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NsiR3, a nitrogen stress inducible small RNA, regulates proline oxidase expression in the cyanobacterium *Nostoc* sp. PCC 7120

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Abbreviations:

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2-OG, 2 oxoglutarate; 5'-UTR, 5' untranslated region; dRNASeq, differential RNA sequencing; FC, fold change; GEO, Gene Expression Omnibus; GFP, Green Fluorescent Protein; GOGAT, glutamine oxoglutarate aminotransferase; GS, glutamine synthetase; GSA, glutamate γ -semialdehyde; LB, Luria Broth; OAA, oxaloacetate; OAC, ornithine-ammonium cycle; P5C, Δ^1 -pyrroline-5-carboxylate; PCR, polymerase chain reaction; Sm, streptomycin; Sp, spectinomycin; sRNA, small RNA; β -Asp-Arg, β -aspartylarginine; STRR, short tandemly repeated repetitive; TCA, tricarboxylic acid cycle.

Keywords:

Regulatory RNA, NtcA, post-transcriptional regulation, heterocyst, PutA.

Conflict of interest

The authors declare no conflict of interest.

Database:

Microarray data are available in GEO database under accession numbers GSE120377 and GSE150191.

Abstract

NsiR3 (nitrogen-stress induced RNA 3) is a small non-coding RNA strongly conserved in heterocyst-forming cyanobacteria. In *Nostoc* sp. PCC 7120, transcription of NsiR3 is induced by nitrogen starvation and depends on the global nitrogen regulator NtcA. A conserved NtcA binding site is centered around position -42.5 with respect to the transcription start site of NsiR3 homologs, and NtcA binds *in vitro* to a DNA fragment containing this sequence. In the absence of combined nitrogen NsiR3 expression is induced in all cells along the *Nostoc* filament but much more strongly in heterocysts, differentiated cells devoted to nitrogen fixation. Co-expression analysis of transcriptomic data obtained from microarrays hybridized with RNA obtained from *Nostoc* wild type or mutant strains grown in the presence of ammonium or in the absence of combined nitrogen revealed that the expression profile of gene *putA* (proline oxidase) correlates negatively with that of NsiR3. Using a heterologous system in *Escherichia coli*, we show that NsiR3 binds to the 5'-UTR of *putA* mRNA, resulting in reduced expression of a reporter gene. Overexpression of NsiR3 in *Nostoc* resulted in strong reduction of *putA* mRNA accumulation, further supporting the negative regulation of *putA* by NsiR3. The higher expression of NsiR3 in heterocysts *versus* vegetative cells of the N₂-fixing filament could contribute to the previously described absence of *putA* mRNA and of the catabolic pathway to produce glutamate from arginine via proline specifically in heterocysts. Post-transcriptional regulation by NsiR3 represents an indirect NtcA-operated regulatory mechanism of *putA* expression.

Introduction

In some filamentous cyanobacteria, the acclimation to nitrogen deficiency involves differentiation of heterocysts, a cell type specialized for the fixation of atmospheric nitrogen [1]. Growth at the expense of N₂ involves complete metabolic remodeling, so that two different cell types with different metabolic capabilities (vegetative cells and heterocysts) cooperate to achieve growth of the filament as a whole [2]. The differentiation of heterocysts involves complex transcriptional changes both in vegetative cells and heterocysts not only to achieve morphological differentiation of heterocysts but also to undergo metabolic adaptations required for growth at the expense of N₂ [3,4].

Small RNAs (sRNAs) constitute a relevant class of post-transcriptional regulators involved in the adaptation of bacterial metabolism to different environmental situations [5]. sRNAs can coordinate changes in gene expression in response to environmental stresses. Similar to the observations made in other groups of bacteria, cyanobacteria exhibit abundant transcription of non-coding RNAs, including antisense RNAs and sRNAs, and transcription of many of them is regulated in response to nitrogen availability [6–9]. Several nitrogen-regulated non-coding RNAs have been identified and characterized in the filamentous, heterocyst-forming cyanobacterium *Nostoc* sp. PCC 7120 [3,10–14]. Furthermore, heterocyst-specific transcription of non-coding RNAs has been described [3,11,15,16], and some of those transcripts affect the process of heterocyst differentiation [17] or the regulation of enzymatic activities whose levels must be adjusted specifically in heterocysts vs. vegetative cells [13].

NsiR3 is an sRNA whose expression is induced upon nitrogen deprivation and depends on NtcA, the global nitrogen regulator in cyanobacteria [9,11]. It was originally described as conserved in 27 heterocyst-forming cyanobacteria but absent in non heterocystous strains [11], suggesting a possible role of NsiR3 related to heterocyst function. However, no regulatory target has so far been identified for NsiR3.

Computational prediction of mRNAs regulated by a given sRNA can be misleading because the interactions between sRNAs and their targets take place through short, often discontinuous complementary sequences [18]. Experimental identification of possible targets based on simple analysis of differences in expression changes upon alteration of the amount of the sRNA is in many cases impeded by secondary effects. Furthermore, such approaches rely on the assumption that the post-transcriptional regulation exerted by the sRNA results in clear changes in the amount of the target mRNA, which is not necessarily true [19]. For this reason, the combination of computational and more sophisticated experimental approaches is often more productive (reviewed in [20]).

