Monoglucosyldiacylglycerol participates in phosphate stress adaptation in *S* sp. PCC 7942

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Cyanobacterial monoglucosyldiacylglycerol (MGlcDG) not only serves as a precursor for monogalactosyldiacylglycerol (MGDG) synthesis, but also participates in stress acclimation. Two genes (*A* and *E*) related to MGDG synthesis of *S* sp. PCC 7942 were identified. The *E*-suppressed mutant (AE) accumulated MGlcDG (4.2%) and showed better growth and photosynthetic activities compared with WT and other mutants (*A*/ *E*-overexpressed and *A*-suppressed strains), which suggested that MGlcDG was involved in phosphate stress adaptation for *S* sp. PCC 7942. A notable increase in contents of 18:1 fatty acid (FA) of MGDG (127%), DGDG (68%), and SQDG (105%) in AE were found under phosphate starvation. However, the expression of △9 desaturase (*C*) was not higher in AE than that in WT during phosphate-starved period. These results suggested that MGlcDG might be involved in the process of FA desaturation, which contributed to membrane fluidity and cell basic metabolism for stress acclimation in cyanobacteria. In complementary experiments of *E.* , although the expression of *A* and *C* in the *A* and *C* coexpressed strain (OEAC) reduced by 22% and 35% compared with that of the strains only overexpressing *A* (OEA) or *C* (OEC), the content of unsaturated FA in OEAC was the highest. This further implied that the accumulation of MGlcDG could prompt FA desaturation in *E.* . Therefore, we propose that an overproduction of MGlcDG is responsible for FA desaturation and participates in phosphate stress adaptation in cyanobacteria.

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1. I

Monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiaclglycerol (SQDG), are restricted mainly to oxygen-evolving photosynthetic organisms [1]. In the biosphere, glycolipid MGDG is the most abundant, which occupies >50% of the total lipid amount of chloroplast and cellular membrane of photosynthetic organisms [2]. Not only does MGDG play a vital in construction of membrane but it is also important for the optimal function of photosynthetic pathways and complex [3].

The biosynthesis of MGDG in plants and cyanobacteria are different. In plants, MGDG synthase (MGD) catalyzes the reaction of diacylaglycerol (DAG) with UDP-galctose in one-step reaction [4]. While in cyanobacteria, DAG is first reacted with UDP-glucose by monoglucosyldiacylglycerol synthase (MgdA) to form monoglucosyldiacylglycerol (MGlcDG), and then converted to MGDG by a glycosyl epimerase (MgdE) rapidly [5].

Recent studies have found that the content of MGlcDG is <1% of total membrane lipid in cyanobacteria under normal conditions [6]. However MGlcDG accumulates when heat stressed, which is important for heat stress adaptation for cyanobacteria [7,8]. Moreover, cyanobacterial unique MGDG synthesis pathway is involved in fatty acid (FA) desaturation and low temperature adaptation [6].

Phosphate-starved environment induces glycolipid

A : DGDG, digalactosyldiacylglycerol; FA, fatty acid; MGDG, monogalactosyldiacylglycerol; MGlcDG, monoglucosyldiacylglycerol; PG, phosphatidylglycerol; SQDG, sulfoquinovosyldiaclglycerol.

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accumulation in cyanobacteria [9,10]. We previously revealed that glycolipid accumulation and FA desaturation were vital for *S* - sp. PCC 7942 to acclimate to phosphate starvation [11]. However, whether MGlcDG participates in the phosphate stress adaptation has not been unveiled. Here, we characterized the function of MGlcDG in phosphate-starved *S* sp. PCC 7942. We found that cyanobacterial MGlcDG played a vital role in FA desaturation and phosphate stress adaptation.

2. M

2.1. G ,

The MGDG synthesis genes (*A* and *E*) of *S* sp. PCC 6803 [5] were used as probes for a BLAST search for the candidate genes of *S* sp. PCC 7942 in Cyanobase (http://genome.kazusa.or.jp/cyanobase) (Table S1).

