Biochemical and Biophysical Research Communications 452 (2014) 873–880

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A novel ether-linked phytol-containing digalactosylglycerolipid in the marine green alga, *Ulva pertusa*

Yohei Ishibashi [a,1], Yusuke Nagamatsu [a,1], Tomofumi Miyamoto [b], Naoyuki Matsunaga [a], Nozomu Okino [a], Kuniko Yamaguchi [a], Makoto Ito [a,*]

[a] Department of Bioscience and Biotechnology, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan
[b] Graduate School of Pharmaceutical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan

ARTICLE INFO

Article history:
Received 9 August 2014
Available online 23 August 2014

Keywords:
Chlorophyll
Digalactosyldiacylglycerol (DGDG)
Endogalactosylceramidase (EGALC)
Marine algae
Phytol
Transglycosylation

ABSTRACT

Galactosylglycerolipids (GGLs) and chlorophyll are characteristic components of chloroplast in photosynthetic organisms. Although chlorophyll is anchored to the thylakoid membrane by phytol (tetramethylhexadecenol), this isoprenoid alcohol has never been found as a constituent of GGLs. We here described a novel GGL, in which phytol was linked to the glycerol backbone via an ether linkage. This unique GGL was identified as an Alkaline-resistant and Endogalactosylceramidase (EGALC)-sensitive GlycoLipid (AEGL) in the marine green alga, *Ulva pertusa*. EGALC is an enzyme that is specific to the R-Galα/β1-6Galβ1-structure of galactolipids. The structure of *U. pertusa* AEGL was determined following its purification to 1-*O*-phytyl-3-*O*-Galα1-6Galβ1-sn-glycerol by mass spectrometric and nuclear magnetic resonance analyses. AEGLs were ubiquitously distributed in not only green, but also red and brown marine algae; however, they were rarely detected in terrestrial plants, eukaryotic phytoplankton, or cyanobacteria.

1. Introduction

Galactosylglycerolipids (GGLs) such as monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) are ubiquitous components of the thylakoid membrane of chloroplasts in photosynthetic organisms [1]. DGDG is composed of an α1,6-linked galactobiose (Galα1-6Gal) and diacylglycerol, the latter of which contains α-linolenic acid (C18:3), linoleic acid (C18:2), or palmitic acid (C16:0) in *Arabidopsis thaliana* [2]. GGLs are thought to be important for stabilizing photosystem II and the light-harvesting chlorophyll-protein complex [3]. Chlorophyll is an essential pigment in photosynthesis that absorbs light and converts it into chemical energy [4]. Chlorophyll is composed of two parts, a porphyrin ring with a magnesium and hydrophobic tail, an phytol (tetramethylhexadecenol), an isoprenoid alcohol that anchors chlorophyll to the thylakoid membrane [4].

Endogalactosylceramidase (EGALC) is an enzyme that is capable of cleaving the β-glycosidic linkage between R-Galα/β1-6Gal and the lipid moiety, thereby releasing intact galactooligosaccharide [5]. The specificity of EGALC is very high for the sugar moiety, but relatively wide for the lipid moiety, i.e., it hydrolyzes GGLs as well as glycosphingolipids (GSLs) [6]. EGALC also catalyzes transglycosylation reaction, in which the intact sugar chains are transferred from GSLs/GGLs to the primary hydroxyl group of various 1-alkanols to generate neoglycoconjugates [7]. We previously developed a sensitive and reliable method to detect GSLs/GGLs that share the R-Galα/β1-6Galβ1-structure, using the transglycosylation reaction of EGALC [6]. When fluorescent alkanol was used as an acceptor substrate, a fluorescent oligosaccharide was generated if the glycolipids contain the R-Galα/β1-6Galβ1-structure. The fluorescent products generated could be detected by a TLC or HPLC system.

6-Gala series GSLs, which possess R-Galα/β1-6Galβ1–1′Cer, are known to be distributed in marine invertebrates such as the turban shell, *Turbo cornutus* [8], which is a marine algae eater; therefore, we examined whether marine algae contained 6-gala series GSLs in this study. We used the EGALC transglycosylation-based assay to detect 6-gala GSLs with 7-nitro-2,1,3-benzoxadiazole (NBD)-labeled pentanol (NBD-pentanol) as the acceptor substrate. The sample was subjected to a mild alkaline treatment prior to being

Abbreviations: AEGL, alkaline-resistant and EGALC-sensitive glycolipid; Cer, ceramide; DGDG, digalactosyldiacylglycerol; EGALC, endogalactosylceramidase; Gal, galactose; GGL, galactosylglycerolipid; GSL, glycosphingolipid; MGDG, monogalactosyldiacylglycerol; NBD, 7-nitro-2,1,3-benzoxadiazole; OPA, *o*-phtalaldehyde; SCDase, sphingolipid ceramide *N*-deacylase.

* Corresponding author. Fax: +81 92 642 2907.
E-mail address: makotoi@agr.kyushu-u.ac.jp (M. Ito).

[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bbrc.2014.08.056
0006-291X/

incubated with EGALC in order to eliminate GGLs such as DGDG. We found that Alkaline-resistant and EGALC-sensitive GlycoLipid (AEGL) was widely distributed in marine algae. However, the AEGL purified from the marine green alga, *Ulva pertusa,* was not GSL, but GGL, which possesses a glycerol backbone bound to Galα1-6Galβ1- and an alkaline-resistant ether-linked phytol.

To the best of our knowledge, this is the first study to identify a phytol-containing GGL that may be present in the thylakoid membrane of chloroplasts in marine algae. AEGLs were ubiquitously distributed in not only green, but also red and brown marine algae; however, they were rarely detected in terrestrial plants, eukaryotic phytoplankton, or cyanobacteria.

2. Materials and methods

2.1. Materials

DGDG was purchased from Matreya LLC (USA). Pre-coated Silica gel 60 TLC plates were obtained from Merck (Germany). 5-Amino-1-pentanol and 4-fluoro-7nitro-2,1,3-benzoxadiazole (NBD-F) were purchased from Tokyo Kasei Kogyo (Japan) and Sigma–Aldrich (USA), respectively.

