Pathway-Level Acceleration of Glycogen Catabolism by a Response Regulator in the Cyanobacterium Synechocystis Species PCC 6803\(^{1[W]}\)

Takashi Osanai, Akira Oikawa, Keiji Numata, Ayuko Kuwahara, Hiroko Iijima, Yoshiharu Doi, Kazuki Saito, and Masami Yokota Hirai*

RIKEN Center for Sustainable Resource Science, Tsurumi-ku, Yokohama, Kanagawa 230–0045, Japan (T.O., A.O., K.N., A.K., H.I., Y.D., K.S., M.Y.H.); PRESTO, Japan Science and Technology Agency, Kawaguchi, Saitama 332–0012, Japan (T.O.); Yamagata University, Faculty of Agriculture, Wakaba-machi, Tsuruoka-shi, Yamagata 997–8555, Japan (A.O.); and Graduate School of Pharmaceutical Sciences, Chiba University, Chuo-ku, Chiba 260–8522, Japan (K.S.)

Response regulators of two-component systems play pivotal roles in the transcriptional regulation of responses to environmental signals in bacteria. Rre37, an OmpR-type response regulator, is induced by nitrogen depletion in the unicellular cyanobacterium Synechocystis species PCC 6803. Microarray and quantitative real-time polymerase chain reaction analyses revealed that genes related to sugar catabolism and nitrogen metabolism were up-regulated by rre37 overexpression. Protein levels of GlgP(slr1367), one of the two glycogen phosphorylases, in the rre37-overexpressing strain were higher than those of the parental wild-type strain under both nitrogen-replete and nitrogen-depleted conditions. Glycogen amounts decreased to less than one-tenth by rre37 overexpression under nitrogen-replete conditions. Metabolome analysis revealed that metabolites of the sugar catabolic pathway and amino acids were altered in the rre37-overexpressing strain after nitrogen depletion. These results demonstrate that Rre37 is a pathway-level regulator that activates the metabolic flow from glycogen to polyhydroxybutyrate and the hybrid tricarboxylic acid and ornithine cycle, unraveling the mechanism of the transcriptional regulation of primary metabolism in this unicellular cyanobacterium.

The study of carbon metabolism of photosynthetic organisms including cyanobacteria is indispensable for biology and biotechnology. Synechocystis sp. PCC 6803 (hereafter Synechocystis 6803) is a non-nitrogen-fixing, unicellular cyanobacterium that performs oxygenic photosynthesis. The Synechocystis 6803 genome was identified in 1996, and the cells synthesize glycogen for carbon storage through the Calvin cycle and gluconeogenesis using CO\(_2\) and light energy (Kaneko et al., 1996). Glycogen synthesis is also activated during nutrient-limited conditions such as nitrogen starvation (Osanai et al., 2006). Glycogen is degraded to supply carbon sources and reductants for producing ATP by respiration under dark conditions (Osanai et al., 2007). Therefore, glycogen metabolism is important for the acclimation of cyanobacteria during environmental changes in light or nutrient conditions.

The transcriptional regulators that control genes related to sugar catabolism in Synechocystis 6803 have been recently revealed (Osanai et al., 2007). The RNA polymerase sigma factor SigE widely regulates sugar catabolism (Osanai et al., 2005, 2011). In particular, SigE regulates the gene expression related to glycogen catabolism, including glycogen phosphorylase (encoded by glgP), isoamylase (encoded by glgX), and the oxidative pentose phosphate (OPP) pathway including glucose-6-phosphate dehydrogenase (G6PD; encoded by zwf), a positive regulator of G6PD (encoded by opcA), 6-phosphogluconate dehydrogenase (6PGD; encoded by gnd), and transaldolase (encoded by tal; Osanai et al., 2005, 2011). SigE also activates polyhydroxyalkanoate (PHA) biosynthetic genes encoding β-ketothiolase (encoded by phaA), acetocetyl-CoA reductase (encoded by phaB), and PHA synthase (encoded by phaC and phaE) and enhances polyhydroxybutyrate (PHB) levels during prolonged nitrogen starvation (Osanai et al., 2013). A gene encoding the response regulator Rre37(sll1330) is induced by the depletion of nitrogen or the addition of Glc (Osanai et al., 2006; Tabei et al., 2012). The disruption of rre37 leads to defective growth under light-activated heterotrophic conditions, while the mutant cells normally grow under photoautotrophic conditions (Tabei et al., 2012). Glycolytic genes including glk (encoding glucokinase), pfkA(sll196) (encoding phosphofructokinase), and fbaA(sll0018) (encoding Fru-bisP aldolase) are down-regulated by rre37 knockout, particularly under...
light and Glc-supplemented conditions (Tabei et al., 2007). Transcriptome analysis revealed that \textit{glgP} (slr1367), \textit{glgX} (slr1857), and \textit{gap1} (encoding glyceraldehyde-3-phosphate dehydrogenase) are repressed in the \textit{rre37}-disrupted mutant, particularly under nitrogen-starved conditions (Azuma et al., 2011). \textit{Rre37} mediates light and nutrient signals to control glycolytic gene expression (Tabei et al., 2007). \textit{Rre37} mediates light and nutrient signals to control glycolytic gene expression (Tabei et al., 2007). \textit{Rre37} mediates light and nutrient signals to control glycolytic gene expression (Tabei et al., 2007).

