.* 2009).

The life cycle of *Porphyra* consists of two completely different morphological phases: a leafy gametophyte (thallus) and a filamentous sporophyte (the shell-boring conchocelis phase; Xie *et al.* 2010). The filamentous sporophyte phase consists of conchocelis, conchosporangial branch and conchospores (Gantt *et al.* 2010). Free conchocelis are generally used for growing seedlings in cultivation of *P. yezoensis* in northern China (Zhu *et al.* 1997). Aeration promotes maturation of suspension cultures of body filaments and release of conchospores from *P. yezoensis*, which then germinate and grow into healthy seedlings (Wang & Jiang 1983). Changes at three different stages of conchocelis in *P. yezoensis* (conchocelis filament, conchosporangial branch and mature conchosporangial cell) have been studied with light and transmission electron microscopy

(Zhou *et al.* 2006). The influence of temperature and light on free-living conchocelis of *P. haitanensis* has previously been investigated (Pan 2006).

In recent years, much progress has been made in metabolomics to provide a global picture of the molecular organisation of multicellular organisms (Hall 2006). Analytical methods have been used for metabolite profiling (Dunn & Ellis 2005) include nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC-MS), flow injection assay-mass spectrometry (FIA-MS) and liquid chromatography-mass spectrometry (LC-MS). LC-MS is an important and widely used method for many biological analyses, including metabolites, mainly due to its superior specificity, sensitivity and efficiency.

Metabolite changes of *P. haitanensis* throughout its life history are unclear, and investigation of such changes would enhance our understanding of its development. In this study, we investigated metabolic changes in *P. haitanensis* during its growth stages using coupling ultra-performance liquid chromatography-quadrupole-time of flight-mass spectrometry (UPLC-Q-TOF-MS). Multivariate data analysis was used to reveal biochemical changes, with principal components analysis (PCA), an unsupervised clustering method that highlights clustering or pattern formations, thus displaying similarities and differences among samples (Van Vliet *et al.* 2008; Farag

et al. 2012), and orthogonal projection to latent structures discriminant analysis (O-PLS-DA). The O-PLS-DA approach, as with all other regression methods, models complexity (Wiklund *et al.* 2008).

MATERIAL AND METHODS

Chemicals

Methanol, acetonitrile, formic acid and ammonium formate (LC-MS grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Distilled water was filtered through a Milli-Q system (Millipore, Bedford, MA, USA). Leucine-enkephalin was purchased from Sigma-Aldrich.

Sample collection

Porphyra haitanensis were collected from Xiangshan, Zhejiang Province, China, at different periods of conchocelis: conchocelis filaments, sporangial branchlets, conchosporangia, discharged conchospores and conchosporangial branchlets after conchospore discharge (Fig. 1).

The freeze-dried samples (0.1 g) were ground with liquid nitrogen and then soaked in 1 ml methanol:formic acid: water (15:1:4, v/v). The homogenate was kept at $-20\text{ }^{\circ}\text{C}$ for at least 16 h. The extract was centrifuged at 1000 g for 10 min. The

supernatant was collected and the extraction repeated twice. The combined solution was evaporated to dryness and dissolved in 0.5 ml MeOH:H₂O:formic acid (90:10:0.05, v/v/v) for UPLC-QTOF-MS analysis. All experiments were performed in nine replicates.

Analysis with UPLC-QTOF-MS/MS

Analyses were carried out using a Waters ACQUITY UPLC BEH C18 analytical column ($2.1 \times 100\text{ mm}$, $1.7\text{ }\mu\text{m}$; Waters, Milford, MA, USA) and an ATLANTIS HILIC analytical column ($4.6 \times 150\text{ mm}$, $5\text{ }\mu\text{m}$; Waters). The BEH C18 column was eluted with a binary solvent system of water (containing 10 mM ammonium formate, solvent A) and acetonitrile (containing 0.1% formic acid, solvent B) at a constant flow rate of $0.2\text{ ml}\cdot\text{min}^{-1}$. The gradient conditions were 2–10% B (0–5 min), 10–100% B (5–10 min), isocratic at 100% B (10–24 min), and 100–2% B (24–26 min). The injection volume was 5 μl . An aliquot of each sample was injected into the column, and 50% of the effluent was split into the mass spectrometer.

The ATLANTIS HILIC column was eluted with binary solvent system of water (containing 10 mM ammonium formate, solvent A) and acetonitrile (containing 0.1% formic acid, solvent B) at a constant flow rate of $0.8\text{ ml}\cdot\text{min}^{-1}$. The gradient conditions were 95–90% B (0–10 min), 90–80% B