2.2. Samples

Marine algae: *U. pertusa*, *Ulva ohonoi*, *Enteromorpha* sp., *Codium fragile*, *Monostroma nitidum*, *Campylaephora hypnaeoides*, *Gracilaria vermiculophylla*, *Grateloupia acuminate*, *Polyopes affinis*, *Porphyra yezoensis*, *Sargassum horneri*, *Sargassum autumnale*, *Sargassum thunbergii*, *Sargassum micracanthum*, *Sargassum nigrifolium*, *Sargassum fusiforme*, *Sargassum ringgoldianum*, *Leathesia difformis*, *Eisenia bicyclis*, *Hydroclathrus clathratus*, *Laminaria japonica*, *Myagropsis myagroides*, and *Undaria pinnatifida* were collected at Shikanoshima Island, Fukuoka, Japan. *Caulerpa racemosa* was collected at Kagoshima bay, Kagoshima, Japan. *A. thaliana*, *Glycine max*, *Lycopersicon esculentum*, *Populus alba*, *Heterosigma akashiwo*, and *Thalassiosira pseudonana* were kindly donated from Drs. M. Inoue, T. Yuasa, Y. Shimasaki, T. Matsushita, and E. Goto, Kyushu University, Japan. *Anabaena* sp. PCC7120 and *Synechocystis* sp. PCC6803 were generously provided by Dr. S. Sato and Ms. R. Muneto, from the Kazusa DNA Research Institute, Japan.

2.3. Lipid extraction

Lipids were extracted by chloroform/methanol (2/1, v/v) from samples, which were previously frozen with liquid nitrogen and crushed to a powder. After an hour incubation at room temperature, extracts were filtrated, evaporated, and dissolved in an appropriate amount of methanol containing 0.2 N KOH and incubated at 37 °C for 2 h. Samples were then neutralized with an appropriate amount of 10 N acetic acid, and partitioned with Folch's method [9]. The organic phase was evaporated and dissolved in chloroform/methanol (2/1, v/v), and applied to the screening for AEGLs with EGALC [6].

2.4. Direct fluorescent labeling of galactolipids by EGALC

Recombinant EGALC and the fluorescent acceptor, NBD-pentanol were prepared using a previously described method [6]. Ten μl of lipid extracts were mixed with 5 μl of 1.5 mM NBD-pentanol, and evaporated, and then dissolved in 20 μl of a reaction mixture containing 150 μU of EGALC in 50 mM sodium acetate buffer, pH 5.5, and incubated at 37 °C for 12 h. The reaction was stopped by adding 80 μl of chloroform/methanol (2/1, v/v), followed by 10 μl of water. After vortexing for a few seconds, the reaction mixture was centrifuged. The upper phase, which contained NBD-pentanol-conjugated oligosaccharides, was collected and dried with a speed vac concentrator. The dried sample was dissolved in 10 μl of methanol, and applied to a TLC plate, which was developed with chloroform/methanol/water (65/25/8, v/v/v). The transglycosylated fluorescent product was visualized by AE-6935B Visirays (ATTO).

2.5. Purification of the EGALC-sensitive glycolipid from U. pertusa

The green alga *U. pertusa* (dry weight 1 kg) was lyophilized using liquid nitrogen, and crushed to a powder, dissolved in chloroform/methanol (2/1, v/v), and filtered to exclude debris. The filtrate was partitioned with Folch's method, and the organic phase was evaporated, and then dissolved in 400 ml of 0.2 N KOH in methanol. After a 120-min incubation, the sample was neutralized by adding 8 ml of 10 N acetic acid, and again partitioned with Folch's method. The organic phase was loaded onto a Silicagel 60 column (2 × 30 cm, Waters) equilibrated with chloroform. The column was eluted with 300 ml of 100/0, 99/1, 98/2, 95/5, 9/1, 8/1, 7/1, 6/1, 4/1, 2/1, and 1/1 of chloroform/methanol (v/v), and finally with methanol. EGALC-sensitive glycolipids were detected in the chloroform/methanol (4/1, v/v) and (2/1, v/v) fractions, which were evaporated, dissolved in chloroform/methanol/water (30/60/8, v/v/v), and loaded onto a Q-Sepharose column (2 × 30 cm, GE healthcare) equilibrated with chloroform/methanol/water (30/60/8, v/v/v). EGALC-sensitive glycolipids were eluted with chloroform/methanol/water (30/60/8, v/v/v), and evaporated and dissolved in methanol/water (2/1, v/v), and then loaded onto Sep-Pak C18 Plus cartridge (Waters) equilibrated with the same solvent. The cartridge was eluted with 2/1, 5/2, 10/3 of methanol/water (v/v) and finally with methanol. The methanol fraction was loaded onto an Iatrobeads (6RS-8060) column (2 × 20 cm, Mitsubishi Chemical) equilibrated with chloroform/methanol (4/1, v/v), and then eluted with the same solvent at a flow rate of 2 ml/min. The EGALC-sensitive fractions were combined and concentrated with a rotary evaporator, then used as purified AEGL. From 1 kg of dried *U. pertusa*, approximately 60 mg of purified AEGL was obtained. AEGL from *C. fragile*, *G. acuminate*, and *S. fusiforme* were isolated by the same procedures.

2.6. Gas chromatographic analysis

Sugar components in purified AEGL were analyzed using gas chromatography (GC) as trimethylsilyl (TMS) derivatives according to a previously described [10].

2.7. Preparation of the oligosaccharide moiety of AEGL

The oligosaccharide moiety was obtained from AEGL by a hydrolysis reaction with EGALC. Purified AEGL was incubated at 37 °C for 12 h in 50 mM sodium acetate buffer, pH 5.5, containing 1 mU of EGALC. The reaction mixture was partitioned with Folch's method, and the oligosaccharide-containing upper phase was dried, dissolved in methanol, and then applied to TLC developed with chloroform/methanol/0.02% $CaCl_2$ (5/4/1, v/v/v). The oligosaccharide was transferred to a PVDF membrane at 180 °C for 30 s with TLC thermal blotter AC-5970 (ATTO) [11].

2.8. Mass spectrometric analysis

AEGL and the AEGL-oligosaccharide were subjected to the MALDI-TOF MS analysis using Voyager-DE mass spectrometer (Life technologies) with 2,5-dihydroxybenzoic acid (DHB, Sigma–Aldrich) as a matrix. Purified AEGL was also subjected to ESI-MS/MS analysis using a 4000Q TRAP (AB SCIEX) at a flow rate of 100 μl/min. The mobile phase was 5 mM ammonium formate in

methanol with 0.2% formic acid. MS/MS analysis was operated in the positive ion mode with a scan range of *m*/*z* 250–715 and collision energy of 15 eV.

