High-Level Accumulation of Triacylglycerol and Starch in Photoautotrophically Grown *Chlamydomonas debaryana* NIES-2212

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(Received July 31, 2015; Accepted October 23, 2015)

Microalgae have the potential to produce triacylglycerol (TAG) and starch, which provide alternative sources of biofuel. A problem in using *Chlamydomonas reinhardtii* as a model for TAG production has been that this alga lacks phosphatidylcholine (PC), which is thought to be important for TAG synthesis in plants. We found that *C. debaryana* is one of the rare species of *Chlamydomonas* having PC. Here we show that this strain, grown under complete photoautotrophic conditions, accumulated TAG and starch up to 20 and 250 pg per cell, respectively, during the stationary phase without nutrient deprivation. Addition of nutrients in this state did not cause loss of TAG, which was found in dilution with fresh medium. The photosynthetically produced TAG contained a high level of monounsaturated fatty acids, which is a preferred property as a material for biodiesel. The oil bodies were present in the cytoplasm, either between the cytoplasmic membrane and the chloroplast or between the chloroplast and the nucleus, whereas the starch granules were present within the chloroplast. Oil bodies were also deposited as a broad layer in the peripheral space of the cytoplasm outside the chloroplast, and might be easily released from the cells by genetic, chemical or mechanical manipulation. These results suggest that *C. debaryana* is a promising seed organism for developing a good biofuel producer.

**Keywords:** Biofuel • *Chlamydomonas debaryana* NIES-2212 • Fatty acid • Starch • Triacylglycerol.

**Abbreviations:** DAG, diacylglycerol; DAPI, 4',6-diamino-2-phenylindole; DGDG, digalactosyl diacylglycerol; DGTS, diacylglyceryl-N,N,N-trimethylhomoserine; FAME, fatty acid methyl ester; GC-MS, gas chromatography–mass spectrometry; MALDI-TOFMS, matrix-assisted laser desorption ionization–time of flight mass spectrometry; MBM, modified Bristol’s medium; MGDG, monogalactosyl diacylglycerol; OS, oxidation state; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; SQDG, sulfoquinovosyl diacylglycerol; TAG, triacylglycerol; 2D-TLC, two-dimensional thin-layer chromatography.

**Introduction**

Microalgae are promising alternatives to land plants in conserving solar energy and recycling CO₂ as reduced carbon such as carbohydrates and oils. Although the efficiency could be ameliorated, a noteworthy advantage of the algae is that they do not compete for agriculture (Dismukes et al. 2008). In addition, the use of microalgae provides the potential to produce various forms of high-added-value chemicals as well as biofuel (Hu et al. 2008, Scott et al. 2010). The potential of algal biofuels as an alternative source of renewable energy has been highlighted repeatedly (Georgianna and Mayfield 2012, Leite et al. 2013, Oncel et al. 2014). Microalgal oil is mainly composed of triacylglycerol (TAG) consisting of long-chain fatty acids, which can be converted to biodiesel [fatty acid methyl esters (FAMEs)] by chemical transesterification. Starch can be converted to ethanol through alcohol fermentation by yeast (Aikawa et al. 2013). The production of storage compounds such as starch and TAG is dependent on the nutritional state of the algae: research on various algae showed that starch and TAG are accumulated under nutrient-limited conditions such as nitrogen deficiency (Tornabene et al. 1983, Hicks et al. 2001, Yussuf 2007, Hu et al. 2008, Aikawa et al. 2012, Msanne et al. 2012). Whether starch synthesis competes with TAG synthesis remains unresolved. The accumulation of TAG could be dramatically increased by limiting starch biosynthesis, if the latter really competed with TAG production (Ball et al. 1991, Zabawinski et al. 2001, Wang et al. 2009, Li et al. 2010, James et al. 2011).

*Chlamydomonas reinhardtii* is a unicellular microalga widely used as a model system for optimizing the production of TAG (Eichenberger 1976, Giroud et al. 1988, Sato 1989, Riekho et al. 2005, Sakurai et al. 2014b) and starch (Ball et al. 1990, Hicks et al. 2001). Various starch-less mutants have been isolated (Ball et al. 1991, Zabawinski et al. 2001, Li et al. 2010, James et al. 2011, Siaut et al. 2011). In addition to starch, *C. reinhardtii* accumulates large amounts of TAG under photoheterotrophic conditions and nutrient starvation (Moller and Benning 2010, Siaut et al. 2011). In comparison with TAG metabolism in higher plants, a number of points remain to be clarified in
the metabolic pathways leading to TAG in this organism. Putative reaction pathways have been estimated based on putative orthologs of metabolic enzymes in bacteria, yeasts, and higher plants (Riekhof et al. 2005).