The schemes for construction of plasmids pET28a_ *A*, pACYC_ *E*, and pUC118_*A MGD1* (pUC118 carrying a gene of *A* MGDG synthase 1) were shown in Figs. S1, S2, and S3. The plasmids were confirmed by enzyme digestion (Fig. S4). Primers were listed in Table S2. All plasmids used in this study were shown in Table S3.

Genes were highly expressed in *E.* grown in LB medium with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) at 30 °C for 9 h [5]. Lipids were extracted [12] and then separated by thin layer chromatography (TLC) using acetone:toluene:water in a ratio of 91:30:7 (v/v/v). Spots were visualized with α-naphthol staining [5]. Spot identification was compared with lipid standard (Avanti Polar Lipids, USA). Lipids extracted from *E.* expressing *A MGD1* were used as positive control.

2.2. C

S sp. PCC 7942 wild-type (WT) was used to generate *A* and *E* Sense/Antisense mutants. All the strains used in the present study were shown in Table S4. Cyanobacteria was air bubbled under 25 ± 2 °C and 140 μmol $m^{-2}s^{-1}$ in 1 L Erlenmeyer flask with 500 mL BG11 medium [9]. Cells were cultivated in the medium with phosphate concentration 0.04 g L^{-1} (+P) or 0 g L^{-1} (−P).

2.3. A E S /A

Plasmid pRL489 is a shuttle expression vector with PsbA promoter and kanamycin (Km^r) selectable marker [13,14]. It was used to construct *A* and *E* Sense/Antisense expression vectors [15]. Vectors were confirmed by restriction enzyme digestion (Figs. S5 and S6). Transformation was performed naturally [16]. The screening was repeated three times with an increasing Km^r concentration to 15 μg mL^{-1}. The genotypes of mutants were confirmed by RT-qPCR analysis (Fig. S7).

2.4. L FA

Lipids of cyanobacteria were extracted [17]. Lipid composition and FA profile analysis were performed by TLC coupled with gas chromatography mass spectrometry (GC-MS) [11]. Briefly, lipids were separated using hexane:chloroform:2-propanol:water:tetrahydrofuran (100:50:80:2:1, v/v/v/v/v) by TLC, staining by α-naphthol. Fatty acid methyl esters (FAMEs) were conducted with 1 mL 1 N methanolic HCl, then incubated at 80 °C for 1 h [18]. The FAMEs were analyzed by AutoSystem XL GC/TurboMass MS (PerkinElmer, Germany) [19].

2.5. M F /F

The chlorophyll extraction from the cells were performed with 90% methanol in dim light. The Calculation of chlorophyll content was from the absorbance of the methanolic extract at 665 nm by spectrophotometer (UV-1000, Techcom, China), using Eq: C (μg mL^{-1}) = $OD_{665\ nm} \times 13.9$ [20].

Pulse Amplitude Modulation fluorometer (PhytoPAM, Walz, Effeltrich, Germany) was used to measure photosystem II quantum yield (F_V/F_M) as described by Pittera et al. [21].

2.6. Q - PCR (RT- PCR)

Total RNA was extracted with Trizol (Takara, China) [15]. Reverse transcription was conducted with PrimeScript RT reagents (Takara, China). RT-qPCR was performed using SYBR Premix Ex Taq II (Takara, China) in Real Time System (CFX Connect™, Bio-Rad). The genes RNase P subunit B (*B*) and *A* were used as the internal gene for *S* sp. PCC 7942 and *E.* , respectively. Primers were listed in Table S2.

3. R

3.1. F MGDG S . PCC 7942

Gene comparison demonstrated that the sequences of Synpcc7942_1083 and Synpcc7942_0861 (Gene ID) in *S* - sp. PCC 7942 were similar to Sll1377 and Sll1376 in *S* - sp. PCC 6803 (Table S1), which might encoded MGlcDG synthase (*A*) and MGlcDG epimerase (*E*), respectively.

