# Engineering Nitrogen Fixation Activity in an Oxygenic Phototroph

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## ABSTRACT

Biological nitrogen fixation is catalyzed by nitrogenase, a complex metalloenzyme found only in prokaryotes. $N_2$ fixation is energetically highly expensive, and an energy generating process such as photosynthesis can meet the energy demand of $N_2$ fixation. However, synthesis and expression of nitrogenase is exquisitely sensitive to oxygen. Thus, engineering nitrogen fixation activity in photosynthetic organisms that produce oxygen is challenging. Cyanobacteria are oxygenic photosynthetic prokaryotes, and some of them also fix $N_2$. Here, we demonstrate a feasible way to engineer nitrogenase activity in the non-diazotrophic cyanobacterium *Synechocystis* sp. PCC 6803 through the transfer of 35 nitrogen fixation (*nif*) genes from the diazotrophic cyanobacterium *Cyanothece* sp. ATCC 51142. In addition, we have identified the minimal *nif* cluster required for such activity in *Synechocystis* 6803. Moreover, nitrogenase activity was significantly improved by increasing the expression levels of *nif* genes. Importantly, the $O_2$ tolerance of nitrogenase was enhanced by introduction of uptake hydrogenase genes, showing this to be a functional way to improve nitrogenase enzyme activity under micro-oxic conditions. To date, our efforts have resulted in engineered *Synechocystis* 6803 strains that remarkably have more than 30% $N_2$-fixation activity compared to that in *Cyanothece* 51142, the highest such activity established in any non-diazotrophic oxygenic photosynthetic organism. This study establishes a baseline towards the ultimate goal of engineering nitrogen fixation ability in crop plants.

## IMPORTANCE

Application of chemically synthesized nitrogen fertilizers has revolutionized agriculture. However, the energetic costs of such production processes as well as the wide spread application of fertilizers have raised serious environmental issues. A sustainable alternative is to endow crop plants the ability to fix atmospheric $N_2$ *in situ*. One long-term approach is to transfer all *nif* genes from a prokaryote to plant cells, and express nitrogenase in an energy-producing organelle, chloroplast or mitochondrion. In this context, *Synechocystis* 6803, the non-diazotrophic cyanobacterium utilized in this study, provides a model chassis for rapid investigation of the necessary requirements to establish diazotrophy in an oxygenic phototroph.

**KEYWORDS** $N_2$ fixation, *Synechocystis*, $O_2$ tolerance, photosynthesis, cyanobacteria.

Enabling crop plants the machinery to fix their own nitrogen via direct transfer of nitrogen fixation (*nif*) genes is envisioned to be key for the next agricultural revolution (1-3). However, engineering diazotrophic plants, attractive a proposition, will be an extreme challenge, due to the complexities in the biosynthesis of active nitrogenase, the enzyme that catalyzes nitrogen fixation, as well as the difficulty of coupling plant metabolism to supply energy and reducing power for the nitrogen fixation process (4). An additional impediment in the scenario is that photosynthesis produces $O_2$, which is highly toxic to synthesis and activity of nitrogenase (5).

Diazotrophy occurs only in limited species of bacteria and archaea (6). Nitrogen fixation is mainly catalyzed by an iron and molybdenum-dependent nitrogenase enzyme complex, with two enzymatic components, an iron protein dinitrogenase reductase (NifH) and an iron-molybdenum protein dinitrogenase (NifDK) (7, 8). Three metal dependent cofactors, the F-cluster, P-cluster, and M-cluster, are necessary to form the holoenzyme for electron transfer to reduce atmospheric $N_2$ to form ammonia, the biologically available form of $N_2$ (9, 10). A significant number of additional *nif* genes are required for the biosynthesis of these metallocluster cofactors and for the maturation of nitrogenase to form a fully functional enzyme (11, 12).

Transferring nitrogen fixation to non-diazotrophs has been attempted for decades. To date, the heterotrophic bacterium *Escherichia coli* has been successfully engineered for nitrogen fixation activity through transfer of *nif* genes from various diazotrophic species (13-17). Engineering eukaryotic species for heterologous nitrogen fixation activity, including the yeast *Saccharomyces* cerevisiae and the green alga *Chlamydomonas reinhardtii*, have been unsuccessful. Limited success was reached only in expressing the NifH component as an active moiety in *Chlamydomonas reinhardtii* (18). While all the Nif components have been successfully expressed in yeast cells, the formation of a fully functional nitrogenase complex has not been achieved yet (19-22).

Expression of nitrogenase components into plants has also been attempted in a few studies. Individually expression of 16 Nif proteins targeted to the plant mitochondria has been reported recently, but none of the structural components showed enzymatic activity (23). Another recent study showed that an active NifH component can be formed

in tobacco chloroplasts (24), indicating that expression of active nitrogenase in chloroplasts might be a viable way in the future to engineer crop plants to fix nitrogen(25). Since it is widely accepted that a cyanobacterial ancestor was the progenitor of chloroplasts (26), engineering a cyanobacterium to fix nitrogen may pave the way to achieve the final goal of engineering nitrogen fixing ability into crop plants. We have utilized the non-diazotrophic cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803) as a chassis to engineer nitrogen fixation activity into an oxygenic photosynthetic organism.

The unicellular diazotrophic cyanobacterium, *Cyanothece* sp. ATCC 51142 (hereafter *Cyanothece* 51142) uses temporal separation as its strategy to protect nitrogenase from $O_2$ produced by photosynthesis (27, 28). Within *Cyanothece,* the two conflicting processes, photosynthesis and $N_2$-fixation, occur sequentially during the diurnal periods, so that photosynthesis and $O_2$ evolution is performed during the day whereas $N_2$ is fixed at night (29). The energy requirements for nitrogenase are met in *Cyanothece* by the catabolism of glycogen. Glycogen is accumulated in the light as the storage form of fixed $CO_2$, and later degraded in the dark to provide energy for nitrogenase. The provision of energy coupled with high rates of respiration ensures a low-oxygen intracellular environment and sufficient supplies of energy for $N_2$ fixation (30). The *Cyanothece* 51142 genome contains the most complete contiguous set of nitrogen fixation and related genes to form a *nif* cluster, which contains 35 genes (cce_0545 to cce_0579), encoding structural proteins, metal cofactor synthesis proteins, ferredoxins, and genes for proteins with unknown but necessary functions (Fig. 1). All 35 genes exhibit similar oscillating diurnal pattern of transcription during light/dark cycles, showing a high level of transcription in the dark and notably reduced levels in light (27). Such a synchronized transcriptional pattern also confirms that all of these genes are related to nitrogen fixation.

