1 **NsiR1, a small RNA with multiple copies, modulates heterocyst**
2 **differentiation in the cyanobacterium *Nostoc* sp. PCC 7120**

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Originality-significance statement:

Heterocysts are nitrogen-fixing specialized cells that appear at semi-regular intervals along cyanobacterial filaments upon nitrogen starvation. Heterocyst differentiation and patterning is a model system to study cell differentiation in multicellular prokaryotes. The differentiation process has a complex regulation, only partially understood, that includes transcriptional changes, factor diffusion between cells, and protein-protein interactions. This work adds to this complexity a small non-coding RNA, NsiR1, that negatively regulates at a post-transcriptional level the expression of *hetF*, a gene that is part of the heterocyst differentiation regulatory network. Thus, regulation by a small RNA is a new element involved in the control of heterocyst differentiation. A better understanding of the regulation of heterocyst differentiation could be useful for the engineering of biological N₂ fixation or the production of O₂-sensitive molecules in cyanobacteria.

Summary

Upon nitrogen starvation filamentous cyanobacteria develop heterocysts, specialized cells devoted to fixation of atmospheric nitrogen. Differentiation of heterocyst at semi-regular intervals along the filaments requires complex structural and functional changes that are under control of the master transcriptional regulator HetR. NsiR1 (nitrogen stress-induced RNA 1) is a HetR-dependent non-coding RNA that is expressed specifically in heterocysts from a very early stage of differentiation. In the genome of *Nostoc* sp. PCC 7120 there are twelve tandem copies of *nsiR1* (*nsiR1.1* to *nsiR1.12*), seven of them with identical sequence (*nsiR1.3* to *nsiR1.9*) and the others slightly divergent. *nsiR1.1* is transcribed antisense to the 5' UTR of *hetF*, a gene required for heterocyst development. Here we show that binding of NsiR1.1 inhibits translation of the *hetF* mRNA by inducing structural changes in its 5' UTR. Altered levels of NsiR1 result in different phenotypic alterations including enlarged cell size and delayed heterocyst development that could be related to a reduced amount of HetF.

Introduction

Cyanobacteria are the only prokaryotes able to perform oxygenic photosynthesis and important contributors to global primary productivity. They constitute a coherent phylogenetic group with diverse morphology and wide ecological distribution. *Nostoc* sp. PCC 7120 (a.k.a *Anabaena* sp. PCC 7120) is a filamentous cyanobacterium that under nitrogen deprivation differentiates heterocysts, specialized cells devoted to fixation of atmospheric nitrogen. In the absence of combined nitrogen, filaments are composed of photosynthetic vegetative cells and regularly spaced (every 10-15 cells) nitrogen fixing heterocysts. Heterocyst differentiation involves a complex developmental program (Flores and Herrero, 2010; Muro-Pastor and Hess, 2012; Herrero *et al.*, 2016; Flores *et al.*, 2019) that is ultimately under control of NtcA, the global regulator of nitrogen assimilation (Herrero *et al.*, 2001), but also under control of HetR, a specific regulator of cell differentiation (Buikema and Haselkorn, 1991; Black *et al.*, 1993). At the initiation of heterocyst differentiation, induction of the expression of NtcA and HetR depends on each other (Muro-Pastor *et al.*, 2002) and leads to increased expression of HetR in those cells becoming heterocysts. Expression of *hetR* is regulated at several levels. *hetR* is transcribed from a complex promoter including several transcriptional start sites (TSSs), one of them specifically induced in cells becoming heterocysts (Rajagopalan and Callahan, 2010; Mitschke *et al.*, 2011). HetR is a DNA-binding protein that positively regulates its own expression and the expression of many genes, including negative elements essential for the maintenance of the pattern of heterocysts along the filaments, such as *patS* (Yoon and Golden, 1998), *hetN*

(Callahan and Buikema, 2001) and *patX* (Elhai and Khudyakov, 2018). The binding of the pentapeptide RGSGR, that is part of the sequence of PatS, blocks the DNA-binding activity of HetR (Huang *et al.*, 2004) and signals its degradation (Risser and Callahan, 2009). HetR is an autoproteolytic protein (Zhou *et al.*, 1998) that seems also to be degraded by the HetF protease (Wong and Meeks, 2001; Risser and Callahan, 2008). A *hetF* mutant accumulates a large amount of HetR, has an altered morphology with enlarged cells and does not differentiate heterocysts (Risser and Callahan, 2008), suggesting HetR turnover is required for differentiation. However, since *hetF* transcription seems unpatterned along the filaments (Risser and Callahan, 2008), the question of how HetF may contribute to the differential accumulation of active HetR in heterocysts versus vegetative cells remains open.

Small non-coding RNAs (sRNAs) are important regulators of gene expression in bacteria, including cyanobacteria (Kopf and Hess, 2015; Muro-Pastor and Hess, 2020). Most frequently they bind to their target mRNAs close to the ribosome binding site therefore inhibiting translation, but a large diversity of alternative regulatory mechanisms operated by sRNAs are known (Wagner and Romby, 2015). They are involved in the regulation of diverse processes related to response to stress or adaptation to a changing environment, including cell differentiation. In *Bacillus* several sRNAs have been identified that are sporulation-specific and with expression restricted to the forespore (Silvaggi *et al.*, 2006; Schmalisch *et al.*, 2010; Marchais *et al.*, 2011; Mars *et al.*, 2016). In heterocyst-forming cyanobacteria, sRNAs and antisense RNAs with heterocyst-specific expression have been identified (Ionescu *et al.*, 2010; Brenes-Álvarez *et al.*, 2016; Brenes-Álvarez *et al.*, 2019). A heterocyst-specific antisense RNA

is involved in carbon fixation shutdown in heterocysts (Olmedo-Verd *et al.*, 2019).

NsiR1 is a sRNA of about 60 nucleotides encoded in 12 tandem copies in the region upstream of *hetF* in *Nostoc* sp. PCC 7120 (Ionescu *et al.*, 2010) (Fig. 1A). NsiR1 is expressed specifically in cells becoming heterocysts before any sign of morphological differentiation is observed (Muro-Pastor, 2014).

Transcription of the different copies of NsiR1 requires HetR (Ionescu *et al.*, 2010) and takes place from individual promoters that contain the DIF1 sequence motif associated to heterocyst-specific transcription (Mitschke *et al.*, 2011; Brenes-Álvarez *et al.*, 2019). It is currently unknown whether transcription of NsiR1 is regulated by HetR directly or indirectly. The central seven copies *nsiR1.3* to *nsiR1.9* (thereafter referred to as *nsiR1.4*) are identical whereas the other copies, located at the periphery of the array, have some sequence divergence. According to the position of the TSS of *hetF* (Ionescu *et al.*, 2010; Mitschke *et al.*, 2011), *nsiR1.1* overlaps the *hetF* 5' UTR. NsiR1.1 could therefore function as an antisense of the *hetF* transcript. As the seven identical copies are very similar to *nsiR1.1*, they could also potentially regulate the expression of *hetF*. In this work we show that *Nostoc* strains constitutively overexpressing *nsiR1.1* or *nsiR1.4* have distinct phenotypes related to heterocyst differentiation, indicating that they both regulate *hetF*, although they could have additional non-overlapping targets.

Results

NsiR1 interacts with the 5' UTR of hetF

NsiR1.1 is transcribed antisense to the 257 nt long 5' UTR of *hetF* in *Nostoc* sp. PCC 7120 and this arrangement is conserved among genomes of heterocystous cyanobacteria (Ionescu *et al.*, 2010) (Supporting Information Fig. S1 and S2), therefore we hypothesized that NsiR1.1 might regulate *hetF* expression. If NsiR1 binding to *hetF* 5' UTR regulates *hetF* expression, we would expect different accumulation of HetF protein in heterocysts versus vegetative cells because *nsiR1* transcription is heterocyst-specific (Ionescu *et al.*, 2010; Brenes-Álvarez *et al.*, 2019). To test this hypothesis, we constructed *Nostoc* sp. PCC 7120 strains that express, under the control of a constitutive promoter (*rnpB*), a translational fusion of the *hetF* 5' UTR plus 60 nucleotides of its coding region to *gfpmut2* (plasmid pMBA93, see a scheme in Fig. 1A). A strain expressing *gfpmut2* and its original 5'UTR from the *rnpB* promoter was constructed as a control (plasmid pMBA92, Fig. 1A). As expected the strain bearing the control plasmid pMBA92 showed similar green fluorescence in vegetative cells or heterocysts (Fig. 1B). However, the strain bearing pMBA93 showed detectable green fluorescence in vegetative cells but not in heterocysts (Fig. 1B), suggesting heterocyst-specific downregulation at the level of the *hetF* 5' UTR.

We then tested the interaction of NsiR1.1 with *hetF* 5' UTR using a heterologous reporter assay in *E. coli* (Urban and Vogel, 2007; Corcoran *et al.*, 2012) (see Materials and Methods for details) in which the 5' UTR of *hetF* plus 60 nucleotides of its coding region was fused to the gene *sfGFP* encoding superfolder GFP (sfGFP). Because identical copies (*nsiR1.3* to *nsiR1.9*) are

very similar to *nsiR1.1* (Ionescu *et al.*, 2010), we also tested the interaction between the *hetF* 5' UTR and the sRNA encoded in one of the identical copies, namely *nsiR1.4* (Supporting Information Fig. S3). We therefore co-expressed in *E. coli* the *hetF::sfgfp* fusion together with NsiR1.1, NsiR1.4 or a control nonsense RNA corresponding to the unrelated terminator of the *rrnB* gene of *E. coli* (transcribed from plasmid pJV300, see Materials and Methods). For this experiment both the *hetF::sfgfp* translational fusion and the different RNAs are transcribed constitutively. Cells carrying the *hetF::sfgfp* translational fusion showed significant fluorescence, indicating that the translational initiation region of *hetF* was functional in *E. coli* (Fig. 1C and D). The fluorescence of cells carrying the *hetF::sfgfp* fusion decreased about 70% or 40% of the control when the fusion was co-expressed with NsiR1.1 or NsiR1.4, respectively, suggesting that each of these versions of NsiR1 was able to interact with the 5' UTR of *hetF* (Fig. 1C and D) when transcribed in a heterologous system.

Taken together, the results obtained in *Nostoc* sp. PCC 7120 and *E. coli* suggested a post-transcriptional negative regulation exerted by NsiR1 on *hetF* expression. The overlapping of NsiR1.1 and the *hetF* 5' UTR (Fig. 1A) extends between positions 20 and 78 of the UTR, 180 nucleotides upstream the start codon. To analyze the possible regulatory mechanism, we carried out *in vitro* footprinting assays of the interaction between *in vitro* transcribed NsiR1.1 and *hetF* 5' UTR (Fig. 2). ³²P-labeled *hetF* 5' UTR (a fragment extending from the TSS at position -257 to +60 with respect to the start of the coding sequence) was incubated with unlabeled NsiR1.1 and probed with RNase T1, RNase A, or lead(II) acetate (Fig. 2A). A footprint was detected between positions 23 and 81 of *hetF* 5' UTR (Fig. 2A, red bar), in agreement with the perfect antisense

sequence complementary to NsiR1.1. In addition to this footprint, we also detected some structural changes when *hetF* 5' UTR was combined with NsiR1.1; a region between positions 98 and 104 of *hetF* 5' UTR became more sensitive to RNase T1, RNase A and lead(II) acetate (Fig. 2A, green bar) and the region including the start codon became less accessible (Fig. 2A, orange bar). These results suggest that upon binding of NsiR1 there is a structural reorganization of the 5' UTR of *hetF* resulting in reduced access to the initiation codon (Fig. 2B and C) that could explain the reduction of HetF expression in heterocysts.