In this work, we show that upon nitrogen deprivation expression of NsiR3 is more strongly induced in heterocysts than in vegetative cells. By using a novel approach based on correlation of expression analysis we identify *putA*, that encodes proline oxidase, as a target of NsiR3 because of the strong negative correlation with NsiR3. Finally, we demonstrate the interaction between NsiR3 and the 5' UTR of the *putA* mRNA, and the negative regulation exerted by NsiR3 on the expression of *putA* in *Nostoc* sp. PCC 7120.

Results

NsiR3 is conserved in heterocyst-forming cyanobacteria and its transcription is induced upon nitrogen stress

The nitrogen-regulated transcription of NsiR3 was discovered in a previous dRNASeq experiment [9]. Phylogenetic conservation of NsiR3 homologs across cyanobacterial genomes was later described [11] and we have now identified NsiR3 homologs in additional cyanobacteria, confirming that NsiR3 appears restricted to heterocyst forming strains (Fig. 1).

In *Nostoc* sp. PCC 7120, NsiR3 is transcribed downstream of *all4558* and in the opposite orientation (Fig. 2A). The predicted secondary structure contains two possible stem-loops acting as transcriptional terminators (Fig. 2B), the second one (T2) is also found in closely related cyanobacterial strains and is formed by STRR (short tandemly repeated repetitive) imperfect 7-nt repeats that are present in genomes of filamentous cyanobacteria usually in intergenic regions but in some case within coding sequences. Their function, if any, is unknown [21,22] (Fig. 2C). Expression of NsiR3 is induced upon nitrogen stress (Fig. 2D-E). Induction upon removal of combined nitrogen is quick and the amounts of NsiR3 remain high up to 24 h. Transfer from ammonium containing medium to nitrate containing medium also induces NsiR3 expression but to lower levels and the induction is transient (Fig. 2D-E), reflecting the fact that nitrogen stress in these conditions is quickly relieved by de-repression of the *nir* operon which encodes nitrate reductase and nitrite reductase, the enzymes involved in assimilation of nitrate [23,24]. Consistent with the predicted secondary structure, two RNAs, of 45 and 115 nucleotides, were detected by Northern hybridization with the NsiR3 probe. The 45 nucleotides RNA (NsiR3S) is of the expected size from the experimentally determined transcriptional start site (TSS) for NsiR3 [9] to the first predicted terminator, T1. The major detected band is 115 nucleotides long (NsiR3L) and matches the calculated length from the TSS to the second predicted terminator, T2, further downstream (Fig. 2B). NsiR3L is the most abundant of the two species of NsiR3, therefore it was used in all the experiments described here.

NtcA directly regulates NsiR3 expression

Transcription of NsiR3 in *Nostoc* sp. PCC 7120 depends on NtcA [9,11]. Alignment of the promoter region from all 70 identified *nsiR3* sequences shows that a putative conserved NtcA binding sequence appears centered around position -42,5 in 67 of them (Fig. 3), which is compatible with direct activation of NsiR3 transcription by NtcA.

To verify the interaction between NtcA and the promoter region of *nsiR3*, we performed an electrophoretic mobility shift assay with purified NtcA protein and a DNA fragment extending from positions -118 to +31 with respect to the TSS of *nsiR3* that includes the possible NtcA binding sequence. In the presence of NtcA, a retarded complex was observed with the fragment containing the wild type promoter sequence (Fig. 4A). However, the fraction of fragment bound by NtcA was strongly (nine-fold) reduced (Fig. 4A-B) when the assay was performed with a DNA fragment containing a version of the *nsiR3* promoter with the NtcA binding site mutated (GTG changed to CAC). These results demonstrate the binding of NtcA to a specific sequence of the *nsiR3* promoter.

NsiR3 accumulates differentially in heterocysts but is not essential for diazotrophic growth

It was previously shown that NsiR3 transcription is dependent on NtcA but independent of HetR [9,11], the master regulator of heterocyst differentiation. Therefore, we expect that NsiR3 transcription would be induced in all cells of the filament upon nitrogen stress. To characterize the pattern of expression of NsiR3 we prepared a plasmid containing a transcriptional fusion of the NsiR3 promoter to the *gfp* gene (pSAM341, see Table S4). The plasmid was introduced in *Nostoc* sp. PCC 7120 by conjugation and expression of GFP was analyzed by confocal fluorescence microscopy (Fig. 5). Filaments growing in ammonium containing medium had very low green fluorescence while filaments growing in medium lacking combined nitrogen showed fluorescence in all cells, but peaks of green fluorescence were associated to cells that were differentiating as heterocysts, as indicated by their lower red autofluorescence. Interestingly, induction of the *nsiR3* promoter occurs early in heterocyst development because significant green fluorescence was already observed in immature heterocysts, still having substantial red fluorescence (not shown).

The conservation of NsiR3 in heterocyst-forming cyanobacteria and its NtcA dependent expression suggest that NsiR3 is involved in some aspect of the regulation of the response to nitrogen stress in these complex cyanobacteria, that includes differentiation of specialized cells. To explore the possible function of NsiR3 we constructed a *Nostoc* strain lacking NsiR3 ($\Delta nsiR3$). This strain grew similarly to wild type in media containing ammonium, nitrate, or lacking combined nitrogen (Fig. 6), therefore the differentiation of functional heterocysts is not compromised in the absence of NsiR3.

