Analysis of triacylglycerol accumulation under nitrogen deprivation in the red alga Cyanidioschyzon merolae

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Triacylglycerol (TAG) produced by microalgae is a potential source of biofuel. Although various metabolic pathways in TAG synthesis have been identified in land plants, the pathway of TAG synthesis in microalgae remains to be clarified. The unicellular rhodophyte Cyanidioschyzon merolae has unique properties as a producer of biofuel because of easy culture and feasibility of genetic engineering. Additionally, it is useful in the investigation of the pathway of TAG synthesis, because all of the nuclear, mitochondrial and plastid genomes have been completely sequenced. We found that this alga accumulated TAG under nitrogen deprivation. Curiously, the amount and composition of plastid membrane lipids did not change significantly, whereas the amount of endoplasmic reticulum (ER) lipids increased with considerable changes in fatty acid composition. The nitrogen deprivation did not decrease photosynthetic oxygen evolution per chlorophyll significantly, while phycobilisomes were degraded preferentially. These results suggest that the synthesis of fatty acids is maintained in the plastid, which is used for the synthesis of TAG in the ER. The accumulated TAG contained mainly 18 : 2(9,12) at the C-2 position, which could be derived from phosphatidylcholine, which also contains this acid at the C-2 position.

INTRODUCTION

Microalgae-based biofuels have attracted attention as an alternative to land plant oils in recent years (Georgianna & Mayfield, 2012; Leite et al., 2013; Oncel et al., 2014). Microalgae can convert solar energy and recycle CO₂ into fuels efficiently (Dismukes et al., 2008). The production of biofuels by land plants competes with the food industry in available area and feedstock. In order to avoid competition with the food industry, it is preferable to use non-arable land or land water, in which various strains of microalgae grow under unique or extreme conditions, such as at high temperatures, at high salinity, or in acidic or alkaline pH. As a biofuel source, microalgae are able to produce triacylglycerol (TAG) and starch (Hu et al., 2008; Scott et al., 2010), which can be converted to biodiesel by chemical transesterification, and to bioethanol by alcohol fermentation, respectively (Aikawa et al., 2012, 2013). In many algae, TAG and starch are accumulated under the conditions of nutrient deficiency (Aikawa et al., 2012; Chisti et al., 2007; Hicks et al., 2001; Hu et al., 2008; Tornabene et al., 1983). Although many metabolic reactions leading to TAG synthesis are identified in land plants (Barron & Stumpf, 1962; Bates et al., 2013; Kennedy, 1961; Lung & Weselake, 2006; Weiss et al., 1960), pathways of TAG synthesis remain to be clarified in microalgae. In plants, TAG is synthesized through the following two pathways (Bates et al., 2013): one is the de novo synthesis of TAG by acylation of glycerol 3-phosphate with acyl-CoAs (Barron & Stumpf, 1962; Kennedy, 1961; Lung & Weselake, 2006; Weiss et al., 1960); another is a more complex pathway in which the membrane lipid phosphatidylcholine (PC) acts as a supplier of fatty acids and/or diacylglycerol (DAG) (Bates et al., 2013). In the PC pool, fatty acids are desaturated,
and polyunsaturated fatty acids are synthesized (Bates & Browse, 2012; Sperling et al., 1993; van de Loo et al., 1995; Wallis et al., 2002). Accordingly, the PC-mediated pathway is important as the supply of diverse fatty acids for TAG.

