A Response Regulator Rre37 and an RNA Polymerase Sigma Factor SigE Represent Two Parallel Pathways to Activate Sugar Catabolism in a Cyanobacterium Synechocystis sp. PCC 6803

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Sugar catabolic genes are induced during nitrogen starvation in a cyanobacterium Synechocystis sp. PCC 6803, but the underlying regulatory mechanism still remains to be completely characterized. In this study, we showed by molecular genetics and transcriptome analyses that a response regulator Rre37 (encoded by sll1330), whose expression is enhanced by nitrogen depletion under the control of NtcA, activates transcript accumulation of sugar catabolic genes, such as gap1, pfkA (sll1196), glgP (slr1367) and glgX (slr1857), mainly during nitrogen starvation. Previously, we reported that a group-2 sigma factor SigE also positively regulates sugar catabolic genes in this strain. Phenotypic analyses using a single or double mutant lacking rre37 and/or sigE indicated that both SigE and Rre37 positively regulate sugar catabolic genes independently. These findings substantiated a regulatory network of sugar catabolic genes in this cyanobacterium.

**Keywords:** Cyanobacteria • Nitrogen starvation • NtcA • Response regulator • Sigma factor • Sugar catabolism.

**Abbreviations:** Cm, chloramphenicol; CRP, cAMP receptor protein; GT, glucose-tolerant Synechocystis strain; IPTG, isopropyl-β-D-thiogalactopyranoside; Km, kanamycin; LAHG, light-activated heterotrophic growth; OD, optical density; 2-OG, 2-oxoglutarate; OPP, oxidative pentose phosphate.

**Introduction**

Cyanobacteria are included in eubacteria characterized by oxygenic photosynthesis. Among them, a unicellular, non-diazotrophic cyanobacterium Synechocystis sp. PCC 6803 (hereafter *Synechocystis*) has been widely studied as a model system with the genomic information (Kaneko et al. 1996), as a glucose-tolerant strain of *Synechocystis* (GT) was isolated for study of photosynthetic genes (Williams 1988). This strain can grow under dark conditions in the presence of glucose and a daily pulse of white or blue light (at least 5 min), which is called light-activated heterotrophic growth (LAHG) (Anderson and McIntosh 1991).

Sugar catabolism, roughly divided into glycolysis, the oxidative pentose phosphate (OPP) pathway and glycogen catabolism, is indispensable for growth under heterotrophic conditions in cyanobacteria including Synechocystis (Osanai et al. 2007). It was previously shown that a *Synechocystis* mutant lacking gap1, encoding glyceraldehyde-3-phosphate dehydrogenase catalyzing the glucose catabolic reaction, could not grow under heterotrophic conditions (Koksharova et al. 1998). Consistently, proteomic analysis showed higher levels of Gap1 and Tal (transaldolase; one of the enzymes of the OPP pathway) under LAHG conditions, compared with those under photautotrophic conditions (Kurian et al. 2006). Metabolic study also indicated that metabolic flow of sugar metabolism was inclined not towards anabolism but catabolism under LAHG conditions (Yang et al. 2002).

