Running title: A galactolipid deficient mutant in cyanobacteria

Corresponding author: Koichiro Awai

255 Shimo-Okubo, Sakura-Ku, Saitama City, Saitama 338-8570 Japan

Tel: +81-48-858-9076

Fax: +81-48-858-3384

E-mail: awai@molbiol.saitama-u.ac.jp

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Digalactosyldiacylglycerol is required for better photosynthetic growth of *Synechocystis* sp. PCC6803 under phosphate limitation

Koichiro Awai[1, 2, *], Hideo Watanabe[1], Christoph Benning[2] and Ikuo Nishida[1]

[1]Graduate School of Science and Engineering, Saitama University, Saitama 338-8570 Japan, [2]Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI 48824, USA

Abbreviations: csMGD1, cucumber MGDG synthase 1; DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; PG, phosphatidylglycerol; SQDG, sulfoquinovosyldiacylglycerol; TLC, thin layer chromatography

Abstract

Digalactosyldiacylglycerol (DGDG) is a typical membrane lipid of oxygenic photosynthetic organisms. Although DGDG synthase genes have been isolated from plants, no homologous gene has been annotated in the genomes of cyanobacteria and the unicellular red alga *Cyanidioschyzon merolae*. Here we conducted a comparative genomics approach and identified a non-plant-type DGDG synthase gene (designated *dgdA*) in *Synechocystis* sp. PCC6803. The enzyme produced DGDG in *Escherichia coli* when co-expressed with a cucumber monogalactosyldiacylglycerol synthase. A *ΔdgdA* knock-out mutant showed no obvious phenotype other than loss of DGDG when grown in a BG11 medium, indicating that DGDG is dispensable under optimal conditions. However, the mutant showed reduced growth under phosphate-limited conditions, suggesting that DGDG may be required under phosphate-limited conditions, like those in natural niches of cyanobacteria.

Keywords: comparative genomics, digalactosyldiacylglycerol, galactolipid, glycosyltransferase, phosphate limitation, thylakoid membrane

Introduction

In oxygenic photosynthetic organisms, such as plants, algae and cyanobacteria, galactosyl-α-1,6-galactosyl-β-D-diacylglycerol or digalactosyldiacylglycerol (DGDG) constitutes a major membrane lipid in the thylakoid membranes, comprising up to 30% of the total lipids (Joyard et al. 1998). DGDG is also important for the assembly and function of photosynthetic complexes in the thylakoid membranes (Dörmann and Benning 2002, Sakurai et al 2007). Crystallographic studies revealed that DGDG is assembled within the photosystem II and light harvesting protein complexes in the thylakoid membranes (Loll et al. 2005, Liu et al. 2004).

In plants, DGDG synthase (E.C. 2.4.6.241) produces a galactose-(α1→6)-galactose linkage by transferring the galactose moiety of UDP-galactose to monogalactosyldiacylglycerol (MGDG) (Kelly and Dörmann 2002, Kelly et al. 2003). DGDG synthase genes have been identified from plants (Dörmann et al. 1999), the moss *Physcomitrella patens* (Nishiyama et al 2003) and the green alga *Chlamydomonas reinhardtii* (Riekhof et al. 2005). Early biochemical studies have demonstrated that both plants and cyanobacteria have similar DGDG biosynthetic pathways (Sato and Murata 1982). However, no homolog of a plant-type DGDG synthase gene has been found in the genomes of cyanobacteria and red algae to date.

DGDG is required for the optimal photosynthetic growth of *Arabidopsis thaliana*. A *dgd1* mutant, which is defective in a plant-type DGDG synthase (Dörmann et al. 1995), shows severe dwarfism with concomitant decreases in DGDG content, chlorophyll content, LHCII complex stability and photosynthetic capability. The mutant phenotypes are almost fully restored by introducing the wild-type gene or a β-glycosyltransferase gene from *Chloroflexus aurantiacus* (Accession number: ZP_00767379), producing glucosyl-β-1,6-galactosyl-β-D-diacylglycerol (Hölzl et al. 2006).

Under phosphate-limited conditions, not only plastidic membranes but also extra plastidic membranes, such as mitochondrial, tonoplasts and plasma membranes, appear to require DGDG (Benning et al. 2006). To cope with phospholipid deprivation caused by phosphate limitation, DGDG is thought to play a complementary role in the extra plastidic membranes, resulting in increased cellular DGDG content (Essigman et al. 1998, Härtel et al. 2000). DGDG content also increases in cyanobacterial cells under phosphate-limited conditions (Güler et al. 1996). However, little is known about the role of DGDG in cyanobacterial membranes under phosphate-limited conditions. To investigate the role of DGDG in photosynthetic membranes, the molecular cloning of cyanobacterial DGDG synthase is essential.

Here we report the identification and characterization of a DGDG synthase gene from the cyanobacterium *Synechocystis* sp. PCC6803. The gene is identified by comparative genomics, a successful method used for the cloning of cyanobacterial monoglucosyldiacylglycerol synthase gene (Awai et al. 2006a). The growth profiles of a knock-out mutant suggest that DGDG is required for better growth under phosphate-limited conditions, which prevail in the natural habitat of cyanobacteria.