In higher plants, TAG is synthesized through two major pathways (Bate et al. 2013). In one of them, TAG is synthesized by acetylation of glycerol-3-phosphate with acyl-CoAs (Weiss et al. 1960, Kennedy 1961, Barron and Stumpf 1962, Lung and Weselake 2006). In another, more complex pathway, the membrane lipid phosphatidylcholine (PC) acts as an intermediate that provides fatty acids and/or diacylglycerol (DAG) for the synthesis of TAG (Bates et al. 2013). TAG synthesized from PC contains high levels of polyunsaturated fatty acids (and other fatty acids depending on the plant) that are modified while they are bound to PC (Saperling et al. 1993, Van de Loo et al. 1995, Wallis et al. 2002, Bates and Browse 2012). PC is thus considered to be important for TAG synthesis in higher plants. In contrast, PC was not detected in C. reinhardtii (Eichenberger 1976, Sato and Furuya 1985). In a previous study from our laboratory, screening for PC in strains of *Chlamydomonas* in the NIES Algal Collection resulted in the identification of four PC-bearing strains: *Chlamydomonas* NIES-2212, *C. applanata* NIES-2202, *C. asymmetrica* NIES-2207 and *C. spheroideas* NIES-2242 (Sakurai et al. 2014a). A strain classified as *C. debaryana* was also isolated as a high TAG producer grown on swine wastewater (Zhang et al. 2014).

In the present study, we focused on the photosynthetic production of TAG and starch from inorganic, non-reduced forms of nutrients in *C. debaryana* NIES-2212. Previous studies on biofuel production in *C. reinhardtii* relied on photoheterotrophic growth using Tris-acetate-phosphate (TAP) medium containing ammonium and acetate (Sueoka 1960). As described previously (Sakurai et al. 2014b), we believe that the use of ammonium [the oxidation state (OS) (Karen et al. 2014) of the N atom is –3; note that the OS is negative in the reduced state] and acetate (the average OS of the C atom is 0), which are both already reduced compounds, in producing starch (the average OS of the C atom is 0) and TAG (the average OS of the C atom is about –1.8) is not justified in view of the use of solar energy (that produces reducing equivalents by photosynthesis) in biofuel production. In this respect, we emphasize the importance of photoautotrophic production of biofuels from oxidized substrates such as CO2 (the OS of the C atom is +4) and NO3− (the OS of the N atom is +5), which can be considered as a net production of bioenergy. Based on this fundamental policy, we are trying to find a condition in which TAG production is optimized under photoautotrophic conditions. In the present study, we observed the accumulation of lipids and starch in *C. debaryana* NIES-2212 under photoautotrophic (dependent only on inorganic nutrients) conditions without deprivation of nutrients. We also analyzed in detail the composition of fatty acids in accumulated TAG, and found that the content of monoenoic acids was high. The usefulness of this strain in large-scale photosynthetic production of TAG is discussed.

### Results

#### Cell growth and oil body accumulation in photoautotrophically grown *C. debaryana*

*Chlamydomonas debaryana* cells displayed logarithmic growth under photoautotrophic conditions on inorganic nutrient medium [modified Bristol’s medium (MBM)] with bubbling with air containing 1.0% (v/v) CO2 with a doubling time of approximately 30 h (Fig. 1A; Supplementary Fig. S1). The culture was started with an inoculum from a 1/20th volume of culture in the late logarithmic phase. The cell density of the culture reached a plateau at about $4 \times 10^6$ cells ml$^{-1}$. The size of the cells at 144 and 240 h was larger than that at 48 h (Supplementary Fig. S2). We observed the accumulation of oil bodies consisting of non-polar lipids such as TAG in the cells by BODIPY staining (Fig. 1B). The culture was sampled for BODIPY staining at 0, 48, 96, 144, 168 and 192 h. The green fluorescence exhibited by lipid-bound BODIPY (Fig. 1B) remained weak in the cells during the logarithmic phase, indicating a low accumulation of oil bodies (Fig. 1B, panels 0 h and 48 h). In contrast, the BODIPY fluorescence began to increase at 96 h and then continued to increase, indicating that oil bodies accumulated to a significantly high level (Fig. 1B, panels 96 h, 144 h, 168 h and 192 h). The BODIPY fluorescence was located preferentially in the periphery of the cell. Not only spherical oil bodies but also flattened oil bodies were observed (Fig. 1C, arrowhead). These results indicated that photoautotrophically grown cells do produce non-polar lipid abundantly under normal nutrient conditions with freshwater inorganic nutrients.

