A Heterocyst-Specific Antisense RNA Contributes to Metabolic Reprogramming in Nostoc sp. PCC 7120

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Upon nitrogen deficiency, some filamentous cyanobacteria differentiate specialized cells, called heterocysts, devoted to N₂ fixation. Heterocysts appear regularly spaced along the filaments and exhibit structural and metabolic adaptations, such as loss of photosynthetic CO₂ fixation or increased respiration, to provide a proper microaerobic environment for its specialized function. Heterocyst development is under transcriptional control of the global nitrogen regulator NtcA and the specific regulator HetR. Transcription of a large number of genes is induced or repressed upon nitrogen deficiency specifically in cells undergoing differentiation. In recent years, the HetR regulon has been described to include heterocyst-specific trans-acting small RNAs and antisense RNAs (asRNAs), suggesting that there is an additional layer of post-transcriptional regulation involved in heterocyst development. Here, we characterize in the cyanobacterium Nostoc (Anabaena) sp. PCC 7120 an asRNA, that we call as_glpX, transcribed within the glpX gene encoding the Calvin cycle bifunctional enzyme sedoheptulose-1,7-bisphosphatase/fructose-1,6-bisphosphatase (SBPase). Transcription of as_glpX is restricted to heterocysts and is induced very early during the process of differentiation. Expression of as_glpX RNA promotes the cleavage of the glpX mRNA by RNase III, resulting in a reduced amount of SBPase. Therefore, the early expression of this asRNA could contribute to the quick shut-down of CO₂ fixation in those cells in the filament that are undergoing differentiation into heterocysts. In summary, as_glpX is the first naturally occurring asRNA shown to rapidly and dynamically regulate metabolic transformation in Nostoc heterocysts. The use of antisense transcripts to manipulate gene expression specifically in heterocysts could become a useful tool for metabolic engineering in cyanobacteria.

Keywords: Anabaena • CO₂ fixation • Fructose-1,6-bisphosphatase • RNA regulation • RNase III • Sedoheptulose-1,7-bisphosphatase.

Introduction

Upon nitrogen starvation, filamentous nitrogen-fixing cyanobacteria develop specialized cells called heterocysts, where nitrogen fixation is confined (Flores and Herrero 2010, Muro-Pastor and Hess 2012, Herrero et al. 2016). The differentiation of vegetative cells regularly spaced in the filaments into heterocysts involves extensive structural and physiological changes to facilitate the microaerobic conditions required for efficient N₂ fixation. The structural changes include modifications of the cell wall, with the deposition of a glycolipid layer and a polysaccharide layer outside the outer membrane, generating a barrier to the diffusion of gas, including oxygen (Wolk 2000). Conspicuous reorganization of the thylakoid membranes, concomitant with loss of photosystem II function (and thus oxygen production), as well as increased respiration in the so-called honeycomb membranes contributes to reduced O₂ levels [for a review see Magnuson and Cardona (2016)]. Early studies showing movement of reduced carbon compounds from the vegetative cells into heterocysts suggested that there was no CO₂ fixation in heterocysts (Fay and Walsby 1966, Wolk 1968). In addition, the CO₂-fixing enzyme ribulose-bisphosphate carboxylase/oxygenase (RuBisCO) is not detected in the heterocyst (Codd and Stewart 1977, Cossar et al. 1987, Elhai and Wolk 1990). Phosphoribulokinase, another key enzyme of the Calvin cycle, is also absent in heterocysts (Codd et al. 1980). In the absence of CO₂ fixation, the photoautotrophic metabolism of vegetative cells becomes a heterotrophic metabolism in heterocysts, that rely on carbohydrates imported via specific transporters (Curatti et al. 2006, Ekman et al. 2013, Nürnberg et al. 2015) and primarily catabolized by the oxidative pentose phosphate pathway (Winkenbach and Wolk 1973, Ow et al. 2009). In the nitrogen-fixing filaments, metabolic division of labor is established between vegetative cells and heterocysts, so that both cell types depend on each other and cooperate to achieve growth of the filament as a whole. Vegetative cells maintain photosynthesis and carbon fixation, providing reduced carbon to the heterocyst, while heterocysts provide nitrogen to the vegetative cells (Flores and Herrero 2010, Muro-Pastor and Hess 2012). Most studies analyzing heterocyst development and function have been performed with the model cyanobacterium Nostoc sp. PCC 7120 (thereafter Nostoc 7120, also known as Anabaena sp. PCC 7120). The structural and metabolic changes summarized above, required for differentiation of functional heterocysts, involve extensive modifications of the gene expression program of vegetative cells (Flaherty et al. 2011, Mitschke et al. 2011b, Flores et al. 2018, Herrero and Flores 2019). For instance, a quantitative proteomic study in Nostoc punctiforme (Sandh...
et al. 2014) identified 106 proteins that accumulate at significantly higher levels in heterocysts than in the vegetative filament while 271 proteins had reduced amounts in the heterocyst, among them most of the enzymes involved in CO₂ fixation, in agreement with the loss of CO₂ fixation described above. At the transcriptional level, heterocyst differentiation is not only under control of the global nitrogen regulator NtcA (Frias et al. 1994, Wei et al. 1994), but also depends on HetR, a specific regulator required for cellular differentiation (Buikema and Haselkorn 1991). Global transcriptomic analysis has defined the HetR regulon in Nostoc 7120 (Mitschke et al. 2011b, 2019) that, interestingly, includes several noncoding trans-acting small RNAs (sRNAs) as well as cis-antisense RNAs (asRNAs) expressed from the opposite strand of coding sequences, that have the potential to form extensive base-pairing interactions with the corresponding sense RNA and could therefore be involved in regulation of the heterocyst differentiation process.

