Transcriptome landscape of *Synechococcus elongatus* PCC 7942 for nitrogen starvation responses using RNA-seq

Sun Young Choi¹,²,³, *¹*, Byeonghyeok Park³,⁷, In-Geol Choi³, Sang Jun Sim²,⁴, Sun-Mi Lee³,⁵, Youngsoon Um¹,⁵ & Han Min Woo¹,²,⁵

The development of high-throughput technology using RNA-seq has allowed understanding of cellular mechanisms and regulations of bacterial transcription. In addition, transcriptome analysis with RNA-seq has been used to accelerate strain improvement through systems metabolic engineering. *Synechococcus elongatus* PCC 7942, a photosynthetic bacterium, has remarkable potential for biochemical and biofuel production due to photoautotrophic cell growth and direct CO₂ conversion. Here, we performed a transcriptome analysis of *S. elongatus* PCC 7942 using RNA-seq to understand the changes of cellular metabolism and regulation for nitrogen starvation responses. As a result, differentially expressed genes (DEGs) were identified and functionally categorized. With mapping onto metabolic pathways, we probed transcriptional perturbation and regulation of carbon and nitrogen metabolisms relating to nitrogen starvation responses. Experimental evidence such as chlorophyll *a* and phycobilisome content and the measurement of CO₂ uptake rate validated the transcriptome analysis. The analysis suggests that *S. elongatus* PCC 7942 reacts to nitrogen starvation by not only rearranging the cellular transport capacity involved in carbon and nitrogen assimilation pathways but also by reducing protein synthesis and photosynthesis activities.

Global concerns about energy security and environmental issues affecting climate changes have focused attention on engineering photosynthetic organisms that are able to sequester and convert CO₂ to organic materials using solar energy¹,². In order to improve the strains, system-wide engineering and optimization is required to reduce the time-, cost-, and labor-intensive process of strain development³. In particular, systems biology including omics analysis has been integrated to strain development.

Nitrogen is an essential element of all the complex macromolecules in a bacterial cell. Anthropogenic nitrogen supply in the presence of CO₂ is one of the critical factors in the occurrence of cyanobacterial blooms mainly caused by *Microcystis* spp.⁴. On the other hand, a nitrogen limitation strategy is commonly used to maximize production of the desired product such as triacylglycerol content in microalgae in the carbon partitioning mechanisms⁵ and to trigger polyhydroxalkanoate (PHA) accumulation as carbon storage in *Ralstonia eutropha*⁶ and in engineered *Escherichia coli*⁷. In addition, improvement of isoprenoid production (astaxanthin in *Haematococcus pluvialis*⁸,⁹ and amorpha-4,11-diene in *E. coli*¹⁰) has been reported under nitrogen limitation. Thus, it is important to understand the positive and negative mechanisms of nitrogen regulation in a cell for system-wide strain development.

Systems biology-based analyses have revealed the cellular mechanisms for nitrogen regulation in industrially-relevant bacteria. Nitrogen assimilation and amino acid biosynthesis of *Corynebacterium*...
Glutamicum, an industrial amino acid producer, have been studied using a DNA microarray. Chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) has been applied to address the links between the nitrogen stress response and stringent response in E. coli by identifying genome-wide DNA binding sites for the global transcriptional regulator NtcC that controls gene expression for nitrogen responses. In addition, metabolic and transcriptomic responses of a wine yeast (Saccharomyces cerevisiae strain EC1118) have been investigated under a nitrogen-limited condition, showing its responses related to wine quality.