Construction of strains with altered levels of different versions of NsiR1

In order to analyze possible phenotypic effects caused by altered levels of NsiR1, we have constructed strains with constitutive high levels of NsiR1.1 (OE_NsiR1.1) or NsiR1.4 (OE_NsiR1.4) transcribed from the strong *trc* promoter to the *T1* terminator of the *rrnB* gene of *E. coli* (Fig. 3A). In addition, because we have introduced a NsiI restriction site (6 bp) at the 5' end of *nsiR1.1* or *nsiR1.4* to facilitate cloning, this 6 nt tag allows the discrimination, based on its length, of molecules of NsiR1.1 and NsiR1.4 transcribed from their native promoters from molecules expressed from the *trc* promoter in the constructs introduced in *Nostoc* sp. PCC 7120.

The location of *nsiR1.1* in the 5' UTR of *hetF* precludes the deletion of all copies of *nsiR1* without removing a segment of *hetF* 5' UTR. As an alternative strategy to deplete NsiR1 without altering the genomic region upstream of *hetF* we have followed a strategy similar to that in Brenes-Álvarez et al., (2020) and generated

a *Nostoc* strain (OE_as_NsiR1) that constitutively expresses the sequence complementary to *nsiR1.4* from the *trc* promoter (Fig. 3A). This antisense RNA is expected to act as a sponge neutralizing the accumulation of *nsiR1*. Using probes specific for NsiR1.1 or NsiR1.4 we have verified by Northern blot the constitutive expression of NsiR1.1 and NsiR1.4 under the control of the *trc* promoter in strains OE_NsiR1.1 and OE_NsiR1.4, respectively (Fig. 3B). In addition, Northern blot hybridization also showed that, in the OE_C strain, signals corresponding to hybridization to the probe for NsiR1.4 (corresponding to transcription of endogenous *nsiR1.3* to *nsiR1.9*) were stronger than those corresponding to *nsiR1.1*, as expected from the different number of identical copies of *nsiR1.4* (seven copies) versus *nsiR1.1* (single copy). Transcripts corresponding to NsiR1.1 and NsiR1.4 whose transcription did not stop at their own Rho-independent terminators but instead were terminated at the *T1* terminator were also observed above the 100 nt long size marker (indicated with black bars in Fig. 3B). Regarding the OE_as_NsiR1 strain, Northern blot hybridization showed that the accumulation of NsiR1.1 and NsiR1.3 to NsiR1.9 was diminished in this strain, suggesting effective removal of endogenous native NsiR1 by overexpression of the antisense sponge (Fig. 3B). Both probes used for hybridization included the *T1* terminator, present also in as_NsiR1 (Fig. 3A), leading to its detection in the OE_as_NsiR1 strain (Fig. 3B). We were unable to quantify *hetF* expression by Northern blot or RT-PCR, suggesting that, similar to the observations previously made in *Nostoc punctiforme* (Wong and Meeks, 2001), the *hetF* transcript is likely unstable or accumulates in small quantity in *Nostoc* sp. PCC 7120. We have instead analyzed the accumulation of *hetF* transcripts and their putative interaction with

the different versions of NsiR1 by primer extension assays (Fig. 3C). The 5'-ends of *hetF* transcripts were analyzed by retrotranscription using an oligonucleotide complementary to *hetF* 5' UTR. Extension products corresponding to the TSS of *hetF*, position 4273166f in the *Nostoc* sp. PCC 7120 genome (Ionescu *et al.*, 2010; Mitschke *et al.*, 2011), were in fact observed using RNA samples isolated from wild type strain or the *hetR* mutant (Supporting Information Fig. S4). In addition, shorter HetR-dependent extension products were also observed around position 4173242, corresponding to the TSS of *nsiR1.1* (Supporting Information Fig. S4). Since, according to dRNA-Seq (Mitschke *et al.* 2011), there are no additional TSS for *hetF* in this region, these shorter extension products could be the result of termination of retrotranscription at processing sites or regions with strong secondary structure. Similar to the observation made in the wild-type strain (Supporting Information Fig. S4), extension products that would correspond to the full-length 5' UTR of *hetF* were observed using RNA from the control strain OE_C as well as from strains OE_NsiR1.4 and OE_as_NsiR1 (indicated with an asterisk in Fig. 3C) but not in strain OE_NsiR1.1 (see below). Extension products that stopped at the position corresponding to the TSS of the chromosomal *nsiR1.1* were observed using RNA isolated from the OE_C, OE_NsiR1.1 and OE_NsiR1.4 strains when the cells were subjected to nitrogen deficiency (Fig. 3C, black triangle). In the OE_as_NsiR1 strain, overexpressing a sponge that is expected to remove endogenous NsiR1, these stops were only observed after 12 hours of nitrogen deficiency and in reduced amounts. Retrotranscriptase stops when it encounters an obstacle, for example, regions of strong secondary structure or a double stranded RNA such as a sRNA-mRNA duplex (Jagodnik *et al.*, 2017).

Because of the location of the additional stops observed, one possible explanation is that they are produced by binding of NsiR1. In fact, and in contrast to the other three strains included in the experiment, no full-length extension products that corresponded to the 5'-end of *hetF* were observed using RNA isolated from strain OE_NsiR1.1. Instead, a strong stop around the start position of *nsiR1.1* was observed irrespective of nitrogen removal (Fig. 3C, green triangle), suggesting that the presence of NsiR1.1 expressed from the constitutive *trc* promoter was responsible for such stops. Similarly, stops independent of the nitrogen condition but weaker than those observed with RNA isolated from strain OE_NsiR1.1 were also observed with RNA isolated from the OE_NsiR1.4 strain (Fig. 3C, red triangle). Taken together, these observations indicate that the HetR-dependent NsiR1.1 sRNA (and possibly NsiR1.4) interacts with the 5' UTR of *hetF* in *Nostoc* sp. PCC 7120.

Based on data from fluorescence microscopy in *Nostoc* strains bearing pMBA93, the observations made in *E. coli*, and *in vitro* footprinting assays (Fig. 1 and 2), NsiR1.1 and, to a lesser extent, NsiR1.4 could exert a negative regulation of *hetF* expression. Because it is known that a *hetF* mutant accumulates more HetR protein than the WT strain (Risser and Callahan, 2008), we quantified the accumulation of HetR in the OE_C, OE_NsiR1.1, OE_NsiR1.4 and OE_as_NsiR1 strains 18 h after combined nitrogen removal as an indication of HetF levels (Fig. 3D and E). Using three independent clones of each strain, we determined that strain OE_NsiR1.1 accumulated about twice more HetR protein than the OE_C strain which could be a consequence of reduced HetF amounts in OE_NsiR1.1. OE_NsiR1.4 also seems to accumulate more HetR protein than the OE_C strain, but in this case, perhaps due to the

effects of NsiR1.4 on HetF accumulation being weaker, the results were not quite statistically significant ($p=0.057$). In order to analyze whether the increased amounts of HetR protein could be instead due to increased transcription of *hetR* in the overexpressing strains, we measured the accumulation of *hetR* transcripts in all four strains analyzed above (OE_C, OE_NsiR1.1, OE_NsiR1.4 and OE_as_NsiR1) (Supporting Information Fig. S5). After 18 h of nitrogen deficiency the amount of *hetR* transcript in the OE_NsiR1.1 strain was not above the amounts observed in the control strain (OE_C), indicating that the increased amount of HetR in strain OE_NsiR1.1 was not the consequence of increased transcription of the *hetR* gene.

Altered heterocyst differentiation in strains OE_NsiR1.1, OE_NsiR1.4 and OE_as_NsiR1

In order to analyze the effects of altered NsiR1 expression at the phenotypic level we tested growth of the OE_C, OE_NsiR1.1, OE_NsiR1.4 and OE_as_NsiR1 strains in solid media containing different nitrogen sources (Fig. 4A). All four strains grew similarly in the presence of ammonium, however strain OE_NsiR1.4 grew worse in the absence of combined nitrogen and a bit slower in the presence of nitrate than the rest of strains. In liquid cultures, strain OE_NsiR1.4 showed slight phenotypic differences (larger cell size) in the presence of ammonium. The differences were more dramatic after combined nitrogen removal (Fig. 4B). After nitrogen removal, strain OE_NsiR1.4 differentiated cells that were stained by Alcian blue (indicative of the presence of heterocyst polysaccharides) but some filaments had enlarged cells which

resembled the phenotype previously described for the *hetF* mutant (Risser and Callahan, 2008). OE_NsiR1.1, also contained some filaments with larger cells under nitrogen deprivation. Frequency of heterocysts in filaments of strains with altered levels of NsiR1 was determined taking samples at different time points after nitrogen removal and staining with Alcian blue. Whereas all four strains reached similar frequency of heterocyst after 72 hours of nitrogen deprivation, different frequencies were observed at an earlier time point (24 h) indicating a delay in differentiation of heterocysts in strains OE_NsiR1.1 and OE_as_NsiR1 (Fig. 4C). This delay was longer in OE_as_NsiR1, the only strain with a statistically significant difference in frequency of heterocyst after 48 hours (Fig. 4C).

Nitrogen fixation is carried out by nitrogenase, and expression of the *nifHDK* genes can be taken as an indication of heterocyst maturity. We therefore analyzed the timing of expression of *nifH* during heterocyst differentiation upon nitrogen removal in the different strains (Fig. 4D). OE_NsiR1.1 and OE_NsiR1.4 showed no difference with respect to the control, however OE_as_NsiR1, depleted of NsiR1, showed a delay in the accumulation of *nifH* mRNA, suggesting a delay in differentiation of mature heterocysts.

Discussion

Heterocyst differentiation is a complex process that requires precise regulatory mechanisms in order to establish exclusive transcriptional programs for different cell types (vegetative cells, immature pro-heterocysts and mature heterocysts).

Heterocyst differentiation is triggered by mutual induction of expression of the transcriptional regulator NtcA and the master regulator HetR in vegetative cells becoming heterocysts (Black *et al.*, 1993; Muro-Pastor *et al.*, 2002; Olmedo-Verd *et al.*, 2006). The accumulation of active HetR in pro-heterocysts seems to trigger the downstream cascade. NsiR1 was the first HetR-dependent sRNA identified in *Nostoc* sp. PCC 7120 (Ionescu *et al.*, 2010), but it is also well conserved in non-heterocyst-forming filamentous cyanobacteria (Brenes-Álvarez *et al.*, 2016). However, while most non-heterocystous strains contain a single copy of NsiR1, heterocystous strains have multiple tandem copies with slight sequence divergence (Supporting Information Fig. S1, S2 and S6). The increase in the number of copies of NsiR1, that correlates with the evolution of the ability to differentiate heterocysts, and their sequence divergence, could allow some degree of functional specialization by regulating a variety of targets. According to the position of the TSS of *hetF* in *Nostoc* sp. PCC 7120 (Ionescu *et al.*, 2010; Mitschke *et al.*, 2011), the *nsiR1* copy closest to *hetF* (*nsiR1.1*) could function as an antisense of *hetF* 5' UTR. This function of NsiR1.1 may be conserved in heterocyst-forming cyanobacteria because most of them have a *nsiR1* homolog in the upstream region of *hetF* in antisense orientation (Supporting Information Fig. S1 and S2). Co-expression experiments in *E. coli* indicate that NsiR1.1, and to a lower extent NsiR1.4, can repress the expression of *hetF* (Fig. 1C and D). A translational fusion of *hetF* 5' UTR to GFP had reduced expression in heterocysts (Fig. 1B), as expected if its translation is repressed by NsiR1, that is expressed at an early stage during heterocyst development (Muro-Pastor, 2014). In addition, primer extension analysis in *Nostoc* sp. PCC 7120 also

suggested a putative interaction of NsiR1.1 with *hetF* 5' UTR based on the stop of retrotranscription (Supporting Information Fig. S4). Several observations point to post-transcriptional regulation of translation rather than transcriptional interference as the mechanism for *hetF* regulation by NsiR1. First, negative regulation of *hetF* expression is observed in the *E. coli* system, in which NsiR1 and the *hetF* 5' UTR are constitutively transcribed from *E. coli* promoters in separate plasmids. Second, the observation that NsiR1.1, which perfectly matches the *hetF* 5' UTR, and NsiR1.4 which contains several nucleotide changes, repress *hetF* translation to a different extent (Fig. 1C), is indicative of post-transcriptional regulation by perfect (NsiR1.1) or imperfect (NsiR1.4) base pairing. Finally, the total quantity of *hetF* mRNA did not significantly decrease when NsiR1 was present (Supporting Information Fig. S4), indicating that NsiR1 did not affect *hetF* transcription.