The unicellular rhodophyte *Cyanidioschyzon merolae*, originally isolated from an Italian acidic hot spring (De Luca et al., 1978), can be cultivated in the places where agricultural use is difficult, because it grows vigorously at high temperatures (up to 50 °C) (Moriyama et al., 2007) and at acidic pH from 1.5 to 2.5. These growth conditions do not allow the growth of most ordinary contaminants. *Cy. merolae* has a very simple cell structure consisting of a single mitochondrion, a single plastid and a single microbody per cell (Kuroiwa, 1998). The nuclear, mitochondrial and plastid genomes have been sequenced (Matsuzaki et al., 2004; Nozaki et al., 2007; Ohta et al., 1998, 2003). A nuclear genetic engineering (Ohnuma et al., 2008) and synchronous culture system (Itoh et al., 1996) have been established. These genetic technologies are expected to improve growth performance and to produce TAG as shown by previous studies (Imamura et al., 2015; Sumiya et al., 2015). The fatty acid composition of lipid classes is very simple, consisting of mainly 16:0, 18:0, 18:1(9) and 18:2(9,12) fatty acids (Sato & Moriyama, 2007). The absence of 18:3 or highly unsaturated fatty acids is a benefit of using this alga to produce lipids. Because it is not only ER lipids but also plastid lipids are synthesized "coupled pathway", namely, using the plastid-derived fatty acids. Consequently, there is a major flow of fatty acids from the plastid to the ER. The biosynthetic pathway of membrane lipids such as phospholipids and galactolipids has been largely clarified, whereas the biosynthesis of TAG has not been studied.

To evaluate the potential in *Cy. merolae* to synthesize high levels of TAG and to initiate analysis of TAG biosynthesis studies, it is important to characterize physiologically the TAG biosynthesis under nitrogen starvation. For this purpose, we observed the accumulation of lipids and starch by microscopy, and analysed the composition of lipids and fatty acids in *Cy. merolae* under nitrogen deprivation. We also measured the photosynthetic activity and the contents of pigments.

**METHODS**

**Growth of Cy. merolae.** Cells of *Cy. merolae* strain 10D (Toda et al., 1998) were grown under continuous white light (50 μmol m⁻² s⁻¹) in 2X Allen’s medium (pH 2.5; Minoda et al., 2004) (hereafter denoted 2X Allen), with aeration using air containing 1% CO₂ at 40 °C. In the nitrogen-deprived medium [hereafter denoted 2X Allen(−N)], 20 mM (NH₄)₂SO₄ was substituted with 20 mM Na₂SO₄. Before deprivation of nitrogen, the cells were grown in the 2X Allen medium for 5 days (OD₅₅₀ of 3–5). For the nitrogen-rich or -free experiments, the culture was divided into two parts, which were washed twice with either 2X Allen or 2X Allen(−N), respectively, and then resuspended in either 2X Allen or 2X Allen(−N). The cells were then grown on 2X Allen and 2X Allen(−N) for 2 days, and were sampled for the analysis of lipids.

**BODIPY staining.** BODIPY staining and fluorescence microscopy were carried out essentially as described previously (Toyoshima & Sato, 2015).

**Measurements of photosynthetic oxygen evolution and chlorophyll content.** All the analytical methods were essentially identical to those described previously (Moriyama et al., 2015; Sakurai et al., 2014). Cells were kept in the dark at 40 °C for 10 min and the oxygen consumption rate (A) was recorded. The culture was then aerated with air containing 1% (v/v) CO₂ for 2 min to supply CO₂. Next, the cells were irradiated by saturating light of 6000 μmol m⁻² s⁻¹ for 2 min and the oxygen evolution rate (B) was recorded. Photosynthetic oxygen evolution rate was calculated as (B−A). For the determination of the chlorophyll content, a 200 μl culture was mixed with 800 μl acetone. After a brief centrifugation in a microfuge at top speed, the supernatant was used for spectrophotometry at 710 and 630 nm using a spectrophotometer (model UV-160A; Shimadzu). Note that red algae have only chlorophyll a (Wolfe et al., 1994). The amount of chlorophyll is the amount of chlorophyll a. Chlorophyll a was determined as described by Porra et al. (1989).

**Absorption spectrum of cell suspension.** Absorption spectra of cell suspensions were measured according to the so-called ‘opal glass method’, with a translucent cuvette placed in front of the detector to minimize the effect of light scattering.

**Detection of phycobiliproteins.** The phycobiliproteins were detected by zinc acetate staining as described by Berkelman & Lagarias (1986). Protein (20 μg) of the whole cell was separated by SDS-PAGE with 15% polyacrylamide gel followed by staining with 10 mM zinc acetate; phycobiliproteins were then detected under UV light at 302 nm. Subsequently, the polyacrylamide gel was stained with Coo massie brilliant blue to visualize all proteins.

