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Profiles of glycerolipids in *Pyropia haitanensis* and their changes responding to agaro-oligosaccharides

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Abstract A sensitive method based on electrospray ionization tandem mass spectrometry was used to profile glycerolipids in *Pyropia haitanensis* and their changes responding to agaro-oligosaccharides. Ten monogalactosyldiacylglycerols (MGDGs), twelve digalactosyldiacylglycerols (DGDGs), five sulfoquinovosyldiacylglycerols (SQDGs), five phosphatidylglycerols (PGs), fifteen phosphatidylcholins (PCs), three phosphatidic acids (PAs), and three phosphatidylethanolamines (PEs) were identified in *P. haitanensis*. We found the SQDG was the most abundant species, followed by MGDG, DGDG, PG, PC, PE, and PA of the total glycerolipids. The predominant lipid species contained C20 fatty acids at *sn*-1/*sn*-2 positions, which was different from higher plants. Changes in membrane lipid species occurred when *P. haitanensis* were treated with agaro-oligosaccharides. At first, agaro-oligosaccharides induced an increase in total glycerolipids including the galactolipids such as MGDG (20:5/20:5) and phospholipids such as PC (18:3/20:5), suggesting that agaro-oligosaccharides caused changes of lipids in chloroplasts and plasma membrane. With increased treatment time, a large decline in major plasma membrane lipids (PCs and PEs) was observed, but not galactolipids (MGDGs and DGDGs), suggesting that the lipid changes occurred mainly at the plasma membrane after prolonged treatment.

Keywords *Pyropia haitanensis* · Agaro-oligosaccharides · Rhodophyta · Glycerolipids · Quadrupole time-of-flight mass spectrometry

Introduction

Lipids are vital cellular constituents. They provide the structural basis for cell membranes and fuels for cell metabolism (Wang 2004). Thus, membrane lipid changes under stress conditions (salt stimulus) will impact on both cell structure and regulatory pathways in plant adaptation and survival. For example, Lu et al. (2012) reported that *Chlamydomonas nivalis* might protect the functions of membrane proteins under stress conditions by regulating the content and composition of galactolipids. Uemura and Steponkus (1997) reported that the degree of fatty acid unsaturation and the content of phospholipids increased in rye during cold acclimation to enhance membrane fluidity and reduce propensity.

Lipids are also used to generate lipid mediators such as phosphatidic acid, lysophospholipids, and free fatty acids, which are involved in plant stress responses (Wang et al. 2006). Laxalt and Munnik (2002) reported phosphatidic acid (PA) as a potential second messenger in plant defense signaling pathways. In addition, PA served as a substrate and/or an activator for enzymes that promote the formation of other lipid regulators, such as lysoPA, free fatty acids, diacylglycerol (DAG), DAG-pyrophosphate, and oxylipins (Wang 2005).

The monogalactosyldiacylglycerols (MGDG), digalactosyldiacylglycerols (DGDG), sulfoquinovosyldiacylglycerols (SQDG), and phosphatidylglycerols (PG) are the main acyl lipids in algal chloroplasts (Benning 1998). Galactolipids play an essential role in chloroplast development and the maintenance of electron transport systems, fatty acid synthesis, and photoreduction of cytochrome (Wang and Lin 2006). Phospholipids are major components of plasma

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membrane and organelle membranes that maintain the integrity of the cell or organelles by creating a semi-impermeable barrier. Phospholipids include phosphatidylcholine (PC), sphingomyelin (SM), phosphatidylserine (PS), and phosphatidylethanolamine (PE) (Fadeel and Xue 2009).

Recently, a profiling strategy based on electrospray tandem mass spectrometry (ESI-MS/MS) has been developed to comprehensively analyze lipid composition in animal and yeast cells (Welti et al. 2002). It requires only simple sample preparation and small samples to identify and quantify lipid species.

Marine red algae emerged as an independent lineage early in the evolution of eukaryotes (Baldauf et al. 2000). The red algal genus *Pyropia* is an important economic marine crop, and *Pyropia haitanensis* is one of the most important species. This species has been cultivated widely along the coasts of South China, especially in Fujian and Zhejiang Provinces (Xie et al. 2009). However, little is known about the profile of membrane lipids of *P. haitanensis* and their changes when induced by elicitors. To reveal the membrane lipid profiles and how lipid species change under stress conditions is important to understand membrane and cell functions. In our previous research, agaro-oligosaccharides induced the oxidative burst and the release of volatile organic compounds in *P. haitanensis* (Wang et al. 2013).

In this study we use ultra-performance liquid chromatography-electrospray ionization-quadrupole-time of flight mass spectrometry (UPLC-ESI-Q-TOF-MS) to qualitatively and quantitatively determine the lipids in *P. haitanensis* and their changes in response to treatment with agaro-oligosaccharides.

