A nitrogen stress-inducible small RNA regulates CO$_2$ fixation in Nostoc

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Abstract

In the absence of fixed nitrogen, some filamentous cyanobacteria differentiate heterocysts, specialized cells devoted to fixing atmospheric nitrogen (N$_2$). This differentiation process is controlled by the global nitrogen regulator NtcA and involves extensive metabolic reprogramming, including shutdown of photosynthetic CO$_2$ fixation in heterocysts, to provide a microaerobic environment suitable for N$_2$ fixation. Small regulatory RNAs (sRNAs) are major post-transcriptional regulators of gene expression in bacteria. In cyanobacteria, responding to nitrogen deficiency involves transcribing several nitrogen-regulated sRNAs. Here, we describe the participation of nitrogen stress-inducible RNA 4 (NsiR4) in post-transcriptionally regulating the expression of two genes involved in CO$_2$ fixation via the Calvin cycle: glpX, which encodes bifunctional sedoheptulose-1,7-bisphosphatase/fructose-1,6-bisphosphatase (SBPase), and pgk, which encodes phosphoglycerate kinase (PGK). Using a heterologous reporter assay in Escherichia coli, we show that NsiR4 interacts with the 5'-untranslated region (5'-UTR) of glpX and pgk mRNAs. Overexpressing NsiR4 in Nostoc sp. PCC 7120 resulted in a reduced amount of SBPase protein and reduced PGK activity, as well as reduced levels of both glpX and pgk mRNAs, further supporting that NsiR4 negatively regulates these two enzymes. In addition, using a gfp fusion to the nsiR4 promoter, we show stronger expression of NsiR4 in heterocysts than in vegetative cells, which could contribute to the heterocyst-specific shutdown of Calvin cycle flux. Post-transcriptional regulation of two Calvin cycle enzymes by NsiR4, a nitrogen-regulated sRNA, represents an additional link between nitrogen control and CO$_2$ assimilation.

Introduction

In response to nitrogen deficiency, some filamentous cyanobacteria are able to differentiate a specialized cell type devoted to fixation of atmospheric nitrogen, the heterocyst (Muro-Pastor and Hess, 2012). Differentiation of functional heterocysts, which takes about 24 h to complete under laboratory conditions, involves a precise gene expression program in which many genes are distinctly regulated in cells undergoing transformation into heterocysts (Brenes-Álvarez et al., 2019). This program is ultimately under the control of the global nitrogen regulator NtcA (Herrero et al., 2004) but also involves HetR, the master regulator of cellular differentiation (Buikema and Haselkorn, 1991) that is required for expression of the heterocyst-specific transcriptome (Brenes-Álvarez et al., 2019). In N$_2$-fixing filaments, composed of vegetative cells and heterocysts, metabolic division of labor is...
established so that photosynthesis and N₂ fixation are spatially separated in the two different cell types that cooperate to achieve growth of the filament as a whole. Whereas the vegetative cells maintain photosynthesis and CO₂ fixation, providing reduced carbon to heterocysts, the heterocysts provide fixed nitrogen through nitrogenase activity (Flores and Herrero, 2010; Herrero et al., 2016). One major aspect of the metabolic reprogramming of vegetative cells undergoing differentiation as heterocysts involves shutdown of oxygentic photosynthesis, which is not compatible with the activity of the O₂-inactivated enzyme nitrogenase. Early studies showing movement of reduced carbon compounds from vegetative cells into heterocysts suggested that there was no CO₂ fixation in heterocysts (Fay and Walsby, 1966; Wolk, 1968), consistent with the absence in those cells of key enzymes of the Calvin cycle, such as ribulose-bisphosphate carboxylase/oxygenase (Rubisco) or phosphoribulokinase (PRK; Codd and Stewart, 1977; Codd et al., 1980; Cossar et al., 1985; Elhai and Wolk, 1990). In agreement with those observations, a quantitative proteomic study in *Nostoc punctiforme* showed that proteins involved in the carbon concentrating mechanism and enzymes involved in CO₂ fixation were present in reduced amounts in the heterocysts versus the vegetative cells (Sandh et al., 2014).

Carbon and nitrogen metabolisms are tightly coupled in cyanobacteria, with regulatory circuits depending on the balance between these two elements (Zhang et al., 2018; Herrero and Flores, 2019; Forchhammer and Selim, 2020). NtcA, the main transcriptional regulator of nitrogen assimilation, is modulated by the level of 2-oxoglutarate, an indicator of the C/N balance that integrates the flow of CO₂ assimilation through the Calvin–Benson cycle and ammonium (NH₄⁺) assimilation through the GS/GOGAT cycle (Vázquez-Bermúdez et al., 2002). Central to C/N homeostasis in cyanobacteria is the nitrogen-regulated expression of elements involved in carbon assimilation, such as the recently described carbon flow regulator CfrA/PirC whose transcription is directly activated by NtcA (Muro-Pastor et al., 2020; Orthwein et al., 2021). In addition to transcriptional regulation, post-transcriptional regulatory mechanisms might also play a relevant role in achieving properly balanced assimilation of carbon and nitrogen (Muro-Pastor and Hess, 2020).