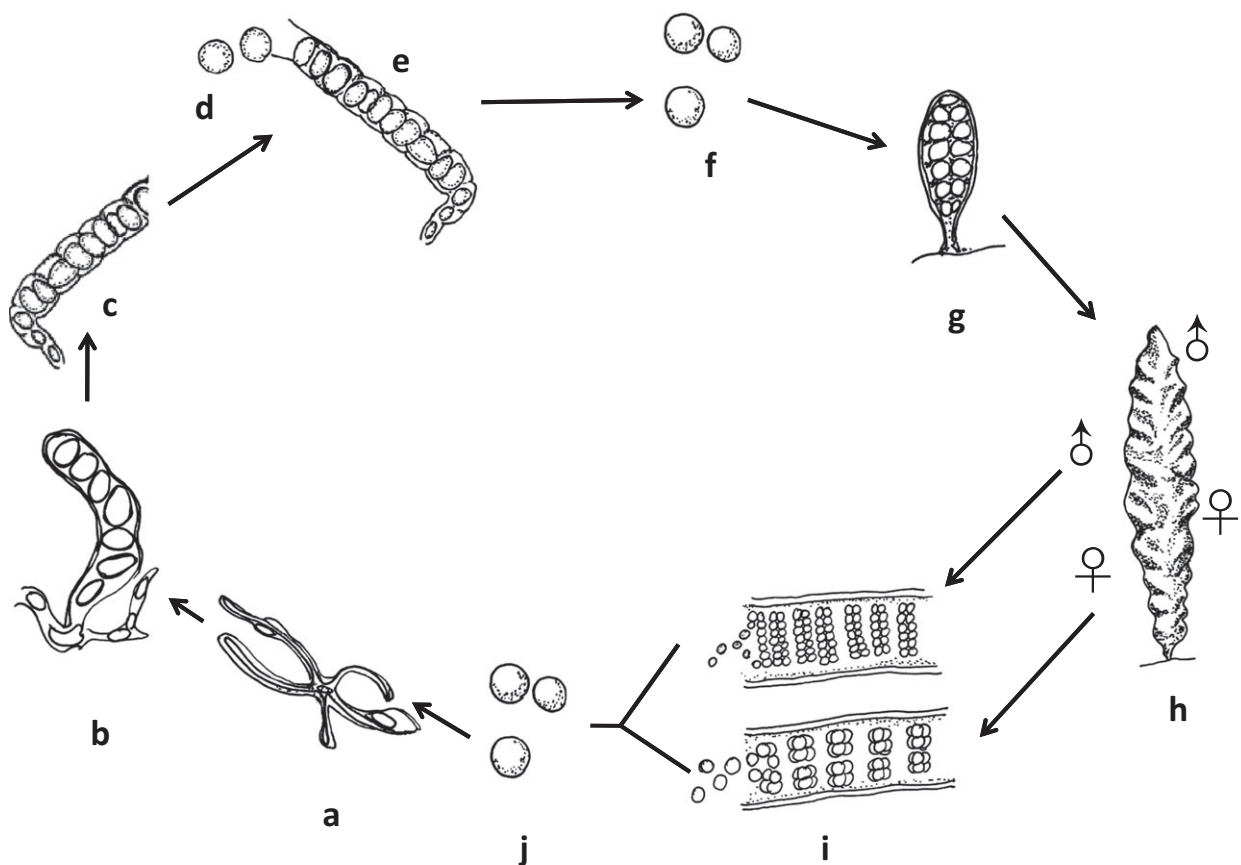


Fig. 1. Life history of *Porphyra* (Graham & Wilcox 2000). a: Conchocelis filaments; b: sporangial branchlets; c: conchosporangia; d: discharged conchospores; e: conchosporangial branchlets after conchospore discharge; f: conchospores; g: young blade; h: blade; i: cross-section of blade margin; j: zygospore.

(10–15 min), 80–70% B (15–20 min), isocratic at 70% B (20–25 min) and 70–95% B (25–27 min). The injection volume was 5 μL . An aliquot of each sample was injected into the column and 25% of the effluent was split into the mass spectrometer.

Mass spectrometry was performed on a Waters Q-TOF Premier mass spectrometer, using electron spraying ionisation (ESI) in positive mode; the TOF detection was set in V mode. The gas flow of desolvation was set at $400 \text{ L}\cdot\text{h}^{-1}$ at 300°C with $50 \text{ L}\cdot\text{h}^{-1}$ cone gas and 120°C source temperature. The sampling cone voltage was set at a ramp of 35–80 V. The capillary voltage was set at 3.0 kV. The collision energy for tandem mass spectrometry (MS) was scanned from 5 to 60 eV. The MS acquisition rate was 0.3 s with a 0.02 s inter-scan delay. Argon was employed as collision gas. Data were collected in centroid mode from 50 to 1200 m/z in MS scanning. Prior to the experiment, the instrument was calibrated with sodium formate, and the lock mass spray for precise mass determination used leucine-enkephalin, at a concentration of $200 \text{ ng}\cdot\text{mL}^{-1}$ and a flow rate of $10 \mu\text{L}\cdot\text{min}^{-1}$, generating an $[\text{M}+\text{H}]^+$ ion at m/z 556.2771 in ESI⁺ mode. The lock spray frequency was set at 10 s.

Data analysis

The UPLC-QTOF-MS data pre-processing was performed with MARKERLYNX 4.1 software (Waters). The Pareto-scaled MarkerLynx matrices with peak numbers (based on the retention time and m/z), sample names and normalised peak intensity were exported and analysed with PCA, projection to latent structures with discriminant analysis (PLS-DA) and orthogonal projection to latent structures with discriminant analysis (O-PLS-DA) using the SIMCA-P⁺ software package (version 12.0; Umetrics AB, Umea, Sweden). After analysis, putative biomarkers were collected and chemically identified using MASS

FRAGMENT software and the public databases HMDB (<http://www.hmdb.ca>), LIPID MAPS (<http://www.lipidmaps.org>) and METLIN (<http://metlin.scripps.edu>).