With respect to the expression of sugar catabolic genes, regulatory proteins for sugar catabolic gene expression have been identified in *Synechocystis*. Disruption of a histidine kinase Hik8 resulted in decreased mRNA levels of pfkA (sll1196) (encoding phosphofructokinase), gap1, zwf (encoding glucose-6-phosphate dehydrogenase; G6PD), gnd (encoding 6-phosphogluconate dehydrogenase; 6PGD) and glgP (encoding glycogen phosphorylase) (Singh and Sherman 2005). The second regulatory protein found for sugar catabolism is an RNA polymerase sigma factor SigE (Osanai et al. 2005a). A mutant lacking SigE exhibited decreased transcript levels of pfkA (sll1196), gap1, pyk1 (sll0587, encoding pyruvate kinase), zwf, opcA (encoding a positive regulator of G6PD), gnd, tal, glgP (sll1356) and glgX (slr0237, encoding glycogen...
isoamylase) under photoautotrophic conditions, as compared with the parental wild-type strain (Osanai et al. 2005a). The third regulatory protein was recently identified, i.e. an OmpR-type response regulator Rre37 (encoded by slr1330) (Tabei et al. 2007). Rre37 activates the expression of glycolytic genes such as glk (encoding glucokinase), pfkA (slr1196), fbaA (slr0018, encoding fructose-bisphosphate aldolase), gpmB (slr1124, encoding phosphoglycerate mutase) and pyk1 in the presence of light and glucose (Tabei et al. 2007). Consistent with these transcript analyses, mutants lacking hik8, sigE or rre37 could not grow under dark or LAHG conditions (Singh and Sherman 2005, Osanai et al. 2005a, Tabei et al. 2007).

Recently, transcriptome analyses have made it clear that sugar catabolic genes were induced during nitrogen starvation (Osanai et al. 2005b, Osanai et al. 2006). Microarray analysis with GT revealed that the transcripts of two genes for glycolysis (gap1 and pyk1), four genes for the OPP pathway (zwf, opcA, gnd and tal) and two genes for glycogen catabolism [ggkX (slr0237) and gglP (slr1367)] were increased >2-fold compared with those under nitrogen-depleted conditions (Osanai et al. 2006). Subsequent analyses indicated the involvement of NtcA in the expression of sugar catabolic genes; NtcA is a transcription factor belonging to the CRP (cAMP receptor protein) family, and binds to specific DNA sequences GTAN₆TAC located in upstream region of genes for nitrogen assimilation to activate transcriptional initiation (Luque et al. 1994, Tanigawa et al. 2002). 2-Oxoglutarate (2-OG), which is a signaling metabolite reflecting the cellular nitrogen status, stimulated NtcA–DNA binding as well as the subsequent transcriptional activation (Muro-Pastor et al. 2001, Tanigawa et al. 2002). Although NtcA deficiency abolished the induction of sugar catabolic genes, bioinformatic analysis revealed that no apparent NtcA-binding sites were found within relevant promoter regions (Su et al. 2005). Thus, NtcA appears to activate sugar catabolic genes indirectly. Among the known positive regulators for sugar catabolic genes, the hik8 mutation did not affect the induction during the nitrogen depletion (Osanai et al. 2006). SigE is known to be under the control of NtcA, and therefore is expected to be responsible for the induction. In fact, the sigE mutation alleviated or abolished the induction of sugar catabolic genes; however, induction of some genes still remained (Osanai et al. 2006). In this study, we characterized the above-mentioned third regulator, Rre37, and investigated its contribution to sugar catabolic gene induction under nitrogen depletion.

**Results**

**NtcA-dependent induction of rre37 during nitrogen depletion**

Previous bioinformatic analysis predicted that the NtcA-binding site is present within the rre37 promoter region and thus rre37 is under the control of NtcA (Su et al. 2005). To examine this, we performed Northern analysis and found that the transcript level of rre37 was increased by nitrogen depletion in GT, whereas the induction was reduced in the ntcA mutant (Fig. 1A). Subsequently, immunoblot analysis revealed that protein levels of Rre37 also increased by nitrogen depletion in GT, but not in the ntcA mutant (Fig. 1B). The protein levels of Rre37 in GT remained high at least 8 h after nitrogen depletion (data not shown). It was also shown that complementation of the ntcA mutant by a wild-type ntcA copy recovered the mRNA and protein levels of Rre37 to the same levels as in GT (Fig. 1B). These results showed that Rre37 is induced during nitrogen depletion dependent on NtcA.