**Extraction and separation of lipids.** Cells were harvested by centrifugation (3000 g, 10 min, at 4 °C). Total lipids were extracted according to the method of Bligh & Dyer (1959). The final chloroform phase was evaporated under vacuum. The lipids were dissolved in 0.2 ml chloroform/methanol (2:1, v/v) and 0.2 ml ethanol, and
stored at −20 °C until use. Lipid classes were separated by 2D-TLC essentially as described previously (Sakurai et al., 2014).

**Analysis of lipids and fatty acids.** All analytical methods were essentially identical to those described previously (Sakurai et al., 2014). Each lipid class was identified by MALDI-TOF MS using a model AXIMA-CFR Plus (Shimadzu). Molecular species of TAG was also analysed by MALDI-TOF MS. Lipids were quantified as amount of fatty acids analysed as methyl esters (FAMEs) by gas chromatography. Identity of FAME or fatty acid pyrolylides that were derived from FAMEs was confirmed by GC-MS (model GCMS-QP2010 Ultra; Shimadzu).

The positional distribution of fatty acids within TAG was analysed by limited hydrolysis of the acyl ester linkage at C-1 and C-3 with the lipase from Rhizopus niveus (62310; Sigma-Aldrich) (Alam et al., 2015; Kohno et al., 1994).

**Lugol staining.** Lugol staining was carried out essentially as described previously (Toyoshima & Sato, 2015). The cells were harvested by centrifugation (600 g, 5 min). Cell pellets were resuspended in 1 ml Lugol solution (I2-KI) to stain starch. The stained cells were examined under bright-field with a BX60 microscope (Olympus).

**Quantification of starch content.** Cells were harvested by centrifugation (1000 g, 5 min). The quantification of starch content was carried out essentially as described previously (Toyoshima & Sato, 2015).

**Transmission electron microscopy.** Transmission electron microscopy was carried out essentially as described previously (Sato et al., 2014).

### RESULTS

**Oil body accumulation in Cy. merolae grown under nitrogen deprivation**

BODIPY staining was used to observe the accumulation of nonpolar lipids consisting of TAG in the *Cy. merolae* cells grown on 2 × Allen or 2 × Allen(−N) (Fig. 1). The culture was sampled for BODIPY staining 2 days after nitrogen deprivation. At the time, the cell densities of both cultures were approximately 1.0 × 10⁹ cells ml⁻¹, whereas OD₇₅₀ of cultures grown on 2 × Allen and 2 × Allen(−N) was 5.1 ± 0.4 and 3.9 ± 0.4, respectively (Fig. S1, available in the online Supplementary Material). No particulate green fluorescence of BODIPY bound to nonpolar lipid, as detected with the NIBA filter, was detected in the cells grown on 2 × Allen, indicating absence of oil bodies. In contrast, distinct particulate green fluorescence was detected in the cells grown on 2 × Allen(−N), indicating that oil bodies accumulated to a significant level. This finding indicated that the *Cy. merolae* cells produce nonpolar lipid abundantly under nitrogen deprivation.

**Physiological changes under nitrogen deprivation**

Chlorophyll content, photosynthetic oxygen evolution rate and absorption spectrum were measured in the cells of *Cy. merolae* grown on 2 × Allen or 2 × Allen(−N), respectively (Fig. 2). Chlorophyll *a* content in the *Cy. merolae* cells grown on 2 × Allen(−N) was significantly lower than that in the cells grown on 2 × Allen (Fig. 2a). The rate of photosynthetic oxygen evolution per cell was also lower in the nitrogen-deficient cells than in the control (Fig. 2b). However, the rate of photosynthetic oxygen evolution per chlorophyll was comparable under both conditions (Fig. 2c).

The absorption spectrum of the cell suspension grown on 2 × Allen exhibited clearly the two peaks of pigments representing phycocyanins (630 nm) and chlorophyll *a* (680 nm) (Fig. 2d). In the cell suspension grown on 2 × Allen(−N), the peaks of these pigments were lower than those in the control (Fig. 2d). Apparently, phycocyanins were mostly lost in the nitrogen-deficient condition. This is consistent with the results of detection of phycobiliproteins by SDS-PAGE (Fig. S2). These results suggest that a low activity of photosynthesis remained at 2 days after nitrogen deprivation, mainly due to the decrease in chlorophyll *a* content in the cell, keeping the photosynthetic activity per chlorophyll *a*.