RESULTS

Analyses of *P. haitanensis* metabolites

Since not all metabolites can be detected effectively on one column, both a reverse phase C18 column and HILIC column were used. The representative total ion current chromatograms are shown in Fig. 2. Based on peak areas, about 11,886 peaks and 8915 peaks were detected using Micromass Markerlynx (Waters, Milford, MA, USA) with reverse phase C18 and HILIC columns, respectively. The peaks were normalised, and the Pareto-scaled data were analysed by PCA, PLS-DA or O-PLS-DA.

In order to understand the metabolite changes of *P. haitanensis* at different stages of the life history, PCA analyses of the metabolite data was carried out. In the PCA score plots, the samples showed a trend of time-dependent cluster formations, both in the reverse phase C18 column (Fig. 3a) and HILIC column (Fig. 3b) data. The samples at different life history phases were clearly separated in the PCA plot, indicating that profiles of metabolites of *P. haitanensis* were obviously different at different life history phases. There was a trend of time-dependent cluster formation in the first two components both in reverse phase C18 (Fig. 3a) and HILIC (Fig. 3b) column data, which explained 61.0% and 69.4% of the total variance, respectively.

Since metabolite profiles of *P. haitanensis* showed the time-dependent trends (Fig. 3), metabolite changes during the whole study period could be seen in changes between conchocelis filaments (group 1) and sporangial branchlets (group 2) and between sporangial branchlets (group 2) and discharged

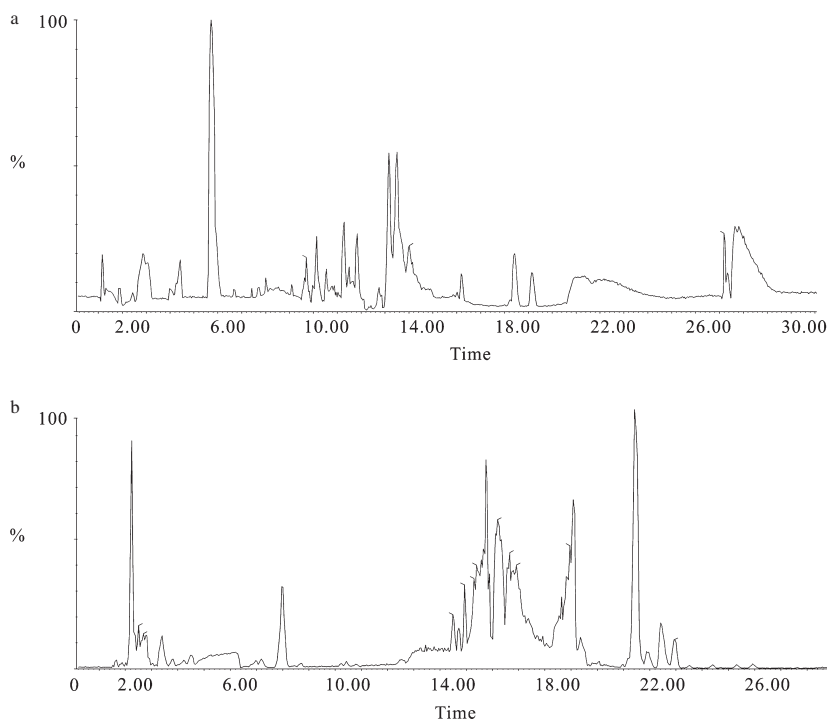


Fig. 2. Typical total ion chromatograms obtained from *P. haitanensis* using reverse phase C18 column (a) and HILIC column (b).