In the current study, we have successfully transferred and expressed this large *nif* gene cluster in non-diazotrophic *Synechocystis* 6803 with a resultant $N_2$-fixation activity. Subsequent engineering of the cluster as well its expression levels have led to nitrogenase activities as high as 30% of that in *Cyanothece* 51142.

## RESULTS AND DISCUSSION

**Introduction of *nif* genes into *Synechocystis* 6803.** *Synechocystis* 6803 has a close phylogenetic relationship with *Cyanothece* 51142 (Fig. 1A) (31). The large *nif* cluster from *Cyanothece* 51142 (28.34 kilobase region of DNA) was transferred into wild type *Synechocystis* 6803 on a single extra-chromosomal plasmid. This large plasmid pSyNif-1 containing the entire *nif* cluster with 35 genes (Fig. 1D) was constructed using the DNA assembler method (32). The chassis of this vector was based on pRSF1010 (33), and this self-replicating plasmid pSyNif-1 was transferred into *Synechocystis* 6803 by conjugation, generating the engineered strain TSyNif-1. Remarkably, over the past four years since its introduction into the heterologous host, pSyNif-1 has been stably maintained in its entirety in *Synechocystis* (Fig. S1). Furthermore, all introduced *Cyanothece* genes were transcribed (Fig. 1E) as detected by RT-PCR, indicating that native promoters in the *nif* cluster from *Cyanothece* 51142 can drive transcription of genes in *Synechocystis* 6803. An acetylene reduction assay method for nitrogen fixation detected nitrogenase activity, under 12h/12h light/dark conditions for strain TSyNif-1 (Fig. 2). Nitrogen fixation reached an activity of 2% relative to that in *Cyanothece* 51142 grown under similar conditions. This is the first time that a non-diazotrophic phototroph has been engineered for biosynthesis of a fully functional nitrogenase enzyme and exhibits detectible and stable nitrogen fixation activity.

**The minimal required gene cluster for nitrogen fixation activity.** Gene expression parameters for *Synechocystis* 6803 are not as well understood as for *E. coli*. Thus, the refactoring of *nif* genes as performed in *E. coli* and *Klebsiella* (16, 34) to determine the minimal requirement of genes for nitrogen fixation in *Synechocystis* 6803 is impractical at this stage. Therefore, we approached the identification of a minimal *nif* cluster for nitrogen fixation using a “top-down” method, which determines the influence of a gene on nitrogenase activity by selectively removing individual genes from the *nif* cluster (Fig. 2). Extrapolating the genetic requirements for nitrogen fixation activity observed in studies in which *nif* genes were introduced in *E. coli* (14, 15), we determined that genes for all homologous proteins introduced into *E. coli* are present in the *Cyanothece* 51142 *nif* cluster between gene *nifT* and *hesB* (Fig. 1). Hence, our second plasmid, pSyNif-2, contains 24 genes in the *nif* cluster between *nifT* and *hesB* (Fig. 2). Eleven genes were

137 removed that presumably encode three metal transporter proteins, two ferredoxins, and
138 six proteins of unknown function, none of which have been analyzed previously,
139 although they are associated with nitrogen fixation. Intriguingly, this second engineered
140 strain TSyNif-2 with a reduced cluster of 24 genes has a 3-fold increase in nitrogen
141 fixation activity when compared to strain TSyNif-1 (Fig. 2). Although plasmids pSyNif-1
142 and pSyNif-2 have the same plasmid backbone (Fig. S2), the transcriptional levels of
143 the structural genes *nifH, nifD* and *nifK* are higher in the TSyNif-2 strain (Fig. 3). This
144 improvement in nitrogenase activity could be the result of removal of one or more
145 regulatory gene(s), which may encode protein(s) that repress expression of genes in the
146 *nif* cluster.

147 However, further removal of genes from both directions, resulted in a decrease of
148 nitrogen fixation activity by more than ten-fold for strains TSyNif-3 to TSyNif-6, in which
149 the genes *hesB*, *hesAB*, *nifT*, and *nifTZ*, were removed, respectively (Fig. 2). Thus, this
150 "top-down" approach determined that an essential minimal cluster from *nifT* to *hesB* is
151 required for nitrogen fixation activity in *Synechocystis* 6803. Additionally, we
152 investigated the removal of two more genes in the cluster, *nifX* and *nifW*, generating the
153 two strains TSyNif-7 and TSyNif-8. Deletion of these two genes did not affect
154 expression of surrounding genes (Fig. S3). However, nitrogen fixation activity dropped
155 100-fold and 10-fold, respectively, in these engineered strains (Fig. 2). We conclude
156 that both *nifX* and *nifW* are important genes for nitrogen fixation. Notably, *nifX* exhibited
157 a positive influence on $N_2$ fixation in cyanobacteria, while it functions as a negative
158 regulator for $N_2$ fixation in the heterotrophic diazotroph *Klebsiella oxytoca* (35).

