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Rre37 stimulates accumulation of 2-oxoglutarate and glycogen under nitrogen starvation in *Synechocystis* sp. PCC 6803

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ABSTRACT

Rre37 (sll1330) in a cyanobacterium *Synechocystis* sp. PCC 6803 acts as a regulatory protein for sugar catabolic genes during nitrogen starvation. Low glycogen accumulation in Δ*rre37* was due to low expression of glycogen anabolic genes. In addition to low 2-oxoglutarate accumulation, normal upregulated expression of genes encoding glutamate synthases (*gltD* and *gltB*) as well as accumulation of metabolites in glycolysis (fructose-6-phosphate, fructose-1,6-bisphosphate, and glyceraldehyde-3-phosphate) and tricarboxylic acid (TCA) cycle (oxaloacetate, fumarate, succinate, and aconitate) were abolished by *rre37* knockout. Rre37 regulates 2-oxoglutarate accumulation, glycogen accumulation through expression of glycogen anabolic genes, and TCA cycle metabolites accumulation.
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1. Introduction

Cyanobacteria, which perform oxygenic photosynthesis like eukaryotic algae and plants, inhabit almost all illuminated ecosystems and play key roles in the global carbon and nitrogen cycles [1,2]. In natural habitats, nitrogen, an essential macronutrient, is limiting factor for cellular growth, and unicellular cyanobacteria have developed mechanisms to respond to nitrogen depletion [3]. Most cyanobacteria, including non-nitrogen fixing, unicellular cyanobacteria, like *Synechocystis* sp., store glycogen under nitrogen or sulfur deficiency [4]. Metabolic changes after nitrogen depletion have been demonstrated [5,6], showing increases in levels of tricarboxylic acid (TCA) cycle metabolites (such as malate, fumarate, succinate) and decreases in purine and pyrimidine nucleotides. Several amino acids including glutamine and glutamate decrease following nitrogen depletion [6], and particularly reflect limitation of available nitrogen sources. Similarly to other bacteria, nitrogen compounds in cyanobacteria are reduced to ammonium and incorporated into 2-oxoglutarate (2-OG) via the glutamine synthetase (GS) and glutamate synthase (GOGAT) cycle, known as the GS–GOGAT pathway [7]. Levels of 2-OG increase during nitrogen depletion, which initiates nitrogen-starvation response in *Synechocystis* cells [8]. Thus, 2-OG is a key metabolite in *Synechocystis* for appropriate acclimation to nitrogen starvation.

Synechocystis sp. PCC 6803 (hereafter *Synechocystis*) is one of the most widely studied cyanobacterial species due to its transformability and availability of its entire genome sequence [9,10]. Changes in gene expression under nitrogen depletion have been investigated by microarray analysis [11,12]. NtcA, which is a transcription factor belonging to the cAMP receptor protein family, regulates OmpR-type response regulator Rre37 and RNA polymerase sigma factor SigE, and binding of NtcA to the *rre37* promoter is

Abbreviations: GS, glutamine synthetase; GOGAT, glutamate synthase; HPLC, high performance liquid chromatography; LC/QqQ-MS, liquid chromatography/triple quadrupole mass spectrometry; OD, optical density; TCA, tricarboxylic acid; 2-OG, 2-oxoglutarate

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enhanced in the presence of 2-OG *in vitro* [13]. PII signaling protein (encoded by *glnB*) in *Synechococcus* sp. PCC 7942 also binds 2-OG in a cooperative manner with ATP and is phosphorylated during nitrogen starvation [2]. PII bound to 2-OG releases PipX, a small protein conserved among several cyanobacteria, and PipX associates with NtcA, possibly controlling the transcriptional activity of NtcA [14,15]. The expression of various sugar catabolic genes and glycogen content are both increased by nitrogen depletion [16]. Rre37 and SigE are involved in nitrogen-induced expression of sugar catabolic genes [13,16,17]. Rre37 activates the expression of two glycogen catabolic genes [*glgX* (slr1857, encoding glycogen isoamylase) and *glgP* (slr1367, encoding glycogen phosphorylase)] and two glycolytic genes [*gap1* (slr0084, glyceraldehyde 3-phosphate dehydrogenase) and *pfkA* (sll1196, phosphofructokinase)] under nitrogen starvation [13]. SigE activates the expression of glycolytic genes (*gap1* and *pfkA*) independently of Rre37 [12,13]. In contrast to studies of mechanism of transcriptional regulation after nitrogen depletion and regulatory factors controlling primary metabolism during nitrogen starvation, few studies of the metabolome have been performed with mutants of nitrogen regulators.