In cyanobacteria, transcriptomic analysis with strand-specific information has been performed in several species, including Nostoc 7120 (Mitschke et al. 2011a, Mitsuoka et al. 2011b, Voß et al. 2013, Pfreundt et al. 2014). In all cases, many antisense transcripts are detected, suggesting a major role of antisense regulation in this group of bacteria (Georg et al. 2009, Georg and Hess 2018). In the case of Nostoc 7120, more than 4,000 transcriptional start sites (TSS) leading to antisense transcripts were identified by dRNAseq (Mitschke et al. 2011b). Another RNAseq study in Nostoc 7120 (Flaherty et al. 2011) also described a large set of asRNA, several of them within genes encoding proteins involved in heterocyst differentiation. However, only a few asRNAs have been functionally characterized in cyanobacteria. IsiR overexpression results in the noncoding strand of the isiA (iron stress induced) gene in Synechocystis sp. PCC 6803. IsiR overexpression induces co-degradation of both RNAs, acting therefore as a regulator of the amount of isiA mRNA (Dühring et al. 2006). Another examples from Synechocystis are AsL_flv4, an asRNA that downregulates the flv4-2 operon (Eisenhut et al. 2012), two asRNAs (PsbA2R and PsbA3R) that upregulate psbA2 and psbA3 expression by protecting the mRNA from RNase E cleavage (Sakurai et al. 2012), RhlR, a positive regulator of RuBisCO (Hu et al. 2017), and pilR, a negative regulator of pilA17 that affects pilus formation and motility (Hu et al. 2018). In Nostoc 7120, an asRNA to furA has been described that modulates the amount of FurA, the master regulator of iron assimilation (Hernández et al. 2006).

Regulated transcription of an asRNA strongly suggests a functional regulatory role for such noncoding transcript. Here, we characterize a nitrogen-regulated, heterocyst-specific asRNA from Nostoc 7120 that is transcribed antisense to glpX, the gene encoding the Calvin cycle bifunctional enzyme sedoheptulose-1,7-bisphosphatase/fructose-1,6-bisphosphatase (SBPase), and leads to reduced accumulation of glpX mRNA and SBPase protein. Transcription of as_glpX could contribute to the shut-down of CO₂ fixation in heterocysts and therefore to the metabolic reprogramming taking place in these specialized cells.

Results

Identification of an asRNA with heterocyst-specific expression in the glpX gene

As mentioned above, analysis of the Nostoc 7120 transcriptome by differential RNAseq revealed several HetR-dependent TSS leading to antisense transcripts (Mitschke et al. 2011b). One of them is in the nonsense strand of the glpX gene (gene id: alr1041) (Fig. 1A). This TSS (1212789r) is located at position +825 from the start codon of glpX. Transcription from this TSS would generate an asRNA that we have called as_glpX. Primer extension analysis (Fig. 1B) confirmed that transcription from position 1212789r producing as_glpX is induced upon removal of combined nitrogen in an HetR-dependent manner.

The 3’-end of as_glpX was identified by 3’-RACE. The most abundant product was 458 nt long, ending at genome position 1212332 (Supplementary Fig. S1). Consistent with RNAseq (Mitschke et al. 2011b) and primer extension results (Fig. 1B), RACE products were detected in RNA samples from wild-type cells subjected to nitrogen deficiency for 9 h but no transcripts corresponding to as_glpX were amplified in wild-type ammonium-grown cells or in hetR mutant cells (Supplementary Fig. S1).

