Chapter 21
Metabolic Engineering of *Cyanidioschyzon merolae*

Nobuko Sumiya and Shinya Miyagishima

Abstract Algae are expected to be promising alternative sources of biofuels, foods, and cosmetics. The unicellular red alga *Cyanidioschyzon merolae* is potentially useful for producing high concentrations of desirable biomaterials by metabolic engineering. *C. merolae* is genetically traceable and can thrive at low pH (1–5) and high temperatures (25–50 °C), which are harmful to many other organisms. Thus, this alga can be suitable for outdoor cultivation without the risk for contamination from other undesirable organisms. Recent studies regarding *C. merolae* have reported enhanced triacylglycerol (TAG) production, which can be used for biodiesel production, by genetic modification. Introducing cyanobacterial acyl-acyl carrier protein (ACP) reductase in *C. merolae* led to temporary TAG accumulation via an artificial metabolic pathway. The omics analyses showed that acyl-ACP reductase expression resulted in upregulating endogenous aldehyde dehydrogenase and the endogenous fatty acid synthetic pathway in chloroplasts. Another study expressed the 12-kDa FK506-binding protein of *Saccharomyces cerevisiae* in *C. merolae* and succeeded in increasing TAG levels by adding rapamycin. The omics analyses suggested that the target of rapamycin (TOR) regulated the expression of TAG-synthesizing enzymes, glycerol-3-phosphate acyltransferase, and acyl-CoA:diacylglycerol acyltransferase. Therefore, the combination of metabolic engineering and the evaluation of the effects in *C. merolae* by omics analyses will help in understanding the regulatory mechanism of metabolism. In addition, recent studies have started to find culture conditions that increase TAG accumulation while maintaining the cellular growth. Combinations of these cultivation techniques and genetic manipulations will leads to production of desirable biomolecules on a large scale in the future.

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T. Kuroiwa et al. (eds.), *Cyanidioschyzon merolae*,
https://doi.org/10.1007/978-981-10-6101-1_21

Keywords Triacylglycerol • Lipid droplets • Acyl-ACP reductase • Aldehyde dehydrogenase • TOR • Rapamycin • Cyanidiales • *Cyanidioschyzon merolae*

21.1 Introduction

Algae are expected to be promising alternative sources of biofuels, foods, and cosmetics (Hlavová et al. 2015). Algae convert 10% of solar energy into biomass, whereas land plants convert only 0.5% (Li et al. 2008). Algal cultivation would complement, rather than compete, with terrestrial plant cultivation because they grow in aquatic environments, even in saline water, heated water, acidic water, and wastewater, where terrestrial crops are difficult to grow (Sheehan et al. 1998; Yan et al. 2016). Thus, many researchers have recently explored and attempted to develop (mostly by genetic manipulation) algae that produce useful materials in high concentrations.

Although studies on the unicellular red alga *Cyanidioschyzon merolae* have just started, the alga is a promising platform organism that can be genetically modified for commercial use. The nuclear (Matsuzaki et al. 2004; Nozaki et al. 2007), mitochondrial (Ohta et al. 1998), and chloroplast (Ohta et al. 2003) genomes of *C. merolae* were completely sequenced. *C. merolae* possesses a simple nuclear genome (16.5 Mbp; 4775 protein-coding genes) with low genetic redundancy (Matsuzaki et al. 2004; Nozaki et al. 2007) (Chap. 5).

Methods for genetic manipulation have been established, such as the transient expression of proteins from plasmids (Ohnuma et al. 2008, 2009; Watanabe et al. 2011) and nuclear or chloroplast gene targeting by homologous recombination (Minoda et al. 2004; Zienkiewicz et al. 2017b). Transgenes are stably overexpressed without any silencing (Fujiwara et al. 2013; Imamura et al. 2013; Miyagishima et al. 2014; Sumiya et al. 2014, 2015, 2016; Watanabe et al. 2014; Zienkiewicz et al. 2017a). Thus, *C. merolae* is a suitable cell factory for metabolic engineering by overexpressing endogenous enzyme genes or introducing artificial metabolic pathways to produce high concentrations of metabolites of interest. In addition, the simple genome architecture of the alga facilitates various omics analyses to evaluate the effects of genetic manipulation on cellular metabolisms.

The hot spring alga *C. merolae* tolerates low pH (>1) and high temperatures ($<50\,^{\circ}C$) and is resistant to high concentrations of metal ions such as Al, Fe, Cu, Ni, Zn, and Mn (Misumi et al. 2008). Therefore, this alga is potentially suitable for outdoor cultivation without the risk for contamination from other neutrophilic organisms.