Results

Cyanobacteria and *Cyanidioschyzon merolae* genomes contain non-plant-type DGDG synthase

A comparative genomic search for a DGDG synthase gene was conducted in the genome of *Synechocystis* sp. PCC6803 (Kaneko et al. 1996) as described previously (Awai et al. 2006a). The *Synechocystis* sp. PCC6803 genes *sll0071*, *sll1004*, *sll1377* and *slr1508* had a glycosyltransferase or related annotation in the pfam database (http://pfam.janelia.org/) and were conserved also in the genome of *Anabaena* sp. PCC7120 (Kaneko et al. 2001). sll1004 and sll1377 were excluded because they belonged to a GT2 transferase family that produces β-glycosidic linkages (Coutinho et al. 2003). sll0071 and slr1508 were retained because they belonged to a GT4 transferase family that produces α-glycosidic linkages. Only slr1508 had a homolog in the plastid genomes of unicellular red algae, such as *Cyanidioschyzon merolae* (Ohta et al. 2003), *Cyanidium caldarium* (Glöckner et al. 2000) and *Galdieria sulphuraria* (Michigan State University Galdieria Database http://genomics.msu.edu/galdieria). Accordingly, we assumed that slr1508 might be a DGDG synthase in *Synechocystis* sp. PCC6803.

The function of slr1508 was confirmed by co-expression analysis in *Escherichia coli* and gene disruption experiments in *Synechocystis* sp. PCC6803.

DGDG was produced in *E. coli* only when *slr1508* were co-expressed with a cucumber MGDG synthase cDNA (*csMGD1*) (Figure 1, Lane 4). Similarly, alr4178, an ortholog of slr1508 in *Anabaena* sp. PCC7120, exhibited DGDG synthetic activity in *E. coli* co-expressing *csMGD1* (data not shown). An insertional gene disruption of *slr1508* in *Synechocystis* sp. PCC6803 (Figure 2) caused no detectable level of DGDG (Figure 3, Lane 2). Thus, we designated the *slr1508* gene *dgdA*.

DGDG is dispensable in *Synechocystis* sp. PCC6803 under optimal growth conditions

To evaluate physiological functions of DGDG in cyanobacteria, the growth profiles of the wild-type and *ΔdgdA* cells were compared. Under optimal growth conditions, we did not see any significant difference in the growth curves between the wild-type and *ΔdgdA* cells (Figure 4, upper panel). This is suggestive of a dispensable role of DGDG in *Synechocystis* sp. PCC6803 under optimal growth conditions. However, under the same conditions, the oxygen evolution rate of the mutant was reduced (Figure 5) and the chlorophyll content was slightly increased compared to the wild type (Figure 6).

DGDG improves the growth of *Synechocystis* sp. PCC6803 under

phosphate-limited conditions

When the growth curves were compared between the wild-type and *ΔdgdA* cells after a shift to a phosphate-depleted medium, the mutant exhibited limited growth (Figure 4, lower panel). After 120 h of phosphate deprivation, the wild-type cells increased sulfoquinovosyldiacylglycerol (SQDG) and DGDG content by 5.9 and 4.3%, respectively, and decreased MGDG content by 10.9% (Table 1). However, PG content was retained under our phosphate-limited conditions. In *ΔdgdA* cells, SQDG and PG content increased by 8.2 and 7.0%, respectively, compared to the wild-type cells. After 120 h of phosphate deprivation, the SQDG content further increased by 11.1%, whereas the PG content decreased to 7.8%, which was below the level of wild-type cells, i.e., 10%.

DgdA is not directly involved in the major potassium transporter complex of *Synechocystis* sp. PCC6803

slr1508 protein (DgdA) was previously annotated as KtrE, a component of the Na^+-dependent K^+ uptake (Ktr) system (Matsuda et al. 2004). *KtrE* forms an operon with *KtrB*, a major facilitator of the Ktr system. It is reported that co-expression of *KtrE* is required for the K^+ transport function of the Ktr system in *E. coli* (Matsuda et al.

2004). Thus, a physiological effect of *dgdA* deletion on the K^+ transport activity was evaluated in *Synechocystis* sp. PCC6803. A knock out mutant of Ktr system is known to be sensitive to osmotic stresses (Berry et al. 2003, Matsuda et al 2004). As shown in Figure 7, a *ktrB*/*ntpJ* (*slr1509*) mutant was sensitive to osmotic stresses imposed by 300 mM sorbitol (Berry et al. 2003, Matsuda et al 2004), whereas the *ΔdgdA* mutant was insensitive to the same osmotic stresses. These results show that the DgdA protein is not directly involved in the K^+ transport activity in *Synechocystis* sp. PCC6803.

Discussion

We herein identified *dgdA* (*slr1508*) as a non-plant-type DGDG synthase gene in *Synechocystis* sp. PCC6803. The gene function has been verified by co-expression analysis with *csMGD1* in *E. coli* and characterization of a *ΔdgdA* mutant.

