

# The sRNA NsiR4 is involved in nitrogen assimilation control in cyanobacteria by targeting glutamine synthetase inactivating factor IF7

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## Abstract

Glutamine synthetase (GS), a key enzyme in biological nitrogen assimilation, is regulated in multiple ways in response to varying nitrogen sources and levels. Here we show a small regulatory RNA, NsiR4 (nitrogen stress induced RNA 4), which plays an important role in the regulation of GS in cyanobacteria. NsiR4 expression in the unicellular *Synechocystis* sp. PCC 6803 and in the filamentous, nitrogen-fixing *Anabaena* sp. PCC 7120 is stimulated through nitrogen-limitation via NtcA, the global transcriptional regulator of genes involved in nitrogen metabolism. NsiR4 is widely conserved throughout the cyanobacterial phylum, suggesting a conserved function. *In silico* target prediction, transcriptome profiling upon pulse overexpression and site-directed mutagenesis experiments using a heterologous reporter system showed that NsiR4 interacts with the 5'UTR of *gifA* mRNA, which encodes glutamine synthetase inactivating factor IF7. In *Synechocystis*, we observed an inverse relationship between the levels of NsiR4 and the accumulation of IF7 *in vivo*. This NsiR4-dependent modulation of *gifA* (IF7) mRNA accumulation influenced the glutamine pool and thus NH<sub>4</sub><sup>+</sup> assimilation via glutamine synthetase. As a second target, we identified *ssr1528*, a hitherto uncharacterized nitrogen-regulated gene. Competition experiments between wild type and an NsiR4 knock-out mutant showed that the lack of NsiR4 led to decreased acclimation capabilities of *Synechocystis* towards oscillating nitrogen levels. These results suggest a role for NsiR4 in the regulation of nitrogen metabolism in cyanobacteria, especially for the adaptation to rapid changes in available nitrogen sources and concentrations. NsiR4 is the first identified bacterial sRNA regulating the primary assimilation of a macronutrient.

**Key words:** regulatory RNA, *Synechocystis*, nitrogen assimilation, glutamine synthetase inactivating factors

## **Significance Statement**

Inorganic nitrogen assimilation is primarily performed through phototrophic organisms that donate organic nitrogen to heterotrophic organisms. A key enzyme in this process, glutamine synthetase, is the target of multiple regulatory mechanisms. Here we describe NsiR4, a small regulatory RNA that reduces the expression of IF7, an inhibitory factor of glutamine synthetase in cyanobacteria. The expression of NsiR4 is under positive control through the transcription factor NtcA. NtcA also induces the transcription of the glutamine synthetase gene and represses the gene encoding IF7. Therefore, NsiR4 is a new player in the NtcA-mediated regulation of nitrogen assimilation, which is important for adaptations to rapid changes in available nitrogen sources and concentrations.

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## Introduction

Cyanobacteria originated oxygenic photosynthesis and are of substantial morphological and physiological diversity. In addition to the photosynthetic fixation of inorganic carbon, some cyanobacteria also fix atmospheric dinitrogen, hence injecting combined nitrogen sources into the biogeochemical cycles. Therefore, cyanobacteria play an important role in global carbon and nitrogen cycles (1, 2). Unicellular strains, such as the non-diazotrophic *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803), and multicellular strains with differentiated cells, such as the diazotrophic *Anabaena* sp. PCC 7120 (hereafter *Anabaena* 7120), have been established as widely accepted model organisms.

**Nitrogen Assimilation in Cyanobacteria.** Cyanobacteria utilize different inorganic and organic sources of combined nitrogen, such as ammonium ( $\text{NH}_4^+$ ), nitrate ( $\text{NO}_3^-$ ), nitrite ( $\text{NO}_2^-$ ) and urea, or some amino acids (3–6). Intracellularly,  $\text{NO}_3^-$ ,  $\text{NO}_2^-$  and urea are converted to  $\text{NH}_4^+$ . The availability of  $\text{NH}_4^+$ , the energetically most favorable nitrogen source, represses the uptake of other nitrogen sources (7). As in eukaryotic algae and plants, the key enzyme of nitrogen assimilation is glutamine synthetase (GS, encoded by *glnA*), which incorporates  $\text{NH}_4^+$  into metabolism via the amidation of glutamate to glutamine. Subsequently, glutamate synthase (glutamine oxoglutarate amidotransferase, GOGAT) catalyzes the transfer of the amide group from glutamine to 2-oxoglutarate (2-OG), producing two molecules of glutamate. The post-translational inactivation of GS is a major regulatory mechanism to limit nitrogen assimilation and maintain C/N balance. Whereas in *E. coli* and most other Gram-negative bacteria, GS activity is modulated through adenylation/deadenylation (8), cyanobacteria have evolved a unique mechanism of GS inactivation mediated through the small inhibitory proteins (inactivating factors) IF7 and IF17, encoded by the genes *gifA* and *gifB*, respectively (9). In addition to its function as substrate of the GS-GOGAT cycle, 2-OG is also a metabolite of the TCA cycle, connecting nitrogen and carbon metabolism at a central point. Depending on the nitrogen or carbon availability, the level of 2-OG varies, making this metabolite an excellent indicator of nitrogen status and the C/N ratio (10, 11).

**The Nitrogen Regulatory Network in Cyanobacteria.** At the molecular level, 2-OG regulates the activity of the main transcriptional regulators of nitrogen assimilation (NtcA, (12, 13))

and carbon metabolism (NdhR/CcmR, (14)). Moreover, 2-OG also modulates the activity of two other regulators of cyanobacterial nitrogen metabolism: the signal transduction protein P<sub>II</sub> and the regulatory factor PipX. Depending on the 2-OG level, which reflects the nitrogen status, PipX interacts with either NtcA or P<sub>II</sub> (15, 16). For a schematic overview of the nitrogen regulatory network, see **Appendix, Figure S1**.

The nitrogen control transcription factor NtcA binds as a homodimer to the conserved palindromic sequence GTA-N<sub>8</sub>-TAC (17–20). Under nitrogen excess (e.g., in presence of NH<sub>4</sub><sup>+</sup>), the 2-OG level is low and NtcA is present in an inactive form, which has low affinity to its target promoters (21). Under nitrogen depletion, however, the 2-OG level increases and stimulates the complex formation between PipX and NtcA and the affinity of the binding of this complex to target promoters (22, 23). Depending on the location of the binding motif relative to the transcriptional start site (TSS), NtcA can act as either an activator or repressor (24). NtcA activates the expression of genes that are important when nitrogen is limiting, such as nitrogen uptake systems (e.g., *amt1*) and components for NO<sub>3</sub><sup>-</sup> (e.g., *nirA*) and NH<sub>4</sub><sup>+</sup> assimilation (e.g., *glnA*) (18). In contrast, upon nitrogen depletion, NtcA acts as a repressor of *gifA* and *gifB*, which encode the GS IFs (24).

**Bacterial Small RNAs with Regulatory Potential.** Bacterial small RNAs (sRNAs) can post-transcriptionally activate or repress gene expression (25). Genome-wide mapping of TSSs revealed a high number of potentially regulatory sRNAs in cyanobacteria (19, 26, 27). One of sRNAs, the light-regulated and widely conserved sRNA PsrR1, is a key regulator of photosynthesis (28).

The expression of some sRNAs increases in nitrogen-limited cells of *Anabaena* 7120 or *Synechocystis* 6803, qualifying these molecules as potential regulators within the nitrogen regulatory network. Accordingly, these sRNAs were named nitrogen stress induced RNAs (NsiR). The expression of NsiR1 is induced upon nitrogen depletion, specifically in cells of *Anabaena* 7120 that are differentiating as nitrogen-fixing heterocysts (29, 30). NsiR2 and NsiR3 have also been identified in *Anabaena* 7120 (19), but the functions of these molecules have remained enigmatic. NsiR4 was first computationally predicted in *Synechocystis* 6803 (31) and subsequently verified as an expressed sRNA (called SyR12) in the genome-wide mapping of TSSs (26). NsiR4 is a 70 nt transcript that becomes strongly induced under nitrogen depletion in *Synechocystis* 6803 (27).

Here we elucidated the molecular functions of NsiR4 through the identification of two different targets, the mRNAs of *gifA* and *ssr1528*, the latter encoding a conserved but uncharacterized protein. We present evidence for an additional layer of GS regulation via the direct interaction of NsiR4 with the 5'UTR of the mRNA of the GS inactivating factor IF7, affecting the expression of this molecule and thereby impacting GS activity and  $\text{NH}_4^+$  assimilation. Thus, NsiR4 is the first example of an sRNA controlling the assimilation of a macronutrient.

## Results

**NsiR4 is a Widely Conserved sRNA in Cyanobacteria.** The phylogenetic conservation of an sRNA is an indicator of functionality. Therefore, we investigated whether NsiR4 homologs are also detected within the more than 200 cyanobacterial genomes publicly available through the Joint Genome Institute (JGI) database in January 2015. Using BlastN, putative *nsiR4* homologs were identified in at least 38 additional genomes, including the closely related *Synechocystis* sp. PCC 6714 (hereafter referred to as *Synechocystis* 6714) (32, 33), more distantly related strains, such as *Synechococcus* sp. PCC 7002, or several *Anabaena* and *Nostoc* species (**Fig. 1A**). Homologs of NsiR4 were not found in  $\alpha$ -cyanobacteria (mainly marine *Prochlorococcus* and *Synechococcus*), *Thermosynechococcus*, *Gloeobacter* as well as some *Oscillatoria* (**Fig. S2**). We concluded that *nsiR4* homologs exist in representatives from all five morphological subsections but not in all cyanobacteria (34), suggesting a widely conserved function.

In addition to *Synechocystis* 6803, the expression of *nsiR4* was observed in the transcriptomic datasets available for *Synechocystis* 6714, *Anabaena* 7120 and *Synechococcus* sp. PCC 7002 (19, 35, 36). The genomic information and mapped TSSs indicated that two distinct NsiR4 forms of different lengths exist in different cyanobacteria. NsiR4 in strains with the longer form (e.g., *Synechocystis*, *Microcystis*, *Cyanothece*) possesses an additional 19-20 nt domain at the 5' end, capable to form a short hairpin (**Fig. 1A,B**). Otherwise, the predicted secondary structures appeared similar for both NsiR4 types, suggesting a potential role for the 16 nt single-stranded region, which is nearly identical in all NsiR4 sequences (**Fig. 1A,B**). Such regions facilitate interactions with the respective target mRNAs.

**NsiR4 Expression is Associated with the Nitrogen Status and Positively Regulated through NtcA.** NsiR4 is one of the most highly induced sRNAs upon nitrogen depletion in *Synechocystis* 6803 (27) and is the second-most abundant sRNA in the primary transcriptome (37). To determine the time course of induction and potential effects of different nitrogen sources, we measured NsiR4 expression in wild-type (WT) *Synechocystis* 6803 grown under standard conditions (nitrogen-replete, 17.6 mM  $\text{NO}_3^-$ ) and after transfer into nitrogen-free medium. NsiR4 was detected at a low level in cells grown in the presence of  $\text{NO}_3^-$ , started to accumulate at a higher level 12 h after removing  $\text{NO}_3^-$ . After 48-72 h, the expression was approximately 10-fold higher than at the start of the experiment (**Fig. 1C**, left panel). To examine the influence of the nitrogen source, three WT cultures were initially grown under standard conditions and then transferred to nitrogen-free medium. Into two of these cultures, we added 10 mM  $\text{NH}_4^+$  or 17.6 mM  $\text{NO}_3^-$ , cultivation was continued for 24 h, and the relative NsiR4 abundance was analyzed. Approximately 10-fold higher NsiR4 expression was observed in nitrogen-depleted cells compared with cells grown in the presence of  $\text{NO}_3^-$ . In contrast, in the presence of  $\text{NH}_4^+$ , NsiR4 levels remained below the detection limit (**Figure 1C**, right panel). We concluded that the expression of NsiR4 is influenced by the nitrogen status of the cell. Moreover, NsiR4 expression is also regulated through nitrogen in *Synechocystis* 6714 and *Anabaena* 7120 (19, 35), suggesting a conserved function associated with nitrogen metabolism.

To examine whether the nitrogen-regulated expression of NsiR4 is under transcriptional control, we conducted reporter gene assays. The upstream sequence of *nsiR4* from *Synechocystis* 6803 was fused to *luxAB* genes encoding luciferase, and expression was measured as bioluminescence *in vivo*. Indeed, the promoter activity showed similar kinetics in response to nitrogen depletion, as observed for the sRNA accumulation (**Fig. 2A**). In NtcA-activated promoters, NtcA-binding sites frequently overlap the -35 region and are centered close to position -41.5 with respect to the TSS (18, 19). Indeed, the sequence 5'-**GTCAAATAGGCTAC**-3', similar to the consensus for NtcA binding sites (17), is located 48-35 nt upstream the TSS of *nsiR4* (at pos. 1289326 on the complementary strand). Moreover, potential NtcA binding sites exist upstream of the putative *nsiR4* homologs in all other strains (**Figure 2C**). We examined the functionality of this site in a reporter gene assay comparing the WT promoter with a mutated version in which three nucleotides were substituted (**GTCAAATAGGCTAC** to **CTCAAATAGGCATC**). Indeed, the activation of *luxAB*

expression through nitrogen depletion was lost in the strain carrying the mutated promoter (**Fig. 2B**). Moreover, NsiR4 expression could not be activated in the nitrogen-depleted cells of an *Anabaena* 7120 *ntcA* mutant (**Fig. 2D**). These data demonstrate that the NsiR4 promoter is under NtcA control in both *Synechocystis* 6803 and *Anabaena* 7120.

**Advanced Target Prediction and Transcriptome Analyses Suggest *gifA* and *ssr1528* as NsiR4 Targets.** To predict potential targets of NsiR4, we used the CopraRNA algorithm, considering the folding, hybridization and conservation of a particular sRNA (38). The highest interaction probability was predicted for the *gifA* (*ss1911*) mRNA (**Table 1**). This gene encodes the GS inactivating factor IF7 and is also, but negatively, regulated through NtcA.

To identify potential target genes experimentally, we generated *Synechocystis* strains with altered NsiR4 expression, i.e., overexpression (NsiR4oex), knockout ( $\Delta$ *nsiR4*) and compensatory ( $\Delta$ *nsiR4::oex*) strains. To achieve inducible expression, we fused the NsiR4 sequence to the *petE* promoter, which is specifically activated in the presence of  $\text{Cu}^{2+}$ . The absence of NsiR4 in the knockout strain and the  $\text{Cu}^{2+}$ -dependent inducibility of NsiR4 in the other two strains were experimentally verified (**Fig. S3**).

The target-sRNA interaction frequently reduces mRNA stability (39). Thus, a microarray experiment was performed with RNA extracted from cells in which NsiR4 was pulse-expressed through the addition of  $\text{Cu}^{2+}$  for 12 h. Compared with WT, in NsiR4oex reduced *gifA* mRNA abundance was observed, for which an interaction was also predicted by the CopraRNA algorithm (compare **Table 1** & **Table S1**). Additionally, *gifA* expression was increased in  $\Delta$ *nsiR4*, but decreased upon introduction of the compensatory construct ( $\Delta$ *nsiR4::oex*) (**Table S1**). Another gene, *ssr1528*, exhibited a similar pattern, suggesting that this gene is a second target of NsiR4. The product of *ssr1528* is a hypothetical protein, with a domain (DUF4090) widely conserved among cyanobacteria (**Fig. S2**). Moreover, similar to *gifA*, the expression of this protein is repressed under nitrogen depletion (40), suggesting a function related to the cellular nitrogen status.

We speculated that the modulation of NsiR4 expression and its potential impact on nitrogen-associated target genes might also affect growth or the tolerance to high concentrations of  $\text{NH}_4^+$ . In short-term growth experiments strains with altered levels of NsiR4 showed no noticeable phenotype regarding  $\text{NH}_4^+$  tolerance (**Fig. S4**). However, in long-term growth competition experiments, especially when the availability of nitrogen is fluctuating, the



$\Delta nsiR4$  mutant strain showed reduced growth rates compared to WT (**Fig. 3**). These findings directly support the physiological importance of NsiR4 in addition to its evolutionary conservation, suggesting that the presence of NsiR4 has been under positive selection in cyanobacteria.

