Expression of Cyanobacterial Acyl-ACP Reductase Elevates the Triacylglycerol Level in the Red Alga Cyanidioschyzon merolae

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Nitrogen starvation is known to induce the accumulation of triacylglycerol (TAG) in many microalgae, and potential use of microalgae as a source of biofuel has been explored. However, nitrogen starvation also stops cellular growth. The expression of cyanobacterial acyl-acyl carrier protein (ACP) reductase in the unicellular red alga Cyanidioschyzon merolae chloroplasts resulted in an accumulation of TAG, which led to an increase in the number and size of lipid droplets while maintaining cellular growth. Transcriptome and metabolome analyses showed that the expression of acyl-ACP reductase altered the activities of several metabolic pathways. The activities of enzymes involved in fatty acid synthesis in chloroplasts, such as acetyl-CoA carboxylase and pyruvate dehydrogenase, were up-regulated, while pyruvate decarboxylation in mitochondria and the subsequent consumption of acetyl-CoA by the tricarboxylic acid (TCA) cycle were down-regulated. Aldehyde dehydrogenase, which oxidizes fatty aldehydes to fatty acids, was also up-regulated in the acyl-ACP reductase expresser. This activation was required for the lipid droplet accumulation and metabolic changes observed in the acyl-ACP reductase expresser. Nitrogen starvation also resulted in lipid droplet accumulation in C. merolae, while cell growth ceased as in the case of other algal species. The metabolic changes that occur upon the expression of acyl-ACP reductase are quite different from those caused by nitrogen starvation. Therefore, there should be a method for further increasing the accumulation of TAG.

Keywords: Acyl-ACP reductase • Aldehyde dehydrogenase • CE-MS • Microarray • Red algae • Triacylglycerol.

Abbreviations: AAR, acyl-ACP reductase; ACCase, acetyl-CoA carboxylase; ACP, acyl carrier protein; BCAA, branched chain amino acid; BCKDH, branched chain α-keto acid dehydrogenase complex; CE-MS, capillary electrophoresis–mass spectrometry; GC-MS, gas chromatography–mass spectrometry; GFP, green fluorescent protein; G6PD, glucose-6-phosphate 1-dehydrogenase; ORF, open reading frame; PDH, pyruvate dehydrogenase complex; RT-PCR, reverse transcription–PCR; TAG, triacylglycerol; TCA, tricarboxylic acid; WT, wild type.

Introduction

Microalgae have been considered to be a source of biofuel because they accumulate triacylglycerol (TAG) in a monolayer membrane organelle, so-called lipid droplets, under autotrophic culture conditions without any competition with the food supply (Hu et al. 2008, Radakovits et al. 2010, Scott et al. 2010, Liu and Benning 2013). However, biodiesel production from algae is as yet far from having achieved commercial viability (Chisti 2008). For economically efficient biofuel production, fast-growing, productive algal strains are required. To acquire such algae, various projects for identifying useful strains have been carried out (Sheehan et al. 1998, Griffiths and Harrison 2009, Rodolfi et al. 2009). However, there is little consensus yet because of the necessary trade-off between either a focus on algal growth or the accumulation of storage lipid. Oleaginous autotrophic algae exhibit a slow growth rate (Chen and Wu 2011). For example, the doubling time of the green alga Botryococcus braunii, which produces up to 80% of the dry cell weight of hydrocarbons, is 72 h (Sheehan et al. 1998). In contrast, fast-growing algae, such as the model green algae Chlamydomonas reinhardtii, accumulate TAG only under certain specific conditions, such as nitrogen-depleted media, under which it is difficult for them to proliferate (Liu and Benning 2013). Therefore, a direct screening of algae from nature is not sufficient for the demands of commercial use, and genetic modification of potentially useful algae is required.

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Several strategies for introducing metabolic modifications have been pursued in bacteria to down-regulate fatty acid degradation or to increase substrates for fatty acid synthesis (Li et al. 2008, Machado and Atsumi 2012, Peralta-Yahya et al. 2012). In contrast, there have been limited numbers of reports on metabolic modifications in eukaryotic algae for the purpose of biofuel production. In the diatoms (stramenopiles) *Cyclotella cryptica* and *Navicula saprophila*, the overexpression of acetyl-CoA carboxylase (ACCase), which is a key enzyme in fatty acid synthesis, failed to increase the lipid level (Sheehan et al. 1998). A starch synthesis mutant of *C. reinhardtii* produces more TAG than the wild type (WT), but the accumulation of TAG is promoted by nitrogen starvation to the point that cell growth ceases (WT, 5-fold; the mutant, 30-fold; Wang et al. 2009, Work et al. 2010).

Recent studies have started to increase the cellular TAG contents by genetic modifications while maintaining cellular growth in eukaryotic algae. It was reported that a knockout line of UDP-glucose pyrophosphorylase, which is involved in starch synthesis, in the diatom *Phaeodactylum tricornutum* accumulates 45-fold more TAG than the WT even in the presence of a nitrogen source in the medium (Daboussi et al. 2014). However, how the mutation affects cellular growth has not been reported. In the same diatom, knockdown of mitochondrial pyruvate dehydrogenase kinase, which inactivates pyruvate dehydrogenase, increased cellular neutral lipid content up to approximately 80% while decreasing the cellular growth rate slightly (Ma et al. 2014). In *C. reinhardtii*, expression of diacylglycerol acyltransferase (DGAT) from *Brassica napus* increased cellular neutral lipid content up to approximately 2-fold compared with the WT cells while maintaining the cellular growth rate similar to that of the WT (Ahmad et al. 2015).

*Chlamydomonas reinhardtii* has been the most extensively studied green alga because it is genetically tractable. However, methods for expressing genes of other organisms and gene targeting in *C. reinhardtii* are still at the developmental stage (Rosales-Mendoza et al. 2012, Liu and Benning 2013). In some other microalgae, methods for transformation have been reported, but as yet there are still far from being ready for practical use (Gong et al. 2011). The unicellular red alga, *Cyanidioschyzon merolae*, is the first alga for which the nuclear, plastid and mitochondrion genomes have been completely determined (i.e. without any gaps) (Ohta et al. 1998, Ohta et al. 2003, Matsuzaki et al. 2004, Nozaki et al. 2007). It possesses a simple nuclear genome (16.5 Mbp; 4,775 protein-coding genes) with low genetic redundancy (Matsuzaki et al. 2004, Misumi et al. 2005, Nozaki et al. 2007). Therefore, this alga is suitable for various "omics" analyses. Methods for genetic manipulation have been established, such as the transient expression of proteins from plasmids (Ohnuma et al. 2008, Ohnuma et al. 2009, Watanabe et al. 2011) and gene targeting by homologous recombination (Minoda et al. 2004). In contrast to *C. reinhardtii*, transgenes (either endogenous or exogenous genes) are stably overexpressed in *C. merolae* (Fujiwara et al. 2013, Sumiya et al. 2014, Watanabe et al. 2014), and are usually used for metabolic manipulation in several other organisms. Thus, *C. merolae* is suitable for an evaluation of genetic and metabolic manipulation for the purpose of enhancement of storage lipid accumulation.