2.9. SCDase assay

Sphingolipid ceramide *N*-deacylase (SCDase) was prepared as described in [12]. Purified AEGL was incubated at 37 °C for 12 h in 25 mM sodium acetate buffer, pH 5.5, containing 0.8 mU of SCDase in the presence of 0.8% Triton X-100. The reaction product was subjected to HPLC equipped with the Inertsil ODS-3 (4.6 × 250 mm, GL Science) column after derivatization with *o*-phthalaldehyde (OPA), eluted with an isocratic mobile phase consisting of methanol/water (9/1, v/v) at a flow rate of 1.0 ml/min, and detected by a fluorescent detector set to excitation and emission wavelengths of 340 and 455 nm [13]. Alternatively, the products were applied to a TLC plate, which was developed using chloroform/methanol/water (65/35/8, v/v/v), and visualized using the orcinol-H_2SO_4 or ninhydrin reagent.

2.10. NMR analysis of AEGL

Purified AEGL was dissolved in 5% D_2O in pyridine-*d5*. NMR spectra of the purified AEGL were recorded on a Varian INOVA 600 spectrometer at 303 k, and the ^1H and ^{13}C NMR chemical shifts were referenced to the solvent peaks for C_5D_5N at δ_H 7.19 and δ_C 123.5.

3. Results and discussion

3.1. Detection of AEGLs from marine algae

Although EGALC degraded both GSLs and GGLs, which share the Galα/β1-6Galβ1-structure, both galactolipids could be distinguished by the alkaline treatment, i.e., GGLs were sensitive, whereas GSLs were resistant to the mild alkaline treatment (Fig. 1A). As shown in Fig. 1B, fluorescent oligosaccharides were generated from the alkaline-resistant glycolipid fractions of several marine algae after the treatment with EGALC in the presence of fluorescent alkanol, NBD-pentanol. Thus, we attempted to purify this alkaline-resistant and EGALC-sensitive glycolipid (AEGL) from the green algae, *U. pertusa,* because, to the best of our knowledge, GSLs possessing the Galα/β1-6Galβ1-structure have yet to be identified in marine algae. The purified *U. pertusa* AEGL (Fig. 2A) generated a NBD-labeled oligosaccharide after the treatment with EGALC in the presence of NBD-pentanol (Fig. 2B). AEGL was stable after the alkaline treatment, in contrast to DGDG (Fig. 2C). MALDI-TOF MS analysis of AEGL revealed $[M+Na]^+$ at *m*/*z* 717 (Fig. 2D).

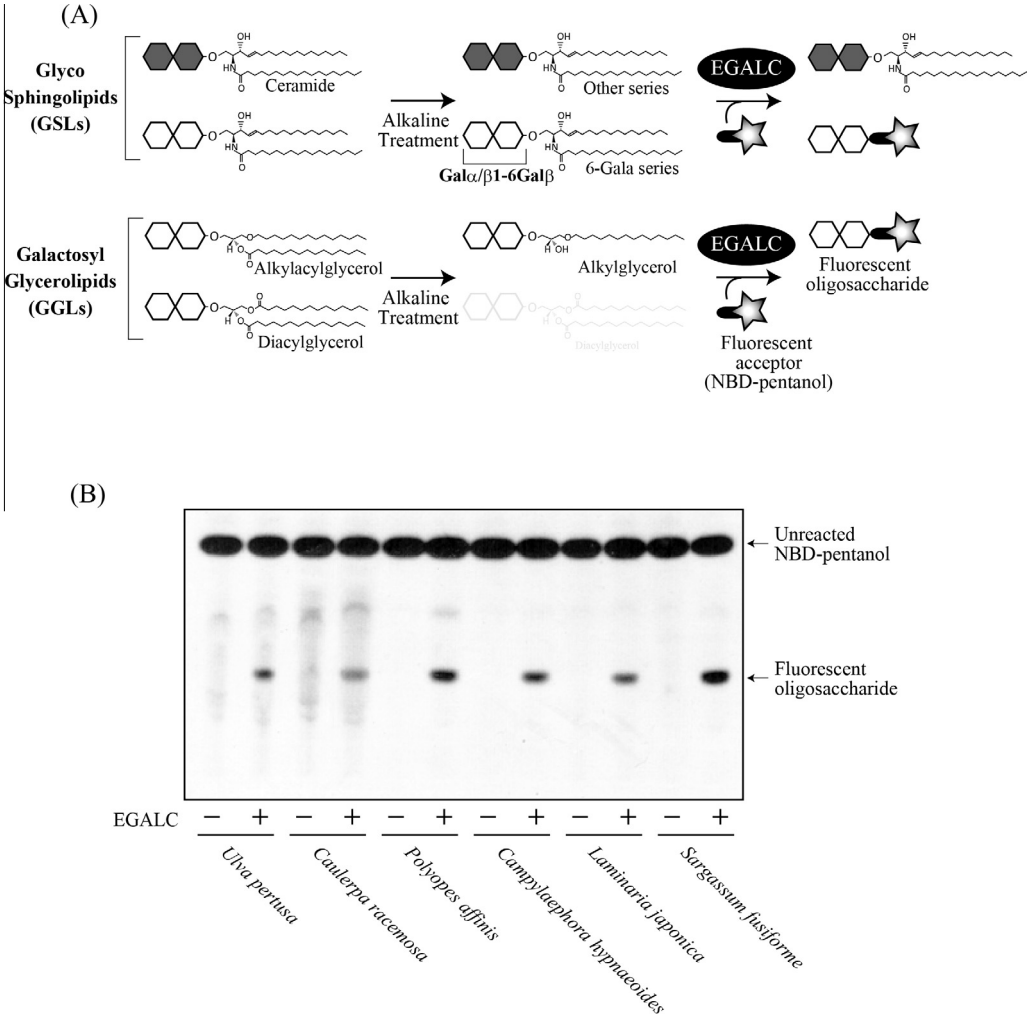

Fig. 1. EGALC transglycosylation-based assay to detect AEGL in marine algae. (A) Scheme for the detection of AEGL by the EGALC transglycosylation-based assay. The alkaline treatment eliminates GGLs that contain diaclyglycerol. EGALC is specific to galactolipids possessing the R-Galα/β1-6Galβ1-structure and it transfers the R-Galα/β1-6Gal from AEGL to fluorescent acceptor, NBD-pentanol. (B) TLC analysis of the NBD-oligosaccharide generated from the alkaline-treated lipid extracts of several marine algae by EGALC.