Post-transcriptional regulation by ncRNAs (small regulatory RNAs [sRNAs] and antisense RNAs) is emerging as an additional level of control of different processes in bacteria. Noncoding RNAs whose transcription is regulated in response to certain environmental changes are candidates for having a role in the adaptation to those changes. For instance, a major transcription factor in cyanobacteria, RpAB, which regulates adaptation to fluctuating light conditions (Wilde and Hihara, 2016), regulates expression of PsrR1 (Kadowaki et al., 2016), an sRNA that controls photosynthetic functions (Georg et al., 2014). Several nitrogen-regulated sRNAs have been identified both in unicellular and heterocystous cyanobacterial strains. In the case of *Nostoc* sp. PCC 7120 (also known as *Anabaena* sp. PCC 7120, hereafter *Nostoc*), sRNAs have been described with NtcA-regulated expression, such as NsiR3, NsiR4, or NsrR1 (Klaßn et al., 2015; Álvarez-Escribano et al., 2018, 2021) or with HetR regulated, heterocyst-specific expression, such as NsiR1 (Ionescu et al., 2010).

NsiR4 is a small RNA widely distributed among cyanobacterial strains. Two versions, which differ in the presence of a 5’-extension capable of forming a short hairpin, have been identified. Long 5’-extended forms are found in unicellular strains, such as the nondiazotrophic strain *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*), whereas shorter versions are present in filamentous strains, including those capable of fixing N₂ via differentiation of heterocysts, such as *Nostoc*. In all cases, the corresponding promoters contain binding sites for NtcA, and NtcA has been shown to activate expression of NsiR4 in response to nitrogen deprivation in both *Synechocystis* and *Nostoc* (Klaßn et al., 2015). In *Synechocystis*, NsiR4 post-transcriptionally regulates accumulation of IF7, one of the inactivating factors that modulate the activity of glutamine synthetase, a key enzyme involved in nitrogen assimilation, therefore participating in a regulatory feed-forward loop operated by NtcA (Klaßn et al., 2015).

In this work, we provide evidence showing that accumulation of bifunctional sedoheptulose-1,7-bisphosphatase/fructose-1,6-bisphosphatase (SBPase) and phosphoglycerate kinase (PGK) is subjected to post-transcriptional regulation involving NsiR4. We also show that expression of NsiR4 is stronger in heterocysts than in vegetative cells, and could therefore contribute to the differential regulation of these two enzymes of the Calvin cycle specifically in heterocysts. Ultimately, regulation of CO₂ fixation by an NtcA-regulated sRNA represents a link between nitrogen availability and carbon assimilation.

**Results**

NsiR4 is conserved in heterocystous cyanobacteria and accumulates differentially in heterocysts

Sequences encoding NsiR4 are strongly conserved among heterocystous cyanobacteria and would produce an sRNA with a single-stranded stretch followed by a terminator stem loop (Supplemental Figure S1). In *Nostoc*, the sequence encoding the NsiR4 homolog is located tail to tail with that of the coding region of *alr3725* (Figure 1A) and is transcribed from a transcriptional start site (TSS) located at position 4499325r (Mitschke et al., 2011), downstream of a NtcA-regulated promoter (Figure 1B). Northern blot hybridization shows that expression of NsiR4 is already observed 3–6 h after nitrogen removal, both in the wild-type (WT) and in a *hetR* mutant strain unable to differentiate heterocysts (Figure 1, C and D), suggesting transcription of NsiR4 would be induced in all cells of the filaments upon nitrogen stress. In order to analyze expression of NsiR4 in specific cells of N₂-fixing filaments, we constructed a fusion between the
the scheme (Figure 2B), showing the presence of combined nitrogen was quantified as indicated in filaments growing in the presence of NH$_4^+$ (Figure 2A). Fluorescence in individual vegetative cells from peaks of strong fluorescence associated with heterocysts showed increased green fluorescence in vegetative cells, with plasmid pELV71 in Figure 1A). Filaments bearing plasmid promoter region cloned upstream of the $gfp$ gene (green arrow) in promoter-probe plasmid pELV71. B, Sequence of the promoter and coding region of NsiR4. The sequence encoding NsiR4 (in orange), the stop codon of $alr3725$ (in red), as well as the NtcA-binding box, the –10 box, the spacing between both boxes (22 nt), and the TSS (black arrow) are indicated. Sequences producing a stem-loop terminator are indicated with orange arrows. C and D, Nitrogen-responsive expression of NsiR4 in Nostoc WT (C) and hetR mutant strain 216 (D). Expression was analyzed by Northern blot in cells grown in the presence of NH$_4^+$ and transferred to a medium containing no source of combined nitrogen for the number of hours indicated. The upper panels show hybridization to a probe for 5S RNA. One of the plasmids encodes $gfp$ (plus the first 60 nucleotides [nt] of the coding sequence) translationally fused to the gene for superfolder green fluorescent protein (sfGFP), whereas the other plasmid encodes NsiR4 or an unrelated RNA used as a control. Cells bearing the fusion of either $glpX$ or $pgk$ to sfGFP showed fluorescence, indicating that both translation initiation regions were functional in E. coli, although to different extents (Figure 3, A–B). In fact, whereas in the case of $pgk$, which produced higher levels of fluorescence even with the native GUG start codon, expression would be consistent with possible negative effects on mRNA stability caused by interaction with a nitrogen stress-inducible sRNA such as NsiR4.

