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Identification of small RNAs involved in nitrogen fixation in *Anabaena* sp. PCC 7120 based on RNA-seq under steady state conditions

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Abstract

Purpose: *Anabaena* sp. PCC7120 is a genetically tractable model organism for nitrogen fixation and photosynthesis research. The importance of small regulatory RNAs (sRNAs) as mediators of a number of cellular processes in bacteria has begun to be recognized. Bacterial sRNA binds to target genes through base pairing, and play a regulatory role. Many studies have shown that bacterial sRNA can regulate cell stress response, carbon and nitrogen fixation, and so on. However, little is known about sRNAs in *Anabaena* sp. PCC 7120 regarded to nitrogen fixation under later steady state.

Methods: To provide a comprehensive study of sRNAs in this model organism, the sRNA (< 200 nt) extracted from *Anabaena* sp. PCC 7120 under nitrogen step-down treatment of 12 days, together with the sRNA from the control, was analyzed using deep RNA sequencing. Possible target genes regulated by all identified putative sRNAs were predicted by IntaRNA and further analyzed for functional categorizations for biological pathways.

Result: Totally, 14,132 transcripts were produced from the de novo assembly. Among them, transcripts that are located either in the intergenic region or antisense strand were kept, which resulted in 1219 sRNA candidates, for further analysis. RPKM-based differential expression analysis showed that 418 sRNAs were significantly differentially expressed between the samples from control (nitrogen addition, N+) and nitrogen depletion, (N-). Among them, 303 sRNAs were significantly upregulated, whereas 115 sRNAs were significantly downregulated. RT-PCR of 18 randomly chosen sRNAs showed a similar pattern as RNA-seq result, which confirmed the reliability of the RNA-seq data. In addition, the possible target genes regulated by unique sRNAs of *Anabaena* sp. PCC 7120 under nitrogen addition (N+) condition or that under nitrogen depletion (N-) condition were analyzed for functional categorization and biological pathways, which provided the evidences that sRNAs were indeed involved in many different metabolic pathways.

Conclusion: The information from the present study provides a valuable reference for understanding the sRNA-mediated regulation of the nitrogen fixation in *Anabaena* PCC 7120 under steady state conditions.

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Introduction

Cyanobacteria are a group of photoautotrophs originated nearly 3.5 billion years ago, during which they have evolved a diverse array of metabolic capabilities (Carr and Whitton 1973; Herrero and Flores 2008; Kaushik et al. 2016; Srivastava et al. 2017). They have been studied as prokaryotic hosts for sustainable biofuel production due to their high photosynthetic efficiency, genetic manipulability, and diverse metabolic pathways (Dismukes et al. 2008; Ducat et al. 2011; Halfmann et al. 2014b; Halfmann et al. 2014a; Chen et al. 2015; Burnat et al. 2018; Singh et al. 2019; Ishikawa et al. 2019). Some filamentous cyanobacteria, including *Anabaena* PCC strain 7120, are able to differentiate heterocyst to fix atmospheric dinitrogen in response to nitrogen deficiency (Flores and Herrero 2010; Kumar et al. 2010; Xu et al. 2015; Esteves-Ferreira et al. 2018; Olmedo-Verd et al. 2019). Understanding of the underlying mechanisms of nitrogen fixation and diazotrophic growth in cyanobacteria will shed light on basic mechanisms of bacterial genetic regulation and physiology character. Besides, it can help the catalog of genetically modified cyanobacterial strains to increase and produce renewable chemicals and biofuels.

In *Anabaena* PCC 7120, 5 to 10% vegetative cells along the filament differentiate into heterocysts in a semi-regular pattern after deprivation of combined nitrogen (Maldener and Muro-Pastor 2001). Metabolite exchange occurs between the heterocysts and neighboring vegetative cells, that is, vegetative cells supply fixed carbon, such as sucrose, to adjacent heterocysts, while heterocysts provide vegetative cells with fixed nitrogen in the form of amino acids (López-Igual et al. 2010; Vargas et al. 2010; Brenes-Álvarez et al. 2019). It is known that the heterocysts differentiate from vegetative cells, involving substantial changes in cell morphology and physiology that result in a micro-oxic environment for the expression and function of oxygen-sensitive nitrogenase (Wolk et al. 2004; Flores and Herrero 2010). The development of heterocysts is mainly regulated by two transcription factors, NtcA and HetR (Buikema and Haselkorn 1991; Herrero et al. 2004; Zhao et al. 2010; Chen et al. 2011; Herrero et al. 2013). Deprivation of combined nitrogen leads to the accumulation of 2-oxoglutarate which is the backbone for nitrogen assimilation (Laurent et al. 2005). The increased cellular levels of 2-oxoglutarate in consequence activates NtcA to initiate the heterocysts differentiation (Zhao et al. 2010). The *hetR* gene is required for an early step of heterocyst differentiation, whenever under N⁻ conditions or N⁺ conditions (Buikema and Haselkorn 1991).