The functions of *7942_1083* (*A*) and *7942_0861* (*E*) were confirmed by coexpression analysis in *E.* . As shown in Fig. 1A, *E.* only expressing *S* *A* accumulated MGlcDG (lane 5), which was similar in the case of *S* sp. PCC 6803 [5]. This result suggested that the candidate gene *A* could catalyze MGlcDG synthesis. Moreover, no MGDG was detected in cells expressing only *E* (lane 6), whereas it was observed when both *A* and *E* were expressed (lane 7). This result indicated that the *E* encoded enzyme responsible for epimerization. Collectively, these results suggested that *S* sp. PCC 7942 synthesized MGDG by two steps (Fig. 1D), which was similar with other cyanobacteria, such as *S* sp. PCC 6803, *A* , *P* , and *G* [22].

It has been reported that cyanobacteria carrying a deleted *A* gene cannot easily survive because of its involvement in MGDG synthesis [23,24]. Previously, Jia et al. [25] antisense expressed the gene of phosphoenolpyruvate carboxylase ( ) of *A* sp. PCC 7120, resulting in an increase of cyclic electron flow and stress tolerance. Gong and Miao [15] investigated the function of β-ketoacyl ACP synthases (*B/F* and *H*) by constructing Sense/Antisense expression strains in *S* sp. PCC 7942. Therefore, in present study, to further understand the function of *A* and *E* in vivo, Sense/Antisense expressed *A* and *E* mutants of *S* sp. PCC 7942 were constructed, i.e., Sense- *A* (SA), Antisense- *A* (AA), Sense- *E* (SE) and Antisense- *E* (AE).

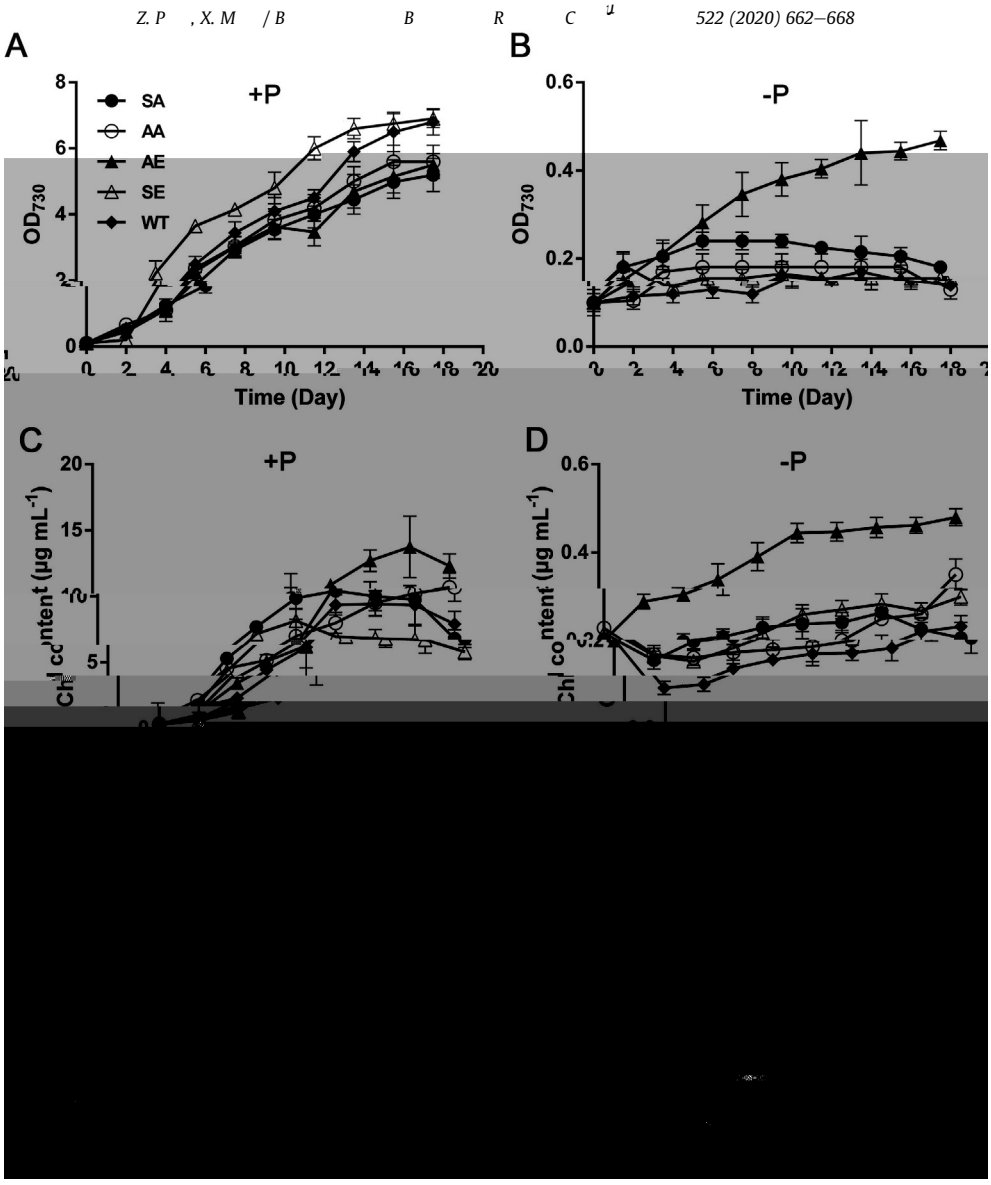
Lipids from WT and different mutants of *S* sp. PCC 7942 were analyzed by TLC and GC-MS. As shown in Fig. 1B and C, different to the other strains, AE accumulated MGlcDG as high as 4.2%. It might be caused by the reduced expression of *E* (Fig. S7), which inhibited the conversion of MGlcDG to MGDG. Similarly, the disruption of *E* and *A* (DGDG synthase gene) also cause the accumulation of MGlcDG and MGDG in