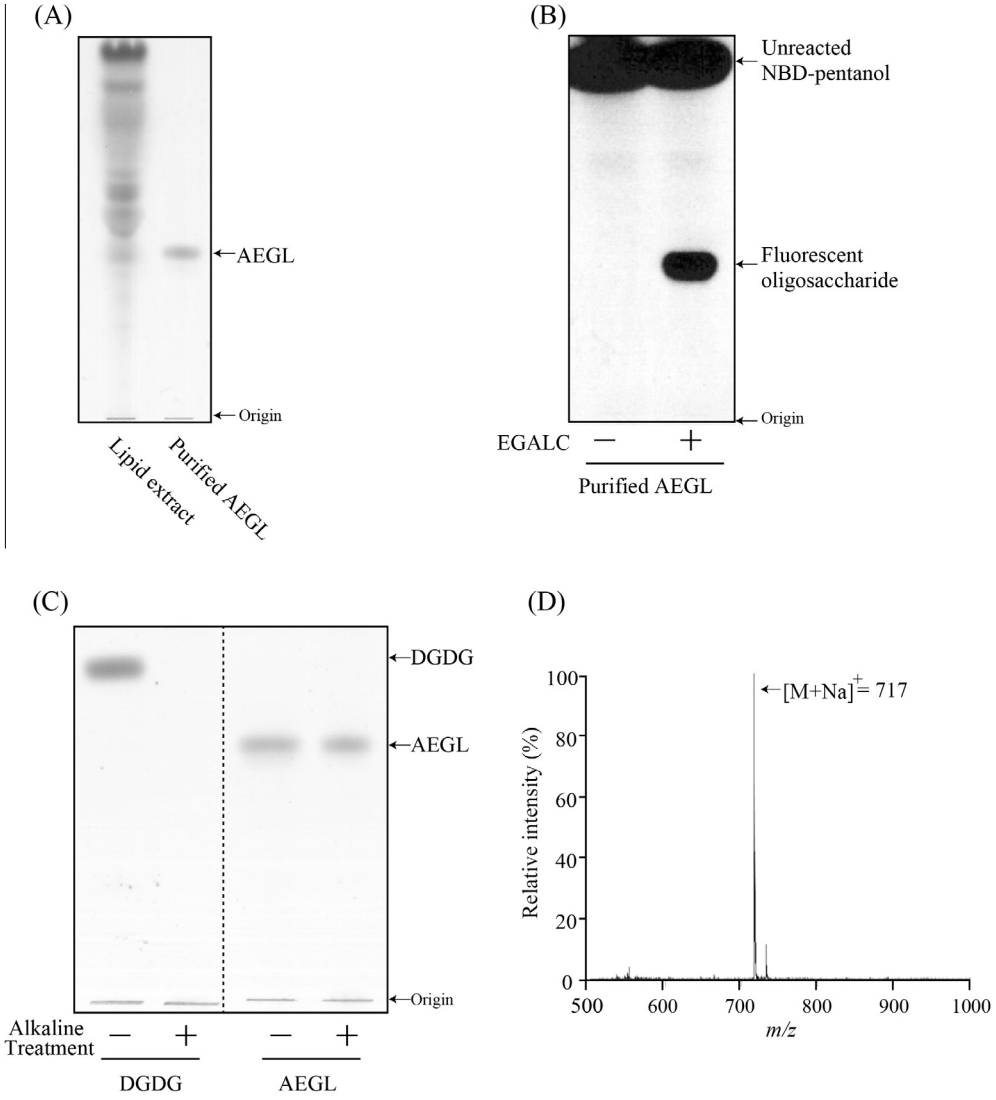

Fig. 2. Purification of AEGL from the green alga, *U. pertusa*. (A) TLC showing the purified AEGL obtained from the alkaline-treated lipid extract of *U. pertusa*. Lane 1, crude lipid fraction from *U. pertusa*, lane 2, purified AEGL. (B) TLC showing the generation of the NBD-oligosaccharide from purified AEGL by EGALC in the presence of NBD-pentanol. (C) TLC showing the alkaline sensitivities of DGDG and AEGL. (D) The MALDI-TOF MS spectrum of purified AEGL.

3.2. Structure of the oligosaccharide moiety of U. pertusa AEGL

The oligosaccharide was obtained from *U. pertusa* AEGL through a hydrolysis reaction with EGALC (Fig. 3A) and subsequently subjected to MALDI-TOF MS analysis. The main molecular ion peak, $[M+Na]^+$, was observed at *m/z* 365 for the AEGL-oligosaccharide, which corresponded to the molecular mass of a disaccharide composed of two hexoses (Fig. 3B). To identify the sugar components of the AEGL-oligosaccharide, its TMS-derivatives were analyzed by GC, and the results obtained indicated that the AEGL-oligosaccharide consisted only of Gal (Fig. 3C). This result was consistent with the specificity of EGALC, which is specific to the R-Galα/β1-6Galβ1-structure [5,6]. These results indicated that the oligosaccharide moiety of the AEGL was estimated to be Galα/β1-6Gal.

3.3. Sensitivity of the U. pertusa AEGL to SCDase

Based on its alkaline resistance, the AEGL from *U. pertusa* appeared to be GSL (Fig. 1A). Thus, we investigated whether AEGL contained ceramide backbone using SCDase, which is an enzyme capable of hydrolyzing the *N*-acyl linkage between the sphingoid base and fatty acid in the ceramide moiety [12,14]. After the treatment of SCDase, the free amino group generated was labeled with OPA and analyzed by HPLC [13]. No peaks were detected on HPLC from *U. pertusa* AEGL after treatment with SCDase, while a peak corresponding to the OPA derivative of Galβ1-4Glcβ1-sphingosine was generated from the authentic GSL, lactosylceramide (LacCer) (Fig. 3D). This result indicated that AEGL was not a GSL, in spite of its tolerance of alkaline conditions.