Genetically modified strains are usually subjected to regulations by laws; however, *C. merolae* can be genetically modified by self-cloning. In some cases, depending on the country, self-cloning organisms are not under the genetically modified organism (GMO) regulation. Genetic modification produces two categories of organism: GMOs, which have heterologous DNA sequences derived from

organisms that are taxonomically different from the host organism, and self-cloning organisms, which do not contain any DNA derived from other organisms. Self-cloning processes are considered the same as naturally occurring gene conversion, such as recombination, deletion, and transposition (Holme et al. 2013). In the case of *C. merolae* transformation using the endogenous *URA* transformation marker, the parental strain is a spontaneous frameshift *URA* mutant or *URA*-deleted mutant (Minoda et al. 2004; Taki et al. 2015). Thus, a gene disruptant or an overexpressor of endogenous genes by endogenous strong promoters does not contain any heterologous DNA sequences and is regarded as a self-cloning organism.

Eukaryotic microalgae accumulate triacylglycerol (TAG) in lipid droplets in cells, which can be used for producing biodiesel (Merchant et al. 2012; Ho et al. 2014). Because TAG comprises a glycerol backbone with three esterified fatty acids, it is a direct precursor of biodiesel via chemical transesterification (Merchant et al. 2012). TAG is synthesized via two pathways. One is the acylation of glycerol-3-phosphate with three acyl-CoAs, which are synthesized via the fatty acid synthesis pathway. In the second pathway, one of the three fatty acids is supplied from membrane lipids. Microalgae usually accumulate TAG when subjected to nutrient stress, such as nitrogen, phosphorus, or sulfur starvation. However, under such nutrient-starved conditions, algal growth ceases. Therefore, for producing TAG without inhibiting cellular growth, algae need to be genetically modified. In this chapter, we introduce two recent studies regarding the metabolic engineering of *C. merolae* as the first step toward producing TAG in the future. In addition to these genetic engineering studies, here we also briefly summarize recent efforts to increase cellular TAG accumulation by changing the culture conditions in *C. merolae*.

21.2 Metabolic Engineering of *C. merolae* for Producing Biofuel

21.2.1 TAG Accumulation Induced by Expressing Acyl-Acyl Carrier Protein (ACP) Reductase

In cyanobacteria, the overexpression of acyl-ACP reductase results in free fatty acid accumulation through the subsequent oxidation of fatty aldehydes by an endogenous aldehyde dehydrogenase (Kaiser et al. 2013). Although the *C. merolae* genome does not encode acyl-ACP reductase, it does encode an aldehyde dehydrogenase (Sumiya et al. 2015). Thus, the introduction of a cyanobacteria-like metabolic pathway in *C. merolae* by expressing cyanobacterial acyl-ACP reductase was intended to increase free fatty acid and TAG levels in *C. merolae* (Fig. 21.1).

To test this possibility, the acyl-ACP reductase gene of *Synechocystis* sp. PCC 6803 (sll0209) was inserted in a *C. merolae* chromosomal neutral locus (Fujiwara et al. 2013) to produce a stable transformant (AAR-3HA). The transgene was

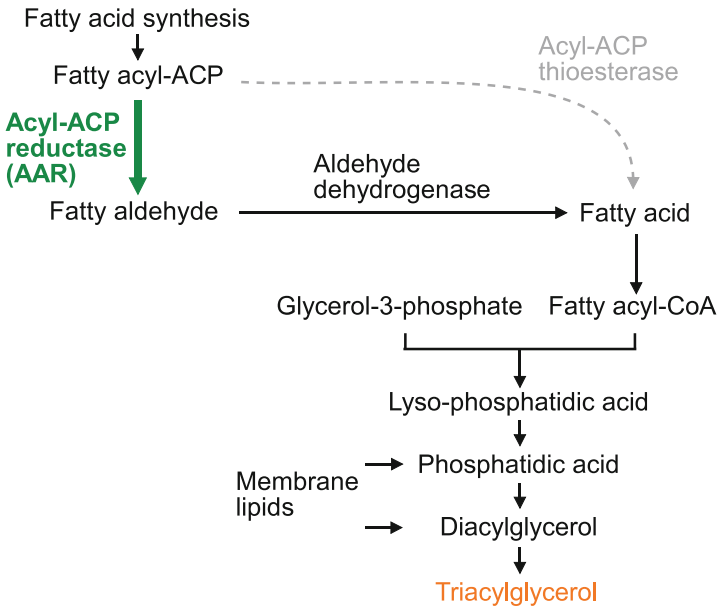

Fig. 21.1 The fatty acid and TAG synthesis pathway in *C. merolae*. *C. merolae* genome does not encode acyl-ACP reductase (*green*) and acyl-ACP thioesterase (*gray*). The acyl-ACP reductase of *Synechocystis* sp. PCC6803 was expressed in *C. merolae* (AAR-3HA strain)