#### Identification of lipids and fatty acids

Total lipids of *C. debaryana* were separated by two-dimensional thin-layer chromatography (2D-TLC) (Fig. 2). Each lipid class was recovered from the plate and its identity was confirmed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOFMS). The presence of the following commonly found classes of lipids was confirmed: phosphatidylinositol (PI), phosphatidylethanolamine (PE), diacylglycerol-N,N,N-trimethylhomoserine (DGTS), phosphatidylglycerol (PG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG), monogalactosyldiacylglycerol (MGDG) and TAG. All these were also detected in *C. reinhardtii* CC1010 (Sakurai et al. 2014b). In *C. debaryana*, however, PC was also detected. Minor components such as phosphatidic acid (PA), wax esters and free fatty acids were also detected, but were not further analyzed.

Fatty acids in *C. debaryana* were identified by gas chromatography–mass spectrometry (GC-MS). The positions of double bonds were estimated by GC-MS analysis of fatty acid pyrroli- dides. In this way, 18:3(5,9,12) and 18:4(5,9,12,15) were identified, but 18:3(6,9,12) and 18:4(6,9,12,15) were not detected even in high sensitivity SIM analysis (Table 1). C$_{20}$ fatty acids that were reported in *C. reinhardtii* 137c (Giroud et al. 1988) and CC1010 (Sakurai et al. 2014b) or fatty acids with odd-numbered chains (Sakurai et al. 2014b) were not detected in *C. debaryana*.  

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Accumulation of TAG in photoautotrophically grown cells

The contents of lipids were determined in the logarithmic and stationary phases (Fig. 3; Supplementary Fig. S3). The total lipid content was approximately 17.2 ± 0.8% of total dry cell weight at 240 h. The content of TAG dramatically increased with culture time, and reached approximately 80 fmol of fatty acid per cell and approximately 80 mol% of total lipids at 240 h. The value was approximately 210-fold with respect to the level at 48 h. It was equivalent to approximately 20 pg of TAG per cell when this value was converted into weight. These results were consistent with the high accumulation of oil bodies in the stationary phase cells (Fig. 1B, panels 144 h, 168 h and 192 h).

Composition of fatty acids

The compositions of fatty acids at 48 and 240 h are shown in Tables 1 and 2, respectively. Data at 144 h are shown in Supplementary Table S1. The specific distribution of fatty acids among lipid classes was found to be as reported previously (Sato 1989, Sakurai et al. 2014b). In the cells sampled at 48 h, 16:4(4,7,10,13) was concentrated in MGDG, while 18:1(11) was detected mainly in PI (Table 1). 18:3(5,9,12) was specific for PC, DGTS and PE, while 18:3(9,12,15) was abundant in DGDG, SQDG and MGDG. These results were similar to those in C. reinhardtii (Sakurai et al. 2014b). In C. reinhardtii, delta5(ω-13) desaturase synthesizes 18:3(5,9,12), which accumulates in DGTS and PE (Kajikawa et al. 2006). Delta 15 desaturase (CrFAD7) is localized to the plastid (Nguyen et al. 2013). Delta 5(ω-13) desaturase was detected in the C. debaryana genome (unpublished results). Unlike the results in C. reinhardtii, delta5(ω-13) was detected at a relatively high level in most classes of lipids (Table 1).
The level is shown as the molar content of fatty acids per cell [FA photoautotrophic conditions at 48, 144 and 240 h after inoculation.