**The *ssr1528* Gene is Directly and Post-Transcriptionally Regulated through NsiR4.** The changes observed in the microarray experiment were verified through a detailed expression analysis. The expression of *ssr1528* was detected in WT under standard conditions (17.6 mM  $\text{NO}_3^-$ ), which was not significantly affected by the addition of  $\text{Cu}^{2+}$  (**Fig. 4A**). However, in strain NsiR4oex carrying an additional, *PpetE*-regulated *nsiR4* copy, *ssr1528* mRNA abundance was diminished upon NsiR4 induction. Consistently, *ssr1528* mRNA abundance increased in strain  $\Delta nsiR4$  compared to WT. In response to nitrogen depletion, the *ssr1528* mRNA levels decreased in WT and NsiR4oex, confirming the N-regulated expression. However, whereas the *ssr1528* mRNA disappeared in WT after 12 h N depletion, the expression of this gene remained at a high level in  $\Delta nsiR4$  (**Fig. 4A**). Moreover, *ssr1528* expression did not increase when 10 mM  $\text{NH}_4^+$  was added to the medium (**Fig. S5**), indicating that the decrease under N depletion is not directly NtcA-dependent and might be exclusively regulated through NsiR4 at the post-transcriptional level.

To examine whether a direct NsiR4:*ssr1528* interaction caused the observed changes in mRNA expression, the *ssr1528* 5'UTR was fused to the gene for the superfolder green fluorescent protein (*sgfp*) and co-expressed with NsiR4 in *E. coli*. GFP fluorescence was measured in strains carrying various combinations of plasmids (41). Compared with the control (pXG-0+pJV300), the strain carrying the *ssr1528-sgfp* fusion showed significant GFP fluorescence, demonstrating that the translation initiation from the *ssr1528* 5'UTR of *Synechocystis* is also functional in *E. coli* (**Fig. 4B**). In the presence of the NsiR4-expressing plasmid, the GFP fluorescence decreased approximately 2-fold, indicating a direct interaction between NsiR4 and the *ssr1528*-5'UTR, which affects translation. To verify the interaction at the predicted site, point mutations were introduced in *ssr1528* (pos. +22 and +23: UA>AU, +1 = TSS) or NsiR4 (pos. +16 and +17: UA>AU, **Fig. 4D**). Indeed, the mutation of either one of these sequences diminished the interaction, indicated as reduced repression (**Fig. 4C**). However, the combination of both mutations restored repression (both mutations

are complementary, **Fig. 4C**). These data confirmed the direct interaction of NsiR4 with *ssr1528* mRNA, which also potentially affects the translation of this protein in *Synechocystis*.

**The *gifA* Gene is Directly and Post-Transcriptionally Regulated through NsiR4.** The *gifA* mRNA was suggested as a direct NsiR4 target through computational prediction and microarray analysis. Similar to *ssr1528*, we performed detailed expression analysis for *gifA* in response to  $\text{Cu}^{2+}$  (for overproduction in NsiR4oex) and N depletion (induction of native *nsiR4* expression) or increased  $\text{NH}_4^+$  availability (for repressing native *nsiR4* and de-repressing *gifA*). As observed for *ssr1528*, *gifA* was expressed in WT strains under standard conditions (17.6 mM  $\text{NO}_3^-$ ) and was not significantly affected when adding  $\text{Cu}^{2+}$  (**Fig. 5A**). However, compared to WT, the *gifA* mRNA level was clearly diminished in NsiR4oex (**Fig. 5A**) and increased in strain  $\Delta\textit{nsiR4}$ . In response to N depletion and due to negative regulation through NtcA, the *gifA* mRNA levels decreased in a similar manner in all strains. However, in response to elevated  $\text{NH}_4^+$  concentrations, the differences between the strains became more evident (**Fig. 5B**). The strong accumulation of *gifA* mRNA, which is typical for WT, was not observed in NsiR4oex, indicating the suppression by high levels of NsiR4. Consistent with previous studies (9), the mRNA induction in WT was rather transient, peaking after 3-6 h. Compared with WT, the maximum *gifA* mRNA level was approximately 6-fold lower in NsiR4oex and approximately 40% higher in  $\Delta\textit{nsiR4}$ . In all cases, the increased levels in  $\Delta\textit{nsiR4}$  were abolished when NsiR4 was ectopically expressed (compensatory strain  $\Delta\textit{nsiR4}::\textit{oex}$ ).

To verify the interaction, the *gifA* 5'UTR was fused to the *sgfp* gene and co-expressed with NsiR4, as done before for *ssr1528*. In the presence of NsiR4, GFP fluorescence decreased 2-3-fold, indicating a direct interaction between NsiR4 and the *gifA*-5'UTR, which affects protein synthesis (**Fig. 5C**). The IntaRNA algorithm (42) predicted the interaction site between NsiR4 and the *gifA* mRNA at positions +28 to +57 nt with respect to the TSS. This site directly overlaps with the *gifA* start codon at positions +52 to +54 (**Fig. 5E**). To verify the interaction at this site, we introduced point mutations in either *gifA* (pos. +30: C>T) or NsiR4 (pos. +31: G>A, **Fig. 5E**). The mutation of either one of these sequences prevented the interaction, whereas the combination of these two mutations, which are complementary, restored repression (**Fig. 5D**). Thus, the direct interaction between both RNAs at the predicted site was confirmed.

The results of the *E. coli* reporter assays suggested a negative impact of NsiR4 on the protein level. To investigate whether the level of IF7 is affected through ectopic NsiR4 expression also in *Synechocystis* 6803, we performed Western blot analysis using antibodies specific for IF7 (and IF17, which should not be influenced through NsiR4). The cells were supplemented with 10 mM  $\text{NH}_4^+$  to de-repress *gifA* expression and induce IF7 accumulation, which was observed in WT as expected (**Fig. 6A**). However, in strain NsiR4oex, the IF7 protein level was lower compared with WT and was not stimulated through  $\text{NH}_4^+$  addition. The IF17 accumulation was barely affected in this strain. In contrast, in  $\Delta\text{nsiR4}$ , strong IF7 accumulation was observed after adding  $\text{NH}_4^+$ , and the steady-state level was approximately 60% higher than in WT, whereas the levels of IF17 were similar to those in the WT experiment. Consistently, this effect was compensated in strain  $\Delta\text{nsiR4}::\text{NsiR4oex}$ , yielding a similar pattern as observed in WT (**Fig. 6B**).

**The Kinetics of Glutamine/Glutamate Levels Suggest an Indirect Impact of NsiR4 on GS Activity and  $\text{NH}_4^+$  Assimilation.** The inactivation of GS upon increased nitrogen supply correlates with the presence of inactivating factors IF7 and IF17 (9). Therefore, the post-transcriptional regulation of *gifA* through NsiR4 could influence GS activity and modulate the rate of nitrogen entry into metabolism. To examine whether the modified *gifA* expression in the *nsiR4* mutant strains also affects GS activity, we examined the substrate and product kinetics after a sudden upshift of the external  $\text{NH}_4^+$  concentration. In cyanobacteria, a rapid but transient change in the intracellular amino acid and 2-OG pools occurs upon changes in nitrogen availability (10).

The primary reaction of  $\text{NH}_4^+$  assimilation is consuming glutamate and producing glutamine. In all strains used here the internal pools of both amino acids strongly changed within minutes after adding 10 mM  $\text{NH}_4^+$ , i.e., the levels of glutamine (produced by GS) increased and glutamate (consumed by GS) decreased. Moreover, the typical restoration of the glutamine/glutamate pool could also be observed, and the steady state levels at 24 h after  $\text{NH}_4^+$  addition were similar in all examined strains. However, in  $\Delta\text{nsiR4}$ , which showed the rapid accumulation of IF7 protein compared with WT (**Fig. 6**), the glutamine level also declined more rapidly (**Fig. 7**). Thus, the higher IF7 level in this strain indeed significantly contributed to GS inactivation. Additionally, an inverse tendency was observed for the glutamate pool, again with differences between WT and  $\Delta\text{nsiR4}$ . However, the kinetics of

both strains harboring the plasmid for ectopic  $\text{Cu}^{2+}$ -regulated expression was similar to WT. This result indicates that the altered rate of glutamine/glutamate pool restoration in  $\Delta\text{nsiR4}$  is compensated through NsiR4 expression. However, the overexpression of NsiR4 and delayed accumulation of IF7 (**Fig. 6**) only slightly impacted GS inactivation, which is additionally achieved through the accumulation of the other inactivating factor IF17 (not affected through altered NsiR4 expression). Interestingly, in addition to the effects on GS and the glutamine/glutamate pool, we also observed differences in response to  $\text{NH}_4^+$  for another amino acid, arginine (**Fig. 7C,D**), when NsiR4 was overexpressed. The changes in the arginine pool appeared delayed compared with the changes in glutamine levels. Moreover, in contrast to glutamine and glutamate, the arginine steady state levels (24 h after  $\text{NH}_4^+$  addition) were also enhanced in the strains expressing NsiR4 ectopically.

## Discussion

**Nitrogen-Regulated sRNAs in Bacteria.** To date, little is known about nitrogen-regulated sRNAs in bacteria. In *Pseudomonas aeruginosa*, the nitrogen-regulated sRNA NrsZ controls the production of rhamnolipid surfactants, as virulence factors required for swarming motility, via the post-transcriptional control of the rhamnolipid synthesis gene *rhlA* (43). The expression of NrsZ is induced through the NtrB/C two component system and is highly conserved among *Pseudomonads* (43). Other sRNAs are indirectly associated with nitrogen metabolism. In many  $\gamma$ -proteobacteria, GcvB acts as a distinct regulatory factor in nitrogen metabolism through the repression of transporters involved in amino acid uptake (44). In cyanobacteria, several 'nitrogen stress-induced RNAs' (NsiR1, NsiR2 and NsiR3) have been described in *Anabaena* 7120 (19, 29), but their functions remain elusive. In the present study, we demonstrate NsiR4 to be directly involved in the regulation of primary nitrogen assimilation.

**NsiR4 Executes the Post-Transcriptional Regulation of Glutamine Synthetase Inactivating Factor IF7 and Interferes with  $\text{NH}_4^+$  Assimilation.** The expression and activity of GS is controlled through the nitrogen status in various ways. The main physiological function of the GS inactivation system in cyanobacteria is to restore amino acid homeostasis upon strong changes in the availability of nitrogen (10). Due to the central role of GS, similar responses to nitrogen changes, such as a decrease in the glutamine pool under nitrogen

starvation, were observed, even in microorganisms as different as *E. coli* and yeast (45). Consistently, a transient increase in glutamine was observed in *E. coli* after the addition of 10 mM  $\text{NH}_4^+$  (46). Interestingly, a  $\Delta gifA\Delta gifB$  double mutant of *Synechocystis* 6803 showed no GS inactivation and lacked the ability to rapidly restore the glutamine pool to initial levels upon  $\text{NH}_4^+$  addition (10). Similarly, in strains bearing a mutant GS protein not susceptible to inactivation through IFs, the same phenotype was observed concerning the restoration of the glutamine pool (47). Because the inactivation of GS requires adequate levels of IF7 and/or IF17 (9), the post-transcriptional regulation of *gifA* through NsiR4 impacts GS activity and hence should codetermine the rate of nitrogen entry into metabolism. Indeed, this assumption is consistent with the rate of glutamine decrease in  $\Delta nsiR4$  observed in the present study (**Fig. 7**). However, IF7 is assumed to have a lower impact on GS inactivation compared with IF17, as GS activity was still reduced to 50% in a  $\Delta gifA$  single mutant in response to excess  $\text{NH}_4^+$ , whereas in the  $\Delta gifB$  single mutant, GS maintained approximately 90% activity (9). These data are consistent with the findings of the present study, as the strain overexpressing NsiR4 (showing reduced IF7 levels) did not show altered glutamine/glutamate kinetics compared with WT because GS inactivation was primarily influenced through the remaining IF7 protein, and IF17 protein levels were not affected. Nevertheless, the evolutionary conservation of NsiR4 and *gifA* indicates that the regulatory interaction of these two molecules is relevant. The changed glutamine kinetics in response to  $\text{NH}_4^+$  excess in the *nsiR4* knockout strain highlights the physiological significance of NsiR4-mediated modulation of the IF7 expression level on GS activity. Assuming that the interaction with NsiR4 reduces the half-life of the *gifA* mRNA, this post-transcriptional mechanism might be faster and more effective for reducing the IF7 protein content and increasing GS activity under nitrogen limitations. Moreover, even subtle changes in GS regulation have a significant impact on the fitness of *Synechocystis* 6803 cells exposed to nitrogen pulses, because the WT with intact IF7-dependent GS regulation outcompeted the  $\Delta nsiR4$  mutant under fluctuating nitrogen conditions, which are characteristic for natural environments.

Interestingly, in addition to the effects on GS and the glutamine/glutamate pool, we also observed differences in response to  $\text{NH}_4^+$  for another amino acid, arginine. However, it is unclear whether this effect is secondary in response to the changed GS activity or highlights a role for Ssr1528 or additional as-yet unidentified targets of NsiR4 (see below).

Nonetheless, the changed arginine pools support a relevant role for NsiR4 in the nitrogen regulatory network, as the N-acetylglutamate kinase, involved in arginine synthesis, is another key enzyme in the nitrogen assimilation network controlled through the regulatory P<sub>II</sub> protein (48).

**Distribution of NsiR4 among Cyanobacteria and Evolution of Target Sets.** In addition to *gifA*, NsiR4 might regulate additional targets (**Table 1**), similar to many other sRNAs, e.g., the cyanobacterial sRNA PsrR1, which regulates a set of photosynthesis genes, thereby executing a global regulatory function (28). In the NsiR4oex strain, decreased *ssr1528* gene expression was observed, whereas increased *ssr1528* gene expression was observed in the  $\Delta$ *nsiR4* knockout strain. The changed expression and the direct interaction between NsiR4 and *ssr1528* were verified in the present study, extending the NsiR4 target set to at least two genes. Unfortunately, the function of *ssr1528* is not known. This gene encodes a small protein of 94 amino acids with a widely conserved domain (DUF4090) among cyanobacteria (**Fig. S2**). Moreover, *ssr1528* expression was repressed under N depletion, similar to *gifA*, suggesting a function associated with the cellular nitrogen status.

Although the microarray analysis of strains with altered sRNA expression has proven valuable for functional studies of sRNA regulators in enterobacteria (39) and cyanobacteria (28), additional targets might have been missed. This approach is limited when a gene is not expressed under the investigated growth conditions, i.e., some targets might be only expressed in N-depleted cells. Furthermore, the regulation through sRNAs at the posttranscriptional level does not necessarily lead to a significant change in the accumulation of the target mRNAs. Therefore, some of the other predicted targets might also represent interaction partners for NsiR4.

NsiR4 is present only in cyanobacteria, suggesting that its target set might also be restricted to these organisms. Indeed, GS inactivation via inhibitory proteins is specific for cyanobacteria, and thus, *gifA*, the main NsiR4 target, is only present in this phylum. Moreover, the presence of *ssr1528* homologs is also restricted to cyanobacteria. However, a direct correlation between the occurrence of NsiR4 and *gifA* or *ssr1528* was not observed (**Fig. S2**). Moreover, in many strains, *nsiR4* is associated with a gene encoding a conserved but unknown protein (DUF362). The DUF362 domain has been associated with iron-sulfur clusters. However, the functional association of this domain with NsiR4 remains elusive.