As a first step towards the production of biofuel, we ectopically overexpressed cyanobacterial acyl-acyl carrier protein (ACP) reductase in *C. merolae*, which does not possess this enzyme, to increase the cellular storage lipid level. This was done for the following reasons. (i) In *Escherichia coli* and cyanobacteria, the overexpression of acyl-ACP thioesterase (for cyanobacteria, an *E. coli* enzyme was expressed) has been successfully used to increase the fatty acid level (Fig. 1) (Liu et al. 2011) and that of other hydrocarbons (Choi and Lee 2013). However, the cyanobacteria and *C. merolae* genomes do not encode this enzyme (Fig. 1) (Sato and Moriyama 2007, Kaczmarzyk and Fulda 2010). (ii) In cyanobacteria, overexpression of the acyl-ACP reductase resulted in free fatty acid accumulation through subsequent oxidation of fatty aldehydes by an endogenous aldehyde dehydrogenase (Fig. 1) (Kaiser et al. 2013). (iii) *Cyanidioschyzon merolae* possesses a putative aldehyde dehydrogenase, but not an acyl-ACP reductase, so the introduction of a cyanobacterial-like metabolic pathway into *C. merolae* by the expression of cyanobacterial acyl-ACP reductase would increase the free fatty acid and TAG levels (Fig. 1). (iv) In addition, a portion of fatty acid is converted to alkanes in cyanobacteria by aldehyde decarbonylase (Schirmer et al. 2010). Although this protein is unique to cyanobacteria and, at least thus far, the enzymes for eukaryotic alkanal synthesis have not been identified, small amounts of alkanes and alkenes were identified in the closely related red alga *Cyanidium caldarium* (Nagashima et al. 1986). If the functional homolog of the aldehyde decarbonylase exists in the *C. merolae* genome,

![Fig. 1](https://academic.oup.com/pcp/article-abstract/56/10/1962/2461010/Expression-of-Cyanobacterial-Acyl-ACP-Reductase/plantcellphysiol-56-10-fig1)

**Fig. 1** The overall pathway of fatty acid and TAG synthesis in *Cyanidioschyzon merolae*. The aldehyde decarbonylase and the acyl-ACP thioesterase that are shown in gray are absent from the *C. merolae* genome. The acyl-ACP reductase of *Synechocystis* sp. PCC6803 which is also absent from the *C. merolae* genome and shown in green is introduced in this study.
the expression of cyanobacterial acyl-ACP reductase in C. merolae would also be expected to lead to an increase in the alkane and/or alkene levels (Fig. 1).

Here we show that the expression of cyanobacterial acyl-ACP reductase in C. merolae yields a 3.0-fold larger amount of TAG than the WT while maintaining algal growth. The expression of acyl-ACP reductase induces the up-regulation of aldehyde dehydrogenase activity. It is also shown that fatty acid synthesis, fatty acid degradation and degradation of branched chain amino acids (BCAAs; Val/Leu/Ile) are all up-regulated. The up-regulation of these pathways required under a nitrogen-depleted condition, which increases the cellular TAG level but suppresses algal growth. These results suggest that there are means of metabolic manipulation that will further increase the storage lipid level without suppressing algal growth unlike the nitrogen-depleted condition.

**Results and Discussion**

**Expression of cyanobacterial acyl-ACP reductase increases the TAG level in C. merolae**

To examine the effect of the expression of a cyanobacterial acyl-ACP reductase on storage lipid accumulation in C. merolae, a stable transformant (AAR-3HA) was produced by inserting the acyl-ACP reductase gene of Synnechocystis sp. PCC 6803 (sil0209) into a C. merolae chromosomal neutral locus (Fujiwara et al. 2013). The transgene was expressed using a C. merolae catalase promoter which has constitutive transcriptional activity (Ohnuma et al. 2008). Because the proteins related to fatty acid synthesis localize in chloroplasts (Ohrrogge and Jaworski 1997), a cleavable transit peptide of CmSecA (Koyama et al. 2011) was fused to the N-terminus so as to target the expressed enzyme into C. merolae chloroplasts. A 3×HA tag was fused to the C-terminus of the enzyme for detection (Supplementary Fig. S1). For the control, we used the green fluorescent protein (GFP) expressing strain (S-200 in Sumiya et al. 2014) to evaluate the effect of the expression of AAR-3HA in the same genetic background (C. merolae M4, which has a mutation in the URA gene). AAR-3HA or GFP were inserted into the same genomic locus. The transcription of GFP is driven by a heat shock-inducible promoter, so this GFP-expressing strain does not express GFP at 42°C, which is the optimal temperature for C. merolae (Sumiya et al. 2014).

Expression of the 3×HA-tagged acyl-ACP reductase was confirmed by reverse transcription–PCR (RT-PCR) and immunoblotting using an anti-HA antibody. The acyl-ACP reductase mRNA was detected for at least 4 d after transfer to fresh medium (Fig. 2A). The anti-HA antibody detected a band of 38 kDa in AAR-3HA, but not in the GFP strain. This is a band which corresponds to the deduced molecular weight of acyl-ACP reductase (37.7 kDa, without the transit peptide) (Fig. 2B). Immunostaining with the anti-HA antibody showed that the expressed enzyme is targeted to chloroplasts (Fig. 2C).

When stationary-phase AAR-3HA cells were inoculated into the fresh medium, the acyl-ACP reductase level increased during a period of 2 d after the inoculation and then decreased (Fig. 2B, Supplementary Fig. S2A). TAG and fatty acids are normally stored in lipid droplets in algal cells (Wang et al. 2009, Moellering and Benning 2010). Consistent with the change in the protein level (Fig. 2B), the number and size of the lipid droplets (detected by the lipid droplet-specific fluorescent dye, BODIPY) increased during the 2 d after the inoculation and then decreased, although a slight increase of lipid droplets was also observed in the control GFP strain (Fig. 2B, D). The fluorescence intensity of BODIPY staining in AAR-3HA cells was approximately three times as high as that in GFP cells 1–2 d after transfer to fresh medium (Supplementary Fig. S2B). These results indicate that the expression of acyl-ACP reductase results in an acceleration of lipid droplet formation in C. merolae. We further investigated the lipid droplet formation under high light conditions, because high light intensity has been reported to increase the amount of TAGs in other algae (Hu et al. 2008). AAR-3HA and the control GFP cells were transferred from low light (100 μE m⁻² s⁻¹) to a high light condition (300 and 500 μE m⁻² s⁻¹). Both AAR-3HA and the control GFP cultures were bleached in 1 d at 500 μE m⁻² s⁻¹ but not at 300 μE m⁻² s⁻¹. The number and size of lipid droplets in AAR-3HA cells increased after the inoculation also at 300 μE m⁻² s⁻¹ (high light condition; Supplementary Fig. S3) but the start of the increase was delayed compared with the low light condition (Supplementary Fig. S2). However, there was no significant difference in the maximal fluorescence intensity of BODIPY staining in AAR-3HA cells between the low and high light (3 d after inoculation for 300 μE m⁻² s⁻¹ and 2 d after inoculation for 100 μE m⁻² s⁻¹ respectively) (Supplementary Figs. S2B, S3B). In a similar manner, there is no significant difference in the fluorescent intensity in the control strain between high and low light (Supplementary Figs. S2B, S3B). Therefore, in C. merolae, high light intensity does not enhance lipid droplet production either in the control strain or in AAR-3HA cells.

These observations suggest that the accumulation of certain kinds of storage lipids is enhanced in AAR-3HA cells. Therefore, we next asked which specific kinds of compounds (i.e. TAGs, free fatty acids, fatty aldehydes and alkanes) are increased by the expression of acyl-ACP reductase 1 d after the inoculation into fresh medium.