**Specific binding of NtcA to the rre37 upstream region**

Since the upstream region of rre37 was proposed to contain a putative NtcA-binding site (from −61 to −74 with respect to the translational start site), the binding of NtcA to the rre37 upstream region was examined by gel mobility shift assay. As shown in Fig. 2A, purified His-NtcA protein retarded the electrophoretic mobility of the upstream fragment of rre37. In addition, the affinity of NtcA–DNA binding increased in the presence of 2-OG (Fig. 2B). Addition of a 10- to 50-fold excess of double-stranded oligonucleotides containing the NtcA-binding sequence reduced the amount of retarded fragment, while a non-specific competitor did not affect the probe–NtcA interaction (Fig. 2C). These results clearly
demonstrated that NtcA specifically binds to the rre37 upstream region.

**Rre37 is involved in induction of glycolytic and glycogen catabolic genes during nitrogen depletion**

In a previous report (Tabei et al. 2007), it was shown that mRNA levels of glk, pfkA (sll1196), fbaA, gpmB and pyk1 decreased under heterotrophic growth conditions in an rre37 null mutant. In this study, we performed a genome-wide analysis to identify the Rre37 regulon. First, the coding region of rre37 was disrupted by insertion of a kanamycin (Km) resistance gene cassette (Supplementary Fig. S1A), and the complete disruption of rre37 was confirmed by PCR (Supplementary Fig. S1B). Then using microarray, the expression profiles of GT and the rre37 mutant under normal growth condition were compared. According to internal control experiment performed previously (Kanesaki et al. 2002), we defined the genes whose expression levels were $>2$ or $<0.5$ as 'induced' or 'repressed', respectively. As a result, the gene expression profile of the rre37 mutant was basically the same as that of GT under normal photoautotrophic growth conditions (data not shown). Subsequently, microarray analysis was performed using cells grown for 4 h after nitrogen depletion. The results of the microarray showed that the expression intensities of 31 genes or 14 genes were decreased or increased by the rre37 disruption, respectively (Table 1 and Supplementary Table S1). Those down-regulated genes included five genes for sugar catabolism, glgP (srl1367), gap1, pfkA (sll1196), glgX (srl1857) and malQ (encoding 4-α-glucanotransferase) (Table 1). Northern analysis further confirmed the dependence of glgP (srl1367), gap1, pfkA (sll1196) and glgX (srl1857) transcript accumulation on rre37 under nitrogen depletion (Fig. 3A). In addition, we found that pfkA (sll1196) expression showed some rre37 dependence even under nitrogen-replete condition.

In order to understand the genetic relationship between Rre37 and SigE, a sigE/rre37 double mutant was constructed. Northern analysis revealed that the expression levels of gap1 and pfkA (sll1196) in the double mutant were lower than those of each single mutant under both nitrogen-replete and nitrogen-depleted conditions (Fig. 3A). In contrast, the induction patterns of glgX (srl1857) and glgP (srl1367) in the double mutant were almost similar to those of the rre37 single mutant (Fig. 3A). Transcript levels of genes for the OPP pathway (gnd and zwf) were not affected by rre37 disruption (Fig. 3B).

In addition to transcript levels, protein levels of Rre37, SigE and Gap1 were examined for the mutants. It was confirmed that Rre37 protein was undetectable in the rre37 mutant as well as in the sigE/rre37 double mutant under both nitrogen-replete...
and nitrogen-depleted conditions (Fig. 4). The protein level of Gap1 decreased under nitrogen-replete conditions by either sigE or rre37 knockout (Fig. 4). The increase in Gap1 by nitrogen depletion remained in the sigE mutant but was diminished in the rre37 mutant. It was also found that the protein level of Gap1 was less in the double mutant than that in either the rre37 or sigE single mutant under both nitrogen-replete and nitrogen-depleted conditions (Fig. 4).

**Growth of mutants under LAHG condition**

To find phenotypes caused by the rre37 mutation with respect to sugar catabolism, growth under LAHG condition was compared between GT and the rre37 mutant. Although GT and mutant strains grew similarly under photoautotrophic conditions (Fig. 5A), disruption of rre37 or sigE retarded growth under LAHG conditions (Fig. 5B). Furthermore, the sigE/rre37 double mutant exhibited less growth under LAHG conditions than each single mutant (Fig. 5B).