cyanobacteria, respectively [5,26]. Moreover, a significant reduction of MGDG in AE (19.3%) and AA (25.2%) were also detected (Fig. 1C), which suggested that the expression level of glycolipid synthesis genes might affect glycolipids assembly (Fig. S7) [27,28]. Therefore, we suggest that candidate genes *A* and *E* in *S* sp. PCC 7942 are involved in MGDG synthesis (Fig. 1D).

3.2. T *MG DG*

MGlcDG accumulates in heat stress and is also involved in cold acclimation in cyanobacteria [6,7]. To further evaluate the function of MGlcDG in phosphate stress adaptation, the growth and photosynthesis activities of different mutants of *S* sp. PCC 7942 were characterized under + P and −P conditions. As shown in Fig. 2A, C, and E, there were almost no significant differences in growth, chlorophyll content or F_V/F_M value among mutants and WT under + P conditions. The possible reason is that galactolipids (MGDG and DGDG) are keys for the better growth of cyanobacteria [5] and MGlcDG is not essential for cyanobacterial growth and photosynthesis under normal conditions [6]. In present study, the total content of galactolipids (>60%) remained constant in mutants and WT (Fig. 1C). It was crucial for stability and functions of photosynthetic apparatus under normal conditions [29].

However, under −P conditions, significant differences in growth (Fig. 2B) and photosynthesis activities (Fig. 2D and F) were observed between AE and the other strains. As shown in Fig. 2B, the growths were retarded under −P conditions for most strains except for AE. AE showed a continuous increasing OD_{730} value under −P conditions and reached to the highest (0.468) on day 18, which was 3.3-fold higher than that of WT (Fig. 2B). Similar patterns were observed for the contents of chlorophyll in −P cells (Fig. 2D). The contents of chlorophyll in most −P strains were almost completely inhibited, however the content of chlorophyll in AE gradually increased and reached to the highest (0.44 μg mL^{-1}) on day 10 (Fig. 2D). Moreover, the F_V/F_M value of AE decreased more slowly than that of the other strains from day 2 to day 10 under phosphate-starved conditions (Fig. 2F). These results demonstrated