Table 1 Composition of fatty acids in cells of Chlamydomonas debaryana grown for 48h in MBM

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Content* (mol%)</th>
<th>PC</th>
<th>DGTS</th>
<th>PE</th>
<th>PI</th>
<th>PG</th>
<th>DGDG</th>
<th>SQDG</th>
<th>MGDG</th>
<th>TAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>0.6 ± 0.2</td>
<td>1.2 ± 0.1</td>
<td>2.4 ± 1.5</td>
<td>7.4 ± 1.5</td>
<td>9.9 ± 1.6</td>
<td>9.2 ± 0.7</td>
<td>9.2 ± 0.7</td>
<td>2.9 ± 1.2</td>
<td>0.6 ± 0.1</td>
<td>11.4 ± 2.5</td>
</tr>
<tr>
<td>16:0</td>
<td>25.0 ± 0.7</td>
<td>29.7 ± 0.9</td>
<td>13.1 ± 4.7</td>
<td>53.6 ± 0.1</td>
<td>31.2 ± 1.3</td>
<td>38.9 ± 2.3</td>
<td>58.7 ± 1.3</td>
<td>2.7 ± 0.8</td>
<td>27.6 ± 5.5</td>
<td></td>
</tr>
<tr>
<td>16(7 + 3c)</td>
<td>0.8 ± 0.2</td>
<td>1.0 ± 0.1</td>
<td>1.6 ± 1.1</td>
<td>0.6 ± 0.3</td>
<td>23.6 ± 2.6</td>
<td>1.1 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>1.8 ± 0.3</td>
<td>3.4 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>16(9)</td>
<td>0.2 ± 0.1</td>
<td>0.1 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>3.0 ± 0.1</td>
<td>0.2 ± 0.2</td>
<td>0.1 ± 0.1</td>
<td>0.0 ± 0.0</td>
<td>0.7 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>16(11)</td>
<td>0.1 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>16(2,7,10)</td>
<td>0.8 ± 0.1</td>
<td>0.8 ± 0.0</td>
<td>0.5 ± 0.2</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>5.2 ± 0.5</td>
<td>2.7 ± 0.4</td>
<td>7.8 ± 0.9</td>
<td>3.1 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>16(3,7,10)</td>
<td>0.0 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>5.6 ± 0.6</td>
<td>1.7 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>16(3,7,10,13)</td>
<td>0.0 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>2.1 ± 0.2</td>
<td>1.3 ± 0.2</td>
<td>1.8 ± 0.0</td>
<td>0.3 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>16(4,7,10,13)</td>
<td>0.1 ± 0.0</td>
<td>0.6 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>1.1 ± 0.4</td>
<td>2.2 ± 0.8</td>
<td>3.1 ± 0.6</td>
<td>7.5 ± 3.3</td>
<td></td>
</tr>
<tr>
<td>18:0</td>
<td>6.6 ± 0.1</td>
<td>2.0 ± 0.6</td>
<td>22.4 ± 2.8</td>
<td>12.1 ± 1.3</td>
<td>3.7 ± 0.2</td>
<td>0.3 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>3.7 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>18(9)</td>
<td>2.0 ± 0.4</td>
<td>1.6 ± 0.2</td>
<td>2.6 ± 1.0</td>
<td>0.0 ± 0.0</td>
<td>7.2 ± 1.8</td>
<td>9.9 ± 0.5</td>
<td>4.4 ± 0.1</td>
<td>2.4 ± 0.2</td>
<td>5.7 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>18(11)</td>
<td>5.2 ± 0.1</td>
<td>2.2 ± 0.3</td>
<td>9.6 ± 1.1</td>
<td>30.3 ± 1.3</td>
<td>2.2 ± 1.0</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>1.7 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>18(2,9,12)</td>
<td>23.2 ± 0.4</td>
<td>14.8 ± 0.7</td>
<td>7.8 ± 0.3</td>
<td>53.2 ± 0.2</td>
<td>16.2 ± 2.3</td>
<td>18.9 ± 1.0</td>
<td>12.4 ± 0.3</td>
<td>14.9 ± 0.5</td>
<td>12.4 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>18(3,5,9,12)</td>
<td>22.0 ± 0.1</td>
<td>24.7 ± 0.8</td>
<td>36.9 ± 2.4</td>
<td>0.4 ± 0.2</td>
<td>0.1 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>0.2 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>5.8 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>18(3,9,12,15)</td>
<td>8.3 ± 0.7</td>
<td>8.7 ± 0.3</td>
<td>0.9 ± 0.4</td>
<td>0.9 ± 0.4</td>
<td>2.2 ± 0.5</td>
<td>12.4 ± 0.7</td>
<td>13.8 ± 1.6</td>
<td>30.3 ± 2.2</td>
<td>12.9 ± 4.9</td>
<td></td>
</tr>
<tr>
<td>18(4,5,9,12,15)</td>
<td>4.2 ± 0.3</td>
<td>12.1 ± 0.4</td>
<td>2.0 ± 0.8</td>
<td>0.0 ± 0.0</td>
<td>0.3 ± 0.2</td>
<td>0.0 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>0.2 ± 0.0</td>
<td>2.1 ± 0.5</td>
<td></td>
</tr>
</tbody>
</table>