159 **Improvement of nitrogen fixation activity.** To increase the RNA expression levels of
160 the nitrogenase related genes, we took advantage of three small endogenous plasmids
161 in *Synechocystis* 6803, pCA2.4, pCB2.4, and pCC5.2. The expression of heterologous
162 genes expressed from these endogenous plasmids maintains higher transcriptional
163 levels than from an pRSF1010 based plasmid, because of the higher copy numbers of
164 these three plasmids within *Synechocystis* (36, 37). First, we replaced the RSF1010
165 backbone of plasmid pSyNif-2 by the entire DNA segment of each of these endogenous
166 episomes and then transferred the plasmids to *Synechocystis* 6803, generating three
167 strains TSyNif-9, TSyNif-10, and TSyNif-11, with the chassis of pCA2.4, pCB2.4, and

pCC5.2, respectively (Fig. 3A and Fig. S4). As expected, the expression levels of genes *nifH*, *nifD* and *nif*K in strain TSyNif-9 showed several fold higher transcription levels compared to in TSyNif-2 (Fig. 3B). In addition, nitrogen fixation activity was increased by another 2 to 3-fold, reaching 13% of the acetylene reduction activity in TSyNif-9 relative to that observed in *Cyanothece* 51142 (Fig. 3C). Next, nitrogenase activity was directly assayed in the engineered strains using a $^{15}N$ assimilation assay method. Remarkably, the highest activity obtained was from strain TSyNif-9, reaching 31% of $^{15}N$ assimilation relative to *Cyanothece* 51142 (Fig. 3D). The activity data presented here is comparable to published data from studies on nitrogen fixation activity in engineered in *E. coli* (Table 1). Additionally, the dinitrogenase protein NifD in whole cell extracts was detected via Western blotting by using antisera from *Rhodospirillum rubrum* (Fig. 3E). Although the MoFe-protein level in *Cyanothece* 51142 is significantly higher than in the engineered *Synechocystis* 6803 strains, the protein level in strain TSyNif-9 reached 10% of total cellular proteins (Fig. S4). It was also evident that the nitrogenase activities in the engineered strains were proportional to the level of nitrogenase structural proteins, which implied that optimizing the expression of nitrogenase proteins is critical for the activity. Most importantly from an evolutionary standpoint, these results highlight the potential for engineering plant chloroplasts to fix nitrogen at a high level of activity, since oxygenic cyanobacteria are the progenitors of chloroplasts.

**Nitrogen fixation activity in *Synechocystis* 6803.** Despite an additional metabolic load of expressing large cohorts of 35 or 24 genes related to nitrogen fixation being introduced in *Synechocystis* 6803, remarkably, the expression and activities of these heterologous proteins did not affect the growth of the engineered strains under diurnal light/dark conditions (Fig. S1). We used strain TSyNif-2 to assess the influence of oxygen and exogenous nitrate on nitrogenase activity under four conditions, BG11, $BG11_0$ (BG11 without nitrate), BG11 with 10 μM DCMU (no $O_2$ evolution) and $BG11_0$ with 10 μM DCMU (Fig. S5). Interestingly, transcript levels of *nifH*, *nifD* and *nifK* genes in the TSyNif-2 strain were downregulated by nitrate, which is similar to that in *Cyanothece* 51142. Specifically, the depletion of nitrate improved the nitrogenase activity over 30-fold in $BG11_0$ with DCMU (Fig. S5). Nitrogen fixation activity was obtained only in anaerobic environment when DCMU was added to the testing culture,

199 although the headspace of all cultures was flushed with pure argon. These data indicate
200 that oxygen generated by photosynthesis directly blocks nitrogenase activity in TSyNif-
201 2, highlighting that one of the biggest challenges for engineering nitrogen fixation in
202 oxygenic phototrophs is the sensitivity of nitrogenase to oxygen.
203 **Improvement of $O_2$ tolerance by introduction of uptake hydrogenase.** In order to
204 test the oxygen sensitivity of nitrogen fixation activity in TSyNif-2, a measured amount of
205 oxygen was added to the headspace to cultures grown in $BG11_0$ media, to generate
206 micro-aerobic conditions of 0.5% and 1.0% of $O_2$ in the sealed testing bottles. The
207 activity dropped more than 10-fold and 60-fold (Fig. 4A), respectively, demonstrating
208 that as expected, nitrogen fixation activity in engineered *Synechocystis* 6803 is highly
209 sensitive to $O_2$. To enhance $O_2$ tolerance under these same conditions, genes coding
210 for the uptake hydrogenase enzyme from *Cyanothece* 51142 were introduced into the
211 chromosome of the TSyNif-2 strain. The uptake hydrogenase is conserved in
212 diazotrophic cyanobacteria (38), and has been shown to be necessary for nitrogen
213 fixation under aerobic conditions in *Cyanothece* (39). The structural genes *hupS* and
214 *hupL*, present together in a single operon in *Cyanothece* 51142, were transformed into
215 TSyNif-2, generating strain TSyNif-12 (Fig. 4B). In addition to the structural genes
216 *hupSL*, a protease encoded by *hupW* is also present in *Cyanothece* 51142. HupW is
217 required for the maturation of HupL protein through the processing of its C-terminus
218 (40). So, the *hupSLW* genes organized in two operons were transformed into TSynif-2
219 to generate the TSyNif-13 strain (Fig. 4B). The expression of *hup* genes in TSyNif-12
220 and TSyNif-13 was assessed by RT-PCR (Fig. S6). The introduction of the uptake
221 hydrogenase did not affect nitrogen fixation activity under anaerobic conditions (Fig.
222 4C). Interestingly, under micro-aerobic conditions, nitrogen fixation activity markedly
223 improved with the expression of uptake hydrogenase, especially for strain TSyNif-13, a
224 2-fold and a 6-fold increase in TSyNif-2 for $O_2$ levels of 0.5% and 1.0%, respectively.
225 The above results suggest that expression of uptake hydrogenase proves to be highly
226 effective in enhancing $O_2$ tolerance of nitrogen fixation activity in the engineered
227 *Synechocystis* strain.
228 In this study, introduction of the *nif* gene cluster from *Cyanothece* 51142 has
229 enabled nitrogen fixation activity in *Synechocystis* 6803. The minimal cluster for 24

genes (Fig. 2) for nitrogenase activity will provide a useful framework for future studies to further enhance such activity by refactoring genes as done in *Klebsiella oxytoca* (34). Although uptake hydrogenase is a complex enzyme, introduction of its structural genes and a protease works as a protecting mechanism from the toxicity of $O_2$. The $O_2$ toxicity to nitrogenase is likely the most difficult aspect to overcome to achieve nitrogen fixation activity under aerobic conditions.