F . 2. The growth (A, B) and photosynthetic activities (C–F) of wild-type (WT) and mutant strains of *S* sp. PCC 7942 cultivated under phosphate replete (+P) and depleted (–P) conditions, respectively. Mean, n = 3. Sense- *A* (SA), Antisense- *A* (AA), Sense- *E* (SE), and Antisense- *E* (AE).

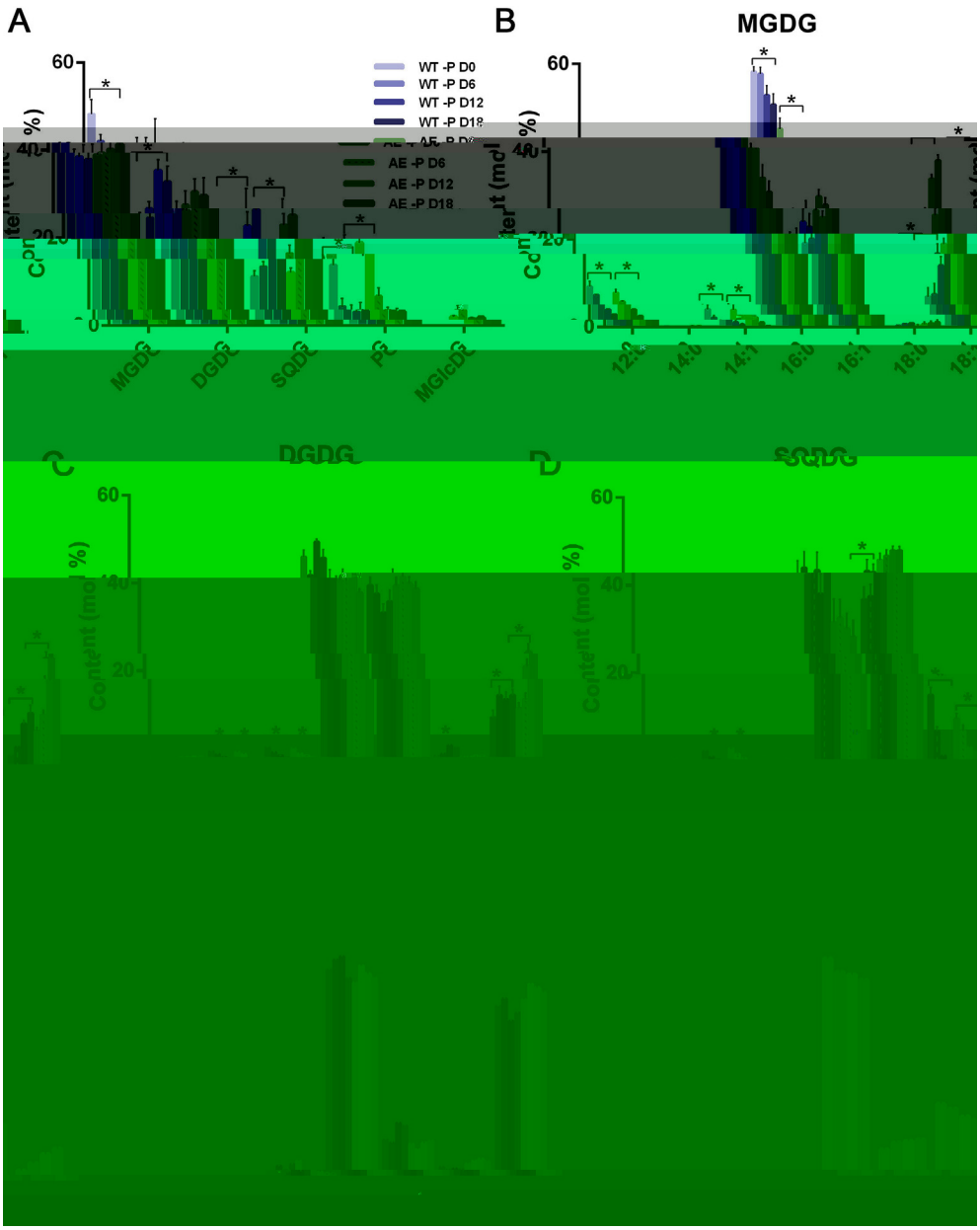
3.3. C FA AE

Cyanobacterial MGlcDG is involved in desaturation of galactolipids, which will elevate membrane fluidity for low-temperature adaptation in cyanobacteria [6]. Previous studies also reported the increasing content of unsaturated FAs in *S* - sp. PCC 7942 [11] and some eukaryotic microalgae [31,32] under –P conditions. To explore whether cyanobacterial MGlcDG participated in phosphate stress adaptation by desaturation of membrane lipids in present study, the dynamic changes of main membrane lipids and their FA compositions in AE and WT under phosphate depletion were analyzed.

As shown in Fig. 3A, the dynamic changes of main membrane lipids (MGDG, DGDG, SQDG and PG) in AE and WT were very similar under –P conditions. However, the content of MGlcDG in AE under –P conditions increased (75%) on day 6 and then decreased to the initial level, whereas no MGlcDG was detected in WT under –P conditions (Fig. 3A). This result suggested that AE regulated MGlcDG synthesis to