In this study, we constructed a strain overexpressing \textit{rre37}, and compared with the wild type, we observed increased expression of genes related to sugar catabolism and nitrogen metabolism and decreased glycogen levels under nitrogen-replete conditions. Metabolome analysis demonstrated changes in carbon metabolites related to sugar metabolism and amino acids in response to the nitrogen status. With biochemical and physiological analyses, we demonstrated that \textit{rre37} overexpression accelerated sugar catabolism, PHB production, and the hybrid tricarboxylic acid and Orn cycle in \textit{Synechocystis} 6803.

**RESULTS**

Construction of the \textit{rre37}-Overexpressing Strain and Microarray Analysis

The \textit{rre37} open reading frame (ORF) was integrated into pTKP2031 fused with the \textit{psbAII} promoter (encoding the PSII D1 protein) and integrated into a neutral site of the \textit{Synechocystis} 6803 genome; the resultant strain was named ROX370 (Fig. 1A). Protein levels of \textit{Rre37} in ROX370 were higher than those of the parental Glc-tolerant (GT) strain under both nitrogen-replete and nitrogen-depleted conditions (Fig. 1B).

Microarray analysis was performed to compare the transcript profiles of GT and \textit{rre37}-overexpressing strains grown under nitrogen-replete conditions (Fig. 1C). Among the top 20 genes, mainly the expression of genes related to primary carbon, nitrogen, and nucleotide metabolism was up-regulated by \textit{rre37} overexpression (Supplemental Table S1). Quantitative real-time PCR was first performed to measure the transcript levels of 14 genes up-regulated by \textit{rre37} overexpression (Supplemental Fig. S1). The transcript levels of five genes down-regulated by \textit{rre37} were also measured by quantitative real-time PCR (Supplemental Fig. S2).

Quantitative real-time PCR was then performed with 12 genes related to sugar catabolism (Fig. 2). It was demonstrated that five of the 12 genes, \textit{glgP} (slr1367), \textit{pfkA} (sll1196), \textit{gap1}, \textit{phaA}, and \textit{phaB}, were up-regulated by \textit{rre37} overexpression (Fig. 2). In contrast, four OPP pathway genes, \textit{zwf}, \textit{opcA}, \textit{gnd}, and \textit{tal}, were down-regulated in the \textit{rre37}-overexpressing strain (Fig. 2).

Changes in the Expression of Sugar Catabolic Genes and Primary Carbon Metabolism by \textit{rre37} Overexpression

Following transcriptome analysis, protein levels of the four glycogen catabolic enzymes [\textit{GlgP} (sll1356), \textit{GlgP} (slr1367), \textit{GlgX} (slr0237), and \textit{GlgX} (slr1857)] under both nitrogen-replete and nitrogen-depleted conditions were measured by immunoblotting. \textit{GlgP} (slr1367) and \textit{GlgX} (slr1857) levels increased as a result of \textit{rre37} overexpression under nitrogen-replete conditions (Fig. 3). \textit{GlgP} (slr1367) protein levels in the \textit{rre37}-overexpressing strain were maintained at a higher level than those in the GT strain under nitrogen starvation, whereas \textit{GlgX} (slr1857) protein levels were less than those in the GT strain after 3 d of nitrogen depletion (Fig. 3). \textit{GlgP} (sll1356) and \textit{GlgX} (slr0237) levels were similar between the two strains under nitrogen-replete and nitrogen-
depleted conditions (Fig. 3). Glycogen levels in the rre37-overexpressing strain decreased to 7.3% of the levels in the GT strain under nitrogen-replete conditions (Table I). Glycogen similarly increased during nitrogen starvation in both the strains, although a 10% decrease by rre37 overexpression was observed after 3 d of nitrogen depletion (Table I).

The levels of G6PD and 6PGD proteins, the two key enzymes of the OPP pathway, were similarly measured by immunoblotting (Supplemental Fig. S3). G6PD proteins were reduced by rre37 overexpression under nitrogen-replete and nitrogen-depleted conditions (1 d), while 6PGD proteins increased under nitrogen-replete conditions (Supplemental Fig. S3). The protein levels of SigE decreased in the rre37-overexpressing strain under nitrogen-replete and nitrogen-depleted conditions (1 d; Supplemental Fig. S3). The protein levels of Gln synthetase (GS; encoded by glnA) were similar between the GT and rre37-overexpressing strains (Supplemental Fig. S3).

To quantify the metabolite levels, capillary electrophoresis-mass spectrometry (CE-MS) analysis was performed using cells grown under nitrogen-replete or nitrogen-depleted conditions for 4 h. A total of 104 peaks were annotated as known metabolites (Supplemental Table S2). Statistical analysis demonstrated that 16 and 34 metabolite levels were altered by rre37 overexpression under nitrogen-replete and nitrogen-depleted conditions, respectively (Supplemental Table S2). After nitrogen depletion, the levels of metabolites of sugar metabolism (Glc-6-P/Fru-6-P/Man-6-P, glucosamine-6-phosphate, N-acetylglucosamine-6-phosphate, and GDP-Man), the OPP pathway (sedoheptulose-7-phosphate), glycolysis (dihydroxyacetone phosphate and 2-phosphoglycerate), and the tricarboxylic acid cycle (malate and fumarate) in the rre37-overexpressing strain were lower than those in the GT strain (Table II). Fru-1,6-bisP/Glc-1,6-bisP increased as a result of rre37 overexpression during nitrogen starvation (Table II). CE-MS analysis also revealed that the seven purine and pyrimidine nucleotide levels (AMP, dAMP, CMP, CTP, GMP, dTDP, and dTTP) in the rre37-overexpressing strain were higher than those in the GT strain after 4 h of nitrogen depletion (Supplemental Table S2).