constitutively expressed under the control of a *C. merolae* catalase promoter (Ohnuma et al. 2009). A cleavable chloroplast transit peptide of CmSecA (Koyama et al. 2011) was fused to the N-terminus of the transgene to transport the protein into the *C. merolae* chloroplast because fatty acid synthesis-related proteins localize in chloroplasts (Mori et al. 2016). To enable detection, a 3 × HA tag was fused to the C-terminus of the enzyme. The S-200 strain, in which the green fluorescent protein (GFP) gene was introduced in the same chromosomal neutral locus, was used as the control strain (Sumiya et al. 2014). Because GFP transcription is driven by a heat shock-inducible promoter, GFP is not expressed at 42 °C, which is the optimal temperature for *C. merolae*.

The staining of neutral lipids with the fluorescent dye BODIPY showed that the number and size of the lipid droplets in AAR-3HA, but not in the control GFP strain, increased 1 day after inoculation (Fig. 21.2a; Sumiya et al. 2015). The TAG level in the AAR-3HA strain also increased to approximately three times that in the control strain 1 day after inoculation (Fig. 21.2b; Sumiya et al. 2015). Moreover, AAR-3HA expression upregulated aldehyde dehydrogenase (CMO345C). In contrast, when AAR-3HA was expressed with a CMO345C knockout background, the TAG level did not increase. These results suggest that AAR-3HA expression in *C. merolae* upregulated aldehyde dehydrogenase, which is also required for increasing the TAG level (Fig. 21.1; Sumiya et al. 2015).

Profiles of the transcriptome (using microarray) and metabolome (by CE-MS analyses) of the AAR-3HA strain showed that AAR-3HA expression upregulated

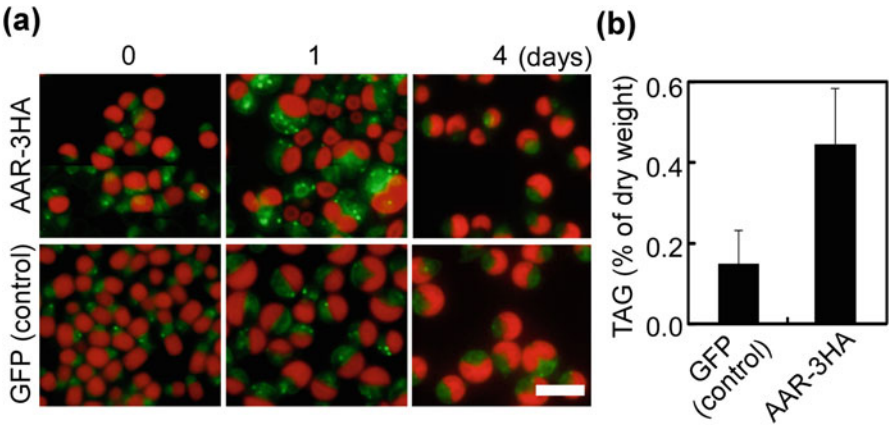

Fig. 21.2 The effect of cyanobacterial acyl-ACP reductase expression on TAG production in *C. merolae*. (**a**) The lipid droplets stained with BODIPY after inoculation of the steady-state AAR-3HA and the control GFP strain. *Green*, lipid droplets stained with BODIPY; *red*, autofluorescence of the chloroplast. Scale bar = 10 μm. (**b**) TAG level in AAR-3HA and GFP cells 1 day after inoculation. The *error bars* represent the standard deviation ($n = 6$) (Reproduced from Sumiya et al. 2015)