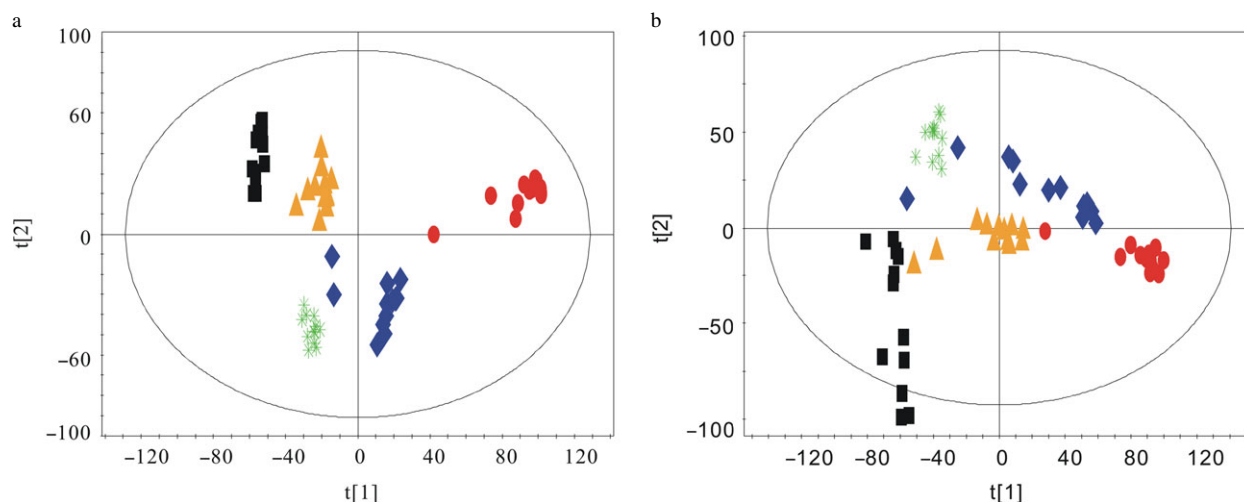


Fig. 3. PCA scores plot using reverse phase C18 column (a) and HILIC column (b) in ESI⁺ mode for the first two components of *P. haitanensis*. Samples were successively harvested at: conchocelis filaments (filled square), sporangial branchlets (filled circle), conchosporangia (filled diamond), discharged conchospores (filled star), conchosporangia branchlets after conchospore discharge (filled triangle).

conchospores (group 4). Pattern recognition based on a supervised projection technique (O-PLS-DA) was accomplished with Pareto scaling. Sevenfold cross-validation (CV) was used to estimate the relevant number of components in the O-PLS-DA model and resulted in one predictive and one orthogonal component in positive ion scan mode. The O-PLS-DA score plot showed significant metabolic differences between the compared groups (Figures S1a–S4a). The parameters of R^2X , R^2Y and Q^2 revealed high discriminative and predictive ability of the model, indicating the positive qualities of these models.

The S-plot visualises covariance and correlation among metabolites, thus it is used to identify important metabolites (Wiklund *et al.* 2008). The loading S-plots showed ions with highest confidence and largest contribution to separation between the compared groups (Figures S1b–S4b). Potential biomarkers were extracted from the sample distribution according to the parameters of variable importance in the

projection [variable importance in the projection (VIP)]. Correlation and covariance were obtained from the O-PLS-DA analysis using data sets in ESI⁺ mode. The relative change of potential biomarkers in the sample was measured according to the correlation and covariance. In comparison of group 2 *versus* group 1, increased metabolites of group 2 are in the upper-right quadrant (with >0 for both correlation and covariance), while decreased metabolites are in the lower-left quadrant (with <0 for both correlation and covariance). In comparison of group 4 *versus* group 2, increased metabolites of group 4 are in the upper-right quadrant (with >0 for both correlation and covariance), while decreased metabolites are in the lower-left quadrant (with <0 for both correlation and covariance). The potential biomarkers from the S-plots (Tables 1 and 3 for reverse phase C18 column, Tables 2 and 4 for HILIC column) were then selected according to parameters of the VIP.

Table 1. Using reverse phase C18 column, potential biomarkers found in conchocelis filaments and sporangial branchlets of *P. haitanensis* samples obtained with O-PLS-DA analysis in S-plot format.

RT (min)	m/z	VIP	correlation	covariance	identification	element composition
10.76	544.3394	31.6818	0.9746	0.2903	Lyso-PC (20:4), [M+H] ⁺	C ₂₈ H ₅₁ NO ₇ P ⁺
11.24	496.3389	26.5886	0.9262	0.2436	Lyso-PC (16:0), [M+H] ⁺	C ₂₄ H ₅₁ NO ₇ P ⁺
10.32	542.3242	22.3374	0.9755	0.2047	Lyso-PC (20:5), [M+H] ⁺	C ₂₈ H ₄₉ NO ₇ P ⁺
5.77	105.0647	17.5519	-0.9575	-0.1607	2,3-Diaminopropionic acid	C ₃ H ₉ N ₂ O ₂ ⁺
11.48	522.3552	16.0484	0.8837	0.1471	Lyso-PC (18:1), [M+H] ⁺	C ₂₆ H ₅₃ NO ₇ P ⁺
10.01	361.2716	9.4257	0.8967	0.0864	unknown	
4.35	120.0771	8.4058	-0.7650	-0.0758	unknown	
1.21	214.9166	8.3099	0.8999	0.0764	unknown	
11.03	546.3545	7.0844	0.8992	0.0649	Lyso-PC (20:3), [M+H] ⁺	C ₂₈ H ₅₃ NO ₇ P ⁺
2.82	86.0887	6.9971	-0.8706	-0.0638	unknown	
10.66	502.2930	6.9435	0.9021	0.0635	Lyso-P E (20:4), [M+H] ⁺	C ₂₅ H ₄₅ NO ₇ P ⁺
12.75	318.2995	6.3137	-0.4843	-0.0568	Sphingaine (d18:0), [M+H] ⁺	C ₁₈ H ₄₀ NO ₃ ⁺
8.39	303.1336	6.2041	0.80203	0.05668	unknown	
11.84	379.2832	5.9916	-0.8065	-0.0544	unknown	
11.82	281.0506	5.9681	-0.7291	-0.0554	unknown	

Lyso-PC = lyso-phosphatidylcholine; Lyso-PE = lyso-phosphatidylethanolamine.