Quantification by gas chromatography showed that the total TAG level in the AAR-3HA cells was 3.0 times larger than in GFP cells (Fig. 3A). In contrast to TAG, the total free fatty acid and total fatty acid levels in AAR-3HA cells did not show any significant differences from those in GFP cells (Student’s t-test, P < 0.05) (Fig. 3A). The total TAG level in AAR-3HA cells was approximately 6.1% of the total cellular fatty acids. The TAG fatty acid profile in the AAR-3HA cells was almost similar to that in the GFP cells, except that the palmitic acid (C16:0) level decreased compared with the GFP cells, while the levels of 17:0, 18:1 and 20:1 of the total fatty acids in AAR-3HA cells were slightly higher than those in the GFP cells (Fig. 3B). There was no significant difference in the levels of fatty aldehydes between the AAR-3HA and GFP cells (Fig. 3C).
suggesting that fatty aldehyde, which is produced by acyl-ACP reductase, was rapidly converted to fatty acids (see below for the up-regulation of the aldehyde dehydrogenase). Gas chromatography–mass spectrometry (GC-MS) analyses detected heptadecane as a major peak. Other alkanes, especially nonadecane, were present in *C. merolae* cells, but the amount was too low to be analyzed (Fig. 3D). *Cyanidioschyzon merolae* is distinct in this regard compared with the two strains of

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**Fig. 2** Expression of the cyanobacterial acyl-ACP reductase increases the number and size of lipid droplets. (A) The level of acyl-ACP reductase (AAR) mRNA expressed from a *C. merolae* chromosomal neutral locus just before (0 d) to 4 d after the inoculation. TIM13 (mitochondrial intermembrane space complex subunit, CMB148C) was used as a quantitative control for the semi-quantitative RT-PCR. (B) Change in the protein level of acyl-ACP reductase (AAR-3HA) after the inoculation. Acyl-ACP reductase has C-terminal 3HA tags, so an anti-HA antibody was used for the detection. Coomassie Brilliant Blue (CBB) staining is shown as a loading control. For the control, a strain in which a GFP gene was inserted at the same chromosomal neutral locus in the same parental strain (M4; the uracil autotrophic parental mutant) was used. (C) Immunofluorescence microscopy showing the localization of AAR-3HA in chloroplasts. A GFP-expressing strain was used as a control, but GFP is not expressed under this condition (see the text). PC, phase contrast; green, AAR-3HA detected with anti-HA antibody; magenta, autofluorescence of Chl. The arrowheads indicate the cell surface. Scale bars = 5 μm. (D) Change in the number and size of lipid droplets in the AAR-3HA and GFP strain. Lipid droplets were stained with BODIPY. Green, lipid droplets stained by BODIPY; magenta, autofluorescence of Chl. Scale bar = 10 μm.
C. caldarium which possess both alkanes and alkenes (Nagashima et al. 1986). The heptadecane level was similar in the AAR-3HA and the GFP cells (Fig. 3E), suggesting that *C. merolae* does not possess a functional homolog of cyanobacterial aldehyde decarbonylase (i.e. the alkane in *C. merolae* is produced by an entirely novel pathway) or that the functional homolog has a much lower activity than that of the aldehyde dehydrogenase. These results show that the

**Fig. 3** The effect of the expression of the cyanobacterial acyl-ACP reductase on the cellular levels of TAG, free fatty acids, total fatty acids, fatty aldehyde and alkanes. The levels in AAR-3HA and GFP cells 1 d after inoculation are shown. (A) The levels of cellular TAG, free fatty acids and total fatty acids per dry cell weight are shown. (B) Relative levels of TAG-derived fatty acids, free fatty acids and total fatty acids extracted from the cells are shown. (C) Relative fatty aldehyde levels per dry cell weight normalized by an internal standard (pentadecane). Blue, GFP cells; red, AAR-3HA cells; DCW, dry cell weight. The error bars represent the SD (n = 6 for TAG and free fatty acids, n = 3 for total fatty acids). Double asterisks indicate a significant difference (Student’s *t*-test, *P* < 0.05). (D) GC-MS spectra of alkanes. The GC-MS spectrum from AAR-3HA cells is shown in red and the spectrum from the retention time index of alkanes is shown in black. (E) Heptadecane level. Blue, GFP cells; red, AAR-3HA cells; DCW, dry cell weight. The error bars represent the SD (n = 3).
expression of acyl-ACP reductase increases the level of TAG, but not free fatty acids, fatty aldehydes or alkanes in \textit{C. merolae} cells.

**Glycolysis/glucconeogenesis are down-regulated and the transcript level of genes directly related to TAG synthesis is down-regulated in the acyl-ACP reductase expresser**

To examine how expression of acyl-ACP reductase changes cellular metabolic activities and eventually leads to the acceleration of TAG accumulation in \textit{C. merolae}, we compared the transcriptome between AAR-3HA and the control GFP cells 1 and 4 d after inoculation by microarray analysis.

Of the 4,946 genes that were examined in the microarray assay, 661 and 785 genes were up-regulated (\(<-1.5\)) and down-regulated (\(>-0.7\)), respectively, in AAR-3HA cells compared with the GFP cells 1 d after inoculation (Fig. 4A; Supplementary Table S1). Of the 661 up-regulated genes, the mRNA levels of most (655) of the genes were then down-regulated in the AAR-3HA cells 4 d after inoculation (Supplementary Table S1). In a similar manner, of the 785 down-regulated genes, the mRNA levels of most (773) of the genes were then up-regulated in the AAR-3HA cells 4 d after inoculation (Supplementary Table S1).

It has been reported that glycolysis/gluconeogenesis and TAG synthesis are up-regulated in other eukaryotic algae during the accumulation of TAG under nitrogen-depleted conditions (Miller et al. 2010, Hockin et al. 2012, Msanne et al. 2012, Ito et al. 2013). In contrast, in AAR-3HA \textit{C. merolae} cells, the transcript level of most of the genes related to carbohydrate metabolism such as glycolysis, the pentose phosphate pathway, pyruvate metabolism and the tricarboxylic acid (TCA) cycle were unchanged or decreased (Fig. 4B). Therefore, we first examined changes in these pathways in detail.

Regarding glycolysis/gluconeogenesis and the pentose phosphate pathway, the pathways exist in both the cytosol and chloroplast in \textit{C. merolae} (Moriyama et al. 2014). The mRNA levels of the genes involved in cytoplasmic glycolysis/gluconeogenesis were down-regulated in the AAR-3HA cells compared with the GFP cells 1 d after inoculation (Fig. 5; Supplementary Table S2). The mRNA level of chloroplastic glucose-6-phosphate 1-dehydrogenase (G6PD, CMR014C), the key enzyme in the pentose phosphate pathway, was down-regulated in AAR-3HA cells compared with GFP cells (Fig. 5; Supplementary Table S3). Most of genes involved in the pentose phosphate pathway in the chloroplast are functionally equivalent to the Calvin cycle. The mRNA level of three Calvin cycle-specific enzymes, i.e. ribulose 1,5-bisphosphate carboxylase/oxygenase (rbcl, CMV013C; rbcS, CMV014C), sedoheptulose-1,7-bisphosphatase (SBPase, CM1996C) and phosphoribulokinase (PRK, CMF117C), in the AAR-3HA cells were comparable with that in GFP cells (Fig. 5; Supplementary Table S4). To examine the metabolite changes in parallel with the increase in TAG level, we further compared the metabolome between AAR-3HA and GFP cells by capillary electrophoresis–mass spectrometry (CE-MS) analyses. Because the mRNA levels of most of the genes were up-regulated or down-regulated in AAR-3HA cells 1 d after inoculation but then returned to the same level by 4 d after inoculation as in GFP cells, we compared the metabolome before and 1 d after inoculation. The metabolic intermediates of glycolysis/gluconeogenesis, the pentose phosphate pathway and the Calvin cycle were increased in the AAR-3HA cells 1 d after inoculation compared with the GFP cells (Fig. 6). Given that glycolysis/gluconeogenesis and the pentose phosphate pathway were down-regulated at the transcript level by the expression of acyl-ACP reductase (Fig. 5), the accumulation of the metabolic intermediates probably resulted from delayed metabolic flow in glycolysis/gluconeogenesis, the pentose phosphate pathway and the Calvin cycle in the AAR-3HA cells.

In terms of the genes directly related to TAG synthesis (from glycerol-3 phosphate to TAG), namely glycerol-3-phosphate O-acyltransferase (GPAT), lysophosphatidyl acyltransferase (LPAAAT), phosphatidic acid phosphatase (PAP) and diacylglycerol acyltransferase (DGAT), these mRNA levels were unchanged or down-regulated (Fig. 5; Supplementary Table S5). The mRNA levels of triacylglycerol lipase homologs (CM5254C, CMP157C and CMT151C), which degrade TAG to diacylglycerol (DAG) in other eukaryotes (Attenstaedt and Daum 2006; the function of the homologs has not been characterized in algae), was up-regulated in AAR-3HA compared with GFP cells 1 d after the inoculation (Fig. 5; Supplementary Table S5). In contrast, 4 d after the inoculation, when the number and size of the lipid droplets had decreased to the original level (Fig. 2D), the mRNA levels related to both TAG synthesis and degradation in AAR-3HA cells came closer to the levels in GFP cells. Therefore, the increase in the TAG level by expression of acyl-ACP reductase does not result from an up-regulation of the TAG synthetic pathway at the transcriptional level.