We found that oxygen evolution rates are reduced in *ΔdgdA* mutants compared to the wild-type. Because DGDG stabilizes the activity of photosystem II in *Synechocystis* sp. PCC6803 (Sakurai et al. 2007), reduced oxygen evolution rate in *ΔdgdA* mutant may be related to the instability of photosystem II. Under low light conditions (< 100 μmol m^{-2} s^{-1}), however, the wild-type and mutant cells showed similar oxygen evolution rates. This may explain why we do not see obvious differences in the growth rate between the wild-type and mutant cells under our growth conditions (Fig. 5). We also found that the *ΔdgdA* mutants accumulate more chlorophyll than the wild-type cells. However, this result is controversial to the previous report in *Arabidopsis* that *dgd1* mutants have much less amount of chlorophyll than the wild type. The reason for this discrepancy is not clear in this study. To understand the exact function of DGDG in the thylakoid membrane, our *ΔdgdA* mutant will provide a useful tool for further analysis.

A *dgdA* ortholog can be found in the plastid genome of the unicellular red alga

Cyanidioschyzon merolae as well as in the genomes of cyanobacteria. However, no *dgdA* ortholog can be found in the plastid genomes of the multicellular red algae *Gracilaria tenuistipitata*, *Porphyra purpurea* and *Porphyra yezoensis*, the glaucophyta *Cyanophora paradoxa*, the green alga *C. reinhardtii*, the moss *P. patens* and plants. Thus, an evolutionary gap remains between unicellular red algae and the higher photosynthetic organisms with respect to DGDG synthases. By contrast, a chloroplast-targeted plant-type MGDG synthase is found in the genome of *Cyanidioschyzon merolae* (Sato and Moriyama, 2007), for which we confirmed the MGDG synthase activity by expression in *E. coli* (data not shown). However no ortholog of plant-type MGDG synthase gene is found in the genomes of cyanobacteria (Shimojima et al. 1997, Awai et al. 2006a). Thus, an evolutionary gap remains between cyanobacteria and *Cyanidioschyzon merolae* with respect to MGDG synthases. These results will stimulate studies with *Cyanidioschyzon merolae* for understanding the evolution from cyanobacteria to higher-plant chloroplasts (Matsuzaki et al. 2004, Sato and Moriyama, 2007).

DgdA protein is expected to utilize MGDG as an accepter of a galactose residue from UDP-Gal. To date, we have not managed to establish an *in vitro* assay system for DgdA protein. Thus, we used co-expression systems in an *E. coli* strain that

accumulate MGDG as a substrate of DgdA protein. When an *E. coli* strain that produces MGlcDG as a substrate for the DgdA protein was used, we did not find detectable levels of DGDG (data not shown), suggesting that MGlcDG is not utilized by DgdA protein. In support of this view, Hölzl et al (2005) reported that α-glucosyl and β-glucosyldiacylglycerol produced in *Synechocystis* sp. PCC6803 are not utilized for the synthesis of α-galactosyl-α-glucosyl- or α-galactosyl-β-glucosyl-diacylglycerol.

We found that *Synechocystis* sp. PCC6803 increases proportions of DGDG and SQDG after a shift to a phosphate-deprived medium, whereas proportion of PG remains constant (Table 1). The cellular lipid content (measured on an optical density base), however, decreased to less than one fifth in the wild-type after the phosphate starvation. Therefore, it seems likely that PG production is also limited in the wild-type cells under phosphate limitation. Increased proportion of DGDG and SQDG may reflect other adaptive responses to phosphate starvation. In *ΔdgdA* mutants, PG and SQDG seems to be substituted for DGDG under optimal growth conditions, and proportion of PG and SQDG increased compared to the wild-type (Table 1). Under phosphate-deprived conditions, the cellular lipid content in the mutant cells also decreased to the same level as in the wild-type cells. However, *ΔdgdA* mutants may be unable to account for the increased PG demand under phosphate starvation, and the PG level decreased below the

wild-type level. As a result, *ΔdgdA* mutant cells showed reduced growth rate under phosphate starvation compared to the wild-type cells (Figure 4). Thus, DGDG at minimum improves optimal growth of *Synechocystis* sp. PCC6803 under phosphate starvation. The *Synechocystis* sp. PCC6803 strain has been isolated from fresh a water lake (Stanier et al. 1971, Rippka et al 1979). Inorganic phosphate concentrations are ~1 μM in mesotrophic lakes, such as Lake Biwa in Japan or Lake Erie in North America, ~3 μM in a eutrophic lake, Lake Mendota, and 2.3 μM in sea water (Bjerrum and Canfield, 2002). These values are much less than that used in our optimal medium i.e., 200 μM. Thus, to cope with low phosphate availability in nature, DGDG synthesis may be advantageous for cyanobacterial species. Our findings provide further evidence for the proposal that utilization of galactolipid in the thylakoid membrane is an adaptive mechanism to phosphate-limited conditions (Dörmann and Benning 2002).

Materials and Methods

Cyanobacteria strains

The wild-type and *ΔgdgA* cells of *Synechocystis* sp. PCC6803 were maintained photoautotorophically (Okazaki et al. 2006). Cells were grown in 300 ml flasks containing 100 ml of BG-11 medium (Stanier et al. 1971) supplemented with 20 mM HEPES-NaOH (pH 7.5) under continuous illumination (50–80 μmol m^{-2} s^{-1}) with rotary shaking (120 rpm) at 30°C. For phosphate limitation, cells grown in the BG11 medium were washed three times and transferred to a phosphate deprived BG11 medium, where K_2HPO_4 was replaced by KCl. To obtain growth curves, bacterial cultures were diluted with a fresh medium to give an initial optical density of 0.05 at 730 nm and measured with a spectrophotometer (Gene Spec III, Hitachi Naka Instruments Co., Ltd).