The nitrogen starvation response of cyanobacteria including Synechococcus elongatus PCC 7942 as a cyanobacterial model organism has been studied to understand the mechanisms of degradation of phycobiliproteins and its consequent induction of chlorosis. Increased expression of NbIa (a small polypeptide) was found to be responsible for degradation of phycobiliproteins using mutant screening under a nitrogen deprivation condition, which is regulated by the global regulator NtcA for nitrogen control. In addition, the signal transduction protein PII and the regulator PipX (PII interacting protein X) also modulate the gene expression in nitrogen control in S. elongatus PCC 7942. The proposed regulatory model of PipX showed that NtcA-independent regulons are involved in the adaption of cyanobacteria for translation and photosynthesis to nutritional changes. However, two-dimensional proteomic analysis has failed to identify proteins for comprehensive nitrogen regulation due to the low amount of protein synthesized during nitrogen starvation. Recently, transcriptomic and phosphoproteomic analyses of Synechocystis sp. PCC 6803 for nitrogen starvation have been reported using DNA microarray and LC-MS/MS, respectively. However, a global transcriptomic analysis of S. elongatus PCC 7942 has not been available yet for the nitrogen starvation response although a comprehensive analysis of S. elongatus PCC 7942 using ChIP-seq, RNA-seq, and tiling expression microarray has been reported.

Here, our study aims to understand the transcriptional landscape of S. elongatus PCC 7942 for the nitrogen starvation response using RNA-seq. Differentially expressed genes were identified with functional categories and nitrogen starvation-related metabolic metabolisms. The transcriptomic patterns and regulation obtained from the RNA-seq analysis were supported with additional experimental evidence. The results provided insight into the nitrogen starvation responses in S. elongatus PCC 7942 for system-wide metabolic engineering.

Results

Nitrogen starvation response of S. elongatus PCC 7942. In order to investigate the nitrogen starvation responses of S. elongatus PCC 7942, cyanobacterial cells were cultivated in either BG-11 medium (N+) or nitrogen-free BG-11 medium (N-). Cyanobacterial cell growth was stopped under a N- condition only, resulting in chlorosis of cells after days of starvation in the medium. However, a global transcriptomic analysis of S. elongatus PCC 7942 has not been available yet for the nitrogen starvation response although a comprehensive analysis of S. elongatus PCC 7942 using ChIP-seq, RNA-seq, and tiling expression microarray has been reported.

Here, our study aims to understand the transcriptional landscape of S. elongatus PCC 7942 for the nitrogen starvation response using RNA-seq. Differentially expressed genes were identified with functional categories and nitrogen starvation-related metabolic metabolisms. The transcriptomic patterns and regulation obtained from the RNA-seq analysis were supported with additional experimental evidence. The results provided insight into the nitrogen starvation responses in S. elongatus PCC 7942 for system-wide metabolic engineering.

Global transcriptomic analysis of S. elongatus PCC 7942 for nitrogen starvation response. Compared to cells grown under a nitrogen repletion condition, differentially expressed genes (DEGs) of S. elongatus PCC 7942 under a nitrogen starvation condition were identified by the Bioconductor RNA-seq analysis pipeline and filtered with criteria of both p-value ≤ 0.01 and log2 fold change (log2FC) ≥ 1. Among 545 differentially expressed genes, 284 genes were down-regulated and 261 genes were up-regulated (Fig. 2 and Supplementary Table S1). The highly up-regulated genes (log2FC ≥ 3) for nitrogen starvation responses were mostly annotated as hypothetical proteins having no COGs (Clusters of Orthogonal Groups). On the other hand, the most down-regulated genes (log2FC ≤ -6) for nitrogen starvation responses were a gene encoding for...
ruberythrin (a non-heme iron protein) that has been related to oxidative stress protection and a gene encoding a hypothetical protein.

In order to find functional categories of DEGs, we performed a GO enrichment analysis over biological processes, molecular functions, and cellular locations. As shown in Fig. 2, many cellular transporter activities were up-regulated for nitrogen starvation responses, i.e. ion, nitrate, and nitrogen compound transport, whereas photosynthesis and protein translation process were down-regulated.

Transcriptomic analysis in specific metabolisms of *S. elongatus* PCC 7942 for nitrogen starvation response.

Stringent responses of nitrogen starvation have been shown in the cellular transporters to adapt environmental changes. Here we described the transcriptional outlook of specific metabolisms over nitrogen starvation responses in *S. elongatus* PCC 7942 with RNA-seq analysis (Fig. 3 and Supplementary Table S1).