Additional CE-MS analysis was then performed using cells grown under nitrogen-replete or nitrogen-depleted conditions for 1 d (Supplemental Table S3). A total of 70 peaks were annotated as known metabolites (Supplemental Table S3). The levels of several sugar metabolites, such as 6-phosphogluconate, ribulose-5-phosphate/xylulose-5-phosphate, and raffinose, were lower in the rre37-overexpressing strain than in the
GT strain after 1 d of nitrogen depletion (Supplemental Table S3). In contrast, sugar metabolites, including Glc-1,6-bisP, lactate, citrate, and GDP-Man, increased by \textit{rre37} overexpression after 1 d of nitrogen depletion (Supplemental Table S3). CE-MS also demonstrated that levels of two pyrimidine nucleotides (dTTP and UTP) in the \textit{rre37}-overexpressing strain were higher than those in the GT strain, while levels of two purine and pyrimidine nucleotides (AMP and CMP) were lower after 1 d of nitrogen depletion (Supplemental Table S3). Levels of 18 amino acids (Cys and Arg could not be measured by our analysis), Orn, and glutathione were then quantified by gas chromatography-mass spectrometry (GC-MS; Fig. 4; Supplemental Table S4). Asp levels increased more than three times by \textit{rre37} overexpression under nitrogen-replete conditions (Fig. 4). Compared with the GT strain, levels of eight amino acids (Ala, Gly, Val, Leu, Ile, Met, His, and Tyr) were lower in the \textit{rre37}-overexpressing strain after 4 or 24 h of nitrogen depletion (Fig. 4).

Physiological and Biochemical Experiment to Demonstrate the Involvement of Rre37 in Sugar Catabolic Regulation

Sugar catabolism mutants fail to grow under mixotrophic and heterotrophic conditions (Osanai et al., 2005, 2011; Singh and Sherman, 2005). The growth of the \textit{rre37}-overexpressing strain was similar to that of the GT strain under photoautotrophic and mixotrophic conditions (addition of 5 mM Glc; Fig. 5). However, the \textit{rre37}-overexpressing cells were not viable under continuous dark conditions (for 4 d), whereas GT cells survived in the presence of Glc (Fig. 5).

Direct involvement of Rre37 in gene expression was examined in vitro. Glutathione S-transferase (GST)-tagged Rre37 proteins (GST-Rre37) were expressed in \textit{Escherichia coli} and were purified by affinity chromatography (Supplemental Fig. S4A). Purified GST-Rre37 bound with the promoter region, containing the −200 to −1-bp region from the translation initiation site of \textit{glgP} (slr1367), unlike GST proteins (Supplemental Fig. S4B). A weak interaction of GST-Rre37 to the promoter region of \textit{pfkA} (sll1196) and \textit{phaA} was observed; however, no interaction was observed in the case of \textit{gap1}.

Table I. Glycogen levels of \textit{rre37} overexpression

Data represent means ± sd from four independent experiments. Glycogen levels were calibrated relative to that of the GT strain under nitrogen-replete conditions (set at 100%). Statistically significant differences between GT and ROX370 are marked by asterisks (Student’s \textit{t}-test; \(*P < 0.05, **P < 0.005*)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relative Glycogen Amount</th>
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<tbody>
<tr>
<td></td>
<td>+Nitrogen</td>
</tr>
<tr>
<td>GT</td>
<td>100 ± 7.4</td>
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<tr>
<td>ROX370</td>
<td>7.3 ± 5.3**</td>
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</table>

Figure 3. Levels of glycogen catabolic proteins. Levels of GlgP (sll1356 and slr1367) and GlgX (sll237 and slr1857) in the GT and ROX370 strains under nitrogen-limited conditions were assayed by immunoblotting (total protein at 14 μg). Bands show the relative expression of the four proteins for the two strains under nitrogen-replete conditions (+N) and after 1 and 3 d of nitrogen depletion (−N). Data represent means ± sd from three to four independent experiments. Protein levels were calibrated relative to that of the GT strain under nitrogen-replete conditions (set at 100%). Statistically significant differences between GT and ROX370 are marked by asterisks (Student’s \textit{t}-test; \(*P < 0.05\).
Pathway-Level Acceleration of Glycogen Catabolism

Table II. Selected metabolite levels of primary metabolism in GT and ROX370

Data represent means ± sd from five independent experiments. The data of the GT strain were obtained from our previous study (Osanai et al., 2014). Metabolite levels were calibrated relative to that of the GT strain under nitrogen-replete conditions (set at 100%). Statistically significant differences between GT and ROX370 are marked by asterisks (Student's t test; *P < 0.05, **P < 0.005).