The promoter of as_glpX contains in the −35 region the sequence ACCTGA, that resembles the DIF1 motif, TCCGGA (Mitschke et al. 2011b), previously found in the promoter regions of several genes for which heterocyst-specific expression has been demonstrated by fusion to gfp, including those for the major regulators ntcA (Olmedo-Verd et al. 2006) and hetR (Rajagopalan and Callahan 2010), sigma factors sigC (Aldea et al. 2007) and sigA (Muro-Pastor et al. 2017), hetC (Muro-Pastor et al. 2009), alr3808 (Li et al. 2015), or the sRNA NsiR1 (Muro-Pastor 2014) (Fig. 1C). The HetR-dependence of as_glpX transcription and the presence of a DIF1-like motif in its promoter sequence suggested heterocyst-specific transcription of as_glpX. To confirm this hypothesis, we constructed a Nostoc 7120 strain bearing the gfpmut2 gene under the control of the as_glpX promoter (sequence from −184 to +5 with respect to the TSS) (see pELV92 in Fig. 1A). GFP fluorescence was analyzed by confocal fluorescence microscopy in a strain bearing the Pa_glpX::gfp fusion (Fig. 1D). Quantification of GFP fluorescence along the filament (Fig. 1E) demonstrated that transcription from the Pa_glpX promoter took place specifically in heterocysts and could be detected in developing heterocysts at a very early stage of differentiation, even before characteristic signs such as increase in size or reduction of red autofluorescence could be observed (Fig. 1D, E, cell # 2). The early expression during heterocyst development of as_glpX is in agreement with the expression pattern of other genes that contain a DIF1 motif in their promoters, such as ntsR1 (Muro-Pastor 2014) or the sigA and sigC genes (Muro-Pastor et al. 2017).

Effect of as_glpX on glpX and SBPase accumulation

We hypothesized that transcription of as_glpX might affect glpX expression. To test this hypothesis, we analyzed the
Fig. 1 Expression of heterocyst-specific $as_{glpX}$. (A) Schematic representation of the $glpX$-$hemA$ genomic region. Bent arrows indicate the position of TSS identified by dRNAseq in this region (Mitschke et al. 2011b). Open reading frames are shown in gray. The segment encoding $as_{glpX}$ is shown as a white arrow. The fragment fused to $gfpmut2$ (green) in pELV92 (positions 1212972 to 1212784) is indicated by the dotted lines. The position of the oligonucleotide used for primer extension, complementary to positions $+75$ to $+56$ with respect to the TSS of $as_{glpX}$, is indicated with a triangle. (B) Primer extension analysis of total RNA from wild type (WT) and hetR mutant strain (DR884a) with the primer complementary to $as_{glpX}$ shown in (A). Cells were grown in the presence of ammonium (0) or deprived of combined nitrogen for 9 h. White triangle points to the extended product of 75 nt. M, size markers. (C) Sequence upstream of the TSS of $as_{glpX}$ (bold, underlined and marked with a bent arrow), is shown together with those upstream the heterocyst-specific transcriptional starts of $ntcA$, $hetR$, $sigC$, $sigA$, $hetC$, $alr3808$ and NsiR1 (bold, underlined). Sequences matching the TCCGGA consensus for the DIF1 motif are shown in red. (D) Expression of $P_{as_{glpX}}::gfp$ transcriptional fusion in nitrogen-fixing filaments of Nostoc sp. PCC 7120 bearing pELV92. Confocal fluorescence image of a filament growing on top of nitrogen-free solid medium (top, autofluorescence; bottom, GFP fluorescence and autofluorescence merged). Mature heterocysts (# 1, 4 and 6), immature heterocysts (# 3 and 5) and a prospective heterocyst (# 2) are indicated with numbers. (E) Quantification of the signals along the filament in (D) is shown separately for the red autofluorescence (top) and GFP fluorescence (bottom). The position of heterocysts # 1 to # 6 is indicated.
effect of constitutive overexpression of as_glpX on the accumulation of glpX mRNA and SBPase protein. As shown above, as_glpX transcription takes place in heterocysts, that constitute only 10–15% of the cells in a N₂-fixing filament, therefore any effect that as_glpX could have on glpX expression would be restricted to a small percentage of the cells and difficult to detect at the whole-filament level. To overcome this difficulty, we have constructed Nostoc 7120 strains that constitutively express as_glpX in all cells of the filament and irrespective of the nitrogen source (Supplementary Table S1). The 458-bp DNA fragment coding for the transcript identified for as_glpX, as defined above by primer extension and 3'-RACE, that we named glpXₖ, was cloned between the trc promoter and the strong T1 transcriptional terminator (plasmid pELV78), and introduced in Nostoc 7120 (generating strain OE_S). A shorter version of the asRNA (as_glpXₖ), corresponding to the sequence coding for the first 100 nucleotides of as_glpX was also cloned in the same way (plasmid pELV78), and introduced in Nostoc 7120 (generating strain OE_S). As a control, a plasmid bearing no insert between the trc promoter and the T1 transcriptional terminator (plasmid pMBAS1) was introduced in Nostoc 7120 (generating strain OE_C) (Fig. 2A). RNA was extracted from cultures of the three strains (OE_C, OE_S and OE_L) grown in nitrate-containing medium, and analyzed by Northern blot with strand-specific probes (Fig. 2B) for glpX or as_glpX (Fig. 2C). While, as expected, no as_glpX RNA was detected in the control strain (OE_C) in the presence of combined nitrogen, bands of the expected sizes were observed in the OE_S and OE_L strains. The 100 nt short version of as_glpX RNA transcribed in strain OE_S accumulated to much higher levels than the full length as_glpX RNA transcribed in the OE_L strain. Since the constructs introduced in both strains drive transcription of different length versions of as_glpX from the same promoter and integrated stably by homologous recombination with the alpha plasmid of Nostoc 7120, the difference in RNA accumulation could be attributed to differences in the stability of the transcripts. The glpXₖ probe hybridized to a major band of about 1.3 kb that could account for the monocistronic transcript from the 1,038 bp glpX gene and to a fainter band of about 2.5 kb that could correspond to a bicistronic transcript containing glpX and the downstream hemA gene (alr1042), encoding glutamyl-tRNA reductase (GluTR). Both OE_S and OE_L strains accumulated significantly lower amounts of the 1.3 and 2.5 kb transcripts than the OE_C strain, and RNA fragments of smaller size were observed, suggestive of hydrolysis of the mRNA induced by the presence of the asRNA. A hemA specific probe also detected a 2.5 kb band that is reduced in the OE_S strain (Supplementary Fig. S2D). In addition, the same probe detected a 1.5 kb band that was not affected by the expression of as_glpX and that could correspond to transcription from the internal promoters in glpX (Supplementary Fig S2A). The reduction in the amount of glpX transcripts was stronger in the OE_S strain that accumulates higher levels of as_glpX. In agreement with the reduction in the amount of glpX mRNA, reduced amounts of SBPase protein were detected in strains OE_S and OE_L using antibodies raised against Synechocystis sp. PCC 6803 SBPase (Fig. 2C, lower panels). Again, the strain OE_S contained less SBPase protein than the OE_L, consistent with the different amounts of antisense transcripts in these two strains. In the strain OE_S, the presumably bicistronic glpX-hemA transcripts were also barely observed when Northern blots were hybridized to probes for the 3’ of the glpX gene (glpX-3’) or the hemA gene (hemA probe) (Supplementary Fig. S2C, D). Bands of about 1.5 kb were detected with these two probes, likely corresponding to a monocistronic hemA transcript (compare Supplementary Fig. S2B and S2C, D). We therefore conclude that expression of the asRNA as_glpX results in cleavage of the glpX mRNA and leads to reduced amounts of SBPase.