Table 2. Using HILIC column, potential biomarkers found in conchocelis filaments and sporangial branchlets of *P. haitanensis* samples obtained with O-PLS-DA analysis in S-Plot format.

RT (min)	m/z	VIP	correlation	covariance	identification	element composition
17.57	830.5726	22.5795	0.9512	0.2472	PC (20:4/20:4), [M+H] ⁺	C ₄₈ H ₈₁ NO ₈ P ⁺
17.59	828.5571	19.9003	0.9597	0.2165	PC (20:5/20:4), [M+H] ⁺	C ₄₈ H ₇₉ NO ₈ P ⁺
17.69	782.5719	16.6196	0.9256	0.1829	PC (16:0/20:4), [M+H] ⁺	C ₄₄ H ₈₁ NO ₈ P ⁺
15.99	105.0652	14.6202	-0.88126	-0.1541	unknown	
17.63	808.5881	13.5552	0.9224	0.1494	PC (20:5/18:0), [M+H] ⁺	C ₄₆ H ₈₃ NO ₈ P ⁺
2.7	842.5823	13.2481	0.9847	0.1402	unknown	
17.73	780.5565	12.6364	0.93678	0.1385	PC (16:0/20:5), [M+H] ⁺	C ₄₄ H ₇₉ NO ₈ P ⁺
17.64	806.5727	11.8318	0.9414	0.1298	PC (20:5/18:1), [M+H] ⁺	C ₄₆ H ₈₁ NO ₈ P ⁺
2.68	844.5977	11.7243	0.9781	0.1254	unknown	
20.56	544.3397	11.5	0.8223	0.1144	Lyso-PC (20:4), [M+H] ⁺	C ₂₈ H ₅₁ NO ₇ P ⁺
17.6	826.5413	10.6709	0.9612	0.1161	PC (20:5/20:5), [M+H] ⁺	C ₄₈ H ₇₇ NO ₈ P ⁺
15.2	274.2726	9.9905	-0.7951	-0.1064	possible common impurity	C ₁₆ H ₃₆ NO ₂ ⁺
21.6	104.1032	8.9752	-0.5508	-0.0941	unknown	
21.92	104.103	8.7100	-0.5483	-0.0916	Choline [M+H] ⁺	C ₅ H ₁₄ NO ⁺
20.8	496.3392	8.5691	0.8659	0.0872	Lyso-PC (16:0) [M+H] ⁺	C ₂₄ H ₅₁ NO ₇ P ⁺

PC = phosphatidylcholine.

Metabolite identification

In order to identify the metabolites, mass accuracy was determined on the Q-TOF-MS system, with 5 ppm tolerance allowed in possible element composition calculation of empirical chemical formulas. Furthermore, a database search was performed with the public databases HMDB, LIPID MAPS and METLIN to elucidate the putative ion structures. For example, glycerol phospholipid compounds were identified from the database search of fragmentation patterns in the mass spectra (Pulfer & Murphy 2003; Hsu & Turk 2003; Xu *et al.* 2010; Yan *et al.* 2010). Taking phosphatidylcholine at m/z 828.5571 (RT 17.59 min) as an example, the verification was carried. First, the precise molecular weight (MW) was determined using the full scan mass spectrum (Fig. 2b). The quasi-molecular [M+Na]⁺ ion (m/z 850.5782) and [M+H]⁺ ion (m/z 828.5574) were found in the low collision spectrum in MS^E data collection mode (Figure S5). Therefore, the MW

of phosphatidylcholine was determined as 827.55 and the accurate m/z of [M+H]⁺ was measured through the Q-TOF premier MS system (828.5574). The high collision spectrum in MS^E data collection mode of phosphatidylcholine and its cleavage pattern are shown in Figure S5b. It contained the characteristic fragmentation ions of phosphocholine head group [C₅H₁₅O₄NP]⁺ at m/z 184.0751 in positive scan mode, indicating that this metabolite was phosphatidylcholine (Hsu & Turk 2003). Moreover, the ions m/z 524, m/z 526, m/z 542 and m/z 544 suggested that the two fatty acyl groups at the sn-1 and sn-2 positions of the glycerol were 20:4 and 20:5, respectively. Furthermore, the abundance of the ion at m/z 542 was higher than that at m/z 544, therefore it was concluded that the fatty acyl (20:5) group was located at sn-1 and the fatty acyl (20:4) group was linked to sn-2, because the fatty acyl at sn-2 was more labile than that at sn-1 (Hsu & Turk 2009). As a result, the putative structure was identified as phosphatidylcholine (20:5/20:4).

Table 3. Using reverse phase C18 column, potential biomarkers found in sporangial branchlets and discharged conchospores of *P. haitanensis* samples obtained with O-PLS-DA analysis in S-plot format.