The predicted interactions between NsiR4 and the 5′-UTR of the mRNAs of $glpX$ and $pgk$ would take place immediately upstream of the translational start codon (GUG in both cases), overlapping the Shine–Dalgarno (SD) sequence (Figure 3), and therefore would affect translation of the mRNAs. In accordance with CopraRNA results, such interactions would be phylogenetically conserved in many heterocystous cyanobacteria (Supplemental Figure S3).

**NsiR4 interacts with $glpX$ and $pgk$ mRNAs**

We have validated the predicted interactions between NsiR4 and the mRNAs of $glpX$ and $pgk$ using a heterologous reporter assay (Corcoran et al., 2012) in which two plasmids, one producing constitutive expression of a predicted target mRNA and the other of the sRNA under study, are combined in Escherichia coli cells. One of the plasmids encodes the 5′-UTR of the predicted $glpX$ or $pgk$ target (plus the first 60 nucleotides [nt] of the coding sequence) translationally fused to the gene for superfolder green fluorescent protein (sfGFP), whereas the other plasmid encodes NsiR4 or an unrelated RNA used as a control. Cells bearing the fusion of either $glpX$ or $pgk$ to sfGFP showed fluorescence, indicating that both translation initiation regions were functional in E. coli, although to different extents (Figure 3, A–B). In fact, whereas in the case of $glpX$, the GUG start codon had to be replaced by AUG to facilitate translation, such a change was not necessary in the case of $pgk$, which produced higher levels of fluorescence even with the native GUG start codon.

In the case of both $glpX$ (Figure 3A) and $pgk$ (Figure 3B), the fluorescence in E. coli cells encoding the corresponding sfGFP fusion significantly decreased when NsiR4 was co-expressed, in comparison with cells expressing the control.

**CopraRNA predicts $glpX$ and $pgk$ mRNAs as possible targets of NsiR4 in Nostoc**

Prediction of mRNAs that might be post-transcriptionally regulated by NsiR4 was carried out using the CopraRNA algorithm, which takes into account comparative phylogenetic information (Wright et al., 2013, 2014). The top 10 predicted interactions using the genomes of 9 heterocystous cyanobacteria, including Nostoc, are shown in Table 1. Because functional enrichment among the possible targets of sRNA regulation can be taken as a further indication of the validity of predictions, two of the best-predicted targets caught our attention, since they correspond to mRNAs encoding two enzymes involved in CO$_2$ assimilation by the Calvin–Benson cycle, namely SBPase, encoded by gene $glpX$ (alr1041), and PGK, encoded by gene $pgk$ (alr14131). In addition, according to transcriptomic analysis, accumulation of $glpX$ and $pgk$ mRNAs is lower under nitrogen deficiency (Brenes-Álvarez et al., 2019; see also Supplemental Figure S2). This observation would be consistent with possible negative effects on mRNA stability caused by interaction with a nitrogen stress-inducible sRNA such as NsiR4.

Figure 1 NsiR4 in Nostoc. A, Schematic representation of the region encoding NsiR4. The flanking annotated open reading frames are indicated (gray arrows), together with NsiR4 (orange arrow) and the promoter region cloned upstream of the $gfp$ gene (green arrow) in promoter-probe plasmid pELV71. B, Sequence of the promoter and coding region of NsiR4. The sequence encoding NsiR4 (in orange), the stop codon of $alr3725$ (in red), as well as the NtcA-binding box, the –10 box, the spacing between both boxes (22 nt), and the TSS (black arrow) are indicated. Sequences producing a stem-loop terminator are indicated with orange arrows. C and D, Nitrogen-responsive expression of NsiR4 in Nostoc WT (C) and hetR mutant strain 216 (D). Expression was analyzed by Northern blot in cells grown in the presence of NH$_4^+$ and transferred to a medium containing no source of combined nitrogen for the number of hours indicated. The upper panels show hybridization to a probe for 5S RNA. One of the plasmids encodes $gfp$ (plus the first 60 nucleotides [nt] of the coding sequence) translationally fused to the gene for superfolder green fluorescent protein (sfGFP), whereas the other plasmid encodes NsiR4 or an unrelated RNA used as a control. Cells bearing the fusion of either $glpX$ or $pgk$ to sfGFP showed fluorescence, indicating that both translation initiation regions were functional in E. coli, although to different extents (Figure 3, A–B). In fact, whereas in the case of $glpX$ the GUG start codon had to be replaced by AUG to facilitate translation, such a change was not necessary in the case of $pgk$, which produced higher levels of fluorescence even with the native GUG start codon. In the case of both $glpX$ (Figure 3A) and $pgk$ (Figure 3B), the fluorescence in E. coli cells encoding the corresponding sfGFP fusion significantly decreased when NsiR4 was co-expressed, in comparison with cells expressing the control.

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Expression of $P_{\text{nsiR4}}$-gfp. A, Confocal fluorescence images of filaments bearing plasmid pELV71 growing on top of medium containing ammonium ($\text{NH}_4^+$) or lacking any source of combined nitrogen (N$_2$) are shown with merged green (GFP fluorescence) and red (autofluorescence, shown in magenta) channels. Quantification of the signal for the green channel along the filaments indicated with white arrows is shown on the right. All images were acquired with the same sensitivity settings for the green channel so that intensities can be compared. Scale bars, 10 $\mu$m. B, Quantification of GFP fluorescence in vegetative cells of the filaments indicated with white arrows in (A). Sixty three regions of interest corresponding to vegetative cells were quantified for each condition (red ovals), as indicated in the scheme on the left. Data are presented on the right as the mean and standard deviation of the fluorescence. T test $****P<0.0001$.