Role of RNase III in the regulation of glpX by as_glpX

RNase III has been extensively identified as the enzyme responsible for the degradation of RNA duplexes generated by overlapping antisense transcription (Lasa et al. 2012). In Nostoc 7120, there are three homologs of RNase III, but only the protein encoded by ORF alr0280 has been shown to have RNase III activity in vitro (Gao et al. 2013). Therefore, we decided to analyze whether Alr0280 was involved in as_glpX-dependent degradation of glpX mRNA. For this purpose, we generated a Δalr0280 strain that lacked a functional Alr0280 protein by deleting an internal fragment containing the RNase III motif from the alr0280 gene (Supplementary Fig. S3). Plasmids pMBAS1 (control), pELV78 (as_glpXₖ), and pELV94 (as_glpXₖ) were introduced in the Δalr0280 strain, generating strains Δalr0280-OE_C, Δalr0280-OE_S and Δalr0280-OE_L, respectively. These strains constitutively expressed a control RNA, the short version and the long version of as_glpX, respectively, in a background lacking the functional RNase III encoded by gene alr0280. Inactivation of alr0280 essentially abolished the observed effect of as_glpX on the accumulation of glpX mRNA and SBPase protein (compare Fig 2C and D), suggesting a direct role of RNase III in the degradation of duplexes involving as_glpX and glpX mRNA. To further verify that alr0280-encoded RNase III was directly related to the instability of glpX mRNA in the presence of as_glpX, we performed genetic complementation of Δalr0280 with the alr0280 gene, generating strain Δalr0280C. Plasmids pELV78, and pELV94 were introduced in the complemented Δalr0280C strain, generating strains Δalr0280C-OE_S and Δalr0280C-OE_L, respectively. Northern blot and Western blot assays confirmed that, in the complemented strains (Fig. 2D), the degradation of glpX mRNA that was induced by as_glpX and the resulting reduction in SBPase amount was similar to those observed in the wild-type background (compare Fig. 2C and D). For the OE_L construct, the amounts of as_glpX appeared reduced in the complemented Δalr0280C background with respect to the mutant Δalr0280 background (about 20% reduction), suggesting coderegulation of both RNAs by RNase III, as expected from the double strand cleavage performed by RNase III. In the OE_S strains, the high accumulation of as_glpXₖ is in too
large excess with respect to glpX mRNA to detect changes in the amount of as_glpX as a result of codegradation of as_glpX/glpX duplex.