Co-expression of plant MGDG synthase and slr1508 in *Escherichia coli*.

Glutathione S-transferase fused to the cucumber MGDG synthase cDNA was amplified from the vector pGEX-GT (Shimojima et al. 1997) using the primer pair 5’-GTCGACGGTTCTGGCAAATATTCTG-3’ and 5’-GTCGACGATCGTCAGTCAGTCACG-3’. The amplified fragment was subcloned into the *Sal*I site of pACYC184 vector and used as a host of co-expression experiments.

The open reading frame of *slr1508* was amplified with the primer pair 5'-GTCGACATGCATATTGCTTGGTTAGG-3' and 5'-GTCGACTCATTCCAGCAGATAAAAATAG-3', and then subcloned into the TA cloning vector pPICT2 (Kawaguchi et al. 2001). The sequence was confirmed. The ORF was subcloned into the *Sal*I site of pQE31 (Qiagen) and used for co-expression experiments with csMGD1 in *E. coli* XL1-Blue. Isopropyl 1-thio-β-D-galactoside (IPTG) was added to over night cultures (final 1 mM) to induce the expression of fusion proteins. Cells were incubated for additional 5 hours. For lipid analysis, cells were harvested by centrifugation and washed with 0.9% NaCl (see below).

Isolation of *ΔdgdA* mutants of *Synechocystis* sp. PCC6803

A 5' region of *slr1508* was amplified by PCR using the primer pair 5'-GTCGACATGCATATTGCTTGGTTAGG-3' (primer #1) and 5'-CGGGATCCTGGACAATAGCGCTCCAC-3'. The resultant 557-bp fragment was subcloned into pPICT2. A 3' region of *slr1508* including a part of the flanking gene *slr1509* was amplified with the primer pair, 5'-CGGGATCCCGGAAAAGAACGTGGAAG-3' and 5'-TCTAGAAGCTTGTTCACATCCACCACAC-3' (primer #2), and the resultant

fragment was subcloned into the vector pGEM-T Easy (Promega). A *Bam*HI-*Hin*dIII fragment of the 3’ region was then inserted into the pPICT2 vector harboring the 5’ region fragment. A kanamycin cassette of the vector pUC4K was inserted into the *Bam*HI site of the plasmid. Transformation of *Synechocystis* sp. PCC6803 was performed as described (Hagio et al. 2000).

Genotyping of the mutant was done using the primer combinations shown in figure 2B: primer #3, 5’-CGTTTCCCGTTGAATATGGC-3’; primer #4, 5’-CAGAGCATTACGCTGACTTG-3’; and primer #5, 5’-CCAGGCCGAACGGTTATTC-3’.

Lipid analysis

Lipids were extracted according to a method of Bligh and Dyer (1959), and separated by TLC using the solvent systems acetone: toluene: water = 90: 30: 7 (Awai et al. 2006b) or chloroform: methanol: 28% NH_4OH = 65: 35: 5 (Wada and Murata 1989). Fatty acids were quantified by gas-liquid chromatography as described (Inatsugi et al. 2002).

Chlorophyll content and oxygen evolution rates

Chlorophyll content was determined as described (Arnon et al. 1974). Oxygen evolution rates of intact cells were measured by a Clark-type oxygen electrode (Rank Brothers). Cells were suspended in a BG-11 medium containing 10 mM $NaHCO_3$.

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References

Arnon, D.I., McSwain, B.D., Tsujimoto, H.Y. and Wada, K. (1974) Photochemical activity and components of membrane preparations from blue-green algae. I. Coexistence of two photosystems in relation to chlorophyll a and removal of phycocyanin. *Biochim. Biophys. Acta* 357: 231–245.

Awai, K., Kakimoto, T., Awai, C., Kaneko, T., Nakamura, Y., Takamiya, K., Wada, H. and Ohta, H. (2006a) Comparative genomic analysis revealed a gene for monoglucosyldiacylglycerol synthase, an enzyme for photosynthetic membrane lipid synthesis in cyanobacteria. *Plant Physiol.* 141: 1120-1127.

Awai, K., Xu, C., Tamot, B. and Benning C. (2006b) A phosphatidic acid-binding protein of the chloroplast inner envelope membrane involved in lipid trafficking. *Proc. Natl. Acad. Sci. USA* 103: 10817-10822.

Benning, C., Xu, C. and Awai, K. (2006) Non-vesicular and vesicular lipid trafficking involving plastids. *Curr. Opin. Plant Biol.* 9: 241-247.

Berry, S., Esper, B., Karandashova, I., Teuber, M., Elanskaya, I., Rögner, M. and Hagemann, M. (2003) Potassium uptake in the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 mainly depends on a Ktr-like system encoded by *slr1509* (*ntpJ*). *FEBS Lett.* 548: 53-58.

Bjerrum, C.J. and Canfield, D.E. (2002) Ocean productivity before about 1.9 Gyr ago limited by phosphorus adsorption onto iron oxides. *Nature* 417: 159-162.

Bligh, E.G. and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37: 911-917.