3.4. NMR analysis of the U. pertusa AEGL

We performed extensive 2D NMR analyses of the *U. pertusa* AEGL (Supporting information and Table 1) to reveal its complete structure. COSY, TOCSY, and NOESY spectra revealed the correlations of H-1 to H_2-6 of β-Gal I, H-1 to H_2-6 of α-Gal II, H_2-1 to H_2-3 of glycerol and H_2-1 to H_2-4 of phytol. The ^{13}C NMR chemical shifts of β-Gal I, α-Gal II, and glycerol were assigned by HSQC spectra, and the chemical shifts of phytyl moiety were good agreement to the reported data of phytol [15]. HMBC correlations between the H-1 of α-Gal II [δ_H 5.48] and C-6 of β-Gal I [δ_C 68.2], and the H-1 of β-Gal I [δ_H 4.78] and C-3 of

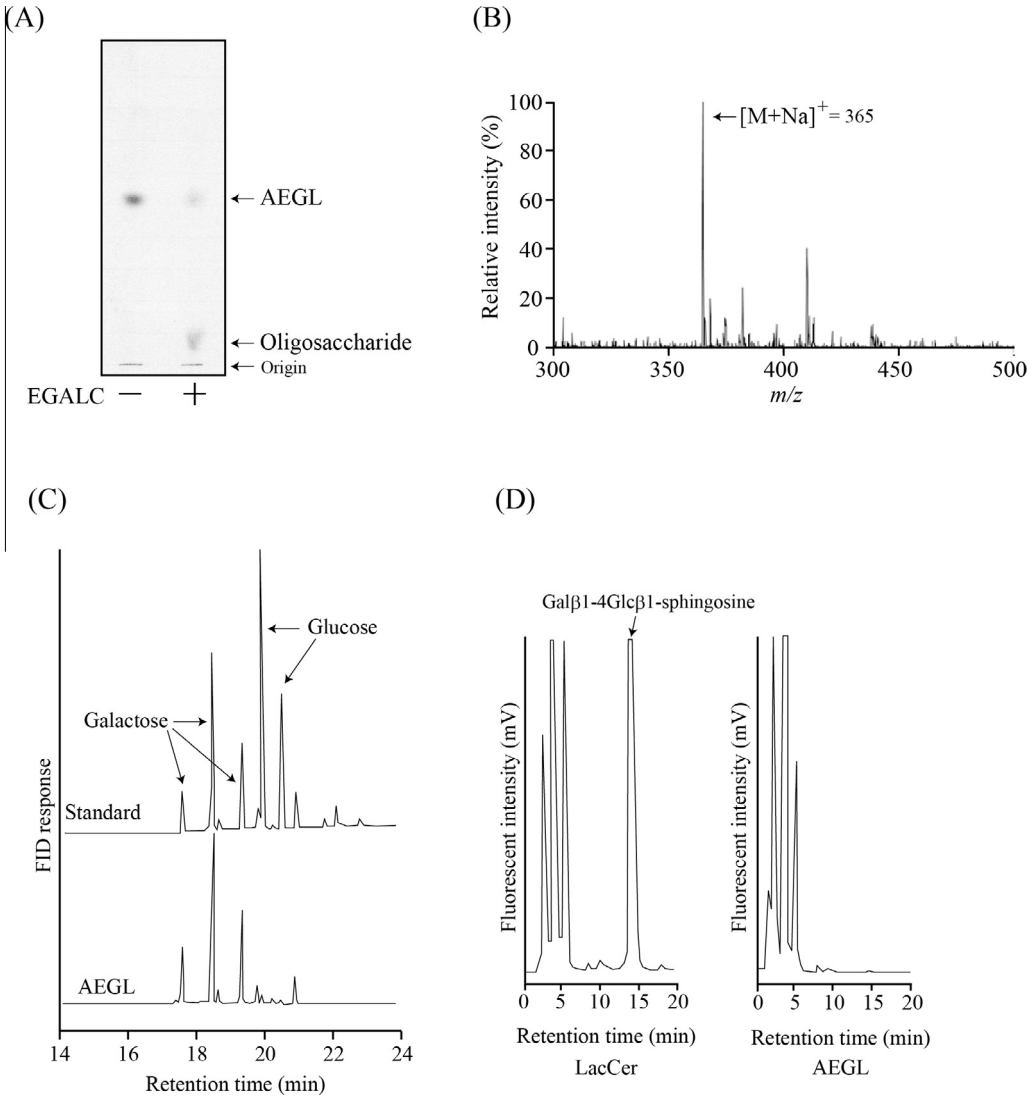

Fig. 3. Structural analysis of the oligosaccharide from the *U. pertusa* AEGL. (A) TLC showing the oligosaccharide released from AEGL by EGALC. Purified AEGL was incubated without (−) or with (+) EGALC (1 mU) in 50 mM sodium acetate buffer, pH 5.5. (B) The MALDI-TOF MS spectrum of the AEGL-derived oligosaccharide in positive ion mode. (C) GC analysis of the sugar components of AEGL using their TMS derivatives. (D) Sensitivity of AEGL to SCDase. After SCDase treatment, LacCer and AEGL were subjected to labeling with OPA and analyzed by HPLC.

Table 1
^{1}H and ^{13}C chemical shifts (ppm) for the *U. pertusa* AEGL.

Position	^{13}C	^{1}H	Position	^{13}C	^{1}H
1	70.0 (t)	4.11 (2H)	β-Gal I-1	105.9 (d)	4.78 (d, 7.6)[a]
2	122.0 (d)	5.51 (brt)	2	72.6 (d)	4.44
3	140.0 (s)	1.99 (2H, m)	3	75.1 (d)	4.18
4	40.1 (t)	1.99 (2H, m)	4	70.3 (d)	4.48
5	25.5 (t)	1.99 (2H, m)	5	75.5 (d)	4.14
6	37.0 (t)	1.99 (2H, m)	6	68.2 (t)	4.30, 4.50
7	33.0 (d)	1.47 (1H, m)			
8	37.7 (t)	1.99 (2H, m)	α-Gal II-1	101.4 (d)	5.48 (d, 3.3)[a]
9	24.8 (t)	1.99 (2H, m)	2	70.6 (d)	4.65
10	37.7 (t)	1.99 (2H, m)	3	71.7 (d)	4.57
11	33.1 (d)	1.47 (1H, m)	4	70.9 (d)	4.68
12	37.5 (t)	1.99 (2H, m)	5	72.7 (d)	4.59
13	25.1 (t)	1.99 (2H, m)	6	62.4 (t)	4.40 (2H)
14	39.6 (t)	1.99 (2H, m)			
15	28.3 (d)	1.47 (1H, m)	Glycerol (g)-1	72.7 (t)	3.77 (2H, d, 5.5)[a]
16	22.8 (q)	0.87 (12H, m)	g-2	70.7 (d)	4.42
A	16.4 (q)	1.61 (brt)	g-3	73.0 (t)	4.12, 4.31
B	19.9 (q)	0.87 (12H, m)			
C	19.9 (q)	0.87 (12H, m)			
D	22.7 (q)	0.87 (12H, m)			

[a] Coupling constant.