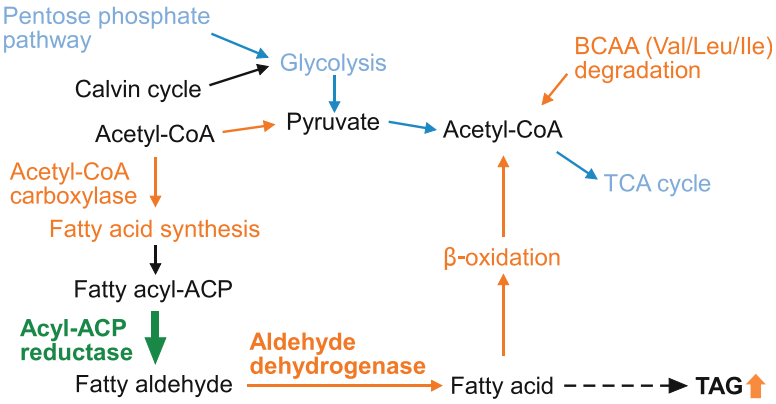

Fig. 21.3 The effects of cyanobacterial acyl-ACP reductase expression on metabolic pathways in *C. merolae*. *Orange* indicates the upregulated pathways, and *blue* indicates the downregulated pathways (Reproduced from Sumiya et al. 2015)

the fatty acid synthetic pathway in the chloroplast, particularly the rate-limiting enzyme acetyl-CoA carboxylase (Fig. 21.3; Sumiya et al. 2015). The branched-chain amino acid (BCAA; Val/Leu/Ile) degradation pathway, which contributes to TAG accumulation in a diatom (Ge et al. 2014), was upregulated by AAR-3HA expression in *C. merolae*. However, the fatty acid degradation pathway (β-oxidation) was also upregulated in the AAR-3HA strain. The number and size of lipid droplets increased and subsequently decreased in the AAR-3HA strain

during culture possibly because of elevated fatty acid degradation (Fig. 21.2a). Thus, further metabolic manipulation is required to maintain elevated TAG levels by expressing acyl-ACP reductase.

21.2.2 TAG Accumulation by Target of Rapamycin Inhibition

Although TAG production is accelerated in eukaryotic microalgae by nutrient starvation, the manner in which nutrient starvation is sensed by cells and that in which the signal is transmitted to the TAG production pathway remains unknown. Target of rapamycin (TOR), a serine/threonine protein kinase, is a candidate for sensing nutrient starvation in microalgae because it senses the nutrient status and regulates cellular growth and metabolism in other eukaryotes (Fig. 21.4; Wullschleger et al. 2006; Laplante and Sabatini 2012). In *Saccharomyces cerevisiae*, TOR inhibits the expression of nitrogen-regulated genes, including the global nitrogen transcription regulator Gln3 (Beck and Hall 1999; Cooper 2002).

The function of TORC1 (TOR of *S. cerevisiae*), as well as mTORC1 and mTORC2 (mammalian TORs), is inhibited by a macrolide compound rapamycin (Heitman et al. 1991; De Virgilio and Loewith 2006; Wullschleger et al. 2006). Rapamycin first binds to the 12-kDa FK506-binding protein (FKBP12), and this complex inhibits the TOR serine/threonine kinase by binding to the FRB domain of TOR. The *C. merolae* genome encodes a single TOR protein (CMR018C, CmTOR) with the FBR domain but not any FKBP12 homologs. Thus, the *C. merolae* wild type is not sensitive to rapamycin.

To produce rapamycin-sensitive *C. merolae*, Imamura et al. (2013) produced a *C. merolae* strain (F12 strain) wherein *S. cerevisiae* FKBP12 is constitutively expressed. Lipid droplet formation was observed in the cytoplasm 24 h after adding rapamycin in the F12 culture. The formation was enhanced by further incubating for 24 h, whereas cellular growth was inhibited. The lipid droplet formation was not observed with a drug solvent/DMSO solution treatment (Fig. 21.5; Imamura et al. 2015). The TAG levels in F12 were increased by approximately 8.8-fold in response to rapamycin treatment compared with the control rapamycin-insensitive C12 strain [the parental plasmid used for constructing the F12 strain was introduced into *C. merolae* M4 (Fig. 21.6; Imamura et al. 2015)].

The TAG level in the F12 strain was also increased in response to nitrogen depletion, as in the wild type, although the level was slightly higher than that under rapamycin treatment (Fig. 21.6; Imamura et al. 2015). TAG accumulation following the inhibition of TOR activity is not an indirect effect that results from growth inhibition because growth inhibition by a topoisomerase I inhibitor, camptothecin, does not result in TAG accumulation (Imamura et al. 2015). These results demonstrate that TAG accumulation is controlled by TOR signaling (Imamura et al. 2015).