**NsiR4 Constitutes a Novel Regulatory Element within the NtcA-Controlled Network.** The assimilation of  $\text{NH}_4^+$  through GS is the port-of-entry for inorganic nitrogen as  $\text{NH}_4^+$  into cellular metabolism. Many of the components involved in nitrogen assimilation including the GS encoding gene *glnA* are controlled through NtcA (18, 24). In the present study, we show strong evidence that NsiR4 expression is activated through NtcA, and accordingly the expression of NsiR4 increases when nitrogen sources are limiting. The dependence on NtcA underlines the importance of NsiR4, consistent with the impact NsiR4 has on  $\text{NH}_4^+$  assimilation and the amino acid pool. The joint regulation of *gifA* through NtcA and NsiR4 establishes a coherent negative feed-forward loop (**Fig. 8**). This type of regulatory motif results in a delayed response to the transcription factor offset (49). Mixed feed-forward loops involving transcription factors and sRNAs are frequent regulatory elements in bacterial gene regulation and have also been described or discussed for other sRNAs, e.g., Spot42 or FnrS (38, 50). A delay in GS inactivation might be of importance in habitats showing strong fluctuations in nitrogen availability.

Through the regulation of IF7, a GS inactivating factor, NsiR4 influences  $\text{NH}_4^+$  assimilation. The mechanism described in the present study represents a regulatory node upstream of amino acid synthesis. With these features, NsiR4 is the first analyzed sRNA for the regulation of macronutrient assimilation in bacteria.

## Materials and Methods

**Strains and growth conditions.** *Synechocystis* 6803 strain GT-Kazusa was grown in  $\text{Cu}^{2+}$ -free BG11 (34) buffered with 20 mM TES, pH 8.0, at 30°C at 50–80  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  of white light. To induce nitrogen deficiency, cells were spin down by centrifugation and resuspended in  $\text{NO}_3^-$ -free BG11. *Anabaena* 7120 WT and the *ntcA* mutant strain CSE2 (51) were grown in  $\text{CO}_2$ -enriched air, 1% v/v, in BG11 without  $\text{NO}_3^-$  and supplemented with 6 mM  $\text{NH}_4\text{Cl}$ , 12 mM TES-NaOH (pH 7.5) and 10 mM  $\text{NaHCO}_3$ . The generation of NsiR4 mutants and further growth conditions are described in the **SI Appendix** in detail.

**Genome and promoter analysis and computational prediction of NsiR4 targets.** The 70 nt sequence of *Synechocystis* NsiR4 (pos. 1289326 – 1289257, complementary strand) was used to search the JGI database. To verify *nsiR4* promoter activity *in vivo* in *Synechocystis*, the nucleotides -130 to +49 (TSS at +1) were fused to *luxAB* reporter genes. The selection of the

reporter strains and bioluminescence measurements were performed as described (52). Target prediction was performed using CopraRNA (38, 42) with the NsiR4 homologs of *Synechocystis* 6803, *Microcystis aeruginosa* NIES-843, *Cyanothece* sp. ATCC 51142, *Cyanothece* sp. PCC 7424, *Cyanothece* sp. PCC 7822, *Synechococcus* sp. PCC 7002, *Pleurocapsa* sp. PCC 7327 and *Stanieria cyanosphaera* PCC 7437 and default settings.

**RNA extraction, microarrays and Northern blots.** The collection of *Synechocystis* cells, the RNA extraction, DNase treatment, labeling and hybridization to microarrays was performed as previously described (55, 56, 57). For Northern hybridization, *Synechocystis* 6803 RNA was separated on denaturing agarose gels, transferred to Hybond-N<sup>+</sup> membranes (Amersham, Germany) and hybridized as previously described (55).

**Protein extraction and immunoblots.** For analysis of IF accumulation, 2  $\mu$ M CuSO<sub>4</sub> was added to induce the *petE* promoter eight hours before addition of 10 mM NH<sub>4</sub>Cl and 20 mM TES-NaOH (pH 7.5). Extracts were prepared using glass beads as previously described (56). Proteins were fractionated by 15% SDS-PAGE and immunoblotted with anti-IF7 (1:2000), anti-IF17 (1:2000) or anti-TrxA (1:3000). Anti-IF7, anti-IF17 and anti-TrxA antisera were obtained from M.I. Muro-Pastor and F.J. Florencio and used as described (57, 58).

**Reporter assays for the *in vivo* verification of targets.** We used the reporter system described before (59) with sGFP plasmid pXG-10-SF (41). The primers used for cloning and the resulting plasmids are given in **Tables S2 and S3**. Further details of the generation of tested constructs can be found in the **SI Appendix**.

**Quantification of amino acids.** Cells were grown in 100 ml BG11 containing 17.6 mM NO<sub>3</sub><sup>-</sup>. After reaching an OD<sub>750</sub> of ca. 0.8, 2  $\mu$ M CuSO<sub>4</sub> and after further 24 h 10 mM NH<sub>4</sub>Cl were added. Samples of 2 ml were taken prior to and in a narrow time series after adding ammonia. Samples were shortly centrifuged and cell pellets frozen in liquid nitrogen. Free amino acids were extracted from frozen cyanobacterial cells with 80% ethanol at 65°C for 3 h. After centrifugation, the extraction was repeated for the remaining cell pellet. Afterwards, both supernatants were combined, dried in a vacuum centrifuge and re-dissolved in sample buffer. Individual amino acids were quantified via HPLC (60).



## **Author contributions**

S.K., J.G., A.M.M.-P. & W.R.H. designed and supervised the study. J.G. & C.S. performed genome analysis and target prediction. S.K. & J.G. generated the *Synechocystis nsiR4* mutants. S.K., D.B. & G.K. performed promoter analysis. C.S. & A.M.M.-P. performed northern blots. A.M.M.-P. performed western blot experiments. C.S. performed target verification experiments. M.H. contributed the amino acid data. S.K. performed the microarray data analysis, growth competition assay and prepared all figures. S.K., A.M.M.-P. & W.R.H. evaluated and interpreted the data and drafted the manuscript with contributions from all authors. All authors proofread the manuscript.

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## Figure Legends

### **Fig. 1: The nitrogen-stress induced RNA 4 (NsiR4) is broadly conserved in cyanobacteria.**

**A:** Alignment of genome loci putatively encoding NsiR4. Verified TSSs are boxed in red (19, 26, 35). Roman Numerals indicate the respective morphological subsections (34). **B:** Conservation of the NsiR4 structure. The consensus structure was predicted using RNAalifold (61) and 30 randomly selected NsiR4 homologs of both types. The short hairpin was predicted for the 5' extension of long NsiR4 forms. The color indicates the number of different interacting pairs (C-G, G-C, A-U, U-A, G-U or U-G) and thereby conservation of these base pairs. The saturation increases with the number of compatible base pairs, thus indicating the structural conservation. **C:** Nitrogen-responsive expression of NsiR4 in *Synechocystis* 6803. Cells were transferred from standard conditions (17.6 mM  $\text{NO}_3^-$ ) to  $\text{NO}_3^-$ -free BG11, total RNA was separated, blotted and hybridized with  $^{32}\text{P}$ -labeled, single-stranded RNA probes. Left panel: Expression kinetics of NsiR4 in response to N-depletion. Right panel: Steady-state levels in response to the nitrogen status mediated through different nitrogen sources or the depletion of N. Cells grown in the presence of  $\text{NO}_3^-$  were washed and resuspended in media containing 17.6 mM  $\text{NO}_3^-$ , 10 mM  $\text{NH}_4^+$  or no nitrogen source. The cells were grown for an additional 24 hours before RNA extraction.

### **Fig. 2. NsiR4 expression is mediated through an NtcA-activated promoter.**

**A:** Bioluminescence of a *Synechocystis* reporter strain harboring a transcriptional fusion of *PnsiR4* (-130 to +49, TSS at +1) and *luxAB* genes in response to N depletion. Initially, cells were grown under standard conditions (17.6 mM  $\text{NO}_3^-$ ) and subsequently transferred to  $\text{NO}_3^-$ -free BG11 medium. **B:** Bioluminescence of a *Synechocystis PnsiR4::luxAB* reporter strain bearing a mutated NtcA motif in the presence of 17.6 mM  $\text{NO}_3^-$  (+N) or under N-depletion for 24 h (-N). To improve and compare bioluminescence mediated through both promoters under +N, 10 mM glucose was added to the cultures. Thus, the values are higher than in panel A. Bioluminescence data are presented as the means  $\pm$  SD of  $n$  independent measurements in at least two independent experiments, including two biological replicates (= independent transformants). In each experiment, a strain carrying a promoterless *luxAB*

was used as a negative control (in each case measured in two independent cultures,  $n=2$ ). **C:** The sequences upstream of *nsiR4* from different cyanobacteria. Putative NtcA binding sites and -10 elements are highlighted. The verified TSSs are shown in red. **D:** Verification of N-regulated and NtcA-dependent NsiR4 expression in *Anabaena* 7120 WT and an *ntcA* insertion mutant through northern blot analysis.

**Fig. 3. Competitive growth analysis of WT and  $\Delta nsiR4$ .** **A:** Experimental setup. Three independent, exponentially growing cultures of WT and  $\Delta nsiR4$  were diluted to an  $OD_{750}$  of 0.1 and mixed 1:1. **B:** Growth performance during long-term cultivation. Cultures were re-diluted every 3 or 4 days to an  $OD_{750}$  of 0.1 and 25  $\mu$ l of a 1:1000 dilution were dropped on BG11 agar plates with and without 40  $\mu$ g/ml kanamycin. Data are the mean  $\pm$  SD of WT and  $\Delta nsiR4$  as well as three mixed WT/ $\Delta nsiR4$  co-cultures. Due to the lowered  $NO_3^-$  content (1 mM instead of 17.6 mM  $NO_3^-$ ), a strong fluctuation in the cellular N status could be assumed, which was also indicated by the cultures switching between green or bleached appearance at lower (0.1-0.8) and higher ( $>0.8$ ) ODs (see also C). **C:** Whole cell absorption spectra of the WT culture indicating cellular bleaching was due to pigment degradation as response to initiated internal N limitation. **D:** Number of generations that were observed over 50 days at average growth rates of  $\mu = 0.751 \pm 0.065$ . **E:** Evolution of the ratio between kanamycin-resistant ( $\Delta nsiR4$ ) and total (WT +  $\Delta nsiR4$ ) colony forming units (CFU) during consecutive cultivation and re-dilution. **F:** Photographs of CFUs at the beginning of the experiment and after 33 and 48 generations (g). **G:** Verification of the  $\Delta nsiR4$  mutant allele reduction (upper band) compared to the WT allele (lower band) by PCR. \*To one of the WT/ $\Delta nsiR4$  co-cultures once per day 100  $\mu$ M  $NH_4Cl$  (f.c.) were added to intensify the fluctuation in the N availability. However, no change in growth performance compared to the other two co-cultures was observed. Thus, all three co-cultures were averaged as shown in panel E.

**Fig. 4. Verification of the post-transcriptional regulation of *ssr1528* through direct NsiR4 interaction.**

**A:** Changes in the mRNA abundance of *ssr1528* in response to altered NsiR4 expression detected by hybridization with a single-stranded  $^{32}P$ -labeled transcript probe. Nitrogen



depletion was induced in cultures grown in the presence of 2  $\mu\text{M}$   $\text{Cu}^{2+}$  for 48 h (= 0 h -N). A representative 5S rRNA loading control hybridization (for WT) is shown. WT - *Synechocystis* 6803 wild type, NsiR4oex – strain carrying pVZ322-PpetE::nsiR4::oop plasmid (overexpressor),  $\Delta\text{nsiR4}$  - deletion mutant,  $\Delta\text{nsiR4}::\text{oex}$  - deletion strain in which NsiR4 expression was restored by the pVZ322-PpetE::nsiR4::oop plasmid. **B:** GFP fluorescence measurements of *E. coli* TOP10 strains with various combinations of plasmids expressing NsiR4 or *ssr1528-sgfp*-fusions. The plasmids pXG-0 (encoding luciferase) and pJV300 (encoding a control RNA) were used as negative controls (for experimental details see (41)). **C:** Repression was calculated by dividing the values measured for *ssr1528-sgfp* in the presence of pJV300 with the corresponding value when NsiR4 was present. Autofluorescence measured for negative control cells was subtracted from every measurement prior to the calculation. The data are presented as the means  $\pm$  SD of 6 independent colonies. **D:** Predicted interaction site between NsiR4 and *ssr1528*, and the respective hybridization energies of the native and mutated sequences at 30°C (the respective nucleotides are boxed in red). The gray box highlights the *ssr1528* start codon. In the RNA sequences, the numbers refer to the TSS at position +1. Predictions were made using IntaRNA (42).

**Fig. 5. Changes in the mRNA abundance of *gifA* in response to altered NsiR4 expression.**

**A:** Expression kinetics was measured after  $\text{Cu}^{2+}$  addition and nitrogen depletion. Nitrogen depletion was induced in cultures grown in presence of 2  $\mu\text{M}$   $\text{Cu}^{2+}$  for 48 h (= 0 h -N). **B:** Expression of *gifA* after adding 10 mM  $\text{NH}_4^+$ . Prior to  $\text{NH}_4^+$  addition, the strains were pre-cultivated for 6 h in presence of 2  $\mu\text{M}$   $\text{Cu}^{2+}$ . The order of blots is the same as in A. For clarity, only a representative 5S rRNA loading control hybridization for NsiR4oex is shown. WT - *Synechocystis* 6803 wild type, NsiR4oex – WT strain carrying pVZ322-PpetE::nsiR4::oop plasmid (overexpression strain),  $\Delta\text{nsiR4}$  - deletion mutant,  $\Delta\text{nsiR4}::\text{oex}$  - deletion strain in which NsiR4 expression was restored by the pVZ322-PpetE::NsiR4::oop plasmid. **C:** Verification of the direct interaction between NsiR4 and *gifA* using an *in vivo* reporter system. GFP fluorescence measurements of *E. coli* TOP10 strains with various combinations of plasmids expressing NsiR4 or *gifA-sgfp*-fusions. The plasmids pXG-0 and pJV300 were used as negative controls (for experimental details see (41)). **D:** Repression calculated by dividing

the values measured for *gifA-sgfp* with the corresponding value when NsiR4 was present. Autofluorescence correction and number of biological replicates as in the legend to **Fig. 4C**. **E:** Predicted interaction site between NsiR4 and *gifA* and the respective hybridization energies of the native and mutated sequences at 30°C (the respective nucleotides are boxed in red). The positions are indicated with respect to the TSS +1 for *nsiR4* (26) and *gifA* (24). The gray box highlights the *gifA* start codon. Predictions were made using the IntaRNA webserver (42).

**Fig. 6. Changes in the IF7 protein levels in response to altered NsiR4 expression.**

**A:** Immunoblots using antibodies specific for inactivating factors IF7 and IF17 (and TrxA which was used as a loading control). **B:** Densitometric evaluation of the signals for IF7 after normalization to the TrxA signal intensity. The data are given as the relative expression compared with the value for strain  $\Delta nsiR4$  at 180 min (= 100%). WT - *Synechocystis* 6803 wild type, NsiR4oex - WT strain carrying pVZ322-*PpetE::nsiR4::oop* plasmid (overexpression strain),  $\Delta nsiR4$  - deletion mutant,  $\Delta nsiR4::oex$  - deletion strain in which NsiR4 expression was restored by the pVZ322-*PpetE::NsiR4::oop* plasmid.

**Fig. 7. Kinetics of intracellular amino acid pools in response to a sudden increase in  $NH_4^+$  availability.**

Kinetics of glutamine (A) and glutamate (B) are shown for WT and the  $\Delta nsiR4$  knockout strain. Additionally, arginine kinetics (C) and steady state levels prior to and 24 h after  $NH_4^+$  upshift (D) are given for  $\Delta nsiR4$ , NsiR4oex and the compensatory strain  $\Delta nsiR4::oex$  compared to WT. The cells were grown in 250 ml Erlenmeyer flasks containing 100 ml BG11 and 17.6 mM  $NO_3^-$ . After reaching an  $OD_{750}$  of ca. 0.8, 2  $\mu M$   $CuSO_4$  was added, and after further incubation for 24 h, 10 mM  $NH_4Cl$  was added to the cultures (indicated with an arrow). Amino acids were extracted from cells prior to and after  $NH_4^+$  addition and quantified through HPLC. The values for each strain are indicated as the relative levels referring to the maximum/minimum amount at 2.5 min (glutamine/glutamate) or at 30 min (arginine) after  $NH_4^+$  addition (= 1). The data are presented as the means  $\pm$  standard deviation of two biological replicates, measured in two technical replicates. Prior to the

calculation of the relative amino acid levels, the absolute amounts were normalized to OD<sub>750</sub> and the total concentration of free amino acids. The sudden upshift/downshift of glutamine/glutamate indirectly represents the glutamine synthetase activity, i.e., the primary reaction of NH<sub>4</sub><sup>+</sup> assimilation.