In this study, an Rre37 mutant of *Synechocystis* was constructed and analysis of gene expression and metabolites were carried out to clarify which pathways or genes are regulated by Rre37 in carbon or nitrogen flow.

2. Materials and Methods

2.1. Strains and growth conditions

The glucose-tolerant (GT) strain of cyanobacterium *Synechocystis* isolated by Williams [18] and the cognate *rre37* knockout mutant (Δ*rre37*) were precultured for 5 days in 1.4 L modified BG-11 medium, which contained (L^{-1}) $NaNO_3$ 1.5 g, K_2HPO_4 0.04 g, $MgSO_4 \cdot 7H_2O$ 0.075 g, $CaCl_2 \cdot 2H_2O$ 0.036 g, citric acid 0.006 g, ferric ammonium citrate 0.006 g, EDTA (disodium salt) 0.001 g, Na_2CO_3 0.02 g, H_3BO_3 2.86 mg, $MnCl_2 \cdot 4H_2O$ 1.81 mg, $ZnSO_4 \cdot 7H_2O$ 0.222 mg, $NaMoO_4 \cdot 2H_2O$ 0.39 mg, $CuSO_4 \cdot 5H_2O$ 0.079 mg, $Co(NO_3)_2 \cdot 6H_2O$ 49.4 μg [19] under continuous illumination at 70 μmol photons m^{-2} s^{-1} at 25 °C with 2% (w/w) CO_2 bubbled at 80 mL min^{-1}. 10.0 g/L agar was added in a solid medium. For culturing Δ*rre37*, 50 μg L^{-1} kanamycin was added to the medium. After cells reached the mid-exponential phase, they were collected by filtration using a 1 μm pore size polytetrafluoroethylene filter (Millipore, Billerica, MA), washed 3 times with BG-11 medium without a nitrogen source ($BG\text{-}11_0$), and then inoculated into fresh $BG\text{-}11_0$ medium at 0.2 g dry-cell weight L^{-1}.

2.2. Glycogen analysis

Glycogen was extracted from dried cells as described previously [20]. Glycogen content was determined by high performance liquid chromatography (HPLC) (LC Prominence, Shimadzu, Kyoto, Japan) using a size exclusion HPLC column (OHpak SB-806M HQ; Shodex, Tokyo, Japan) and a reflective index detector (RID-10A; Shimadzu) as previously described [21].

2.3. Quantitative gene expression analysis

Cells were harvested at different time points by centrifugation. RNA was extracted using a Nucleospin RNAII kit (Macherey–Nagel GmbH & Co. KG Düren, Germany) and cDNA was synthesized using a ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan) according to the manufacturers' instructions. Total RNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and loaded in an Agilent 2100 bioanalyzer (Agilent Technologies, La Jolla, CA) to check purity, concentration and integrity. To investigate expression of *rre37*, reverse transcription PCR was performed using cDNA and *rre37* primers (Supplementary Table S1). Real time quantitative PCR was performed with the gene-specific oligonucleotides (Supplementary Table S1) using Thunderbird SYBR qPCR mix (Toyobo). The transcript level of target genes was quantified using an Mx3000P qPCR system (Agilent Technologies). The *rnpB* gene, which encodes RNase P subunit B, was used as a housekeeping gene. The amplification efficiency (E) of each oligonucleotide was calculated using the equation $E = 10^{(-1/\text{slope})}$, and expression of target gene relative to reference gene was calculated using Pfaffl method [22].