**Composition of lipids and fatty acids in Cy. merolae**

Total lipids of *Cy. merolae* were separated by 2D-TLC. Fig. S3 shows the fractionation of total lipids from the cells grown on 2 × Allen(−N). In addition to PC, phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidyglycerol (PG), digalactosyl diacylglycerol (DGDG), sulfoquinovosyl diacylglycerol (SQDG) and MGDG that were detected in a previous study (Sato & Moriyama, 2007), phosphatidic acid (PA) and TAG were also detected. Each lipid class was recovered from the...
plate and its identity was confirmed by MALDI-TOF MS. Other spots that were not further analysed included putative wax esters and free fatty acids.

The recovered lipid classes were converted to FAMEs, which were identified by GC-MS. In addition to fatty acids described in a previous report (Sato & Moriyama, 2007), 17:1(9), 19:0, 19:1(9), 20:0, 20:1(11) and 20:2(11,14) fatty acids were also detected as minor components (Tables S1 and S2). The presence of C20 acids is consistent with the presence of an elongation pathway in Cy. merolae, which was suggested from the genomic data.

**Accumulation of TAG in Cy. merolae grown on 2 × Allen(– N)**

The contents of lipids were determined in the culture after 2 days of growth in 2×Allen and 2×Allen(–N) (Fig. 3). The total lipid contents were 18.9 ± 1.6 % and 21.3 ± 2.0 % of total dry cell weight in 2×Allen and 2×Allen(–N), respectively (Fig. S4). The content of total lipids per cell also increased in the cells grown on 2×Allen(–N). In the total lipids, the contents of ER lipids such as PC, PE, PA and TAG increased. The content of TAG in the cells grown on 2×Allen(–N) was 45.6 ± 0.4 pmol (10^6 cells)^{-1}, which corresponded to 26 mol% of total lipids. This value was 14 times higher with respect to the TAG level in the control cells [3.4 ± 0.4 pmol (10^6 cells)^{-1}]. These results were consistent with the observation of a high accumulation of oil bodies in the nitrogen-limited cells (Fig. 1). The contents of plastid membrane lipids such as PG, SQDG, MGDG and DGDG did not decrease under nitrogen deprivation.

**Composition of fatty acids in Cy. merolae grown on 2 × Allen(– N)**

The compositions and contents of major fatty acids in the cells grown on 2×Allen and 2×Allen(–N) are shown in Tables S1 and S2, and summarized in Table 1. Specific distribution of fatty acids among lipid classes was found to be as reported previously (Sato & Moriyama, 2007). In the cells grown on 2×Allen, 16:0 and 18:2(9,12) were detected at relatively high levels in most classes of lipids. 18:1(9) was abundant in PC, PE, PA and PI (Tables 1 and 2).
Table 1. Composition and content of fatty acids in cells of *Cy. merolae* grown on 2 × Allen and 2 × Allen (− N) for 2 days