The interaction site of NsiR1 in the *hetF* 5' UTR was located relatively far upstream from the *hetF* start codon, precluding repression of translation by direct interaction of NsiR1 with the translation initiation region and pointing instead to a translational regulation based on a possible conformational change of the *hetF* 5' UTR. *In vitro* footprinting data suggest that the *hetF* 5' UTR is highly structured and the interaction with NsiR1.1 provokes a conformational change in which the region of the start codon of *hetF* becomes less accessible (Fig. 2), providing a mechanistic explanation for the negative regulation of *hetF* expression by NsiR1.1.

In order to study the function of NsiR1, we created strains of *Nostoc* sp. PCC 7120 with altered levels of different versions of NsiR1 (Fig. 3). The overexpression of NsiR1.1 or NsiR1.4 under the control of the *trc* promoter was

irrespective of the nitrogen source and strong enough to overpass the endogenous expression of NsiR1, that was induced upon nitrogen removal. As expected, the OE_as_NsiR1 strain had reduced amounts of NsiR1, implying that the interaction between NsiR1 and its antisense results in its degradation (Fig. 3B). Primer extension assays with RNA extracted from strains with altered levels of different versions of NsiR1 confirmed the interaction of NsiR1.1 and NsiR1.4 with *hetF* 5' UTR (Fig. 3C). We were unable to quantify changes in *hetF* expression in our mutant strains, likely because expression of *hetF* is low and the transcripts are unstable (Wong and Meeks, 2001; Risser and Callahan, 2008). Instead, because one of the features described for a *hetF* mutant is the accumulation of higher levels of HetR protein (Risser and Callahan, 2008), we analyzed HetR protein levels as an indication of HetF function. In fact, strains OE_NsiR1.1 and, to a lesser extent, OE_NsiR1.4, had higher amounts of HetR, as expected for a strain with a reduced amount of HetF (Fig. 3D and E). This higher amount of HetR was not due to increased *hetR* transcription in strains OE_NsiR1.1 and OE_NsiR1.4 (Supporting Information Fig. S5). Finally, OE_NsiR1.1 had a phenotype partially similar in some respects to that described for a *hetF* mutant. A *hetF* mutant grows poorly in media without combined nitrogen, has enlarged cells and cannot differentiate heterocysts (Risser and Callahan, 2008). OE_NsiR1.1 showed some filaments with larger cells in liquid cultures growing without combined nitrogen (Fig. 4). Taken together, all these *in vivo* and *in vitro* results strongly support a role for NsiR1 in reducing the amount of HetF in cells becoming heterocysts. Proper HetR turnover seems to be required for heterocyst differentiation, since *hetF* mutants accumulating HetR are unable to differentiate (Risser and Callahan, 2008).

Similarly, strains bearing several alleles of *hetR* that result in reduced turnover are also unable to differentiate (Risser and Callahan, 2007). Specific NsiR1 expression in developing heterocysts would reduce HetF accumulation resulting in increased amount of HetR in these cells, favoring expression of HetR-dependent transcripts and progression of differentiation (Fig. 5). Finally, despite the small difference in sequence between NsiR1.1 and NsiR1.4 (Supporting Information Fig. S3), strains OE_NsiR1.1 and OE_NsiR1.4 had slightly different phenotypes. They had some growth difference in solid media with different nitrogen sources (Fig. 4A). In fact, the observation that the effect of OE_NsiR1.4 is more important than OE_NsiR1.1, while NsiR1.4 is less efficient in repressing *hetF* expression, suggests that NsiR1.1 and NsiR1.4 might have additional non-overlapping targets related to heterocyst differentiation. On the other hand, the observation that the strain OE_as_NsiR1, depleted of NsiR1, showed delayed expression of *nifH* transcripts encoding nitrogenase indicates NsiR1 influences the overall timing of heterocyst maturation. Further study of additional potential targets for NsiR1, and the characterization of additional heterocyst-specific non-coding RNAs (Brenes-Álvarez *et al.*, 2019) could reveal the importance of these type of molecules in the regulation of this developmental process.

Materials and Methods

Strains and growth conditions

For Northern blot and primer extension analysis, cultures of wild-type, *hetR* mutant 216 (Buikema and Haselkorn, 1991) and different derivative strains of *Nostoc* sp. PCC 7120 (Supporting Information Table S1) were bubbled with an air/CO₂ 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, lacking NaNO₃ but containing 6 mM NH₄Cl, 10 mM NaHCO₃, and 12 mM *N*-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid-NaOH buffer (pH 7.5) (BG11₀C + 6 mM NH₄Cl). Nitrogen deficiency was induced by filtering, washing and resuspending cells in nitrogen-free-medium with 10 mM NaHCO₃ (BG11₀C). Chlorophyll concentration of the cultures was measured as described (MacKinney, 1941). For Western blot analysis, cultures of different derivative strains of *Nostoc* sp. PCC 7120 (Supporting Information Table S1) were grown in flasks at 30°C in liquid BG11 medium for one week. Nitrogen deficiency was induced as described above. OE_C, OE_NsiR1.1, OE_NsiR1.4 and OE_as_NsiR1 strains were grown in the presence of streptomycin (Sm) and spectinomycin (Sp), 2 µg/ml each (liquid medium) or 5 µg/ml each (solid medium). *Nostoc* strains bearing Nm^R plasmids (pMBA92, pMBA93 or pMBA94) were grown in the presence of neomycin (Nm), 5 µg/ml (liquid medium) or 25 µg/ml (solid medium). *E. coli* strains were grown in LB medium, supplemented with appropriate antibiotics (Sambrook and Russell, 2001).

Construction of Nostoc strains with altered levels of NsiR1

Plasmids and oligonucleotides used in this work are described in Supporting Information, Tables S2 and S3. The sequences of all fragments amplified by

PCR were entirely verified by sequencing. We have used pMBA37 (Olmedo-Verd *et al.*, 2019) as a backbone for overexpressing different copies of *nsiR1* or an antisense to *nsiR1*. pMBA37 contains the *trc* promoter and the *T1* terminator from *E. coli rrnB* gene. The *trc* promoter from pMBA37 was amplified using oligonucleotides 573 and 574. This product and oligonucleotide 573 were later used for PCRs with oligonucleotides 757, 758 or 512 generating fragments in which the *trc* promoter was fused respectively to *nsiR1.1* (fragment 1), *nsiR1.4* (fragment 2) or *as_nsiR1* (fragment 3). After digestion of fragments 1-3 with *Clal* and *XhoI* in the sites provided by oligonucleotides 573, 757, 758 and 512, these fragments were cloned into *Clal*-*XhoI* digested pMBA37 vector, rendering respectively pMBA77, pMBA78 and pMBA42 (Supporting Information Table S2). pMBA77, pMBA78 and pMBA42 were introduced in *Nostoc* sp. PCC 7120 wild type by conjugation (Elhai and Wolk, 1988) generating strains OE_NsiR1.1, OE_NsiR1.4 and OE_as_NsiR1, respectively (Supporting Information Table S1).

Construction of Nostoc strain with 5'UTR of hetF fused to gfp

We have used pMBA91, a derivative of pMBA20 (Olmedo-Verd *et al.*, 2019), as a backbone for the constitutive overexpression from *rnpB* promoter of a translational fusion to GFP of the 5'UTRs of *hetF*. As a control, the *gfpmut2* gene was cloned with its own 5'UTR. pMBA91 is similar to pMBA20 but with a Nm resistance gene instead of a SmSp resistance cassette. The Nm resistance gene from pRL278 (Black *et al.*, 1993) was amplified using oligonucleotides 892 + 893. After digestion with *Bam*HI, this product was cloned in *Bam*HI-digested

pMBA20, rendering pMBA91 (Supporting Information Table S2). The *hetF* 5'UTR was amplified using as template genomic DNA and oligonucleotides 555 + 895. *gfpmut2* gene was obtained by PCR using as template pSAM270 (Brenes-Álvarez *et al.*, 2019) and oligonucleotides 896 + 894. A PCR product with a translational fusion between the *hetF* 5'UTR plus sequences encoding the first 20 amino acids of HetF and *gfpmut2* was amplified using as templates the two above-described PCR fragments and oligonucleotides 555 + 894. The PCR product was digested with NsiI and XhoI and cloned in NsiI-XhoI digested pMBA91, rendering pMBA93. The 5'UTR of *gfpmut2* was used as control. The *gfpmut2* gene plus its 5'UTR were amplified using as template pSAM270 (Brenes-Álvarez *et al.*, 2019) and oligonucleotides 894 + 899. The PCR product was digested with NsiI and XhoI and cloned in NsiI-XhoI digested pMBA91, rendering pMBA92 (Supporting Information Table S2). pMBA92 and pMBA93 were introduced in *Nostoc* sp. PCC 7120 by conjugation (Elhai and Wolk, 1988). Sequences of inserts in plasmids expressing GFP translational fusions in *Nostoc* sp. PCC 7120 are shown in Supporting Information Table S4.

In vitro synthesis and labelling of RNA

RNA transcripts were generated *in vitro* with MEGAscript High Yield Transcription Kit (AM1333, Ambion). The DNA templates for the transcription of *hetF* 5'UTR, NsiR1.1 and NsiR1.4 were generated by PCR with a primer that includes a T7 promoter sequence upstream the 5'-end of the coded RNA, and a primer matching the 3'-end of the RNA (Supporting Information Table S3). The templates used for these PCR amplifications were genomic DNA (for *hetF*

5'UTR), or only the overlapping oligonucleotides (for NsiR1.1 and NsiR1.4). The sequences of the RNAs transcribed *in vitro* are shown in Supporting Information Table S5. Three (*hetF*) or one (*nsiR1*) non-encoded guanosines were added at the 5' end of each template for efficient T7 transcription. After *in vitro* transcription, RNAs were treated with DNase I and purified by phenol, phenol/chloroform and chloroform extractions, ethanol-precipitated at -20 °C, and washed with 70% ethanol. 100 pmol of RNAs were labeled at the 5'-end with [γ^{32} P]ATP and polynucleotide kinase and purified in a 6% polyacrylamide gel as described (Álvarez-Escribano *et al.*, 2018).

In vitro structure probing and footprinting

For structure probing of NsiR1.1 and NsiR1.4, 0.1 pmol of labeled NsiR1.1 and NsiR1.4 RNAs in a 7 μ l volume were denatured for 1 min at 95 °C and chilled on ice for 5 min. For the footprinting of NsiR1.1 on *hetF* 5'UTR, 0.1 pmol of labeled *hetF* 5'UTR RNA was mixed in 7 μ l with water (-) or 1 pmol NsiR1.1 (+), denatured for 1 min at 95 °C and chilled on ice for 5 min. After denaturing and chilling steps, we added to all samples 1 μ l of 1 mg/ml yeast RNA (Ambion AM7118) and 1 μ l of 10x structure buffer (Ambion). The samples were incubated further for 15 min at 37 °C. RNase T1, RNase A and lead(II) acetate treatment were performed as described (Álvarez-Escribano *et al.*, 2018). Alkaline and RNase T1 G ladders were generated as described (Álvarez-Escribano *et al.*, 2018). All samples were run on 6% polyacrylamide, 7 M urea gels and bands visualized with a Cyclone Storage Phosphor System.