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Relative Metabolite Levels</th>
<th>GT +Nitrogen</th>
<th>GT −Nitrogen, 4 h</th>
<th>ROX370 +Nitrogen</th>
<th>ROX370 −Nitrogen, 4 h</th>
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</thead>
<tbody>
<tr>
<td>Glc-1-P</td>
<td>100 ± 29.1</td>
<td>119.1 ± 27.1</td>
<td>87.9 ± 17.8</td>
<td>96.6 ± 20.1</td>
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<tr>
<td>Glc-6-P/Fru-6-P/Man-6-P</td>
<td>100 ± 27.5</td>
<td>148.2 ± 12.6</td>
<td>60.8 ± 10.8*</td>
<td>118.0 ± 16.0*</td>
<td></td>
</tr>
<tr>
<td>Glucosamine-6-phosphate</td>
<td>100 ± 39.7</td>
<td>64.7 ± 25.6</td>
<td>99.4 ± 48.7</td>
<td>8.7 ± 51.2**</td>
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<tr>
<td>Gluconate</td>
<td>100 ± 28.9</td>
<td>172.7 ± 10.1</td>
<td>78.5 ± 14.1</td>
<td>118.3 ± 7.4**</td>
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<tr>
<td>GlcNAc</td>
<td>100 ± 35.0</td>
<td>57.1 ± 23.8</td>
<td>159.0 ± 15.3*</td>
<td>54.3 ± 6.6</td>
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<tr>
<td>GlcNAc-6-P</td>
<td>100 ± 45.9</td>
<td>87.2 ± 19.9</td>
<td>87.0 ± 21.6</td>
<td>59.6 ± 21.4*</td>
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<td>GDP-Man</td>
<td>100 ± 34.7</td>
<td>168.0 ± 12.7</td>
<td>88.6 ± 26.5</td>
<td>98.1 ± 12.1**</td>
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<td>Glycolysis</td>
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<td>Fru-1,6-bisP/Glc-1,6-bisP phosphate</td>
<td>100 ± 32.4</td>
<td>82.2 ± 6.9</td>
<td>111.3 ± 35.7</td>
<td>150.9 ± 18.1**</td>
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<td>Dihydroxyacetone</td>
<td>100 ± 29.7</td>
<td>149.8 ± 14.3</td>
<td>76.5 ± 9.3</td>
<td>118.6 ± 18.3*</td>
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<td>OPP pathway</td>
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<tr>
<td>Sedoheptulose-7-phosphate</td>
<td>100 ± 35.9</td>
<td>121.0 ± 5.8</td>
<td>70.3 ± 12.9</td>
<td>107.1 ± 9.6**</td>
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<td>Tricarboxylic acid cycle</td>
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<td>Citrate</td>
<td>100 ± 32.4</td>
<td>59.1 ± 38.8</td>
<td>91.0 ± 25.9</td>
<td>92.4 ± 29.1</td>
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<td>Cis- and trans-aconitate</td>
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<td>114.8 ± 55.4</td>
<td>53.9 ± 28.7</td>
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<tr>
<td>Isocitrate</td>
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<td>197.0 ± 157</td>
<td>31.2 ± 29.0</td>
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<td>Succinate</td>
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<td>97.0 ± 19.5</td>
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<td>Fumarate</td>
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<td>146.0 ± 89.3</td>
<td>386.7 ± 58.0**</td>
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<td>Malate</td>
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<td>875.6 ± 83.4</td>
<td>172.3 ± 83.1</td>
<td>372.9 ± 32.6**</td>
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</table>

Increased PHB Biosynthesis during Nitrogen Starvation by rre37 Overexpression

Following transcriptome analysis (Figs. 1C and 2), protein levels of PHA biosynthetic enzymes (PhaA, PhaB, PhaC, and PhaE) in the rre37 overexpression strain were measured by immunoblotting. PhaC and PhaE levels were quantified with insoluble fractions because they were integrated into the PHB supercomplex (Osanai et al., 2013). PhaB protein levels increased by rre37 overexpression under nitrogen-replete conditions, whereas PhaA, PhaC, and PhaE levels were enhanced after nitrogen depletion by rre37 overexpression (Supplemental Fig. S6). PHB levels after nitrogen starvation increased by rre37 overexpression (Supplemental Fig. S7A). The M, and monomer units of PHB were not affected by rre37 overexpression (Supplemental Fig. S7, B and C).

A strain overexpressing both rre37 and sigE was generated to investigate the relationship between Rre37 and SigE (Fig. 6A). Immunoblotting confirmed increased levels of SigE and Rre37 compared with the GT strain (Fig. 6B). The transcripts of all four PHA biosynthetic enzymes (phaA, phaB, phaC, and phaE) increased by double overexpression of rre37 and sigE (Fig. 6C). Compared with the rre37-overexpressing strain, PHB levels were further increased by double overexpression of rre37 and sigE (Supplemental Fig. S7A). The M, and monomer units of PHB were not altered by the double overexpression (Supplemental Fig. S7, B and C).
In this study, *rre37* overexpression altered transcript profiles and, particularly, activated glycogen catabolic genes (Fig. 2; Supplemental Table S1). Glycogen levels decreased severely under nitrogen-replete conditions owing to *rre37* overexpression (Table I), and metabolites in the sugar catabolic pathway decreased after 4 h of nitrogen depletion (Fig. 7; Table II; Supplemental Table S2). Compared with the GT strain, metabolites including citrate, lactate, *N*-acetyl-Glu, and PHB increased in the *rre37*-overexpressing strain after prolonged nitrogen depletion (Fig. 7; Supplemental Table S3; Supplemental Fig. S7). Our results revealed that sugar catabolism is accelerated by genetic engineering of the Rre37 response regulator at the pathway level in *Synechocystis* 6803 (Fig. 7). Rre37 is important for regulating the proper distribution of carbon storage during nitrogen starvation in this cyanobacterium.