RT (min)	m/z	VIP	correlation	covariance	identification	element composition
10.76	544.3394	32.9174	-0.9730	-0.3017	Lyso-PC (20:4), [M+H] ⁺	C ₂₈ H ₅₁ NO ₇ P ⁺
11.24	496.3389	26.7355	-0.9126	-0.2447	Lyso-PC (16:0), [M+H] ⁺	C ₂₄ H ₅₁ NO ₇ P ⁺
10.32	542.3242	23.4478	-0.9752	-0.2150	Lyso-PC (20:5), [M+H] ⁺	C ₂₈ H ₄₉ NO ₇ P ⁺
11.48	522.3552	16.3714	-0.8699	-0.1498	Lyso-PC (18:1), [M+H] ⁺	C ₂₆ H ₅₃ NO ₇ P ⁺
5.77	105.0647	10.8933	0.9888	0.0996	2,3-Diaminopropionic acid, [M+H] ⁺	C ₃ H ₉ N ₂ O ₂ ⁺
1.37	277.0886	10.2422	0.7563	0.0938	Galactosylglycerol [M+Na] ⁺	C ₉ H ₁₈ O ₈ Na ⁺
10.01	361.2716	9.8603	-0.8939	-0.0905	unknown	
12.75	318.2995	8.8236	0.8845	0.0810	Sphingaine (d18:0), [M+H] ⁺	C ₁₈ H ₄₀ NO ₃ ⁺
12.62	274.2733	8.7064	0.6629	0.0791	Sphingaine (d16:0), [M+H] ⁺	C ₁₆ H ₃₆ NO ₂ ⁺
4.35	120.0771	7.9275	0.9415	0.0723	unknown	
11.03	546.3545	7.1991	-0.8847	-0.0658	Lyso-PC (20:3), [M+H] ⁺	C ₂₈ H ₅₃ NO ₇ P ⁺
10.66	502.293	7.0468	-0.8886	-0.0644	Lyso-PE (20:4), [M+H] ⁺	C ₂₅ H ₄₅ NO ₇ P ⁺
2.82	86.0887	6.3081	0.9310	0.0580	unknown	
8.39	303.1336	-6.2549	-0.9284	-0.0564	unknown	

PC = phosphatidylcholine; PE = phosphatidylethanolamine.

Table 4. Using HILIC column, potential biomarkers found in sporangial branchlets and discharged conchospores of *P. haitanensis* samples obtained with O-PLS-DA analysis in S-plot format.

RT (min)	m/z	VIP	correlation	covariance	identification	element composition
17.57	830.5726	22.0465	-0.98728	-0.2337	PC (20:4/20:4), [M+H] ⁺	C ₄₈ H ₈₁ NO ₈ P ⁺
17.59	828.5571	19.585	-0.8943	-0.2077	PC (20:5/20:4), [M+H] ⁺	C ₄₈ H ₇₉ NO ₈ P ⁺
21.92	104.103	18.0049	0.9959	0.1905	Choline [M+H] ⁺	C ₅ H ₁₄ NO ⁺
17.69	782.5719	16.0086	-0.8253	-0.1687	PC (16:0/20:4), [M+H] ⁺	C ₄₄ H ₈₁ NO ₈ P ⁺
17.63	808.5881	13.0414	-0.8239	-0.1381	PC (20:5/18:0), [M+H] ⁺	C ₄₆ H ₈₃ NO ₈ P ⁺
2.28	320.2568	12.7006	0.9110	0.1343	unknown	
17.73	780.5565	12.2584	-0.8499	-0.1298	PC (16:0/20:5), [M+H] ⁺	C ₄₄ H ₇₉ NO ₈ P ⁺
17.64	806.5727	11.594	-0.8608	-0.1230	PC (20:5/18:1), [M+H] ⁺	C ₄₆ H ₈₁ NO ₈ P ⁺
2.7	842.5823	11.3878	-0.9490	-0.1214	unknown	
20.56	544.3397	11.2968	-0.8253	-0.1205	Lyso-PC (20:4), [M+H] ⁺	C ₂₈ H ₅₁ NO ₇ P ⁺
17.6	826.5413	10.5673	-0.9002	-0.1123	PC (20:5/20:5), [M+H] ⁺	C ₄₈ H ₇₇ NO ₈ P ⁺
2.68	844.5977	9.9237	-0.9063	-0.1054	unknown	
23.52	118.0817	9.3570	0.9637	0.0988	unknown	
15.99	105.0652	9.0649	0.9966	0.0959	unknown	
15.02	284.3295	8.3801	0.9881	0.0887	unknown	

PC = phosphatidylcholine.

Using the procedures described above, the metabolites in *P. haitanensis* throughout its life history were identified and included phosphatidylcholine, lyso-phosphatidylcholine, lyso-phosphatidylethanolamine, sphingaine, 2,3-diaminopropionic acid and choline (Tables 1–4).

Metabolite changes in *P. haitanensis*

The metabolite changes of *P. haitanensis* during different life history periods were analysed using PCA, which was able to identify differences among different groups during its life history. The data were analysed with O-PLS-DA and compared in pairs, the first group (conchocelis filaments) *versus* the second group (sporangial branchlets) and the second group (sporangial branchlets) *versus* the fourth group (discharged conchospores). The identified biomarkers of *P. haitanensis* were mainly phospholipids, including phosphatidylcholine and lyso-phosphatidylcholine. The levels of phosphatidylcholine, lyso-phosphatidylcholine and lyso-phosphatidylethanolamine increased in sporangial branchlets, compared with conchocelis filaments, while levels of sphingaine (d18:0) decreased (Tables 1–4), indicating that synthesis of membrane lipids had increased. However, levels of phosphatidylcholine, lyso-phosphatidylcholine and lyso-phosphatidylethanolamine decreased in discharged conchospores compared with the sporangial branchlets, whereas levels of sphingaine (d18:0) and sphingaine (d16:0) increased (Tables 1–4). These results indicate that membrane lipids are increasingly synthesised in sporangial branchlets.