adapt to –P conditions. The change of lipid composition is vital for cells to adapt to stress environments [11]. A higher amount of MGlcDG in AE can maintain membrane integrity by strong bilayer stabilizing propensity for stress adaptation [33,34].

The FA compositions of WT and AE varied significantly under –P conditions (Fig. 3B–F). After 18 days phosphate starvation, the amount of 18:1 FA in glycolipids (MGDG, DGDG and SQDG) of AE reached to the highest, which was 127%, 68%, and 105% higher than that of WT, respectively (Fig. 3B–D). The increased content of unsaturated FAs in glycolipids might improve membrane fluidity [11,35] and further affect photosynthetic activities for phosphate stress adaptation (Fig. 2D and F) [36].

The decreasing pattern of FAs C12, C14 and 16:0 in glycolipids (MGDG, DGDG and SQDG), and 18:0 in SQDG were observed in both WT and AE under –P conditions (Fig. 3B–D). While the content of 18:1 in these glycolipids increased significantly in both strains (Fig. 3B–D). These results suggested that FAs C12, C14, 16:0 and 18:0 participated in 18:1 synthesis under –P conditions for both strains. However, as for AE, high proportions of 16:0 (~60%) and 18:0 (~20%) in MGlcDG were observed during the entire –P period, which was different from WT (Fig. 3F). As MGlcDG is a precursor for