RNA isolation, Northern blot analysis and primer extension assays

Total RNA was isolated using hot phenol as described (Mohamed and Jansson, 1989) with modifications (Brenes-Álvarez *et al.*, 2016). Samples for Northern blot hybridization of NsiR1 were separated in 10% urea-polyacrylamide gels as described (Steglich *et al.*, 2008) (7 µg of total RNA). Samples for Northern blot hybridization of longer transcripts (*hetR* or *nifH*) were separated in 1% agarose denaturing formaldehyde gels (10 µg of total RNA) and transferred to Hybond-N+ membrane (GE Healthcare) with 20X SSC buffer. Strand-specific ³²P-labeled probes were prepared with Taq DNA polymerase using a PCR fragment as template and the oligonucleotides specified in Supporting Information Table S3 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 for NsiR1.1 and NsiR1.4 probes were amplified with oligonucleotides 506 + 573 and pMBA77 (NsiR1.1) or pMBA78 (NsiR1.4) as templates. PCR fragments for *hetR* and *nifH* probes were amplified from genomic DNA using oligonucleotides hetR8 + hetR9 and 787 + 788 respectively. Hybridization to *mpB* (Vioque, 1992) or 5S rRNA probes was used as a loading and transfer control. Primer extension of 5' ends of *hetF* was performed as previously described (Muro-Pastor *et al.*, 1999) using 10 µg of total RNA and oligonucleotide 105 labeled with [γ -³²P]ATP.

Western blot analysis

For HetR Western blot analysis, crude extracts were prepared using glass beads. Cells from 25 ml cultures of OE_C, OE_NsiR1.1, OE_NsiR1.4 and OE_as_NsiR1 subjected to 18 h of nitrogen deficiency were harvested by filtration, washed with TE50/100 buffer (50 mM Tris pH 8, 100 mM EDTA) and resuspended in 500 µl of resuspension buffer (50 mM Tris-HCl pH 8.0, 2 mM 2-mercaptoethanol) containing a protease inhibitor cocktail (Roche). The cellular suspension was mixed with glass beads (SIGMA, 200 µm) in an Eppendorf tube and subjected to 7 cycles of 1 min vortexing plus 1 min of cooling on ice. Cell extract was separated from cell debris and unbroken cells by centrifugation (3 min at 3000 x g at 4 °C). The soluble fraction was obtained by centrifugation of the crude extract at 16.000 x g for 30 min at 4 °C. The protein concentration was determined by the Lowry procedure (Markwell *et al.*, 1978). 40 µg of proteins were fractionated on 10% SDS-PAGE. Antibodies against *Nostoc* sp. PCC 7120 HetR (Valladares *et al.*, 2016) and *E. coli* GroEL (Sigma-Aldrich) were used.

Reporter assay for in vivo verification of targets

We used the GFP reporter system described in (Urban and Vogel, 2007) with the superfolder GFP (sfGFP) and plasmid pXG10-SF (Corcoran *et al.*, 2012) for the experimental verification of targets in *E.coli*. The 5'UTR of *hetF* from the TSS at position –257 (4273166f) plus 60 nucleotides within its coding region was amplified using oligonucleotides 555 + 557. The GTG start codon was changed to ATG using overlapping PCRs and oligonucleotides 555, 556, 557 and 558. The information about TSS was taken from Mitschke *et al.* (2011). To

express NsiR1.1 and NsiR1.4 in *E. coli*, the genes were amplified from genomic DNA using primers 559 (5' phosphorylated) + 561 or 560 (5' phosphorylated) + 562, respectively. These PCR products were digested with XbaI and fused to a plasmid backbone that was amplified from pZE12-luc with primers PLlacOB and PLlacOD and digested with XbaI, rendering pMML3 and pMML4 (Supporting Information Table S2). Different combinations of plasmids were introduced in *E. coli* DH5 α and GFP fluorescence measurements were carried out by either flow cytometry or with a microplate reader (Varioskan) using liquid cultures from individual colonies, as previously described (Wright *et al.*, 2013). Plasmid pJV300 (Supporting Information, Table S2) was used as a control. Sequences of inserts from plasmids used in the heterologous reporter system are shown in Supporting Information Table S6.

Fluorescence microscopy

Fluorescence of *Nostoc* sp. PCC 7120 filaments carrying plasmid pMBA92, pMBA93 or pMBA94 (Supporting Information Table S2) growing on top of solidified nitrogen-free medium, was analyzed and quantified as described (Muro-Pastor, 2014) using a Leica HCX PLAN-APO 63 $\times$ 1.4 NA oil immersion objective attached to a Leica TCS SP2 laser-scanning confocal microscope. Samples were excited at 488 nm by an argon ion laser and the fluorescent emission was monitored by collection across windows of 500–538 nm (GFP) and 630–700 nm (photosynthetic pigment autofluorescence). Filaments were stained with Alcian blue as described (Olmedo-Verd *et al.*, 2005) and visualized using a Olympus BX60 microscope. The frequency of

heterocysts was calculated as the number of cells stained by Alcian blue per at least five hundred individual cells counted. All results are expressed as the average of two biological replicates $\pm$ SD.

Computational and statistical methods

To detect *nsiR1* homologs in the upstream region of *hetF*, the HetF sequence of *Nostoc* sp. PCC 7120 was used in a blastp search in IMG (Markowitz *et al.*, 2014) against 100 cyanobacterial genomes, those used in Shih *et al.* (2013), excluding the genomes corresponding to the fast-evolving *Prochlorococcus* clade. 50 HetF homologs were identified. We then used one of the identical repeats of *nsiR1* in *Nostoc* sp. PCC 7120 as input in a blastn search to predict putative *nsiR1* homologs in the region (4 kb) upstream each of the 50 predicted *hetF* homologs. Sequences of *nsiR1* homologs that were not in the upstream region of *hetF* were taken from (Brenes-Álvarez *et al.*, 2016). All the NsiR1 homologs identified are shown aligned in Supporting Information Fig. S6. The Student's t test was used to determine statistical significance. Number of biological samples can be found in the corresponding figure legends. Models of RNA secondary structures were generated with Mfold (Zuker, 2003) implementing restrictions according to the information obtained from *in vitro* structure probing.

Supporting information:

Appendix S1: Supporting pdf file, including supplementary Figures S1 to S6
and supplementary Tables S1 to S6.

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Author contributions

M.B-A., A.V., and A.M.M-P. designed research; M.B-A., M.M., A.V., and
A.M.M-P. performed research; M.B-A., A.V., and A.M.M-P. analyzed data; and
M.B-A., A.V., and A.M.M-P. wrote the paper.

Declaration of interest

The authors declare no conflict of interest.

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Figure legends

Fig. 1. NsiR1 interacts with *hetF* 5' UTR.

A. Schematic representation of the genomic region encoding NsiR1 in *Nostoc* sp. PCC 7120 and constructs in plasmids pMBA92 and pMBA93. Bent arrows indicate the transcriptional start sites. Identical repeats of *nsiR1* (3-9, e.g. *nsiR1.4*) are shown as red arrows. Repeats of *nsiR1* with some sequence divergence (1, 2, 10, 11 and 12) are shown in light blue except repeat 1 (*nsiR1.1*), antisense to *hetF*, that is shown in green.

B. Top, confocal fluorescence images (green channel, red channel and bright field) of filaments from *Nostoc* strains bearing plasmids pMBA92 (control, *gfp* gene expressed from the *mpB* promoter) or pMBA93 (translational fusion, expressed from the *mpB* promoter, of the *hetF* 5' UTR to the *gfp* gene) growing on top of nitrogen-free medium. Bottom, quantification of the signals for the green (GFP) and red (autofluorescence) channels. The loss of autofluorescence from photosynthetic pigments is a characteristic feature of heterocyst differentiation. Heterocysts are indicated with numbers.

C. GFP fluorescence of *E. coli* cultures bearing different combinations of a plasmid expressing 5' UTR *hetF::sfgfp* with plasmids expressing NsiR1.1, NsiR1.4 or an unrelated control RNA (pJV300, see Materials and Methods for details). The data are represented as mean and standard deviation of results from cultures corresponding to four independent colonies after normalization for cell density and subtraction of background fluorescence in cells bearing pXG0. *T*-test P-value <0.05*; < 0.01**.

D. FACS-based reporter assays. The plot shows the distribution of fluorescence in 10,000 *E. coli* cells bearing plasmids expressing 5' UTR *hetF*:*sfgfp* and the unrelated control RNA (black), NsiR1.1 (green) or NsiR1.4 (red). The inset shows the average fluorescence of each sample.

Fig. 2. *In vitro* footprinting assay of the interaction between NsiR1.1 and the *hetF* 5' UTR.

A. 5'-labeled *hetF* 5' UTR RNA was treated with RNase T1, RNase A, or lead(II) acetate (Pb^{+2}) in the absence (-) or presence (+) of NsiR1.1. Samples were loaded on two gels that were run to different lengths. Vertical bars indicate the NsiR1.1 binding site (red), a region with increased sensitivity to the probes in the presence of NsiR1.1 (green) and the region including the start codon (orange). Nucleotides with differential sensitivity to RNase T1 or RNase A in the presence or absence of NsiR1.1 are indicated with colored triangles. Nucleotide positions of *hetF* 5' UTR are shown on the left. C, untreated control; OH, alkaline ladder; T1s, RNase T1 sequencing ladder.

B, C. Models of the secondary structure of *hetF* 5' UTR from *Nostoc* sp. PCC 7120 without (B) or with bound NsiR1.1 (C). Nucleotides corresponding to the areas highlighted by vertical bars in the gels are colored in the same way. Nucleotides with differential sensitivity to RNase T1 or RNase A outside the NsiR1 binding site are indicated with colored triangles. Start codon is in purple with a purple frame. The three non-encoded guanosines added at the 5' end of *hetF* 5'UTR for efficient transcription are shown in lowercase.

Fig. 3. *Nostoc* sp. PCC 7120 strains with altered levels of NsiR1.

A. Scheme of DNA fragments cloned in the plasmids used to generate the different strains. Transcription start site (bent arrows), Rho-independent

terminator of NsiR1 (small stem loop), *T1* terminator (large stem loop), *trc* promoter, strand specific probes for NsiR1.1 (green line) and for NsiR1.4 (red line) are indicated.

B. Northern blots using RNA extracted from the different strains at different times after nitrogen removal hybridized with probes for NsiR1.1 (top), NsiR1.4 (middle) and 5S rRNA (bottom) as a loading control. Endogenous NsiR1 (black triangles) and the three RNA species constitutively expressed from the *trc* promoter, NsiR1.1 (green triangle), NsiR1.4 (red triangle) and as_NsiR1 (grey triangles), are indicated. Black bars indicate transcripts whose transcription did not stop at the Rho-independent terminators of *nsiR1*.

C. Primer extension analysis of *hetF* transcripts using RNA isolated from the mutant strains at different time points after combined nitrogen removal. The position of the TSS of *hetF* (4273166f) is indicated with an asterisk. The position corresponding to the TSS of *nsiR1.1* (4273242r) is indicated with a black triangle. Additional stops provoked by constitutive overexpression of NsiR1.1 (green triangle) and NsiR1.4 (red triangle) from the *trc* promoter are indicated. On the right, a sequencing reaction performed with the same oligonucleotide used for primer extension.

D. Accumulation of HetR in the mutant strains 18 h after combined nitrogen removal. Western blots were carried out with proteins extracted from three independent cultures of each strain using antibodies against HetR or GroEL (used as loading control).

E. Quantification of Western blot shown in (D). The data are presented as the mean $\pm$ standard deviation of the HetR signal normalized to the GroEL signal in the three independent clones of each strain shown in (D). The mean of HetR

accumulation of the OE_C strain in each gel is used as 100%. ***T*-test *P*-value < 0.01.

Fig. 4. Phenotype of OE_C, OE_NsiR1.1, OE_NsiR1.4 and OE_as_NsiR1 strains.

A. Growth of solid media. Cells from liquid cultures of the indicated strains grown in the presence of nitrate were collected and resuspended in BG11₀ at an OD₇₅₀ = 0.17. Five-fold serial dilutions were prepared and 10 µl of each dilution plated on BG11₀ plates containing ammonium (NH₄⁺), nitrate (NO₃⁻) or lacking combined nitrogen (N₂). Pictures were taken after 10 days of incubation at 30°C.