2.4. Metabolic profile analysis using LC/QqQ-MS

Cell sampling was performed as described previously [5]. Dried extracts were dissolved in Milli-Q water and applied to an LC/QqQ-MS system (HPLC system: Agilent 1200 series, MS system: Agilent 6460 with Jet Stream Technology, Agilent Technologies) controlled with MassHunter Workstation Data Acquisition software v. B.04.01 (Agilent Technologies). LC/QqQ-MS was performed with multiple reaction monitoring as described previously [5].

3. Results

3.1. Delay of glycogen accumulation in Δrre37 under nitrogen starvation

During nitrogen starvation for 72 h, *rre37* was expressed in GT, whereas Δ*rre37* lacked expression of the *rre37* gene, confirming disruption of *rre37* in the mutant (data not shown). Disruption of *rre37* did not influence cell growth in Δ*rre37* during nitrogen depletion (data not shown).

After nitrogen depletion, *Synechocystis* started to accumulate glycogen and the glycogen content reached 44% of dry-cell weight after 72 h [5]. Glycogen accumulation was delayed in Δ*rre37* for 24 h, although the glycogen content in both GT and Δ*rre37* increased to 40% of dry-cell weight after 48 h (Fig. 1). Rre37 appears to have a functional role for glycogen accumulation up to 12 h after nitrogen depletion in GT.

3.2. Positive regulation of glycogen anabolic genes by rre37 under nitrogen starvation

To reveal the role of Rre37 during nitrogen depletion, changes in both metabolite content (related to glycogen catabolism and anabolism, glycolysis, pentose phosphate pathway, TCA cycle, and GS–GOGAT pathway) and gene expression, which are involved

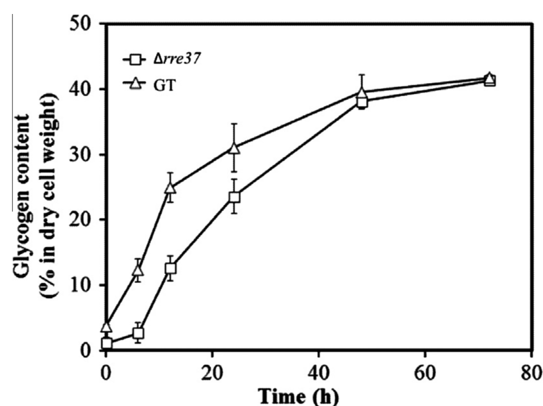

Fig. 1. Glycogen content in glucose-tolerant strain of *Synechocystis* sp. PCC 6803 (GT) (Δ) and *rre37* mutant (□) after nitrogen depletion.

in glycogen accumulation and the signaling pathway involved in nitrogen starvation, were compared for GT and Δ*rre37* after 6 and 12 h (Fig. 2, Supplementary Fig. S1, and Table S2). Expression of two glycogen anabolic genes, *glgA* (encoding glycogen synthase) and *glgC* (encoding glucose-1-phosphate adenyltransferase) were about 2-fold upregulated after 6 h in GT, and ADP-glucose, a precursor of glycogen, accumulated; however, expression in Δ*rre37* was unaltered and the ADP-glucose level decreased (Fig. 2 and Supplementary Fig. S2). Also, expression of *glgX* and *glgP* increased after 6 h in GT and the glucose-1-phosphate level increased, whereas in Δ*rre37*, expression of these genes was lower than in GT and the amount of glucose-1-phosphate did not change. After 12 h, glycogen started to be accumulated in Δ*rre37* in accordance with the increase in expression of *glgA* and *glgC*, which reached a level similar to their expression in GT (Supplementary Figs. S1 and S2). However, expression of *glgP* and *glgX* in Δ*rre37* were lower than in GT after 12 h. These results suggest that a delay in upregulation of expression of genes, *glgA* and *glgC*, caused a delay in glycogen accumulation in Δ*rre37*.