Table 1 List of predicted interaction partners for NsiR4 based on the CopraRNA algorithm

| Rank | $P$-value | Locus Tag | Gene | Annotation |
|------|-----------|-----------|------|------------|
| 1    | 1.65E-15  | pcc7120delta_rs22335 (all4121) | petH | Ferredoxin-NADP$(\cdot\cdot\cdot)$ reductase |
| 2    | 1.74E-10  | pcc7120delta_rs22380 (all4131) | pgk | Phosphoglycerate kinase |
| 3    | 1.68E-09  | pcc7120delta_rs07060 (alr1041) | glpX | Fructose-1,6-bisphosphatase |
| 4    | 5.55E-08  | pcc7120delta_rs04675 (alr0549) | NA  | Four helix bundle protein |
| 5    | 4.36E-06  | pcc7120delta_rs04815 (all0578) | dnaB | Intein-containing replicative DNA helicase |
| 6    | 6.05E-06  | pcc7120delta_rs05000 (alr0617) | cpcS | Phycocyanobilin lyase |
| 7    | 8.24E-06  | pcc7120delta_rs04560 | NA  | Hypothetical protein |
| 8    | 2.23E-05  | pcc7120delta_rs05130 (alr0643) | NA  | MotA/TolQ/ExbB proton channel family protein |
| 9    | 2.49E-05  | pcc7120delta_rs21105 (all3880) | cphB | Cyanophycinase |
| 10   | 2.73E-05  | pcc7120delta_rs11495 (all1943) | NA  | Hypothetical protein |

NsiR4 sequences from the following strains were used: *Nostoc* sp. PCC 7120, *T. variabilis* ATCC 29413, *Calothrix* sp. PCC 6303, *C. stagnale* PCC 7417, *N. azollae* 0708, *N. punctiforme* PCC 73102, *Nostoc* sp. PCC 7524, *Rivularia* sp. PCC 7116, and *Nostoc* sp. PCC 7107. NA, not available. Genes studied in this work are indicated in bold.
RNA, indicating that the interaction of NsiR4 with either the glpX or the pgk mRNAs negatively affected their translation. To verify that the interactions took place at the predicted sites, a point mutation that would destabilize interaction with both the glpX and pgk mRNAs was introduced into NsiR4 (nt 13, C to G, Mut13). The corresponding hybridization energies to the WT and the mutated version of NsiR4 are indicated (Figure 3A and B). Mutation of NsiR4 diminished the interaction with both mRNAs, as indicated by a weaker reduction of fluorescence compared with cells expressing native NsiR4. We additionally constructed a mutated version of the mRNA of glpX containing a compensatory change (nt −20, G to C, Comp13) that would restore interaction with the Mut13 version of NsiR4. In this case, the fluorescence of cells bearing the compensatory change in the 5′-UTR of glpX and expressing the Mut13 version of NsiR4 was reduced with respect to that of cells bearing NsiR4, indicating interaction between NsiR4 (Mut13) and glpX (Comp 13). The absolute fluorescence values of cells bearing the compensated version of glpX were in all cases

**Figure 3** Verification of NsiR4 interaction with the 5′-UTR of glpX and pgk using an in vivo reporter system in *E. coli*. A, Left, predicted interaction between NsiR4 and the 5′-UTR of glpX according to IntaRNA (Mann et al., 2017). Nucleotides in the 5′-UTR are numbered with respect to the start of the coding sequence (shaded). The mutation introduced in NsiR4 at position 13 (C to G, Mut13) and the corresponding compensatory mutation in glpX 5′-UTR at position −20 (G to C, Comp13) are indicated in blue and purple, respectively. Hybridization energies of the interaction between the glpX mRNA and the WT (black) or mutated (blue) versions of NsiR4 are indicated. Right, fluorescence measurements of *E. coli* DH5α cultures bearing combinations of plasmids expressing different versions of NsiR4 (WT or Mut13) and glpX::sfGFP fusions (WT or Comp13). Plasmid pJV300 was used as control. B, Left, predicted interaction between NsiR4 and the 5′-UTR of pgk according to IntaRNA (Mann et al., 2017). Nucleotides in the 5′-UTR are numbered with respect to the start of the coding sequence (shaded). The mutation introduced in NsiR4 at position 13 (C to G, Mut13) is indicated in blue. Hybridization energies of the interaction between the pgk mRNA and the WT (black) or mutated (blue) versions of NsiR4 are indicated. Right, fluorescence measurements of *E. coli* DH5α cultures bearing combinations of plasmids expressing different versions of NsiR4 (WT or Mut13) and the pgk::sfGFP fusion. Plasmid pJV300 was used as control. A and B, Putative SD sequences are boxed. The data are presented as the mean ± standard deviation of the results from eight independent colonies after subtraction of fluorescence in cells bearing plasmid pXG0. Fluorescence is normalized to the A600 of each culture. T test ***P < 0.001; *P < 0.05.
Higher than those of the native version, presumably due to better efficiency in the translation of the compensated version versus the WT version. A similar compensatory mutation could not be analyzed in the case of the pgk mRNA because the fluorescence of the strain bearing the modified version of the mRNA was too low for accurate measurement.