To understand the association between nitrogen and TOR signaling, Imamura et al. (2015) compared transcriptome between nitrogen-depleted and TOR-

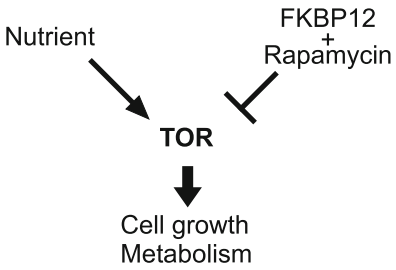

Fig. 21.4 TOR kinase signaling. TOR senses nutrient concentration and regulates cell growth and metabolism. The rapamycin-FKBP12 complex inactivates TOR

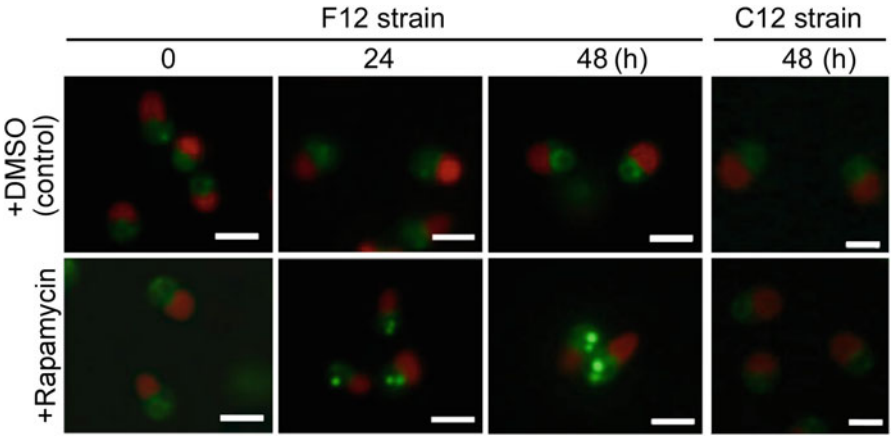

Fig. 21.5 BODIPY staining of the FKBP12-introduced F12 and control C12 strain after rapamycin or DMSO treatment. *Green*, lipid droplets stained with BODIPY; *red*, autofluorescence of the chloroplast. Scale bars = 2 μm (Reproduced from Imamura et al. 2015)

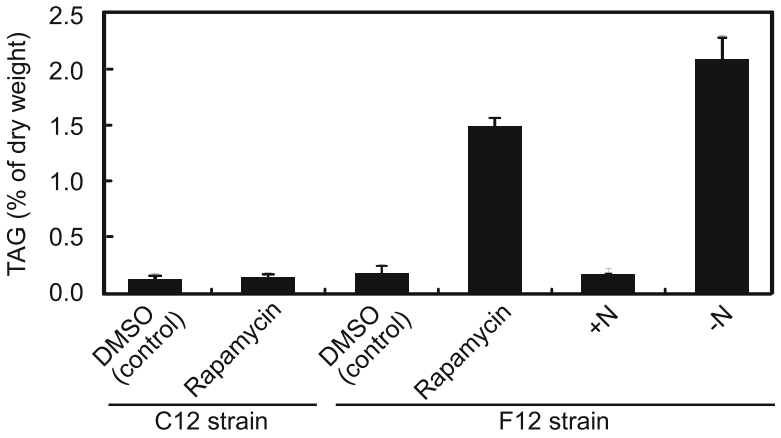

Fig. 21.6 TAG level 48 h after rapamycin treatment or nitrogen depletion. The *error bars* represent the standard deviation ($n = 3$) (Reproduced from Imamura et al. 2015)

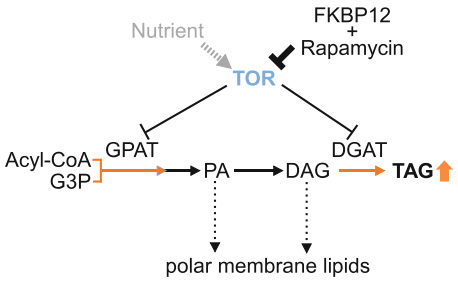

Fig. 21.7 A proposed model for the relationship between TOR signaling and TAG synthesis. By nutrient depletion or rapamycin treatment, TOR is inactivated, and the TAG synthesis pathway, especially glycerol-3-phosphate acyltransferase (GPAT) and acyl-CoA:diacylglycerol acyltransferase (DGAT), is activated (Reproduced from Imamura et al. 2016)