3.3. Effect of Rre37 on glycolysis, pentose phosphate pathway, and TCA cycle

For metabolites related to glycolysis pathway, fructose-6-phosphate, fructose-1,6-bisphosphate, and glyceraldehyde-3-phosphate were increased and 1,3-bisphosphoglycerate was decreased in GT after 6 h of nitrogen depletion; however, contradictory tendencies of these metabolites were observed in Δ*rre37*, showing that drastic change in glycolytic pathway by disruption of Rre37 (Fig. 2). For metabolites related to pentose phosphate pathway, erythrose 4-phosphate and sedoheptulose-7-phosphate were increased and ribose 5-phosphate was decreased in GT after 6 h of nitrogen depletion; however, contradictory tendencies of these metabolites were observed in Δ*rre37*, showing that metabolites nearly associated with glycolytic pathway were changed in pentose phosphate pathway. For genes related to glycolysis pathway, expression of *gap1* was 5.2-fold increased in GT, however this was 2.5-fold increased in Δ*rre37* (Fig. 2). Expression of *gap2* was slightly increased (1.2-fold) in GT, but was slightly decreased (0.9-fold) in Δ*rre37*, correlating with increase of glyceraldehyde-3-phosphate level in GT. For genes related to pentose phosphate pathway, expressions of glucose-6-phosphate dehydrogenase (*zwf*) and 6-phosphogluconate dehydrogenase (*gnd*) were not so changed between GT (2.4, and 1.6-fold, respectively) and Δ*rre37* (1.4, and 1.5-fold, respectively) after 6 h of nitrogen starvation.

All the metabolites of the TCA cycle except for citrate increased during nitrogen depletion in the *Synechocystis* cells, as previously described [6], but oxaloacetate, fumarate, succinate, aconitate, and 2-OG decreased in Δ*rre37* (Fig. 2). The expression of *icd*, encoding isocitrate dehydrogenase, which catalyzes the production of 2-OG, was examined in both GT and Δ*rre37* cells. As previously reported [11,23], *icd* gene expression was upregulated in GT (1.5-fold) after 6 h of nitrogen depletion but it was downregulated (0.7-fold) in Δ*rre37* (Fig. 2). *icd* gene expression was upregulated