**glpX and pgk are post-transcriptionally regulated by NsiR4 in Nostoc**

In order to analyze the effects of NsiR4 in Nostoc, we prepared strains with altered levels of NsiR4. In the overexpressor strains (OE_NsiR4), constitutive high levels of NsiR4 were transcribed from the strong trc promoter to the T1 terminator of rnb of *E. coli* (Figure 4A). Because of the proximity of nsiR4 with the gene located downstream (Figure 1), and in order to deplete NsiR4 while preserving the structure of the region, we took an approach based on the overexpression of an antisense sequence (complementary to NsiR4), which would act as a sponge to neutralize NsiR4 (OE_as_NsiR4). This approach has been successfully used to deplete other sRNAs in *Nostoc*, such as Yfr1, which is involved in cell wall homeostasis (Brenes-Álvarez et al., 2020b) or the nitrogen-stress-inducible NsiR1, which modulates heterocyst differentiation (Brenes-Álvarez et al., 2020a). We verified the accumulation of NsiR4 in Nostoc strains bearing the above-described constructs by Northern blot. In the overexpressor strains, NsiR4 molecules transcribed from the trc promoter bear a 6-nt tag so that they can be distinguished from native endogenous NsiR4 molecules based on their lengths. Expression of NsiR4 from the trc promoter clearly exceeded endogenous NsiR4 expression, whereas transcription of the sequence antisense to NsiR4 led to complete depletion of NsiR4 (Figure 4B). Both OE_NsiR4 and OE_as_NsiR4 strains could grow on agar plates, similarly to the OE_C control strain, in the presence of combined nitrogen, however, OE_NsiR4, unlike OE_as_NsiR4 or the control strain, could not grow under N2-fixing conditions (Supplemental Figure S4).

We then analyzed the amount of SBPase and PGK in the strains with altered levels of NsiR4. Using antibodies against *Synechocystis* SBPase, we detected reduced amounts of SBPase protein in the strain overexpressing NsiR4 (OE_NsiR4), whereas a slightly increased amount of protein was observed in the strain depleted of NsiR4 (OE_as_NsiR4; Figure 4C). Similarly, reduced PGK activity was present in crude extracts of the strain overexpressing NsiR4, while higher PGK activity was found in crude extracts of the strain depleted of NsiR4 (OE_as_NsiR4; Figure 4D).

We also tested the accumulation of the mRNAs of glpX and pgk in the OE_C (control), OE_NsiR4 and the OE_as_NsiR4 strains. The accumulation of both mRNAs was significantly diminished in the strain overexpressing NsiR4 (OE_NsiR4) with respect to the control strain (Figure 4, E and F), and there was also a slightly increased accumulation of both mRNAs in the OE_as_NsiR4, which was depleted of NsiR4. Taken together, these results indicated that NsiR4 negatively affects the accumulation of glpX and pgk mRNAs in *Nostoc*. This is the expected result if inhibition of their translation by NsiR4 results in destabilization of the mRNAs.

**Discussion**

Cyanobacteria have evolved sophisticated regulatory networks in order to keep a proper carbon/nitrogen balance (Forchhammer and Selim, 2020). In addition to transcriptional regulation, mechanisms involving post-transcriptional regulation by noncoding RNAs appear to play a role in the coordination of carbon and nitrogen assimilation in these photosynthetic bacteria (Muro-Pastor and Hess, 2020). Here we describe the participation of NsiR4, a NtcA-regulated sRNA, in the post-transcriptional regulation of glpX and pgk. The conserved binding site of NsiR4 on the translation initiation region of these two mRNAs overlaps the predicted ribosome binding site (Figure 3; Supplemental Figure S3), suggesting that NsiR4 inhibits the initiation of translation of these two mRNAs in heterocyst-forming cyanobacteria. As shown in Figure 4, both the accumulation of the glpX and pgk mRNAs and the accumulation or activity of the corresponding enzymes are diminished in *Nostoc* strains bearing high levels of NsiR4. A secondary consequence of translation inhibition would be destabilization of the mRNAs, resulting in their degradation (Figure 4, E and F). According to a global analysis of the nitrogen-responsive transcriptome of *Nostoc*, glpX and pgk belong to a group of genes with a similar pattern of repression upon nitrogen deprivation (Brenes-Álvarez et al., 2019), suggesting the possible implication of common factors. The NtcA-regulated sRNA NsiR4 could be one such factor participating in the post-transcriptional regulation of these two genes.