Materials and methods

Acetonitrile, isopropanol, formic acid, and sodium formate (liquid chromatography-mass spectrometry (LC-MS) grade) were from Sigma-Aldrich (USA). Distilled water was filtered through a Milli-Q system (Millipore, USA). Leucine-enkephalin was from Sigma-Aldrich. Standards (phosphatidylcholine (18:1/14:0), phosphatidylglycerol (16:0/18:1), and phosphatidylethanolamine (18:1/18:1)) were obtained from the Avanti Polar Lipids, Inc. (USA). Glycolipids standards including monogalactosyldiacylglycerol, digalactosyldiacylglycerol, and sulfoquinovosyldiacylglycerol were from Lipid Products (UK).

Plant material and cultivation treatments

The marine red alga *P. haitanensis* was obtained from the coast of Xiangshan, Zhejiang Province, China. Young fronds (2–10 cm in length) were collected and transported to the laboratory. They were kept in flasks (20–30 mg fresh weight of algae per flask) containing 30 L of 0.45-μm filtered seawater at 18±1 °C, under constant aeration and photon flux density of 45 to 54 μmol photons m^{-2} s^{-1} with a photoperiod of 12-h light/12-h dark for 2 days. Just before the start of the experiment, five individuals were harvested as control material. Agaro-oligosaccharides (100 μg mL^{-1}) were added in the culture medium to elicit defense responses in *P. haitanensis*. Algal samples were harvested after 1 and 3 h of treatment with agaro-oligosaccharides. No nutrients were added during the experiment. All experiments were performed in five replicates and reported as average±one standard deviation. At the end of the experiment, the algae were frozen in liquid nitrogen and stored at −80 °C for analysis.

Lipid analysis

Samples were extracted according to Bligh and Dyer (1959). The sample was dried under nitrogen gas and dissolved in 0.5-mL methanol for UPLC-qTOF-MS analysis.

Chromatographic separation was performed on an ACQUI-TY UPLC BEH C8 analytical column (100× 2.1 mm, 1.7 μm, Waters, USA) using an ACQUITY UPLC system (Waters). Optimal separation was achieved with a gradient elution using (A) water (containing 0.1 %*v/v* formic acid and 0.001 %*v/v* sodium formate) and (B) a mixture of methanol/acetonitrile/isopropanol (1:2:1, *v/v/v*, containing 0.1 %*v/v* formic acid and 0.001 %*v/v* sodium formate) at a flow rate of 0.35 mL min^{-1}. The gradient (time, %B) was set as (0, 5), (5, 50), (30, 100), (35, 100), and (36, 5). 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 Q-TOF Premier (Waters) operating in both negative ion and positive ion electrospray ionization (ESI) modes. The ESI conditions were capillary voltage 3.0 kV (positive modes) and 2.6 kV (negative modes), sampling cone voltage 30–60 V, source temperature 120 °C, desolvation temperature 250 °C, nebulization gas flow 400 L h^{-1}, and cone gas flow 50 L h^{-1}. The collision energy for tandem mass spectrometry (MS) was scanned from 15 to 55 eV. The MS acquisition rate was 0.3 s with a 0.02-s inter-scan delay. Data were collected in the centroid mode from 80 to 1,200*m/z* in MS scanning. All analyses were performed using the lock spray to ensure accuracy and reproducibility. Leucine-enkephalin was used as the lock mass at a concentration of 200 ng mL^{-1} and a flow rate of 10 μL min^{-1}, generating an $[M+H]^+$ ion of 556.2771 Da in ESI^+ mode, and an $[M-H]^-$ ion of 554.2615 Da in ESI^- mode. The lock spray frequency was set at 10 s.

Data analysis

The UPLC/MS data preprocessing was performed with MarkerLynx 4.1 software (Waters, USA). For each sampling

time, differences between the treatments and control were analyzed via one-way analyses of variance (ANOVA) followed by a post hoc multiple comparisons test (Dunnett's test). The content of lipid in *P. haitanensis* was assessed by ANOVA. Results are presented as the mean±SD. Differences were considered significant at level $P < 0.05$. Analysis was undertaken using SPSS 13.0 for Windows.

Results

Profiling the lipid species and semi-quantitative determination of the lipids

Lipid samples extracted from the control group (without agaro-oligosaccharide treatment) and treatment group (with agaro-oligosaccharide treatment) of *P. haitanensis* were analyzed under the optimal UPLC-qTOF-MS conditions. Both positive and negative total ion current (TIC) chromatograms were shown in Fig. 1.