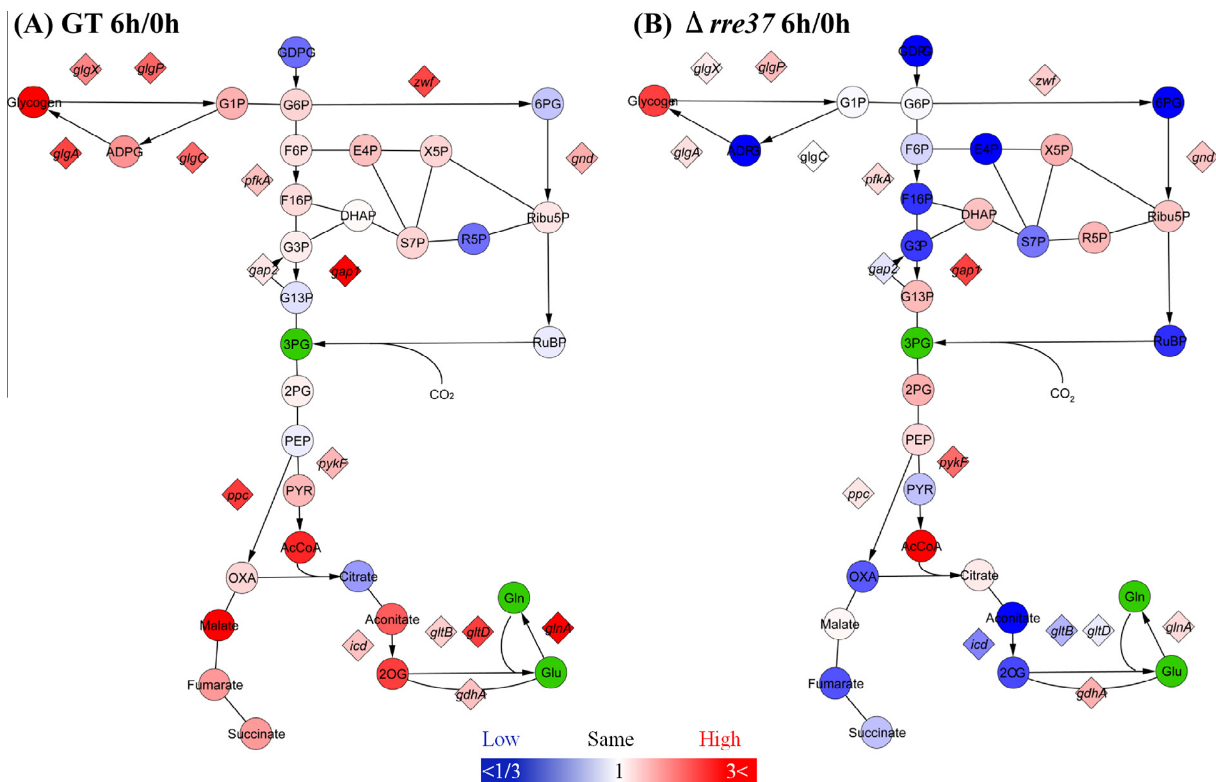

Fig. 2. Change in metabolite concentration (○) and gene expression (◇) in (A) *Synechocystis* sp. PCC 6803 (GT) and (B) *rre37* mutant (Δ*rre37*). Ratio of values after 6 h of nitrogen depletion to those at the starting point (0 h) were obtained. Fold change increases or decreases are indicated by shades of red and blue according to the scale bar. Metabolites: GDPG, GDP-glucose; ADPG, ADP-glucose; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; F16P, fructose-1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; G3P, glyceraldehyde-3-phosphate; G13P, 1,3-bisphosphoglycerate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; PYR, pyruvate; AcCoA, acetyl-CoA; 2-OG, 2-oxoglutarate; OXA, oxaloacetate; 6PG, 6-phosphogluconate; E4P, erythrose 4-phosphate; X5P, xylulose 5-phosphate; Ribu5P, ribulose-5-phosphate; R5P, ribose 5-phosphate; S7P, sedoheptulose-7-phosphate; RuBP, ribulose-1,5-bisphosphate; Gln, glutamine; Glu, glutamate. (Clear signals for 3PG, Gln, and Glu were not obtained and are shown in green.) Genes: *glgA*, glycogen (starch) synthase; *glgC*, glucose-1-phosphate adenylyltransferase; *glgX*, glycogen isoamylase; *glgP*, glycogen phosphorylase; *pfkA*, phosphofructokinase; *gap1*, glyceraldehyde 3-phosphate dehydrogenase (catabolic reaction); *gap1*, glyceraldehyde 3-phosphate dehydrogenase (NADP+); *ppc*, phosphoenolpyruvate carboxylase; *icd*, isocitrate dehydrogenase; *gdhA*, NADP-specific glutamate dehydrogenase; *gltB*, NADH-dependent glutamate synthase large subunit; *gltD*, NADH-dependent glutamate synthase small subunit; *glnA*, glutamate-ammonia ligase; *pykF*, pyruvate kinase 1; *zwf*, glucose-6-phosphate dehydrogenase; *gnd*, 6-phosphogluconate dehydrogenase.

1.4-fold in GT after 12 h of nitrogen depletion, but in Δ*rre37*, it was downregulated except at 24 h (Supplementary Fig. S3). These data suggest that downregulation of *icd* gene expression is one reason for the delayed accumulation of 2-OG in Δ*rre37*. In *Synechocystis*, the gene product of *ppc* (phosphoenolpyruvate carboxylase) converts phosphoenolpyruvate to oxaloacetate [24]. Expression of *ppc* was upregulated in GT (2.5-fold) and unchanged in Δ*rre37* (1.2-fold), which showed decreased accumulation of oxaloacetate in Δ*rre37* after 6 h of nitrogen depletion (Fig. 2). The observed differences in TCA cycle metabolites between GT and Δ*rre37* are likely partially due to differential expression of *ppc*.