Expression of NsiR4 is induced in vegetative cells upon removal of combined nitrogen (Figure 2), which is what would be expected for a gene positively regulated by NtcA. In addition, there is a stronger induction in heterocyst than in vegetative cells (Figure 2A). This complex pattern of expression is similar to that of NsiR3, another NtcA-regulated sRNA (Álvarez-Escríbano et al., 2021) and could ultimately be a consequence of the higher concentration of NtcA present in heterocysts versus vegetative cells (Olmedo-Verd et al., 2006; Sandh et al., 2014). The differential accumulation of NsiR4 in heterocysts could result in differential regulation of glpX and pgk in these cells, as summarized in the proposed model (Figure 5). A quantitative proteomic study in *N. punctiforme* (Sandh et al., 2014) showed that the amount of several proteins involved in CO2 fixation through the Calvin cycle were reduced in heterocysts and, in fact, SBPase was the protein with the lowest ratio of abundance in heterocyst versus vegetative cells. Similarly, a transcriptomic study carried out in *Anabaena variabilis* reported that the amount of pgk transcript was reduced in heterocysts versus vegetative cells (Park et al., 2013). In the case of SBPase, two post-transcriptional regulatory mechanisms would contribute to
Figure 4. Effect of NsiR4 on the amount of SBPase protein, PGK activity, and the accumulation of glpX and pgk mRNAs in Nostoc. A, Scheme of the DNA fragments cloned into the plasmids used to generate the different strains with altered levels of NsiR4 (orange arrows). TSS (bent arrows), Rho-independent terminator of NsiR4 (small stem-loop), T1 terminator (large stem-loop) and the trc promoter are indicated. B, Northern blot using RNA extracted from strains OE_C, OE_NsiR4, and OE_as_NsiR4 grown in the presence of nitrate (0) or 24 h after nitrogen removal (24), hybridized with a probe for NsiR4 (top) or 5S RNA (bottom) used as loading control. Endogenous NsiR4 (black triangles) and the 6-nt-longer NsiR4 version constitutively expressed from the trc promoter (red triangle) are indicated. Samples contained 10 µg of total RNA. C, Accumulation of SBPase in strains with altered levels of NsiR4 was analyzed by Western blot in samples containing 40 µg of soluble fraction from cells grown in the presence of nitrate and transferred to medium containing no source of combined nitrogen for 24 h. Western blots were quantified with ImageLab software (Bio-Rad, Hercules, CA, USA) and normalized to Ponceau Red staining. The position of size markers (in kilodalton) is indicated on the right side. Four technical replicates of two different clones of each strain were quantified. Values are expressed as percentage of the normalized SBPase amount with respect to the amount in the control strain (OE_C), which was considered 100%. D, PGK activity in strains with altered levels of NsiR4 was measured in crude extracts from cells analyzed in (C). Assays were performed in duplicate with two different clones of each strain. Specific activity is expressed as percentage of the activity (2.1 U/mg protein) in the control strain (OE_C), which was considered 100%. E and F, Accumulation of glpX (E) or pgk (F) mRNAs in strains with altered levels of NsiR4 were analyzed by Northern blot in the same cells analyzed in (C) and (D). The position of size markers (in kilobase) is indicated on the left side. Data from two different clones of each strain were quantified and the amount of glpX or pgk mRNA was normalized to the amount of mpB RNA. Values are expressed as percentage of the glpX or pgk RNA amount, normalized with respect to RNA amount in the control strain (OE_C), which was considered 100%. Samples contained 3.5 µg of total RNA. C–F, The data are presented as the mean ± standard deviation. T test *P < 0.05; **P < 0.01; ***P < 0.001.
such differential accumulation, namely, the previously described operation of a heterocyst-specific antisense RNA (as_glpX; Olmedo-Verd et al., 2019) and the negative regulation by NsiR4 described here. Because SBPase has been identified as a rate-limiting enzyme in the Calvin cycle in cyanobacteria and plants (Ding et al., 2016; De Porcellinis et al., 2018), reduced amounts of SBPase in heterocysts likely contribute to shutdown of CO$_2$ fixation in these specialized cells. In the case of PGK, speculation on the redistribution of metabolic fluxes in heterocysts versus vegetative cells may be more complex, since PGK is not only involved in the Calvin cycle but is also required for glycolysis that, in principle, is active in heterocysts. In any case, the negative regulation exerted by native amounts of NsiR4 must be subtle and perhaps required for fine-tuning PGK activity under N$_2$-fixing conditions.

Strains overexpressing NsiR4 from a strong constitutive promoter are not able to grow under N$_2$-fixing conditions. Because the amount of NsiR4 in these strains clearly exceeds that present in the WT irrespective of the nitrogen status (Figure 4B), this observation can be a consequence of excessive reduction of PGK activity or pleiotropic effects due to interactions of NsiR4 with additional targets yet to be identified.

sRNA regulation of enzymes involved in primary carbon metabolism seems to be widespread in cyanobacteria. In Prochlorococcus, a cyanobacterium distantly related to Nostoc, several enzymes of carbon primary metabolism, including pgk, might be regulated by sRNAs and antisense RNAs (Lambrecht et al., 2019). Our results indicate that CO$_2$ fixation can be modulated by a nitrogen stress-inducible sRNA that downregulates enzymes of the Calvin cycle. Modulation of glpX and pgk in vegetative cells might also be necessary to fine-tune carbon assimilation rate to nitrogen availability while mature heterocysts become functional and under N$_2$-fixing conditions (Figure 5). The observed induction of expression of NsiR4 also in vegetative cells, although much weaker than that observed in heterocysts, suggests NsiR4 could be a relevant factor for adapting carbon metabolism to nitrogen deficiency.