The results of this study indicate a correlation among transcripts, proteins, metabolites, and phenotypes. Both the transcript and protein levels of *glgP* (sll1356) increased by *rre37* overexpression (Figs. 2 and 3), and the glycogen levels decreased in the *rre37*-overexpressing strain (Table I). Thus, glycogen phosphorylase encoded by *glgP* (sll1356) is a key enzyme in determining glycogen levels in *Synechocystis* 6803. On the other hand, GlgX (slr1857) proteins increased as a result of *rre37* overexpression (Fig. 3), whereas the transcripts decreased (Fig. 2), suggesting that GlgX (slr1857) is regulated at posttranscriptional levels. Glycogen levels decreased by less than one-tenth by *rre37* overexpression (Table I); however, there was a relatively lower induction of glycogen catabolic enzymes (Fig. 3). This pathway-level acceleration of sugar catabolism, including the activation of glycolysis, may be a reason for decreased glycogen in the *rre37*-overexpressing strain. The protein levels of three PHA biosynthetic enzymes (PhaA, PhaC, and PhaE) were enhanced by *rre37* overexpression after nitrogen depletion (Supplemental Fig. S6), which is positively correlated with the increase in PHB levels (Supplemental Fig. S7). Compared with the wild type, Fru-1,6-bisP levels were higher in the *rre37*-overexpressing strain after nitrogen depletion (Table II; Fig. 7). The transcription of *pfkA* (sll1196) is activated by Rre37 (Fig. 2), possibly leading to the accumulation of Fru-1,6-bisPs in the *rre37*-overexpressing strain. Phosphofructokinase is known to catalyze one of the rate-limiting steps in glycolysis (Bosca and Corredor, 1984), and our metabolome analysis also suggests that phosphofructokinase is a key enzyme in glycolysis in this cyanobacterium. The dark-sensitive
phenotype of the rre37-overexpressing strain (Fig. 5) seems to be due to aberrant sugar catabolism, which has been demonstrated by several mutants in Synechocystis 6803 (Osanai et al., 2005, 2011; Singh and Sherman, 2005; Kahlon et al., 2006; Nagarajan et al., 2012).

Redundant glycogen catabolic and glycolytic genes are present in the Synechocystis 6803 genome. The two glycogen phosphorylase genes glgP(sll11356) and glgP (sllr1367) are present in Synechocystis 6803, and Re37 selectively up-regulates glgP(sllr1367) (Fig. 2; Supplemental Table S1). In contrast, SigE preferentially activated glgP (sll11356) expression (Osanai et al., 2011); therefore, the two glgPs are differentially regulated by two transcriptional regulators. Biochemical analysis revealed that GST-Re37 was directly associated with the glgP (sll1356) promoter (Supplemental Fig. S4B), although the binding sequence was not clearly identified. In the nitrogen-fixing cyanobacterium Anabaena sp. PCC 7120, an ortholog of Re37, named NrrA, bound to the promoter regions of nitrogen-fixing and sugar catabolic genes [including CTT (A/G)AT(G/T)T] (Ehira and Ohmori, 2006, 2011). A similar AT-rich tandem repeat of octamers T(C/T)AAC (T/A)(G/A)T occurs within the promoter region of glgP (sll1356) (Supplemental Fig. S4D), and further analysis is required to determine the Re37-binding sequences. rre37 overexpression up-regulated the glycolytic genes pfkA (sll1196) and gap1, whereas the OPP pathway genes were repressed (Fig. 2). SigE particularly activated gene expression in the OPP pathway at the mRNA, protein, and enzymatic activity levels (Osanai et al., 2011); therefore, the balance of Re37 and SigE protein levels may determine which Glc degradation pathway (either glycolysis or the OPP pathway) is activated. Re37 preferentially activates the expression of phaA and phaB, whereas SigE specifically up-regulates the expression of phaC and phaE (Osanai et al., 2013; Fig. 2), indicating that multiple transcriptional regulators are involved in PHB biosynthesis.

Figure 5. Growth on a modified BG-11 plate. Each spot contains 2 µL of culture diluted to A730 = 1, 0.5, or 0.2. The cells were grown with or without 5 mM Glc under continuous light conditions for 3 d (top) or under continuous dark for 4 d and light for 5 d (bottom).

Figure 6. Construction of the strain overexpressing both rre37 and sigE. A, Plasmid for sigE overexpression in Synechocystis 6803. The sigE coding region was ligated with the psbAII promoter. A gentamycin resistance cassette is located upstream of the psbAII promoter and spans the sll0945 ORF. B, SigE and Re37 protein levels in the GT strain and the rre37/sigE double-overexpressing strain. C, Quantitative real-time PCR analysis with the rre37/sigE double-overexpressing strain. The relative transcript levels of genes involved in PHB biosynthesis (phaA, phaB, phaC, and phaE) are shown. Data represent means ± so from five to six independent experiments. Levels were calibrated relative to that of the GT strain (set at 100%). Statistically significant differences are marked by asterisks (Student’s t test; **P < 0.005).
The network of sigma factors in *Synechocystis*, which is studied by many researchers (Foster et al., 2007; Pollari et al., 2008, 2011), may also be important for regulating primary metabolism.