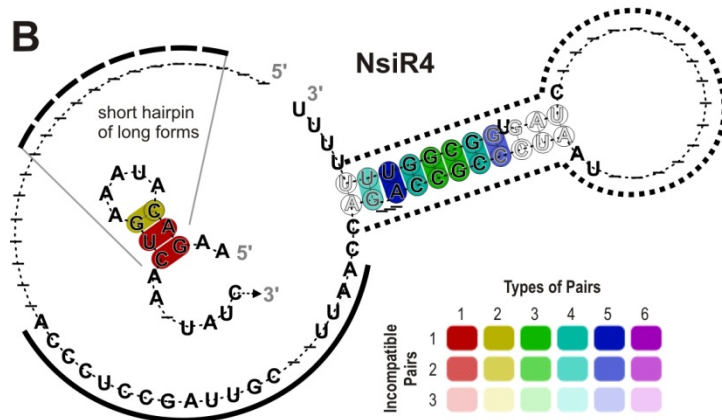
**Fig.8. NsiR4 is involved in regulating NH<sub>4</sub><sup>+</sup> assimilation.**

Schema illustrating the N-regulated feed-forward mechanism for the direct regulation of the glutamine synthetase inactivating factor IF7, thereby also affecting GS activity. AMT – ammonium transporter, NRT – nitrate transporter, GS – glutamine synthetase, GOGAT – glutamine oxoglutarate amidotransferase (Glutamate synthase), NR – nitrate reductase, NiR – nitrite reductase, CM – cytoplasmic membrane.

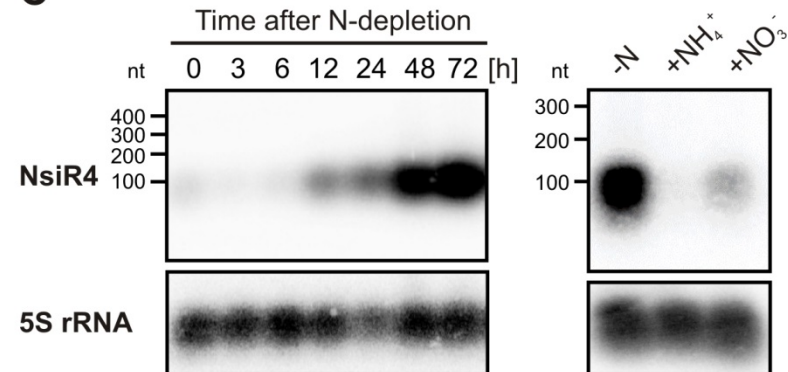
**A**

	short hairpin	single-stranded part	terminator loop	
I <i>Synechocystis</i> sp. PCC 6803	AAGACATAAAGTCAAT . ATC	ACCCTCCGATTGCTA . . . . .	GAGGTCGCCCAT . . . . . TGCTTCAGTGGCGGCTTT . . . . .	long form
I <i>Synechocystis</i> sp. PCC 6714	AAGACATAAAGTCAAT . ATC	ACCCTCCGATTGCTA . . . . .	GAGGTCGCCCGT . . . . . TGCTTCAGTGGCGGCTTT . . . . .	
I <i>Cyanothece</i> sp. PCC 7424	AAGACATAAAGTCAAT . ATC	ACCCTCCGATTGCTTATC . . . . .	CAGGCCGCCA . . . . . TAGCTAGGGCGGCTTT . . . . .	
I <i>Cyanothece</i> sp. ATCC 51472	AAGACATAAAGTCAATTATC	ACCCTCCGATTGCTTATT . . . . .	GCAACCGCCAC . . . . . ACTTCAGTGGCGGTTTT . . . . .	
I <i>Cyanothece</i> sp. PCC 7822	AAGACATAAAGTCAAT . ATC	ACCCTCCGATTGCTTATC . . . . .	CAAGCCGCCG . . . . . TTGTCTAGGGCGGCTTT . . . . .	
I <i>Cyanothece</i> sp. ATCC 51142	AAGACATAAAGTCAATTATC	ACCCTCCGATTGCTTATT . . . . .	GCAACCGCCAC . . . . . ACTTCAGTGGCGGTTTT . . . . .	
I <i>Microcystis aeruginosa</i> NIES-843	AAGACATAAAGTCAAT . ATC	ACCCTCCGATTGCAATGAAAAATGTTTGGATCGCTTAAGGTTTAAGCCTTT	CAGACATTTTT . . . . .	
II <i>Pleurocapsa</i> sp. PCC 7327	AAGATATAGAGTCAAT . ATC	ACCCTCCGATTGCTGGAAATTGACCTCCTTAATCTGTCGAATGTAAGATTAGAGGTC	CAATTTT . . . . .	
I <i>Gloeocapsa</i> sp. PCC 7428		ACCCTCCGATTGCTCAAG . . . . .	CAACCGCCATC . . . . . AAGCTAGCTAGTGGCGGTTTTTT . . . . .	
I <i>Synechococcus</i> sp. PCC 7002		ACCCTCCGATTGCTTATCAAGCTAGACCGTCTC . . . . .	AC . . . . . CAGTGGCGGTTTTTT . . . . .	
II <i>Chroococcidiopsis thermalis</i> PCC 7203		ACCCTCCGATTGCTCAA . . . . .	CAACCGCCATC . . . . . TCCTGGCGGTTTT . . . . .	
II <i>Stanieria cyanosphaera</i> PCC 7437		ACCCTCCGATTGCTTATT . . . . .	CAGACCGCCAA . . . . . AT . . . . . CTAGAGCGGTTTTTT . . . . .	
III <i>Microcoleus</i> sp. PCC 7113		ACCCTCCGATTGCTTAA . . . . .	CAGCCGCCCTA . . . . . TTTCTAGAGCGGTTTTTT . . . . .	
III <i>Crinalium epipsammum</i> PCC 9333		ACCCTCCGATTGCTTAA . . . . .	ACGACCGCCCTC . . . . . TT . . . . . TCTAGTGGCGGTTTTTT . . . . .	
III <i>Leptolyngbya</i> sp. PCC 7376		ACCCTCCGATTGCTTATCAAGCTAGACCGTCTA . . . . .	AC . . . . . CAGCGACGGTTTTTTTT . . . . .	
III <i>Spirulina subsalsa</i> PCC 9445		ACCCTCCGATTGCTTATT . . . . .	CAGACCGCCCTC . . . . . ACACCAAGTGGCGGTTTTTT . . . . .	
III <i>Calothrix</i> sp. PCC 7103		ACCCTCCGATTGCTTAA . . . . .	TAGACTGCCCCCAAATAAGTGGGGCGGTTTTTT . . . . .	
III <i>Calothrix</i> sp. PCC 6303		ACCCTCCGATTGCTTAA . . . . .	CAGACCGCCCT . . . . . TATCTAGAGCGGTTTTTT . . . . .	
IV <i>Anabaena (Nostoc)</i> sp. PCC 7120		ACCCTCCGATTGCTTAA . . . . .	CAGACCGCCCTA . . . . . ATCTAGTGGCGGTTTTTT . . . . .	
IV <i>Anabaena variabilis</i> ATCC 29413		ACCCTCCGATTGCTTAA . . . . .	CAGACCGCCCTA . . . . . ATCTAGTGGCGGTTTTTT . . . . .	
IV <i>Anabaena</i> sp. PCC 7108		ACC . . . . . TCTGATTGCTTAA . . . . .	CATACCGCCTT . . . . . GTACAAAGCGGTTTTTT . . . . .	
IV <i>Nostoc punctiforme</i> PCC 73102		ACCCTCCGATTGCTTAA . . . . .	CAGACCGCCCTA . . . . . TTATATTTAGTGGCGGTTTTTT . . . . .	
IV <i>Nostoc</i> sp. PCC 7524		ACCCTCCGATTGCTTAA . . . . .	CAAACCGCCCTA . . . . . TAGTGGCGGTTTTCT . . . . .	
IV <i>Nostoc</i> sp. PCC 7107		ACCCTCCGATTGCTTAA . . . . .	CAGACAACCGCC . . . . . AT . . . . . CATCTAGTGGCGGTTTTTT . . . . .	
IV <i>Scytonema hofmanni</i> UTEX2349		ACCCTCCGATTGCTTAA . . . . .	CAAACCGCCCTA . . . . . TT . . . . . ATAGTGGCGGTTTTTT . . . . .	
IV <i>Cylindrospermum stagnale</i> PCC 7417		ACCCTCCGATTGCTTAA . . . . .	CAAACCGCCCT . . . . . CATCTTGTGGCGGTTTTTT . . . . .	
IV <i>Microchaete</i> sp. PCC 7126		ACCCTCCGATTGCTTAA . . . . .	TAGACCGCCCT . . . . . CATCTAGTGGCGGTTTTTT . . . . .	
IV <i>Rivularia</i> sp. PCC 7116		ACCCTCCGATTGCTTAA . . . . .	CAGACCGCCCTA . . . . . TTTTAGTGGCGGTTTTTT . . . . .	
V <i>Chlorogloeopsis</i> sp. PCC 7702		ACCCTCCGATTGCTTAA . . . . .	CAGACCGCCCTA . . . . . ACCTACTAGCGGCGGTTTTTT . . . . .	
V <i>Chlorogloeopsis fritschii</i> PCC 6912		ACCCTCCGATTGCTTAA . . . . .	CAGACCGCCCTA . . . . . TTTTACTAGCGGCGGTTTTTT . . . . .	
V <i>Chlorogloeopsis</i> sp. PCC 9212		ACCCTCCGATTGCTTAA . . . . .	CAGACCGCCCTA . . . . . TTTTACTAGCGGCGGTTTTTT . . . . .	
V <i>Mastigocladopsis repens</i> PCC 10914		ACCCTCCGATTGCTTAA . . . . .	CAGACCGCCCTA . . . . . ATTAAGTGGCGGTTTTTT . . . . .	
V <i>Fischerella</i> sp. PCC 9605		ACCCTCCGATTGCTTAA . . . . .	CAGACCGCCCTA . . . . . CTCTACTAGTGGCGGTTTTTT . . . . .	
V <i>Fischerella</i> sp. JSC.11		ACCCTCCGATTGCTTAA . . . . .	CAGACCGCCCTA . . . . . CTCTACTAGTGGCGGTTTTTT . . . . .	
V <i>Fischerella thermalis</i> PCC7521		ACCCTCCGATTGCTTAA . . . . .	CAGACCGCCCTA . . . . . CTCTACTAGTGGCGGTTTTTT . . . . .	
V <i>Fischerella muscicola</i> PCC7414		ACCCTCCGATTGCTTAA . . . . .	CAGACCGCCCTA . . . . . CTCTACTAGTGGCGGTTTTTT . . . . .	
V <i>Fischerella muscicola</i> SAG1427		ACCCTCCGATTGCTTAA . . . . .	CAGACCGCCCTA . . . . . CTCTACTAGTGGCGGTTTTTT . . . . .	
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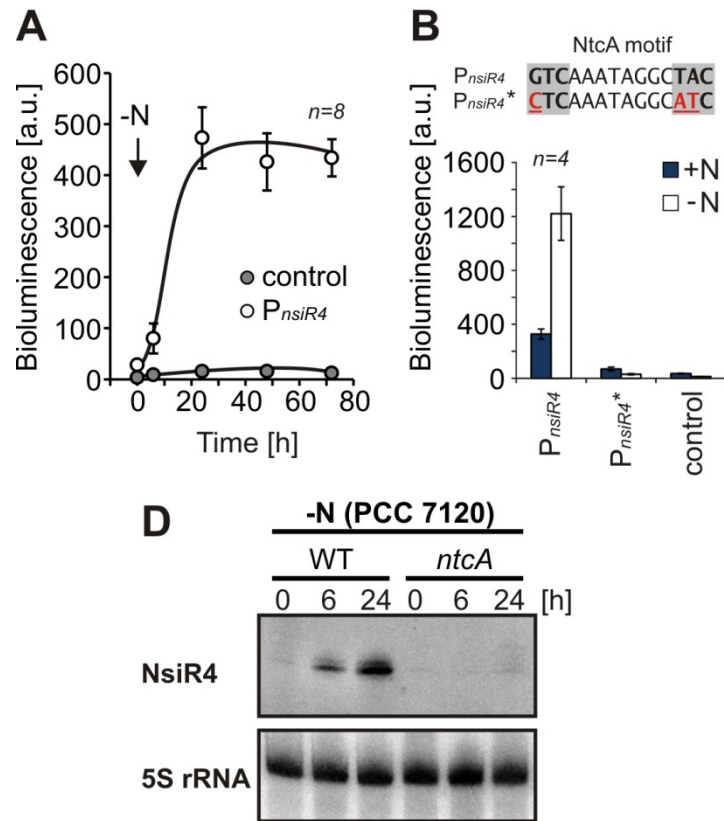
**B**



**C**



**Fig. 1**



**C**

NtcA consensus motif

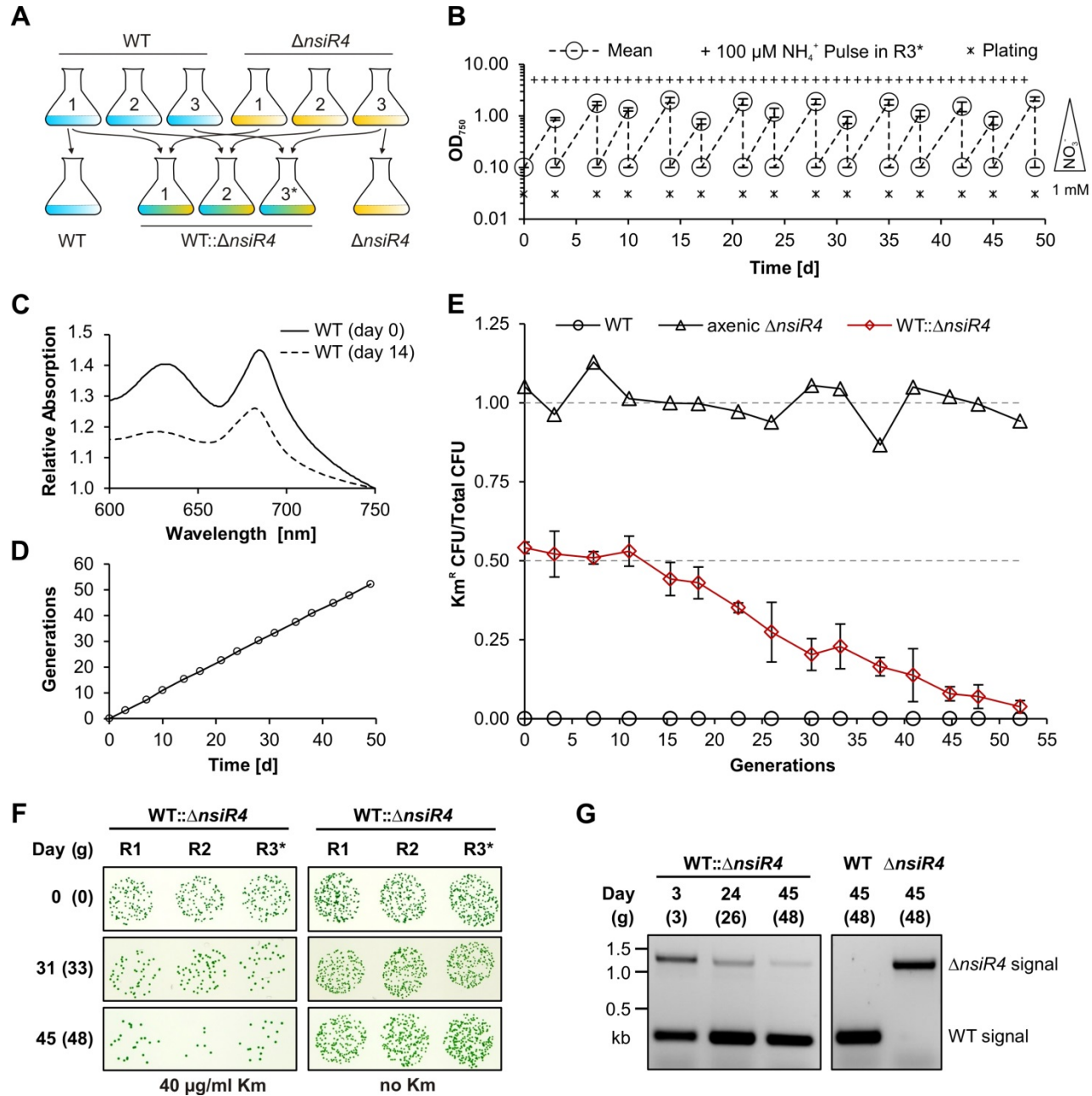
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*Synechococcus* sp. PCC 7002  
*Chroococcidiopsis thermalis* PCC 7203  
*Stanieria cyanosphaera* PCC 7437  
*Microcoleus* sp. PCC 7113  
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*Leptolyngbya* sp. PCC 7376  
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*Nostoc* sp. PCC 7524  
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*Cylindrospermum stagnale* PCC 7417  
*Microchaete* sp. PCC 7126  
*Rivularia* sp. PCC 7116  
*Cyanobacterium* PCC 7702  
*Chlorogloeopsis fritschii* PCC 6912  
*Mastigocladopsis repens* PCC 10914  
*Fischerella thermalis* PCC 7521  
*Fischerella muscicola* PCC 7414