Coutinho, P.M., Deleury, E., Davies, G.J. and Henrissat, B. (2003) An evolving hierarchical family classification for glycosyltransferases. *J. Mol. Biol.* 328: 307-317.

Dörmann, P., Balbo, I. and Benning, C. (1999) Arabidopsis galactolipid biosynthesis and lipid trafficking mediated by DGD1. *Science* 284: 2181-2184.

Dörmann, P. and Benning, C. (2002) Galactolipids rule in seed plants. *Trends Plant Sci.* 7: 112-118.

Dörmann, P., Hoffmann-Benning, S., Balbo, I. and Benning C. (1995) Isolation and characterization of an Arabidopsis mutant deficient in the thylakoid lipid digalactosyl diacylglycerol. *Plant Cell* 7: 1801-1810.

Essigmann, B., Güler, S., Narang, R.A., Linke, D. and Benning, C. (1998) Phosphate availability affects the thylakoid lipid composition and the expression of *SQD1*, a gene required for sulfolipid biosynthesis in *Arabidopsis thaliana. Proc. Natl. Acad. Sci. USA* 95: 1950–1955.

Glöckner, G., Rosenthal, A. and Valentin, K. (2000) The structure and gene repertoire of an ancient red algal plastid genome. *J. Mol. Evol.* 51: 382-390.

Güler, S., Seeliger, A., Härtel, H., Renger, G. and Benning C. (1996) A null mutant of *Synechococcus* sp. PCC7942 deficient in the sulfolipid sulfoquinovosyl diacylglycerol. *J. Biol. Chem.* 271: 7501-7507.

Hagio, M., Gombos, Z., Várkonyi, Z., Masamoto, K., Sato, N., Tsuzuki, M. and Wada, H. (2000) Direct evidence for requirement of phosphatidylglycerol in photosystem II of photosynthesis. *Plant Physiol.* 124: 795-804.

Härtel, H., Dörmann, P. and Benning, C. (2000) DGD1-independent biosynthesis of extraplastidic galactolipids after phosphate deprivation in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* 97: 10649-10654.

Hölzl, G., Witt, S., Kelly, A.A., Zähringer, U., Warnecke, D., Dörmann, P. and Heinz, E. (2006) Functional differences between galactolipids and glucolipids revealed in photosynthesis of higher plants. *Proc. Natl. Acad. Sci. USA* 103: 7512-7517.

Hölzl, G., Zähringer, U., Warnecke, D. and Heinz, E. (2005) Glycoengineering of cyanobacterial thylakoid membranes for future studies on the role of glycolipids in photosynthesis. *Plant Cell Physiol.* 46: 1766-1778.

Inatsugi, R., Nakamura, M. and Nishida, I. (2002) Phosphatidylcholine biosynthesis at

low temperature: differential expression of CTP:phosphorylcholine cytidylyltransferase isogenes in *Arabidopsis thaliana*. *Plant Cell Physiol.* 43: 1342-1350.

Joyard, J., Maréchal, E., Miège, C., Block, M.A., Dorne, A.J. and Douce, R. (1998) Structure, distribution and biosynthesis of glycerolipids from higher plant chloroplasts. In *Lipids in photosynthesis: Structure, function and genetics*. Edited by Siegenthaler, P.A. and Murata, N. pp. 21-52. Kluwer Academic Publishers, Dordrecht, Netherlands.

Kaneko, T., Nakamura, Y., Wolk, C.P., Kuritz, T., Sasamoto, S., Watanabe, A., Iriguchi, M., Ishikawa, A., Kawashima, K., Kimura, T., Kishida, Y., Kohara, M., Matsumoto, M., Matsuno, A., Muraki, A., Nakazaki, N., Shimpo, S., Sugimoto, M., Takazawa, M., Yamada, M., Yasuda, M. and Tabata, S. (2001) Complete genomic sequence of the filamentous nitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC 7120. *DNA Res.* 8: 205-213; 227-253.

Kaneko, T., Sato, S., Kotani, H., Tanaka, A., Asamizu, E., Nakamura, Y., Miyajima, N., Hirosawa, M., Sugiura, M., Sasamoto, S., Kimura, T., Hosouchi, T., Matsuno, A., Muraki, A., Nakazaki, N., Naruo, K., Okumura, S., Shimpo, S., Takeuchi, C., Wada, T., Watanabe, A., Yamada, M., Yasuda, M. and Tabata, S. (1996) Sequence

analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. *DNA Res.* 3: 109-136; 185-209.

Kawaguchi, J., Kii, I., Sugiyama, Y., Takeshita, S. and Kudo, A. (2001) The transition of cadherin expression in osteoblast differentiation from mesenchymal cells: consistent expression of cadherin-11 in osteoblast lineage. *J. Bone Miner. Res.* 16: 260-269.

Kelly, A.A. and Dörmann, P. (2002) DGD2, an *Arabidopsis* gene encoding a UDP-galactose-dependent digalactosyldiacylglycerol synthase is expressed during growth under phosphate-limiting conditions. *J. Biol. Chem.* 277: 1166-1173.

Kelly, A.A., Froehlich, J.E. and Dörmann, P. (2003) Disruption of the two digalactosyldiacylglycerol synthase genes *DGD1* and *DGD2* in Arabidopsis reveals the existence of an additional enzyme of galactolipid synthesis. *Plant Cell* 15: 2694-2706.