**Aldehyde dehydrogenase activity is up-regulated in the acyl-ACP reductase expresser**

The above results show that glycolysis/gluconeogenesis and the pathway directly related to TAG synthesis are down-regulated in AAR-3HA cells, in contrast to other algae that accumulate TAG under a nitrogen-depleted condition. In order to obtain insights into how TAG is accumulated in AAR-3HA cells, we examined the changes in other metabolic pathways.

In cyanobacteria, fatty aldehydes are degraded to fatty acids by aldehyde dehydrogenase (Fig. 1) (Kaiser et al. 2013). The \textit{C. merolae} genome encodes a putative homolog (CMO345C) which is closely related to the aldehyde dehydrogenase of \textit{Synechococcus elongatus} PCC 7942 (identity, 37%; similarity, 51%; AldE in Fig. 5). Acyl-ACP reductase synthesizes fatty aldehydes from fatty acyl-ACP. It is known that fatty aldehydes are toxic to organisms (Laurenzi et al. 1996, Kaiser et al. 2013). As shown above, the aldehyde level in AAR-3HA was similar to that in GFP cells (Fig. 3C), so the fatty aldehydes produced by acyl-ACP reductase are probably rapidly converted to fatty acids in AAR-3HA.

To confirm that CMO345C possesses fatty aldehyde dehydrogenase activity in \textit{C. merolae} cells, 6×His-tagged CMO345C (deduced mol. wt, 58.3 kDa) was expressed in \textit{E. coli} and purified (Fig. 7A). The aldehyde dehydrogenase
activity was examined using decanal (C10 aldehyde) and octadecanal (C18 aldehyde) as substrates in the CMO345C-expressing E. coli crude lysate and in the CMO345C purified fraction. The crude cell lysate exhibited the aldehyde dehydrogenase activity and the activity was enriched by purification (Fig. 7A). $K_{cat}/K_m$ of the recombinant CMO345C was $61.4 \pm 8.6$ and $17.7 \pm 4.0$ min$^{-1}$ $mM^{-1}$ for decanal and octadecanal, respectively. The $K_m$ of CMO345C was $169.1 \pm 3.9$ and $48.4 \pm 6.3$ $mM$ for decanal and octadecanal, respectively. These values were similar to the values of S. elongatus aldehyde dehydrogenase, AldE (Kaiser et al. 2013). These data show that CMO345C possesses fatty aldehyde dehydrogenase activity.

Transcriptome analyses showed that the CMO345C mRNA level (AldE in Fig. 5) is up-regulated in AAR-3HA compared with the GFP cells both 1 and 4 d after inoculation ($1.86 \pm 0.09$ and $1.33 \pm 0.06$ times larger than the GFP cells, respectively). Therefore, the transient increase in the fatty aldehyde level probably leads to an increase in aldehyde dehydrogenase activity so as to avoid fatty aldehyde accumulation by the activity of acyl-ACP reductase.

Then, we produced a CMO345C knockout strain ($\Delta$CMO345C) and an acyl-ACP reductase-expressing strain on a $\Delta$CMO345C background (AAR-3HA $\Delta$CMO345C, Supplementary Fig. S4) to examine the contribution of CMO345C to the total cellular aldehyde dehydrogenase activity and TAG accumulation by the expression of acyl-ACP reductase. Transcription (Supplementary Fig. S5A) and translation (Supplementary Fig. S5B) of acyl-ACP reductase were confirmed in the AAR-3HA $\Delta$CMO345C cells as in the AAR-3HA cells (Fig. 2). Fatty aldehyde dehydrogenase activity in the crude cell extract was measured by using decanal (C10 aldehyde) and octadecanal (C18 aldehyde) as substrates. The expression of cyanobacterial acyl-ACP reductase up-regulated fatty aldehyde dehydrogenase activity (AAR-3HA compared with the GFP strain) (Fig. 7B). In contrast, with the $\Delta$CMO345C background, this up-regulation was not observed (AAR-3HA $\Delta$CMO345C compared with AAR-3HA $\Delta$CMO345C) (Fig. 7B). The obvious increase of lipid droplets was not observed in $\Delta$CMO345C and AAR-3HA $\Delta$CMO345C (Fig. 7C), in contrast to AAR-3HA (Fig. 2D).

The cellular concentration of most of the fatty aldehydes is higher in $\Delta$CMO345C than in GFP cells and higher in AAR-3HA $\Delta$CMO345C than in $\Delta$CMO345C (Fig. 7D). These results suggest that CMO345C degrades aldehydes and AAR-3HA produces aldehydes.

Among the aldehydes examined, the long chain fatty aldehydes (C16, C18 and C22) in particular accumulated in $\Delta$CMO345C and AAR-3HA $\Delta$CMO345C compared with AAR-3HA and GFP cells (Fig. 7D). In AAR-3HA $\Delta$CMO345C, the accumulation of octadecanal (C18) and docosanal (C22) was especially evident (Fig. 7D). The carbon number of these accumulated fatty aldehydes is $\geq 16$ (Fig. 7D), which is the number of newly synthesized fatty acids by the fatty acid synthesis pathway, suggesting that CMO345C contributes to fatty acid production. Therefore, the up-regulation of the aldehyde dehydrogenase (CMO345C) activity contributes to the formation of fatty aldehydes.
Fig. 5 Effect of the expression of the cyanobacterial acyl-ACP reductase on the transcriptome related to carbon metabolism. The large green arrow indicates the acyl-ACP reductase which is expressed in C. merolae AAR-3HA cells. The orange arrows indicate the transcripts which are up-regulated (>1.5 times) in AAR-3HA compared with GFP cells 1 d after inoculation. The blue arrows indicate the transcripts down-regulated (<0.7 times) in AAR-3HA compared with GFP cells 1 d after inoculation. G6PD, glucose-6-phosphate dehydrogenase; SBPase, sedoheptulose-1,7-bisphosphatase; PRK, phosphoribulokinase; KCS, β-ketoacyl-CoA synthase; ACX, fatty acyl-CoA oxidase; AldE, aldehyde dehydrogenase; AAR, acyl-ACP reductase; PDH, pyruvate dehydrogenase; ACCase, acetyl-CoA carboxylase; BCKDH, branched chain α-keto acid dehydrogenase; IDH, isocitrate dehydrogenase; (NAD+); Glu, glucose; G6P, glucose-6-phosphate; G1P, glucose-1-phosphate; F6P, fructose-6-phosphate; F1,6BP, fructose 1,6-bisphosphate; GADP, glyceraldehyde 3-phosphate; 1,3BPG, 1,3-bisphospho-glycerate; 3PG, 3-phosphoglyceric acid; 2PG, 2-phosphoglyceric acid; PEP, phosphoenolpyruvic acid; Pyr, pyruvate; G1,5LP, D-glucono-1,5-lactone-6-phosphate; 6PDG, 6-phospho-D-gluconate; S7P, sedoheptulose-7-phosphate; R5P, ribose-5-phosphate; RuBP, ribulose-1,5-bisphosphate.
Fig. 6 Effect of the expression of the cyanobacterial acyl-ACP reductase on the levels of the metabolites related to carbohydrate metabolism. The content of the metabolites per dry cell weight was determined by anionic and cationic CE-MS analyses. The levels in AAR-3HA against GFP cells just before (0 d) and 1 d after inoculation are visualized in color. Red indicates the increased metabolites in AAR-3HA compared with GFP cells, and blue indicates the decreased metabolites in AAR-3HA compared with GFP cells. Diagonal lines indicate the metabolites that were not detected in AAR-3HA or GFP cells. Asterisks indicate a significant difference (Student's t-test, \( P < 0.05, n = 3 \)). The orange arrows indicate the transcripts which are up-regulated (>1.5 times) in AAR-3HA compared with GFP cells 1 d after inoculation. The blue arrows indicate the transcripts down-regulated (<0.7 times) in AAR-3HA compared with GFP cells 1 d after inoculation. G1, 5LP, glucono-1,5-lactone-6-phosphate; 6PDG, 6-phosphogluconic acid; erythrose-4P, d-erythrose 4-phosphate; SBP, sedoheptulose 1,7-bisphosphate; S7P, d-sedoheptulose 1970

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by guest on 16 September 2017
increase of the number and size of lipid droplets in AAR-3HA cells.