**Nitrogen assimilation.** For ammonium uptake, three cyanobacterial ammonium transporters (Amt1, AmtB, and Amt1-like) were annotated in *S. elongatus* PCC 7942. The expression of gene encoding NtcA-regulated AmtB (encoded by locus Synpcc7942_2279) is responsible for nitrogen limitations, which was up-regulated by 1.7 of logFC after 24 h of nitrogen starvation. On the other hand, transcriptional levels of genes encoding for Amt1 (encoded by locus Synpcc7942_0442) and Amt1-like (encoded by locus Synpcc7942_0965) were not differently altered. Besides ammonium, nitrate and urea are commonly used by cyanobacteria as a nitrogen source. An ABC-type transporter constituted by NrtABCD (encoded by locus Synpcc7942_1239-1236), which is involved in nitrate-nitrite uptake, were up-regulated (1.1 of logFC). Also, a NtcA-regulated ferredoxin-nitrite reductase NirA (encoded by locus Synpcc7942_1240) was up-regulated (1.9 of logFC) to the nitrogen starvation response. However, a ferredoxin-nitrate reductase NarB (encoded by locus Synpcc7942_1235) was not up-regulated. Genes encoding for urease and urea transporters meanwhile have not identified in *S. elongatus* PCC 7942 and their orthologous genes predicted by the orthologs of *Synechocystis* sp. PCC 6803 were not differently expressed upon nitrogen starvation. Interestingly, an ABC-type cyanate transporter and a cyanate lyase (encoded by the cynDBAS genes; Synpcc7942_2107-2104) were up-regulated (1.1 of logFC). Thus, nitrogen-starved cyanobacterial cells transport cyanate and irreversibly catalyze bicarbonate-dependent conversion of cyanate to ammonium.

Further assimilation of ammonia into glutamine and glutamate, the GS/GOGAT pathway was used to subsequently distribute nitrogen to other cellular components in *S. elongatus* PCC 7942. Expressions of the *glnN*
(Synpcc7942_0169) gene encoding for glutamine synthase (Type III GS) were differentially up-regulated by 1.5 of logFC due to transcriptional activation by NtcA. However, expressions of the glnA (Synpcc7942_2156) gene encoding for the type I GS and the glsF (Synpcc7942_0890) gene encoding for a ferredoxin-dependent glutamate synthase (GOGAT; Glutamine oxoglutarate aminotransferase) were not differentially up-regulated by 0.9 and 0.4 of logFC, respectively.

Nitrogen control is regulated by a transcriptional regulator NtcA (encoded by locus Synpcc7942_0965). As a RNA-seq result, NtcA was positively auto-regulated (1.4 of logFC) to the nitrogen starvation response and regulated transcriptional expressions of the amtB, nirA, glnN, and cynDBAS genes by activating NtcA-promoters. Another regulon of NtcA is the glnA (Synpcc7942_2156) and glnB (Synpcc7942_0321) genes encoding for glutamine synthase (GS) and FII protein, respectively. However, RNA-seq showed that the amt1, glnA, and glnB genes were not transcriptionally activated after 24 h of nitrogen starvation. Gene expression of NtcB (LysR family of transcriptional regulator), encoded by locus Synpcc7942_1242 and PipX (encoded by Synpcc7942_2061), a co-activator of NtcA, was not differentially changed.