DISCUSSION

Metabolites change in different growth stages of plants. Profiling metabolites in *P. haitanensis* revealed strong and consistent changes during its life history. In this study, *P. haitanensis* showed a circular trend in time-dependent cluster formation during its life history, both in reverse phase C18 column and HILIC column data. Conchocelis filaments and

conchosporangial branchlets after conchospore discharge were classified into one cluster, indicating that they had similar metabolite profiles as the conchocelis filaments. Conchosporangia and discharged conchospores were classified into one cluster, indicating that conchosporangia had a similar metabolite profile as the discharged conchospores. The potential biomarkers of *P. haitanensis* at different life history stages were mainly phospholipids: phosphatidylcholine (20:5/20:4), lyso-phosphatidylcholine (20:4), *etc.* Phospholipids are more than just structural components of membranes; they can be co-factors for membrane enzymes, signal precursors or signalling molecules (Laxalt & Munnik 2002). Phosphatidylcholine is a major constituent of cell membranes, and an important structural component that contributes to the integrity and function of membranes. The levels of phosphatidylcholine (16:0/20:4, 16:0/20:5, 20:5/18:0, 20:5/20:4, 20:5/20:5, 20:4/20:4), lyso-phosphatidylcholine (16:0, 18:1, 20:3, 20:4, 20:5) and lyso-phosphatidylethanolamine (20:4) increased in sporangial branchlets, indicating that *P. haitanensis* synthesised membrane lipids and stored energy at this stage. Phosphatidylcholine (16:0/20:4, 16:0/20:5, 20:5/18:0, 20:5/18:1, 20:5/20:4, 20:5/20:5, 20:4/20:4), lyso-phosphatidylcholine (16:0, 18:1, 20:3, 20:4, 20:5) and lyso-phosphatidylethanolamine (20:4) decreased in discharged conchospores, indicating that *P. haitanensis* consumed energy during this stage.

Sphingolipids are essential for establishment and maintenance of cell polarity *via* control of the actin cytoskeleton, and accumulation of ceramide is likely responsible for arresting the cell cycle in G1 stage (Cheng *et al.* 2001). We found the levels of sphingaine (d18:0) decreased in the sporangial branchlets stage, compared with the conchocelis filaments, indicating sphingaine (d18:0) might be involved in meiosis.

Eukaryotic membranes contain diverse lipid molecular species, and the lipid composition changes in response to both internal and external cues. Understanding how lipids change and what causes the change is important for the study of membrane and cell functions (Welti *et al.* 2002). Our metabolite profiling study showed that metabolite changes during the life

history of *P. haitanensis* were mainly related to phospholipids in the plasma membrane. This analysis of putative biomarkers provides insights into the lipid metabolism at different growth stages of *P. haitanensis*.

ACKNOWLEDGEMENTS

This work was supported by the Scientific Research Fund of Zhejiang Provincial Education Department Y201121083, Zhejiang Marine Biotechnology Innovation Team (2010-R50029) and Ningbo Marine Algae Biotechnology Team (201-1B81007).