A fully functional nitrogenase holoenzyme, requires 8 electrons and 16 ATPs to reduce one molecule of $N_2$ to ammonia. Thus, metabolism within cells needs to be adjusted to supply enough reducing power and energy for nitrogen fixation. Biosynthesis of fully functional nitrogenase is a complex process. This complexity increases the difficulty to find the minimal genes and best ratios between each protein expressed from the *nif* cluster in *Synechocystis* 6803. The same gene designations in different species occasionally have alternative functions (Table S1). An example is the gene *nifX*, which functions as a negative regulator in *Klebsiella oxytoca (35)*. Gene *nifX* is of importance in *Cyanothece*, since deletion of *nifX* affects nitrogenase activity.

Although multiple challenges and many barriers exist to enable plants to efficiently fix atmospheric nitrogen, we have engineered an oxygenic photosynthetic cell to fix $N_2$ by reconfiguring the genetic processes for nitrogen fixation from *Cyanothece* 51142 to function in *Synechocystis* 6803. Our studies to date have established the highest rate of engineered nitrogen fixation activity in any non-diazotrophic oxygenic organism.

## MATERIALS AND METHODS

**Microorganisms, culture conditions and media.** All cyanobacterial strains, including *Cyanothece* 51142, *Synechocystis* 6803, and the engineered strains (listed in Table S2), were cultured in 100-mL flasks of fresh BG11 medium (41) with appropriate antibiotics (20 μg/ml kanamycin, 15 μg/ml chloramphenicol, or 20 μg/ml spectinomycin). As a pre-culture, cells were grown at 30˚C, with 150 rpm shaking, and under 50 μmol photons·$m^{-2}$·$s^{-1}$ constant light. For the nitrogen fixation activity assay, unless otherwise stated, pre-cultured cells were collected and washed with fresh BG11 without nitrate ($BG11_0$) medium and resuspended in 500 ml fresh $BG11_0$ medium. Cells were then grown at 30˚C with air bubbling under 12h light/dark conditions with 50 μmol photons ·$m^{-2}$·$s^{-1}$ of light. Yeast and *E. coli* strains (listed in Table S2) used for construction of recombinant plasmids were grown with 200 rpm shaking in YPAD (42) and LB medium at 30˚C and 37˚C, respectively.

**Construction of recombinant plasmids and engineered strains.** Plasmids and strains used in this study are listed in Table S2, and all primers are listed in Table S3. Two methods were used to construct the plasmids, DNA assembler (32) and Gibson assembly (43). To build the large pSyNif-1 and pSyNif-2 plasmids containing the *nif* genes, genomic DNA from *Cyanothece* 51142 was used as the template for PCR, and all DNA fragments were combined using the DNA assembler method to construct the plasmids in the yeast *S. cerevisiae*. For the other recombinant plasmids listed in Table S2, Gibson assembly method was used to construct them with DNA fragments amplified by PCR. Genomic DNA from *Cyanothece* 51142 and the large plasmid pSyNif-2 were used as templates for PCR to construct the plasmids for backbone replacements, the plasmids containing the uptake hydrogenase genes, and the plasmids used to remove specific *nif* genes, respectively.

Plasmids pSyNif-1 and pSyNif-2 were introduced into *Synechocystis* 6803 wild-type strain through the method of tri-parental conjugation (44) to form strains TSyNif-1 and TSyNif-2, respectively. The other recombinant plasmids were transformed into *Synechocystis* 6803 by natural transformation (45), and double homologous recombination integrated the fragments into the chromosome (for the uptake

hydrogenase genes) or the plasmid (for the plasmid backbone replacement, and specific removal of *nif* genes).

**Reverse transcription-PCR (RT-PCR) and Quantitative-PCR (q-PCR).** RT-PCR analysis was performed using RNA samples isolated from culture grown in $BG11_0$ medium at time point D1 (1 hour into the dark period) under light/dark conditions. After extraction and quantification of RNA (46), 100 ng of DNase-treated RNA samples were used for reverse transcription, using the Superscript II Reverse Transcriptase and random primers (Invitrogen) according to the manufacturer's instructions. cDNA generated after reverse transcription was used as the template for PCR to evaluate the transcription levels of various individual genes.

The q-PCR was performed on RNA samples extracted from culture grown in $BG11_0$ medium under light/dark conditions as previously described (27). Briefly, QRT-PCR SYBR green dUTP mix (Abgene) was used for the assay on an ABI 7500 system (Applied Biosystems). Each reaction was performed in three replicates, and the average $C_T$ was used to calculate the relative transcriptional levels for the amount of RNA. All primers used for RT-PCR and q-PCR are listed in Table S3.

**Measurement of nitrogen fixation activity.** Nitrogen fixation activity was measured by an acetylene reduction assay (47), modified from a previously published method (48). Unless otherwise stated, the activity assay was performed as follows: 25 ml of cyanobacterial culture were grown in $BG11_0$ medium with air bubbling under light/dark conditions as mentioned above, and transferred to a 125 ml air-tight glass vial. 10 μM DCMU was added to the culture, vials were flushed with pure argon, and cultures were incubated in 12h light/dark conditions. Cells in the sealed vials were cultured overnight and at the time point D1, 5 ml acetylene was added into the sealed vials, followed by 3 hours of incubation in light at 30˚C. 200 μl of gas was sampled from the headspace and injected into an Agilent 6890N Gas Chromatograph equipped with a Poropak N column and a flame ionization detector, using argon as the carrier gas. The temperature of the detector, injector, and oven were 200°C, 150°C and 100°C, respectively.