Carbon assimilation. Compared with bicarbonate transporters of Synechocystis sp. Strain PCC 6803, single bicarbonate component BicA (encoded by locus Synpcc7942_1380), a constitutive high-flux low-affinity Na+/HCO3− symport, SbtA (encoded by locus Synpcc7942_1475), an inducible low-flux high-affinity Na+/HCO3− symport were differently expressed (logFC ≥1) in a nitrogen starvation condition. Also, a multicomponent ATP-binding bicarbonate transporter BCT1 (encoded by the cmpABCD genes; Synpcc7942_1488-1491) was differently expressed (logFC ≥1) in a nitrogen starvation condition. SbtA and CmpABCD were up-regulated.
regardless of inorganic carbon conditions. Besides bicarbonate transporters, the multiple NADH:ubiquinone oxido-reductase complex is essential to the CO₂ uptake system in cyanobacteria. In S. elongatus PCC 7942, a small NADH:ubiquinone oxido-reductase (NDH-1S) encoded by the ndhF3-ndhD3-cupAS (Synpcc7942_2091-2094) gene clusters plays a CO₂ uptake system, which were not down-regulated in nitrogen starvation (N⁻) compared with nitrogen (N+), although the genes were down-regulated in nitrogen starvation (N⁻) compared with a nitrogen starvation control (N⁻c0₂) (see the details in Supplementary Table S1). Furthermore, genes encoding for NDH-1L and NDH-1M were not altered.

**Carbon assimilation pathway and central metabolisms.** Nitrogen starvation in S. elongatus PCC 7942 did not alter significant expression of genes encoding for β-carboxysome, which acts as a microcompartment to supply CO₂ to Rubisco along with carboxysomal CA activity. No particular changes were shown for expression of carbonic anhydrase encoded by caa (Synpcc7942_1447), caaA (Synpcc7942_1388), and ccmM (Synpcc7942_1423). The carbon fixing enzyme of the Calvin-Basham-Benson (CBB) cycle, Rubisco encoded by the cbbL (Synpcc7942_1426-1427) gene, is crucial for carbon assimilation in cyanobacteria. Expression of the cbbL genes was not differentially expressed for the nitrogen starvation. Unlike CbbL, expression of the prk (Synpcc7942_0977) encoding for phosphoribulokinase and fbpI (Synpcc7942_0505) encoding for the type I fructose-1,6-bisphosphatase in the CBB pathway was down-regulated in both nitrogen starvation (N⁻) compared with nitrogen (N+) and nitrogen starvation (N⁻) compared with a nitrogen starvation control (N⁻c0₂) by logFC ≤ -1. However, expressions of the remaining genes in the CBB pathway were not differentially changed.

In the glycolytic pathway, the pentose phosphate pathway, and the TCA cycle, no differentially expressed genes were found except genes encoding enzymes that involved a reaction with G3P as either a substrate or a product such as Gap3 (encoded by locus Synpcc7942_1939), Eda (encoded by locus Synpcc7942_0017), or Tal (encoded by locus Synpcc7942_2297). Furthermore, expressions of genes in glycogen biosynthesis were not changed.

**Secondary metabolic pathways.** S. elongatus PCC 7942 possesses the methylerythritol phosphate (MEP) pathway, which utilizes glyceraldehyde-3-phosphate and pyruvate to produce IPP and DMAPP for terpenoids biosynthesis. The terpenoid pathway in cyanobacteria is linked to chlorophyll biosynthesis.Geranylgernaly reductase converts GGPP to phytyl diphosphate (Pdh), which is used as a substrate for chlorophyll synthase (ChlG encoded by locus Synpcc7942_2084) to produce a chlorophyll a. Another co-substrate for ChlG is a chlorophyllide a, which is produced from L-glutamate via both chlorophyllide a biosynthesis and tetrapyrrole biosynthesis.

As a result of RNA-seq for nitrogen starvation response, expressions of most genes involved in the MEP pathway were not changed except the dxa (Synpcc7942_0430) encoding for deoxyxysulose-5-phosphate synthase, which is the first enzyme of the MEP pathway. Dxa was down-regulated (logFC ≤ -1) in S. elongatus PCC 7942 for the nitrogen starvation condition. On the other hand, genes in involved in chlorophyllide a and tetrapyrrole biosynthesis were significantly changed by logFC ≤ -1.