inactivated conditions using microarray analyses. In the rapamycin-treated F12 strain, 148 and 64 genes were up- and downregulated, respectively. Of the 148 upregulated genes, 71 were also upregulated under nitrogen-depleted conditions. These 71 upregulated genes, which are shared by nitrogen depletion and TOR inactivation under nitrogen-replete conditions, include nitrogen assimilation-related genes, suggesting that TOR is involved in the signaling of nitrogen starvation in *C. merolae* (Imamura et al. 2015). In terms of TAG synthesis, CMA017C and CMK217C, which encode glycerol-3-phosphate acyltransferase (GPAT) and acyl-CoA:diacylglycerol acyltransferase (DGAT), respectively, are upregulated by both nitrogen depletion and TOR inactivation under nitrogen-replete conditions (Imamura et al. 2015). These results suggest that following nitrogen starvation, TOR is inactivated, which in turn upregulates GPAT and DGAT, as well as TAG accumulation (Fig. 21.7; Imamura et al. 2015).

TAG accumulation by TOR inactivation requires the addition of rapamycin and inhibits cellular growth. To enhance TAG synthesis while maintaining cellular growth for commercial use, further studies, particularly in understanding the signaling pathway from TOR to TAG synthesis, are required.

21.3 Future Perspectives

Although >10 years have passed following the discovery of a homologous recombination event of introduced DNA in *C. merolae* (Minoda et al. 2004), metabolic engineering trials have only just started. By combining a *URA* transformation marker (Minoda et al. 2004; Fujiwara et al. 2013) and the recently reported new transformation marker chloramphenicol acetyltransferase (Zienkiewicz et al. 2017a), it is feasible to edit multiple chromosomal loci in *C. merolae*. In addition, a chloroplast transformation system was recently developed (Zienkiewicz et al. 2017b), which leads to the expression of larger amounts of transgene products than

the nuclear transformation in other organisms. These recently developed techniques should facilitate further metabolic manipulations in *C. merolae*.

In the two examples of metabolic engineering of *C. merolae* described here, the heterologous sequences (i.e., acyl-ACP reductase gene of *Synechocystis* sp. PCC 6803 or *S. cerevisiae* FKBP12 gene) were integrated into a *C. merolae* chromosome. However, metabolically manipulating *C. merolae* by self-cloning should be feasible in the future. In this regard, the group of Drs. N. Sato and T. Moriyama in the University of Tokyo has extensively analyzed the lipid synthetic pathway in *C. merolae* (Chap. 19) and the information will be useful for further metabolic engineering.

Besides the genetic manipulations, recent studies have tried to increase cellular TAG accumulation in the wild-type *C. merolae* by adding chemicals to the culture or changing the environment for cultivation. The group of Drs. K. Tanaka and S. Imamura in Tokyo Institute of Technology found that inhibition of histone deacetylase (HDAC) with addition of trichostatin A or other inhibitors increase the number of lipid droplets and TAG contents (Japan patent JP 2014-144756). The group of Dr. Misumi tested the wavelength of illumination that is suitable for TAG accumulation. As a result, it was found that the cultivation under red light (600 nm∼700 nm) increases the cellular TAG accumulation while maintaining the cellular growth. In addition, changing the light for cultivation from blue (400 nm∼500 nm) to red further increased the TAG contents (Japan patent JP 2014-152585). The group of Dr. Kuroiwa tested the formula of media that are suitable for accumulation of lipid droplets. By using media, in which sulfate concentration was reduced and sodium concentration was increased compared to conventional media (e.g. Allen's medium and M4 medium), they succeeded in increasing the cellular storage lipids while maintaining the cellular growth (Japan patent JP 2014-071081). Thus, combinations of these techniques and genetic manipulations will yield high production of TAG in *C. merolae* in near future.

The ability of *C. merolae* to thrive at low pH and in the presence of high metal concentrations is potentially ideal for open pond cultivation without the risk for contamination from undesirable organisms. Further studies on metabolic engineering of *C. merolae* will lead to, for example, the production of biofuels and other biomaterials using acidic drainage derived from hot springs, mines, and industrial plants.

Although here we have focused on issues of *C. merolae*, the group of Drs. Tanaka and Imamura found that TOR inhibition with rapamycin (Japan patent JP 2013-142173) or HDAC inhibition with several inhibitors (Japan patent JP 2014-144756) also leads to TAG accumulation in green algae. Thus, further studies on the mechanisms of TAG accumulation under these conditions will also give important information for biofuel production in other algal lineages.

Acknowledgments Our study was partly supported by Japan Society for the Promotion of Science Grant-in-Aid for Scientific Research 25251039 (to S.M.) and by the Core Research for Evolutional Science and Technology Program of the Japan Science and Technology Agency (to S.M.).

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