NsiR3 is a regulator of *putA* (*alr0540*)

Bacterial sRNAs impact their target mRNAs often by repressing the initiation of translation via binding to their 5'-UTRs [5]. Hence, the mRNAs do not associate with ribosomes and may become more vulnerable to ribonucleases, leading to reduced mRNA level. To identify potential targets of NsiR3 whose expression would be negatively regulated by NsiR3 in *Nostoc* sp. PCC 7120 we decided to search for genes whose expression profile showed a negative correlation with the expression of NsiR3. For this purpose, we have used a novel approach based on correlation analysis of data obtained from hybridization of high density microarrays [3]. In order to identify those genes with negative correlation with NsiR3 we analyzed data obtained from hybridization of RNA samples extracted from the $\Delta nsiR3$ mutant grown in ammonium containing medium (2 replicates), or from the $\Delta nsiR3$ mutant after 8 h of incubation in medium lacking combined nitrogen (2 replicates). In addition, in order to increase the sensitivity of the correlation analysis, previously available data from hybridization of the same microarray platform with other twenty samples (both from the wild type and a *hetR* mutant strain either in the presence of ammonium or after different periods of nitrogen deprivation) [3] were included in the analysis. The expression data from the 24 samples (Table S1) were analyzed as described [3] and a correlation table was obtained for NsiR3 (Table S2). The probe with the strongest negative correlation with NsiR3 (-0.943) was that of gene *alr0540* (*putA*), encoding proline oxidase. Moreover, another probe corresponding to the 5'-UTR of *alr0540* had a strong negative correlation as well (-0.884). Fig. 7A shows the expression profile of NsiR3 and *putA* in the 24 samples hybridized to the arrays. We also confirmed by Northern blot the opposite expression dynamics of *putA* with NsiR3 that was deduced from the co-expression analysis (Fig. 7B).

The results described above strongly suggest that the expression of *putA* might be regulated by NsiR3. In fact, we could predict with IntaRNA [25] an interaction between NsiR3 and the 5'-UTR of *putA* mRNA (Fig. 8A). NsiR3 would bind to positions -5 to -17 with respect to the initiation codon of *putA*, occluding the ribosome binding site.

To verify the interaction between NsiR3 and the 5'-UTR of *putA* we used a heterologous reporter system [26] in which the 5'-UTR plus the first 60 nt of the *putA* coding sequence were translationally fused to the gene encoding superfolder GFP (*sfgfp*). This construct was co-expressed in *E. coli* either with NsiR3 or with a control, unrelated RNA. The initiation codon of *putA* was changed from GTG to ATG for optimal expression in *E. coli*. The fluorescence of cells carrying *sfgfp* fusions significantly decreased when NsiR3 was co-expressed, indicating a negative effect of NsiR3 on expression of sfGFP (Fig. 8B).

To verify the interactions between NsiR3 and the mRNA of *putA* we created a mutated version of NsiR3 altered in position 14 (G to C, NsiR3 Mut14) (Fig. 8A). The mutation in NsiR3 eliminated the interaction between NsiR3 and the mRNA of *putA* (Fig. 8B). We designed a compensatory mutation in the 5'-UTR of *putA* (Comp-14*17) that would restore the interaction with NsiR3 Mut14 (Fig. 8A). When NsiR3 (Mut14) was combined with the mutated version of the 5'-UTR of *putA*, the reduction of fluorescence was restored (Fig. 8B). All together these results verify the interaction of NsiR3 with the 5'-UTR of *putA* at the positions predicted by IntaRNA. In consequence, an inhibitory effect of NsiR3 on *putA* in the *in vivo* context of the heterologous *E. coli* system is supported.

NsiR3 represses the expression of *putA* in *Nostoc* sp. PCC 7120

To analyze the regulatory effect of NsiR3 on *putA* in *Nostoc* sp. PCC 7120 we generated a *Nostoc* strain with controlled expression of NsiR3 by complementing strain $\Delta nsiR3$ with a plasmid containing *nsiR3* under control of the copper-inducible *petE* promoter ($\Delta nsiR3 + P_{petE}::nsiR3$). Accumulation of the mRNA of *putA* was analyzed by Northern blot in the wild type, $\Delta nsiR3$ and $\Delta nsiR3 + P_{petE}::nsiR3$ strains at different times after nitrogen removal and copper addition (Fig. 9A). A stronger reduction in the amount of the full-length *putA* mRNA was observed in the $\Delta nsiR3 + P_{petE}::nsiR3$ strain than in the wild type, in agreement with the observation that $\Delta nsiR3 + P_{petE}::nsiR3$ accumulated about 40 times more NsiR3 than the wild type (Fig. 9A-B). The deletion of *nsiR3* in the $\Delta nsiR3$ strain resulted in levels of the *putA* mRNA, that were not significantly reduced after 24 h in the

absence of combined nitrogen. These results indicate a strong negative correlation between the amount of NsiR3 and the amount of the *putA* mRNA. As a control, we hybridized the same filter with a probe for gene *agrE* encoding the bifunctional enzyme arginine dihydrolase/ornithine cyclodeaminase, that acts upstream proline oxidase in the catabolic pathway from arginine to proline [27]. The dynamic of changes in the amount of *agrE* mRNA upon nitrogen removal was different to that of *putA*. In the wild type strain, whereas the amount of *putA* mRNA was reduced to about 40% after 24 h in the absence of combined nitrogen, no significant change was observed in the case of *agrE*. In addition, the levels of *agrE* mRNA did not correlated to changes in the amount of NsiR3 (Fig. 9), as predicted for a mRNA that is not a target of NsiR3.