**Chloroplast-localized acetyl-CoA carboxylase is up-regulated in the acyl-ACP reductase expresser**

It is expected that the up-regulation of aldehyde dehydrogenase activity in AAR-3HA cells stimulates the synthesis of their substrate, fatty acyl-ACP, for the following reasons: (i) fatty acyl-ACP is produced by the fatty acid synthesis pathway and the rate-limiting enzyme in this pathway is ACCase, which catalyzes the carboxylation of acetyl-CoA to malonyl-CoA (Bao and Ohlrogge 1999). (ii) Acyl-ACP inhibits the transcription and activity of ACCase (Davis and Cronan 2001, Sasaki and Nagano 2004, Zhang and Rock 2009, Andre et al. 2012). Therefore, the expression of acyl-ACP reductase in *C. merolae* was expected to reduce the acyl-ACP level and thus affect ACCase activity. There are two types of ACCase in plants. One is a chloroplast-localized heteromeric ACCase, which is composed of four subunits, and the other is a cytoplasmic homomeric ACCase, which is composed of a single polypeptide (Sasaki and Nagano 2004). Fatty acid synthesis occurs predominantly in the chloroplasts. In contrast, malonyl-CoA synthesized by cytoplasmic ACCase is consumed as the precursor for flavonoids, anthocyanins, very long chain fatty acids, and so on (Sasaki and Nagano 2004). The transcriptome analyses showed that there is no significant difference in the cytoplasmic homomeric ACCase (CMM188C) mRNA level between AAR-3HA and GFP cells both 1 and 4 d after inoculation (Supplementary Table S6). Among the components of the chloroplast heteromeric ACCase, the mRNA levels of three components, i.e. accA, accB and accD (CMV056C, CMV134C and CMV207C, respectively), which are encoded in the chloroplast genome, were up-regulated in AAR-3HA cells compared with GFP cells 1 d after inoculation (Fig. 5; Supplementary Table S6).

Consistent with the transcriptional up-regulation of the chloroplastic ACCase, the ACCase activity in the total cell extract in AAR-3HA increased and was 4.4 times higher than that in GFP cells 1 d after inoculation (Fig. 8A). This up-regulation of ACCase activity by the expression of acyl-ACP reductase was not observed in AAR-3HA ΔCMO345C (Fig. 8A). By 4 d after inoculation, when the number of the lipid droplets had decreased (Fig. 2D), the ACCase activity decreased to the same level as in GFP cells. These results suggest that the up-regulation of aldehyde dehydrogenase activity by the expression of acyl-ACP reductase stimulates the transcription and activity of chloroplastic ACCase.

**Chloroplastic pyruvate dehydrogenase is up-regulated in the acyl-ACP reductase expresser**

Given that the chloroplastic fatty acid synthesis pathway is up-regulated, the production of acetyl-CoA, which is the substrate for ACCase, is also likely to be stimulated in AAR-3HA. Acetyl-CoA is supplied by the pyruvate decarboxylation catalyzed by the pyruvate dehydrogenase complex (PDH). Pyruvate dehydrogenase is a multienzyme complex, and photosynthetic eukaryotes including *C. merolae* possess both mitochondrial and chloroplast complexes (Tovar-Méndez et al. 2003, Moriyama et al. 2014). The acetyl-CoA for the fatty acid synthesis pathway is produced by the chloroplastic complex in both plants and algae (Tovar-Méndez et al. 2003, Shitaide et al. 2015). The mRNA levels of the chloroplast PDH E1 component α- and β-subunit genes (CMV153C and CMV154C) were up-regulated (2.18 ± 0.10 and 1.79 ± 0.05 times, respectively) in AAR-3HA compared with GFP cells 1 d after inoculation (Fig. S5; Supplementary Table S7).

The PDH activity in the total AAR-3HA cell extract was 3.6 times higher than that in the GFP extract 1 d after inoculation (Fig. 8B). The PDH activity was examined by using the total cell lysate, so the up-regulation of PDH activity was probably due to changes in mitochondrial PDH activity. However, the mRNA levels of the mitochondrial PDH E1α, β and E2 components were down-regulated in AAR-3HA compared with GFP cells 1 d after inoculation (Fig. 5; Supplementary Table S7), in contrast to chloroplastic PDH. The acetyl-CoA produced by the mitochondrial complex is subsequently consumed by the TCA cycle (Tovar-Méndez et al. 2003). mRNA levels of some of the enzymes in the TCA cycle, which includes two copies of the rate-limiting enzyme, isocitrate dehydrogenase (NAD\(^+\)) subunit (IDH in Fig. 5; CMT412C, and CMS272C (Wiskich and Dry 1985), were also down-regulated in AAR-3HA cells compared with GFP cells 1 d after inoculation (Fig. 5; Supplementary Table S8). In addition, metabolites in the TCA cycle and most of the amino acids derived from the TCA cycle metabolites (Fig. 6) were increased in AAR-3HA cells compared with GFP cells 1 d after inoculation. These results suggest that the up-regulation of PDH activity in the total cell lysate in AAR-3HA cells resulted from the up-regulation of chloroplastic but not mitochondrial PDH activity.

The increase in the total cellular PDH activity was not observed in ΔCMO345C and AAR-3HA ΔCMO345C, suggesting that the up-regulation of the PDH activity in AAR-3HA cells requires the up-regulation of aldehyde dehydrogenase (Fig. 8B).

In summary, in AAR-3HA, (i) chloroplastic PDH, which produces acetyl-CoA for fatty acid synthesis, is up-regulated, while mitochondrial PDH, which produces acetyl-CoA for the TCA cycle, is down-regulated; (ii) chloroplastic ACCase, but not cytoplasmic ACCase, is up-regulated; and (iii) aldehyde dehydrogenase activity is up-regulated, and it is suggested that chloroplastic fatty acid synthesis is up-regulated by the expression of cyanobacterial acyl-ACP reductase in *C. merolae* chloroplasts (Fig. 9).
Fig. 7 Aldehyde dehydrogenase activity is up-regulated by the expression of the cyanobacterial acyl-ACP reductase and this up-regulation is required for the increase in the number and size of lipid droplets. (A) Aldehyde dehydrogenase activity of CMO345C protein in vitro. *Cyanidioschyzon merolae* putative aldehyde dehydrogenase homolog CMO345C was expressed in *E. coli* and purified. Coomassie Brilliant Blue (CBB)-stained SDS–polyacrylamide gel is shown on the left. Aldehyde dehydrogenase activities of CMO345C-expressing *E. coli* crude cell lysate and CMO345C protein purified from *E. coli* using decanal and octadecanal as substrates are shown on the right. The lysate of *E. coli* containing the empty pET-100 vector was used as a negative control. Error bars represent the SD (n = 3). (B) Aldehyde dehydrogenase activity using decanal or octadecanal as a substrate. The activity was determined in crude cell extract prepared from AAR-3HA, GFP, AAR-3HA ΔCMO345C and ΔCMO345C. The boiled lysate was used as a negative control. Error bars represent the SD (n = 5). Double asterisks indicate a
The total free amino acid level is increased, but BCAA degradation is up-regulated in the acyl-ACP reductase expresser

Recently, Ge et al. (2014) reported that the up-regulation of genes related to BCAA degradation promotes TAG accumulation, and suggested that the products of BCAA degradation are converted to acetyl-CoA via malate and pyruvate in Phaeodactylum tricornutum. In AAR-3HA cells, the mRNA of the genes related to Val/Leu/Ile (BCAA) degradation were increased in AAR-3HA cells compared with GFP cells 1 d after the inoculation (Figs. 5, 6; Supplementary Table S10). The key enzyme in this pathway is the branched chain α-keto acid dehydrogenase complex (BCKDH in Fig. 5; Mooney et al. 2002). The BCKDH activity in the total cell extract of AAR-3HA cells was three times higher than that of GFP cells 1 d after inoculation (Fig. 8C). By 4 d after inoculation, the activity decreased to the same level as in the GFP cells. The up-regulation of BCKDH activity was not observed in ΔCMO345C or AAR-3HAΔCMO345C cells, suggesting that the up-regulation of aldehyde dehydrogenase is required for the up-regulation of BCKDH activity (Fig. 8C). These results suggest that the up-regulation of BCAA degradation is also likely to contribute to the accumulation of TAG in AAR-3HA cells.