.....GTA...Ng...TAC.....N22-23.....TA N<sub>3</sub> T.....put. TSS

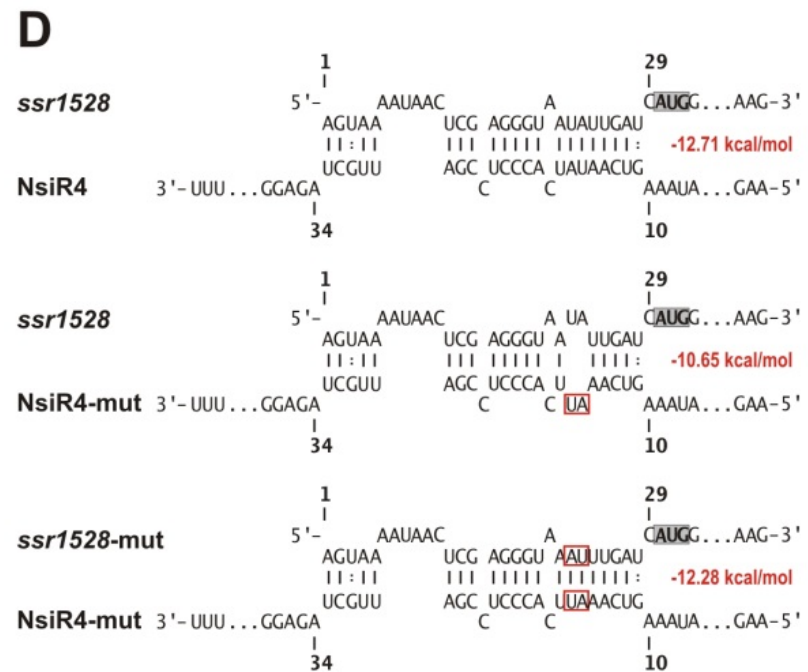
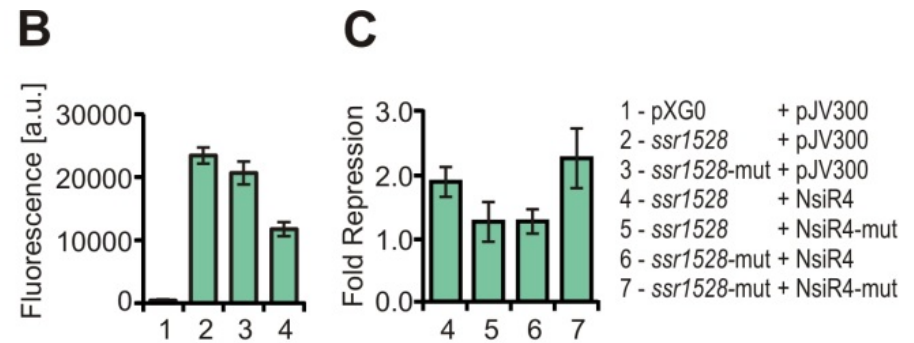
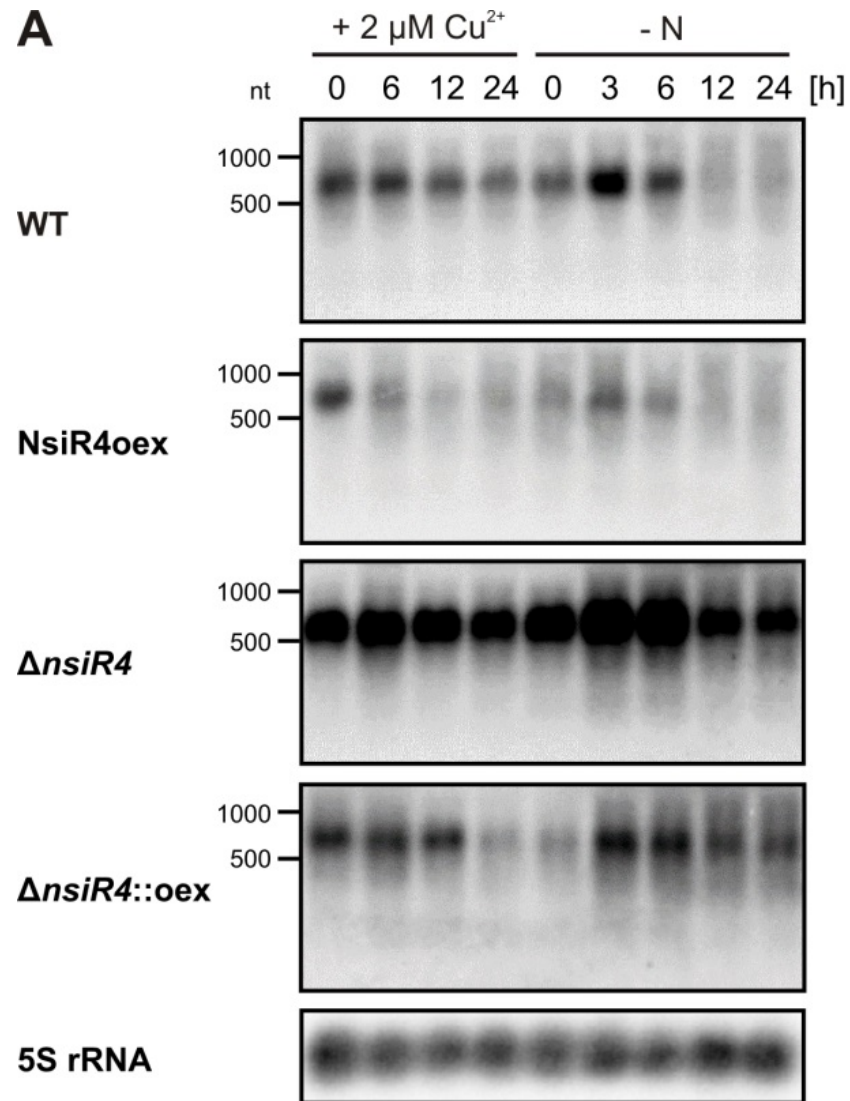
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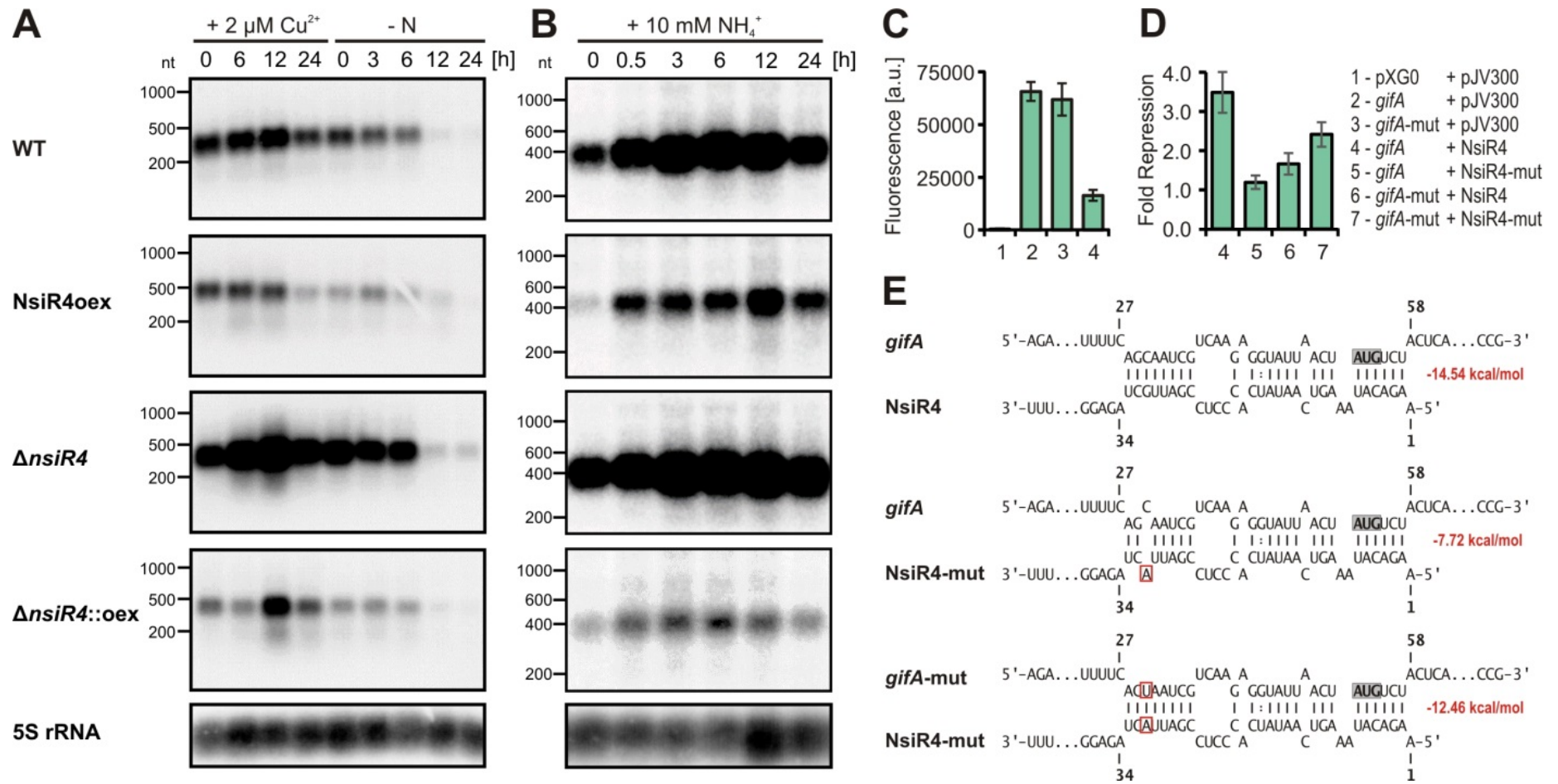
Fig. 2