Total protein levels were determined on a plate reader (Bio-Tek Instruments, Winooski, VT) using a BCA-assay kit (Pierce, Rockford, IL) according to the

manufacturer's instructions. Total chlorophyll *a* was methanol extracted and quantified on an Olis DW2000 spectrophotometer (On-Line Instrument Systems, Inc., GA).

***In vivo* $^{15}N_2$ incorporation assay.** All strains were grown under light/dark conditions as mentioned above, and 50 mL cultures were transferred into 125 mL airtight glass vials. 10 μM DCMU was added to the cultures, vials were flushed with pure $N_2$, and cultures were incubated under light/dark conditions. Cells in the sealed vials were cultured overnight and at the time point D1, 8 mL of headspace gas was removed, followed by injection of 8 mL of $^{15}N_2$ gas (98%$^+$; Cambridge Isotope Laboratories, Inc.). After 8 h of incubation at 30˚C in light (50 μmol photons·$m^{-2}$·$s^{-1}$), the cultures were collected and dried in a laboratory oven at 50˚C-60˚C for 24 h. The dried pellets were ground, weighed, and sealed into tin capsules. Isotope ratios were measured by Elemental Analyzer-Isotope Ratio Mass Spectrometry (EA-IRMS, Thermo Fisher Scientific), and are showed as $\delta^{15}N$ (‰), of which the number is a linear transform of the isotope ratios $^{15}N/^{14}N$, representing the per mille difference between the isotope ratios in a sample and in atmospheric $N_2$ (49). Data presented are mean values based on at least two biological replicate cultures.

**Western blot analysis.** The coding region of the *nifD* gene (cce_0560) from Cyanothece 51142 was PCR-amplified from the genomic DNA of *Cyanothece* 51142, using the primers shown in Table S3. The PCR fragment was ligated into the expression vector pET28a cleaved by *Nde*I and *BamH*I. The resulting plasmid pET28a-nifD was used to produce the NifD protein with an N-terminal $His_6$ tag. For overproduction of the NifD protein, *Escherichia coli* BL21 (DE3) was transformed with plasmid pET28a-nifD and cultivated in LB medium at 37 °C to an optical density at 600 nm ($A_{600}$) of 0.3. Protein expression was induced by the addition of 0.2 mM isopropyl-*β*-D-thiogalactopyranoside, and the culture was incubated for another 18 h at 20 °C. After the cells were harvested, purification of NifD by nickel-nitrilotriacetic acid affinity chromatography was performed. Briefly, harvested *E. coli* cells were resuspended in 20 mM HEPES buffer (pH 7.0) containing 100 mM NaCl, and 2 mM *β*-mercaptoethanol supplemented with a protease inhibitor cocktail (Sigma-Aldrich). Lysozyme was added to a concentration of 1 mg/ml, and the cells were lysed by freezing-thawing, followed by sonication. After cells were centrifuged at 13,000 rpm, Tris-HCl buffer (pH 8.0) was

added to the supernatant (final concentration, 50 mM), and it was loaded onto a Ni-NTA agarose column (0.2 ml). After bound proteins were washed with the starting buffer containing 1 M NaCl, they were eluted with 0.3 ml of the starting buffer containing 250 mM imidazole. The purified protein was stored at -20 °C and used as the positive control for western blot assay.

Cyanobacterial cells cultured in N-free medium under L/D cycles were collected at time point D4 (4 hours after dark phase) and resuspended in 0.5 mL TG buffer (10 mM Tris-HCl [pH 8.0], 10% glycerol) containing a protease inhibitor cocktail (Sigma-Aldrich). A 0.5 mL volume of sterilized, acid-washed glass beads was added to the cells, and the mixture was disrupted using a bead beater (BioSpec Products). The resultant mixture was centrifuged for 10 min at 7,500 × g, and the supernatant was transferred into a new tube. The amount of protein was determined using a bicinchoninic acid (BCA) protein assay reagent (Thermo Scientific).

Fifteen μg of total protein extract from each sample was solubilized with 8 × sample buffer (10 ml of 0.5 M Tris [pH 6.8], 15 ml of 70% glycerol, 8 ml of 20% sodium dodecyl sulfate, 4 ml of *β*-mercaptoethanol, 4 ml of 0.1% bromophenol blue) at 70 °C for 10 min and fractionated on a sodium dodecyl sulfate (0.1%, wt/vol)-polyacrylamide (12.5%, wt/vol) gel by electrophoresis. After electrophoresis, proteins were eletrotransferred to a polyvinylidene fluoride (PVDF) membrane (Millipore), blocked with 5% bovine serum albumin (BSA) for 2 hours at room temperature, and then incubated with the primary antibodies against the NifD protein from *Rhodospirillum rubrum* (50, 51) diluted in 1.5% BSA (1:2,000) overnight at 4 °C. The HRP-conjugated secondary antibody Goat Anti-Rabbit IgG (H + L)-HRP Conjugate (Bio-Rad) was diluted at 1:5,000 in 1.5% BSA. Immunodetection was performed using Western blotting Luminol Reagent (Millipore).

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**Conflict of Interest**: The authors declare no conflict of interest.

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524
525

526 **Table 1. Nitrogen fixation activity in diazotrophs and engineered strains.**

| Strain | Nitrogenase activity (nmol $C_2H_4$/mg protein/h) | % activity (based on $C_2H_2$ reduction assay) | % activity (based on $^{15}N$ assimilation assay) | Reference |
|---|---|---|---|---|
| Diazotrophs | | | | |
| *Cyanothece* 51142 | 1262 | 100 | 100 | This study |
| *Azotobacter vinelandii* | 3300 | 100 | 100 | (52) |
| *Paenibacillus* sp. WLY78 | 3050 | 100 | 100 | (14) |
| *Pseudomonas stutzeri* A1501 | 1050 | 100 | ND | (53) |
| *Klebsiella oxytoca* M5a1 | 3708 | 100 | ND | (54) |
| Engineered strains | | | | |
| TSyNif-1 | 20 | 2 | 6 | This study |
| TSyNif-9 | 166 | 13 | 31 | This study |
| Engineered *E. coli*[a] | 180 | 5 | 35 | (15) |
| Engineered *E. coli*[b] | 300 | 10 | 30 | (14) |
| Engineered *E. coli*[c] | 105 | 10 | ND | (53) |
| Engineered *E. coli*[d] | 740 | 20 | ND | (16) |

527 [a]Nitrogen fixation genes from *A. vinelandii* and *K. oxytoca*.