**Discussion**

This study demonstrated that the nitrogen-regulated response regulator Rre37 is controlled by NtcA and activates sugar catabolic genes in a manner different from other transcriptional regulators.

In this study, genetic and biochemical analysis showed that Rre37 is regulated by NtcA. Binding of NtcA to the rre37

<table>
<thead>
<tr>
<th>ORF No.</th>
<th>Gene</th>
<th>Gene function</th>
<th>Ratio (rre37 mutant/GT after N depletion)</th>
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<tr>
<td>srl2501</td>
<td>ssl2501</td>
<td>Hypothetical protein</td>
<td>0.11 ± 0.02</td>
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<td>srl1367</td>
<td>glgP</td>
<td>Glycogen phosphorylase</td>
<td>0.20 ± 0.02</td>
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<tr>
<td>srl1855</td>
<td>Hypothetical protein</td>
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</tr>
<tr>
<td>slr2008</td>
<td>Processing protease</td>
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<td></td>
</tr>
<tr>
<td>sll0185</td>
<td>Hypothetical protein</td>
<td>0.22 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>srl0084</td>
<td>gap1</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
<td>0.22 ± 0.03</td>
</tr>
<tr>
<td>slr1498</td>
<td>carA</td>
<td>Carbamoyl-phosphate synthase small chain</td>
<td>0.26 ± 0.02</td>
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<td>Low affinity sulfate transporter</td>
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<tr>
<td>slr1993</td>
<td>phaA</td>
<td>Acetyl coenzyme A acetyltransferase (thiolase)</td>
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<tr>
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<td>phaB</td>
<td>3-Oxocarboxy[-acyl-carrier protein] reductase 2</td>
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<tr>
<td>sll1196</td>
<td>pfkA</td>
<td>Phosphofructokinase</td>
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<td>slr1854</td>
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<tr>
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<td>argJ</td>
<td>Glutamate N-acetyltransferase</td>
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<td>ctaDI</td>
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<tr>
<td>sll1676</td>
<td>malQ</td>
<td>4-α-Glucanotransferase</td>
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<tr>
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<td>Cytochrome c oxidase subunit III</td>
<td>0.45 ± 0.10</td>
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<td>0.46 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>slr1856</td>
<td>Phosphoprotein substrate of icfG gene cluster</td>
<td>0.47 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>sll1783</td>
<td>Hypothetical protein</td>
<td>0.47 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>sll1251</td>
<td>Hypothetical protein</td>
<td>0.48 ± 0.09</td>
<td></td>
</tr>
</tbody>
</table>

This table showed genes that are down-regulated by rre37 disruption. The data represent the average and standard error of the expression ratio obtained by three independent experiments.

This study demonstrated that the nitrogen-regulated response regulator Rre37 is controlled by NtcA and activates sugar catabolic genes in a manner different from other transcriptional regulators.

In this study, genetic and biochemical analysis showed that Rre37 is regulated by NtcA. Binding of NtcA to the rre37
promoter region in vitro was sequence specific, and 2-OG enhanced the NtcA–DNA binding affinity (Fig. 2A, B). In Synechocystis, the intracellular concentration of 2-OG increased in response to nitrogen depletion; the range of 2-OG concentration in vivo was estimated to be from 0.06 mM (ammonium-grown cells) to 0.44 mM (15 min after nitrogen depletion) (Muro-Pastor et al. 2001, Tanigawa et al. 2002). This range of 2-OG concentration coincides well with the experimental data (Fig. 2B), indicating that an increase of 2-OG is responsible for the \( \text{rre}37 \) transcriptional activation in the case of nitrogen depletion.