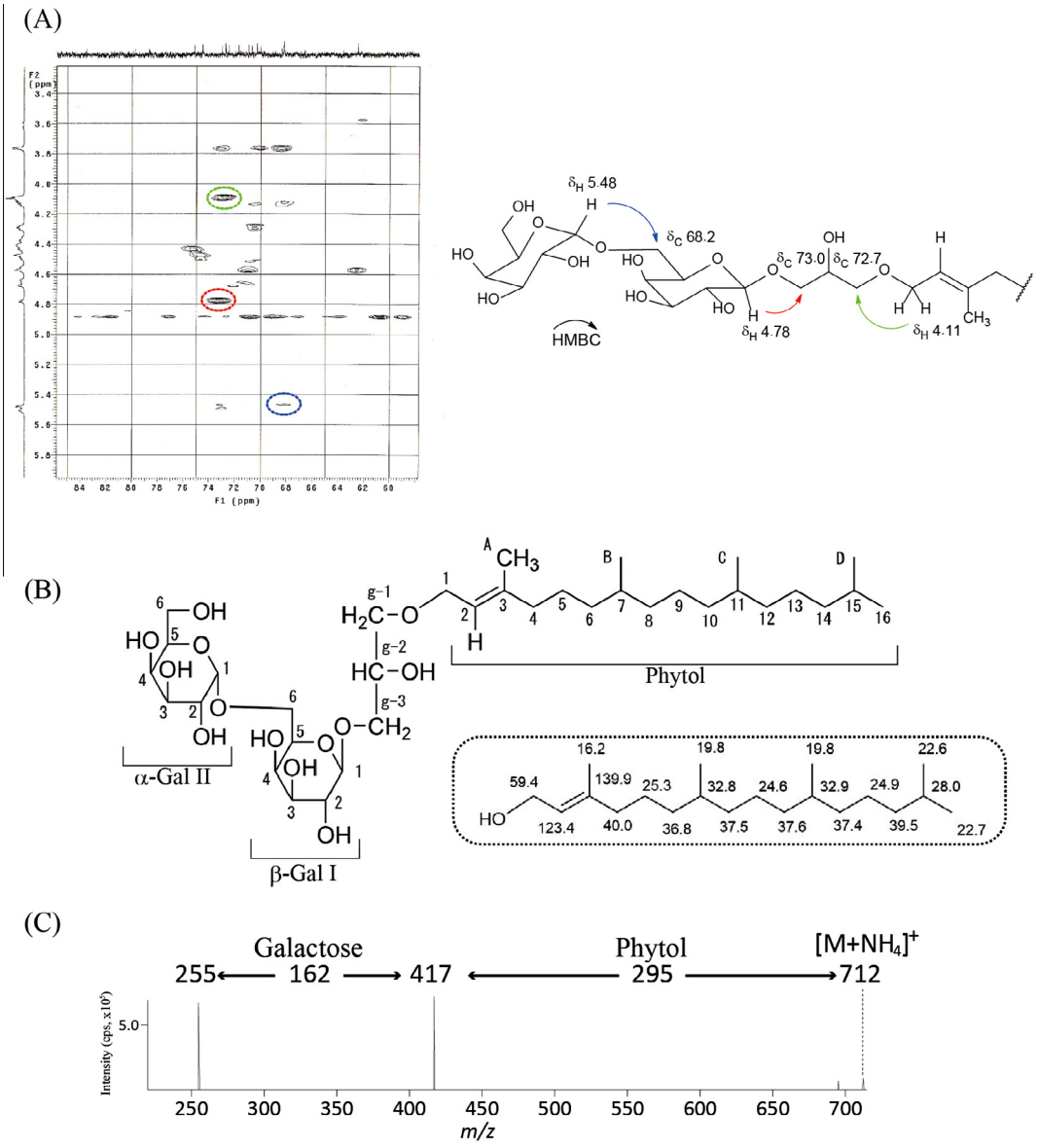

Fig. 4. Determination of the structure of the *U. pertusa* AEGL. (A) HMBC spectra and selected correlations of AEGL. (B) The proposed structure of AEGL determined by ^{1}H and ^{13}C NMR. Chemical shifts in each atom of β-Gal I, α-Gal II, glycerol, and phytol in AEGL are summarized in Table 1. The ^{13}C-NMR chemical shifts assignment of phytol [15] are shown in a dotted box. (C) The MS/MS spectrum of AEGL.

glycerol [δ_C 73.0] revealed that AEGL had a glycerol backbone to which Galα1-6Gal was connected via a β-linkage; the oligosaccharide structure of AEGL was identical to that of DGDG (Fig. 4A). However, the hydrophobic chain of AEGL at *sn*-1 was completely different from that of DGDG; the chain of AEGL was shown to be the phytol, whereas that of DGDG was a fatty acid (Fig. 4B). The HMBC correlation between the H_2-1 of phytol [δ_H 4.11] and C-1 of glycerol [δ_C 72.7] revealed the presence of an ether-linked phytol (Fig. 4A,B), which was not contradictory to the alkaline tolerance of AEGL (Fig. 1A). Fragment ions from AEGL also indicated the presence of phytol in ESI-MS/MS analysis (Fig. 4C). These results indicated that the structure of *U. pertusa* AEGL was 1-*O*-(2E)-3,7,11,15-tetramethyl-2-hexadecenyl-3-*O*-(α-D-galactopyranosyl-(1→6)-β-D-galactopyranosyl)-*sn*-glycerol, and it is classified as 'glycosylmonoalkylglycerol' in LIPID MAPS. To the best of our knowledge, AEGL is the first GGL to be identified that contains an ether-linked phytol.