528 [b]Nitrogen fixation genes from *Paenibacillus*.

529 [c]Nitrogen fixation genes from *Pseudomonas*.

530 [d]Nitrogen fixation genes from *K. oxytoca*.

531 ND-Not determined

## FIGURE LEGENDS

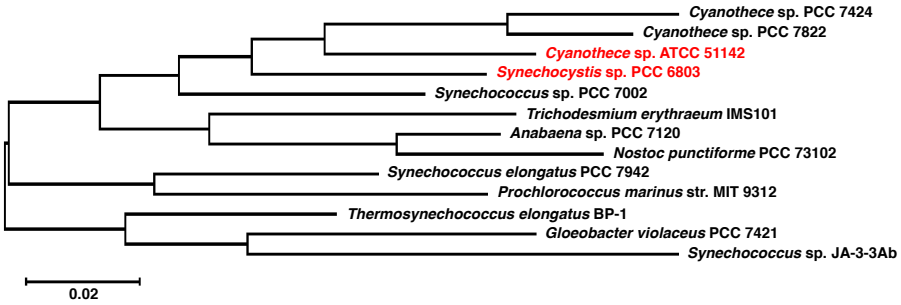
**FIGURE 1** Introduction of *nif* genes into *Synechocystis* 6803. (A) Maximum likelihood 16S rDNA phylogeny of cyanobacteria. (B) Scheme showing the genetic organization of the *nif* cluster and (C) the role of each gene product in *Cyanothece* 51142. Shown are the genes for the three structural proteins, *nifHDK* (green), necessary cofactors (blue), accessory proteins (orange), ferredoxins (purple), and hypothetical proteins (brown). Gene names and annotation are from Genbank (https://www.ncbi.nlm.nih.gov/genbank/) and Cyanobase (http://genome.microbedb.jp/cyanobase). (D) A schematic map of the plasmid pSyNif-1 containing the entire *nif* cluster. The backbone (gray) is from the broad host plasmid pRSF1010, which can replicate in *Synechocystis* 6803. The yeast helper fragment (black) contains *CEN6* and *ARS* as an *ori*, and *ura3* as a selection marker. (E) Transcription of all 35 genes in engineered *Synechocystis* 6803. Each lane represents a gene in the *nif* cluster, as numbered in panel B. Total RNA was extracted from cells cultured in $BG11_0$ medium under 12h light/dark conditions, and cDNA was used as the template for PCR.

**FIGURE 2** The minimal *nif* cluster required for nitrogen fixation activity in *Synechocystis* 6803. (A) Scheme showing the top-down method to determine the minimal *nif* cluster. The hollow rectangles represent the genes deleted from the cluster, and the colored rectangles represent the remaining genes. (B) Nitrogen fixation activity in engineered strains. Samples were collected from cultures under 12h light/dark conditions in $BG11_0$ medium. Nitrogen fixation activity was assayed by acetylene reduction, and error bars represent the standard deviation observed from at least three independent experiments.

**FIGURE 3** Enhancement of transcription levels of *nif* genes leads to higher nitrogen fixation activity. (A) A schematic map of the plasmid pSyNif-9 containing the *nif* cluster with 24 genes from *nifT* to *hesB*. The backbone (dark blue) is from the endogenous plasmid pCA2.4 of *Synechocystis* 6803. (B) Comparison of transcription levels of the *nif* structural genes in engineered strains through quantitative PCR (q-PCR). (C and D) Comparison of nitrogen fixation activities in engineered strains, as measured by (C) $C_2H_2$ reduction assay as well as (D) $^{15}N$ assimilation assay. (E) Western blot showing

563 the presence of NifD protein in engineered *Synechocystis* 6803 strains. Lanes 1-6
564 represent 0.5 μg purified NifD-His protein from *E. coli*, 15 μg whole cell extracts of
565 *Cyanothece* 51142, *Synechocystis* 6803 wild-type, TSyNif-1, TSyNif-2, and TSyNif-9,
566 respectively. Cyanobacterial samples were collected from cultures under 12h light/dark
567 conditions in $BG11_0$ medium. Error bars represent the standard deviation of at least
568 three independent experiments.
569
570 **FIGURE 4** Expression of uptake hydrogenase improves $O_2$ tolerance of nitrogenase. (A)
571 Effect of $O_2$ on nitrogen fixation activity of the TSyNif-2 strain. (B) Schematic showing
572 the insertion of uptake hydrogenase genes *hupSL* and *hupW* from *Cyanothece* 51142
573 into the chromosome of the TSyNif-2 strain. (C) Comparison of nitrogen fixation activity
574 under different micro-oxic conditions. Samples were collected from cultures under 12h
575 light/dark conditions in $BG11_0$ medium. Nitrogen fixation activity was assayed by
576 acetylene reduction, and error bars represent the standard deviation of at least three
577 independent experiments.