* Each value represents the average ± SE of the results obtained from three independent experiments.

16(17) and 16(13) were not clearly separated by the GC system, but 16(13) was found to be localized to PG. In other classes of lipids, this peak certainly represents 16(17).

During the stationary phase, the proportions of 16:0 and 18:2(9,12) increased, whereas those of 18:0, 18:3(5,9,12), 18:3(9,12,15) and 18:4(5,9,12,15) decreased in PC and DGTS with respect to the exponential growth phase (48 h) at a confidence level of 95% (Table 2; Supplementary Table S1). In PG, DGDG, SQDG and MGDG, the proportions of 14:0, 16:0, 18:0 and 18:1(9) increased, whereas those of 16:3(7,10,13) and 16:4(7,10,13) decreased (Table 2; Supplementary Table S1). In TAG, the levels of 16:1(7) and 18:1(9) increased. 16:1(3c), which was not clearly separated from 16:1(7) in the GC system employed, was considered to be absent in the TAG fraction, judging from the results of detailed GC-MS analysis of fatty acid pyrrolidides prepared from the TAG. Compared with the TAG in C. reinhardtii CC1010 (Sakurai et al. 2014b), the proportion of monounsaturated fatty acids in TAG was high in C. debaryana NIES-2212, and that of saturated fatty acids and polyunsaturated fatty acids was low (Table 2; Supplementary Table S1).

**Accumulation of starch granules**

We examined the accumulation of starch, which is a potential source of biofuel as well as lipids (Fig. 4). Lugol solution stains crystallized starch and exhibits blue-violet coloration. The cells at 48 h were hardly stained with Lugol (Fig. 4A). The cells at 144 and 240 h became intensely violet with Lugol staining, indicating that starch accumulated to significant levels at these time points (Fig. 4B, C). The content of starch (Fig. 4D) was about 15 pg per cell at 48 h. It reached about 250 pg per cell at 144 h and then decreased at 240 h. Under nitrogen-limited conditions, the content of starch per cell was not significantly different (data not shown).

**Morphology of oil bodies and starch granules**

Fig. 5 shows the ultrastructure of C. debaryana cells. Within a representative cell taken from the culture at 48 h, a nucleus (labeled 'N'), a large chloroplast having stacked thylakoids (labeled 'T'), vacuoles (labeled 'V'), significantly developed...
Table 2 Composition of fatty acids in cells of *Chlamydomonas debaryana* NIES-2212 grown for 240h in MBM

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Content* (mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PC</td>
</tr>
<tr>
<td>14:0</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>16:0</td>
<td>36.3 ± 0.6 c</td>
</tr>
<tr>
<td>16:1(7,3t)</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>16:1(9)</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>16:1(11)</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>16:2(7,10)</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>16:3(7,10)</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>16:4(7,10,13)</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>18:0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>18:1(9)</td>
<td>1.1 ± 0.2 b</td>
</tr>
<tr>
<td>18:1(11)</td>
<td>2.1 ± 0.4</td>
</tr>
<tr>
<td>18:2(9,12)</td>
<td>37.5 ± 1.3 a</td>
</tr>
<tr>
<td>18:3(5,9,12)</td>
<td>12.4 ± 1.7 b</td>
</tr>
<tr>
<td>18:3(9,12,15)</td>
<td>3.8 ± 0.7 b</td>
</tr>
<tr>
<td>18:4(5,9,12,15)</td>
<td>1.0 ± 0.1 b</td>
</tr>
</tbody>
</table>

* Each value represents the average ± SE of the results obtained from three independent experiments.

b Fatty acids that showed an increase in composition with respect to the time point at 48 h at a confidence level of 95%.

c Fatty acids that showed an increase in composition with respect to the time point at 48 h at a confidence level of 95%.