The transcript levels of glycolytic genes \[ \text{gap}1 \] and \[ \text{pfkA} \] (sll1196) and the protein levels of Gap1 in the \( \text{sigE} / \text{rre}37 \) double mutant were lower than those of the \( \text{rre}37 \) or \( \text{sigE} \) single mutants (Figs. 3A, 4). The growth under LAHG conditions was severely repressed in the double mutant, as compared with the \( \text{rre}37 \) or \( \text{sigE} \) single mutant (Fig. 5B). These results indicate that each of Rre37 and SigE could be involved in the transcriptional activation of these genes independently (Fig. 6). In addition, since Rre37 is an OmpR-type response regulator that has an N-terminal receiver domain, cognate histidine kinase may phosphorylate the aspartate residue for the activation. A recent study revealed that both SigE and NtcA are regulated by protein–protein interaction with ChlH and PipX, respectively (Espinosa et al. 2006, Osanai et al. 2009), and thus further biochemical analysis searching for an Rre37-binding protein(s) is significant for uncovering transcriptional mechanisms for sugar catabolic genes. Rre37 has a C-terminal DNA-binding domain, and the transcriptional activation under the control of Rre37 was considered to be through the specific binding to the uncharacterized motif sequence. These subjects have not been clarified yet, and the elucidation would lead to further understanding of sugar catabolic gene expression in this cyanobacterium.

Induction of sugar catabolic genes during nitrogen depletion is widely observed among cyanobacteria. In the nitrogen-fixing cyanobacterium \( \text{Nostoc punctiforme} \) strain ATCC 29133 and \( \text{Anabaena} \) sp. PCC 7120, the transcripts of genes such as \( \text{fbp} \) (encoding fructose bisphosphatase), \( \text{tal}, \text{zwf}, \text{opcA} \) and \( \text{glgP} \) increased under nitrogen-fixing conditions (Summers and Meeks 1996, Ehira and Ohmori 2006). In addition, the \( \text{zwf} \) mutant ceased to grow under nitrogen-fixing conditions or heterotrophic growth conditions (Summers et al. 1995). In the same report, the authors mentioned that the OPP pathway and oxidative phosphorylation were important for producing NADPH and ATP in dark heterotrophic conditions (Summers et al. 1995). \( \text{rre}37 \) orthologs were found from at least 10 other

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**Fig. 3** Northern analysis of sugar catabolic genes. GT and the \( \text{rre}37 \), \( \text{sigE} \) and \( \text{sigE} / \text{rre}37 \) mutant cells were grown under nitrogen-replete condition and were transferred to nitrogen-depleted medium. RNAs were isolated either 0 or 4 h after nitrogen depletion, and subjected to Northern analyses using probes for glycolytic and glycogen catabolic genes (A), and OPP pathway genes (B). Arrowheads indicate the positions of molecular size standards (in kb), and 10 \( \mu \)g of total RNA was put on each lane. The lower panels show rRNA stained with methylene blue as a loading control. The experiments were performed three times to confirm the reproducibility.

**Fig. 4** Immunoblotting with antisera to \( \text{Rre}37 \), \( \text{SigE} \) and \( \text{Gap}1 \). GT and the \( \text{rre}37 \), \( \text{sigE} \) and \( \text{sigE} / \text{rre}37 \) mutant cells was harvested at 0 and 4 h after nitrogen deprivation, and total protein (10 \( \mu \)g) was subjected to immunoblot analysis.
for 15 min each day. Plates were photographed (A) 4 d or (B) 9 d after inoculation. (Muro-Pastor et al. 2006). In particular, NrrA, the rre37, and their putative promoter regions as in Synechocystis cyanobacteria, and they have an NtcA-binding sequence in their putative promoter regions as in Synechocystis (Muro-Pastor et al. 2006). In particular, NrrA, the rre37 ortholog in Anabaena sp. PCC 7120, is involved in induction of genes, including glgP and gnd, during nitrogen-fixing conditions and is essential for normal heterocyst development (Ehira and Ohmori 2006). Thus, transcriptional regulation of sugar catabolic genes by Rre37 homologs could be conserved among cyanobacteria. The group-2 sigma factor SigE is also widespread among cyanobacteria (Osanai et al. 2008), and therefore it would be reasonable to consider that these two transcriptional regulators are playing major roles in the nitrogen regulation of sugar catabolic genes among divergent cyanobacteria.