**Fig. 3**

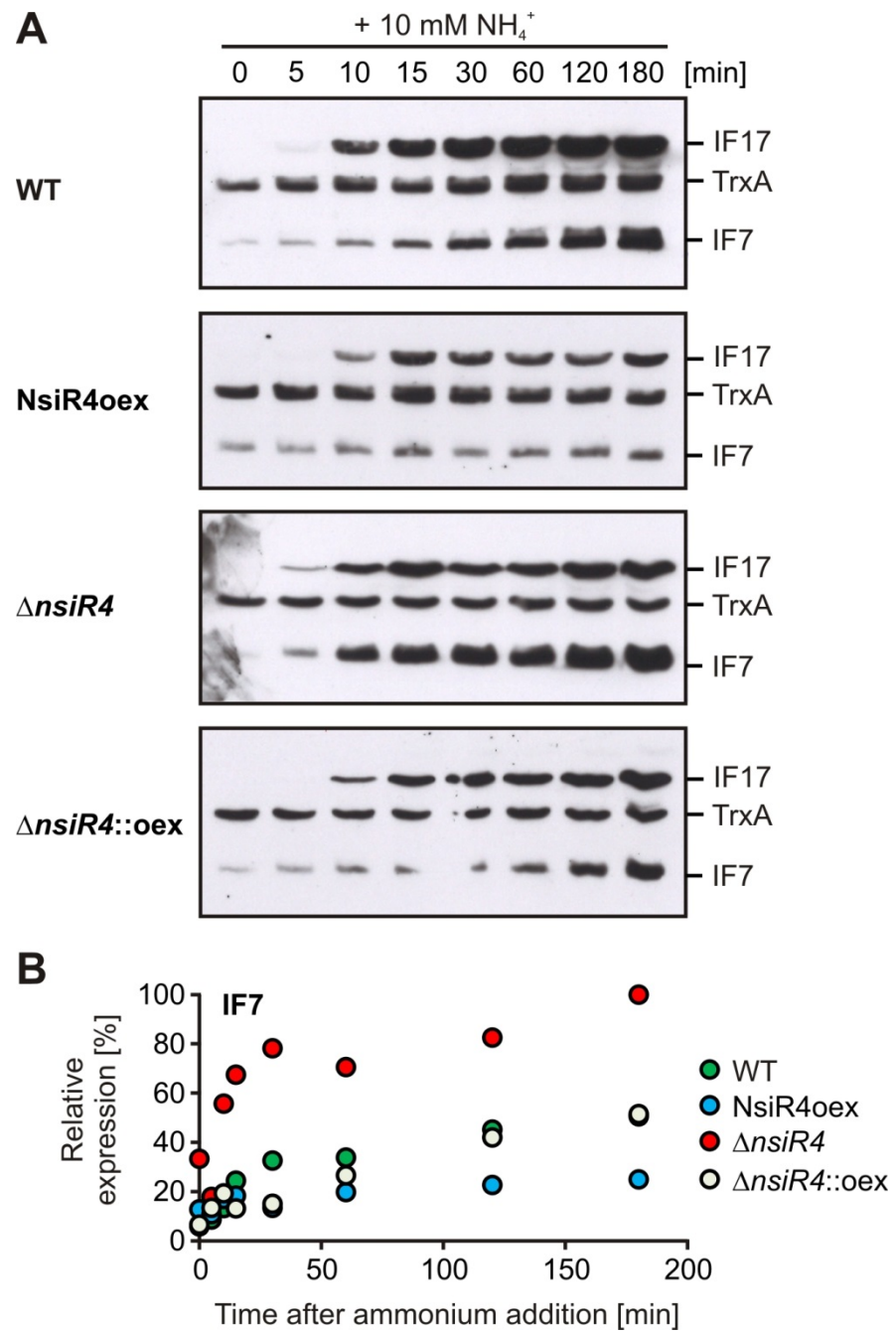


**Fig. 4**

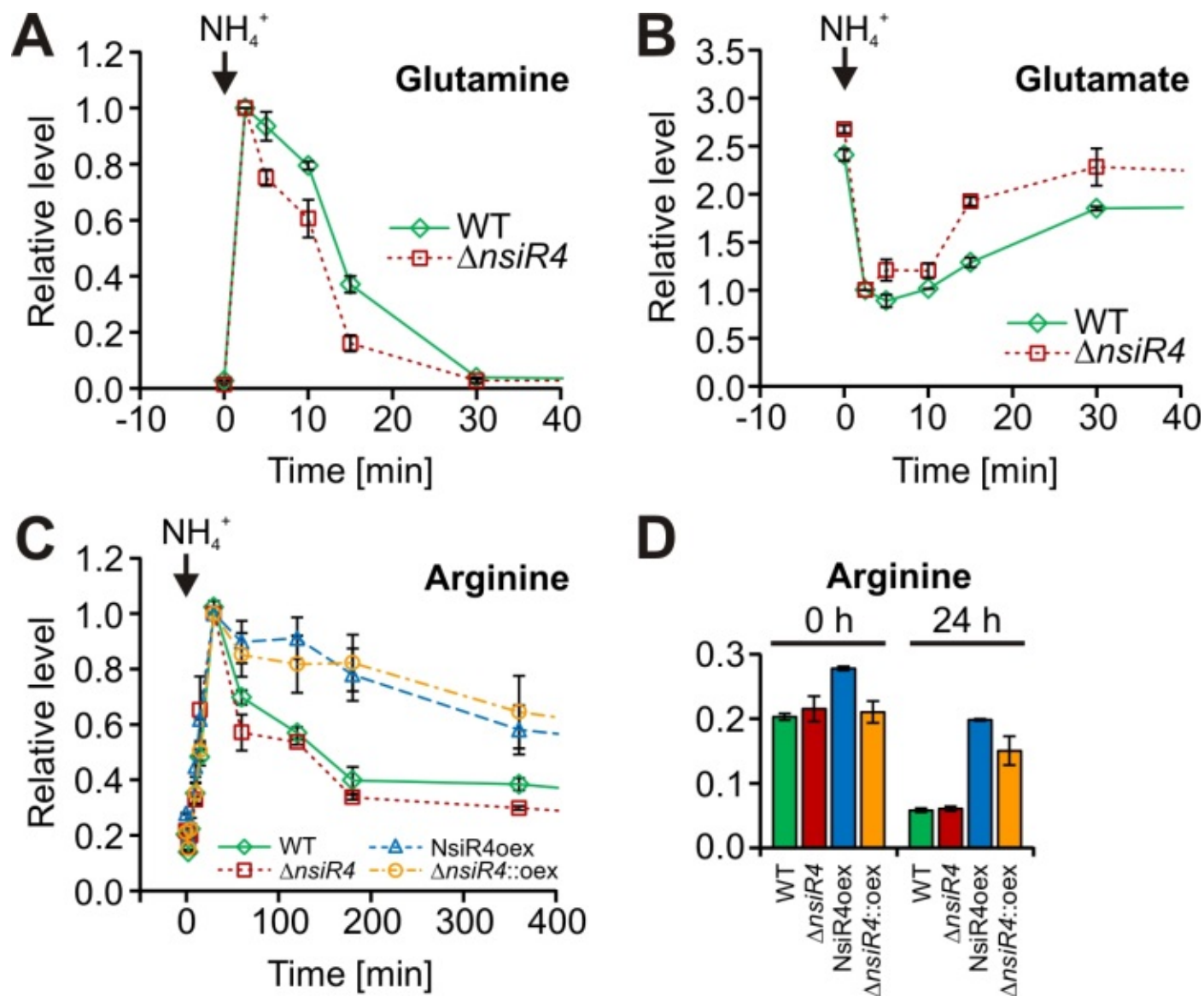


**Fig. 5**





**Fig. 6**



**Fig. 7**

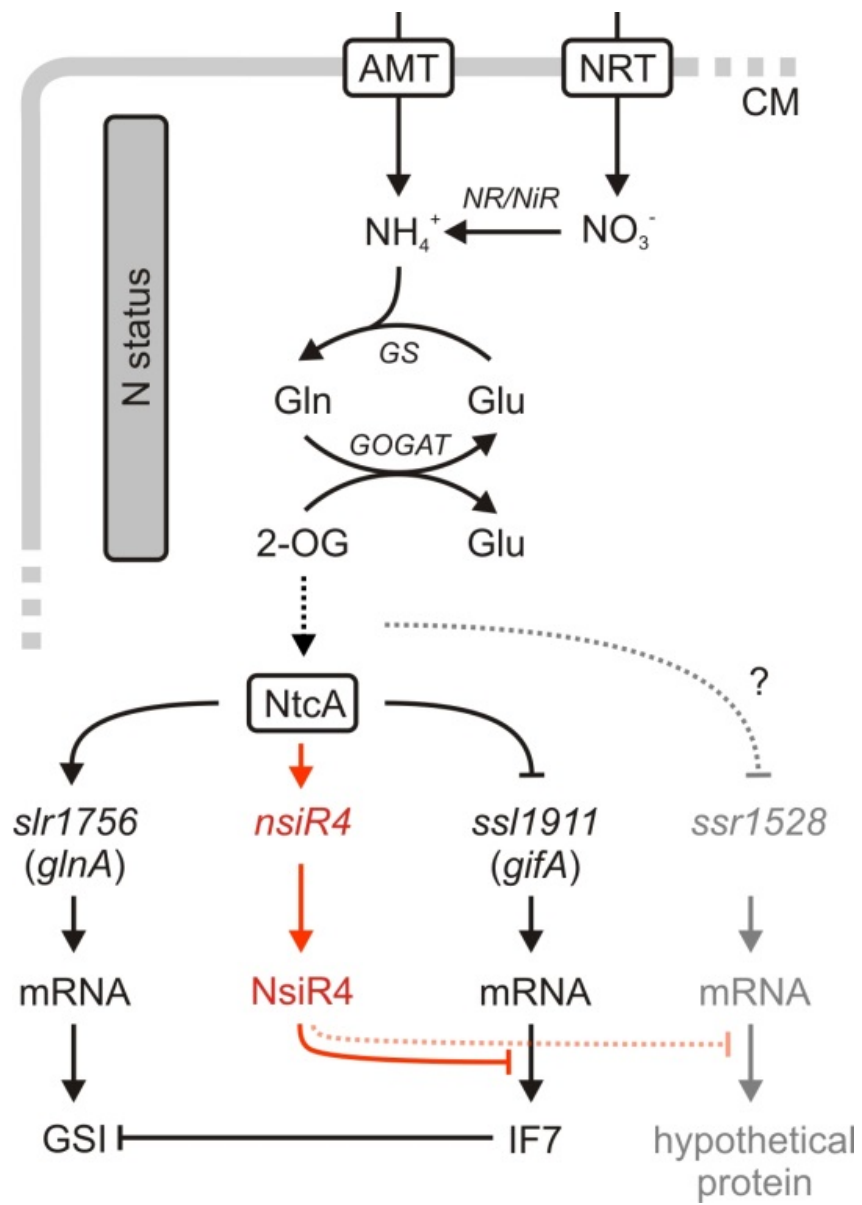


Fig. 8

# The sRNA NsiR4 is involved in nitrogen assimilation control in cyanobacteria by targeting glutamine synthetase inactivating factor IF7

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## Supplementary Material

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## Supplementary Materials and Methods

**Strains and growth conditions.** For the generation of *nsiR4* mutants, we used the glucose-tolerant strain *Synechocystis* 6803 (GT-Kazusa) provided from N. Murata (National Institute for Basic Biology, Okazaki, Japan). Cultivation was performed in Cu<sup>2+</sup>-free BG11 medium (1) buffered with 20 mM TES, pH 8.0 at 30°C under continuous white light illumination of 50–80  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  and gentle agitation. Mutant strains were grown in presence of the corresponding antibiotics. To induce nitrogen deficiency, the cells from liquid cultures were harvested through centrifugation (for 5 min at 4,000 rpm and room temperature), resuspended in NO<sub>3</sub><sup>-</sup>-free BG11 and cultivated further. To re-establish N-replete conditions, NH<sub>4</sub>Cl or NaNO<sub>3</sub> were added to the respective experiments. To induce the ectopic expression of NsiR4, 2  $\mu\text{M}$  CuSO<sub>4</sub> was added to the cultures. *Anabaena* 7120 WT and the mutant strain CSE2 (carrying a streptomycin resistance cartridge within the *ntcA* coding region; (2)) were grown with bubbling (CO<sub>2</sub>-enriched air, 1% vol/vol) in BG11 without NO<sub>3</sub><sup>-</sup> and supplemented with 6 mM NH<sub>4</sub>Cl, 12 mM TES-NaOH (pH 7.5) and 10 mM NaHCO<sub>3</sub>. For nitrogen step-down experiments, the *Anabaena* cells were collected through filtration, washed and re-suspended in nitrogen-free medium (BG11 without NO<sub>3</sub><sup>-</sup>). For long-term growth competition experiments, three independent, exponentially growing cultures of *Synechocystis* 6803 WT and  $\Delta\textit{nsiR4}$  were diluted to an OD<sub>750</sub> of 0.1 and mixed in equal numbers. Cultures were grown in 20 ml BG11 in 100 ml Erlenmeyer flasks with 1 mM NO<sub>3</sub><sup>-</sup> (instead of the usual 17.6 mM). After 3 or 4 days cultures were re-diluted to an OD<sub>750</sub> of 0.1 and 25  $\mu\text{l}$  of a 1:1000 dilution were dropped on BG11 agar plates with and without 40  $\mu\text{g/ml}$  kanamycin. Amplification of the *nsiR4* locus was made using primers SyR12\_ko\_seg\_for/rev and genomic DNA of WT and  $\Delta\textit{nsiR4}$  cultures and a representative, mixed culture after 3, 24 and 45 days.

**Genome and promoter analysis.** The 70 nt of *Synechocystis* NsiR4 (pos. 1289326 – 1289257, complementary strand) was used as a reference for BlastN searches in the JGI database. However, the identification of an sRNA gene based on sequence alone is not straightforward due to the short length and little sequence conservation. Therefore, the sequences of the resulting hits were extended 100 bp in both directions and further analyzed in multiple alignments using ClustalW (3). The presence of *nsiR4* in a given genome was regarded as positive when the sequence aligned to the corresponding sequence from *Synechocystis* and contained a potential terminator hairpin. Initially, the MEME search tool (4) was used for the comparative analysis of sequences identified upstream of the identified *nsiR4* homologous genes. To verify the activity of the *nsiR4* promoter in *Synechocystis* *in vivo* a sequence spanning the range between -130 to +49 (relative to the TSS at +1) was fused to *luxAB* reporter genes. The fragment was amplified from the gDNA of *Synechocystis* (for oligonucleotides see **Table S2**), followed by restriction digestion with *KpnI* and cloning into the promoter-probe vector pILA (5). For mutagenesis of the NtcA motif, the corresponding plasmid was re-amplified using the primers prNtc\_mut\_fw/rev (**Table S2**). The resulting plasmids, containing either the native or a mutated NsiR4 promoter, were used to transform a *Synechocystis* host strain carrying the *luxCDE* operon, which encodes enzymes for the synthesis of decanal, the substrate for the

luciferase reaction. The selection of the reporter strains and bioluminescence measurements were performed as described (6).

**Generation of NsiR4 mutant strains.** A schematic presentation of the cloning strategies is shown in **Supplementary Figure S6**. To generate the NsiR4 knockout strain ( $\Delta nsiR4$ ), two fragments covering the adjacent genes *sll1697* and *sll1698* were amplified from gDNA using the primer combinations 5'SyR12\_for/5'SyR12\_BsrGI\_rev and 3'SyR12\_PstI\_for/3'SyR12\_rev (**Supplementary Table S2**). The PCR products were digested with the restriction endonucleases *BsrGI* and *PstI*, respectively. A kanamycin resistance cartridge ( $Km^R$ ) was amplified from the vector pVZ322 using the primers Kan\_PstI\_for and Kan\_BsrGI\_rev, and subsequently digested with *BsrGI* and *PstI* and ligated to the compatible ends of both fragments using T4 DNA ligase (Thermo Scientific). The resulting construct comprising a  $Km^R$  flanked by sequences homologous to the genes *sll1697* and *sll1698* was re-amplified using the primers 5'SyR12\_for and 3'SyR12\_rev and introduced into the cloning vector pJET1.2. This plasmid was used to transform WT *Synechocystis*. The mutant cells were initially selected on BG11 agar plates (0.9% Kobe I agar, Roth, Germany) supplemented with 10  $\mu\text{g ml}^{-1}$  kanamycin and subsequently grown in the presence of 50  $\mu\text{g ml}^{-1}$  in liquid cultures.

To establish the ectopic expression of *nsiR4*, a self-replicating plasmid carrying the *nsiR4* gene under control of the *petE* promoter, which mediates  $\text{Cu}^{2+}$ -regulated transcription in *Synechocystis* (7), was prepared. The genomic sequence of *nsiR4* was amplified from *Synechocystis* gDNA using the primers SyR12\_EcoRI\_for and SyR12\_EcoRI\_rev. The product was digested with *EcoRI* and introduced into a vector as previously described (8). This plasmid is based on pJET1.2 and contains an *EcoRI* site between the *petE* promoter from *Synechocystis* (ranging from nucleotide -235 to -1 with respect to the TSS at +1), (9) and the oop-terminator. The entire construct was integrated into the *Synechocystis* chromosome via homologous recombination into the *spkA* locus, which is a neutral site in the WT strain used here (8). However, divergent from the initial idea of chromosomal integration we cloned the cassette *PpetE::nsiR4::oop* into the replicative broad-host vector pVZ322. The construct was re-amplified using the primers spk\_km\_hindIII\_for and spk\_km\_xhoI\_rev, subsequently digested with *HindIII* and *XhoI* and introduced into the plasmid pVZ322, digested with the same enzymes (note that the  $Km^R$  of pVZ322 was deleted after *HindIII/XhoI* treatment). The resulting plasmid was transferred into WT *Synechocystis* and  $\Delta nsiR4$  via conjugal transfer from *E. coli* (10), resulting in the strains NsiR4oex (in WT) and  $\Delta nsiR4::oex$  (in  $\Delta nsiR4$ ), respectively. The recombinant strains were selected on BG11 agar containing 1  $\mu\text{g ml}^{-1}$  gentamycin and also grown in presence of the same concentration in liquid cultures.

**RNA extraction, microarrays and Northern blots.** The collection of *Synechocystis* cells and RNA extraction was performed as previously described (6, 11). Prior to the microarray analysis, 10  $\mu\text{g}$  of total RNA were treated with Turbo DNase (Invitrogen) according to the manufacturer's protocol and precipitated with ethanol/sodium acetate. Labeling and hybridization were performed as previously described (12), using 3  $\mu\text{g}$  of RNA for the labeling reaction and 1.65  $\mu\text{g}$  of labeled RNA for the hybridization. For Northern hybridization,

*Synechocystis* 6803 RNA was separated on denaturing agarose gels and transferred to Hybond-N<sup>+</sup> membranes (Amersham, Germany) through capillary blotting with 20x SSC buffer. The membranes were hybridized with [ $\alpha$ -<sup>32</sup>P]-UTP incorporated single-stranded RNA probes generated through *in vitro* transcription as previously described (13). The signals were detected using a Personal Molecular Imager system (Pharos FX, BIO-RAD, Germany) and analyzed using Quantity One software (BIO-RAD, Germany). RNA from *Anabaena* was isolated using hot phenol (14). Total RNA was separated on urea-acrylamide gels and transferred to Hybond N<sup>+</sup> membranes with 1x TBE buffer in a semi-dry blotter. The membranes were hybridized with oligonucleotides labeled with  $\gamma$ -<sup>32</sup>P-dATP and polynucleotide kinase (probe for NsiR4) or with probes labeled with  $\gamma$ -<sup>32</sup>P-dCTP and Ready-to-go DNA labeling kit (Amersham) (probe for 5S rRNA).