In addition to sugar catabolism, Rre37 plays pivotal roles in nitrogen metabolism. The levels of Asp increased by *rre37* overexpression under nitrogen-replete conditions (Fig. 4), which may be due to the up-regulation of the expression of *aspC* (encoding Asp aminotransferase), indicating the positive correlation between transcripts and metabolites related to Asp biosynthesis (Fig. 7; Supplemental Fig. S1). The data combining transcriptome and metabolome analyses suggest the activation of nitrogen assimilation by the GS-Glu synthase cycle and carbamoyl phosphate synthase, constituting "the hybrid tricarboxylic acid and ornithine cycle" during nitrogen depletion (Fig. 7). This hybrid tricarboxylic acid and Orn cycle is likely to increase ammonium assimilation during early nitrogen starvation because a single round of this cycle assimilates two molecules of ammonium ions and generates an Arg, which could be used for storing nitrogen (Fig. 7). Future metabolomic analysis is required to support the rationale of metabolic flow.

CE-MS analysis revealed that several purine and pyrimidine nucleotides increased as a result of *rrc37* overexpression under nitrogen-replete or early nitrogen-starved conditions (Supplemental Table S2). Transcriptome analysis revealed that the expression of *carA* and *carB*, encoding a subunit of a carbamoyl phosphate synthase, which catalyzed the first reaction of pyrimidine biosynthesis from Gln, increased by *rre37* overexpression (Supplemental Table S1; Supplemental Fig. S1). Carbamoyl phosphate is used to produce orotate via three enzymatic reactions, and orotate and 5-phospho-α-D-ribosyl 1-pyrophosphate produce orotidine-5-phosphate, which is a precursor of UMP. The levels of 5-phospho-α-D-ribosyl 1-pyrophosphate were not increased in the *rre37*-overexpressing strain (Supplemental Tables S2 and
S); therefore, the reason for increased pyrimidine nucleotides may be the increased supply of carbamoyl phosphate as a result of rre37 overexpression.

**CONCLUSION**

We demonstrated that the metabolic flow from glycogen to PHB or the hybrid tricarboxylic acid and Orn cycle was regulated at the pathway level by Rre37. The study of global regulators of primary metabolism is useful for basic science to help better understand the regulation of primary metabolism and possibly for applied science to increase interest in metabolites such as bioplastics and biofuels in the future.

**MATERIALS AND METHODS**

**Bacterial Strains and Culture Conditions**

Among the GT strains of *Synechocystis* sp. PCC 6803 (isolated by Williams [1988]), the GT-I strain was used in this study (Kanesaki et al., 2012). The cells were grown in modified BG-11 medium (Rippka, 1988), which is BG-11, liquid medium containing 5 mM NH₄Cl (buffered with 20 mM HEPES-KOH, pH 7.8). Liquid cultures were bubbled with 1% (v/v) CO₂ in air at 30°C under continuous white light (approximately 50–70 μmol photons m⁻² s⁻¹). Growth and cell densities were measured at 680 nm using the Hitachi U-3310 spectrophotometer.

**Plasmid Construction for rre37 Overexpression**

A region of the *Synechocystis* 6803 genome encoding the rre37 (sll1330) ORF was amplified by PCR using KOD polymerase (Toyobo) and the specific primers 5'-ATTATTCATAGATCCGATCTCACA-3' and 5'-AAAGGGGTAAAACC-TAGTTAAGTACAGAAC-3'. The amplified PCR fragments were digested with NdeI and HpaI (Takara Bio) and inserted into the NdeI-HpaI sites of the pTKP2031V vector (Takara Bio) using the DNA Ligation Kit (Takara Bio) and inserted into the NdeI-HpaI sites of the pTKP2031V vector (Takara Bio) using the DNA Ligation Kit (Takara Bio). The resultant plasmid was confirmed by sequencing. The plasmids were integrated into the GT-I strain by natural transformation as described previously (Osanai et al., 2011). The rre37-overexpressing strain was named ROX370.

**RNA Isolation**

Cells were suspended in 400 μL of TES buffer (5 mM Tris-HCl, pH 8, 0.5 mM EDTA, and 0.5% (v/v) SDS) followed by the addition of 400 μL of acid-phenol. After incubation for 10 min at 65°C, the mixtures were centrifuged at 20,500 g for 5 min and the supernatants were transferred to a 1.5-ml tube. A 400-μL aliquot of phenol:chloroform:isoamyl alcohol (25:24:1) was then added, and the tubes were centrifuged for an additional 3 min. RNAs were concentrated by isopropanol precipitation, followed by adding 100 μL of TE buffer (10 mM Tris-HCl, pH 8, and 1 mM EDTA). Following this, 1 mL of isogen (Wako) was added, and the tubes were incubated for 5 min at room temperature, followed by centrifugation for 15 min. The supernatants were transferred to a 1.5-ml tube, topped up with 400 μL of phenol:chloroform:isoamyl alcohol (25:24:1), and centrifuged at 20,500 g for 3 min. Eight extractions with phenol:chloroform:isoamyl alcohol were performed in total. RNAs were resuspended in 20 μL of sterilized water after isopropanol precipitation.