The *sn*-2 position of the glycerol backbone in AEGL was determined to be a free hydroxyl group (Fig. 4B) and lyso-forms of AEGL lacking fatty acids at sn-2 were not found in the marine alga *U. pertusa* without alkaline treatment. These results suggest that ester-linked fatty acids were detached from the native AEGL after alkaline treatment. This treatment is, however, integral to remove bulky DGDG from the glycolipid fraction of *U. pertusa* (Fig. 2C), and, thus, the fatty acid at *sn*-2 in native AEGL was not determined in this study.

3.5. Distribution of AEGL in photosynthetic organisms

We isolated AEGL fractions, which contain alkaline-resistant and EGALC-sensitive glycolipids with the same R_f value of *U. pertusa* AEGL, from the green alga *C. fragile*, red alga *G. acuminate*, and brown alga *S. fusiforme*. To examine whether the AEGL fractions contain GSLs, we used SCDase, which hydrolyzes GSLs, but

Table 2
Screening of AEGLs from various photosynthetic organisms. Fluorescent-labeled oligosaccharides were detected by TLC after the treatment of samples with EGALC in the presence of NBD-pentanol. ++, highly positive; +, weakly positive; –, not detected.

Green algae		Brown algae		Terrestrial plant	
Genus and species	EGALC-sensitivity	Genus and species	EGALC-sensitivity	Genus and species	EGALC-sensitivity
Ulva pertusa	++	*Sargassum horneri*	++	*Spinacia oleracea*	+
Ulva ohonoi	++	*Sargassum autumnale*	++	*Eucalyptus camaldulensis*	+
Enteromorpha sp.	++	*Sargassum thunbergii*	++	*Arabidopsis thaliana*	–
Codium fragile	++	*Sargassum micracanthum*	++	*Brassica oleracea*	–
Caulerpa racemosa	++	*Sargassum nigrifolium*	++	*Raphanus sativus*	–
Monostroma nitidum	++	*Sargassum fusiforme*	++	*Glycine max*	–
Red algae		*Sargassum ringgoldianum*	++	*Populus alba*	–
Genus and species	EGALC-sensitivity	*Leathesia difformis*	++	Eukaryotic phytoplankton	
Campylaephora hypnaeoides	++	*Eisenia bicyclis*	++	Genus and species	EGALC-sensitivity
Gracilaria vermiculophylla	++	*Hydroclathrus clathratus*	++	*Heterosigma akashiwo*	–
Grateloupia acuminata	++	*Laminaria japonica*	++	*Thalassiosira pseudonana*	–
Polyopes affinis	++	*Myagropsis myagroides*	++	Cyanobacteria	
Porphyra yezoensis	++	*Undaria pinnatifida*	++	Genus and species	EGALC-sensitivity
				Synechocystis sp.	–
				Anabaena sp.	–

not AEGL, to generate lyso-GSLs and fatty acids. As shown in Supplemental Fig. 1, AEGL fractions from the 4 genera of marine algae were insensitive to SCDase, indicating that these fractions did not contain GSLs. These results indicate that AEGL is distributed not only in green algae, but also in red and brown algae. Furthermore, AEGL was found in 6 species of green algae, 5 species of red algae, and 13 species of brown algae; however, it was rarely found in the terrestrial plants, eukaryotic phytoplankton, or cyanobacteria under the conditions tested in this study (Table 2). These results suggest that AEGL may be a characteristic component of multicellular marine algae.

The synthesis pathway of AEGL is unclear at present. In mammals, ether-linked lipids are generated via the dihydroxyacteone phosphate (DHAP) pathway. In this pathway, DHAP is converted to acyl-DHAP by DHAP acyltransferase [16], followed by exchanging the acyl group to alkyl group, generating ether-linked lipids. Judging from the genome database [17], DHAP acyltransferase homologues are not present in the marine alga, and, thus, AEGL is not likely to be generated by this pathway. Alternatively, we hypothesize that the ether-linked phytol is generated from the geranylgeranyl-diphosphate (GGPP) pathway, which is shown in the synthesis pathway of ether-lipids in Archaea [18]. GGPP is converted in Archaea to an ether-linked glycerolipid, 3-*O*-geranylgeranylglyceryl phosphate (GGGP), by 2,3-di-*O*-geranylgeranylglyceryl phosphate synthase (DGGGPS). In the synthesis pathway of AEGL, GGPP could be converted to phytyl-diphosphate (phytyl PP) by GGPP reductase (GGPPR) [19], and then incorporated into a glycerol backbone. It is worth noting that homologues of DGGGPS and GGPPR are present in the marine algal genome database, although the function of these homologues are entirely unknown.

The function of AEGLs currently remains unclear; however, a functional relationship/interaction may exist between AEGL and DGDG/MGDG or AEGL and chlorophyll in thylakoid membranes. Since DGDG and MGDG have been shown to have different roles in the organization and stabilization of the thylakoid membrane [20,21], the novel GGL in this study could have a specific role in photosynthesis in marine algae. AEGL may contribute to effective light-harvesting in marine environments, in which photosynthetic organisms must collect photics efficiently because light intensity is weaker than that in land environments. The fatty acid composition of GGL was previously shown to be altered by light intensity [22], suggesting that the structure of the lipid moiety of GGL may be somewhat related to the environmental adaption of photosynthetic organisms. In this context, it is worth noting that the *U. pertusa* AEGL was detected in the chloroplast-enriched fraction (Supplemental Fig. 2A,B).

One distinct feature of the *U. perutusa* AEGL is the presence of an ether linkage in the glycerol backbone; however, its biological significance is unclear. The loss of ether-linked fatty acid-containing phospholipids affect membrane fluidity and traffic in mammalian cells, which suggested a specific role of ether-linked lipids in membrane functions [23,24].

AEGL may act as a reservoir for free phytol in marine algae when chlorophyll are decomposed into chlorophyllide under stress or aging conditions [25], because free phytol is thought to be highly toxic to proteins in the thylakoid membrane. In plant cells, free phytol was shown to be converted to fatty acid phytyl esters for detoxification [26].

In conclusion, we identified a novel GGL consisting of an α1,6-galactobiose and glycerol backbone bound to an ether-linked phytol from marine green alga. To understand the functions of AEGL *in vivo* and elucidate why AEGL was exclusively present in multicellular marine algae, the identification of enzymes involved in the metabolic pathways of AEGL, especially the enzyme(s) responsible for the formation of ether-linked phytol, is required.