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**Fig. 4** Starch accumulation of *C. debaryana* under photoautotrophic conditions. (A – C) Detection of the starch that accumulated in the cells by Lugol staining. Panels a, b, and c are bright-field images without DIC for the cells sampled at 48, 144 and 240 h after inoculation, respectively. The color of Lugol solution bound to starch granules was detected as blue-violet spots. Scale bars = 20 μm. (D) The contents of starch in a cell of *C. debaryana* NIES-2212 grown under photoautotrophic conditions. Each value represents the average ± SE of three independent experiments.

Golgi apparatus (labeled ‘G’), which appeared as stacks of flattened membranes, and endoplasmic reticulum (labeled ‘ER’) were observed (Fig. 5A, C, D). The chloroplast envelope membrane (labeled ‘CEnv’ or marked with small arrowheads), which surrounded the chloroplast as two parallel membranes, was also observed clearly (Fig. 5D, arrowhead). Unlike *C. reinhardtii*, an eyespot (labeled ‘E’) was observed near the base of flagella. The entire cell was surrounded by a thick layer of cell wall (labeled ‘CW’). Starch granules (labeled ‘S’) were present within the chloroplast, and some of them surrounded the pyrenoid (labeled ‘P’). Oil bodies were barely observed.

Within the cells sampled from the culture at 144 and 240 h, numerous starch granules and oil bodies (labeled ‘O’) were found (Fig. 5B, E–G). The standard components of the cell, such as the nucleolus, pyrenoid, eyespot, flagella and thylakoid membranes, were always observed throughout the growth phase. This is in contrast to the nutrient-deficient, oil-accumulating *C. reinhardtii* cells, in which thylakoid membranes and the eyespot were degraded (Ball et al. 1990). Flagella were also lacking in nutrient-deficient cells. These results confirmed that the *C. debaryana* cells retain normal metabolic activities and motility even in the stationary phase.

Many oil bodies were located in the periphery of the cells (Fig. 5B, E–G), as observed by light microscopy (Fig. 1). Close examination of the localization of starch granules and oil bodies with respect to the chloroplast envelope membrane showed that the chloroplast envelope membrane was observed between the thylakoid membranes and oil bodies, but not observed between thylakoid and starch (Fig. 5E, G). A thin layer of cytoplasm was observed between the cell wall and...
oil body (Fig. 5E, labeled ‘Cyto’). These results indicated that the oil bodies were present in the cytoplasm, whereas the starch granules were present within the chloroplast. We frequently found a large oil body in the peripheral space of the cytoplasm between the cytoplasmic membrane and the chloroplast as a broad flattened layer in the cells at 240 h (Fig. 5F, G). The flattened oil bodies were clearly located outside the envelope membranes but inside the cytoplasmic membrane.

**Discussion**

*Accumulation of TAG in C. debaryana NIES-2212 under photoautotrophic conditions*

In the present study, we showed that photoautotrophically grown *C. debaryana* produce non-polar lipid abundantly under normal growth conditions with freshwater inorganic nutrients. This is in contrast to *C. reinhardtii*, which accumulates non-polar lipids only under nutrient deprivation (Moellering...
The content of TAG in C. debaryana, which amounted to approximately 20 pg per cell (Fig. 3), is equal to or greater than the values reported in previous studies on C. reinhardtii that were performed with genetic engineering and/or photobioreactor growth and nutrient deprivation (Moellerling and Benning 2010, Siaut et al. 2011, Iwai et al. 2014). When we tested a nitrogen-limited condition in C. debaryana, the content of TAG per cell increased considerably, although the proliferation of cells was arrested (Supplementary Figs. S4, S5). The time of TAG accumulation did not change markedly between the normal medium and under nitrogen deprivation (Supplementary Fig. S5). The content of TAG per culture volume was, however, roughly equivalent to that under normal conditions, in which cell growth continued.