**Materials and Methods**

**Bacterial strains and culture conditions**

Cyanobacterial strains used in this study are listed in **Supplementary Table S2**. The glucose-tolerant strain of *Synechocystis* (GT) isolated by Williams (1988) and the cognate sigE, rre37 and ntcA mutants were grown in BG-11 liquid medium (BG-11 without a nitrogen source) with 5 mM NH₄Cl (buffered with 20 mM HEPES-KOH, pH 8.0), which was named modified BG-11 medium. Liquid cultures were incubated under normal growth conditions bubbled with 2% (v/v) CO₂ in air, at 30°C under continuous white light (~70 μmol photons m⁻² s⁻¹) (Rippka 1988). For plate cultures, BG-11 medium (17.5 mM NaNO₃ and 20 mM HEPES-KOH, pH 8.0) was solidified using 1.5% (w/v) agar (Nissui), and incubated in air containing 2% (v/v) CO₂ at 30°C under continuous white light (~70 μmol photons m⁻² s⁻¹). For sigE and rre37 mutants, 50 μg ml⁻¹ Km (Sigma) was supplemented in modified BG-11 liquid medium. Similarly, the ntcA knockdown mutant was grown in modified BG-11 liquid medium supplemented with 80 μg ml⁻¹ chloramphenicol (Cm; Sigma). Growth and cell densities were estimated by measuring the A₇₅₀ [optical density (OD)₇₅₀]. To observe the growth under LAHG, cells of *Synechocystis* (2 μl each) were spotted on modified BG-11 plates (supplemented with 5 mM glucose). For LAHG, modified BG-11 plates containing 5 mM glucose were incubated in the dark with a daily pulse of white light for 15 min.

**Construction of rre37 and sigE/rre37 mutants**

To construct the rre37 mutant, the rre37 coding region was amplified by PCR with primer sets 5'-GGCAAGCTTGTGATCCAGTCTACATATCA-3' (forward primer 1) and 5'-GTTGGATCCCTAGTAAAGTACAGACTCC-3' (reverse primer 1). Each primer contained a HindIII and BamHI restriction site, respectively (underlined). PCR fragments were purified with a Wizard SV Gel and PCR Clean-up System (Promega) and the rre37
fragment was digested with \textit{BamH} and \textit{HindIII}. The resulting fragment was cloned into \textit{pUC119} (Clontech) digested with \textit{BamH} and \textit{HindIII}. Then, a Km-resistant cassette obtained by digesting \textit{pKR}11 (Reece and Phillips 1995) with \textit{SphI} was inserted into the \textit{SphI} site of \textit{rre37}. The resultant vector was introduced into \textit{GT} to make the \textit{rre37} mutant (GR37). The Km-resistant (5 \( \mu \)g ml\(^{-1}\)) cells were isolated and streaked on plates supplemented with 50 \( \mu \)g ml\(^{-1}\) Km several times for segregation. For the construction of the \textit{sigE/rre37} double mutant (G3750), the \textit{sigE} coding region was amplified by PCR with specific primer sets, 5'-GAGGCCCAGCAAGGATGATCTGC TTCC-3' and 5'-AGCTACGCTAACTTGAAG-3', and cloned into pGEM T-easy vector (Promega) to make \textit{psigE-TA}. The Cm' cassette from \textit{pKR}10 (Reece and Phillips 1995) was excised with \textit{BamH} and inserted into the \textit{psigE-TA} BglII site. GR37 was transformed with the resultant plasmid, and transformants were selected on plates containing Km (5 \( \mu \)g ml\(^{-1}\)) and Cm (5 \( \mu \)g ml\(^{-1}\)). Construction of the \textit{sigE} knockout mutant and the \textit{nctA} knockdown mutant (named G50 and GN20, respectively) and complementation of GN20 (GN20Cp) were as described (Osanai et al. 2005a, Osanai et al. 2006).