B. Bright field images of filaments of OE_C, OE_NsiR1.1, OE_NsiR1.4 and OE_as_NsiR1 strains growing in liquid media containing ammonium (NH₄⁺) or growing in liquid media without combined nitrogen 24 h after nitrogen removal (N₂). Filaments with enlarged irregular cells are pointed by black arrows.

Heterocysts were stained with Alcian Blue. Scale bars, 25 µm.

C. Frequency of heterocysts in OE_C, OE_NsiR1.1, OE_NsiR1.4 and OE_as_NsiR1. The data are presented as the mean and standard deviation of two cultures from two independent colonies. *T*-test *P*-value <0.05*; < 0.01**.

D. Northern blots with RNA isolated from the indicated strains at different time points (indicated in hours) after nitrogen removal and hybridized with probes for *nifH* (upper panels) and *mpB* (bottom panels) as a loading control. Sizes are indicated on the left in kb.

Fig. 5. Model of the function of NsiR1 in the regulation of heterocyst differentiation. Positive regulation is indicated by a blue arrow. Negative post transcriptional regulation by NsiR1 is indicated by a red line with a blunt end.

907 Different sizes indicate the relative amount of HetF and HetR in vegetative cells
908 versus heterocysts.
909

Figure 1

Figure 2

A

B

C

Figure 3

Figure 4

Figure 5

Supplementary Information for

NsiR1, a small RNA with multiple copies, modulates heterocyst differentiation in *Nostoc* sp. PCC 7120

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Supplementary Figures

Fig. S1. Genomic regions containing *nsrR1* repeats in 40 cyanobacterial genomes. *nsrR1* repeats are indicated by small arrows with solid colors (red for identical repeats in each genome, light blue for repeats with sequence divergence and green for repeats that might be antisense to *hetF* 5'-UTR). Genes are indicated by arrows with faded colors. Homologous genes are shown in the same color. *hetF* homologs are shown by dark grey arrows on the right side of the regions. The TSS of *hetF* in *Nostoc* sp. PCC 7120 is indicated by a bent arrow. The coherent clade of heterocyst-forming cyanobacteria is highlighted by a red frame. *Nostoc* sp. PCC 7120 is indicated in red.

Organism	hetF	nsiR1	Organism	hetF	nsiR1
<i>Trichodesmium erythraeum</i> IMS101	+	1	<i>Crocospaera watsonii</i> WH0003	-	-
<i>Arthrospira platensis</i> str. Paraca	+	-	<i>Crocospaera watsonii</i> WH8501	-	-
<i>Arthrospira platensis</i> NIES-39	+	-	<i>Cyanothece</i> sp. PCC 8802	-	-
<i>Arthrospira</i> sp. PCC 8005	+	1	<i>Cyanothece</i> sp. PCC 8801	-	-
<i>Arthrospira maxima</i> CS-328	+	-	<i>Synechocystis</i> sp. PCC 6803	-	-
<i>Oscillatoria nigroviridis</i> PCC 7112	+	3	<i>Pleurocapsa</i> sp. PCC 7327	-	-
<i>Microcoleus vaginatus</i> FGP-2	+	4	<i>Microcystis aeruginosa</i> NIES-843	-	-
<i>Oscillatoria</i> sp. PCC 6506	+	2	<i>Cyanothece</i> sp. PCC 7822	-	-
<i>Oscillatoria formosa</i> PCC 6407	+	-	<i>Cyanothece</i> sp. PCC 7424	-	-
<i>Oscillatoria</i> sp. PCC 10802	+	2	<i>Gloeocapsa</i> sp. PCC 73106	-	-
<i>Oscillatoria acuminata</i> PCC 6304	+	2	<i>Synechococcus</i> sp. PCC 7002	-	-
<i>Fischerella</i> sp. 9605	+	5	<i>Leptolyngbya</i> sp. PCC 7376	+	-
<i>Fischerella</i> sp. PCC 9431	+	6	<i>Cyanobacterium stanieri</i> PCC 7202	-	-
<i>Fischerella</i> sp. PCC 9339	+	5	<i>Geminocystis herdmanii</i> PCC 6308	-	-
<i>Fischerella</i> sp. PCC JSC-11	+	3	<i>Cyanobacterium aponinum</i> PCC 10605	-	-
<i>Cyanobacterium</i> PCC 7702	+	2	<i>Stanieria cyanosphaera</i> PCC 7437	-	-
<i>Mastigocladopsis repens</i> PCC 10914	+	6	<i>Pleurocapsa</i> sp. PCC 7319	-	-
<i>Calothrix</i> sp. PCC 7103	+	8	<i>Xenococcus</i> sp. PCC 7305	-	-
<i>Calothrix</i> sp. PCC 6303	+	8	<i>Lyngbya majuscula</i> 3L	+	1
<i>Nostoc</i> sp. PCC 7524	+	12	<i>Microcoleus chthonoplastes</i> PCC 7420	+	1
<i>Nostoc</i> sp. PCC 7120	+	12	<i>Microcoleus</i> sp. PCC 7113	+	1
<i>Anabaena variabilis</i> ATCC 29413	+	12	<i>Crinalium epipsammum</i> PCC 9333	+	1
<i>Nostoc</i> sp. PCC 7107	+	13	<i>Chamaesiphon minutus</i> PCC 6605	-	-
<i>Cylindrospermum stagnale</i> PCC 7417	+	10	<i>Geitlerinema</i> sp. PCC 7105	+	1
<i>Anabaena cylindrica</i> PCC 7122	+	8	<i>Synechococcus elongatus</i> PCC 7942	-	-
<i>Anabaena</i> sp. PCC 7108	+	8	<i>Synechococcus elongatus</i> PCC 6301	-	-
<i>Raphidiopsis brookii</i> D9	+	2	<i>Prochlorothrix hollandica</i> PCC 9006	+	-
<i>Cylindrospermopsis raciborskii</i> CS-505	+	3	<i>Leptolyngbya</i> sp. PCC 6406	+	-
<i>Nostoc azollae</i> 0708	+	7	<i>Nodosilinea nodulosa</i> PCC 7104	+	-
<i>Nodularia spumigena</i> CCY 9414	+	11	<i>Synechococcus</i> sp. PCC 7335	+	-
<i>Calothrix</i> sp. PCC 7507	+	3	<i>Leptolyngbya</i> sp. PCC 7375	+	4
<i>Microchaete</i> sp. PCC 7126	+	11	<i>Geitlerinema</i> sp. PCC 7407	+	1
<i>Nostoc punctiforme</i> PCC 73102	+	9	<i>Leptolyngbya boryana</i> PCC 6306	+	1
<i>Scytonema hofmanni</i> UTEX 2349	+	2	<i>Thermosynechococcus elongatus</i> BP-1	-	-
<i>Rivularia</i> sp. PCC 7116	+	12	<i>Synechococcus</i> sp. PCC 6312	-	-
<i>Synechocystis</i> sp. PCC 7509	-	-	<i>Cyanothece</i> sp. PCC 7425	-	-
<i>Gloeocapsa</i> sp. PCC 7428	-	-	<i>Acaryochloris marina</i> MBIC11017	-	-
<i>Chroococcidiopsis thermalis</i> PCC 7203	-	-	<i>Acaryochloris</i> sp. CMEE 5410	-	-
<i>Dactylococcopsis salina</i> PCC 8305	-	-	<i>Synechococcus</i> sp. PCC 7502	-	-
<i>Halothece</i> sp. PCC 7418	-	-	<i>Pseudanabaena</i> sp. PCC 6802	-	-
<i>Spirulina subsalsa</i> PCC 9445	+	1	<i>Pseudanabaena biceps</i> PCC 7429	-	-
<i>Spirulina major</i> PCC 6313	+	-	<i>Pseudanabaena</i> sp. PCC 7367	-	-
<i>Cyanothece</i> sp. CCY0110	-	-	<i>Synechococcus</i> sp. JA-3-3Ab	-	-
<i>Cyanothece</i> sp. ATCC 51472	-	-	<i>Synechococcus</i> sp. PCC 7336	-	-
<i>Cyanothece</i> sp. ATCC 51142	-	-	<i>Gloeobacter violaceus</i> PCC 7421	-	-

Figure S2. Co-occurrence of *nsiR1* and *hetF*. Genomes are arranged and color-coded as in (Shih *et al.*, 2013) as follows: filamentous non-heterocystous strains in green, heterocystous ramified strains in blue, heterocystous non-ramified strains in pink, baeocystous strains in orange and unicellular strains in black. *Nostoc* sp. PCC 7120 is indicated in red. Presence or absence of a *hetF* homolog is indicated. Presence or absence of homologs of *nsiR1*, number of putative *nsiR1* copies and genomic location of the *nsiR1* arrays upstream to *hetF* (light green) or in a different genomic region (yellow) are also indicated.

A

NsiR1.1 GGUAGAUGCACCUUGAUUUUAACUCCCUAGUUGGUUGAUAGCCACUGGGGGUUUUUU--
 NsiR1.4 GGUAGAUGCACCCUGAUAACUAACUCCCUAGCUGGCUAACACCGACUGGGGGCUUUUUUU

B

C

Figure S3. Sequence and structure probing of NsiR1.1 and NsiR1.4. A. Comparison of the sequences of NsiR1.1 and NsiR1.4. Nucleotide differences are highlighted in red. B. Secondary structure model of NsiR1.1 and NsiR1.4. Black and white triangles indicate the positions sensitive to hydrolysis by RNase T1 or lead(II) acetate (Pb^{+2}), respectively. The guanosine added at the 5' end for T7 transcription efficiency is in lower case. C. 5'-end labelled NsiR1.1 (left) or NsiR1.4 (right) were incubated with RNase T1 or lead(II) acetate and the resulting fragments analyzed on an 8% polyacrylamide sequencing gel. C, untreated control; OH, alkaline ladder; T1s, RNase T1 ladder. Nucleotide positions of NsiR1.1 or NsiR1.4 are shown on the right.

Figure S4. Primer extension analysis of the *hetF* transcript. Total RNA was isolated from *Nostoc* sp. PCC 7120 wild type strain (WT) or mutant strain 216 (*hetR*) at different times after removal of combined nitrogen. Primer extension assays were carried out using an oligonucleotide complementary to the 5' UTR of *hetF*. The position of the TSS of *hetF* (4273166f) is indicated with an asterisk. The position corresponding to the TSS of *nsiR1.1* (4273242r) is indicated with a black triangle. On the right, a sequencing reaction performed with the same oligonucleotide used for primer extension is included. The relative position of *hetF* (black arrow) and *nsiR1.1* transcripts (green arrow) is indicated.

Figure S5. Accumulation of *hetR* transcripts in OE_C, OE_NsiR1.1, OE_NsiR1.4 and OE_as_NsiR1 strains. A. *hetR* expression. Northern blots with RNA from the indicated strains at different time points (indicated in hours) after removal of combined nitrogen were hybridized with probes for *hetR* (top panels) and *rnpB* (bottom panels), used as a loading control. Membranes are the same used in Fig 4. B. Quantification of *hetR* expression. The data are presented as *hetR/rnpB* expression.