Discussion

In this work we characterize NsiR3, a highly conserved sRNA in heterocystous cyanobacteria. NsiR3 was identified in the genomes of seventy heterocyst-forming cyanobacteria but was not found in unicellular or filamentous strains that do not develop heterocysts. This distribution of the *nsiR3* gene, in contrast to that of other NtcA-regulated sRNAs such as NsiR4, also found in unicellular strains [10], suggests a relevant function related to the specific metabolic traits of heterocyst-forming strains. *nsiR3* is not transcribed in the presence of ammonium but its transcription is strongly induced upon transfer to nitrogen-free medium, suggesting a possible function in the response to nitrogen stress. Furthermore, *nsiR3* transcription is also induced upon transfer from ammonium to nitrate containing medium. However, in this case the induction is lower and transient. This can be explained because the nitrogen stress is quickly relieved upon induction of the nitrate assimilation pathway in the presence of nitrate [23]. We show that the global regulator of nitrogen assimilation, NtcA, binds directly to the *nsiR3* promoter, explaining the previous observation that NtcA is required for expression of NsiR3 [9,11]. By means of a fusion to the *gfp* gene, we show that expression of NsiR3, although induced in all cells of the filament under nitrogen deprivation, is much stronger in heterocysts than in vegetative cells. This observation might be explained by the higher concentration of NtcA present in the heterocyst *versus* in vegetative cells [28,29]. Alternatively, the *nsiR3* promoter might contain a response element for an unknown heterocyst-specific factor in addition to the NtcA binding site.

However, neither the DIF1 nor the DIF2 sequence motifs previously described for heterocyst specific expression [3,9] are found in the region upstream of the *nsiR3* gene. The observation that a NtcA-regulated RNA, although not exclusively expressed in heterocysts, exhibits differential expression in this particular cell type suggests the possibility of heterocyst-specific regulation perhaps based on different levels of NtcA and/or 2-oxoglutarate.

One of the main challenges in the study of sRNAs is the identification of their regulated targets. A number of computational and experimental methods have been developed for this purpose [20]. Here we took advantage of a co-expression analysis, based on hybridization of RNA samples obtained from wild type, *hetR*, and $\Delta nsiR3$ strains grown under different conditions, to analyze the correlation in expression profiles between *nsiR3* and every gene in *Nostoc* sp. PCC 7120. *putA* (encoding proline oxidase) showed the strongest negative correlation with NsiR3 and was confirmed in this work as a true target of NsiR3 by means of several experimental approaches both in *E. coli* and *Nostoc*. Co-expression analysis could be a good alternative to identify sRNA targets, provided that enough global data (from microarrays or RNA-Seq) are available to compute the correlation index in expression changes between the sRNA under study and every gene expressed in the organism of interest, as shown here for NsiR3. This was especially relevant in this study because *putA* was not identified as a high-ranking potential candidate target of NsiR3 by predictive computational approaches (not shown). A simple differential expression analysis between wild type and $\Delta nsiR3$ strain would not allow the identification of *putA* as a potential target of NsiR3 because the fold change of *putA* mRNA levels between $\Delta nsiR3$ strain and WT after 8 h of nitrogen starvation was not significant enough ($\log_2$ fold change=0.85) (see Table S1). It is relevant to mention that the approach we have used relies on the post-transcriptional regulation by the sRNA on its target having a quantitative impact on the amount of the target mRNA, as is the case for NsiR3 and *putA*.

A *Nostoc* strain lacking NsiR3 ($\Delta nsiR3$) didn't show distinct phenotypic alterations under standard laboratory growth conditions. This is an observation that has been made with null mutants of many other regulatory sRNAs such as NsiR4 [10], or IsaR1 as well [30]. These mutants do not have a distinct phenotype under standard laboratory growth conditions but eventually might reveal phenotypic alterations in a natural environment,

where cells are subjected to fluctuating conditions, or the presence of specific substances in the medium, or a combination of factors.

NsiR3 has a clear impact on *putA* expression. In the wild type strain the amount of *putA* mRNA was reduced upon nitrogen stress (Fig. 7 and Fig. 9). However, this reduction was less profound in the $\Delta nsiR3$ mutant (Fig. 9). In contrast, over-expression of NsiR3 from the *petE* promoter resulted in a strong reduction of *putA* mRNA levels, that even became undetectable. We have not studied the molecular mechanism of the post-transcriptional regulation exerted by NsiR3 on *putA* expression. However, the binding site of NsiR3 on the *putA* mRNA overlaps with the predicted ribosome binding site, therefore a reasonable assumption is that NsiR3 inhibits the initiation of *putA* translation. A secondary consequence of translation inhibition would be destabilization of the *putA* mRNA, resulting in its degradation. An active mechanism by which NsiR3 recruits an RNase to degrade *putA* mRNA cannot be excluded.