In the unicellular strain Synechocystis, the NsiR4 homolog has been described to regulate the accumulation of IF7 (Klähn et al., 2015). Such a regulatory circuit constitutes a feed-forward loop, since transcription of gifA, encoding IF7,
is also under control of NtcA (García-Domínguez et al., 2000). In the case of *Nostoc*, the mRNA of *gifA* is not predicted as a target of NsiR4 in the informatic screening. In fact, NsiR4 from *Nostoc* and homologs identified in heterocystous strains (Supplemental Figure S1) lack the 19 nt 5′-extension that in NsiR4 from *Synechocystis* is involved in the interaction of NsiR4 and the *gifA* mRNA, encoding IF7 (Klähn et al., 2015). These observations suggest functional diversification of NsiR4, with different targets being modulated in different groups of cyanobacteria.

In the context of heterocyst differentiation, posttranscriptional regulation of gene expression would represent an additional level of control of the metabolic transformation of vegetative cells into heterocysts. As described above for NsiR4, the observed strong expression of other NtcA-regulated sRNAs in heterocysts could contribute to differential gene expression in these specialized cells. In this context, NsiR3, another NtcA-regulated sRNA that shows stronger expression in heterocysts than in vegetative cells, would contribute to differential regulation of proline oxidase, whose diminished levels in heterocysts could facilitate channeling of arginine to the biosynthesis of cyanophycin granules (Álvarez-Escribano et al., 2021). Mixed regulatory circuits involving transcription factors and non-coding RNAs could eventually achieve a more efficient and fine-tuned response than can be expected from purely transcriptional regulation (Nitzan et al., 2017), thus improving the fitness of microorganisms exposed to highly dynamic environments.

**Materials and methods**

**Strains and growth conditions**

Cultures of *Nostoc* WT and *hetR* derivative strain 216 were bubbled with an CO2/air mixture (1% v/v) and grown photoautotrophically at 30°C in BG11 medium (Rippka et al., 1979) containing ferric citrate instead of ammonium ferric citrate and lacking NaNO3 but containing 6 mM NH4Cl, 10 mM NaHCO3 (BG11C). For the cultures, BG11 medium and nitrogen deficiency was induced as described in Supplemental Table S1.

**Nostoc strains construction**

Plasmids and oligonucleotides used in this work are described in Supplemental Tables S2 and S3. The sequences of all fragments amplified by polymerase chain reaction (PCR) were entirely verified by sequencing. We have used pMBA37 (Olmedo-Verd et al., 2019) as a backbone for overexpressing NsiR4 or an antisense to NsiR4. The sequence encoding NsiR4 or as-NsiR4 was amplified using pSAM302 as a template and oligonucleotides 919 and M13 universal or 920 and 921, respectively. The products were digested with NsiI and XhoI and cloned between the NsiI and XhoI sites in pSAM349 (NsiR4) and pSAM350 (as-NsiR4) (Supplemental Table S2). pMBAS1, a plasmid that overexpresses a control RNA corresponding only to the T1 terminator under the trc promoter (Olmedo-Verd et al., 2019), pSAM349 and pSAM350 were introduced in *Nostoc* WT by conjugation (Elhai and Wolk, 1988) generating strains OE_C, OE_NsiR4, and OE_as_NsiR4, respectively (Supplemental Table S1). The inserts in pMBAS1, pSAM349, and pSAM350 are shown in Supplemental Table S4.

To generate a plasmid bearing a fusion between the promoter of NsiR4 and a promoterless *gfp*mut2 gene in *Nostoc*, the promoter region (−129 to +9 with respect to the TSS of NsiR4) was amplified with primers 406 and 407 and cloned into pSpark, rendering pELV56. The Clal-Xhol fragment containing *PnsiR4* was cloned into Clal-Xhol-digested pCSAM201 (Ionescu et al., 2010), rendering pELV64. The EcoRI fragment of pELV64 containing the transcriptional fusion between *PnsiR4* and *gfp*mut2 was cloned into EcoRI-digested pCSEL24 (Olmedo-Verd et al., 2006), rendering pELV71.

**Reporter assay for in vivo verification of targets in E. coli**

We used a previously described reporter assay (Urban and Vogel, 2007) and fusions to the gene *sfGFP* in plasmid pXG10-SF (Corcoran et al., 2012) for experimental target verification in *E. coli*. In this system both the GFP fusions and NsiR4 are transcribed constitutively. 5′-UTR of *glpX* and *pgk* were cloned into pXG10-SF from their corresponding TSS, according to Mitschke et al. (2011), to 60 nt within the coding region. To facilitate translation in *E. coli*, the GTG start codon of *glpX* mRNA was replaced by ATG using overlapping PCR and oligonucleotides specified in Supplemental Table S3. PCR fragments containing the region to be cloned were amplified using genomic DNA as template and oligonucleotides specified in Supplemental Table S3. Fragments were digested with NsiI and HpaI and cloned into pXG10-SF treated with the same enzymes, resulting in translational fusions of the different targets to sfGFP (Supplemental Table S5).

To express NsiR4 in *E. coli*, the sequence encoding NsiR4 was amplified from genomic DNA using primers 274 (5′-phosphorylated) and 275. The PCR product was digested with XbaI and fused to a plasmid backbone that was amplified from pZE12-luc with primers PLlacOB and PLjacOD.
Alternatively, oligonucleotide 47 was end-labeled with M13 universal and M13 reverse and pSAM302 as template. The PCR fragment for the NsiR4 probe was amplified with oligonucleotides of the sRNA or mRNA to be detected. The PCR fragment was cloned in the same way as the corresponding WT versions (see Figure 3).