**Effect of reduced amount of SBPase on growth and photosynthesis**

SBPase has been identified as a rate-limiting enzyme in the Calvin cycle in plants and cyanobacteria (Ding et al. 2016; Liang and Lindblad 2016; De Porcellinis et al. 2018), therefore we analyzed the impact of reduced levels of this enzyme on growth and photosynthetic activity in Nostoc 7120. Because a stronger effect on SBPase accumulation was observed in the OE_S strain, further experiments were only performed with this strain.

Growth curves were obtained for OE_C and OE_S strains in liquid cultures grown in the presence of nitrate and bubbled...
with 1% CO₂ both under relatively low light intensity (75 μE m⁻² s⁻¹) and under a more demanding high light intensity (400 μE m⁻² s⁻¹). Under low light there were no significant differences in growth rates between both strains (Fig. 3A). However, under high light conditions that promoted faster growth of the control strain, the growth of the strain overexpressing the as_glpX_S was very similar to that under low light conditions at least for the first 70 h after transfer to high light.

Photosynthetic capacity of OE_C and OE_S strains was estimated from oxygen evolution rates at different light intensities (Fig. 3B). While in the OE_C control strain oxygen evolution rates saturated at around 500 μE m⁻² s⁻¹, in the OE_S strain overexpressing as_glpX_S, oxygen evolution rates saturated at significantly lower light intensities (<200 μE m⁻² s⁻¹). At saturating light, oxygen evolution rate in the OE_S strain was about 50% of that observed in the control strain, suggesting a reduced photosynthetic capacity and providing an explanation for the differences in growth rates observed above for both strains under high light.

Discussion

The asRNAs encoded on plasmids, phages and transposons were among the first regular RNAs to be studied, long before chromosomally encoded asRNAs were described (Thomason and Storz 2010). Only with the advent of global approaches to the analysis of bacterial transcriptomes it was widely recognized that a substantial proportion of transcripts constitute asRNAs, and that up to 75% of all genes are associated to asRNAs in certain bacteria (Georg and Hess 2018). Although antisense transcription is widespread in bacteria (Dornenburg et al. 2010, Thomason and Storz 2010, Georg and Hess 2018), it is a matter of debate whether pervasive transcription has a physiological meaning or asRNAs are just the result of noisy transcription (Wade and Grainger 2014). However, evidences for possible regulatory roles of antisense transcription are accumulating (Lasa et al. 2011, Lasa et al. 2012, Lioliou et al. 2012).

In contrast to pervasive, constitutive antisense transcription, regulated transcription of asRNAs and sRNAs points to a functional role of these molecules in the adaptation to certain environmental changes. For instance, in Listeria monocytogenes and Staphylococcus aureus a subset of asRNAs are dependent on the alternative sigma factor SigB, suggesting these transcripts might be functional (Lasa et al. 2011, Wurtzel et al. 2012). Similarly, the stress-related SigB operon in Bacillus subtilis includes 136 putative regulatory RNAs (Mars et al. 2015, Georg and Hess 2018), suggesting a possible role for regulatory RNAs in the transition from standard growth conditions to specific stress conditions. In this context, the observation that the HetR-dependent, nitrogen-regulated transcriptome of Nostoc 7120 includes abundant sRNAs and asRNAs (Mitschke et al. 2011b, Brenes-Alvarez et al. 2019) suggests a possible role of these molecules specifically in the process of heterocyst differentiation and/or function. In fact, several heterocyst-specific nitrogen-stress induced sRNAs have been identified, including NsrR1, an early marker of heterocyst differentiation (Ionescu et al. 2010, Muro-Pastor 2014), or NsrR8 (Brenes-Alvarez et al. 2016). Here, we characterize a heterocyst-specific asRNA, as_glpX. The observation that transcription of as_glpX is regulated in a similar way to that of key elements in the process of differentiation, such as HetR or the heterocyst-specific sigma factor SigC, points to a functional role in the regulation of some process involved in the reprogramming of the vegetative cells becoming heterocysts.