**Photosynthetic and respiratory electron transport pathways.** Cyanobacteria utilize redox-active components in thylakoids for both photosynthesis and respiration. For oxygenic photosynthesis, both photosystem I and II are required. In photosystem II (PSII), allophycocyanin (APC) and phycocyanin (PC) are a light-harvesting pigment-protein complex of the phycobilisome of cyanobacteria. Type-I NADPH dehydrogenase oxides NADPH and reduces plastoquinone (PQ) with PS II. Electrons are transported from the PQ pool to the cytochrome b6f complex. Soluble electron carriers such as plastocyanin or cytochrome c reduce the oxidized photosystem I (PSI) plastoquinone to NADP. Reducible NADP can be used for CO₂ fixation. A proton gradient from the electron transport pathway is used for ATP synthesis.

RNA-seq for the nitrogen starvation response revealed that genes encoding for PSII and its phycobilisome in S. elongatus PCC 7942 were differentially expressed by logFC ≤ -4.2. Also, several genes encoding for F3,F5, ATPase were altered by logFC ≤ -1. Genes encoding for NDH-1L and NDH-1M complexes, cytochrome b6f complex, PS I, and cytochrome c oxidase were not significantly altered under nitrogen starvation. Interestingly, NblA, a phycobilisome degradation protein encoded by the nbla (Synpcc7942_0430), was significantly up-regulated by 1.96 of logFC, resulting in chlorosis in S. elongatus PCC 7942 under nitrogen starvation.

**Biological evidences for down-regulated photosynthesis and up-regulated bicarbonate transporter of S. elongatus PCC 7942 for nitrogen starvation response.** Based on the transcriptomics analysis, two distinct features were shown for nitrogen starvation responses in S. elongatus PCC 7942. First, genes encoding for APC and PC and its biosynthesis pathway (chlorophyll a biosynthesis) were significantly down-regulated. On the other hand, genes encoding for bicarbonate uptake transporters were up-regulated for nitrogen starvation. Thus, we performed biological experiments to support the findings from the RNA-seq analysis.

To confirm whether the levels of Chlorophyll a (Chl a) and phycobiliproteins (APC and PC) were decreased under nitrogen starvation, we determined the levels of Chl a and PC content in S. elongatus PCC 7942 using a spectrophotometer. As a result, both levels of Chl a and PC content of S. elongatus PCC 7942 were gradually decreased after nitrogen starvation stress was induced (Fig. 4), consistent with previous results. Eventually, chlorosis of the cyanobacterial cells occurred. Subsequently, we investigated whether the CO₂ fixation rate was changed in S. elongatus PCC 7942 under a nitrogen starvation condition. Thus, we performed the measurement in a controlled photobioreactor supplied with bubbled air containing 5% CO₂ (Fig. 5). The results showed that the CO₂ fixation rates for the nitrogen starvation condition were statistically higher than for the nitrogen repletion
Discussion
Although there are many questions with respect to the regulation of nitrogen control, there is evidence that the levels of chlorophyll a and phycocyanin contents (mg/gDCW) were measured by the previous spectrophotometric assay under either nitrogen starvation (N−; white bar) or nitrogen repletion (N+; black bar) condition. Note that '24 h' and '48 h' on the time axis corresponds to the time point of the arrow 1 and arrow 2 of the Fig. 1 as the cyanobacterial culture time (h), respectively. Data are presented as the mean of at least three independent experiments. The error bars represent the standard deviation of samples.

Figure 4. Decreased chlorophyll a and phycocyanin contents of S. elongatus PCC 7942 under nitrogen starvation condition. The levels of chlorophyll a and phycocyanin contents (mg/gDCW) were measured by the previous spectrophotometric assay under either nitrogen starvation (N−; white bar) or nitrogen repletion (N+; black bar) condition. Note that '24 h' and '48 h' on the time axis corresponds to the time point of the arrow 1 and arrow 2 of the Fig. 1 as the cyanobacterial culture time (h), respectively. Data are presented as the mean of at least three independent experiments. The error bars represent the standard deviation of samples.