**Microarray Analysis**

Residual DNA was excluded by reaction with TURBO DNase for 5 h at 37°C. Complementary DNA was synthesized from 2 μg of total RNA using the SuperScript III First-Strand Synthesis System (Life Technologies Japan). Quantitative real-time PCR was performed using StepOne Plus (Life Technologies Japan), according to the manufacturer’s instruction, using the primers listed in Supplemental Table S5. The expression level of rnpF (encoding RNaseP subunit B) was used as an internal standard as described previously (Schlebusch and Forchhammer, 2010).

**Production of Antisera and Immunoblotting**

We previously produced antisera against Rre37, GlgX(sll0237), GlgX (sll1857), GlgP(sll1356), GlgP(sll1367), PbaA, PbaB, PbaC, PbaE, and SigE (Azuma et al., 2013; Osanai et al., 2011, 2013). Cells were dissolved in 0.5 mL of PBS-T (3.2 mM Na₂HPO₄, 0.5 mM KH₂PO₄, 1.3 mM KCl, 135 mM NaCl, and 0.05% (w/v) Tween 20, pH 7.4) supplemented with the protease inhibitor Complete Mini (Roche Diagnostics; one tablet per 10 mL in PBS-T). Cells were disrupted by sonication using the Bioruptor UCD-250 (CosmoBio) instrument. Following centrifugation at 20,500 g for 5 min, 300 μL of the supernatant was mixed with 100 μL of the SDS-PAGE sample buffer (250 mM Tris-HCl, pH 6.8, 20% (v/v) 2-mercaptoethanol, 8% (w/v) SDS, 20% (w/v) Suc, and 1% (w/v) bromophenol blue) and boiled for 4 min at 98°C. The centrifuged pellet was resuspended in 300 μL of PBS-T with 0.5% (w/v) Tween 20 (Wako) for insoluble fraction immunoblotting. In total, 200 μL of the suspension was mixed with 200 μL of water and 400 μL of SDS-PAGE sample buffer, followed by 4 min of boiling at 98°C. Protein concentrations were measured using the BCA Protein Assay Reagent (Thermo Scientific) with bovine serum albumin as a standard. Immunoblotting was performed as described previously (Osanai et al., 2014). Proteins were visualized using one-step nitroblue tetrazolium-/5-bromo-4-chloro-3-indolyl phosphate (Thermo Scientific) by alkaline phosphatase reaction. Bands were quantified with NIH Image software.

**Glycogen Measurement**

Glycogen levels were measured by the Biotechnology Center of Akita Prefectural University. Cells grown under nitrogen-replete or nitrogen-depleted conditions were concentrated in 1 mL of methanol with A₅₇₀ = 6, followed by mixing for 10 min using a vortex mixer. After centrifugation, the supernatant was transferred to a 1.5-ml tube and dried at 65°C. The dried glycogen was enzymatically degraded with glucoamylase, and the resultant Glcs were quantified by measuring changes in A₅₇₀ during reactions with hexokinase and G6PD.

**CE-MS Analysis**

Cells were collected by centrifugation at 9,800 g for 2 min, followed by freezing in liquid nitrogen. Cells (50–100 mg fresh weight) were suspended in 600 μL of a mixture containing 60% (v/v) methanol and 200 μM each 10-camphorsulfonic acid and trimesic acid as internal standards, and it was mixed using an MT-200 microtube mixer (Tomy) for 20 min at room temperature, followed by centrifugation at 20,500 g for 5 min at 4°C. A 300-μL aliquot of the supernatant was centrifuged through a Millipore 5-kD cutoff filter at 10,000 g for 30 min. A 250-μL aliquot of the filtrate was dried by centrifugal concentrator (drying time, 120 min). The residue was dissolved in 20 μL of water and subjected to CE-MS analysis. The CE-MS system and conditions were as described previously (Okawa et al., 2011).

**Amino Acid Analysis with GC-MS**

Equal amounts of cells (50 mL of cell culture with A₅₇₀ = 1) were harvested by rapid filtration using a previously described method (Osanai et al., 2014). In total, 300 μL of the upper phase was transferred to a new tube and vacuum dried. Samples were suspended in 500 μL of methanol with 1 μM nor-Val as an internal standard and derivatized using ImaGene software version 4.0 (BioDiscovery). Data were deposited to the Gene Expression Omnibus public database (accession no. GSE47091).

**Quantitative Real-Time PCR**

Residual DNA was excluded by reaction with TURBO DNase for 5 h at 37°C. Complementary DNA was synthesized from 2 μg of total RNA using the SuperScript III First-Strand Synthesis System (Life Technologies Japan). Quantitative real-time PCR was performed using StepOne Plus (Life Technologies Japan), according to the manufacturer’s instruction, using the primers listed in Supplemental Table S5. The expression level of rnpF (encoding RNaseP subunit B) was used as an internal standard as described previously (Schlebusch and Forchhammer, 2010).
20°C min⁻¹. The interface and ion source temperatures were 280°C and 240°C, respectively.