The use of Q-TOF analyzer operating in MS^E mode allows the selection and isolation of precursor ions with high efficiency, sensitivity, and mass accuracy. Characteristic fragment ions can be used to establish the component of lipid and their acyl composition. For galactolipids and phospholipids, the metabolites are identified by comparison with the fragmentation pathways and characteristic ions from the literature. In positive ion mode, the galactosyl head group of $[C_9H_{16}O_6+Na]^+$ at *m/z* 243.09 was used as the characteristic fragment ion for the MGDG and DGDG families of lipids. DGDG generated another characteristic fragment ion, $[C_{15}H_{26}O_{11}+Na]^+$ at *m/z* 405.14, which was used to distinguish MGDG and DGDG (Xu et al. 2010). $[C_5H_{15}O_4NP]^+$ at *m/z* 184.07 was considered as the characteristic ion of PC (Yan et al. 2010). In negative ion mode, *m/z* 225.01 $[C_6H_9O_7S]^-$ (the sulfoquinovosyl head group), *m/z* 153.00 $[C_3H_6O_5P]^-$, and *m/z* 168.04 $[C_4H_{11}O_4NP]^-$ were used as the characteristic fragment ion for SQDG, PA, and PC, respectively (Xu et al. 2010; Yan et al. 2010). $[C_6H_{12}O_7P]^-$ at *m/z* 227.028 and $[C_3H_8O_6P]^-$ at *m/z* 171.01 were used as the characteristic fragment ions for PG (Xu et al. 2010), while ions *m/z* 140.01 $[C_2H_7O_4NP]^-$ and *m/z* 196.04 $[C_5H_{11}O_5NP]^-$ were used for PE identification (Yan et al. 2010). Furthermore, the regionchemical distribution of lipid acyl chains was determined by the ratio of the abundance of *sn*-1 carboxylate fragment ions to that of *sn*-2 carboxylate fragment ions (Han and Gross 1995; Hvattum et al. 1998; Xu et al. 2010; Yan et al. 2010). Detailed process for the identification of PC (20:5/20:4) is provided in Text S1 as an example of lipid identification. Quantitative determination of lipids requires the addition of internal standards because the ionization efficiency of different classes of lipids by ESI-MS may differ significantly with respect to experimental conditions. The lipids in each class were quantified with an internal standard of that class.

A simple and robust LC-MS-based methodology using external standards for semiquantitative characterization of the total lipids was also described by Sommer et al. (2006). In our work, the dynamic range/linearity of each lipid class was determined with mixed standard lipids up to 5 nmol mL^{-1}. The lipids in each class were quantified in comparison with internal standards. Ten MGDGs, twelve DGDGs, five SQDGs, five PGs, fifteen PCs, three PAs, and three PEs were unequivocally identified in *P. haitanensis* (Tables 1 and 2).

The proportion of each class in total lipids (nmol g^{-1} fresh weight algae) was reported in Tables 1 and 2. The anionic sulfoquinovosyldiacylglycerol (SQDG) was the most abundant species, followed by monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), anionic phosphatidylglycerol (PG), phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidic acid (PA) of the total membrane lipids in *P. haitanensis*.

The total amount of each class of lipids exhibited marked variations, with MGDG in the range of 9.91–328.54 nmol g^{-1} fresh algae, DGDG in the range of 0.49–25.25 nmol g^{-1} fresh algae, SQDG in the range of 26.65–1318.22 nmol g^{-1} fresh algae, PG in the range of 9.96–210.54 nmol g^{-1} fresh algae, PC in the range of 0.35–20.73 nmol g^{-1} fresh algae, PA in the range of 2.53–13.46 nmol g^{-1} fresh algae, and PE in the range of 2.89–26.36 nmol g^{-1} fresh algae (Tables 1 and 2).