3.4. Expression of nitrogen assimilatory genes is reduced by rre37 knockout

Since 2-OG accumulation differed between GT and Δ*rre37*, the transcript levels of genes related to nitrogen assimilation were examined. Expression of *glnA* (encoding glutamate-ammonia ligase) was upregulated 6-fold under nitrogen starvation in GT, consistent with previous studies [11,16] whereas *glnA* was upregulated only 1.2-fold in Δ*rre37* after 6 h of nitrogen depletion (Fig. 2 and Supplementary Fig. S3). Expression of *gdhA*, which encodes glutamate dehydrogenase, catalyzing the reversible reaction between 2-OG and glutamate [25], was upregulated in GT and in Δ*rre37* (1.4 and 1.6-fold, respectively) after 6 h of nitrogen depletion. *Synechocystis* possess two types of GOGATs; one using ferredoxin and a second type using NADH as electron donors [7]. Both *gltD* (encoding NADH dependent glutamate synthase small subunit) and *gltB* (NADH-dependent glutamate synthase large subunit) were upregulated in GT (2.4 and 1.7-fold, respectively) but in Δ*rre37*, these genes were downregulated (0.8-fold) after 6 h of nitrogen depletion. On the other hand, expression of *glsF* (encoding ferredoxin-dependent glutamate synthase) was upregulated in both GT and Δ*rre37* (3.0 and 3.4-fold, respectively). These results suggested that expression of genes in the GS and NADH–GOGAT is partly inhibited by *rre37* knockout.

3.5. Expression analysis of genes related to nitrogen regulators

Since *rre37* knockout widely affected primary carbon and nitrogen metabolism, the transcript levels of four nitrogen regulators, *ntcA*, *ntcB*, *sigE* and *glnB* were measured for GT and Δ*rre37* cells. Under nitrogen starvation in GT, *ntcA* was upregulated 2.3-fold and *glnB* 4.6-fold after 6 h of nitrogen depletion, whereas *ntcA* and *glnB* were slightly increased in Δ*rre37* (1.4 and 2.2-fold, respectively), (Fig. 3). In Δ*rre37*, the level of *ntcA* and *glnB* transcripts increased gradually, a similar pattern to that of glycogen catabolic and anabolic genes (*glgA*, *glgC*, *glgP*, and *glgX*). SigE showed a similar expression pattern in GT and Δ*rre37* until 24 h of nitrogen depletion. In *Synechocystis*, expression of *ntcB*, a nitrate assimilation transcriptional activator, is enhanced even in the absence of nitrite [26]. In GT, *ntcB* was not upregulated at 6 h, corresponding with previous results [11], but in Δ*rre37*, *ntcB* expression decreased in response to nitrogen depletion, suggesting that *rre37* affects nitrate assimilation.

4. Discussion

This is the first metabolomics-based report showing changes in carbon and nitrogen metabolism of a GT mutant lacking a response regulator, Rre37. Our studies showed that nitrogen depletion led to 2-OG accumulation and expression of genes related to *icd* and the GS–GOGAT pathway, such as *glnA*, *gltB*, and *gltD*, were upregulated in GT, whereas in Δ*rre37*, there was delayed accumulation of 2-OG and relatively low expression of these genes (Fig. 4). *icd* and *glnA* are reported to have NtcA-activated promoters [7,27], and low upregulation of *icd* and *glnA* gene expression in Δ*rre37* is partially due to low upregulation of *ntcA* gene expression. However, NtcA promoter for *gltB* and *gltD* genes, related to the GOGAT system, has not been found [7,27]. Therefore, regulatory mechanism on expression of *gltB* and *gltD* during nitrogen starvation is interesting and should be investigated.