<i>Cylindrospermum stagnale</i> PCC 417 #1	GGUAGAUGCACC UU- GAUCUUC AAC- -UCCCCUGUUGGCUAACAGC CAC- AGGGGU- UUUU-
<i>Fischerella</i> sp. JSC-11 #1	GGUAGAUGCACC UU- GAUCUUC AAC- -UCCCCUGUUGGCUAACAGC CAC- AGGGGU- UUUU-
<i>Fischerella</i> sp. PCC 9339 #1	GGUAGAUGCACC UU- GAUCUUC AAC- -UCCCCUGUUGGCUAACAGC CAC- AGGGGU- UUUU-
<i>Fischerella</i> sp. PCC 9431 #1	GGUAGAUGCACC UU- GAUCUUC AAC- -UCCCCUGUUGGCUAACAGC CAC- AGGGGU- UUUU-
<i>Mastigocladopsis repens</i> PCC 10914 #1	GGUAGAUGCACC UU- GAUCUUC- AA- -C UCCCCAUGUAGGCUAACACCUUC- UGGGAG- UUUU-
<i>Anabaena</i> sp. PCC 7108 #1	GGUAGAUGCACC UU- GAUCUUC AAA- U- CCCCCUGGAGG- UUAACACCGCC CACAGGG- UUU
<i>Anabaena cylindrica</i> PCC 7122 #1	GGUAGAUGCACC UU- GAUCUUC AAC- U- CCCCCUGGAGG- CUACACCCGC- CGGGGG- UUU
<i>Nostoc azollae</i> 0708 #1	GGUAGAUGCACC UU- GAUCUUC AAC- U- C C U C C U G G A G G- UUAACACCCAC- CGGAGG- UUU
<i>Microchaete</i> sp. PCC 7126 #1	GGUAGAUGCACC UU- GAUCUUC CAC- U- C C C U G G U U U G G- UGAAGCGCCGC- CAGAGG- AUUU
<i>Calothrix</i> sp. PCC 7507 #1	GGUAGAUGCACC UU- GAUCUUC CAC- U- C C C U G G U U U G G- UGAAGCGCCGC- CAGAGG- AUUU
<i>Nostoc</i> sp. PCC 7524 #1	GGUAGAUGCACC UU- GAUCUUC AAC- U- C C C C C G G U U G G- CUAACCCUAC- U- UGGGGG- UUUU
<i>Nostoc punctiforme</i> PCC 73102 #1	GGUAGAUGCACC UU- GAUCUUC AAC- U- C C C C C A U G G G- CUAACCCUAC- UAGGGG- UUUU
<i>Nostoc</i> sp. PCC 7107 #1	GGUAGAUGCACC UU- GAUCUUC AAC- U- C C C C C U A G C U G G- UUAACACCCAC- UGGGGG- AUUU
<i>Raphidiopsis brookii</i> D9 #1	GGUAGAUGCACC UU- GAUCUUC AAC- U- C C C C U A A C U G G- UUAUGCGCCUU- UAGGGG- UUUU
<i>Cylindrospermopsis raciborskii</i> CS505 #1	GGUAGAUGCACC UU- GAUCUUC AAC- U- C C C C U A G C U G G- CUGAGGCCUC- UAGGGG- UUUU
<i>Nostoc</i> sp. PCC 7120 #1	GGUAGAUGCACC UU- GAUCUUC AAC- U- C C C C U A U G U G G- UUGAUGCCAC- UGGGGG- UUUU
<i>Anabaena variabilis</i> ATCC 29413 #1	GGUAGAUGCACC UU- GAUCUUC AAC- U- C C C C U A U G U G G- UUGAUGCCAC- UGGGGG- UUUU
<i>Scytonema hofmanni</i> PCC 9009 #1	GGUAGAUGCACC UU- GAUCUUC AAU- U C C C C C G G U G G C- U U U A C A C C C A A G U G G G G- G G U U

Spirulina subsalsa PCC 9445 #1
Raphidiopsis brookii D9 #2
Nostoc sp. PCC 7107 #13
Nostoc punctiforme PCC 73102 #4
Cylindrospermum stagnale PCC 7417 #8
Nodularia spumigena CCY9414 #11
Nostoc sp. PCC 7524 #12
Mastigocladopsis repens PCC 10914 #5
Anabaena cylindrica PCC 7122 #8
Nodularia spumigena CCY9414 #9
Nostoc sp. PCC 7524 #10
Microchaete sp. PCC 7126 #11
Oscillatoria acuminata PCC 6304 #1
Oscillatoria acuminata PCC 6304 #2
Geitlerinema sp. PCC 7407 #1
Oscillatoria sp. PCC 10802 #1
Oscillatoria sp. PCC 10802 #2
Arthrospira sp. PCC 8005 #1
Trichodesmium erythraeum IMS101 #1
Geitlerinema sp. PCC 7105 #1
Oscillatoria sp. PCC 6506 #1
Oscillatoria sp. PCC 6506 #2
Microcoleus vaginatus FGP-2 #4
Oscillatoria nigroviridis PCC 7112 #2
Oscillatoria nigroviridis PCC 7112 #3
Microcoleus vaginatus FGP-2 #2
Microcoleus vaginatus FGP-2 #3
Oscillatoria nigroviridis PCC 7112 #1
Microcoleus vaginatus FGP-2 #1
Nostoc punctiforme PCC 73102 #5
Chlorogloeopsis sp. PCC 7702 #1
Nostoc punctiforme PCC 73102 #2
Anabaena sp. PCC 7108 #2

GGUAGAUGCACCCU-GAUAAGCUCU---CCCAUGUCAUUUCCCUAGAUGGCAUUGGAUUUU
GGUAGAUGCACCCU-GAUAACUAAUUCUCCCCUAGUCGGACAAACCCGU-GGGGG-UUUU
GGUAGAUGCACCCU-GAUACUUAAU-UCCCUGAAUAGCUAACA-CUUUC-UUGGAG-AUUU
GAUAGAUGCACCCU-GAUAACUAGC-UCCCUAGUUCGCUAACACCCACU---GAGG-AUUU
GGUAGAUGCACCCU-GAUAACUAAU-UCCCUUAGCUGUCUAACACCCAAU---GGGGC-UUUU
AGUAGAUGCACCCU-GAUAACUAAU-UCCCUUUGCCGCAAGAUUCGAU---GGAGUUUUU
GGUAAAUGCACCCU-GAUAACUAAU-UCCCAUUUUAGUUCUAUCUAUU---GGAGUAUUU
GGUAGAUGCACCCU-GAUAACUAAU-UCCCUUGGUGGCUACCAUUGGUAAGAG---AUUU
GCUAUAUUGCACCCU-GAUAACUAAU-UCCCUUAGCUGACCAACACUCU-UGAGAG-UUUU
GAUAAAUGGUGUCCU-GAUAUUUAAU-UCCCUUAGACUGCCA---CCAGU-GAGGG-AUUU
GAUAGAUAGACCCU-AAUCAAUAAU-UCCCUAUAUUGGUGCCA---CGGAUAGGAG-CUUU
UGUAGAUGCACCCU-GAUAACUAGC---UCCUGGUAAGACCAAGUUUU-UGAGGGAUUU
GGUAGAUGCACCCU-GAUGGAU-A---UAUCCCGGCACCAAA---ACGCC-GGGA-A-UUU
GGUAGAUGCACCCU-GAUGAC-G---UUUCCCGGCUCUUUU---GAGCC-GGUU-U-UUU
GGUAGAUGCACCCU-GAUUAU-U---UCCCGAGC-CCAC-C---CGGAC-UGGGGA-UUU
GGUAGAUGCACCCU-GAUGAU-G---GUCUGGCUUUU-U-C---UGGC-CGGGAU-UUU
GGUAGAUGCACCCU-GAUAAC-----UUCUCCCGCCGAC-C---CGGGC-GGGGU-UUU
AGUAGAUGCACCCU-GAUGGU-----UUCUCCUGGCUUC---GGCUG-GGGAU-UUU
AGUAGAUGCACCCU-GAUA-U-A-C---GACUCCAGCGUCAU---AGGUG-GGGGU-UUU
GGUAGAUGCACCCU-GAUGUU-A---ACCCCGGCACGACAC---CCAGC-CGGGU-UUU
AGUAGAUGCACCCU-GAUGGAUA---CC-CCCAACCAUUC---UGG-C-UGGGGU-UUU
AGUAGAUGCACCCU-GAUGAUUA---AC-CCUACCAAUUA---CGG-C-UAGGGU-UUU
AGUAGAUGCACCCU-GAUUAUA---AC-CCCAACCAAUUU---UGG-C-CGGGU-UUU
AGUAGAUGCACCCU-GAUUAUA---ACUCCCGACCAAUUU---UGG---UGGGU-UUU
GGUAGAUGCACCCU-GAUUAUA---ACUCCCGACCAAUUU---UGG---CCGGU-UUU
AGUAGAUGCACCCU-GAUUAUA---ACUCCCGACCAAUUU---UGG---CCGGU-UUU
GGUAGAUGCACCCU-GAUUAUA---ACUCCCGACCAAUUU---UGG---CCGGU-UUU
GGUAGAUGCACCCU-GAUUAUA---ACUCCCGACCAAUUU---UGGUV-UGGGG-UUU
AGUAGAUGCACCCU-GAUUAUA---ACUCCCGACCAAUUU---UGGUV-UGGGG-UUU
GAAACCAACA-UUC-GAUAACUAAU-UCCCUUGGUGGUAACAACU---UUAGGGUUUU
GGUAGAUGCACCCU-GAUAUUAAU-UCCCUAGCUGGCUUACAGCU---CAUCAUGCCUA
GAUAGAUGCACCCU-GAUUUUAAU-UCCCUAUAUAGUUAUAUCUAU-UGAG-UUUC
-----CAAACCCU-GAUAACUAAU-UCCCUAACCAGCUAACACGA---CAGGA-UUUU

Figure S6. Alignment of 132 repeats of *nsiR1* generated with mLocARNA (Will *et al.*, 2012). Each repeat is represented by the name of the organism and the number of the repeat. Identical copies of *nsiR1* for each genome are included only once and marked “identical”. *nsiR1.1* homologs that might be transcribed antisense to *hetF* 5’UTR in heterocystous cyanobacteria are framed in purple. Nucleotides are shown in different colors in order to facilitate visualization of the alignment.

Supplementary tables

Table S1. Strains

Strain	Description	Reference
<i>Escherichia coli</i>		
DH5 α	Used for routine transformation	(Hanahan, 1983)
<i>Nostoc sp.</i>		
PCC 7120	Wild type	Pasteur Culture Collection
216	Non-functional HetR. S179N mutation.	(Buikema and Haselkorn, 1991)
OE_C	Sm ^R Sp ^R , pMBA51 inserted in plasmid alpha. <i>T1</i> terminator of <i>E. coli</i> <i>rrnB</i> gene expressed constitutively from the <i>trc</i> promoter.	(Olmedo-Verd <i>et al.</i> , 2019)
OE_NsiR1.1	Sm ^R Sp ^R , pMBA77 inserted in plasmid alpha. NsiR1.1 transcribed constitutively from the <i>trc</i> promoter.	This work
OE_NsiR1.4	Sm ^R Sp ^R , pMBA78 inserted in plasmid alpha. NsiR1.4 expressed constitutively from <i>trc</i> promoter.	This work
OE_as_NsiR1	Sm ^R Sp ^R , pMBA42 inserted in plasmid alpha. Antisense to NsiR1.1 transcribed constitutively from the <i>trc</i> promoter.	This work