The content of MGDG and PG decreased during the stationary phase (Fig. 3). The contents of other lipids did not change much between the logarithmic and stationary stage (Fig. 3). During the stationary stage, the proportion of polyunsaturated fatty acids in PC, DGTS and chloroplast-related lipids decreased. This is consistent with the results in C. reinhardtii, in which PC is not present (Sakurai et al. 2014b). 18:2(9,12) increased in PC (Table 2; Supplementary Table S1). In TAG, 16:1(7) and 18:1(9) increased. These results suggest that PC does not appear to be related directly to TAG synthesis, and de novo synthesis of TAG continued during the stationary phase in the normal medium, although the conversion of MGDG to TAG was also possible. However, a role(s) for PC cannot be excluded in the abundant accumulation of TAG in C. debaryana.

Peripheral cytoplasmic localization of oil
Within the cells at 144 and 240 h, the nucleolus, pyrenoid and thylakoids were clearly observed, suggesting that the cell still performs metabolism and photosynthesis actively. This is in contrast to C. reinhardtii where a reduction in thylakoid membranes was observed under nitrogen-limited conditions (Siaut et al. 2011). We noticed that oil bodies protruded into the peripheral space of the cytoplasm (Fig. 5G). Such a protrusion of oil bodies was also confirmed under an optical microscope after BODIPY staining (Fig. 1C, arrowhead). Such a peripheral cytoplasmic localization of oil could be an artifact of fixation, but the fact that such a protrusion has never been observed in other strains before suggests that this property is specific to the strain NIES-2212. We call such oil bodies ‘peripheral, flattened oil bodies’. If the cytoplasmic membrane and the cell wall could be made more fragile by genetic engineering or other means, C. debaryana would release the oil easily out of the cell. Such a property could be beneficial for mass production of biofuel.

The cells are not suffering from nutrient starvation during the stationary phase
A question arises as to whether the cells in the stationary phase were not suffering from nutrient starvation. To answer this question, we supplied nutrients (phosphate and nitrate) to the culture in the stationary phase. We also diluted the stationary phase culture with fresh medium as a control. In the control experiment, non-polar lipid was consumed rapidly as the cells at 48 h (Fig. 1). When the nutrients were added to the stationary phase culture, TAG remained at a high level (Supplementary Fig. S6). The cells did not proliferate significantly upon addition of nitrogen and phosphate. The addition of A5 microelement solution gave similar results (data not shown). In contrast, when nitrate was added to the culture of MBM-nON for 240 h, the cells began proliferating and TAG was consumed (Supplementary Fig. S6). Additionally, non-polar lipids accumulated when the cells in the logarithmic phase were concentrated and grown for 24 h, whereas non-concentrated cells did not accumulate non-polar lipids (Supplementary Fig. S7). These results may suggest that the non-polar lipid accumulation is dependent on the density of cells per ml. TAG may be synthesized using the excess energy generated by arrest of cell proliferation.

Accumulation of starch in C. debaryana
We also showed that C. debaryana accumulated starch, which amounted to about 250 pg per cell at 144 h after the inoculation (Fig. 4). The content of starch in C. debaryana was greater than the value in C. reinhardtii (Siaut et al. 2011). Although starch can be converted to ethanol by fermentation (Aikawa et al. 2012), the accumulation of TAG could be increased if the synthesis of starch could be obstructed by genetic engineering, as it is thought that the synthesis of TAG competes with starch. In fact, some starch-less mutants of C. reinhardtii accumulated TAG more than the wild type (Wang et al. 2009, Li et al. 2010, James et al. 2011).

In our results, the content of TAG continued increasing from the time point 144 h onwards, whereas the content of starch decreased. This result may suggest that starch was converted into TAG within the cell. If this is the case, the content of TAG might be increased further by optimizing the harvesting time.
Concluding remarks

Cells of *C. debaryana* NIES-2212 grown under complete photoautotrophic conditions massively accumulated TAG and starch in the stationary growth phase without deprivation of nutrients. In particular, oil bodies including TAG accumulated as a broad layer in the peripheral space of the cytoplasm. A high content of monoenoic acids in the accumulated TAG is suited for production of good quality biodiesel. The productivity of TAG and starch of this strain was superior to most of the previously reported values in the genus *Chlamydomonas*. Moreover, this strain has the potential to increase the productivity of TAG further and to facilitate easy release of TAG by genetic engineering and/or optimization of the culture conditions. These results indicate that *C. debaryana* could be a new seed organism as a biofuel producer.