Using a constitutive promoter, our results demonstrate that overexpression of the asRNA as_glpX results in cleavage of the glpX mRNA and leads to reduced amounts of SBPase. Because natural transcription of as_glpX takes place quickly upon initiation of nitrogen deficiency and specifically in heterocysts, we conclude that its regulatory effects will be restricted to differentiating cells, therefore contributing to the metabolic transformation of vegetative cells into heterocysts as summarized in the model (Fig 4). In fact, in a proteomic study carried out in the filamentous heterocyst-forming cyanobacteria N. punctiforme, SBPase is the protein whose amount is more strongly
reduced in heterocysts with respect to vegetative cells (log₂ ratio = −5.36) (Sandh et al. 2014). This observation suggests that besides the inactivation of CO₂ fixation and lack of RuBisCO expression, suppression of SBPase activity is also a critical metabolic adaptation taking place in the heterocysts. The physiological characterization of the strain that overexpresses as_glpX_S demonstrates that the reduced amounts of SBPase in the OE_S strain becomes limiting for photosynthetic activity and growth under high light conditions. This observation further supports the hypothesis that the regulation of the levels of SBPase by as_glpX specifically in heterocysts might in fact contribute to the reduced photosynthetic activity in these cells.

It has been discussed that post-transcriptional regulation by an antisense transcript might be an easy way to fine-tune gene expression under specific environmental conditions, or perhaps in specific cell types in the case of heterocystous cyanobacteria, without altering the promoter of the sense transcript, that eventually integrates other signals (Brophy and Voigt 2016). as_glpX is the first HetR dependent, heterocyst-specific asRNA whose function is described. Participation of heterocyst-specific regulatory transcripts (both sRNAs and asRNAs) in the post-transcriptional control of gene expression would represent an additional level of control of a process that leads to the complete structural and metabolic transformation of only certain cells of cyanobacterial filaments. In addition, since as_glpX RNA induces degradation of glpX mRNA, the kinetics of inhibition of protein accumulation would be much quicker that could be achieved by transcriptional repression only.

The interest in the use of cyanobacteria as factories for the production of useful metabolites has driven the development of RNA tools for metabolic engineering in Nostoc sp. PCC 7120 (Higo et al. 2017) and the manipulation of gene expression specifically in heterocysts (Higo et al. 2018). The natural occurrence of heterocyst-specific antisense regulatory transcripts such as as_glpX points to asRNAs as a useful addition to these tools.

Materials and Methods

Strains and growth conditions

Nostoc sp. PCC 7120 and derivative mutant strains (Supplementary Table S1) were grown photoautotrophically at 30°C in BG11 (Rippka et al. 1979) (containing 17.6 mM NaNO₃), BG11₀ (free of combined nitrogen), or BG11₀ plus ammonium (BG11₀ containing 4 mM NH₄Cl and 8 mM N-[tri(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES)-NaOH buffer, pH 7.5) under continuous illumination (40–75 µM m⁻² s⁻¹) with white LED light in liquid cultures bubbled with CO₂-enriched air (1% v/v) or in medium solidified with 1% Difco agar. Liquid cultures bubbled with CO₂-enriched air were buffered with 10 mM NaHCO₃ (BG11C). Nitrogen deficiency was induced as described (Brenes-Álvarez et al. 2016). Mutant strains were grown in the presence of appropriate antibiotics at the following concentrations: streptomycin (Sm), 2–3 µg/ml each (liquid medium) or 3–5 µg/ml each (solid medium), neomycin (Nm), 5 µg/ml (liquid medium) or 25 µg/ml (solid medium).

To test growth of OE_C and OE_S strains under different light intensities, BG11C liquid bubbled cultures grown at 75 µM m⁻² s⁻¹ were diluted to A₇₁₀ 0.05, split into two, and one was maintained at 75 µM m⁻² s⁻¹ (low light) and the other was incubated at 400 µM m⁻² s⁻¹ (high light). 0.25 ml samples were taken at different times and the A₇₁₀ determined in 1 ml suspensions containing 25% glycerol.

Escherichia coli strain DH5α was grown in LB medium supplemented with appropriate antibiotics.

Strains construction

Plasmids used in this work are described in Supplementary Table S2, and oligonucleotides used for PCR and other purposes are detailed in Supplementary Table S3. Plasmids were introduced into E. coli by transformation and into Nostoc 7120 by conjugal transfer.

To generate a vector for the expression of a fusion between the as_glpX promoter and a promoterless gfpmut2 gene in Nostoc 7120, the promoter region (~184 to +5 with respect to the TSS of as_glpX) was amplified with primers #712 and #713 and ligated to Clal-XhoI digested pSAM270 (Supplementary Table S2), generating plasmid pELV92.