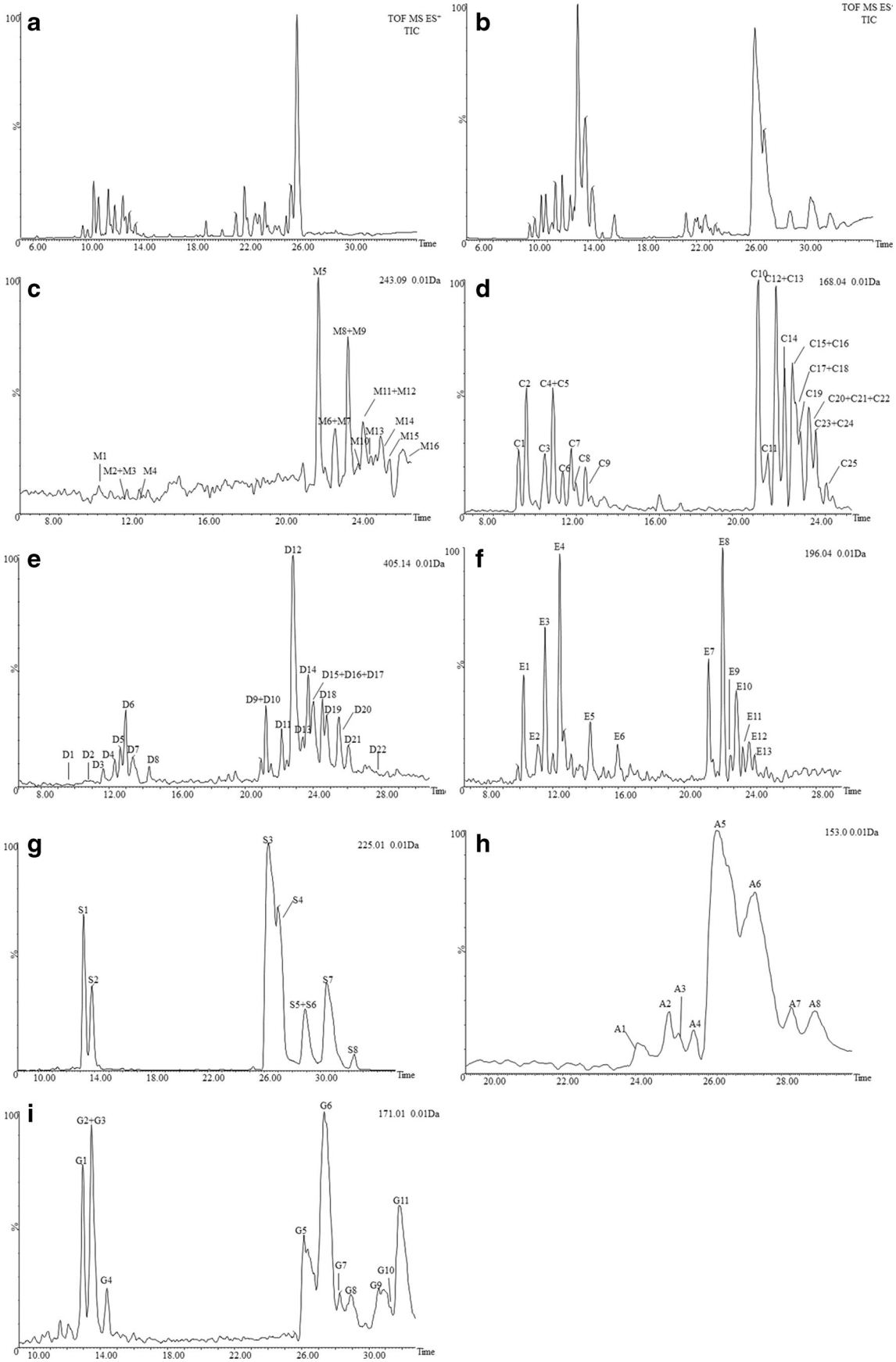
Composition of lipid species

The predominant species of MGDG were those containing $C_{20:5}/C_{20:5}$, $C_{20:5}/C_{16:0}$, and $C_{20:4}/C_{16:0}$; three main species of DGDG contained $C_{20:5}/C_{16:0}$, $C_{16:0}/C_{18:2}$, and $C_{20:5}/C_{20:5}$; the major species of SQDG contained combinations of $C_{20:5}/C_{16:0}$, $C_{16:0}/C_{16:0}$, and $C_{20:4}/C_{16:0}$; the major species of PG class contained $C_{20:5}/C_{16:0}$; the major species of PC class contained $C_{20:5}/C_{20:5}$; and the major species of PA and PE class contained $C_{20:5}/C_{20:4}$ (Tables 1 and 2).

Changes of lipid species after treatment with agaro-oligosaccharides

ESI-MS/MS analysis was used to probe the changes of lipids in *P. haitanensis* after treatment with agaro-oligosaccharides. Compared with control, treatment with agaro-oligosaccharides produced significant changes in membrane lipids (Tables 1 and 2).

There was a general trend toward an increase in the total amount of each lipid class, such as PG (20:5/16:0), SQDG (20:4/16:0), MGDG (20:5/20:5), DGDG (20:5/16:0), PC (18:3/20:5), PA (20:5/20:4), and PE (20:5/20:5), after 1 h of agaro-oligosaccharide treatment (Tables 1 and 2). After 3 h of


a
TOF MS ES+
TIC
b
TOF MS ES+
TIC
c
243.09 0.01Da
M1
M2+M3
M4
M5
M6+M7
M8+M9
M10
M11+M12
M13
M14
M15
M16
d
168.04 0.01Da
C1
C2
C3
C4+C5
C6
C7
C8
C9
C10
C11
C12+C13
C14
C15+C16
C17+C18
C19
C20+C21+C22
C23+C24
C25
e
405.14 0.01Da
D1
D2
D3
D4
D5
D6
D7
D8
D9+D10
D11
D12
D13
D14
D15+D16+D17
D18
D19
D20
D21
D22
f
196.04 0.01Da
E1
E2
E3
E4
E5
E6
E7
E8
E9
E10
E11
E12
E13
g
225.01 0.01Da
S1
S2
S3
S4
S5+S6
S7
S8
h
153.0 0.01Da
A1
A2
A3
A4
A5
A6
A7
A8
i
171.01 0.01Da
G1
G2+G3
G4
G5
G6
G7
G8
G9
G10
G11
Time