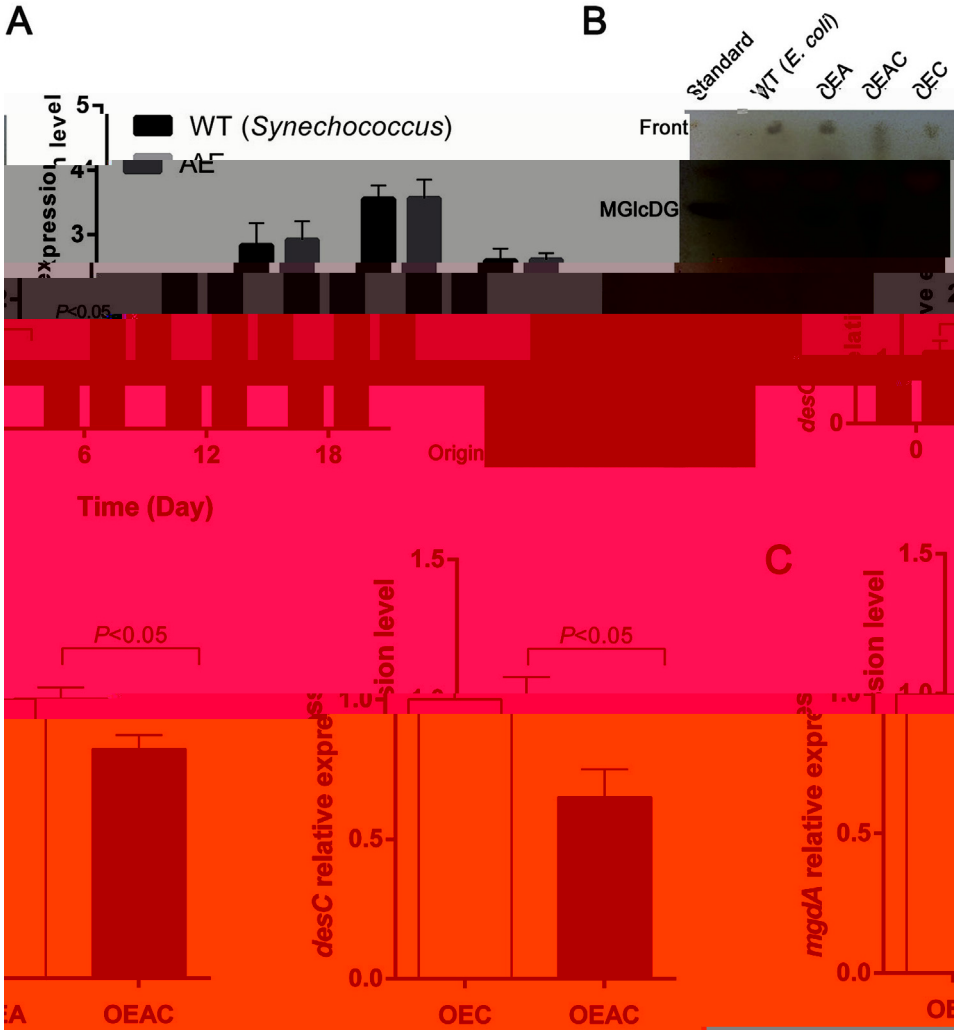
F . 3. Contents of membrane lipids (A) and their fatty acid compositions (B–F) in wild-type (WT) and Antisense- E (AE) strains of *S* u sp. PCC 7942 under –P conditions. WT and AE were initially grown under + P conditions, then transferred to –P conditions and kept for 0 day, 6 days, 12 days, and 18 days. Mean, n = 3. Student's -test (*P < 0.05).

MGDG synthesis (Fig. 1D) and MGDG also acts as a substrate for DGDG assembly [26]. It was possible that the larger pool size of saturated MGlcDG in AE than that in WT might serve as the most preferable substrate for desaturation. It has also been reported that MGlcDG prompts desaturation of 18:2 to 18:3 in MGDG in *S* - sp. PCC 6803 [6]. Above all, these results implied that MGlcDG could participate in phosphate stress adaptation by prompting desaturation of membrane lipids.

3.4. MG DG FA u

AE and WT were C16 and C18 rich, and the unsaturation of which changed significantly in –P cells (Fig. 3). △9 desaturase (*C*) catalyzes the double bound formation of 16:0 and 18:0 [37,38]. Therefore, the expressions of *C* in both strains were then characterized. The expression of *C* in –P cells (Fig. 4A, day 6, 12 and 18) were higher (>3-fold) than those cultivated under + P conditions for both strains (Fig. 4A, day 0), representing that the increased amount of unsaturated FAs were caused by the enhanced *C* expression. Under + P conditions (Fig. 4A, day 0), *C* expression of AE was lower (38%) than that of WT. During phosphate-starved period (Fig. 4A, day 6, 12 and 18) the expression of *C* showed almost no difference between AE and WT. These results suggested that the higher unsaturated FAs in glycolipids in AE than that in WT (Fig. 3B–D) was not caused by the increased *C* expression. Based on the lipid analysis above, we suggested that an adequate MGlcDG supplement might be involved in the activation of FA desaturation process.