Plasmids for the constitutive expression of long and short versions of as_glpX in Nostoc 7120 were constructed based on vectors pMBA20 and pMBA37 (described below) previously designed for constitutive expression of a segment of the Nostoc 7120 chromosome, which is here replaced by the segment encoding as_glpX. These vectors contained either the rnap promoter (pMBA20) from Nostoc 7120 (Vioque 1992) or the trc promoter (pMBA37).
overlapping fragments were amplified by PCR using as template pMBA20 with rnpB promoter and the T1 terminator was generated in the following way. Two terminator were constructed as controls. Plasmid pMBA50, containing only the rnpB in the fusion of the fragments corresponding to the /C1 and /C1, respectively, using as template pMBA37. The resulting products were used in an overlapping PCR with oligonucleotides #501 and #504, rendering fragment 3. The T1 terminator was amplified using as template pJE12luc (Urban and Vogel 2007) and oligonucleotides #505 and #506, generating fragment 4. Finally, fragments 3 and 4 were used as templates in an overlapping PCR with oligonucleotides #501 and #506, rendering fragment 5. After digestion with Clal and SacI at the sites provided by oligonucleotides #501 and #506, fragment 5 was cloned into Clal-Sacl digested pSAM303 vector (see Supplementary Table S2) rendering pMBA20. To generate plasmid A37, the rnpB promoter in pMBA20 was replaced by the trc promoter. The trc promoter was amplified from plasmid pTrc99A (Aumann et al. 1998) with oligonucleotides #573 and #574 and, after digestion with Clal and NsiI at the sites provided by oligonucleotides #573 and #574, the fragment was cloned into Clal-NsiI digested vector pMBA20 rendering pMBA37. pMBA37 contains the same Nostoc 7120 chromosome as pMBA20 flanked by unique NsiI and XhoI sites between the trc promoter and the T1 terminator. A fragment corresponding to the 458 bp full length as_glxX sequence was amplified with oligonucleotides #624 and #821, digested with NsiI and XhoI and cloned into pMBA37 digested with NsiI and XhoI, rendering pELV94. Similarly, the first 100 bp of as_glxX were amplified with oligonucleotides #624 and #625 and cloned into pMBA37 digested with NsiI and XhoI, rendering pELV78. pELV94 was introduced by conjugation in Nostoc 7120 and its derivative strains Δalr0280 and Δalr0280Δ (see below for Δalr0280 and Δalr0280Δ construction), generating strains OE_L, Δalr0280–OE_L and Δalr0280Δ–OE_L, respectively. pELV78 was introduced by conjugation in Nostoc 7120 and its derivative strains Δalr0280 and Δalr0280Δ, generating strains OE_S, Δalr0280–OE_S and Δalr0280Δ–OE_S, respectively. Plasmids containing only the promoter (rnpB or trc) and the T1 terminator were constructed as controls. Plasmid pMBA50 containing only the rnpB promoter and the T1 terminator was generated in the following way. Two overlapping fragments were amplified by PCR using as template pMBA20 with oligonucleotides #501 and #509 and oligonucleotides #600 and #506. The resulting products were used for another PCR with oligonucleotides #501 and #506 resulting in the fusion of the fragments corresponding to the rnpB promoter and the T1 terminator. After digestion with Clal and SacI at the sites provided by oligonucleotides #501 and #506 this fragment was cloned into Clal-Sacl digested vector pMBA20 rendering pMBA50. Plasmid pMBA51, containing only the trc promoter and the T1 terminator, was generated in the following way. Two overlapping fragments, containing both the trc promoter and the T1 terminator were amplified by PCR with oligonucleotides #573 and #601 and oligonucleotides #602 and #506, respectively, using as template pMBA37. The resulting products were used for another PCR with oligonucleotides #573 and #506 resulting in the fusion of the fragments. After digestion with Clal and SacI at the sites provided by oligonucleotides #573 and #506 this fragment was cloned into Clal-Sacl digested pMBA37, rendering pMBA51. Plasmid pMBA51 was introduced by conjugation in Nostoc 7120 wild type and Δalr0280 generating strains OE_C and Δalr0280–OE_C, respectively.

**Construction of RNase III mutant and complemented strains**

The RNase III gene (alr0280) was inactivated by deletion of an internal sequence of 378 bp that encodes the RNase III motif (Supplementary Fig. S3). Using genomic DNA as template, two overlapping fragments were amplified by PCR with oligonucleotides #592 and #594, and #593 and #595, respectively. The resulting products were used as templates for a third PCR with primers #592 and #593, resulting in the fusion of both fragments and the deletion of the sequence encoding amino acids 27 to 159 of Alr0280. The fragment was digested with BamHI and cloned into BamHI-digested pCSRO (Merino-Puerto et al. 2010), producing pELV74, which was transferred to Nostoc 7120 by conjugation with selection for resistance to Sm and Sp. Cultures of the exconjugants obtained were used to select for clones with the alr0280 gene replaced by the mutant version by selection for resistance to sucrose (Cai and Wolk 1990), and individual sucrose resistant colonies were checked by PCR (Supplementary Fig S3B). The resulting mutant strain was called Δalr0280.