**A.**

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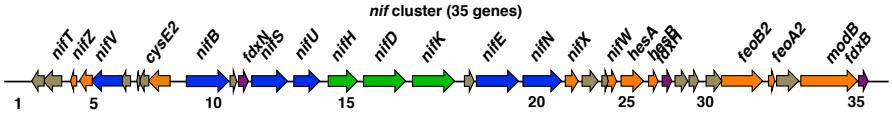
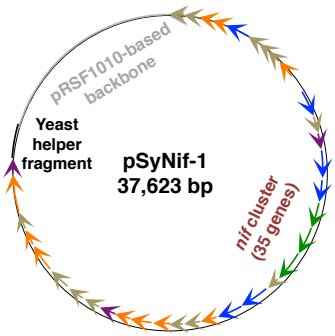



**C.**

| Gene | Role of gene product |
|---|---|
| *nifH* | Nitrogenase Fe protein (*γ* subunit), provides electrons and ATP for catalysis |
| *nifDK* | Nitrogenase MoFe protein (*α* and *β* subunits), catalyzes reduction of nitrogen to ammonia |
| *nifV* | Homocitrate synthase, involved in FeMo-cofactor synthesis |
| *nifB* | Nitrogenase cofactor biosynthesis protein, required for FeMo-cofactor synthesis |
| *nifS* | Cysteine desulfurase, transfers sulfur from cysteine to NifU |
| *nifU* | Fe–S cluster assembly scaffold protein |
| *nifEN* | FeMo-cofactor assembly proteins, putative function as a scaffold for synthesis of FeMo-cofactor |
| *nifT* | Unknown function, nitrogen fixation related protein |
| *nifZ* | Putative Fe-S cofactor synthesis protein |
| *cysE2* | Serine *O*-acetyl transferase, involved in biosynthesis of cysteine, a sulfur source of Fe–S cluster biosynthesis |
| *nifX* | Putative involved in FeMo-cofactor synthesis |
| *nifW* | Putative involved in stability of MoFe protein |
| *hesA* | Putative molybdenum cofactor biosynthesis |
| *hesB* | Putative involved in Fe-S cluster biosynthesis |
| *feoB2* | Ferrous iron transport protein B |
| *feoA2* | Ferrous iron transport protein A |
| *modB* | Putative molybdate ABC transporter |
| *fdxN* | 4Fe-4S ferredoxin |
| *fdxH* | 2Fe-2S ferredoxin |
| *fdxB* | 4Fe-4S ferredoxin |