Small regulatory RNAs (sRNAs) are known to be key genetic regulators in many organisms including bacteria, plants, and animals (Babski et al. 2014; Morris and

Mattick 2014; Higo et al. 2017; Lambrecht et al. 2018). They perform many important regulatory functions, such as in stress response, regulation of virulence genes, carbon source uptake, and metabolism (Gottesman 2005; Gottesman and Storz 2011). In bacteria, sRNAs typically range from 50 to 200 nt in length (Storz et al. 2011), and are often encoded in intergenic regions in trans to their target genes or in cis on the opposite strand of their target gene (Babski et al. 2014). The former ones usually interact with their target mRNAs near the ribosomal binding site (RBS) through imperfect base-pairing. The latter ones have full complementarity to their target mRNA. The interactions between sRNA and its targeted mRNA can result in gene translational repression by masking the RBS or activation by making the RBS accessible, as well as affecting mRNA degradation and mRNA stability (Storz et al. 2011; Babski et al. 2014).

sRNAs in cyanobacteria are frequently implicated in photosynthesis, stress responses, and nitrogen fixation (Nakamura et al. 2007; Hernández et al. 2010; Ionescu et al. 2010; Gong and Xu 2012; Georg et al. 2014; Muro-Pastor 2014; Klähn et al. 2015; de Porcellinis et al. 2016; Higo et al. 2018). For example, in *Synechocystis* sp. PCC 6803, the small RNA PsrR1 is a regulatory factor controlling photosynthetic functions. In filamentous cyanobacterium *Anabaena* PCC 7120, *furA*, the gene for the ferric uptake regulator, is interfered by a cis-acting antisense RNA whose knockout mutation results in an iron deficiency phenotype (Hernández et al. 2006; Hernández et al. 2010). In unicellular cyanobacterium *Synechocystis*, the antisense RNA IsrR regulates the expression of iron stress-induced protein IsiA in a co-degradation mechanism under iron stress (Dühning et al. 2006).

Examples of sRNA involved in nitrogen fixation in cyanobacterium are nitrogen stress-induced RNAs (NsiR). NsiR1 is the first known bacterial non-coding RNA specifically upregulated in response to nitrogen step-down, and its expression requires NtcA and HetR (Ionescu et al. 2010; Mitschke et al. 2011a), it has been suggested to be an early marker for cells undergoing differentiation (Muro-Pastor 2014). NsiR2 and NsiR3 that were identified in a differential RNA-seq analysis to *Anabaena* PCC 7120 annotated an unknown function (Mitschke et al. 2011a). NsiR4 was firstly found to be strongly upregulated in *Synechocystis* sp. PCC 6803 under nitrogen depletion (Mitschke et al. 2011b; Kopf et al. 2014). Later, the ortholog in *Anabaena* PCC 7120 was found to play an important role in the regulation of glutamine synthetase, a key enzyme in biological nitrogen assimilation (Klähn et al. 2015). Recently, using the Northern blot, a study showed that NsiR8 and NsiR9 were strongly induced in the WT control, but not in a HetR mutant subjected to a 24-h nitrogen step-down

treatment, suggesting they could be related to heterocysts differentiation or function (Brenes-Álvarez et al. 2016).

Although many sRNAs related with nitrogen fixation have been identified in *Anabaena* PCC 7120 using RNA sequencing-based approaches (Flaherty et al. 2011; Mitschke et al. 2011a). They were all identified from cells at the early stage (1–24 h) of nitrogen depletion. However, up to now, there has not been any report on the characterization of the sRNA regulation profiles in *Anabaena* PCC 7120 under steady state after nitrogen step-down. In this study, we have employed the RNA-seq method to analyze the sRNA transcripts (< 200 nt) in *Anabaena* PCC 7120 after 12 d treatment of nitrogen step-down. By applying this approach, 1219 sRNA candidates were identified. Among them, 1124 were shared by the control of nitrogen addition (N+) and the treatment of nitrogen depletion (N-); 46 ones were unique in the N- group and 49 in the control group. Possible target genes regulated by all identified putative sRNAs were predicted by IntaRNA and further analyzed for functional categorizations for biological pathways. Overall, our results provide a new insight towards understanding the complex regulatory network of sRNAs in *Anabaena* sp. PCC 7120 after combined nitrogen depletion under steady state conditions.

Materials and methods

Strains, growth conditions, and sample preparations

Anabaena PCC 7120 was grown in AA/8 medium with KNO₃ and NaNO₃ as the combined nitrogen source (Allen and Arnon 1955) and incubated at 30 °C with shaking at 120 rpm in a shaker under continuous white-light (ca. 50 µE m⁻² s⁻¹) illumination. To conduct the nitrogen step-down treatment, *Anabaena* PCC 7120 grown in AA/8(N+) medium were collected by centrifugation at 5000 × *g* for 10 min and washed 3 times in AA/8 nitrate free medium AA/8(N-). Then cells were resuspended and grown in 300-ml AA/8(N-) medium until OD₇₀₀ was around 0.5. The nitrogen step-down treatments were repeated 4 times. After culturing 12 d, cells were harvested by centrifugation at 5000 × *g* for 10 min, cell pellets were quickly frozen in liquid nitrogen and stored at - 80 °C for later use. The control group was prepared similarly as the nitrogen-deprived samples except grown in AA/8(N+) medium.