Figure 5. Alleviated initial CO2 fixation rate of S. elongatus PCC 7942 under nitrogen starvation condition. (a) a cartoon of the controlled photobioreactor setting for cultivation of S. elongatus PCC 7942 in 1.8 L BG-11 medium either with or without nitrogen sources, supplied with bubbled air containing 5% CO2 at a flow rate of 140 mL min−1 under constant illumination light (100 μmol photons m−2 s−1). The concentration of CO2 was measured using an infrared by CO2 analyzer (Q-S153, Qubit systems, Kingston, ON, Canada) with a digital data recording computer. (b) Measurement of CO2 fixation rates of S. elongatus PCC 7942 under either nitrogen starvation (N−; white bar) or nitrogen repletion (N+; black bar) condition in a controlled photobioreactor. Note that '24 h' and '48 h' on the time axis corresponds to the time point of the arrow 1 and arrow 2 of the Fig. 1 as the cyanobacterial culture time (h), respectively. The calculation of CO2 fixation rate (mg/l/d) was described in the section of method. Data are presented as the mean of at least duplicate independent experiments. The error bars represent the standard deviation of samples (*p-value < 0.01).
Moreover, regulation of NtcA is also involved in ammonium assimilation into carbon metabolism. Nitrogen starvation is perceived as an increase in the concentration of intracellular 2-oxoglutarate, which acts as a signal molecule for intracellular sensor PII protein. Accumulation of the levels of 2-oxoglutarate (2-OG) modulates the phosphorylation status of PII protein to interact with PipX for further transcriptional regulation with NtcA. Besides serving as a substrate for the GS/GOGAT pathway, 2-OG is a product or substrate in the 2-OG decarboxylating TCA cycle. When expressions of metabolic enzymes that consume or produce 2-OG were investigated, expressions of genes encoding for isocitrate dehydrogenase (Synechocystis sp. 6803) and 2-OG decarboxylase (Synechocystis sp. 7942) were not changed. Thus, increased levels of 2-OG must be due to high expression of the GS/GOGAT activity in nitrogen starved Synechocystis sp. 7942, which was also confirmed by our RNA-seq analysis. Moreover, NtcA regulates OmpR-type regulator Rre37, which is involved in nitrogen-expression of sugar catabolic genes in Synechocystis sp. 6803. Homology of Rre37 in Synechocystis sp. 7942 is annotated in a transcriptional regulator but it has not been characterized yet.

Furthermore, protein PII co-regulates inorganic carbon uptake in cyanobacteria. Given that phosphorylated PII is related to high CO2 concentration (5% CO2) and low nitrogen availability, the mutant ΔghbB (encoding for PII of Synechocystis sp. 6803) showed high affinity to high CO2 concentration. In addition, decreased phyco- cyanin content and increased glycogen content have been shown in the mutant ΔghbB. However, the effect is limited on the metabolome and the inorganic carbon uptake in Synechocystis sp. 7942. Similarly, expression of the ghb genes in this study was not significantly changed for the nitrogen starvation condition. Thus, the role of PII signaling protein is important to decipher carbon and nitrogen metabolisms with the status of phosphorylation, which could be useful for metabolic engineering of cyanobacteria.

The global regulatory model for carbon and nitrogen assimilation involves the levels of 2-OG in expression of the high affinity carbon concentrating mechanism. It has been reported that CcmR of Synechocystis sp. 6803 negatively regulates expression of genes for SbtA and NDH-1S transporters in the presence of co-repressors (NADP+ and 2-OG). Interestingly, our RNA-seq study showed that gene expressions for carbon uptake transport (BicA, SbtA, and BCT1) were up-regulated for the nitrogen starvation condition in Synechocystis sp. 7942. CmpR of Synechocystis sp. 7942 has been shown to be a transcriptional activator for the cmp operon encoding for a BCT1 transporter in the presence of 2-phosphoglycerate as a co-activator. Up-regulation of SbtA under nitrogen starvation remains unclear because CcmR could be missing in Synechocystis sp. 7942. These up-regulations of bicarbonate transporters under nitrogen starvation led us to investigate the CO2 uptake rate, and thereupon a significant increase of the CO2 uptake rate was measured under a nitrogen starvation condition in a controlled photobioreactor. Interestingly, genes of a distinct cyanate transporter of Synechocystis sp. 7942 were highly up-regulated, which might also be involved in an increase of the CO2 uptake rate by catalyzing a bicarbonate-dependent conversion of cyanate to supply ammonium. Thus, an increased CO2 uptake rate could be an initial nitrogen starvation response.