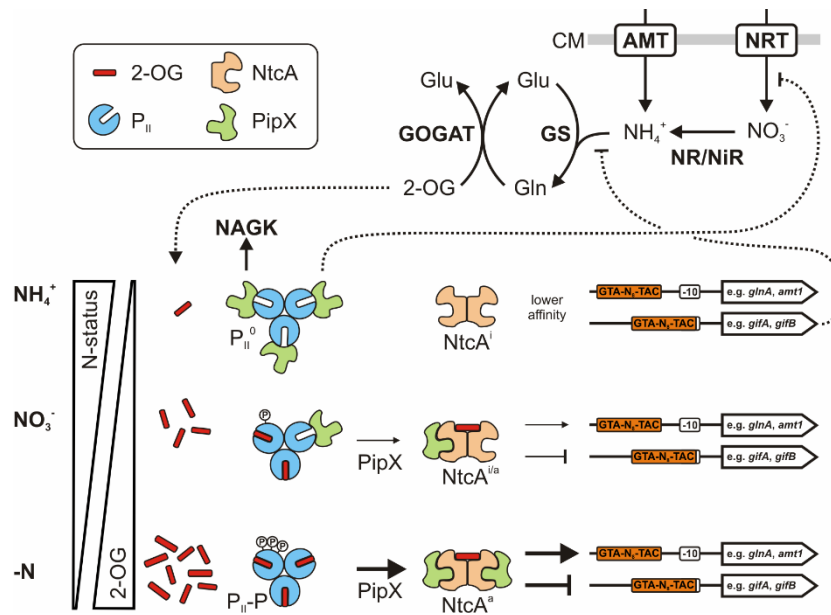
**Protein extraction and immunoblots.** *Synechocystis* and derivative strains were grown in NO<sub>3</sub><sup>-</sup>-containing, Cu<sup>2+</sup>-free medium. For analysis of IF accumulation, 2  $\mu$ M CuSO<sub>4</sub> was added to induce transcription from the *petE* promoter eight hours before addition of 10 mM NH<sub>4</sub>Cl and 20 mM TES-NaOH (pH 7.5). Samples were taken prior to and in a narrow time series after NH<sub>4</sub><sup>+</sup> addition. Cells from different time points were collected by centrifugation and frozen until protein extraction. Extracts were prepared using glass beads as previously described (15) in 50 mM Hepes-NaOH buffer (pH 7.0), 50 mM KCl, 1 mM EDTA. For Western blot analysis proteins were fractionated on 15% SDS-PAGE and immunoblotted with anti-IF7 (1:2000), anti-IF17 (1:2000) or anti-TrxA (1:3000). Anti-IF7, anti-IF17 and anti-TrxA antisera were obtained from M.I. Muro-Pastor and F.J. Florencio and used as described (16, 17). The ECL Plus immunoblotting system (GE Healthcare) was used to detect the different antigens with anti-rabbit secondary antibodies. Densitometric evaluation was performed with Quantity One software.

**Reporter assays for the *in vivo* verification of targets.** For the experimental target verification, we used the reporter system described by (18) and the sGFP plasmid pXG-10-SF introduced by (19). The primers used for cloning and the resulting plasmids are given in **Tables S2 and S3**. The entire 5'UTR containing the predicted NsiR4 interaction sequence and a part of the coding region were amplified from gDNA using the primer combinations tv\_ssl1911\_gifA\_for/tv\_ssl1911\_gifA\_rev or tv\_ssr1528\_fw/tv\_ssr1528\_rev and which covered ranges from +1 to +123 (*gifA*) or +1 to +119 (*ssr1528*) with respect to the TSS at position +1. The first nucleotide of the *gifA* start codon is at +52, for *ssr1528* at +30. For *gifA* the information about the TSS has been taken from (20), for *ssr1528* it was extracted from (9). The corresponding PCR product was cloned into the vector pXG-10-SF via the endonuclease sites *NsiI/NheI* resulting in a translational fusion of the sGFP with a truncated IF7 or Ssr1528 protein. The transcription is mediated by the constitutive promoter P<sub>LtetO-1</sub>. For the preparation of the plasmid establishing P<sub>LlacO-1</sub>-mediated sRNA expression in *E. coli* the *nsiR4* gene was amplified from gDNA using the primers 5\_SyR12\_long\_phos/3\_SyR12\_xbaI, digested with *XbaI* and fused to a plasmid backbone which was amplified from pZE12-*luc* (by using the primer combination PLLacOB/PLacOD) and also digested with *XbaI*.

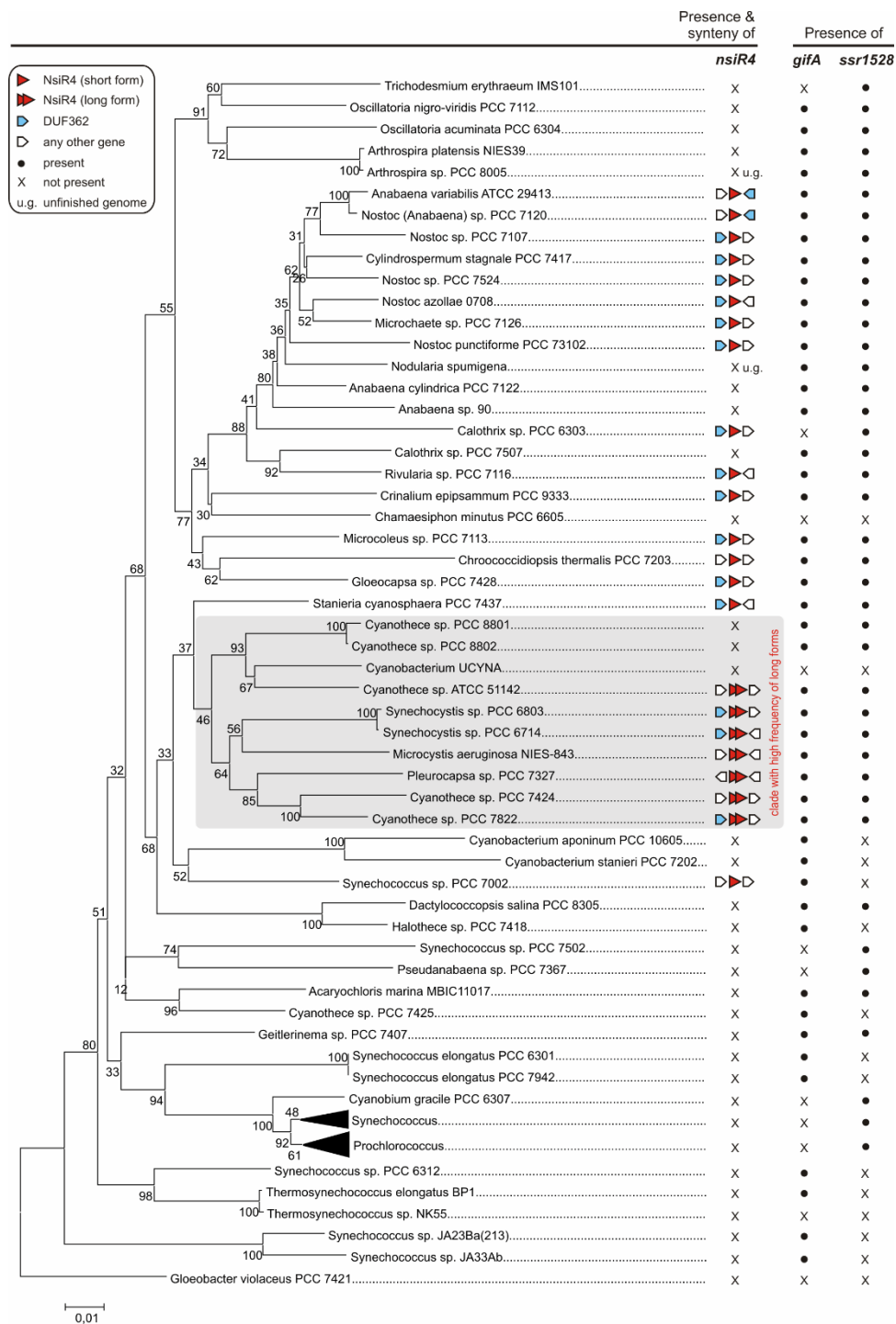
For the mutagenesis of NsiR4 and the 5'UTRs of *gifA* and *ssr1528*, the plasmids harboring the native versions were re-amplified using the primers SyR12\_1911\_mut\_fwd/SyR12\_1911\_mut\_rev or SyR12\_1528\_mut\_fwd/SyR12\_1528\_mut\_rev (for mutating NsiR4 parts interacting with *gifA* and *ssr1528*, respectively) and PXG10\_1911\_mut\_fwd/PXG10\_1911\_mut\_rev (*gifA*) or PXG10\_1528\_mut\_fwd/PXG10\_1528\_mut\_rev (*ssr1528*) (for the respective 5'UTRs) and introduced into *E. coli*. Positions for mutations were selected on the basis of lowered hybridization energies predicted by IntaRNA (21) while keeping the secondary structures as calculated with RNApdist (22). For testing various combinations of both plasmids, these were introduced into *E. coli* TOP10 (Invitrogen): e.g. pXG0 + pJV300, pXG10-*gifA* + pJV300/pZE12-NsiR4. The plasmids pJV300 and pXG-0 were used as negative control plasmids. The fluorescence measurement was done as described previously (23).



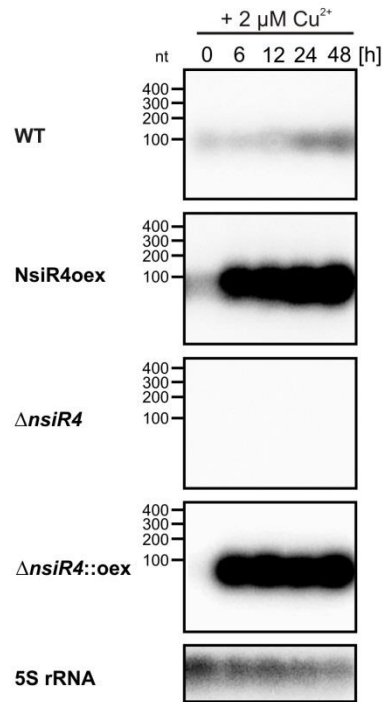
## Supplementary Figures



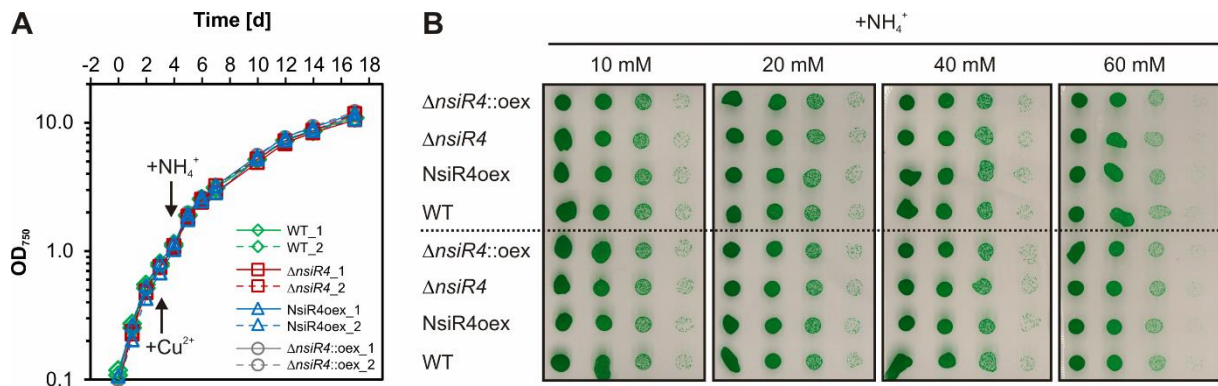
**Fig. S1: The nitrogen regulatory network in cyanobacteria.** The scheme was prepared based on references (20, 24–27).



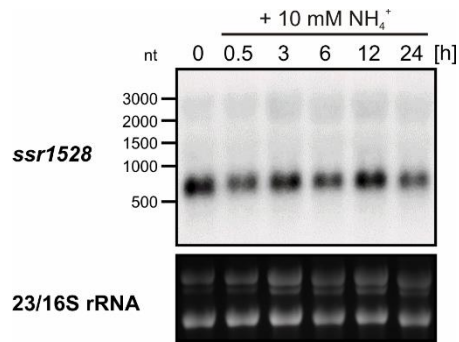
**Fig. S2: Presence and synteny of the genomic locus for *nsiR4* among the cyanobacterial phylum in combination with the presence of *gifA* and *ssr1528* homologous genes.** The representative phylogenetic tree was generated by using the neighbor joining algorithm based on cyanobacterial 16S rRNA sequences that were extracted from the SILVA database (28). By using BlastN and the JGI database (<https://img.jgi.doe.gov/cgi-bin/w/main.cgi>) all available genomes were screened for a sequence similar to NsiR4 from *Synechocystis* 6803. In order to sustain clarity, several genomes which also harbor short forms of NsiR4 were not included in the phylogenetic tree (e.g. *Leptolyngbya* strain PCC7376, *Fischerella thermalis* PCC7521). Presence of *gifA* and *ssr1528* genes was analyzed using the BlastP algorithm and the corresponding amino acid sequences from *Synechocystis* 6803 (E-value cutoff of 1e-5).



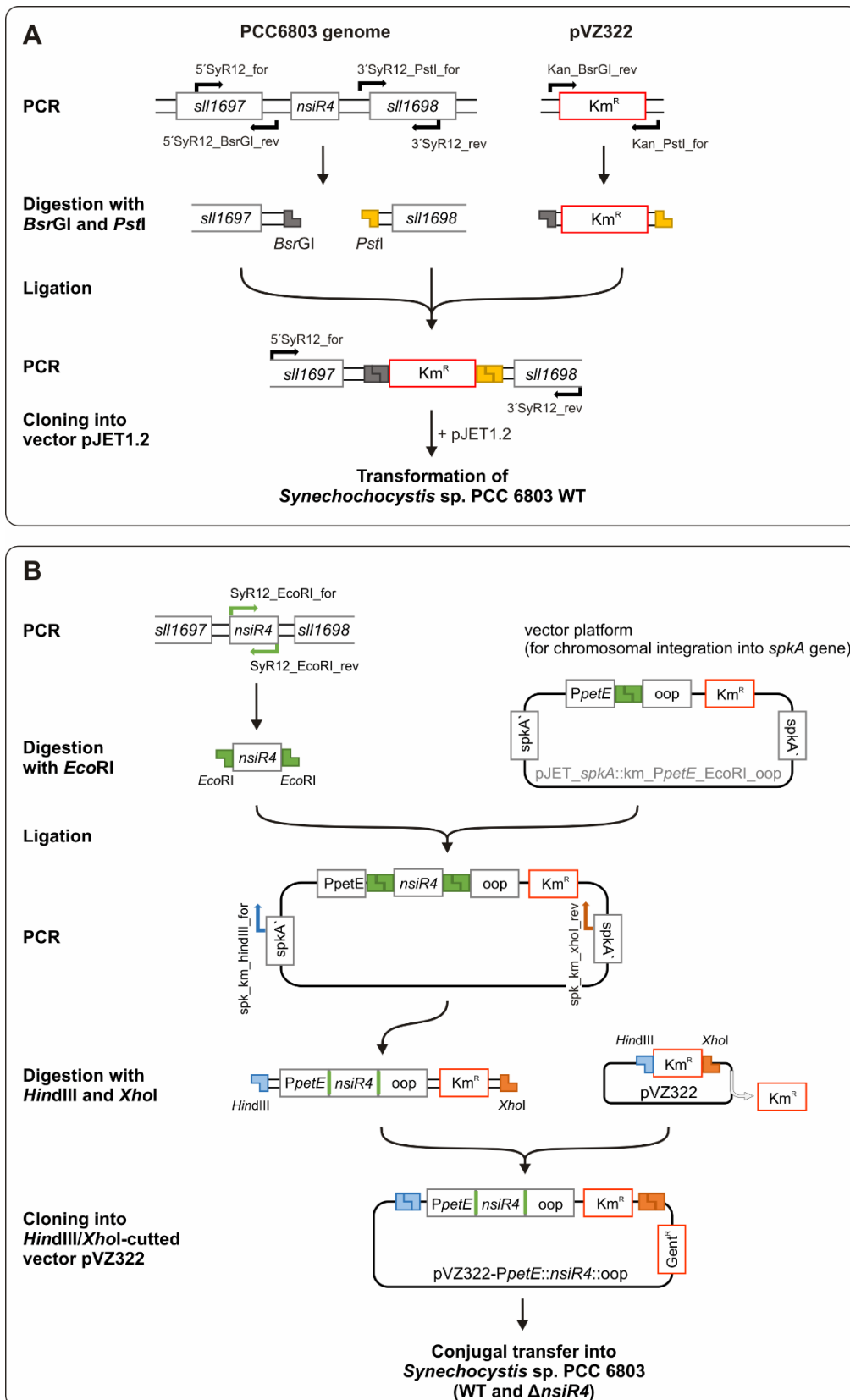
**Fig. S3: Verification of the *nsiR4* mutant strains through northern blot analysis.** Expression kinetics of NsiR4 was measured in cells grown in the presence of 17.6 mM NO<sub>3</sub><sup>-</sup> and after the addition of 2 μM Cu<sup>2+</sup>. For clarity, only one representative 5S rRNA loading control hybridization for NsiR4oex is shown. WT - *Synechocystis* 6803 wild type, NsiR4oex – WT strain carrying pVZ322-*PpetE*::*nsiR4*::*oop* plasmid (overexpression strain), Δ*nsiR4* - deletion mutant, Δ*nsiR4*::*oex* - deletion strain in which NsiR4 expression was restored through the pVZ322-*PpetE*::*nsiR4*::*oop* plasmid.