To genetically complement the Δalr0280 strain, a DNA fragment containing the alr0280 gene and flanking sequences (595 bp upstream and 113 bp downstream) was amplified with primers #716 and #717 and ligated to BamHI-digested pRL424, generating plasmid pELV89, that was transferred to strain Δalr0280 by conjugation. pELV89 was integrated in the alr0280 genomic region yielding a strain resistant to Nm that contains a wild-type copy of alr0280 (Δalr0280::strain; Supplementary Fig S3A).

**RNA isolation, Northern blot, primer extension and 3’ RACE**

Total RNA was isolated using hot phenol as described (Mohamed and Jansson 1989) with modifications (Brenes-Alvarez et al. 2016). For Northern blot, RNA (7.5 μg) was separated on 1% agarose denaturing formaldehyde gels, transferred to Hybond-N+ membrane (GE Healthcare, Chicago, IL, USA) with 20x SSC buffer and hybridized at 65°C according to the manufacturer’s recommendations with single stranded 32P-labeled probes. The single stranded probes were prepared by asymmetric amplification in the presence of [α-32P]dCTP with one single primer (primer #259 for glpX-S probe; primer #602 for glpX-S probe; primer #694 for hemA probe; primer #346 for as_glxX) using as templates PCR fragments obtained with the primers indicated in Supplementary Table S3. The position of the probes used is shown in Fig. 2B and Supplementary Fig S2A. As a control for RNA loading and transfer efficiency, the filters were hybridized with a probe of the rnpB gene (Vioque 1992).

Primer extension analyses of 5'-ends were performed as previously described (Muro-Pastor et al. 1999) with 5 μg of total RNA and oligonucleotide #346 labeled with [γ-32P]ATP. 3' RACE assays were performed essentially as described (Ionescu et al. 2010), with 6 μg of dephosphorylated total RNA (RNAsin Alkaline phosphatase, Roche, Basel, Switzerland). The 3'-RNA-Adapter (50 pmol) was ligated to the Nostoc 7120 total RNA at 25°C for 2 h with T4 RNA ligase (New England Biolabs, Ipswich, MA, USA). After phenol/chloroform extraction and ethanol precipitation, the RNA was reverse-transcribed with Superscript III (Thermo Fisher, Waltham, MA, USA; 200 U) using 10 pmol of primer #281, complementary to the 3'RNA-adapter. The products of reverse transcription were amplified by PCR using MyTag DNA Polymerase (Bioline, London, UK) with primers #282 (nested to #281) and gene-specific primer #523 using 1 μl of the reverse transcription reaction as template. PCR products were cloned into pMBL-T cloning vector (Canvac Biotech, Córdoba, Spain) and 12 clones sequenced.

**Fluorescence microscopy**

Fluorescence of Nostoc 7120 filaments carrying plasmid pELV92 (P_glpX::gfp), growing on top of solidified nitrogen-free medium, was analyzed and quantified as described (Muro-Pastor 2017) using a Leica DMLB-AOPLAF (magnification ×14 NA 0.12). Emmission objective attached to a Leica TCS SP2 confocal laser-scanning 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 (cyanobacterial autofluorescence).

**Cell fractionation and Western blotting**

Crude extracts were prepared using glass beads. Cells from 20 ml cultures grown to 4–5 μg Chl/ml were harvested by filtration, washed with 50 mM Tris-HCl buffer (pH 7.5) 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, Saint Louis, MO, USA, 212–300 μm) and subjected to seven cycles of 1 min vortexing followed by 1 min of cooling on ice. Cell extract was separated from cell debris and unbroken cells by centrifugation (3 min at 3,000 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 Bradford procedure (Bradford 1976).

For Western blot analysis, 30 μg of proteins from soluble fraction were fractionated on 10% SDS/PAGE, transferred to nitrocellulose membranes (Transblot Turbo RTA Transfer Kit, BioRad, Hercules, CA, USA) and immunoblotted with antibodies against Synechocystis sp. PCC 6803 sedoheptulose-1,7-bisphosphatase. The ECL Plus immunoblotting system (GE Healthcare, Chicago, IL, USA) was used to detect the different antibodies, using anti-rabbit (Sigma-Aldrich, Saint Louis, MO, USA) horseradish peroxidase conjugated secondary antibodies (1:20,000). Ponceau Red staining was used as loading and transfer control.
Oxygen evolution

Oxygen evolution at different light intensities was measured at 30°C in a Clark-type oxygen electrode (Hansatech Chlorolab 2, Norfolk, UK) with 2 ml of a suspension of cells (4.5–5 μg Chl a/ml) that had been grown in BG11C medium at a light intensity of 75 μE m⁻² s⁻¹. Chlorophyll content was determined from methanolic extracts as described (MacKinney 1941).

Supplementary Data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

References