◀ **Fig. 1** Total ion chromatogram of the lipid mixture extracted from *Pyropia haitanensis* at a low collision energy of 5 V in the positive mode with the MS^E technique **(a)**; total ion chromatogram of the lipid mixture extracted from *Pyropia haitanensis* at a low collision energy of 5 V in the negative mode with the MS^E technique **(b)**; extracted ion chromatogram of *m/z* 243.08 for characteristic product ion of MGDG in *Pyropia haitanensis* from the high collision energy scans (ramp of 15–55 V) in the positive mode with the MS^E technique **(c)**; extracted ion chromatograms of *m/z* 168.04 for characteristic product ion of PC in *Pyropia haitanensis* from the high collision energy scans (ramp of 15–55 V) in the negative mode with the MS^E technique **(d)**; extracted ion chromatogram of *m/z* 405.14 for characteristic product ion of DGDG in *Pyropia haitanensis* from the high collision energy scans (ramp of 15–55 V) in the positive mode with the MS^E technique **(e)**; extracted ion chromatograms of *m/z* 196.04 for characteristic product ion of PE in *Pyropia haitanensis* from the high collision energy scans (ramp of 15–55 V) in the negative mode with the MS^E technique **(f)**; extracted ion chromatograms of *m/z* 225.01 for characteristic product ion of SQDG in *Pyropia haitanensis* from the high collision energy scans (ramp of 15–55 V) in the negative mode with the MS^E technique *(g)*; extracted ion chromatograms of *m/z* 153.00 for characteristic product ion of PA in *Pyropia haitanensis* from the high collision energy scans (ramp of 15–55 V) in the negative mode with the MS^E technique **(h)**; extracted ion chromatogram of *m/z* 171.01 for characteristic product ion of PG in *Pyropia haitanensis* from the high collision energy scans (ramp of 15–55 V) in the negative mode with the MS^E technique **(i)**. A1: PA (20:5/20:5), A2: PA (20:5/20:4), A3: unknown, A4: PA (20:4/20:4), A5: unknown, A6: unknown, A7: unknown, A8: unknown; C1: unknown, C2: LysoPC (20:5), C3: unknown, C4: LysoPC (18:2), C5: LysoPC (20:4), C6: unknown, C7: LysoPC (20:3), C8: LysoPC (18:3), C9: LysoPC (18:1), C10: PC (20:5/20:5), C11: unknown, C12: PC (20:5/20:4), C13: PC (18:2/20:5), C14: PC (20:5/20:3), C15: PC (16:0/20:5), C16: PC (20:4/20:4), C17: PC (20:5/18:1), C18: PC (18:3/20:5), C19: PC (20:4/20:3), C20: PC (16:0/20:4), C21: PC (16:0/18:2), C22: PC (20:4/18:1), C23: PC (20:3/20:3), C24: PC (16:0/20:3), C25: PC (16:0/18:1); D1: LysoDGDG (20:5), D2: LysoDGDG (18:2), D3: LysoDGDG (16:0), D4: LysoDGDG (18:1), D5: unknown, D6: unknown, D7: unknown, D8: unknown, D9: DGDG (16:0/16:0), D10: DGDG (20:5/20:5), D11: DGDG (20:5/18:2), D12: DGDG (20:5/16:0), D13: DGDG (20:4/16:0), D14: DGDG (16:0/18:2), D15: DGDG (18:1/18:2), D16: DGDG (20:2/18:2), D17: DGDG (16:0/20:3), D18: DGDG (16:0/18:1), D19: DGDG (20:2/18:1), D20: unknown, D21: unknown, D22: DGDG (18:0/18:2); E1: LysoPE (20:5), E2: unknown, E3: LysoPE (20:4), E4: unknown, E5: unknown, E6: unknown, E7: PE (20:5/20:5), E8: PE (20:5/20:4), E9: PE (20:4/20:4), E10: unknown, E11: unknown, E12: unknown, E13: unknown; G1: unknown, G2: LysoPG (16:1), G3: LysoPG (16:0), G4: unknown, G5: PG (20:5/16:1), G6: PG (20:5/16:0), G7: unknown, G8: PG (20:4/16:0), G9: unknown, G10: PG (20:2/16:0), G11: PG (20:1/16:0), M1: LysoMGDG (20:5), M2: LysoMGDG (18:2), M3: LysoMGDG (20:4), M4: LysoMGDG (16:0), M5: MGDG (20:5/20:5), M6: MGDG (20:4/20:5), M7: MGDG (20:5/18:2), M8: MGDG (20:5/16:0), M9: MGDG (20:4/18:2), M10: MGDG (20:4/18:1), M11: MGDG (20:4/16:0), M12: MGDG (16:0/18:2), M13: MGDG (16:0/20:3), M14: MGDG (16:0/18:1), M15: unknown, M16: unknown, S1: LysoSQDG (16:0), S2: unknown, S3: SQDG (20:5/16:0), S4: unknown, S5: SQDG (20:4/16:0), S6: SQDG (18:2/16:0), S7: SQDG (16:0/16:0), S8: SQDG (18:0/16:0)