Liu, Z., Yan, H., Wang, K., Kuang, T., Zhang, J., Gui, L., An, X. and Chang, W. (2004) Crystal structure of spinach major light-harvesting complex at 2.72 Å resolution. *Nature* 428: 287-292.

Loll, B., Kern, J., Saenger, W., Zouni, A. and Biesiadka, J. (2005) Towards complete cofactor arrangement in the 3.0 Å resolution structure of photosystem II. *Nature* 438: 1040-1044.

Matsuda, N., Kobayashi, H., Katoh, H., Ogawa, T., Futatsugi, L.**,** Nakamura, T., Bakker E.P. and Uozumi N. (2004) Na^{+}-dependent K^{+} uptake Ktr system from the cyanobacterium *Synechocystis* sp. PCC6803 and its role in the early phases of cell adaptation to hyperosmotic shock. *J. Biol. Chem.* 279: 54952-54962.

Matsuzaki, M., Misumi, O., Shin-i, T., Maruyama, S., Takahara, M., Miyagishima, S., Mori, T., Nishida, K., Yagisawa, F., Nishida, K., Yoshida, Y., Nishimura, Y., Nakao, S., Kobayashi, T., Momoyama, Y., Higashiyama, T., Minoda, A., Sano, M., Nomoto, H., Oishi, K., Hayashi, H., Ohta, F., Nishizaka, S., Haga, S., Miura, S., Morishita, T., Kabeya, Y., Terasawa, K., Suzuki, Y., Ishii, Y., Asakawa, S., Takano, H., Ohta, N., Kuroiwa, H., Tanaka, K., Shimizu, N., Sugano, S., Sato, N., Nozaki, H., Ogasawara, N., Kohara, Y. and Kuroiwa, T. (2004) Genome sequence of the ultrasmall unicellular red alga *Cyanidioschyzon merolae* 10D. *Nature* 428: 653-657.

Nishiyama, T., Fujita, T., Shin-I, T., Seki, M., Nishide, H., Uchiyama, I., Kamiya, A., Carninci, P., Hayashizaki, Y., Shinozaki, K., Kohara, Y. and Hasebe, M. (2003)

Comparative genomics of *Physcomitrella patens* gemetophytic transcriptome and *Arabidopsis thaliana*: Implication for land plant evolution. *Proc. Natl. Acad. Sci. USA* 100: 8007-8012.

Ohta, N., Matsuzaki, M., Misumi, O., Miyagishima, S., Nozaki, H., Tanaka, K., Shin-i, T., Kohara, Y. and Kuroiwa, T. (2003) Complete sequence and analysis of the plastid genome of the unicellular red alga *Cyanidioschyzon merolae*. *DNA Res.* 10: 67-77.

Okazaki, K., Sato, N., Tsuji, N., Tsuzuki, M. and Nishida, I. (2006) The significance of C16 fatty acids in the *sn*-2 positions of glycerolipids in the photosynthetic growth of *Synechocystis* sp. PCC6803. *Plant Physiol.* 141: 546-556.

Riekhof W.R., Sears, B.B. and Benning, C. (2005) Annotation of genes involved in glycerolipid biosynthesis in *Chlamydomonas reinhardtii*: discovery of the betaine lipid synthase $BTA1_{Cr}$. *Eukaryot. Cell* 4: 242-252.

Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M. and Stanier, R.Y. (1979) Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J. Gen. Microbiol.* 111: 1-61.

Sakurai, I., Mizusawa, N., Wada, H. and Sato, N. (2007) Roles of digalactosyldiacylglycerol in photosynthesis. *Plant Cell Physiol.* 48: S172.

Sato, N. and Moriyama, T. (2007) Genomic and biochemical analysis of lipid biosynthesis in the unicellular rhodophyte *Cyanidioschyzon merolae*: lack of a plastidic desaturation pathway results in the coupled pathway of galactolipid synthesis. *Eukaryot. Cell* 6: 1006-1017.

Sato, N. and Murata, N. (1982) Lipid biosynthesis in the blue-green alga, *Anabaena variabilis*. I. Lipid classes. *Biochim. Biophys. Acta* 710: 271-278.

Shimojima, M., Ohta, H., Iwamatsu, A., Masuda, T., Shioi, Y. and Takamiya, K. (1997) Cloning of the gene for monogalactosyldiacylglycerol synthase and its evolutionary origin. *Proc. Natl. Acad. Sci. USA* 94: 333-337.

Stanier, R.Y., Kunisawa, R., Mandel, M. and Cohen-Bazire, G. (1971) Purification and properties of unicellular blue-green algae (order *Chroococcales*). *Bacteriol. Rev.* 35: 171-205.

Wada, H. and Murata, N. (1989) *Synechocystis* sp. PCC6803 mutants defective in desaturation of fatty acids. *Plant Cell Physiol.* 30: 971-978.