Purification of GST-Tagged Rre37 and EMSA

The region of the Synechocystis 6803 genome encoding rre37 was amplified by PCR using KOD polymerase and the specific primers 5'-GAAGGT-CCGTTGGATCATGAACTGGCTACATA-3' and 5'-GATGGCGGCCGTCGAGCTCGAAGTCGAGAC-3'. The amplified DNA fragments were digested with BamHI and Xhol and inserted into the BamHI-Xhol sites of pCEXX-1 (GE Healthcare Japan) using the In-Fusion HD Cloning Kit (Takara Bio). Purification was performed as described previously (Osanai et al., 2009). Double-stranded DNAs were synthesized by PCR (for 200-bp fragments) or annealing of synthesized primers (for 30- or 40-bp fragments) with the primers listed in Supplemental Table S5. PCR was performed with KOD plus neo polymerase (Toyobo), and purification from agarose gels was performed using the Wizard SV Gel and PCR Clean-up System (Promega). The primers (final 10 pmol μL⁻¹) for each primer were suspended in TE buffer (10 mM Tris-HCl and 1 mM EDTA) with 100 mM NaCl for annealing and incubated for 10 min at 96°C, followed by switching off the incubator to gradually bring the temperature down to room temperature.

The double-stranded DNAs and GST-Rre37 proteins were mixed in 15 μL of EMSA buffer (20 mM Tris-HCl, pH 8, 50 mM NaCl, 10 μg MgCl₂, 1 μM dithiothreitol, and 4% (w/v) glycerol) and incubated for 20 min at room temperature. After adding 1.5 μL of loading buffer (40% (w/v) Suc, 0.02% (w/v) xylene cyanol, and 0.02% (w/v) bromphenol blue), the mixtures were resolved by acrylamide gel electrophoresis in 0.5× TBE buffer (44.5 mM Tris, 44.5 mM borate, and 1 mM EDTA) using precast gels (SuperSepAce 5% L; Wako). DNA bands were visualized by ethidium bromide and UV illumination.

Extraction, Purification, and Analysis of PHB

Precultured cells were diluted to A₅₇₀ = 0.2 in 70 mL of BG-11 medium containing 3 mM NH₄Cl. Cells were cultured for 9 d and collected by centrifugation at 18,000g for 2 min. Cells were freeze dried with FDU-2200 (EYELA) at −80°C for 3 d. Extraction, quantification, and determination of the Mₛ and monomer units of PHB have been described (Osanai et al., 2013).

Construction of a Strain Overexpressing Both rre37 and sigE

First, the kanamycin resistance cassette of pTKP2031V was deleted by digestion with Xhol and AflII (Takara Bio). The gentamycin resistance cassette from pVZ222 (Zinchenko et al., 1999) was amplified by PCR using KOD polymerase and the specific primers 5'-AATAATTCTCGAGTGAACGACAGATTATA-3' and 5'-AACCCCGAGCCTCTTAGGTGCGCCGACTT-3', digested with Xhol and AflII, and inserted into the Xhol-AflII sites of pTKP2031V. The resultant plasmid was named pTKP2031. A region of the Synechocystis 6803 genome encoding one of two glycogen synthases, glgA (AndIVES), including +1 to +1,180 bp from the translation initiation codon was amplified by PCR using KOD polymerase and the specific primers 5'-TTGCGCTATCGAGAATTGTTATGTCG-3' and 5'-TTAAGAATCTTATGAGAATGAGCGAATGAA-3'. The amplified PCR fragments were digested with SphI and EcoRI (Takara Bio) and inserted into the SphI-EcoRI sites of the pUC19 vector (Takara Bio). The resultant plasmid was digested with AplI, and the region including the gentamycin resistance cassette, pBluescript KI promoter, and NdeI-Hpal cloning sites of pTKP2031 was amplified using KOD polymerase and the specific primers 5'-TTGCTTCTATCGCTCGAG-3' and 5'-ATCCAATTTGAGCACTGAA-3' and integrated into the AplI site of the plasmid. The resultant plasmid was named pTKP9495. The sigE coding region was obtained by PCR as described previously (Osanai et al., 2011), except for the reverse primer (5'-AAGGGGTTAATCCATAACACATTTGGA-3'), which was newly synthesized and contained the Hpal site. The amplified sigE coding region digested with NdeI and Hpal was integrated into the NdeI-Hpal sites of pTKP9495 (Fig. 6A). Transformation was performed as well as with pTKP2031V-rre37, except that 3 μg μL⁻¹ gentamycin was used instead of 50 μg μL⁻¹ kanamycin.

Statistical Analyses

Statistical analyses were performed using StatPlus:macLE software for MacOSX (Analyst Soft) or Microsoft Excel version 14.3.2 (Microsoft). P values were determined using paired two-tailed Student's t tests. A 95% confidence interval was used to determine significance.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Results of quantitative real-time PCR 1.

Supplemental Figure S2. Results of quantitative real-time PCR 2.

Supplemental Figure S3. Levels of G6PD, 6PGD, SigE, and Gs.

Supplemental Figure S4. EMSA analysis 1.

Supplemental Figure S5. EMSA analysis 2.

Supplemental Figure S6. Levels of PHA biosynthetic proteins.

Supplemental Figure S7. Levels of PHB.

Supplemental Table S1. Results of microarray analysis.

Supplemental Table S2. Results of CE-MS analysis (4 h after nitrogen depletion).

Supplemental Table S3. Results of CE-MS analysis (1 d after nitrogen depletion).

Supplemental Table S4. Levels of amino acids.

Supplemental Table S5. Primers used for quantitative real-time PCR.

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LITERATURE CITED


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