agaro-oligosaccharide treatment, massive declines in the levels of PC such as PC (18:3/20:5) and PE such as PE (20:5/20:4) occurred, but PA such as PA (20:4/20:4), lysoPC such as LysoPC (20:5), and lysoPE such as LysoPE (20:5) increased dramatically (Tables 1 and 2). These changes suggested a rise in lipolytic activities after treatment with agaro-oligosaccharides. The loss occurred in almost all species of PC and PE, with most decreases in PE (20:5/20:4) and PC (18:3/20:5). The PG, MGDG, SQDG, and DGDG levels, such as PG (20:5/16:1), SQDG (20:5/16:0), MGDG (20:5/20:5), and DGDG (20:5/20:5), tended to decline, 3 h after agaro-oligosaccharide treatment, comparing with 1 h after agaro-oligosaccharide treatment. The galactolipids such as MGDGs did not change much, 3 h after agaro-oligosaccharide treatment, comparing with 1 h agaro-oligosaccharide treatment. The large decline in the major types of membrane phospholipids (such as PC (18:3/20:5), PE (20:5/20:5)) but not galactolipids (such as MGDGs) after 3 h of treatment with agaro-oligosaccharides suggested that phospholipases were activated to a greater extent than galactolipases. The increase in PA and lysophospholipid levels indicated that the hydrolytic activity was increased by exposure to agaro-oligosaccharides.

Discussion

We used the ESI-MS/MS method for rapid analysis of the composition of membrane lipids that include glycolipids in photosynthetic membrane and plasma membrane. The easy sample preparation and relatively short analysis time permit the quick profiling of membrane lipids in *P. haitanensis* and their changes triggered by agaro-oligosaccharides. Our results indicated that the content of acidic lipids (SQDG) in *P. haitanensis* was significantly higher than in higher plants and strongly resembles the lipid composition of cyanobacteria. In higher plants, lipids are usually synthesized by two distinct pathways, the prokaryotic and eukaryotic pathways. The synthesized lipids by the prokaryotic pathway have exclusively C16 fatty acids at the *sn*-2 position of glycerol, while the lipids synthesized by the eukaryotic pathway have C18 fatty acids (Xu et al. 2002). The positional distribution of fatty acids of the individual lipid class of *P. haitanensis* indicated that MGDG and DGDG have a typical mixed biosynthetic pathway including both prokaryotic pathway and eukaryotic pathway, because the fatty acids at *sn*-2 position include both C16 and C18 fatty acids. SQDG and PG are biosynthesized through the prokaryotic pathway exclusively within the chloroplast because the fatty acids at *sn*-2 position are C16 fatty acids in SQDG and PG. We found that the *sn*-2 position of the glycerol backbone in MGDG and DGDG was also occupied by C20 fatty acids, *sn*-2 position of PC was occupied by C18 fatty acids or C20 fatty acids, and *sn*-2 position of PE and PA was occupied by C20 fatty acids in *P. haitanensis*. These findings provide clues on the evolutionary process of plant. During the evolution of eukaryotes to higher plant, the 20 carbon metabolism may be discarded, and only the C18 fatty acid metabolism is retained. The results reported here suggest that red algal *P. haitanensis* is an ancient plant species.

Table 1 Content of photosynthetic glycerolipids in *Pyropia haitanensis*. The same superscripted letter indicates no significant difference (Dunnett's test, $P<0.05$) after treatment with agaro-oligosaccharides