Legends to figures

Fig. 1. Accumulation of DGDG in *E. coli* co-expressing cucumber MGDG synthase (csMGD1) and slr1508. Extracted lipids were separated by TLC using solvent system acetone: toluene: water = 90: 30: 7. Spots were stained with alpha naphthol. 1, *E. coli* with an empty vector for csMGD1; 2, *E. coli* with csMGD1; 3, *E. coli* with csMGD1 and pQE31, an empty vector for slr1508; 4, *E. coli* with csMGD1 and slr1508; and 5, *Synechocystis* sp. PCC6803. MGlcDG, monoglucosyldiacylglycerol. *E. coli* cells that solely expressed *slr1508* did not accumulate DGDG (data not shown).

Fig. 2. A. Schematic representation of gene disruption for *slr1508*. Numbers 1 through 5 show the positions of the primers #1 to #5 used for genotyping (see Materials and Methods). Km: kanamycin resistance gene. B. Genotyping of the slr1508 mutant. WT, the wild type; *ΔdgdA*, the *slr1508* mutant.

Fig. 3. Lipid analysis of wild-type and *ΔdgdA* cells grown under optimal and phosphate-deprived conditions. Lipids were separated by the solvent system chloroform: methanol: 28% NH_4OH = 65: 35: 5 and visualized by 50% sulfuric acid. 1.

wild-type in the BG11 medium, 2. *ΔdgdA* in the BG11 medium, 3. wild-type in the phosphate-deprived BG11 medium, 4. *ΔdgdA* in the phosphate-deprived BG11 medium.

Fig. 4. Growth curves of wild-type and *ΔdgdA* cells. Optimal (Pi +, upper panel) and phosphate deprived (Pi -, lower panel) media were used. Filled circles and squares indicate wild-type and *ΔdgdA* cells, respectively. Errors bars indicate SD based on three independent experiments.

Fig. 5. Photosynthetic activity of the wild-type and *ΔdgdA* cells grown in optimal medium. Oxygen evolution rates were measured using cells of OD_{730} = 1.0. Filled circles and squares indicate the wild-type and *ΔdgdA* cells, respectively. Errors bars indicate SD based on three independent experiments.

Fig. 6. Chlorophyll contents of the wild-type and *ΔdgdA* cells. Cells grown in optimal (Pi +) and phosphate deprived (Pi -) medium are shown. Chlorophyll was extracted and measured based on optical density at 730 nm of 1.0. Black and gray bars indicate chlorophyll contents of wild-type and *ΔdgdA* cells, respectively. Errors bars indicate SD based on three independent experiments.

Fig. 7. Growth of the wild-type and *ΔdgdA* cells on solid media containing indicated concentrations of sorbitol. 1, wild-type; 2, *ΔdgdA*; and 3, *Δslr1509* (*ntpJ*/*KtrB*).

Table I Lipid compositions of the wild-type and *ΔdgdA* cells grown in optimal and phosphate-deprived media. Each value is represented as mol%. SD is based on three independent experiments.

	Pi	MGDG	DGDG	SQDG	PG
wild-type	+	49.7±1.9	25.9±3.8	14.3±1.3	10.1±2.5
	-	38.8±2.5	30.2±1.7	20.2±1.3	10.8±2.8
ΔdgdA	+	60.4±3.7	nd	22.5±3.0	17.1±0.7
	-	58.5±2.4	nd	33.6±3.9	7.8±1.5

nd, not detected (< 0.1 mol%).

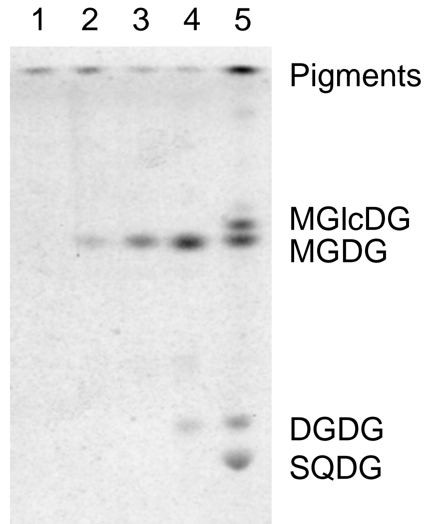


Fig. 1 Awai et al.

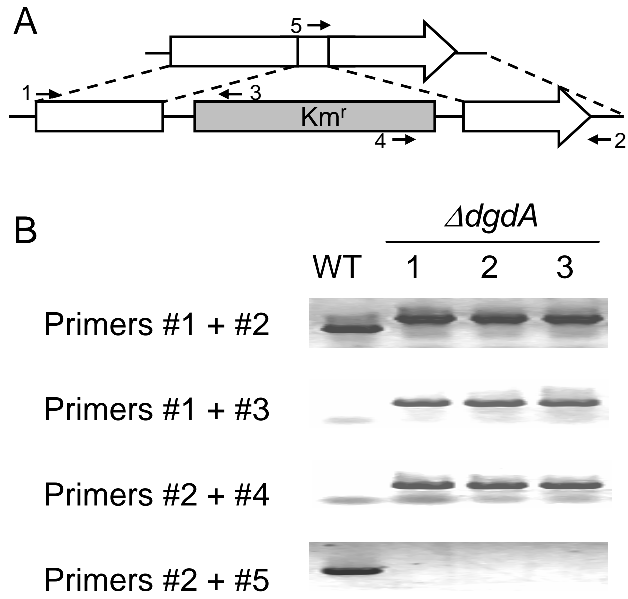


Fig. 2 Awai et al.