β-Oxidation is up-regulated in the acyl-ACP reductase expresser

Although the number and size of lipid droplets increased for 2 d after inoculation in the acyl-ACP reductase expresser, they then decreased. A possible reason for the decrease is activation of a lipid degradation pathway in AAR-3HA cells. In support of this idea, fatty acid degradation (β-oxidation) was also up-regulated in AAR-3HA cells compared with GFP cells 1 d after inoculation at the transcript level (Fig. 5; Supplementary Table S11). Even 4 d after inoculation, the
Comparison of the pathways up- and down-regulated by the expression of acyl-ACP reductase or nitrogen depletion

Many algal species accumulate cellular storage lipids (mainly TAGs) in lipid droplets when the cells are transferred to nitrogen-depleted media, although cell growth ceases. However, the mechanism for sensing nitrogen starvation and the mechanisms to up-regulate TAG synthesis are still unknown. As shown in other algal species (Wang et al. 2009, Moellering and Benning 2010, Liu and Benning 2013), nitrogen depletion from the medium resulted in an accumulation of lipid droplets in both AAR-3HA and GFP cells (Fig. 10A). The average intensity of BODIPY fluorescence per cell in AAR-3HA was higher than in GFP cells throughout the culture in the nitrogen-depleted medium (Fig. 10A; Supplementary Fig. S6A). However, the size of the AAR-3HA cells was larger than that of GFP cells (Fig. 10A) and the average intensity of BODIPY fluorescence per area in AAR-3HA was almost the same as that in GFP cells (Fig. 10A; Supplementary Fig. S6B).

To examine whether the up- and down-regulated pathways brought about by the expression of acyl-ACP reductase are similar to those under nitrogen depletion, we compared the AAR-3HA and GFP cells under a nitrogen-depleted condition with a nitrogen-replete condition. Regarding the growth rate, the AAR-3HA and GFP cells in the nitrogen-depleted medium stopped their growth 4 d after inoculation. In contrast, AAR-3HA and GFP cells in the nitrogen-replete medium continued to grow until 7 d after inoculation, a point at which AAR-3HA cells reached a level 0.7-fold of that of the GFP cells (Fig. 10B).

Chl a and the total protein level were decreased in the GFP cells under the nitrogen-depleted condition (Fig. 10C, D), as in the case of green algae (Li et al. 2008, Cakmak et al. 2012, Msanne et al. 2012, Rismani-Yazdi et al. 2012). However, these levels in AAR-3HA decreased more slowly than in the GFP cells, exhibiting 2.3 (Chl a) and 1.7 (total protein) times the levels in the GFP cells 5 d after nitrogen depletion (Fig. 10C, D). In terms of the Chl level, the difference was also evident in the red autofluorescence in Fig. 10A. In contrast to the nitrogen-depleted condition, the levels in AAR-3HA changed in a similar manner to the GFP cells under the nitrogen-replete condition (Fig. 10C, D). Consistent with the pattern of Chl a (Fig. 10C), the mRNA levels of Chl a/b-binding protein (cab, CMN234C and CMN235C) and Chl a synthase (ChIG, CMT220C) were down-regulated under the nitrogen-depleted condition in the GFP cells (Fig. 10E).

As shown above (Fig. 5; Supplementary Table S6), the mRNA level of chloroplast-localized ACCase was up-regulated in AAR-3HA cells compared with GFP cells under the nitrogen-replete condition (Fig. 10E). In contrast, the levels were down-regulated in the GFP cells, but not in AAR-3HA cells, under the nitrogen-depleted condition (Fig. 10E).

It was previously reported that the pentose phosphate pathway is up-regulated upon nitrogen depletion to supply reduced equivalents to the fatty acid synthesis pathway in two green algal species (Miller et al. 2010, Rismani-Yazdi et al. 2012). In a similar manner, the transcript level of G6PD (an enzyme involved in the pentose phosphate pathway, CMI224C and CMR014) was up-regulated in both the AAR-3HA and GFP cells under the nitrogen-depleted condition (Fig. 10E). In contrast, the level was down-regulated in the AAR-3HA cells compared with the GFP cells under the nitrogen-replete condition (Figs. 5, 10E; Supplementary Table S3).

A down-regulation of carbon fixation under a nitrogen-depleted condition is common in green algae (Miller et al. 2010). In both AAR-3HA and GFP cells, the mRNA levels of Rubisco (rbcL, CMV013C; and rbcS, CMV014C) were down-regulated under a nitrogen-depleted condition (Fig. 10E).
Fig. 10 Differences between acyl-ACP reductase-expressed and GFP cells under nitrogen-replete and nitrogen-depleted conditions. (A) Change in the number and size of lipid droplets in nitrogen-depleted AAR-3HA and GFP cells before (0 d) to 5 d after inoculation. Lipid droplets were stained with BODIPY. Green, lipid droplets stained by BODIPY; magenta, autofluorescence of Chl; gray, phase-contrast. Scale bar = 10 μm. (B–D). Changes in the cell number (B), Chl a level (C) and total protein level (D) in nitrogen-replete AAR-3HA cells (magenta), nitrogen-depleted AAR-3HA cells (blue), nitrogen-replete GFP cells (light green) and nitrogen-depleted GFP cells (dark green) after inoculation are shown. The error bars represent the SEs (n = 4). The cell number at day 0 (5 × 10^6 cells ml^−1 for each culture) was defined as 1.0 in (B). (E) Semi-quantitative RT-PCR showing the relative mRNA levels of the genes related to the fatty acid synthesis pathway, photosynthesis (Chl a-binding protein, cab, CMN234C and CMN235C), Chl synthesis (Chl a synthase, Chlg), pentose phosphate pathway (glucose-6 phosphate dehydrogenase, G6PD, CMI224C and
These results suggest that the expression of cyanobacterial acyl-ACP reductase under a nitrogen-replete condition changes the activity of the metabolic pathways in a manner quite different from nitrogen starvation.

In eukaryotic algae under nitrogen depletion, TAG is believed to be synthesized at the expense of Chl and proteins (Mribbon et al. 2012, Levitan et al. 2015). TAG in *C. merolae* nitrogen-depleted GFP cells is probably synthesized in a similar manner to that in other algae, because we have observed a decrease of the Chl and protein levels. In contrast, the milder decrease in Chl and protein levels in AAR-3HA than in GFP cells under the nitrogen-depleted condition (both yielded approximately the same level of lipid droplets) probably indicates the existence of an additional carbon source for TAG synthesis under nitrogen-depleted conditions, at least in AAR-3HA cells. The up-regulation of chloroplastic ACCase in the nitrogen-depleted AAR-3HA but not GFP cells suggests that fatty acids synthesized in the chloroplast may be an additional carbon source for TAG synthesis.

In this study, the expression of cyanobacterial acyl-ACP reductase in *C. merolae* cells resulted in lipid droplet (TAG) accumulation without a severe cell growth defect. Nitrogen starvation also resulted in lipid droplet accumulation, while cell growth ceased, as in the case of other algae. The cellular responses and metabolic changes upon the expression of acyl-ACP reductase are quite different from those observed upon nitrogen starvation. Therefore, there should be a method for further increasing the storage lipid level while still maintaining cell growth that is different from the metabolic response to nitrogen starvation.