**D.**




**E.**

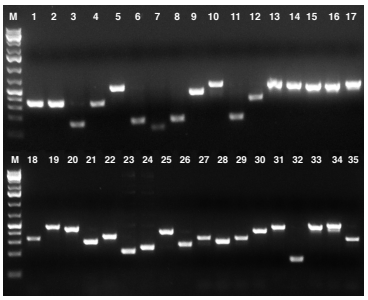



**FIG 1**

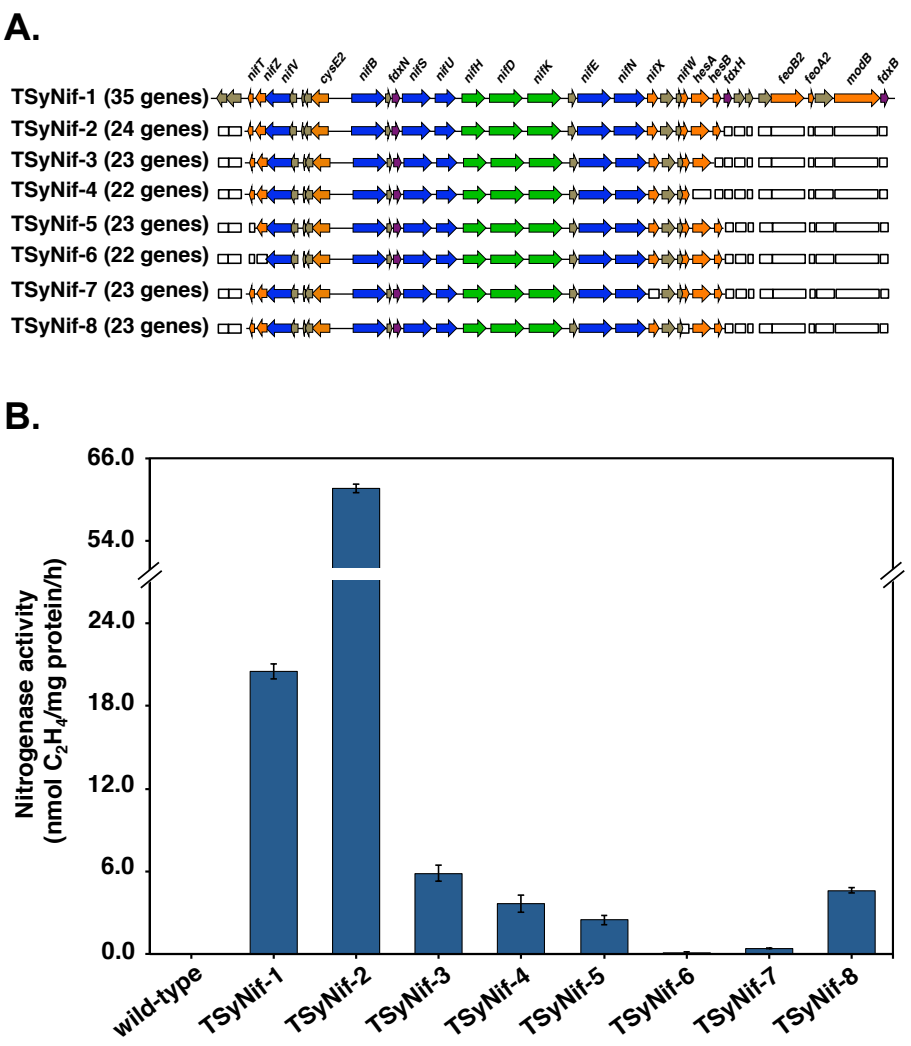



**FIG 2**

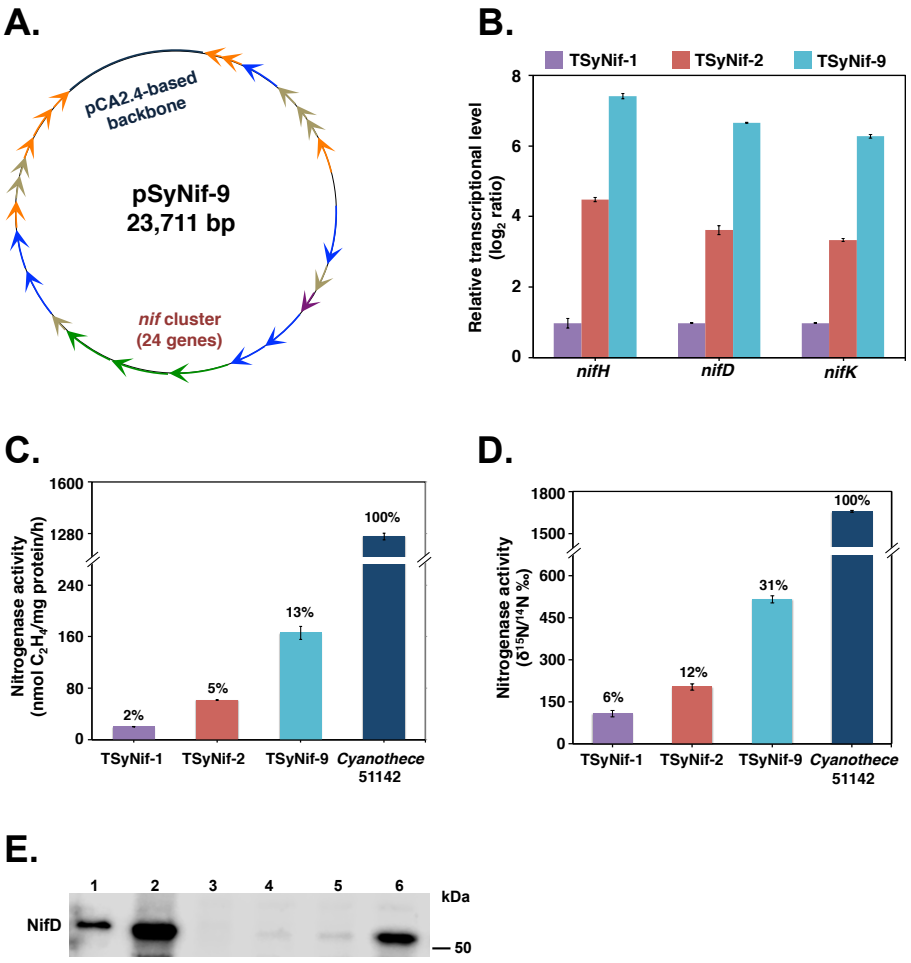



**FIG 3**

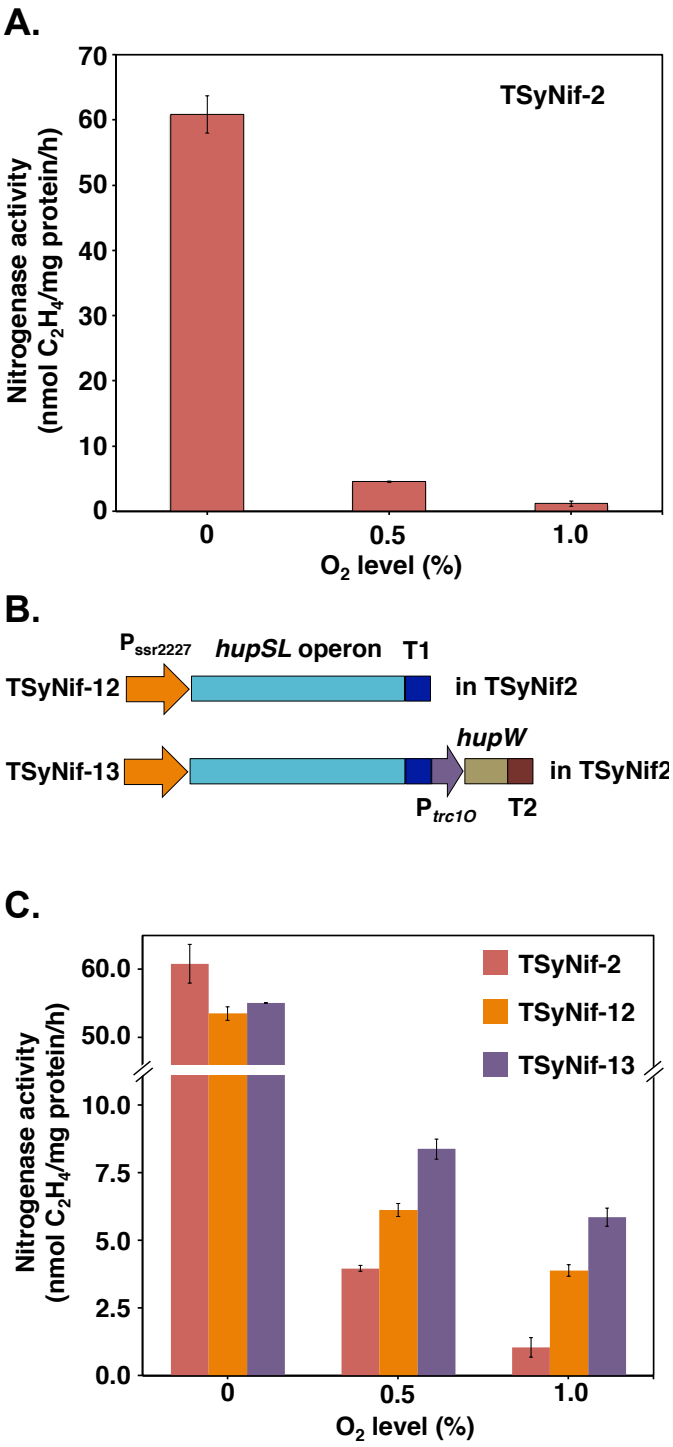



**FIG 4**