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>PC</th>
<th>PE</th>
<th>PA</th>
<th>PI</th>
<th>PG</th>
<th>DGDG</th>
<th>SQDG</th>
<th>MGDG</th>
<th>TAG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ N</td>
<td>− N</td>
<td>+ N</td>
<td>− N</td>
<td>+ N</td>
<td>− N</td>
<td>+ N</td>
<td>− N</td>
<td>+ N</td>
</tr>
<tr>
<td>16 : 0</td>
<td>30.7</td>
<td>29.6</td>
<td>38.7</td>
<td>35.3</td>
<td>33.3</td>
<td>30.8</td>
<td>36.9</td>
<td>33.0</td>
<td>44.8</td>
</tr>
<tr>
<td>16 : 1(3t)</td>
<td>0.0</td>
<td>0.2</td>
<td>0.0</td>
<td>0.1</td>
<td>0.0</td>
<td>1.0</td>
<td>0.0</td>
<td>0.0</td>
<td>1.6</td>
</tr>
<tr>
<td>17 : 0</td>
<td>2.2</td>
<td>3.4†</td>
<td>2.5</td>
<td>3.7†</td>
<td>4.0</td>
<td>2.1†</td>
<td>4.4</td>
<td>5.4†</td>
<td>0.4</td>
</tr>
<tr>
<td>18 : 0</td>
<td>4.3</td>
<td>5.7</td>
<td>5.1</td>
<td>7.0</td>
<td>17.6</td>
<td>6.1†</td>
<td>14.9</td>
<td>17.4†</td>
<td>1.5</td>
</tr>
<tr>
<td>18 : 1(9)</td>
<td>27.3</td>
<td>16.6*</td>
<td>30.9</td>
<td>34.2</td>
<td>26.1</td>
<td>14.5*</td>
<td>24.8</td>
<td>17.8*</td>
<td>6.6</td>
</tr>
<tr>
<td>18 : 2(9,12)</td>
<td>30.4</td>
<td>41.8†</td>
<td>18.9</td>
<td>16.0</td>
<td>16.6</td>
<td>42.1†</td>
<td>16.5</td>
<td>24.6†</td>
<td>44.0</td>
</tr>
<tr>
<td>20 : 1(11)</td>
<td>0.8</td>
<td>0.3*</td>
<td>0.4</td>
<td>0.1</td>
<td>0.0</td>
<td>0.8</td>
<td>0.0</td>
<td>0.1</td>
<td>0.0</td>
</tr>
<tr>
<td>20 : 2(11,14)</td>
<td>1.9</td>
<td>0.9</td>
<td>1.1</td>
<td>2.8</td>
<td>0.0</td>
<td>0.2</td>
<td>1.5</td>
<td>1.4</td>
<td>0.2</td>
</tr>
<tr>
<td>Amount of lipids</td>
<td>10.0</td>
<td>14.7</td>
<td>8.3</td>
<td>10.1</td>
<td>0.6</td>
<td>0.7</td>
<td>1.9</td>
<td>2.2</td>
<td>9.8</td>
</tr>
</tbody>
</table>

*Fatty acids that showed a decrease in content with respect to those in cells grown on 2 × Allen at a confidence level of 95 %.
†Fatty acids that showed an increase in content with respect to those in cells grown on 2 × Allen at a confidence level of 95 %.

Positional distribution of fatty acids in TAG

The positional distribution of fatty acids in TAG of *Cy. merolae* grown on 2 × Allen (− N) was examined by limited hydrolysis of the acyl ester linkage at the C-1 and C-3 positions with the lipase from *R. niveus*. The results of TLC (Fig. S6) of the reaction products showed the presence of remaining TAG, free fatty acid and 1,2/2,3-DAG from the top to the bottom. The individual components were converted to FAMEs and analysed to determine the positional distribution of fatty acids in the original TAG. The results showed that the TAG contained mainly 18 : 2(9,12) at the C-2 position (Table 2).

Accumulation of starch in *Cy. merolae* grown on 2 × Allen (− N)

We measured the content of starch, which is thought to compete with the synthesis of lipids (Fig. 4). Lugol solution stains crystallized starch in red–violet. The cells grown in 2 × Allen did not show specific staining with Lugol (Fig. 4a), whereas the cytosol of the cells grown on 2 × Allen (− N) became significantly red–violet with the staining, indicating that starch accumulated under nitrogen deprivation. The content of starch in the cells grown on 2 × Allen (− N) was approximately 0.96 pg cell−1, whereas that in the cells grown on 2 × Allen was approximately 0.19 μg cell−1 (Fig. 4b).

Ultrastructure of the cells

Fig. 5 shows the ultrastructure of the *Cy. merolae* cells grown under the two conditions. In the cells grown on 2 × Allen, a nucleus (labelled ‘N’), a mitochondrion (labelled ‘Mt’), a chloroplast (labelled ‘Cp’), a lysosome (labelled ‘L’) and a vacuole (labelled ‘V’) were observed (Fig. 5a–d). In the chloroplast, thylakoid membranes were observed clearly as an annual-ring-like form. Depending on the orientation of sectioning, arrays of phycobilisomes were found (Fig. 5d). Starch granules and oil bodies were rarely observed.

In the cells grown on 2 × Allen (− N), numerous starch granules (labelled ‘S’) and some oil bodies (labelled ‘O’) were rarely observed.