REFERENCES

- Brodie J., Zuccarello G.C. (2007) Systematics of the species-rich algae: red algal classification, phylogeny and speciation. In: Hodkinson R.T., Parnell J., Waldren S. (Eds), *The taxonomy and systematics of large and species-rich taxa: building and using the tree of life*. Systematics Association Series CRC Press, Boca Raton, FL, USA, pp 317–330.
- Butterfield N.J. (2000) *Bangiomorpha pubescens* n. gen., n. sp.: implications for the evolution of sex, multicellularity and the Mesoproterozoic-Neoproterozoic radiation of eukaryotes. *Paleobiology*, **26**, 386–404.
- Cheng J.J., Park T.-S., Fischl A.S., Ye X.S. (2001) Cell cycle progression and cell polarity require sphingolipid biosynthesis in *Aspergillus nidulans*. *Molecular and Cellular Biology*, **21**, 6198–6209.
- Dunn W.B., Ellis D.I. (2005) Metabolomics: current analytical platforms and methodologies. *Trends in Analytical Chemistry*, **24**, 285–294.
- Farag M.A., Porzel A., Schmidt J., Wessjohann L.A. (2012) Metabolite profiling and fingerprinting commercial cultivars of *Humulus lupulus* L. (hop): comparison of MS and NMR methods in metabolomics. *Metabolomics*, **8**, 492–507.
- Gantt E., Berg G.M., Bhattacharya D., Blouin N.A., Brodie J., Chan C.X., Collén J., Cunningham F.X., Gross J., Grossman A.R., Karpowicz S., Kitade Y., Klein A.S., Levine I.A., Lin S., Lu S., Lynch M., Minocha S.C., Müller K.M., Neefus C.D., Oliveira M.C., Rymarquis L., Smith A., Stiller J.W., Wu W.-K., Yarish C., Zhuang Y.Y., Brawley S.H. (2010) *Porphyra*: complex life histories in a harsh environment: *P. umbilicalis*, an intertidal red alga for genomic analysis. In: Seckbach J., Chapman D.J. (Eds), *Red algae in the genomic age*. Springer, Berlin, Germany, pp 129–148.
- Graham L.E., Wilcox L.W. (2000) *Introduction to the Algae*. Prentice-Hall, Upper Saddle River, NJ, USA, pp 1–20.
- Hall R.D. (2006) Plant metabolomics: from holistic hope, to hype, to hot topic. *New Phytologist*, **169**, 453–468.
- Hsu F.F., Turk J. (2003) Electrospray ionisation/tandem quadrupole mass spectrometric studies on phosphatidylcholines: the fragmentation processes. *Journal of the American Society for Mass Spectrometry*, **14**, 352–363.
- Hsu F.F., Turk J. (2009) Electrospray ionisation with low-energy collisionally activated dissociation tandem mass spectrometry of glycerophospholipids: mechanisms of fragmentation and structural characterization. *Journal of Chromatography B*, **877**, 2673–2695.
- Laxalt A.M., Munnik T. (2002) Phospholipid signalling in plant defence. *Current Opinion in Plant Biology*, **5**, 332–338.
- Pan S.Y. (2006) Influence of temperature and light on free-living conchocelis of *Porphyra haitanensis*. *Hebei Fisheries*, **2**, 17–20.
- Pulfer M., Murphy R.C. (2003) Electrospray mass spectrometry of phospholipids. *Mass Spectrometry Review*, **22**, 332–364.
- Van Vliet E., Morath S., Eskes C., Linge J., Rappsilber J., Honegger P., Hartung T., Coecke S. (2008) A novel in vitro metabolomics approach for neurotoxicity testing, proof of principle for methyl mercury chloride and caffeine. *NeuroToxicology*, **29**, 1–12.
- Wang S.P., Jiang H.R. (1983) Studies on the ecology of the free conchocelis of *Pyropia yezoensis*. *Marine Fisheries Research*, **5**, 79–94.
- Welti R., Li W.Q., Li M.Y., Sang Y., Biesiada H., Zhou H.E., Rajashekar C.B., Williams T.D., Wang X. (2002) Profiling membrane lipids in plant stress responses. *Journal of Biological Chemistry*, **227**, 31994–32002.
- Wiklund S., Johansson E., Sjöström L., Mellerowicz E.J., Edlund U., Shockcor J.P., Gottfries J., Moritz T., Trygg J. (2008) Visualization of GC/TOF-MS-based metabolomics data for identification of biochemically interesting compounds using OPLS class models. *Analytical Chemistry*, **80**, 115–122.
- Xie C.T., Chen C.S., Ji D.H., Xu Y. (2009) Characterization, development and exploitation of EST-derived microsatellites in *Porphyra haitanensis* Chang et Zheng (Bangiales, Rhodophyta). *Journal of Applied Phycology*, **21**, 367–374.
- Xie C.T., Chen C.S., Xu Y., Ji D.H. (2010) Construction of a genetic linkage map for *Porphyra haitanensis* (Bangiales, Rhodophyta) based on sequence-related amplified polymorphism and simple sequence repeat markers. *Journal of Phycology*, **46**, 780–787.
- Xu J.L., Chen D.Y., Yan X.J., Chen J.J., Zhou C.X. (2010) Global characterization of the photosynthetic glycerolipids from a marine diatom *Stephanodiscus* sp. by ultra performance liquid chromatography coupled with electrospray ionization-quadrupole-time of flight mass spectrometry. *Analytica Chimica Acta*, **663**, 60–68.
- Yan X.J., Li H.Y., Xu J.L., Zhou C.X. (2010) Analysis of phospholipids in microalga *Nitzschia closterium* by UPLC-Q-TOF-MS. *Chinese Journal of Oceanology and Limnology*, **28**, 106–112.
- Zhou W.J., Li Y., Dai J.X. (2006) Observation on free-living conchocelis of *Porphyra yezoensis*. *Journal of Fishery Science of China*, **13**, 217–220.
- Zhu J.Y., Zheng Q.S., Lu Q.Q., Zhu M.X., Xu P. (1997) Studies on suspending culture of conchocelis of *Porphyra yezoensis*. *Journal of Aquaculture*, **2**, 12–14.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1–S4. Scores for scatter plot of O-PLS-DA (a) and OPLS loadings S-plot of *P. haitanensis* (b).

Figure S5. The mass spectra of phosphatidylcholine (20:5/20:4) in the TIC of the lipid mixture extracted from *P. haitanensis* at the low collision energy of 5 V in the positive mode with MS^E technique (a); MS2 spectrum of the [M+H]⁺ at m/z 828.5 of phosphatidylcholine (20:5/20:4) in the lipid mixture extracted from *P. haitanensis* (b).