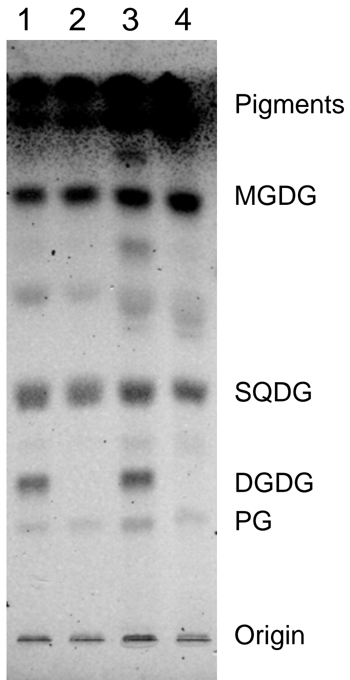
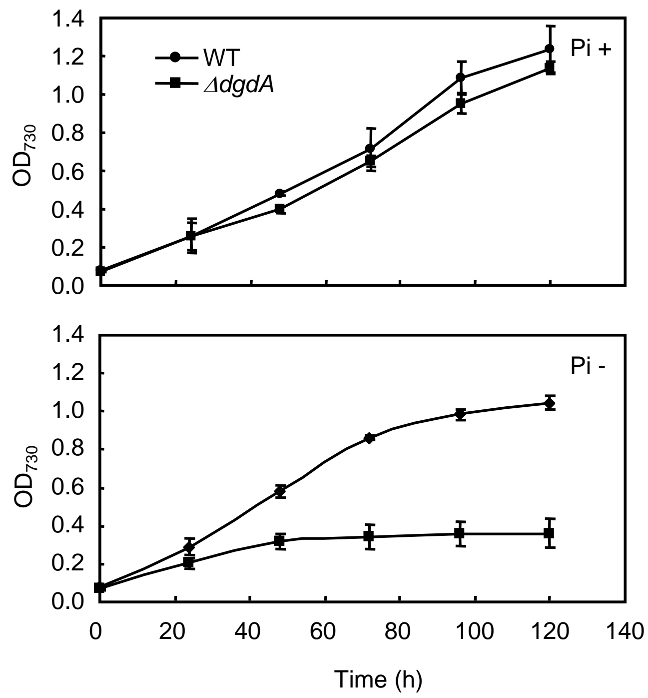


Fig. 3 Awai et al.



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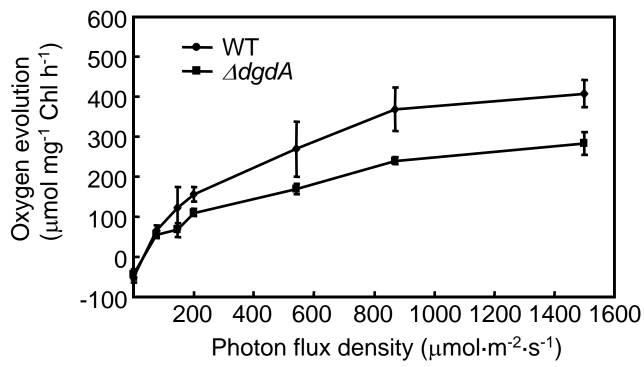

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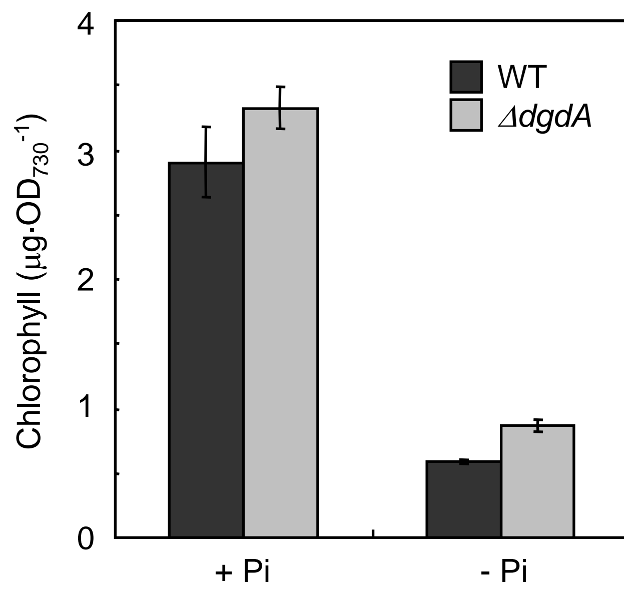

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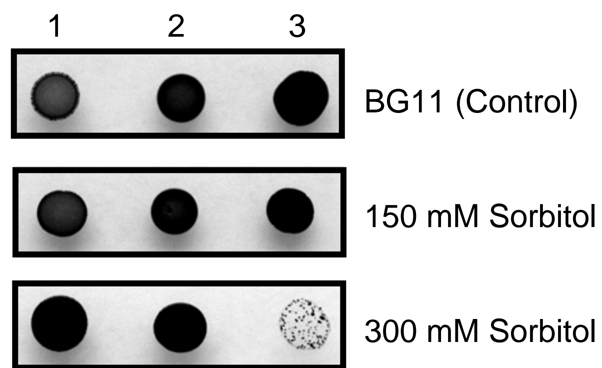

Fig. 7 Awai et al.