A final question relates to the possible biological relevance of the repression of *putA* upon nitrogen stress. *putA* encodes proline oxidase, a bifunctional enzyme that converts proline to glutamate. Therefore, *putA* is important for proline utilization and also for arginine catabolism, that produces proline by the bifunctional enzyme ArgE [27]. Interestingly, *putA* mRNA is not detected in *Nostoc* heterocysts [27]. The higher transcription of NsiR3 in heterocysts (Fig. 5) could result in complete degradation of *putA* mRNA specifically in this cell type and could explain why *putA* is not detected in heterocysts. AgrE is also absent in heterocysts [27], therefore the main arginine catabolism pathway is blocked in these cells (Fig. 10), facilitating the channeling of arginine to the biosynthesis of cyanophycin granules, that accumulate at high levels in heterocysts and are a source, together with glutamine, of combined nitrogen for vegetative cells (Fig. 10). Modulation of proline oxidase in vegetative cells during diazotrophic growth could also be necessary to avoid excessive glutamate accumulation under conditions in which there is a net gain of glutamate in vegetative cells through GOGAT acting on glutamine provided by the heterocyst (Fig. 10).

It is unknown if transcriptional regulation is involved in the absence of *putA* mRNA in the heterocyst, but in any case the expression of NsiR3 at initial stages of heterocyst differentiation could facilitate faster shutdown of *putA* expression than can be achieved by transcriptional regulation only.

NsiR3 adds to a growing number of sRNAs with functions related to nitrogen assimilation in different groups of bacteria, including *E. coli* [31], or *Pseudomonas* [32,33]. In cyanobacteria, several NtcA-regulated sRNAs have been described to regulate responses to nitrogen deficiency, including phycobilisome degradation [12] or glutamine synthetase activity [10]. Recently, NsiR1, a heterocyst-specific sRNA, has been described to modulate heterocyst differentiation [17].

We have previously identified an antisense RNA that shuts down, specifically in heterocyst, the expression of sedoheptulose-1,7-bisphosphatase, a key Calvin cycle enzyme [13]. Here we describe a trans-acting sRNA that could be relevant for the inhibition of proline oxidase expression specifically in heterocysts, reinforcing the relevance that non-coding RNAs could have in the metabolic adaptations required for heterocyst function. Global analysis [3] suggest that more antisense and sRNA, still to be characterized, could be involved in the heterocyst specific regulation of key enzymes of the intermediary metabolism. Post-transcriptional RNA regulation could be an important layer of regulation in the metabolic adaptations of heterocysts, together with better known transcriptional regulation, that is worth exploring.

Methods

Strains and growth conditions

Nostoc sp. PCC 7120 wild type, $\Delta nsiR3$ and $\Delta nsiR3 + P_{petE}::nsiR3$ strains (Table S3) were grown photoautotrophically at 30°C in BG11 medium [34] containing ferric citrate instead of ammonium ferric citrate. For Northern blot analysis of expression under different conditions, cultures of *Nostoc* sp. PCC 7120 were bubbled with an air/CO₂ mixture (1% v/v) and grown photoautotrophically at 30°C in BG11 medium supplemented with 10 mM NaHCO₃ (BG11C) lacking NaNO₃ but containing 6 mM NH₄Cl and 12 mM *N*-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid-NaOH buffer (pH 7.5) (BG11C + NH₄⁺). To induce nitrogen deficiency, filaments were collected by filtration, washed and resuspended in nitrogen-free BG11 medium containing 10 mM NaHCO₃ (BG11₀C). To induce the expression of the *petE* promoter 1.5 μM CuSO₄ was added to the cultures at the time of nitrogen removal. To test growth of different strains on plates under different conditions, liquid cultures of these strains growing in BG11 media were diluted to A₇₅₀

0.17 and 10 μ l of serial 5-fold dilutions were spotted on plates containing different nitrogen sources.

Nostoc sp. PCC 7120 derivative strains bearing Sm^RSp^R plasmids were grown in the presence of streptomycin (Sm) and spectinomycin (Sp), 2 μ g/ml each (liquid medium) or 5 μ g/ml each (solid medium).

E. coli strains (Table S3) were grown in LB medium, supplemented with appropriate antibiotics [35].

Electrophoresis mobility shift assay

NtcA was purified as described [12]. Electrophoresis mobility shift assays were carried out as described [36] with a 150-bp PCR fragment amplified with oligonucleotides 23 and 469, encompassing positions -118 to +31 with respect to the TSS of *nsiR3*. A mutated version of the same DNA fragment was obtained by overlap-extension PCR using mutagenic complementary oligonucleotides 420 and 421 and flanking oligonucleotides 23 and 469. The DNA fragment was labelled with [γ -³²P]ATP and polynucleotide kinase and 2,000-6,000 cpm (<30 pM) were used in each assay.

Reporter assay for *in vivo* verification of targets

We used the reporter assay described in [37] and fusions to the gene encoding superfolder GFP (*sfGFP*) in plasmid pXG10-SF [26] for experimental target verification in *E. coli* (Table S4). In this system both the GFP fusions and NsiR3 are transcribed constitutively.

5'-UTR of *putA* was cloned in pXG10-SF from its TSS, taken from [9], to 60 nucleotides within the coding region. To facilitate translation in *E. coli*, the GTG start codon of *putA* was replaced by ATG using overlapping PCR and oligonucleotides specified in Table S5. PCR fragments containing the region to be cloned were amplified using genomic DNA as template and oligonucleotides specified in Table S5. Fragments were digested with NsiI and NheI and cloned into pXG10-SF treated with the same enzymes, resulting in translational fusions to sfGFP (Table S6).