Table S2. Plasmids

Name	Description	Reference
pJV300	Control plasmid expresses a ~50 nt nonsense RNA derived from the <i>T1</i> terminator of <i>E. coli rrnB</i> gene.	(Sittka <i>et al.</i> , 2007)
pXG0	Cm ^R , control plasmid without GFP	(Urban and Vogel, 2007)
pXG10-SF	Cm ^R , plasmid for construction of translational sfGFP fusions, under control of the P _{LtetO-1} promoter.	(Corcoran <i>et al.</i> , 2012)
pZE12-luc	Ap ^R , plasmid used for the expression of NsiR1 under control of the P _{Llac-O} promoter.	(Lutz and Bujard, 1997)
pRL278	Nm ^R , shuttle vector for conjugation in <i>Nostoc</i> bearing a Neomycin resistance gene.	(Black <i>et al.</i> , 1993)
pSAM270	Plasmid bearing a transcriptional fusion of the <i>hetR</i> promoter to <i>gfpmut2</i>	(Brenes-Álvarez <i>et al.</i> , 2019)
pMBA20	Ap ^R Sm ^R Sp ^R , plasmid for the overexpression of transcripts from the <i>rnpB</i> promoter and followed by the <i>T1</i> transcriptional terminator of <i>E. coli rrnB</i> gene.	(Olmedo-Verd <i>et al.</i> , 2019)
pMBA37	Ap ^R Sm ^R Sp ^R , plasmid for the overexpression of transcripts from the <i>trc</i> promoter and followed by the <i>T1</i> terminator of <i>E. coli rrnB</i> gene, used as transcriptional terminator.	(Olmedo-Verd <i>et al.</i> , 2019)
pMBA51	Ap ^R Sm ^R Sp ^R , control plasmid, expresses a 56 nt transcript derived from the <i>T1</i> terminator of <i>E. coli rrnB</i> gene from the <i>trc</i> promoter.	(Olmedo-Verd <i>et al.</i> , 2019)
pMBA42	Ap ^R Sm ^R Sp ^R , plasmid based on pMBA37 for the overexpression of an antisense to NsiR1.1.	This work
pMBA77	Ap ^R Sm ^R Sp ^R , plasmid based on pMBA37 for the overexpression of NsiR1.1.	This work
pMBA78	Ap ^R Sm ^R Sp ^R , plasmid based on pMBA37 for the overexpression of NsiR1.4.	This work
pMBA91	Ap ^R Nm ^R , Kanamycin/ Neomycin resistance version of pMBA20.	This work
pMBA92	Ap ^R Nm ^R , plasmid based on pMBA91 expressing from the <i>rnpB</i> promoter the gene <i>gfpmut2</i> plus its 5'UTR.	This work
pMBA93	Ap ^R Nm ^R , plasmid based on pMBA91 expressing from the <i>rnpB</i> promoter a translational fusion of <i>hetF</i> 5'UTR plus sequences encoding the first 20 amino acids of HetF fused to GFP.	This work
pMML2	Cm ^R , sfGFP reporter plasmid based on pXG10-SF containing the <i>hetF</i> 5'UTR plus sequences encoding the first 20 amino acids of HetF (with mutation in start codon GTG -> ATG).	This work
pMML3	Ap ^R , plasmid based on pZE12-luc expressing NsiR1.1.	This work
pMML4	Ap ^R , plasmid based on pZE12-luc expressing NsiR1.4.	This work

Table S3. Oligonucleotides

Name	Sequence (5'-3')	Used for
105	CGCCAAAGTTTCAATTCGATTTCC	Primer extension of <i>hetF</i>
189 (PLlacOB)	CGCACTGACCGAATTCATTAA	Plasmid backbone amplification from pZE12- luc
190 (PLlacOD)	GTGCTCAGTATCTTGTTATCCG	
506	GTTTTGAGCTCAGGTCTAGGGCGGCGGATTT	Probes for <i>nsiR1.1</i> and <i>nsiR1.4</i>
512	TTTTATCTCGAGGTAGATGCACCCTGATAACTAACTC CCCTAGCTGGCTAACACCGACTGGGGGCTTTTTATGC ATGCTCCAGTGAATC	Cloning of <i>as_nsiR1</i> fused to the <i>trc</i> promoter in pMBA37
555	GTTTTATGCATAAGTATATGCTAGTTATAGAAAAAC CCCC	Cloning of <i>hetF</i> 5'UTR with mutation in start codon (GTG->ATG) in pXG10-SF
556	GAAATTCCTGGGACATAACTTCTCCTATC	
557	GTTTTGCTAGCCCGCACCAAGTAGTCATTCTGCC	
558	GATAGGAGAAGTTATGTCCCAGGAATTC	
559	5' P-GGTAGATGCACCTTGATCTTTAA	Cloning of <i>nsiR1.1</i> in pZE12-luc
561	GTTTTTCTAGACCCAAGACAGTAGAATAATCTAAG	
560	5' P-GGTAGATGCACCCTGATAACTA	Cloning of <i>nsiR1.4</i> in pZE12-luc
562	GTTTTTCTAGACGGATGAATCTGCACAATGATG	
573	GTTTTATCGATGCGCCGACATCATAACGGTT	Cloning of the <i>trc</i> promoter
574	GTTTTATGCATTCCACACATTATACGAGCCG	
757	GTTTTATCTCGAGAAAAACCCCCAGTGGCTATCAAC CAACTAGGGGAGTTAAAGATCAAGGTGCATCTACCAT GCATTCCACACATTATACG	Cloning of <i>nsiR1.1</i> fused to the <i>trc</i> promoter in pMBA37
758	GTTTTATCTCGAGAAAAAGCCCCAGTCGGTGTTAGC CAGCTAGGGGAGTTAGTTATCAGGGTGCATCTACCAT GCATTCCACACATTATACG	Cloning of <i>nsiR1.4</i> fused to the <i>trc</i> promoter in pMBA37
787	GTA CTGCAAGGGGCGTGGCT	Probe for <i>nifH</i>
788	CCTATTGGTAGCTTCTGCGGG	
831	TAATACGACTCACTATAGGGAAGTATATGCTAGTTAT AGAAAAACCCCC	PCR of template for <i>in</i> <i>vitro</i> transcription of <i>hetF</i> 5'UTR
873	CCGCACCAAGTAGTCATTCTG	
833	TAATACGACTCACTATAGGGTAGATGCACCTTGATCT TTAACTCCCCTAGTTGGTTG	PCR of template for <i>in</i> <i>vitro</i> transcription of <i>NsiR1.1</i>
834	AAAAAACCCCCAGTGGCTATCAACCAACTAGGGGAGT TAAAGATC	
835	TAATACGACTCACTATAGGGTAGATGCACCCTGATAA CTAACTCCCCTAGCTGGCTAAC	PCR of template for <i>in</i> <i>vitro</i> transcription of <i>NsiR1.4</i>
836	AAAAAAGCCCCAGTCGGTGTTAGCCAGCTAGGGGAG TTAG	
hetR8	GCGCTCTGGTGGCACATCG	Probe for <i>hetR</i>
hetR9	CATGAGGACGAGCGGGCATA	
892	GTTTTGGATCCCTGCATCCCTTAACTTACTTAT	Cloning of Nm resistance cassette in pMBA20
893	GTCTAGAGGATCCCCGGTGGG	
894	GTTTTCTCGAGGCATGCCTGCAGGTCTGGACAT	Cloning of translational fusions to GFP in pMBA91
895	CTCCTTTACTCCGCACCAAGTAGTCATTCTGC	Cloning of <i>hetF</i> 5'UTR fused to GFP
896	TACTTGGTGCGGAGTAAAGGAGAAGAAGTTTTC	
899	GTTTTATGCATGCGAAAGCTTGCATGCCTGCAGG	Cloning of <i>gfpmut2</i> 5'UTR

Restriction sites used for cloning (underlined), point mutations made in *hetF* start codon (red) and 5' monophosphate (5'P) are indicated. The T7 promoter sequence in oligonucleotides 831, 833 and 835 is in bold.

Table S4. Sequences of inserts in plasmids expressing GFP translational fusions in *Nostoc*.

Plasmid	Sequence	Description
pMBA92	<p>GTCCCTTCACTATCAAAAACTACCTTGGACTT ATGCCCTACCCTGGAATAAAAAGAAATAAGCAA AACAGACACAAGACACCAACGAAGATTACACTG GAGCatgcatgcgaaagcttgcatgcctgcagg tcgactctagaggatcctctagatttaagaagg agatatacatatgagtaaggagaagaactttt cactggagttgtcccaattcttggtgaattaga tggatgatgtaaatgggcacaaatcttctgtcag tggagagggatgaaggtgatgcaacatacggaaa acttacccttaaattttatttgcactactggaaa actacctgttccatggccaacacttgtcactac tttcgcgtatgggtcttcaatgctttgcgagata cccagatcatatgaaacagcatgactttttcaa gagtgccatgcccgaaggttatgtacaggaaaag aactatatttttcaaagatgacgggaactacaa gacacgtgctgaagtcaagtttgaaggtgatac ccttggttaatagaatcgagttaaaaggtattga ttttaaagaagatggaacattcttggacacaa attggaatacaactataactcacacaatgtata catcatggcagacaaaacaaagaatggaatcaa agttaacttcaaaattagacacaacattgaaga tggaagcgttcaactagcagaccattatcaaca aaataactccaattggcgatggccctgtcctttt accagacaaccattacctgtccacacaatctgc cctttcgaaagatcccaacgaaaagagagacca catggtccttcttgagtttgaacagctgctgg gattacacatggcatggatgaactatacaata aatgtccagacctgcaggcatgcctcgag</p>	<i>gfpmut2</i> plus its original 5'UTR downstream the <i>mpB</i> promoter
pMBA93	<p>GTCCCTTCACTATCAAAAACTACCTTGGACTT ATGCCCTACCCTGGAATAAAAAGAAATAAGCAA AACAGACACAAGACACCAACGAAGATTACACTG GAGCatgcatAAGTATATGCTAGTTATAGAAAA AACCCCAAGTGGCTATCAACCAACTAGGGGAGT TAAAGATCAAGGTGCATCTACCGTTAAACTGTT CTGGGCAGATGCTAACCAATCCGGATAAAGTTG TAGATGTAGAACCAGCAAAACCTTTGAGTGGAG AAACATTCAATGGCTTGCTGCATCTAGATTTAC AGAAAAAATGGAAATCGAATTGAACTTTGGCG TTTCAAGCTAGATTTAGGAGGCAGATAGGAGAA GTTGTGTCCAGGAATTTACATTTCTGTAACC CCAGTAGGGCAGAATGACTACTTGGTGCAGgag aaaggagaagaacttttctactggagttgtccca attcttggtgaattagatgggtgatgtaaatggg cacaaatcttctgtcagtgagagggatgaaggt gatgcaacatacggaaaacttacccttaaattt atcttgcactactggaaaactacctgttccatgg ccaacacttgtcactacttttcgcgtatgggtctt caatgctttgcgagataccagatcatatgaaa cagcatgactttttcaagagtgccatgccgaa ggttatgtacaggaaagaactatatttttcaaa gatgacgggaactacaagacacgtgctgaagtc aagtttgaaggtgatacccttggttaatagaatc gagttaaaaggtattgatttttaagaagatgga aacattcttggacacaaattggaatacaactat aactcacacaatgtatacatcatggcagacaaa</p>	Translational fusion of <i>hetF</i> 5'UTR plus sequences encoding the first 20 amino acids of HetF fused to GFP, downstream the <i>mpB</i> promoter

	caaaagaatggaatcaaagttaacttcaaaatt agacacaacattgaagatggaagcgttcaacta gcagaccattatcaacaaaatactccaattggc gatggccctgtccttttaccagacaaccattac ctgtccacacaatctgccctttcgaaagatccc aacgaaaagagagaccacatggtccttcttgag tttgtaacagctgctgggattacacatggcatg gatgaactatacaataaaatgtccagacctgca ggcatgcctcgag	
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Nostoc sequences are capitalized, with black letters corresponding to 5'UTR, blue letters corresponding to *hetF* coding sequences and orange letters corresponding to the *rnpB* promoter, respectively. NsiI and XhoI sites that were used for cloning are highlighted in magenta and red, respectively. Start codons are shown in bold and underlined. The coding sequence of *gfpmut2* is in green.

Table S5. RNAs used for *in vitro* footprinting assays.