Identification	Content (nmol g^{-1})	Treated group (1 h, nmol g^{-1})	Treated group (3 h, nmol g^{-1})
PG (20:2/16:0)	9.96±1.86^{a}	18.82±1.05^{b}	17.02±0.94^{b}
PG (20:4/16:0)	16.86±1.26^{a}	32.44±2.35^{b}	32.60±2.26^{b}
PG (20:1/16:0)	105.76±5.89^{a}	138.43±11.56^{b}	145.68±13.65^{b}
PG (20:5/16:1)	152.51±8.04	169.43±11.39	162.12±7.79
PG (20:5/16:0)	210.54±13.10^{a}	271.97±37.33^{b}	296.70±17.60^{b}
Lyso-PG (16:1)	72.77±6.55^{a}	44.75±8.70^{b}	56.80±9.90^{ab}
Lyso-PG (16:0)	99.06±11.24^{a}	86.87±8.27^{a}	128.02±10.00^{b}
SQDG (18:0/16:0)	33.86±1.89	36.80±2.13	34.49±0.72
SQDG (18:2/16:0)	26.65±2.36^{a}	39.60±3.48^{b}	42.43±2.47^{b}
SQDG (20:4/16:0)	152.65±11.88^{a}	264.28±13.96^{b}	252.39±14.30^{b}
SQDG (16:0/16:0)	361.13±15.82^{a}	501.28±33.01^{b}	521.11±49.78^{b}
SQDG (20:5/16:0)	1318.22±227.16^{ab}	1529.17±72.31^{a}	1213.39±22.93^{b}
Lyso-SQDG (16:0)	651.28±54.40^{a}	496.51±34.27^{b}	699.43±30.25^{a}
MGDG (20:4/18:1)	9.91±1.51^{a}	15.31±0.28^{b}	15.06±2.34^{b}
MGDG (16:0/20:3)	16.93±3.26^{a}	23.25±2.06^{ab}	26.05±4.82^{b}
MGDG (20:4/20:5)	25.67±1.72^{a}	37.86±3.79^{b}	31.41±2.71^{a}
MGDG (20:5/18:2)	24.95±3.78^{a}	28.74±3.99^{ab}	32.63±2.99^{b}
MGDG (20:4/18:2)	16.66±1.70^{a}	31.97±3.17^{b}	33.42±4.25^{b}
MGDG (16:0/18:2)	36.69±2.64^{a}	45.91±0.48^{b}	47.46±3.53^{b}
MGDG (16:0/18:1)	43.62±3.27^{a}	49.32±3.61^{ab}	55.14±5.29^{b}
MGDG (20:4/16:0)	65.01±11.24^{a}	114.96±11.80^{b}	107.87±5.12^{b}
MGDG (20:5/16:0)	231.32±23.69^{a}	273.95±8.08^{b}	275.49±16.05^{b}
MGDG (20:5/20:5)	328.54±20.96^{a}	404.97±29.90^{b}	384.84±26.92^{b}
Lyso-MGDG (20:4)	49.51±4.52^{a}	58.63±4.61^{b}	64.41±4.34^{b}
Lyso-MGDG (18:2)	53.15±3.78^{a}	49.13±6.86^{a}	73.08±4.33^{b}
Lyso-MGDG (16:0)	211.91±19.01^{a}	239.53±15.33^{ab}	267.67±12.39^{b}
Lyso-MGDG (20:5)	287.09±33.48^{a}	317.06±26.67^{ab}	342.63±15.79^{b}
DGDG (16:0/16:0)	0.49±0.16^{a}	2.77±0.47^{b}	0.94±0.23^{a}
DGDG (16:0/20:3)	3.36±0.25	3.50±0.16	3.54±0.33
DGDG (18:1/18:2)	3.46±0.38	4.09±0.29	4.12±0.41
DGDG (20:2/18:2)	3.67±0.38	4.13±0.34	4.16±0.39
DGDG (20:5/18:2)	4.43±0.48	4.83±0.25	4.93±0.24
DGDG (20:2/18:1)	4.97±0.37	5.76±0.26	5.97±0.88
DGDG (20:4/16:0)	6.40±0.70^{a}	8.18±0.04^{b}	8.09±0.48^{b}
DGDG (18:0/18:2)	7.07±0.74 ^{a}	8.31±1.61 ^{a}	12.93±1.33^{b}
DGDG (16:0/18:1)	10.62±1.00^{a}	13.89±0.93^{b}	13.76±1.29^{b}
DGDG (16:0/18:2)	12.90±0.57^{a}	15.32±0.55^{b}	14.67±0.34^{b}
DGDG (20:5/20:5)	11.34±0.82^{a}	15.52±2.00^{b}	15.07±1.26^{b}
DGDG (20:5/16:0)	25.25±0.44^{a}	32.30±4.02^{b}	30.02±3.14^{ab}
Lyso-DGDG (20:5)	2.20±0.21	2.19±0.21	2.66±0.40
Lyso-DGDG (18:1)	9.43±0.79	8.30±0.26	9.59±1.18
Lyso-DGDG (18:2)	9.56±1.53	9.82±0.75	10.62±1.30
Lyso-DGDG (16:0)	67.34±6.61^{a}	73.66±4.68^{ab}	82.73±6.72^{b}

Significant changes in membrane lipid species occurred when *P. haitanensis* were treated with agaro-oligosaccharides. The results demonstrated that *P. haitanensis* had developed mechanisms to alter lipid compositions to cope with elicitor through lipid synthesis and degradation. At first, agaro-oligosaccharides induced an increase in total membrane lipids including the galactolipids and phospholipids, while the levels of lyso-galactolipids and lyso-phospholipids decreased. These

Table 2 Content of glycerolipids of plasma membrane in *Pyropia haitanensis*. The same superscripted letter indicates no significant difference (Dunnett's test, $P<0.05$) after treatment with agaro-oligosaccharides