The stringent response in photosynthesis is regulated with C and N metabolism in Synechocystis sp. 7942 under a nitrogen starvation condition. Previously, accumulation of intracellular glycogen has been observed in nitrogen-starved cyanobacteria, in contrast with decreased levels of total lipid and total protein contents. However, our experiment did not show the DEGs for glycogen biosynthesis. However, genes coding for Gap3, Eda, and Tal (G3P consuming or producing enzymes) were highly up-regulated, which might also be involved in an increase of the CO2 uptake rate by catalyzing a bicarbonate-dependent conversion of cyanate to supply ammonium. Thus, an increased CO2 uptake rate could be an initial nitrogen starvation response.

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Conclusion
A transcriptome analysis of Synechocystis sp. 7942 under a nitrogen starvation condition was provided in the point of the global regulatory network of carbon and nitrogen assimilation. Genes involved in carbon and nitrogen assimilation were highly up-regulated. Photosynthesis and its related metabolic genes were significantly down-regulated. Detailed information of RNA-seq for nitrogen starvation in Synechocystis sp. 7942 could be useful for metabolic engineering to accelerate the development of biosolar cell factories to re-direct carbon flux of CO2 to desired products under a nitrogen limiting condition.

Methods
Cyanobacterial strain and growth conditions. Synechocystis sp. 7942 strain was grown at 30 °C while shaking under constant illumination light (100µmol photons m−2 s−1) in BG-11 medium as described previously. For nitrogen starvation experiments, cyanobacterial cells were grown in 50 mL BG-11 for 24 h as a control (N+) and the cells at OD730 of 1.0 were harvested by centrifugation at 13 000 rpm for 5 min. Then, cells pellets were re-suspended either in fresh BG-11 medium (N+) or nitrogen-free BG-11 medium (N−) after washing twice (refer ‘arrow 1’ in Fig. 1a). The cells were cultivated for 24 h and the biological duplicated samples were prepared for RNA-seq (refer ‘arrow 2’ in Fig. 1a) and stored at −80°C until they were analyzed.

RNA preparation and RNA-seq. 5 mL of Trizol reagent (California, Carlsbad, USA) was added to the cyanobacterial cell pellet from 50 mL culture under liquid nitrogen. To break the cell wall, the quick frozen cell was
grinded using a mortar and pestle and aliquot into 1 mL tube. After addition 200 µl of 1-bromo-3-chloropropane in a 1 mL tube and incubation for 3 min at room temperature, the extraction mixture was centrifuged at 13 000 rpm for 15 min at 4 °C. Retrieved aqueous phase was mixed with the equal volume of isopropanol and incubated for 10 min at room temperature and centrifuged to precipitate RNA at 4 °C. The RNA pellet was washed using 75% (v/v) ethanol (1 mL), air dried and finally dissolved in 30 µl RNase-free water.

The quality and quantity of total RNA were evaluated using RNA electropherograms (Agilent 2100 Bioanalyzer, Agilent Technologies, Santa Clara, USA). Total RNA (10 µg) from each sample was used as a starting material to prepare sequencing libraries. The Ribo-Zero rRNA removal kit (Epitome, USA) was used for ribosomal RNA depletion according to manufacturer instructions. Libraries for illumination sequencing were made with the TrueSeq Stranded mRNA sample prep kit (Illumina, USA) following the manufacturer’s protocol. RNA sequencing was performed on the Illumina HiSeq 2500 platform using single-end 50bp sequencing. All RNA-sequencing and alignment procedures were conducted by ChunLab (Seoul, South Korea). All data sets have been uploaded to the Gene Expression Omnibus under accession (GSE79726) and all sequencing reads were submitted to NCBI Sequence Read Archive (SRP072154).