Glycogen accumulation was delayed by low expression of glycogen anabolic genes, such as *glgA* and *glgC* in particular, after nitrogen depletion in Δ*rre37*. Reportedly, expression of *rre37* is correlated with an increase in expression of glycogen catabolic genes (*glgX* and *glgP*) under nitrogen depletion [13]. Our research showed that expression of *rre37* is also correlated with an increase in expression of glycogen anabolic genes, resulting in glycogen accumulation at the initial stage of nitrogen depletion.

Rre37 increases expression of sugar catabolic genes [13]. Decrease in downstream glycolytic metabolites by *rre37* knockout correlates with previous research that increase in metabolites such as glucose-6-phosphate, fructose-6-phosphate, and dihydroxyacetone phosphate, was observed after 4 or 6 h of nitrogen depletion in GT [6,28]. Decreased glycolytic metabolites caused decrease of erythrose 4-phosphate and sedoheptulose-7-phosphate in pentose phosphate pathway, which are connected with glycolysis pathway. Rre37 activates *gap1* in glycolysis under nitrogen starvation [13] and higher expression of *gap1* was observed in GT, compared with Δ*rre37*. Similar expression levels of *zwf* and *gnd* in pentose phosphate pathway between GT and Δ*rre37* correlate with the previous research that SigE activates *zwf* and *gnd* [13].

Moreover, accumulation of TCA cycle metabolites was abolished in Δ*rre37*. TCA cycle metabolites play a role as carbon sink under nitrogen starved condition and the levels of succinate, fumarate, malate, and 2-OG increased [6]. Also under photomixotrophic conditions, *Synechocystis* increased the levels of malate, fumarate, and isocitrate [29]. Low level of 2-OG in Δ*rre37* was due to low expression of *icd* as described above. Expression level of *ppc*, was 1.4-fold in wild type after 12 h of nitrogen starvation [11], correlating with our research (1.9-fold after 12 h of nitrogen starvation) (Supplementary Fig. S1). Similar expression level of *ppc* in Δ*rre37* after nitrogen starvation was one reason of decreases in oxaloacetate, fumarate, and succinate. Decreased sugar catabolism in Δ*rre37* would also affect low accumulation of TCA cycle metabolites.

In the presence of excess 2-OG under nitrogen limited conditions, the PII protein is phosphorylated on binding 2-OG and ATP, a phenomenon that has been well investigated in *Synechococcus* sp. PCC 7942, and 2-OG also activates NtcA [30–32]. Our results showed that *ntcA* and *glnB* transcription correlates 2-OG accumulation, which agrees with the 2-OG dependence of NtcA mediated gene expression. Similar expression pattern of *sigE* in GT and Δ*rre37* was consistent with *sigE* and *rre37* working independently [13].

Metabolomics and quantitative gene expression analysis using the *rre37* mutant showed the role of Rre37 under nitrogen starvation. (1) Rre37 positively regulated glycogen anabolic genes, thereby accumulating glycogen. (2) Rre37 positively regulated sugar catabolism, and was involved in 2-OG accumulation. (3) Rre37 urged accumulation of TCA cycle metabolites such as malate, fumarate, and succinate. In addition to sugar metabolism, previous study showed that expressions of *phaA* and *phaB* genes (encoding β-ketothiolase and acetoacetyl-CoA reductase, respectively), related to polyhydroxybutyrate accumulation, were decreased (about 0.3-fold) in Δ*rre37*, compared with GT after 4 h of nitrogen depletion [13]. Here we showed that Rre37 is also involved in the regulation of nitrogen metabolism (Supplementary Fig. S3), and therefore, future studies could extend the roles of Rre37 into areas other than sugar metabolism in this cyanobacterium.