Furthermore, the involvement of desaturation of MGlcDG was also verified by comparing the FA profiles of different recombinant



F . 4. Dynamic expressions of *C* in wild-type (WT) and Antisense- *E* (AE) strains of *S* u sp. PCC 7942 grown under −P conditions (A), lipid analysis by TLC (B) and the expression of *A* and *C* (C) in *E.* mutants. *A*-overexpressed *E.* (OEA); *C*-overexpressed *E.* (OEC); *A* and *C* coexpressed *E.* (OEAC). Cyanobacteria were initially grown under + P conditions, then transferred to −P conditions and kept for 0 day, 6 days, 12 days, and 18 days. *C* expressions in cyanobacteria were calculated relative to that of WT grown under + P conditions (day 0). *B* and *A* were used as the internal gene for *S* u sp. PCC 7942 and *E.* , respectively. Mean, n = 3.

E. strains, i.e., *A*-overexpressed *E.* (OEA); *C*-overexpressed *E.* (OEC); *A* and *C* coexpressed *E.* (OEAC). As shown in Table 1 and Fig. 4B, the amount of 16:1 increased (71%) in OEA (MGlcDG accumulated strain) than that in WT, which indicated that the overproduction of MGlcDG in OEA could elevate the desaturation of 16:0. As expected, there was a significant increase in contents of 16:1 (4.5-fold) and 18:1 (1.1-fold) in OEC than that in WT (Table 1), which was consistent with the study described by Woelke et al. [38]. When both *A* and *C* were overexpressed (OEAC), although the expression of *A* and *C* in OEAC reduced by 22% and 35% compared with that in OEA and OEC, respectively (Fig. 4C), unsaturated FA was the highest in OEAC (Table 1). The amount of 16:1 and 18:1 were 5.0-fold and 1.2-fold higher in OEAC than that in WT (Table 1). These results further provided evidence that MGlcDG could prompt FA desaturation in *E.* . In summary, we propose that the accumulation of MGlcDG is involved in lipid desaturation and participates in phosphate stress acclimation in *S* u sp. PCC 7942.

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FA compositions of total membrane lipids extracted from wild-type (WT) and recombinant *E.* . Mean, n = 3. *, P < 0.05, Student's -test (mutants vs. WT).

FAs (mol %)	WT (*E.*)	OEA	OEC	OEAC
14:0	3.18 ± 0.37	4.1 ± 0.01	3.31 ± 0.05	3.60 ± 0.19
16:0	35.24 ± 1.59	37.18 ± 1.16	32.98 ± 0.11*	27.81 ± 0.30 *
16:1	2.91 ± 0.30	4.98 ± 0.29 *	12.95 ± 0.09 *	14.46 ± 0.21 *
17:1	17.94 ± 0.35	15.06 ± 1.02 *	13.92 ± 0.15 *	11.01 ± 0.06 *
18:0	1.39 ± 0.12	1.65 ± 0.14	0.98 ± 0.03 *	2.00 ± 0.03 *
18:1	31.43 ± 1.52	31.39 ± 3.30	33.35 ± 0.12	37.68 ± 0.38 *
18:2	n.d.	n.d.	n.d.	0.51 ± 0.01 *
19:1	7.49 ± 0.12	4.39 ± 0.26 *	2.52 ± 0.03 *	1.99 ± 0.49

n.d., not detected; OEA, *A*-overexpressed *E.* ; OEC, *C*-overexpressed *E.* ; OEAC, *A* and *C* coexpressed *E.* .

A

Miao and Peng designed the study. Peng executed the experiments, analyzed the data, and wrote the manuscript. Miao made a critical revision of the manuscript. Both authors read and approved the manuscript for submission and publication.

D

The authors declare that there is no conflict of interest.

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