To express NsiR3 in *E. coli*, the sequence encoding NsiR3 was amplified from genomic DNA using primers 195 (5' phosphorylated) and 283. The PCR product was digested with

XbaI and fused to a plasmid backbone that was amplified from pZE12-luc with primers PLLacOB and PLLacOD [37] and digested with XbaI, rendering pIAE19 (Table S6).

For the mutagenesis of NsiR3 and the 5'-UTR of *putA*, mutations were introduced by overlapping PCR with primers containing the desired changes (Table S5) and the fragments were cloned in the same way as the corresponding wild type versions.

Combinations of plasmids bearing fragments encoding NsiR3 (or its mutated versions) and the 5' UTRs of target genes (or mutated versions) were introduced in *E. coli* DH5 α . Plasmid pJV300, expressing an unrelated RNA, was used as a control. Fluorescence measurements were done with a microplate reader (Varioskan) using liquid cultures from eight individual colonies of cells carrying each plasmid combination, as previously described [38].

Fluorescence microscopy

Plasmid pSAM341, containing the promoter of *nsiR3* fused to *gfpmut2* (see Table S4), was introduced in *Nostoc* by conjugation [39] with selection for resistance to Sm and Sp. Images of filaments containing pSAM341 were taken four days after plating in media with ammonium or without combined nitrogen. The accumulation of GFP along the filaments was analyzed and quantified using a Leica (Wetzlar, Alemania) TCS SP2 confocal laser scanning microscope as described [16]. GFP was excited at 488 nm by an argon ion laser, and the fluorescence emission was monitored by collection across windows of 500-538 nm (GFP imaging) and 630-700 nm (cyanobacterial autofluorescence). At least 10 fields of each strain were analyzed. Representative images are shown in Fig. 5. Images were treated with ImageJ 1.45 s software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, MD, <http://imagej.nih.gov/ij/>, 1997–2018).

Generation of *Nostoc* strains with altered levels of NsiR3

To generate a strain lacking NsiR3 (Δ *nsiR3*), two overlapping fragments encompassing flanking sequences around NsiR3 were amplified by PCR using as template genomic DNA with oligonucleotides 127 and 129 and oligonucleotides 128 and 130, respectively. The resulting products were then used as templates for a third PCR with oligonucleotides 127 and 128, resulting in the fusion of both fragments and the deletion of the sequences encoding NsiR3. The fragment was cloned into pSpark (Canvax Biotech, Córdoba,

Spain), rendering pELV16 and its sequence was verified by sequencing. After digestion with BamHI at the sites provided by oligonucleotides 127 and 128, the fragment was cloned into BamHI-digested, *sacB* containing Sm^RSp^R vector pCSRO [40], rendering pELV17, which was transferred to *Nostoc* sp. PCC 7120 by conjugation [39] with selection for resistance to Sm and Sp. Cultures of the exconjugants obtained were used to select for clones resistant to 5% sucrose [41], and individual sucrose resistant colonies were checked by PCR. Clones lacking the deleted *nsiR3* region were named $\Delta nsiR3$. To establish controlled expression of NsiR3 in *Nostoc*, the *nsiR3* gene was placed under control of the *petE* promoter, which mediates Cu²⁺-regulated transcription [42] and was cloned in a self-replicating plasmid. The *petE* promoter of *Nostoc* sp. PCC 7120 (genomic coordinates 278185 to 277848) was amplified with oligonucleotides 299 and 302 and fused to the *nsiR3* fragment amplified with oligonucleotides 301 and 300 by a third PCR using the two fragments as templates and oligonucleotides 299 and 300. After digestion with ClaI and XhoI at the sites provided by oligonucleotides 299 and 300, the fragment was cloned into pSAM221 [12], rendering pIAE24. pIAE24 was introduced into the $\Delta nsiR3$ strain by conjugation as described above with selection for resistance to Sm and Sp.

RNA isolation and Northern blot analysis

Total RNA was isolated using hot phenol as described [43] with modifications [11]. Northern blot detection of NsiR3 (Fig. 2) was performed using 10% urea-polyacrylamide gels as described [6] and 7.5 µg of total RNA. Northern blot hybridization of mRNAs (Fig. 7 and Fig. 9) was performed using 1% agarose denaturing formaldehyde gels and 10 µg of total RNA. All RNA samples were then transferred to Hybond-N+ membrane (GE Healthcare, Chicago, Illinois, USA) with 20XSSC buffer. Strand-specific ³²P-labeled probes were prepared with Taq DNA polymerase using a PCR fragment as template and oligonucleotides specified in Table S5 in a reaction with [α-³²P]dCTP and one single oligonucleotide as primer (corresponding to the complementary strand of the sRNA or mRNA to be detected). PCR fragments used as templates for NsiR3, *putA*, and *agrE* probes were amplified from genomic DNA using oligonucleotides pairs 125/126, 645/646 and 909/910, respectively. Hybridization to *mnpB* [44] or 5S rRNA was used as a loading and transfer control for agarose or urea-polyacrylamide gels, respectively.