As in the eukaryotic alga *C. merolae*, the overexpression of acyl-ACP reductase increases free fatty acid synthesis in cyanobacteria (Kaiser et al. 2013). In addition, overexpression of acyl-ACP reductase and aldehyde dehydrogenase together resulted in greater accumulation of free fatty acids than the overexpression of acyl-ACP reductase alone. Thus, the expression of acyl-ACP reductase (and up-regulation of aldehyde dehydrogenase) enhances fatty acid production in both cyanobacteria and eukaryotic red algal chloroplasts. Although acyl-ACP reductase is not encoded in the genomes of eukaryotic phototrophs, including *C. merolae*, putative aldehyde dehydrogenase is well conserved in eukaryotic algae and land plants.

### Materials and Methods

#### Algal culture

*Cyanidioschyzon merolae* 10D and its derivatives were grown in Allen’s medium in a 1 liter flat bottle (60 mm thick; containing 1 liter of culture) with 5 l min⁻¹ aeration with ambient air or in a 100 ml test tube (30 mm thick; containing ~50 ml of culture) at 100 or 300 µE m⁻² s⁻¹ (high light condition) with 250 ml min⁻¹ aeration with ambient air under continuous light at 42°C. The walls of the culture bottles or test tubes were directed to fluorescent lamps so that the self-shading of the cells is minimized (Miyagishima et al. 2012). For nitrogen starvation, nitrogen-free medium, which has the same components as Allen’s medium except for 20 mM Na₂SO₄ instead of (NH₄)₂SO₄ (Imamura et al. 2009), was used.

#### Plasmid construction

To express cyanobacterial acyl-ACP reductase in *C. merolae*, we constructed a plasmid containing the catalase promoter, the N-terminal 130 amino acids of *CmSecA*, acyl-ACP reductase of *Synechocystis* sp. PCC6803 (sib0209), 3× HA tags and the Nos terminator, in this order. The primer sequences are listed in Supplementary Table S12. The 1.500 bp upstream of the catalase promoter (CM050C) ORF (open reading frame) with a 3× HA tag was amplified by the primers CatPro_HinSphI_bla_Nter and CatPro_H3HA_Not_C_C1, and the product was further amplified with the primers CatPro_HinSphI_bla_Nter and CatPro_H3HA_Not_C_2. The PCR product was digested with HindIII and NotI, and inserted into the HindIII and NotI sites of a pBluescript-GFP vector (Ohnuma et al. 2008). The resultant plasmid was digested with BglII and BamHI and the CmSecA fragment (amplified by primer sets SecAnuc_1-BglII_1 and SecAnuc-130-BamHI_2) inserted using an In-fusion cloning kit (TAKARA BIO INC.). The result plasmid was digested with BamHI and the PCC6803 acyl-ACP reductase gene (amplified with the primers orf1594_BamHI_Nter and orf1594_BamHI_Cter) was inserted with an In-fusion cloning kit to produce pUC18_CatP_SecA_AAR-3HA_Nos. The region from the catalase promoter to the Nos terminator (amplified by the primers CMD184D_+25-M13F_Inf1 and Nos_Inf2 from the plasmid pUC18_CatP_SecA_AAR-3HA_Nos) was then inserted into the linearized vector that was amplified from the plasmid pD184-O250-EGFP-UraCm-Cm (Fujiiwara et al. 2013) using the primers Nos-CmUra_Inf1 and CMD184C_+25_Inf2 with the In-fusion cloning kit to produce pQE80_D184_CatP_SecA_AAR-3HA_Ura_D184 plasmid.

To produce CM0345C, the CM0345C ORF was replaced by the *C. merolae* URA gene by homologous recombination. The upstream region, ORF and downstream regions of CM0345C were amplified using the primers CMD0345C_−2000_F and CMD0345C_+2000_R, and the amplified fragment was cloned into the pGEM-T easy vector (Promega). The resultant plasmid was amplified with the primers CmUra_CM0345C_−5′_Inf_F and CM0345C_−5′_CmUra_Inf_R, and the fragment containing the 3′ region, vector and 5′ region of CM0345C was used to clone the URA gene amplified with the primers CmUra−897_F_inf and CmUra−3′_747_R_inf by using an In-Fusion cloning kit to make a pGEMT_O345cm_URA_O345 plasmid.

To produce the AAR-3HA CM0345C, the CM0345C ORF was replaced by the *C. merolae* URA gene and the cyanobacterial acyl-ACP reductase gene by homologous recombination. The catalase promoter, the N-terminal 130 amino acids of *CmSecA*, acyl-ACP reductase, 3× HA tags and theNos terminator were inserted into the plasmid pGEMT_O345cm_URA_O345 between the URA gene and CM0345C 3′ region. The fragment containing the CM0345C 3′ region, vector, CM0345C 5′ region and URA was amplified with the primers Nos_CM0345C_−5′_Inf_F and CatPro_CmUra_Inf_R from the plasmid pGEMT_O345cm_URA_O345. The fragment was used to clone the region from the catalase promoter to the Nos terminator that was amplified with the primers CatPro_for_Inf_F and Nosoter_R from the plasmid pUC18_CatP_SecA_AAR-3HA_Nos by using an In-Fusion cloning kit to make a pGEMT_O345cm_URA_CatP_SecA_AAR-3HA_Nos plasmid.

To express the 6× His-tagged CM0345C protein in *E. coli*, the CM0345C ORF was amplified with the primers pet100_0345SOF_R and pet100_0345SORF_R and cloned into a pET100 expression vector (Life Technologies) (amplified by the primers pet100_F1 and pet100_R1) by using an In-Fusion cloning kit to produce a O345cm_pET100 plasmid.

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**Fig. 10** Continued

CM0104C, and carbon fixation (ribulose bisphosphate carboxylase large chain, *rbcL*, and ribulose bisphosphate carboxylase small chain, *rbcS*). RNA extracted from the nitrogen-replete AAR-3HA cells [AAR-3HA (N⁺)] nitrogen-depleted AAR-3HA cells [AAR-3HA (N⁻)], nitrogen-replete GFP cells [GFP (N⁺)] and nitrogen-depleted GFP cells [GFP (N⁻)] 1 d after inoculation was examined. Tim13 was used as a quantitative control for the semi-quantitative RT-PCR.
All of the constructs were verified by DNA sequencing before transformation.

**Transformation of C. merolae**

To produce AAR-3HA stable transformants, 6 µg of the PCR product obtained from the plasmid pQE80_D184_Catp_SeCa_AAR-HA_Ura_D184 by the primers D184(1270F) and D184(+1448R) were introduced by the polyethylene glycol (PEG)-mediated protocol into C. merolae M4, which has a mutation in the URA gene (Minoda et al., 2004), and the transformants were selected as described previously (Kobayashi et al., 2010). To produce :CMO345S and AAR-3HA :CMO345SC, the PCR products were obtained from the plasmid pGEMT_O345_URA_O345 for CMO345_-1978_F and CMO345_-1953_R.

**Semi-quantitative RT-PCR**

Cells were harvested by centrifugation, frozen in liquid nitrogen and stored at –80°C until use. Total RNA was extracted with the Trizol/RNeasy hybrid protocol (Trizol, Life Technologies; RNeasy Mini Kit, Qiagen) (Yu et al. 2013). cDNA was synthesized using six nucleotide random primers with ThermoScript RT (Life Technologies). The primers for semi-quantitative RT-PCR are listed in Supplementary Table S12.