Table 2. Positional distribution of fatty acids in TAG

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Content (mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C-1/C-3</td>
</tr>
<tr>
<td>16 : 0</td>
<td>22</td>
</tr>
<tr>
<td>18 : 0</td>
<td>31</td>
</tr>
<tr>
<td>18 : 1(9)</td>
<td>16</td>
</tr>
<tr>
<td>18 : 2(9,12)</td>
<td>31</td>
</tr>
</tbody>
</table>

and S1). The fatty acid contents were similar to one another in PG, DGDG and MGDG. 16 : 0 was abundant in TAG.

In the cells grown on 2 × Allen (− N), the proportion of 18 : 2(9,12) was higher in PC and PA, whereas that of 18 : 0 and 18 : 1(9) was lower with respect to the control at a confidence level of 95 % (Tables 1 and S2). PC and PA showed similar fatty acid contents. In PI, the proportion of 18 : 0 and 18 : 2(9,12) was higher, whereas that of 16 : 0 and 18 : 1(9) was lower in the cells grown on 2 × Allen (− N).

The fatty acid contents in PE, PG, DGDG, SQDG and MGDG did not change significantly with or without nitrogen supply. In TAG, the proportion of 18 : 0 and 18 : 2(9,12) was higher, whereas that of 14 : 0 and 16 : 0 was lower in the nitrogen-deprived cells. Fig. S5 shows a MALDI-TOF MS spectrum of the TAG, which exhibits two intense signals that correspond to the Na+ adducts of TAG 52 : 2 and 54 : 4, indicating that major molecular species of TAG were 16 : 0/18 : 0/18 : 2, 18 : 0/18 : 1/18 : 2, 18 : 1/18 : 0/18 : 2, 18 : 1/18 : 1/18 : 2 and 18 : 1/18 : 1/18 : 2. The positional distribution of fatty acids in TAG of *Cy. merolae* grown in red–violet. The cells grown on 2 × Allen did not show specific staining with Lugol (Fig. 4a), whereas the cytosol of the cells grown on 2 × Allen (− N) became significantly red–violet with the staining, indicating that starch accumulated under nitrogen deprivation. The content of starch in the cells grown on 2 × Allen (− N) was approximately 0.96 pg cell−1, whereas that in the cells grown on 2 × Allen was approximately 0.19 μg cell−1 (Fig. 4b).
were found in the cytosol (Fig. 5e–i). In the chloroplast, thylakoid membranes were observed (Fig. 5e, g). Phycobilisomes were, however, rarely observed, confirming the spectroscopic data showing that phycocyanins were almost lost in the nitrogen-deprived cells. This is different from the situation in other microalgae under nutrient deficiency, in which thylakoid membranes were extensively degraded (Ball et al., 1990; James et al., 2011).

Interestingly, a part of the nucleus invaginated into the mitochondrion with or without nitrogen deficiency (Fig. 5c, f). In addition, close contact of the chloroplast with the nucleus was found, especially in the nitrogen-deprived cells (Fig. 5g). In this case, a part of the chloroplast was elongated alongside the mitochondrion to touch the nucleus. In the nitrogen-replete cells, the chloroplast and the nucleus were located in association, but the mitochondrion was always located between them.

Both oil bodies and starch granules were significantly smaller than those found in other microalgae such as Chlamydomonas (Iwai et al., 2014; Ho et al., 2015; Siaut et al., 2011; Toyoshima & Sato, 2015). The starch granules were about 100–300 nm in diameter and present in the periphery of the cytoplasm extending ahead of the nucleus. Oil bodies, about 500 nm in diameter, were also found in the cytoplasm, surrounded by numerous starch granules. The limiting membrane of each oil body was not clearly observed in general, maybe because of the high curvature of the surface of the oil bodies within the sections (about 70–100 nm thick). However, only some parts of the limiting membrane of the oil body were seen in Fig. 5(h) (upper part) and (i) (lower part).