Computational methods

Sequences of homologs of NsiR3 were taken from reference [11]. Additionally, NsiR3 sequences were identified by Blast search of the NCBI genomic database [45]. The prediction of the interaction between NsiR3 and the 5'-UTR of predicted targets in *Nostoc* sp. PCC 7120 was performed using IntaRNA [25]. Alignment of NsiR3 homologs was made using Clustal Omega [46]. Secondary structure of NsiR3 was predicted by RNAalifold [47].

Processing of microarray data and comparison of expression between samples was performed as described [3]. Raw data was extracted with the read.maimages function of the *limma* R package [48] and the average expression of elements with multiple probes was calculated using the avereps function. We performed the following comparisons of expression between two samples to extract differentially expressed probes: every sample *versus* the absolute reference (wild type 0 h from the -N experiment = ammonium-grown cells), every sample *versus* its own reference (for instance $\Delta nsiR3$ 8 vs. $\Delta nsiR3$ 0) and finally sample of the $\Delta nsiR3$ 8 h after nitrogen removal *versus* the wild-type 8 h after nitrogen removal. A total of 6,048 genetic elements (genes, putative sRNAs, putative asRNAs, etc.) that exhibited a $\log_2FC$ (fold change) >1.5 in at least one of the comparisons shown in Table S1 were selected for further analysis. A Pearson correlation analysis was performed between the expression profile of NsiR3 and the expression profile of any other feature (Table S2). The microarray data can be accessed in the GEO database under the accession **GSE120377** and **GSE150191**.

Author contributions

A.V., and A.M.M-P. planned experiments; I.Á-E., E.O.-V., A.V., and A.M.M-P. performed experiments; M.B-A., J.G., A.V., and A.M.M-P. analyzed data; and A.V., W.R.H., and A.M.M-P. wrote the paper.

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Supporting Information

Supplementary Dataset file (excel) containing:

Table S1. Differentially_expressed_genes_all_samples

Table S2. Pearson_correlation_of_NsiR3_and_all_genes

Supplementary information file (pdf) containing:

Table S3. Strains

Table S4. Plasmids

Table S5. Oligonucleotides

Table S6. Sequences

Figure legends

Fig. 1. Alignment of NsiR3 RNA. The sequences of NsiR3S from different cyanobacteria were aligned with Clustal Omega. Nucleotide positions conserved in the 70 strains where NsiR3 was found are indicated by asterisks. Each sequence is indicated by the organism name followed with the GenBank accession number (www.ncbi.nlm.nih.gov/genbank).

The NsiR3 sequence from *Nostoc* sp. PCC 7120 is highlighted in bold.

Fig. 2. The nitrogen-stress induced sRNA NsiR3. (A) Schematic representation of the region encoding NsiR3 in *Nostoc* sp. PCC 7120. The two flanking genes are indicated. The bent arrow represents the transcriptional start at position 5452083f, and the stem loops represent the two transcriptional terminators of NsiR3. The position of the NtcA binding site is also indicated. (B) Secondary structure model of NsiR3 from *Nostoc* sp. PCC 7120 based on the consensus obtained with RNAalifold from the alignments in Fig. 1 and Fig. 2C. The heptanucleotide repeats (STRR) are framed. (C) The sequences of NsiR3 encoded in those strains that could have a second transcriptional terminator (T2) further downstream T1 were aligned using Clustal Omega. The predicted seed region is highlighted in yellow. Accession numbers are indicated in Fig. 1. (D) Nitrogen-responsive expression of NsiR3 in *Nostoc* sp. PCC 7120. Expression was analyzed by Northern blot in cells grown in the presence of ammonium and transferred to medium containing no source of combined nitrogen (N₂) or containing nitrate (NO₃⁻) for the number of hours indicated. The upper panel shows hybridization to the NsiR3 probe. The lower panel shows hybridization to a probe for 5S RNA used as loading and transfer control. Sizes (nt) are indicated on the left. The experiment was repeated four times with similar results. The northern blot containing the highest number of time points is shown. (E) Quantification of NsiR3L in the blot shown in (D) upon nitrogen removal (black, N₂) or nitrate addition (red, NO₃⁻).

Fig. 3. Alignment of the promoter sequences of *nsiR3*. The sequences upstream *nsiR3* were aligned with Clustal Omega. Nucleotide positions fully conserved are indicated by asterisks. The consensus sequence is represented with WebLogo [49]. The likely -10 elements are boxed and two experimentally determined TSS from *Nostoc* sp. PCC 7120 [9] and *Nodularia spumigena* CCY9414 [50] are highlighted in red. Accession numbers are indicated in Fig. 1.

Fig. 4. NtcA binds to the *nsiR3* promoter. (A) Electrophoretic mobility shift assays showing binding of purified His-tagged NtcA protein to a DNA fragment containing the wild type promoter of NsiR3 from *Nostoc* sp. PCC 7120 (left) or a mutated version altered in the positions indicated in red (right). Three times more probe was used in the assay with the mutant fragment than with the wild type fragment. A representative experiment is shown. (B) Quantification of the autoradiogram presented in panel (A). The fraction of probe bound by NtcA was calculated for the wild type (black) or the mutated (red) version.