Data analysis and Statistical analysis. The quality of raw reads was checked by Fast QC and the reads with low quality (Q < 30) were eliminated using HTQC22. Bowtie227 software was used to align the reads with the S. elongatus PCC 7942 reference genome. The relative transcript abundance was quantified as raw read counts and reads per kilobase per million mapped reads (RPMK). edgeR28, a Bioconductor components in R packages was used for differential gene expression analysis. Cyanobacterial gene ontology analysis was performed by assigning COG (Clusters of Orthologous Groups) categories with KEGG (Kyoto Encyclopedia of Genes and Genomes) database. In addition, cyanobacterial clusters of orthologous groups of proteins (CyOG)44 categories to each gene of S. elongatus PCC 7942 were assigned (Supplementary Table S1). Heat maps generated by MeV (MultiExperiment Viewer ver. 4.8) showed differential expressed genes for nitrogen starvation responses in Fig. 3. For gene ontology enrichment analysis, a GO term annotation file obtained by IPR2GO (http://genome.microbedb.jp/CyanoBase) was used to perform an hypergeometric test with unsupported model organisms using the GOSTat45 of R package. Then, a custom data structure of the GO ontologies was created.

Measurement of Chlorophyll a and phycocyanin contents using spectrophotometer. For the measurement of Chlorophyll a (Chl a) and phycocyanin (PC) contents, cyanobacterial cell cultures (1 mL) were harvested at the cyanobacterial culture time and the pigments of cell pellets were extracted in 100% acetone at 50 °C for 15 min. After centrifugation (13 000 rpm for 3 min), the pigment extract was analyzed for Chl a (mg per mL) by following the previous spectrophotometric assay44. The supernatant was obtained and the absorbance was measured at 620, 680, and 750 nm on Cary 60 UV-Vis spectrophotometer (Agilent technologies, CA, USA). For PC measurement, harvested cell suspension (1 mL) was treated either with or without heating at 75 °C for 8 min. PC was determined by measuring the absorbance at 620 nm and 750 nm and PC content (mg per mL) was calculated by following the previous spectrophotometric assay44.

Calculations for carbon fixation rate using CO2 analysis. Cyanobacterial cells were inoculated to OD730 of 1 in 1.8 L of either BG-11 medium (N+) or nitrogen-free medium (N−) in a flat-panel photobioreactor (Labfors 5 Lux-LED flat-panel option [637 mm (L) × 298 mm (W) × 79 mm (D)]; INFORS-HT, Bottmingen, Switzerland) under constant illumination light (100 μmol photons m−2 s−1). 5% CO2 (v/v) and 95% air (v/v) was supplied directly into the medium at flow rate of 140 mL min−1 (0.08 vvm) by gas bubbling and 5 N NaOH was used to adjust pH 7 in the medium. CO2 analysis was performed by continues monitoring CO2 concentration from off-gas line before and after cell inoculation. CO2 concentration (ppm) was digitally recorded per every second by CO2 analyzer (Q-S153, Qubit systems, Kingston, ON, Canada).

The carbon fixation rate in a unit of mg of CO2 per liter per day was calculated from the previous calculation4. Briefly, CO2 value was converted from ppm into μmol CO2 L−1 using an equation of 

$$\Delta\text{CO}_2 (\mu\text{mol CO}_2 \text{ L}^{-1}) = \frac{\Delta\text{CO}_2 (\text{ppm})}{22.441 (T + G) \times 7 \times 10^{-4}} 
$$

where C refers to the temperature in °C and T refers to the absolute temperature (273K). Based on the flow rate (in L min−1) (i.e., 140 mL min−1) and the volume of the culture medium (i.e., 1.8 L), the CO2 fixation rate can be obtained with units of mg L−1 d−1.

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Author Contributions
S.Y.C. carried out the biological experiments and B.P. performed the bioinformatics analysis. S.Y.C., B.P., I.-G.C., H.M.W. designed the experiments, analyzed the data, and wrote the manuscript. S.J.S., S.-M.L., Y.U. and H.M.W. guided the scope of the project, provided critical input for the manuscript. All authors read and approved the final manuscript.

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