**Immunoblot analyses**

Cells (5 × 10^6) were harvested by centrifugation and stored at –80°C. Cells were suspended in SDS–PAGE sample buffer (50 mM Tris, pH 6.8, 6% 2-mercaptoethanol, 2% SDS, 10% glycerol) and sonicated. After centrifugation, the supernatant fraction was separated by SDS–PAGE and analyzed by immunoblotting with a mouse monoclonal anti-HA antibody (HA-7, Sigma-Aldrich) as the primary antibody at a dilution of 1:1000 and goat anti-mouse IgG–horseradish peroxidase (HRP) antibody (Life Technologies) as the secondary antibody at a dilution of 1:2000.

**Immunofluorescence microscopy**

Immunofluorescence staining of cyanobacterial acyl-ACP reductase was performed as described (Nishida et al. 2005). Mouse monoclonal anti-HA antibody (HA-7) was used as the primary antibody at a dilution of 1:100 and the Alexa Fluor 488 goat anti-mouse IgG antibody (Life Technologies) was used as the secondary antibody at a dilution of 1:500.

**Lipid droplet staining with BODIPY**

Lipid droplets were stained with BODIPY as described (Kuroiwa et al. 2012). The fluorescence intensity was determined by Metamorph software (Molecular Devices).

**TAG and fatty acid analyses**

Cells were harvested by centrifugation, frozen immediately in liquid nitrogen and stored at –80°C until use. After freeze-drying the cells, approximately 15 mg of the cells were used for total lipid extraction. For TAG analysis, the total lipids were extracted according to the method described previously (Bligh and Dyer 1959). In the case of the analyses of total fatty acids and free fatty acids, the total lipid was extracted by the method described in the Plant Organelle Database 2 (Mano et al. 2011) [Ikuko Nishida, Lipid extraction from Arabidopsis mitochondria, http://podb.nibbc.jp/Organelle/FunctionalPDF/Organelle_lipids_F_nishida_0818.pdf]. The lipids were separated by one-dimensional thin-layer chromatography (TLC) with hexane:dichethyl ether:acetone:light petroleum ether (3:4:1:1, by vol.) and then analyzed with gas chromatography (GC-2014, Shimazu) equipped with a flame ionization detector (FID), along with an ULBON HR-SS-10 capillary column (25 m × 0.25 mm). Helium was used as a carrier gas at a linear velocity of 20 cm s⁻¹. The detector temperature was 250°C. The column temperature, after an initial isothermal period of 5 min at 140°C, was increased to 200°C at a rate of 4°C min⁻¹ and maintained for 10 min after reaching 200°C. The fatty acid methyl esters were identified in comparison with the retention time of fatty acid methyl ester standards (Supelco 37-component FAME Mix and methyl cis,cis-11,14-eicosadienoate). Pentadecanoic acid, which is not detected in C. merolae, was used as an internal standard for calculation of the concentration of each fatty acid methyl ester based on the peak area. All experiments were performed at least three times independently.

**Alkane and aldehyde (hydrocarbons) analysis by GC-MS**

Total lipid was extracted from 15 mg (dry cell weight) of WT and transgenic lines by the Bligh–Dyer method (Bligh and Dyer 1959). After drying under a stream of nitrogen gas, the purified total lipid was resolved in 1 ml of hexane, and 1 µg of pentadecane was added as an internal standard for relative quantification by gas chromatography–mass spectrometry (GC-MS) analysis. The prepared sample was diluted 1:20 (v/v) with hexane, and the identification of alkane and aldehyde was performed using a GCMS-QP2010 Ultra (Shimadzu), equipped with a factor FOUR VF-5 ms column (30 m, 0.20 mm i.d., 0.33 µm film thickness; Varian). A 1.0 µl aliquot of the diluted samples was automatically injected in splitless mode. The carrier gas was helium at a flow rate of 1 ml min⁻¹. The column temperature was initially 40°C for 2 min, and then heated to 310°C at a rate of 9°C min⁻¹ and finally 310°C for 10 min. The temperatures of the injector, interface and ion source were 250, 300 and 200°C, respectively. A voltage of 70 eV was used for ionization, and EI mass spectra were obtained with a scan range of 55–465 m/z.

**Microarray analyses**

Cells were harvested by centrifugation, frozen in liquid nitrogen and stored at –80°C until use. Total RNA was extracted as above. A 50 ng aliquot of total RNA was subjected to transcriptome analyses by using a customized oligo DNA microarray (8 × 15 k, 4 947 probes × 3, Agilent Technologies), according to the manufacturer’s instructions. For the preparation of fluorescent cRNA, random primers and Cy3 for one-color microarray analyses were used. All of the microarray data are shown in Supplementary Table S1. All of the gene functions were classified according to the KEGG pathway database (http://www.genome.jp/kegg/tool/map_pathway1.html).

**CE-MS metabolite analyses**

CE-MS analyses for cationic and anionic metabolite were performed as described previously (Hasunuma et al. 2013), with minor modifications. A total of 1 × 10⁵ to 10⁶ cells, an amount which is equivalent to 5 mg (DW), were harvested by centrifugation and then dissolved in 1 ml of pre-cooled (–30°C) methanol containing 37.3 µM l-methionine sulfone and 37.3 µM PIPES as internal standards for mass analyses. Cells were suspended by vortexing, and then 500 µl of ice-cold chloroform and 200 µl of ice-cold distilled water were added to 500 µl of the cell suspension. After vortexing the mixture for 30 s, the mixture was centrifuged at 14,000 × g for 5 min at 4°C. The aqueous layer was filtered with Amicon Ultra-0.5 ml Centrifugal Filters (3 KDa cut-off; Millipore) by centrifugation at 14,000 × g for 90 min at 4°C. A 300 µl aliquot of the filtrates was dried in a vacuum evaporator (CVE-3000, Tokyo Rikakikai). Dried extracts were stored at –80°C until use. Cationic and anionic CE-MS analyses were performed as described previously (Hasunuma et al. 2013).

**Enzyme activity assays**

To determine the aldehyde dehydrogenase activity of C. merolae CM0345C protein in vitro, O345_pET100 plasmid was transformed into the E. coli strain Rosetta (DE3) and the N-terminal 6× His-tagged CM0345C was expressed by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside) to the medium. As a negative control, Rosetta (DE3) transformed with an empty pET-100 plasmid was used. The recombinant CM0345C protein was purified with a HiTrap HP column (GE Healthcare). Aldehyde dehydrogenase assay was performed according to the methods of Kaiser et al. (2013) with a slight modification. The reaction medium contained 50 mM potassium phosphate buffer (pH 7.4),
1.0 mM NAD, 1.0 mM diethiothreitol (DTT), 100 mM NaCl, 0.2–0.8 mM decanal or octadecanal, and 27 µg of purified 6x-His-tagged CMO345C or 100 µg of C. merolae cell extract. Cyanidioschyzon merolae cell extract was prepared by sonication and further centrifugation at 15,000 × g for 10 min at 4 °C. As a negative control, cell extract boiled at 95 °C for 5 min was used.

ACCase activity was determined according to Willis et al. (2008) with minor modifications. MgCl₂ at 5 mM was used instead of 5 mM MnCl₂.

Acyl-CoA oxidase activity was determined according to Adham et al. (2005) without the 4-hydroxybenzhydrazide and protease inhibitor cocktail. Oleoyl coenzyme A lithium was used as the substrate.

PDH and BCKDH activity were determined according to Taylor et al. (2004) with a slight modification. Potassium phosphate buffer (pH 8.0 for PDH and pH 7.4 for BCKDH) and the total cell extract were used instead of TES-KOH (pH 7.4) with a slight modification. Potassium phosphate buffer (pH 8.0 for PDH and pH 7.4) and isolated mitochondria, respectively. As the substrate of BCKDH complex, 8.5 mM α-ketoisocapric acid was used.

Quantification of Chl a and cellular total protein

Cells were harvested by centrifugation and the Chl a level was determined according to Porra et al. (1989). For the protein assay, 5 × 10⁸ cells were harvested by centrifugation and stored at ~80 °C. Cells were suspended in 8 M urea, 0.1% Triton X-100 and sonicated. After centrifugation at 15,000 × g for 10 min at 4 °C, the protein content in the supernatant fraction was determined by Protein Assay (Bio-Rad).

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

References