Fig. 5. Expression pattern of the *nsiR3* promoter in *Nostoc* filaments. Confocal fluorescence images (red channel, green channel, both channels merged, and bright field image) of *Nostoc* filaments carrying the *gfp* gene under the control of the *nsiR3* promoter (plasmid pSAM341) and growing on top of medium containing ammonium, NH_4^+ (A) or lacking any source of combined nitrogen, N_2 (B). Quantification of the signals for the green channel (GFP) is shown for the segments indicated by a blue line in the corresponding bright field images. All images were acquired with the same sensitivity settings so that intensities can be compared. Scale bars, 20 μm .

Fig. 6. The $\Delta\textit{nsiR3}$ strain can differentiate heterocysts. Cells were grown in the presence of nitrate, washed with BG11₀ and resuspended at 0.1 μg chlorophyll/ml. Five-fold serial dilutions of liquid cultures of wild type or 4 independent $\Delta\textit{nsiR3}$ isolates were prepared. 10 μl of the cell suspension and of three five-fold serial dilutions were plated on BG11₀ plates containing ammonium (NH_4^+), nitrate (NO_3^-), or no source of combined nitrogen (N_2). Pictures were taken after 10 days of incubation at 30°C.

Fig. 7. Negative correlation of *putA* and *nsiR3* expression. (A) Expression level of *nsiR3* (red) and *putA* (*alr0540*, black), in the 24 RNA samples hybridized to the microarrays used for the co-expression analysis. (B) *putA* expression after removal of combined nitrogen. RNA was extracted from *Nostoc* cultures at different times after nitrogen removal and subjected to Northern blot. The filter was hybridized with probes for *putA* (top), *nsiR3* (middle), and *rnpB* (bottom), that was used as loading control. The experiment was repeated three times with similar results. The northern blot containing the highest number of time points is shown. (C) Quantification of the blot shown in (B). The amount of *putA* mRNA (black) and NsiR3 (red) are expressed as percentage of the

maximum. For *putA* the full-length transcript (arrow in B) was used in quantification. Sizes of ribosomal RNAs are indicated in (B).

Fig. 8. Verification of NsiR3 interaction with the 5'-UTR of *putA* using an *in vivo* reporter system. (A) Predicted interaction between NsiR3 and the 5'-UTR of *putA* mRNA according to IntaRNA [25]. Nucleotides are numbered with respect to the start of the coding sequence (initiation codon is underlined). The ribosome binding site in the *putA* mRNA is highlighted in yellow. A mutation introduced in NsiR3 at position 14 (G to C, Mut14) and the corresponding compensatory mutation in *putA* 5'-UTR position -17 (C to G, Comp-14*17) are indicated in red and blue, respectively. (B) Fluorescence measurements of *E. coli* DH5 α cultures bearing combinations of plasmids expressing different versions of NsiR3 and *putA::sfGFP* fusions. Plasmid pJV300 (encoding a control RNA) was used as control. The data are presented as the mean $\pm$ standard deviation of cultures from eight independent colonies after subtraction of fluorescence in cells bearing pXG-0 (***) $p < 0.0001$, Student t-test).

Fig. 9. Expression of *putA* and *agrE* in strains with altered levels of NsiR3. (A) Expression was analyzed by Northern blot in wild type, a mutant strain lacking NsiR3 ($\Delta nsiR3$) and in the same strain complemented with a plasmid bearing *nsiR3* under control of the Cu²⁺-regulated promoter of the *petE* gene ($\Delta nsiR3 + P_{petE}::nsiR3$). Cells were grown in the presence of ammonium in medium lacking Cu²⁺ (to prevent expression of the *petE* promoter) and transferred to medium containing no source of combined nitrogen (N₂) and 1.5 μ M Cu²⁺ for the indicated number of hours. The filter was hybridized with probes for *putA* (top), *agrE*, *nsiR3*, and *rnpB* (bottom), that was used as loading control. Size standards are indicated on the right in kb. The experiment was performed twice with similar results. (B) Quantification of the blots shown in (A). The full-length transcripts (arrows in A) were used in quantification. RNA amounts of *putA* and *agrE* mRNAs in wild type (black), $\Delta nsiR3$ (red) and $\Delta nsiR3 + P_{petE}::nsiR3$ (green) strains are expressed relative to the amount present in the wild type at time 0. NsiR3 amounts are expressed as absolute values.

Fig. 10. Model of *putA* regulation by NsiR3. Blue arrows represent the most relevant enzymatic reactions in the metabolism of arginine and proline in vegetative cells and heterocysts that are discussed in the text. (1) cyanophycinase, (2) isoaspartyl dipeptidase, (3) nitrogenase, (4) glutamine synthetase (GS), (5) glutamine oxoglutarate

aminotransferase (GOGAT), (6) cyanophycin synthetase, bifunctional arginine dihydrolase/ornithine cyclodeaminase (AgrE) and proline oxidase (PutA). The positive transcriptional regulation of NsiR3 by NtcA is represented by green arrows. NtcA and NsiR3 are more abundant in the heterocyst. Red truncated lines indicate the inhibition of PutA by NsiR3. The red crosses illustrate the absence of AgrE and PutA in heterocysts [27]. The meaning of the abbreviations used is as follows: β -Asp-Arg, β -aspartylarginine; P5C, Δ^1 -pyrroline-5-carboxylate; GSA, glutamate γ -semialdehyde; 2-OG, 2 oxoglutarate; OAA, oxaloacetate; OAC, ornithine-ammonium cycle; TCA, tricarboxylic acid cycle.

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