**Affinity purification of His-Rre37 and production of Rre37 and Gap1 antiserum**

The \textit{rre37} open reading frame was amplified by PCR with specific primer sets: 5'-GGCCCCTCATATGGTGAATCCAGTCTAC AT-3' and 5'-AGCTACGCTAACTTGAAG-3'; each primer had a \textit{NdeI} or \textit{BamH} restriction site, respectively (underlined). The PCR product was digested with \textit{NdeI} and \textit{BamH} and then cloned into pET15b (Novagen) digested with \textit{NdeI} and \textit{BamH} to make the \textit{Rre37} expression vector. After introduction into \textit{Escherichia coli} strain BL21 (DE3) pLysS (Novagen), transformed cells were cultured in LB medium containing ampicillin (50 \( \mu \)g ml\(^{-1}\)) at 30°C, and over-expression of histidine-tagged \textit{Rre37} (His-Rre37) was induced by addition of 1 mM isopropyl-\( \beta \)-\( \delta \)-thiogalactopyranoside (IPTG) (Nacalai tesque) at a cell density OD\(_{600}\) = 0.4. After incubation at 30°C for 4 h, cells were harvested by centrifugation and suspended by lysis buffer [50 mM Tris–HCl (pH 8.0), 300 mM NaCl, 0.1% Triton X-100, one tablet of protease inhibitor (complete EDTA-free, Roche)]. Then, cells were disrupted by sonication (10 s, 20 min, 4°C). The soluble fraction was subjected to SDS–PAGE, and fractions containing only His-Rre37 were applied to an Ni-affinity column (Sigma) equilibrated with wash buffer [50 mM Tris–HCl (pH 8.0), 300 mM NaCl], and the column was rinsed with wash buffer several times. His-Rre37 proteins were obtained by serial rinsing of the column with wash buffer containing 10, 20, 50, 75, 100, 125, 150, 175 and 200 mM imidazole. Each collected fraction was subjected to SDS–PAGE, and fractions containing only His-Rre37 were dialyzed against storage buffer [50 mM Tris–HCl (pH 8.0), 300 mM NaCl, 50% glycerol]. The protein concentrations and purities were examined by Bio-Rad protein assay and SDS–PAGE with Coomassie Brilliant staining, respectively. Antiserum to Rre37 was produced by Tampaku Seisei Kogyo (Gunma, Japan) with 1.35 mg of purified His-Rre37 proteins. Antiserum to Gap1 was produced with the synthesized peptide SNLAYLKYDSTHGSC and injected into rabbits, as was antiserum to Rre37. Design and synthesis of the peptide as well as the antiserum production were by Tampaku Seisei Kogyo.

**Affinity purification of His-NtcA**

To construct the NtcA expression vector, the \textit{nctA} coding region was amplified by PCR using primers 5'-GGCCCAT ATGATGATCAGTCCCTAAC-3' and 5'-GGCTCTAGATTAG GTAAGACTGTTGACTGAG-3' from \textit{Synechocystis} genomic DNA (underlining indicates \textit{NdeI} and \textit{XbaI} restriction sites, respectively). The product was cloned into the corresponding sites of the pCOLD I vector (TAKARA) digested with \textit{NdeI} and \textit{XbaI}. The resultant plasmid was transformed into BL21 (DE3) pLysS, and the cells were cultivated at 37°C in LB medium. After the OD\(_{600}\) reached 0.5, the culture was placed at 15°C for 30 min. Then, the culture was supplemented with 1 mM IPTG and incubated at 15°C for 24 h. Cells were harvested by centrifugation, and NtcA protein was purified from the soluble fraction using the same method as for His-Rre37 protein purification.