DISCUSSION

In the present study, we showed that Cy. merolae accumulates nonpolar lipid abundantly under nitrogen deprivation (Fig. 1). This is consistent with the reports on other microalgae including Cy. merolae, which accumulate nonpolar lipids under nutrient deprivation (Chisti, 2007; Hu et al., 2008; Imamura et al., 2015; Iwai et al., 2014; Moellering & Benning, 2010; Msanne et al., 2012; Sakurai et al., 2014; Siaut et al., 2011; Tornabene et al., 1983). Compared with other microalgae, Cy. merolae exhibits a very simple composition of fatty acids [mainly 16 : 0, 18 : 0, 18 : 1(9) and 18 : 2(9,12)], and did not contain trienoic or more unsaturated fatty acids in all lipid classes (Table S1). This is in contrast with various microalgae (Iwai et al., 2014; Moellering & Benning, 2010; Msanne et al., 2012; Sakurai et al., 2014; Siaut et al., 2011). Because it grows in habitats with high temperature and stable environments (De Luca et al., 1978), Cy. merolae might not need trienoic or more unsaturated fatty acids, which are normally required to maintain the fluidity and function of the membrane at low temperatures. Nevertheless, the level of the four major fatty acids changed dramatically under nitrogen deprivation (Table 1).

Role of plastids and ER in the synthesis of TAG

The cell density (in cells ml$^{-1}$) of the culture grown on 2 × Allen(−N) for 2 days was not significantly different from that of the culture grown on 2 × Allen (Fig. S1). This shows that the cells divided even under nitrogen deprivation during the 2 days. This is in contrast with Chlamydomonas reinhardtii, which does not proliferate under nitrogen-limited conditions (Iwai et al., 2014; Sakurai et al., 2014). The activity of photosynthesis as measured by the rate of photosynthetic oxygen evolution per chlorophyll a was maintained in both nitrogen-replete and -deplete cells, whereas the actual rate of photosynthetic oxygen evolution per cell was lower in the nitrogen-deplete cells because of the lower content of chlorophyll a (Fig. 2a–c). This is in contrast with Ch. reinhardtii, where a remarkable decrease in photosynthetic oxygen evolution per chlorophyll was observed under nitrogen deprivation (Sakurai et al., 2014). Fig. 2(d) and Fig. 5(e) show that phycobilisomes were mostly degraded under nitrogen deficiency in Cy. merolae, indicating that this alga maintains metabolic activity by using phycobiliproteins as both nitrogen and carbon sources, as well as using the remaining activity of photosynthesis.

An interesting observation in Cy. merolae is that the contents of plastid membrane lipids did not decrease (Fig. 3) and that the thylakoid membranes were not degraded under nitrogen deprivation (Fig. 5). This is in contrast with Ch. reinhardtii, in which extensive breakdown of
chloroplast lipids and thylakoid membranes was found under nitrogen deprivation (Ball et al., 1990; James et al., 2011). *Cy. merolae* is thought to maintain photosynthesis by using nitrogen provided from phycobiliproteins under nitrogen deprivation. The synthesis of fatty acids in the plastids maintained by the photosynthesis provides fatty acids to ER. By contrast, *Ch. reinhardtii* degrades plastid membrane lipids and synthesizes TAG. The increase of 18:2(9,12) in PC under nitrogen deprivation is consistent with the results of microarray in which the expression of