Acknowledgments

This work was supported in part by Basic Research B from the Japanese Ministry of Education, Culture, Sports, Science and Technology (to M.I.), and the Japan Society for the Promotion of Science for Young Scientists (to Y.I.). We thank Drs. Hiroyuki Wariishi and Shigeo Kawaguchi, Kyushu University for their technical assistances. We are deeply grateful to Drs. Mari Inoue, Takashi Yuasa, Yohei Shimasaki, Tomonao Matsushita, and Eiji Goto, Kyushu University, and Dr. Shusei Sato and Ms. Reiko Muneto, the Kazusa DNA Research Institute, for donating photosynthetic organisms.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.08.056.

References

[1] G. Holzl, P. Dormann, Structure and function of glycoglycerolipids in plants and bacteria, Prog. Lipid Res. 46 (2007) 225–243.
[2] K. Kobayashi, M. Kondo, H. Fukuda, et al., Galactolipid synthesis in chloroplast inner envelope is essential for proper thylakoid biogenesis, photosynthesis, and embryogenesis, Proc. Natl. Acad. Sci. U.S.A. 104 (2007) 17216–17221.
[3] L. Boudiere, M. Michaud, D. Petroutsos, et al., Glycerolipids in photosynthesis: composition, synthesis and trafficking, Biochim. Biophys. Acta 1837 (2014) 470–480.
[4] R.E. Blankenship, Molecular Mechanisms of Photosynthesis, Blackwell Scientific, 2002.
[5] Y. Ishibashi, T. Nakasone, M. Kiyohara, et al., A novel endoglycoceramidase hydrolyzes oligogalactosylceramides to produce galactooligosaccharides and ceramides, J. Biol. Chem. 282 (2007) 11386–11396.
[6] Y. Ishibashi, Y. Nagamatsu, S. Meyer, et al., Transglycosylation-based fluorescent labeling of 6-gala series glycolipids by endogalactosylceramidase, Glycobiology 19 (2009) 797–807.
[7] Y. Ishibashi, M. Kiyohara, N. Okino, et al., Synthesis of fluorescent glycosphingolipids and neoglycoconjugates which contain 6-gala oligosaccharides using the transglycosylation reaction of a novel endoglycoceramidase (EGALC), J. Biochem. 142 (2007) 239–246.
[8] T. Matsubara, A. Hayashi, Structural studies on glycolipid of shellfish. III. Novel glycolipids from *Turbo cornutus*, J. Biochem. 89 (1981) 645–650.
[9] J. Folch, M. Lees, G.H. Sloane Stanley, A simple method for the isolation and purification of total lipides from animal tissues, J. Biol. Chem. 226 (1957) 497–509.
[10] N. Matsunaga, S.-I. Chisada, H. Fujioka, et al., Glycosphingolipid receptors for pathogenic vibrios in intestines of mariculture fish, Fish. Sci. 77 (2011) 583–590.
[11] T. Taki, T. Kasama, S. Handa, et al., A simple and quantitative purification of glycosphingolipids and phospholipids by thin-layer chromatography blotting, Anal. Biochem. 223 (1994) 232–238.
[12] M. Furusato, N. Sueyoshi, S. Mitsutake, et al., Molecular cloning and characterization of sphingolipid ceramide *N*-deacylase from a *Marine bacterium*, *Shewanella alga* G8, J. Biol. Chem. 277 (2002) 17300–17307.
[13] K. Zama, Y. Hayashi, S. Ito, et al., Simultaneous quantification of glucosylceramide and galactosylceramide by normal-phase HPLC using *O*-phtalaldehyde derivatives prepared with sphingolipid ceramide *N*-deacylase, Glycobiology 19 (2009) 767–775.
[14] M. Ito, T. Kurita, K. Kita, A novel enzyme that cleaves the *N*-acyl linkage of ceramides in various glycosphingolipids as well as sphingomyelin to produce their lyso forms, J. Biol. Chem. 270 (1995) 24370–24374.
[15] R.A. Goodman, E. Oldfield, A. Allerhand, Assignments in the natural-abundance carbon-13 nuclear magnetic resonance spectrum of chlorophyll a and a study of segmental motion in neat phytol, J. Am. Chem. Soc. 95 (1973) 7553–7558.
[16] A.K. Hajra, Dihydroxyacetone phosphate acyltransferase, Biochim. Biophys. Acta 1348 (1997) 27–34.
[17] Y. Nakamura, N. Sasaki, M. Kobayashi, et al., The first symbiont-free genome sequence of marine red alga, susabi-nori (*Pyropia yezoensis*), PLoS One 8 (2013) e57122.
[18] Y. Koga, H. Morii, Biosynthesis of ether-type polar lipids in archaea and evolutionary considerations, Microbiol. Mol. Biol. Rev. 71 (2007) 97–120.
[19] T. Ischebeck, A.M. Zbierzak, M. Kanwischer, et al., A salvage pathway for phytol metabolism in *Arabidopsis*, J. Biol. Chem. 281 (2006) 2470–2477.
[20] A.G. Lee, Membrane lipids: it's only a phase, Curr. Biol. 10 (2000) R377–R380.
[21] M.S. Webb, B.R. Green, Biochemical and biophysical properties of thylakoid acyl lipids, Biochim. Biophys. Acta 1060 (1991) 133–158.
[22] A. Burgos, J. Szymanski, B. Seiwert, et al., Analysis of short-term changes in the *Arabidopsis thaliana* glycerolipidome in response to temperature and light, Plant J. 66 (2011) 656–668.
[23] A. Hermetter, B. Rainer, E. Ivessa, et al., Influence of plasmalogen deficiency on membrane fluidity of human skin fibroblasts: a fluorescence anisotropy study, Biochim. Biophys. Acta 978 (1989) 151–157.
[24] T.P. Thai, C. Rodemer, A. Jauch, et al., Impaired membrane traffic in defective ether lipid biosynthesis, Hum. Mol. Genet. 10 (2001) 127–136.
[25] S. Hortensteiner, Chlorophyll breakdown in higher plants and algae, Cell. Mol. Life Sci. 56 (1999) 330–347.
[26] F. Lippold, K. vom Dorp, M. Abraham, et al., Fatty acid phytyl ester synthesis in chloroplasts of Arabidopsis, Plant Cell 24 (2012) 2001–2014.