Identification	Control (nmol g^{-1})	Treated group (1 h, nmol g^{-1})	Treated group (3 h, nmol g^{-1})
PC (20:3/20:3)	0.35±0.04[a]	0.31±0.01[ab]	0.26±0.03[b]
PC (20:4/18:1)	2.51±0.36	2.43±0.44	1.94±0.55
PC (16:0/18:1)	2.35±0.13	2.22±0.32	1.97±0.22
PC (16:0/18:2)	1.80±0.28[a]	2.49±0.34[b]	2.04±0.20[ab]
PC (20:4/20:3)	2.62±0.47	3.23±0.35	3.03±0.13
PC (20:4/20:4)	2.83±0.19[a]	4.19±0.29[b]	3.04±0.51[a]
PC (16:0/20:3)	3.21±0.44	3.22±0.49	3.19±0.34
PC (16:0/20:4)	5.15±0.47[ab]	5.60±0.37[a]	4.70±0.26[b]
PC (18:2/20:5)	4.25±1.16	5.73±0.50	5.12±0.69
PC (18:3/20:5)	5.40±0.45[a]	7.94±0.57[b]	5.54±0.98[a]
PC (16:0/20:5)	6.95±0.54[a]	7.12±0.30[a]	5.71±0.34[b]
PC (20:5/18:1)	6.34±0.32	6.28±0.57	6.23±0.59
PC (20:5/20:3)	7.78±1.03	8.54±0.82	8.25±0.56
PC (20:5/20:4)	13.19±1.35	14.86±0.56	13.50±0.67
PC (20:5/20:5)	20.73±2.43	22.26±0.91	20.45±2.36
Lyso-PC (18:2)	9.09±0.53[a]	7.58±1.08[a]	12.56±1.90[b]
Lyso-PC (18:1)	12.23±0.73	12.16±2.26	13.95±1.48
Lyso-PC (18:3)	12.88±0.25[a]	14.72±3.09[ab]	18.42±1.75[b]
Lyso-PC (20:3)	15.04±1.78[a]	10.46±2.05[b]	19.65±1.90[c]
Lyso-PC (20:4)	34.24±4.68[a]	25.34±3.46[b]	38.71±3.26[a]
Lyso-PC (20:5)	44.83±1.98[a]	28.83±3.15[b]	46.96±4.91[a]
PA (20:4/20:4)	2.53±0.21[a]	39.23±7.62[b]	52.91±2.97[c]
PA (20:5/20:5)	12.59±2.14[a]	71.14±9.02[b]	76.84±6.66[b]
PA (20:5/20:4)	13.46±3.08[a]	103.84±5.76[b]	106.78±7.57[b]
PE (20:4/20:4)	2.89±0.30[ab]	3.30±0.35[a]	2.46±0.26[b]
PE (20:5/20:5)	14.70±1.76[a]	22.31±2.05[b]	17.06±3.04[a]
PE (20:5/20:4)	26.36±3.57[a]	35.55±1.66[b]	26.29±3.02[a]
Lyso-PE (20:4)	8.68±1.54[a]	11.53±1.90[ab]	12.19±1.73[b]
Lyso-PE (20:5)	8.23±0.92[a]	11.51±2.06[a]	15.19±2.08[b]

results also suggested that agaro-oligosaccharides induced changes of lipids in both chloroplasts and plasma membranes. But with the increased time of treatment, a large decline was observed in major types of membrane phospholipids but not galactolipids, suggesting that the lipid change occurred mainly at the plasma membrane and phospholipases were activated to a greater extent than galactolipases.

Signal-induced production of PA in the cell is accomplished by two principal routes: phospholipase D (PLD)-mediated hydrolysis of membrane lipids and DAG-kinase-mediated phosphorylation of DAG (Wang 2004). Laxalt and Munnik (2002) reported that phosphatidic acid (PA) was a second messenger in plant defense signaling pathways. In addition, PA serves as a substrate and/or an activator for enzymes that promote the formation of other lipid regulators, such as lysoPA, free fatty acids, diacylglycerol (DAG), DAG-pyrophosphate, and oxylipins (Wang 2005).

Zhang et al. (2003) reported that PA is involved in the alleviation of H_2O_2-induced cell death in plants. Increases in PA concentration have been observed under various stress conditions, such as chilling, freezing, wounding, pathogen elicitation, nutrient starvation, oxidative stress, and so on (Welti et al. 2002). Our previous study found that the agaro-oligosaccharides induced the production of hydrogen peroxide (H_2O_2). In this study, we found that the levels of PA (20:5/20:4, 20:4/20:4, 20:5/20:5) displayed a continuous increase after treatment with agaro-oligosaccharides, which suggested that agaro-oligosaccharides might induce the production of PA to alleviate the deleterious effects of H_2O_2.

In summary, the profile of membrane lipids *P. haitanensis* was obtained, and their changes in response to agaro-oligosaccharides were demonstrated. The lipid profile of *P. haitanensis* provides important clues on its evolutionary process, and the changes of lipids facilitate the understanding of membrane lipids of algae in response to elicitor.

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