**Fig. S4: Growth performance of *nsiR4* mutant strains.** **A:** Growth curve of two replicates per strain. To induce NsiR4 overexpression (in strains NsiR4oex and  $\Delta nsiR4::oex$ ) and to maximize the effect on *gfiA* repression, 2  $\mu\text{M}$   $\text{Cu}^{2+}$  and 10 mM  $\text{NH}_4^+$  were added to the cultures (indicated by arrows) that were initially inoculated in copper-free BG11 medium containing 17.6 mM  $\text{NO}_3^-$ . **B:** Drop dilution assays for two biological replicates and various  $\text{NH}_4^+$  concentrations. Cells were grown in the presence of nitrate and the corresponding antibiotics. Cells were washed and resuspended at a concentration of 1  $\mu\text{g}$  chlorophyll *a*/ml. Three 10-fold serial dilutions were prepared and 5  $\mu\text{l}$  of each dilution were plated. All plates were supplemented with 2  $\mu\text{M}$   $\text{Cu}^{2+}$ . Photograph was taken after 5 days of growth. WT - *Synechocystis* 6803 wild type, NsiR4oex – WT strain strain carrying pVZ322-*PpetE::nsiR4::oop* plasmid (overexpression strain),  $\Delta nsiR4$  - deletion mutant,  $\Delta nsiR4::oex$  - deletion strain in which NsiR4 expression was restored by the pVZ322-*PpetE::NsiR4::oop* plasmid.



**Fig. S5: Expression of *ssr1528* in *Synechocystis* WT after adding 10 mM ammonium.** Prior to ammonium addition the strains were pre-cultivated for 6 h in presence of 2  $\mu\text{M}$   $\text{Cu}^{2+}$ . Total RNA was extracted, gel-separated, blotted onto Hybond-N<sup>+</sup> nylon membranes and hybridized with specific, <sup>32</sup>P-labelled, single-stranded RNA-probes. As loading control a part of the corresponding agarose gel 23S and 16S rRNA is shown.



**Fig. S6: Schematic view of the cloning strategies. A:** The knockout mutant  $\Delta$ *nsiR4*. **B:** The overexpression strain *NsiR4*oex and the compensatory strain  $\Delta$ *nsiR4*::oex.

## Supplementary Tables

**Table S1: List of genes with a lowered expression level in strain NsiR4oex.** Given are log<sub>2</sub> fold changes in the corresponding strains vs. WT. Additionally to the sorted list of fold changes in NsiR4oex, the corresponding log<sub>2</sub> values are also given for the strains  $\Delta$ nsiR4 and  $\Delta$ nsiR4::oex. The two genes shown in bold were the only examples for which log<sub>2</sub> fold changes <-1 were obtained in the NsiR4oex strain and for which additionally an opposite change was observed in the  $\Delta$ nsiR4 knockout strain.

NsiR4oex	$\Delta$ nsiR4	$\Delta$ nsiR4::oex	Locus Tag	Gene Name	Annotation
<b>-1.18</b>	<b>1.04</b>	<b>-0.68</b>	<i>ssr1528</i>	N/A	hypothetical protein
<b>-1.10</b>	<b>1.12</b>	<b>-0.86</b>	<i>ssl1911</i>	<i>gifA</i>	<b>glutamine synthetase inactivating factor IF7</b>
-0.69	-0.80	-1.29	<i>slr0447</i>	<i>urtA</i>	urea transport system substrate-binding protein
-0.69	-0.44	-0.92	<i>sll0108</i>	<i>amt1</i>	ammonium transporter Amt family
-0.54	-0.43	-0.16	<i>slr1513</i>	N/A	hypothetical protein
-0.51	0.05	-0.48	<i>ssr0692</i>	N/A	hypothetical protein
-0.49	-0.41	-0.23	<i>sll0822</i>	<i>abrB</i>	AbrB-like transcriptional regulator
-0.49	-0.31	-0.10	<i>slr1512</i>	<i>sbtA</i>	sodium-dependent bicarbonate transporter
-0.48	-0.34	0.11	<i>slr0041</i>	<i>cmpB</i>	bicarbonate transport system permease protein
-0.46	0.38	-0.16	<i>slr0904</i>	N/A	magnesium chelatase family protein

**Table S2: List of oligonucleotides.**

Name of Oligonucleotide	Sequence (in 5' – 3' direction)	Application
<b>Generation of <i>nsiR4</i> mutant strains in <i>Synechocystis</i> sp. PCC 6803</b>		
5'SyR12_for	CTCCGGTCCAATCCTACGAAGC	Amplification of sequence flanking <i>nsiR4</i> upstream region
5'SyR12_BsrGI_rev	GAATGTACAGGCCGGATCGGTAGGCTTTATGTAG	
3'SyR12_PstI_for	GAAGTGCAGCCCATTGCTTCAGTGGCGGCTTTC	Amplification of sequence flanking <i>nsiR4</i> downstream region
3'SyR12_rev	GCCGTACAGACCAACGCAGACC	
Kan_PstI_for	GAAGTGCAGAATAAAAAACGCCCGGCGGCAACCGAGCGAATCCCGTCAAGTCAGCGTAATGCTC	Amplification kanamycin resistance cassette from pVZ322
Kan_BsrGI_rev	GAATGTACACAAAGCCACGTTGTGTCTCAAATCTCTG	
SyR12_ko_seg_for	CGTCCCAAATCGAGCAGTGCATG	Verification of $\Delta$ <i>nsiR4</i> knockout mutants
SyR12_ko_seg_rev	CTAGGGTGTTCGCTCCACGTTTC	
SyR12_EcoRI_for	GAAGAATTCAAGACATAAAGTCAATATCACCTCCGATTGC	Amplification of <i>nsiR4</i> from <i>Synechocystis</i> sp. PCC 6803 for generating an NsiR4 expressing plasmid
SyR12_EcoRI_rev	GAAGAATTCGCATGGCAGCTTCTAAAGGACTAA TAAACTC	
spk_km_hindIII_for	GAAAAGCTTCATTTCGACACCGAGAAAACC	Amplification of insert ( <i>PpetE</i> -NsiR4- <i>oop</i> ) from shuttle vector pJET_spkA::km_ <i>PpetE</i> _ecoRI_ooop for ligation into pVZ322
spk_km_xhoI_rev	GAAGTGCAGTGGATGATGGGGCGATTTCAG	
<b>Oligonucleotides used for Northern blots (T7 promoters are underlined)</b>		
fw_pro_ssr1528	GATCGCCGCTGGCATTGATTTTGATGGC	Amplification of PCR template used for <i>in vitro</i> transcription generating probes for <i>ssr1528</i>
rev_pro_ssr1528	<u>TAATACGACTCACTATAGGGGCGGGAGCGCAT</u> GGTATTACTGACCCC	
rev_pro_ssl1911	ATGTCTACTCAACAACAGGCTCGCGCT	Amplification of PCR template used for <i>in vitro</i> transcription generating probes for <i>gifA</i> ( <i>ssl1911</i> )
fw_pro_ssl1911	<u>TAATACGACTCACTATAGGGAGCGGCAGCGCG</u> GGACAACATGGA	
5sRNA_for	<u>TAATACGACTCACTATAGGAGAAAGAGGAACTT</u> GGCATCGGAC	Amplification of PCR template used for <i>in vitro</i> transcription generating probes for 5S rRNA
5sRNA_rev	GTCATGGAACCACTCCGATCCC	
Oligo probe for NsiR4 ( <i>Anabaena</i> 7120)	GGTCTGGTTAAGCAATCGGAGGGTAAT	Northern blot for NsiR4 detection in <i>Anabaena</i> 7120
7120-rrn5Sa-1	AGTTTTCTGGTGCTATG	PCR fragment probe for detection of 5S rRNA in <i>Anabaena</i> 7120
7120-rrn5Sa-2	ACCTGGCACCGAGCGATTG	
<b>LuxAB reporter assays</b>		
Syr12-KpnI_fw	GGTACCCACGTTCAAACACTTTTACATTTCG	Amplification of the <i>nsiR4</i> promoter from <i>Synechocystis</i> 6803 for cloning into pILA reporter-plasmid
Syr12-KpnI_rev	GGTACCGCAATGGGCGACCTCTAGC	
prNtc_mut_fw	CTCAAATAGGCATCATAAAGCCTACCGATC	Mutation of the putative NtcA motif upstream of <i>nsiR4</i>
prNtc_mut_rev	GATGCCTATTTGAGGAAAGTTCCCGTAAC	
<b>Target verification in <i>E. coli</i> using a Gfp Reporter system (nucleotides underlined and shown in bold refer to the introduced point mutations)</b>		
tv_ssr1528_fw	ATGCATAGTAAAATAACTCGAGGGTAATATTGATCATGG	Amplification of putative target gene-sequence containing the predicted interaction-region with
tv_ssr1528_rev	GCTAGCCTTGGCTTCGGGAATGGCACTG	



tv_ssl1911_gifA_for	ATGCATAGAGGGTAATTAACCAAACTTTTTCA G	NsiR4. The sequence consists of the respective 5' UTR and part of the coding region
tv_ssl1911_gifA_rev	GCTAGCGGATTGTTGACGGTTTTTGATGAATTG	
PLlacoB	CGCACTGACCGAATTCATTAA	Amplification of fragment from plasmid pZE12_luc
PLlacoD	GTGCTCAGTATCTTGTTATCCG	
5_SyR12_long_phos	AAGACATAAAGTCAATATCACCTCC	Amplification of <i>nsiR4</i> (long version) for ligation into pZE12-luc; phosphorylated
3_SyR12_xbal	GTTTTTCTAGATAAAGGACTAATAAACTCTAAA AAGAAAGCC	Reverse primer for the amplification of <i>nsiR4</i> for ligation into pZE12-luc
PXG10_1911_mut_rev	TGACGATTACTGAAAAAAGTTTTGGTTAATTAC	Introduction of a point mutation into <i>gifA</i> sequence
PXG10_1911_mut_fwd	TTCAGTAATCGTCAAGAGGTATTA ACTAT	
PXG10_1528_mut_rev	CCATGATCAAATTACCCTCGAGTTATTTTA	Introduction of a point mutation into <i>ssr1528</i> sequence
PXG10_1528_mut_fwd	GTAAATTGATCATGGCTAATACA ACTAAAGGA	
SyR12_1911_mut_rev	CGACCTCTAGTAATCGGAGGGTGATATTG	Introduction of compensatory mutation into <i>nsiR4</i> sequence ( <i>gifA</i> mRNA as target)
SyR12_1911_mut_fwd	CGATTACTAGAGGTCGCCCAT TGCTT	
SyR12_1528_mut_rev	TGAAATTGACTTTATGTCTTGCTCAGT	Introduction compensatory mutation into <i>nsiR4</i> sequence ( <i>ssr1528</i> mRNA as target)
SyR12_1528_mut_fwd	ATAAAGTCAAATTCACCCTCCGATTGCTA	

**Table S3: List of plasmids.**

Plasmid	Plasmid backbone	Description	Marker	Reference
pJET_spkA::km_PpetE_ecoRI_oop	pJET1.2	Shuttle vector for insertion of <i>nsiR4</i> -sequence. Generation of NsiR4 expressing plasmid	Km <sup>R</sup>	This study
pJET-spkA::km_PpetE_ <i>nsiR4</i> _oop	pJET1.2	Shuttle vector for re-amplification of cassette <i>PpetE::nsiR4::oop</i>	Km <sup>R</sup>	This study
pVZ322-PpetE_NsiR4_ oop_km	pVZ322	Plasmid for copper-inducible NsiR4 expression in <i>Synechocystis</i> sp. PCC 6803	Gen <sup>R</sup> , Km <sup>R</sup>	This study
pJET-ssl1697::km::ssl1698	pJET1.2	Generation of <i>nsiR4</i> knockout strain	Km <sup>R</sup>	This study
pILA		Promoter probe vector harbouring the promoterless <i>luxAB</i> genes encoding luciferase, contains recombinations sites for integration into the <i>Synechocystis</i> chromosome	Km <sup>R</sup> , Amp <sup>R</sup>	(5)
pILA-P <i>nsiR4</i>	pILA	pILA harbouring <i>luxAB</i> under control of the <i>nsiR4</i> promoter (range between -130 to +49 with respect to the transcriptional start siteTSS at +1 was used)	Km <sup>R</sup> , Amp <sup>R</sup>	This study
pILA-P <i>nsiR4</i> _mut	pILA	same as pILA-P <i>nsiR4</i> but carrying a mutated NtcA binding motif	Km <sup>R</sup> , Amp <sup>R</sup>	This study
pZE12-luc		General expression plasmid	Amp <sup>R</sup>	(29)
pJV300	pZE12-luc	Control plasmid, expressing a ~50 nt nonsense transcript derived from <i>rrnB</i> terminator	Amp <sup>R</sup>	(30)
pXG0	pZA31-luc	Plasmid expressing luciferase used as a negative control; cell autofluorescence	Cm <sup>R</sup>	(18)
pXG10	pXG0	Plasmid for synthesis of translational sfGFP fusions	Cm <sup>R</sup>	(19)
pXG10_ssl1911	pXG10-SF	GFP reporter plasmid containing the <i>gifA</i> 5'UTR plus the initial part of the coding region	Cm <sup>R</sup>	this study
pXG10_ssr1528	pXG10-SF	GFP reporter plasmid containing the <i>ssr1528</i> 5'UTR plus initial part of the coding region	Cm <sup>R</sup>	this study
pXG10_ssl1911_mut	pXG10-SF	GFP fusion plasmid containing the <i>gifA</i> 5'UTR with mutation in the interacting region with NsiR4	Cm <sup>R</sup>	this study
pXG10_ssr1528_mut	pXG10-SF	GFP fusion plasmid containing the <i>ssr1528</i> 5'UTR with mutation in the interacting region with NsiR4	Cm <sup>R</sup>	this study
pZE12_NsiR4	pZE12-luc	Plasmid expressing NsiR4	Amp <sup>R</sup>	this study
pZE12_NsiR4_mut1911	pZE12-luc	Plasmid expressing NsiR4 with a compensatory mutation for the mutation in <i>gifA</i>	Amp <sup>R</sup>	this study

pZE12_NsiR4_mut1528	pZE12-luc	Plasmid expressing NsiR4 with a compensatory mutation for the mutation in <i>ssr1528</i>	Amp <sup>R</sup>	this study
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