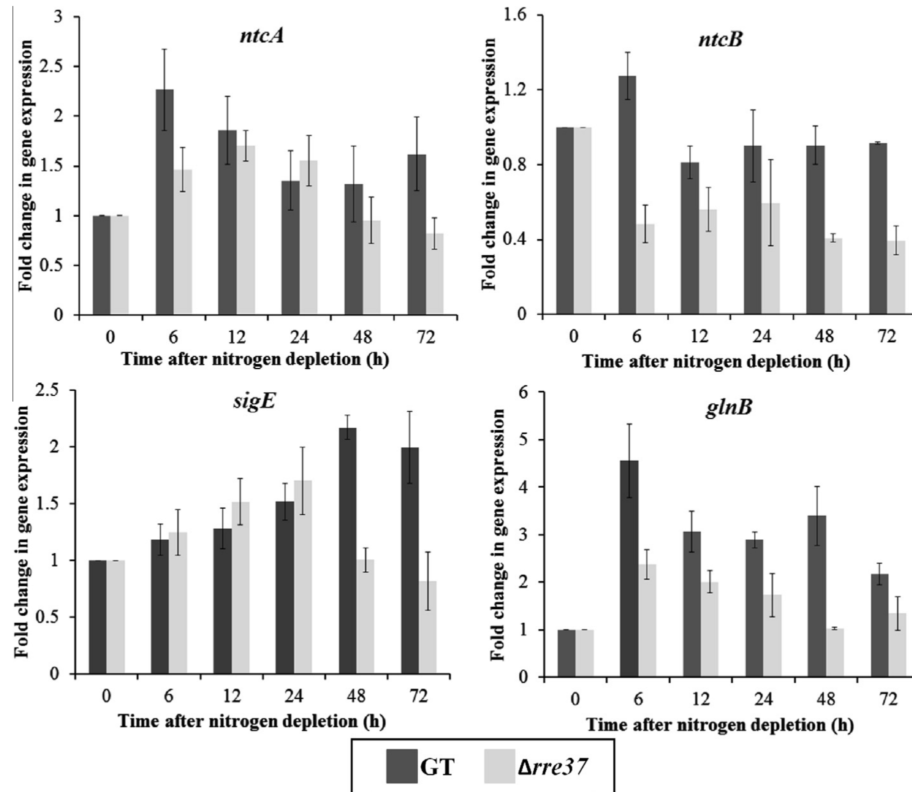

Fig. 3. Expression analysis of genes involved in nitrogen regulation during nitrogen depletion in *Synechocystis* sp. PCC 6803 (GT) and *rre37* mutant (Δ*rre37*). *ntcA* (global nitrogen regulator), *ntcB* (nitrate assimilation transcription factor), *sigE* (RNA polymerase group 2 sigma factor), *glnB* (nitrogen regulatory protein P II).

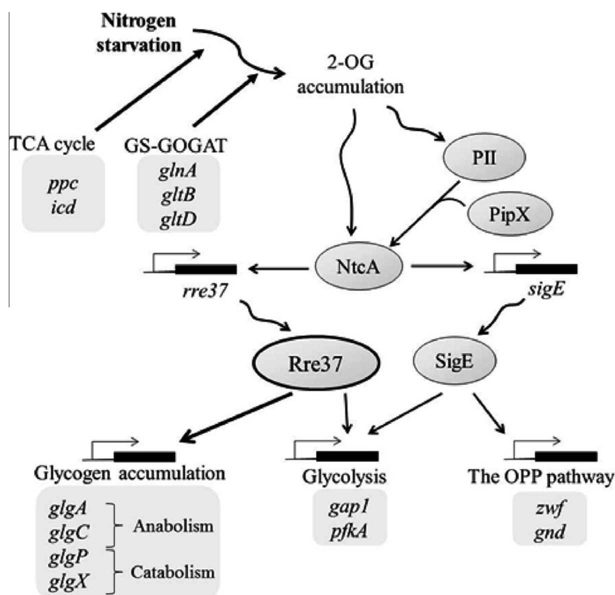

Fig. 4. Schematic model for Rre37 function under nitrogen starvation in *Synechocystis* sp. PCC 6803 [Partially revised from Azuma et al. [13]. In this research, Rre37 positively regulates glycogen anabolic genes (*glgA* and *glgC*). Expression of *icd* gene and activation of GS-GOGAT system urges 2-OG accumulation.]. OPP, oxidative pentose phosphate.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.12.008.

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