Materials and Methods

Organism and growth conditions

*Chlamydomonas debaryana* NIES-2212 (Yumoto et al. 2013) was obtained from the Microbial Culture Collection at the National Institute for Environmental Studies, Japan. The cells were grown in MBM (Watanabe 1960) at 25 °C under continuous illumination provided by fluorescent lamps at a fluence rate of 50 μmol m⁻² s⁻¹, with bubbling with air containing 1.0% (v/v) CO₂. The detailed constituents of the MBM were described previously (Sakurai et al. 2014b).

Measurement of cell growth

After fixation with 0.25% glutaraldehyde, the cells were counted in a Fuchs–Rosenthal hemocytometer (Kayagaki). The optical density of the culture was measured at 750 nm (OD₇₅₀) (Supplementary Fig. S1). Because a linear correlation was seen between the OD₇₅₀ and the cell density of *C. debaryana* (Supplementary Fig. S8), the cell number was estimated by measuring the OD₇₅₀.

BODIPY staining

To 100 μl of the suspension of glutaraldehyde-fixed cells was added 1 μl of 1 mg ml⁻¹ BODIPY 493/503 (Molecular Probes). A 4 μl aliquot of BODIPY-stained cell suspension was placed on a slide glass, and mixed with 4 μl of TAN buffer (Sakurai et al. 2014b) containing 1% glutaraldehyde, and 4 μl of TAN buffer containing 1 μg ml⁻¹ 4,6-diamino-2-phenylindole (DAPI). The stained cells were viewed under a model BX60 microscope (Olympus) using a ×100 objective. Images were captured with a digital camera (Olympus DP70). For BODIPY fluorescence images, the mirror unit, U-MWU (Olympus; excitation, 470–490 nm; emission, 515–550 nm) was used. For DAPI and Chl fluorescence images, the mirror unit, U-MWU (Olympus; excitation, 330–385 nm; emission, >420 nm) was used.

Extraction and separation of lipids

The cells grown under photoautotrophic conditions were harvested by centrifugation (1,600 × g, 10 min, at 4 °C) at 48, 144 and 240 h after inoculation (Supplementary Fig. S3). Total lipids were extracted by the method of Bligh and Dyer (1959) as described in Sakurai et al. (2014b). The final chloroform phase was evaporated under vacuum. The lipids were dissolved in 0.4 ml of ethanol, and stored at −20 °C until use. Lipid classes were separated by 2D-TLC essentially as described in Sakurai et al. (2014b) as shown in Fig. 2.

Analysis of lipids and fatty acids

All analytical methods used were essentially identical to those described in Sakurai et al. (2014b). Briefly, identity of individual lipid classes was confirmed by MALDI-TOFMS. Lipids were quantified as the amount of fatty acids analyzed as methyl esters by GC. The identity of fatty acids was confirmed by GC-MS analysis as methyl esters (FAMEs) and pyrrolidides.

Starch staining

The cells were harvested by centrifugation (600 × g, 5 min). Cell pellets were washed with 1 ml of 90% ethanol for pigment removal. Pellets were resuspended in 1 ml of 1/5th strength diluted Lugol solution (1:10) for staining starch. The stained cells were examined with a BX60 microscope (Olympus).

Determination of starch content

The cells were harvested by centrifugation (1,000 × g, 5 min). Cell pellets were washed twice with 1 ml of 90% ethanol for pigment removal, mixed vigorously, incubated at 60 °C for 5 min and centrifuged. Cell pellets were resuspended in 10 N KOH. The suspension was incubated at 100 °C for 5 min to solubilize starch. After neutralization with 43.8 N H₃PO₄, and centrifugation (10,000 × g, 2 min), the supernatants were used for quantitation of starch, using a commercial starch assay kit (K648-100, BioVision).

Transmission electron microscopy

The cells were first moderately fixed in 0.125% glutaraldehyde and harvested by centrifugation (700 × g, 10 min), and then fixed with 1% glutaraldehyde for 2 h and post-fixed with 1% osmium tetroxide. The procedure for transmission electron microscopy was carried out essentially as described in Sato et al. (2014).

Supplementary data

Supplementary data are available at PCP online.

Funding

This work was supported in part by the Japan Science and Technology Agency [a Grant-in-Aid for Core Research for Evolutional Science and Technology (CREST)].

Disclosures

The authors have no conflicts of interest to declare.

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