Name	Sequence (5'-3')
NsiR1.1	gGGUAGAUGCACCUUGAUCUUUAACUCCCCUAGUUGGUUGAUAGCCACUGGGGG UUUUUU
NsiR1.4	gGGUAGAUGCACCCUGAUAAACUAACUCCCCUAGCUGGCUAACACCGACUGGGGG CUUUUUU
<i>hetF</i> 5'UTR	gggAAGUAUAUGCUAGUUAUAG AAAAAACCC CAGUGGC UAUCAACCAACUAGG GGAGUUAAGAUCAAGGUGCAUCUACC GUUAAACUGUUCUGGGCAGAUAGCUAAC CAAUCCGGAUAAAGUUGUAGAUGUAGAACCAGCAAAACCUUGAGUGGAGAAAC AUUCA AUGGCUUGCUGCAUCUAGAUUUACAGAAAAAUGGAAUUGGAAUUGAAA CUUUGGCGUUUCAAGCUAGAUUUAGGAGGCAGAUAGGAGAAGUU <u>GUG</u> UCCAGG AAUUUCACAUUUCUGUAACCCAGUAGGGCAGAAUGACUACUUGGUGCGGUUCU GUAACCCAGUAGGGCAGAAUGACUACUUGGUGCGG

Nostoc sequences are capitalized, guanosines added for T7 transcription efficiency are in lower case. Predicted interaction site of *hetF* 5'UTR with different versions of NsiR1 is highlighted in yellow. Start codon is shown in bold and underlined.

Table S6. Sequences of inserts in plasmids used for verification of sRNA-mRNA interactions in *E. coli*.

Plasmid	Sequence	Description
pMML2	atgcatAAGTATATGCTAGTTATAGAAAAACCCCCAGTGGCTATC AACCAACTAGGGGAGTTAAAGATCAAGGTGCATCTACCGTTAAACT GTTCTGGGCAGATGCTAACCAATCCGGATAAAGTTGTAGATGTAGA ACCAGCAAAACCTTTGAGTGGAGAAACATTCAATGGCTTGCTGCAT CTAGATTTACAGAAAAAATGGAAATCGAATTGAACTTTGGCGTTT CAAGCTAGATTTAGGAGGCAGATAGGAGAAGTT ATGTCC CAGGAAT TTCACATTTCTGTAACCC CAGTAGGGCAGAATGACTACTTGGTGCG Ggctagc	<i>hetF</i> 5'UTR
pMML3	GGTAGATGCACCTTGATCTTTAACTCCCCTAGTTGGTTGATAGCCA CTGGGGGTTTTTTCTATAACTAGCATATACTTAGATTATTCTACTG TCTTGGG tctaga	NsiR1.1
pMML4	GGTAGATGCACCCGATAACTAACTCCCCTAGCTGGCTAACACCGA CTGGGGGCTTTTTTATGTTTCATAAATAACCAGCATCATTGTGCAGA TTCATCCGTCAGATCTAGAT tctaga	NsiR1.4

Nostoc sequences are capitalized, with black letters corresponding to 5'UTR and blue letters corresponding to coding sequences, respectively. NsiI and NheI sites that were used for cloning are highlighted in magenta and yellow, respectively. Nucleotide changed with respect to wild type sequence is indicated in red. Grey shadowed letters indicate the NsiR1.1 and NsiR1.4 sequences. XbaI restriction sites used for cloning are highlighted in blue. The start codon of *hetF* is shown in bold and underlined.

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Figure S1

Figure S2

	Organism	hetF	nsiR1
	<i>Trichodesmium erythraeum</i> IMS101	+	1
	<i>Arthrospira platensis</i> str. Paraca	+	-
	<i>Arthrospira platensis</i> NIES-39	+	-
	<i>Arthrospira</i> sp. PCC 8005	+	1
	<i>Arthrospira maxima</i> CS-328	+	-
	<i>Oscillatoria nigroviridis</i> PCC 7112	+	3
	<i>Microcoleus vaginatus</i> FGP-2	+	4
	<i>Oscillatoria</i> sp. PCC 6506	+	2
	<i>Oscillatoria formosa</i> PCC 6407	+	-
	<i>Oscillatoria</i> sp. PCC 10802	+	2
	<i>Oscillatoria acuminata</i> PCC 6304	+	2
	<i>Fischerella</i> sp. 9605	+	5
	<i>Fischerella</i> sp. PCC 9431	+	6
	<i>Fischerella</i> sp. PCC 9339	+	5
	<i>Fischerella</i> sp. PCC JSC-11	+	3
	<i>Cyanobacterium</i> PCC 7702	+	2
	<i>Mastigocladopsis repens</i> PCC 10914	+	6
	<i>Calothrix</i> sp. PCC 7103	+	8
	<i>Calothrix</i> sp. PCC 6303	+	8
	<i>Nostoc</i> sp. PCC 7524	+	12
	<i>Nostoc</i> sp. PCC 7120	+	12
	<i>Anabaena variabilis</i> ATCC 29413	+	12
	<i>Nostoc</i> sp. PCC 7107	+	13
	<i>Cylindrospermum stagnale</i> PCC 7417	+	10
	<i>Anabaena cylindrica</i> PCC 7122	+	8
	<i>Anabaena</i> sp. PCC 7108	+	8
	<i>Raphidiopsis brookii</i> D9	+	2
	<i>Cylindrospermopsis raciborskii</i> CS-505	+	3
	<i>Nostoc azollae</i> 0708	+	7
	<i>Nodularia spumigena</i> CCY 9414	+	11
	<i>Calothrix</i> sp. PCC 7507	+	3
	<i>Microchaete</i> sp. PCC 7126	+	11
	<i>Nostoc punctiforme</i> PCC 73102	+	9
	<i>Scytonema hofmanni</i> UTEX 2349	+	2
	<i>Rivularia</i> sp. PCC 7116	+	12
	<i>Synechocystis</i> sp. PCC 7509	-	-
	<i>Gloeocapsa</i> sp. PCC 7428	-	-
	<i>Chroococcidiopsis thermalis</i> PCC 7203	-	-
	<i>Dactylococcopsis salina</i> PCC 8305	-	-
	<i>Halothece</i> sp. PCC 7418	-	-
	<i>Spirulina subsalsa</i> PCC 9445	+	1
	<i>Spirulina major</i> PCC 6313	+	-
	<i>Cyanothece</i> sp. CCY0110	-	-
	<i>Cyanothece</i> sp. ATCC 51472	-	-
	<i>Cyanothece</i> sp. ATCC 51142	-	-

	Organism	hetF	nsiR1
	<i>Crocospaera watsonii</i> WH0003	-	-
	<i>Crocospaera watsonii</i> WH8501	-	-
	<i>Cyanothece</i> sp. PCC 8802	-	-
	<i>Cyanothece</i> sp. PCC 8801	-	-
	<i>Synechocystis</i> sp. PCC 6803	-	-
	<i>Pleurocapsa</i> sp. PCC 7327	-	-
	<i>Microcystis aeruginosa</i> NIES-843	-	-
	<i>Cyanothece</i> sp. PCC 7822	-	-
	<i>Cyanothece</i> sp. PCC 7424	-	-
	<i>Gloeocapsa</i> sp. PCC 73106	-	-
	<i>Synechococcus</i> sp. PCC 7002	-	-
	<i>Leptolyngbya</i> sp. PCC 7376	+	-
	<i>Cyanobacterium stanieri</i> PCC 7202	-	-
	<i>Geminocystis herdmannii</i> PCC 6308	-	-
	<i>Cyanobacterium aponinum</i> PCC 10605	-	-
	<i>Stanieria cyanosphaera</i> PCC 7437	-	-
	<i>Pleurocapsa</i> sp. PCC 7319	-	-
	<i>Xenococcus</i> sp. PCC 7305	-	-
	<i>Lyngbya majuscula</i> 3L	+	1
	<i>Microcoleus chthonoplastes</i> PCC 7420	+	1
	<i>Microcoleus</i> sp. PCC 7113	+	1
	<i>Crinalium epipsammum</i> PCC 9333	+	1
	<i>Chamaesiphon minutus</i> PCC 6605	-	-
	<i>Geitlerinema</i> sp. PCC 7105	+	1
	<i>Synechococcus elongatus</i> PCC 7942	-	-
	<i>Synechococcus elongatus</i> PCC 6301	-	-
	<i>Prochlorothrix hollandica</i> PCC 9006	+	-
	<i>Leptolyngbya</i> sp. PCC 6406	+	-
	<i>Nodosilinea nodulosa</i> PCC 7104	+	-
	<i>Synechococcus</i> sp. PCC 7335	+	-
	<i>Leptolyngbya</i> sp. PCC 7375	+	4
	<i>Geitlerinema</i> sp. PCC 7407	+	1
	<i>Leptolyngbya boryana</i> PCC 6306	+	1
	<i>Thermosynechococcus elongatus</i> BP-1	-	-
	<i>Synechococcus</i> sp. PCC 6312	-	-
	<i>Cyanothece</i> sp. PCC 7425	-	-
	<i>Acaryochloris marina</i> MBIC11017	-	-
	<i>Acaryochloris</i> sp. CCME 5410	-	-
	<i>Synechococcus</i> sp. PCC 7502	-	-
	<i>Pseudanabaena</i> sp. PCC 6802	-	-
	<i>Pseudanabaena biceps</i> PCC 7429	-	-
	<i>Pseudanabaena</i> sp. PCC 7367	-	-
	<i>Synechococcus</i> sp. JA-3-3Ab	-	-
	<i>Synechococcus</i> sp. PCC 7336	-	-
	<i>Gloeobacter violaceus</i> PCC 7421	-	-

Figure S2. Co-occurrence of *nsiR1* and *hetF*. Genomes are arranged and color-coded as in (26) as follows: filamentous non-heterocystous in green, heterocystous ramified in blue, heterocystous non-ramified in pink, baeocystous in orange and unicellular in black. *Nostoc* sp. PCC 7120 is indicated in red. Presence or absence of a *hetF* homolog is indicated. Presence or absence of homologs of *nsiR1*, number of putative *nsiR1* copies and genomic location of the *nsiR1* arrays upstream to *hetF* (light green) or in a different genomic region (yellow) are also indicated.

Figure S3

A

NsiR1.1 GGUAGAUGCACCUUGAUCUUUAACUCCCCUAGUUGGUUGAUGCCACUGGGGGUUUUUU--
 NsiR1.4 GGUAGAUGCACCUUGAUAACUAACUCCCCUAGCUGGCUAACACCGACUGGGGGCUUUUUUU

B

C

Figure S3. Sequence and structure probing of NsiR1.1 and NsiR1.4. (a) Comparison of the sequences of NsiR1.1 and NsiR1.4. Nucleotide differences are highlighted in red. The region involved in the interaction with *alr3234* mRNA is underlined. (b) Secondary structure model of NsiR1.1 and NsiR1.4. Black and white triangles indicate the positions sensitive to hydrolysis by RNase T1 or lead (II) acetate (Pb), respectively. (c) 5'-end labelled NsiR1.1 (left) or NsiR1.4 (right) were incubated with RNase T1 or lead (II) acetate and the resulting fragments analyzed on a 8% polyacrilamide sequencing gel. C, untreated control; OH, alkaline ladder; T1s, RNase T1 ladder. Nucleotide positions of NsiR1.1 or NsiR1.4 are shown on the left.

Figure S4

Figure S4. Primer extension analysis of the *hetF* transcript. Total RNA was isolated from *Nostoc* sp. PCC 7120 wild type strain (WT) or mutant strain 216 (*hetR*) at different times after removal of combined nitrogen. Primer extension assays were carried out using an oligonucleotide complementary to the 5' UTR of *hetF*. The position of the TSS of *hetF* (4273166f) is indicated with an asterisk. The position corresponding to the TSS of *nsiR1.1* (4273242r) is indicated with a black triangle. On the right, a sequencing reaction performed with the same oligonucleotide used for primer extension is included. The relative position of *hetF* (black arrow) and *nsiR1.1* RNAs (green arrow) is indicated.

Figure S5

Figure S5. Accumulation of *hetR* transcripts in OE_C, OE_NsiR1.1, OE_NsiR1.4 and OE_as_NsiR1 strains. (a) *hetR* expression. Northern blots with RNA from the indicated strains at different time points (indicated in hours) after removal of combined nitrogen were hybridized with probes for *hetR* (top panels) and *rnpB* (bottom panels), used as a loading control. Membranes are the same used in Fig 7. (b) Quantification of *hetR* expression. The data is presented as *hetR/rnpB* expression.