Combinations of plasmids bearing nsiR4 (or its mutated version) and the 5′-UTRs of target genes (or the mutated version) were introduced into E. coli DH5α. Plasmid pV300 expressing an unrelated RNA was used as a control. Fluorescence measurements were carried out with a microplate reader (VarioskanLux; Thermo Fisher Scientific, Waltham, MA, USA) using liquid cultures from eight individual colonies of cells carrying each plasmid combination, as previously described (Wright et al., 2013).

**Cell fractionation, Western blotting, and enzymatic assays**

For Western blot analysis, crude extracts were prepared using glass beads. Cells from 25 mL cultures of OE_C, OE_NsiR4 and OE_as_NsiR4 subjected to 24 h of nitrogen deficiency were harvested by filtration, washed with 50 mM Tris–HCl pH 8, and resuspended in 500 μL of resuspension buffer (50 mM Tris–HCl pH 8, 2 mM 2-mercaptoethanol) containing EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland). The cellular suspension was mixed with glass beads (SIGMA, St Louis, MO, USA; 200 μm) in an Eppendorf tube and subjected to seven cycles of 1 min vortexing plus 1 min of cooling on ice. Crude extract was separated from cell debris and unbroken cells by centrifugation (3 min at 3,000 g at 4°C) and used for PGK activity assay. The soluble fraction obtained by centrifugation of the crude extract at 16,000 g for 30 min at 4°C was used for Western blot. The protein concentration was determined by the Bradford method (Bradford, 1976) or the Lowry procedure (Markwell et al., 1978). PGK activity was determined in crude extracts by measuring NADH oxidation in a coupled assay with glyceraldehyde-3-phosphate dehydrogenase (Kuntz and Krietsch, 1982). Reaction mixtures contained 50 mM Tris–HCl pH 7.5, 1 mM MgCl₂, 0.3 mM NADH, 4 mM ATP, 1 mM DTT, 10 mM 3-phosphoglycerate, 5 U/mL glyceraldehyde-3-phosphate dehydrogenase from rabbit (SIGMA), and 0.3–0.6 μg of crude extract. The decrease in absorbance at 366 nm was monitored at 30°C on 96-wells microtiter plates using a VarioskanLux. For Western blot, 40 μg of protein in the soluble fraction were fractionated by electrophoresis on 10% (w/v) SDS–polyacrylamide gels. Antibodies against *Synechocystis* SBPase were used.

**Computational and statistical methods**

Homologs of *Nostoc* NsiR4 among cyanobacteria in the Nostocales group were identified by BLAST search (Altschul et al., 1990) at National Center for Biotechnological Information (NCBI) sequence database. ContraRNA (Wright et al., 2013, 2014) was used for the prediction of the targets of NsiR4, using the homologs in the genomes of *Nostoc* sp. PCC 7120, *Trichormus variabilis* ATCC 29413, *Calothrix* sp. PCC 6303, *Cylindrospermum stagnale* PCC 7417, *Nostoc* azollae 0708, *N. punctiforme* PCC 73102, *Nostoc* sp. PCC 7524, *Rivularia* sp. PCC 7116, and *Nostoc* sp. PCC 7107. Prediction of the interaction site between NsiR4 and the 5′-UTR of *g1px* and *pgk* in *Nostoc* and other filamentous cyanobacteria was performed using IntaRNA and a segment covering positions –100 to +100 with respect to the start codon of the corresponding mRNAs (Mann et al., 2017). Alignment of NsiR4 homologs was made using Clustal Omega (Sievers and Higgins, 2014). Secondary structure of NsiR4 was predicted with RNAalifold (Gruber et al., 2008).

The Student’s t test was used to determine statistical significance. Number of replicates can be found in the corresponding figure legends.
sRNA regulation of two Calvin-Benson cycle enzymes

Accession numbers
Sequence data and accession numbers for nsiR4, glpX, and pgk from Nostocales cyanobacteria are indicated in Supplemental Data sets S1, S2, and S3, respectively.

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

Supplemental Figure S1. The nitrogen stress-inducible RNA 4 (NsiR4).
Supplemental Figure S2. Nitrogen-responsive expression of glpX, pgk, and NsiR4 in Nostoc.
Supplemental Figure S3. Conservation of the predicted interaction between NsiR4 and the glpX or pgk mRNAs in cyanobacteria.
Supplemental Figure S4. Growth of OE_NsiR4, OE_as_NsiR4 and OE_C strains in the presence or absence of combined nitrogen.
Supplemental Table S1. Strains.
Supplemental Table S2. Plasmids.
Supplemental Table S3. Oligonucleotides.
Supplemental Table S4. Sequences of inserts in plasmids used for overexpression of RNAs in Nostoc.
Supplemental Table S5. Sequences of inserts in plasmids used for verification of sRNA–mRNA interactions in E. coli.
Supplemental Data set S1. Sequences and accession numbers for nsiR4.
Supplemental Data set S2. Sequences and accession numbers for glpX (from −100 to the stop codon).
Supplemental Data set S3. Sequences and accession numbers for pgk (from −100 to the stop codon).

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