**Gel mobility shift assays**

A 100 bp DNA fragment containing the region upstream from a translation start site of \textit{rre37} was obtained by PCR using primers 5'-TTTTGAAAGATTTGCCCCCATGCAGTA-3' and 5'-CG ATCTAGACCCCTGACTGAAAGTTCCCTG-3'. The amplified DNA fragment was purified from polyacrylamide gels and the 5' ends were labeled with \( [\gamma^{32}P] \)ATP by T4 polynucleotide kinase (TAKARA). A \( 32P \)-labeled DNA fragment (1 nM) was incubated with various concentrations of His-NtcA in 20 \( \mu \)l of binding buffer [25 mM Tris–HCl (pH 8.0), 50 mM KCl, 4 mM spermidine, 20% glycerol, 0.5 mg of poly(dI–dC)] for 30 min at 30°C. The mixtures were subjected to electrophoresis on a native 4% polyacrylamide gel at 70 V, then placed over Whatman 3MM paper and dried. Analyses of gel images were carried out with a BAS 1000 image analyzer (Fuji). For the competition experiment, a specific competitor DNA was prepared by annealing the oligonucleotides 5'-ATGCAGGTTACGTGTTG TACAAGCCTTA-3' and 5'-TCAAGGGTGTGTTACACAGT TACCTGCT-3'. A non-specific competitor DNA was similar.

**Isolation of RNA and microarray analysis**

Cells of mid-exponential phase cultures of \textit{Synechocystis} \( (A_{750} = 0.5–0.6) \) grown in modified BG-11 medium were collected by centrifugation at 6,600 \times 5 \text{ min. RNA was}
isolated by the hot phenol method as follows: cells were sus-
pended with 0.5% SDS, mixed with an equal volume of acid phenol
and incubated at 65 °C for 10 min. After centrifugation
(17,400 × g, 10 min, 4 °C), the supernatant was obtained and
proteins were eliminated from the supernatant by addition of
an equal volume of phenol–chloroform–isoamylalcohol
(25 : 24 : 1, by vol.) several times. Finally, total RNA was obtained
by ethanol precipitation. Extracted RNA was treated with
proteinase K (Inviogen) and DNase I (Promega) for further
purification. Microarray analysis was performed as described
(Suzuki et al. 2004). Signals were quantified using the
ImaGene version 4.0 software (Bio Discovery)
(Kanesaki et al. 2002).

Northern blot analysis and immunoblotting
Cells of mid-exponential phase cultures of
Synechocystis (A₅₅₀ = 0.5–0.6) grown in modified BG-11 medium were col-
lected by filtration with 0.45 µM MF-membrane filters
(Milli pore) and resuspended in BG-11g medium. After culti-
vation for 0 or 4 h, cells were collected, and RNA was ex-
tracted by the hot phenol method (see above). For
extraction of total protein, cells were suspended in ice-cold
phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl,
10 mM Na₂HPO₄, 1.76 mM KH₂PO₄) and disrupted by son-
ication (15 s × 7 times). Soluble fractions were obtained by
centrifugation at 14,970 g for 15 min at 4 °C. Northern
blot analysis was as described (Kanamaru et al. 2001).
Digoxigenin (DIG)-labeled gene-specific probes were gener-
ated using a DIG DNA labeling mix (Roche). Primer sets for
constructing probes of gap1 (slr0884), pfkA (slr1196), glgX
(slr1857), glgP (slr1367), gnd (slr0329) and zwf (slr1843) were
described previously (Osanai et al. 2005a). Immunoblot anal-
ysis was performed as described previously (Tanaka et al.
1993). Antiserum against SigE was produced previously
(Osanai et al. 2005a).

Supplementary data

Supplementary data are available at PCP online.

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