![Fig. 5. Electron micrographs of *Cy. merolae* cells. (a–d) Cells grown in the presence of ammonium; (e–i) cells grown without ammonium. (a) Whole cell image; (b) whole cell image of a dividing cell; (c, f) nucleo-mitochondrial contact site; (d, g) contact of a nucleus, a mitochondrion and a chloroplast; (e) a starch-accumulating cell; (h, i) an oil body. Cp, Chloroplast; L, lysosome; Mt, mitochondrion; N, nucleus; O, oil body; S, starch granule; V, vacuole. Bars, 200 nm (a, b, e) and 100 nm (c, d, f–i).](http://mic.microbiologyresearch.org/809)
the CMK291C gene encoding microsomal Δ12 desaturase (Sato & Moriyama, 2007) increased under nitrogen deprivation (Imamura et al., 2010). The composition of fatty acids in PA was similar to that in PC in the cells grown under nitrogen deprivation (Table 1). This result may suggest that the composition and proportions of fatty acids in the acyl-CoA pool became identical to that in the PC pool due to abundant flow of linoleic acid from PC to the acyl-CoA pool under nitrogen deprivation (Fig. 6). This is again consistent with the result of microarray in which the expressions of CMT312C and CMR500C genes encoding phospholipase A2, presumably providing linoleic acid from the PC pool to the acyl-CoA pool, increased under nitrogen deprivation (Imamura et al., 2010). In addition, PA might be converted to PC by phospholipase D (PLD), which cleaves the phosphoric ester bond and releases PA and an alcohol (Bates et al., 2012; Lee et al., 2011). However, no homologue of PLD is detected in the Cy. merolae genome. The fatty acid contents of PE did not change significantly, whereas those of PC and PA changed dramatically, indicating that the PC synthesis pathway is independent of the flow of unsaturated fatty acids through the PC pool. The fatty acid contents in the plastid membrane lipids such as MGDG, DGDG and SQDG did not change significantly, whereas those of ER lipids such as PC, PA, PI and TAG changed dramatically under nitrogen deprivation. Thus, the PC-mediated TAG synthesis pathway is driven actively by the supply of fatty acids from the plastid under nitrogen deprivation.

Structure of TAG and role of PC in TAG synthesis

The contents of fatty acids in TAG were similar to those in PC, except the reversed contents of 16:0 and 18:0 (Table 1). The result of MALDI-TOF MS analysis showed that major TAG species were 52:2 and 54:4 (Fig. S5), indicating that fatty acid combinations of TAG are 16:0/18:0/18:2 and 16:0/18:1/18:2 for 52:2, and 18:0/18:2/18:0/18:1 for 54:4. The result of lipase treatment showed that the C-2 position was mainly occupied by 18:2(9,12) in TAG (Fig. S6 and Table 2). Thus, the major molecular species compositions of TAG are 16:0/18:2/18:0, 18:0/18:2/18:2 and 18:1/18:2/18:1. In a previous study, 16:0 was bound to the C-1 position and 18:2(9,12) was bound to the C-2 position in PC (Sato & Moriyama, 2007). These results suggest that PC supplies the DAG moiety to TAG. However, homologues of phosphatidylcholine diacylglycerol cholinephosphotransferase (Lu et al., 2009) and phospholipase C (Nakamura et al., 2005), which can mediate the synthesis of DAG from PC, are not detected in the Cy. merolae genome. If TAG is to be synthesized from the backbone of C-1 and C-2 acyl groups in PC, DAG should mainly be acylated with 18:0. The preferential use of 18:0 is due to the affinity of diacylglycerol acyltransferase (DGAT) (Lung & Weselake, 2006; Turchetto-Zolet et al., 2011). In fact, the substrate specificity and selectivity of DGAT in higher plants were reported previously (Lung & Weselake, 2006). We still need to know which of the three DGATs in Cy. merolae (CME100C, CMJ162C and CMQ199C) (Imamura et al., 2015; Sumiya et al., 2015) determines the specificity of acylation.

In the present study, we showed the physiological properties of Cy. merolae grown under nitrogen deprivation. We also analysed the contents of lipids and the composition of fatty acids in detail. Cell proliferation and synthesis of
fatty acids in the plastid are maintained in Cy. merolae at least during the 2 days of nitrogen deprivation, in clear contrast with other eukaryotic microalgae and plants. The presence of a large amount of phycobilisomes in the plastid of Cy. merolae could account for this continued metabolic activity. The absence of major desaturases in the plastid of Cy. merolae necessitates an abundant flow of fatty acids through the PC in the ER for desaturation, and this flow efficiently feeds the TAG synthesis under nitrogen deprivation (Fig. 6). These results are useful in engineering for biofuel production by elucidating and modifying the pathway of TAG synthesis in Cy. merolae.

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REFERENCES


Nozaki, H., Takano, H., Misumi, O., Terasawa, K., Matsuzaki, M., Maruyama, S., Nishida, K., Yagisawa, F., Yoshida, Y. & other authors (2007). A 100%-complete sequence reveals unusually high levels of mitochondrion-encoded genes in the hot-spring red alga Cyanidioschyzon merolae. BMC Biol 5, 28.


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