RNA isolation and sequencing

Total RNA from *Anabaena* sp. PCC 7120 strain samples cultured on media of AA/8(N+) and AA/8(N-) were separately extracted using TRIzol® reagent (INVITROGEN, Carlsbad, CA, USA). The *Anabaena* sp. PCC 7120 strain samples above cultured on media of AA/8(N+) and AA/8(N-) were mixtures of three repeated samples.

RNA quantity and quality including concentration, RIN value, and 23S/16S ratio were assessed using Agilent 2100 Bioanalyzer (Agilent Technologies). Samples with high quality of 260/280 and 260/230 ratios were selected for Illumina sequencing library construction. RNA samples were treated with DNase I (Promega), and 3 µg of total RNA from each sample was electrophoresed by agarose gel electrophoresis for RNA fractionation, then the electrophoretic bands of 18–200 nt RNA segments were excised from the PAGE gel. The library was constructed according to Illumina TruSeq™ Small RNA Sample Preparation protocol. The sequencing run was conducted on an Illumina HiSeq™ 2000 platform at the Beijing Genome Institute, Shenzhen, China. The deep sequencing data have been submitted to the NCBI Sequence Read Archive with the accession number SUB1682364.

Sequence data processing, assembly, and annotation

The raw reads generated from the high-throughput sequencing were firstly cleaned by removing adaptor sequences. Then sequences were filtered off based on low quality value (Q_{20} ratio > 40%) and the high *N* numbers in the reads (> 10%). Then, all the clean reads from all replications were merged and subjected to de novo assembly using the Trinity program (Haas et al. 2013). The assembled transcripts were mapped onto the *Anabaena* sp. PCC 7120 genome and its six megaplasmids using BWA (Li and Durbin 2009). For the annotation of candidate sRNAs, we searched databases of sRNAMap (Huang et al. 2009), sRNATarBase (Cao et al. 2010), SIPHI (<http://newbio.cs.wisc.edu/sRNA/>), and BSRD (Li et al. 2012) based on the similarity of sequences using Blast (*e* value < 0.00001).

Identification of differentially expressed sRNA

The RPKM method (reads per kilo base per million mapped reads) was used for length normalization and calculation of the transcript expression levels (Mortazavi et al. 2008). An FDR (false discovery rate) of < 0.001 was used as the threshold *p* value in multiple tests to judge the degree of differences in gene expression (Reiner et al. 2003). In this study, the sRNA differential expression between two groups was considered when the *p* value was less than 0.001 and the expression level was at least a two-fold change between the two groups.

Target prediction

To obtain the target information, we searched the target genes of all the putative sRNAs by using the program IntaRNA (Busch et al. 2008; Wright et al. 2014) to take the sRNAs as query with default parameters. GO annotations with the default parameters were performed with the functional annotation tool WEIGO (Ye et al. 2006).

All putative sRNA/target pairs were subsequently filtered by correlation analysis of the sRNA and transcript expression. Only sRNA/target pairs with strong negative correlation were retained as putatively sRNA regulated transcripts.

qRT-PCR validation

Total RNA was treated with RNase-free DNase I (FERMENTAS, Life Sciences, Germany), then it was used as a template to synthesize first-strand cDNA using a One Step SYBR PrimeScript RT-PCR kit (TAKARA) following the manual. We selected 18 differentially expressed sRNA for qRT-PCR analysis to evaluate our Illumina sequencing result. The sRNA primers were designed using Primer 5 and listed in Table 1. The expression level of the 18 sRNA was analyzed by using the comparative CT method ($2^{-\Delta\Delta CT}$ method). The housekeeping gene *rrn16Sa* in *Anabaena* sp. PCC7120 was used as the internal control for normalization (Pinto et al., 2012), and used the Mix of AceQ™ qPCR SYBR® Green Master to establish the qRT-PCR reaction system. qRT-PCR reactions were carried out by using BIO-RAD CFX96 in three biological repeats for every treatment. Primers, reaction systems, and programs are listed in Additional file. GraphPad Prism 6 was applied to analysis and construction.

Results

Strain growth observations on different media

When cultured on different media, filament cells of *Anabaena* sp. PCC 7120 showed different growth, heterocysts differentiated directly on AA/8(N-) medium (Fig. 1a, c) after 3 d culturing; however, only the vegetative cells grown on AA/8(N+) medium (Fig. 1b, d). It is obvious that nitrogen step-down of the medium induced the heterocysts differentiation.

sRNA assembly and functional annotation

To comprehensively identify sRNA involved in nitrogen fixation at a steady state, we compared the sRNAs profile (18–200 nt) of *Anabaena* sp. PCC 7120 grown on media of AA/8(N+) and AA/8(N-) after 12 d nitrogen step-down treatment. A total of 13,219,856 clean reads were obtained from the control group (N+), and 13,778,584 reads from the nitrogen-depleted group (N-); while $Q \geq 20$. Totally, 14,132 transcripts were produced from the de novo assembly. These assembled transcripts were then mapped onto the *Anabaena* sp. PCC 7120 genome (92.96%) and its 6 megaplasmids (7.04%). The mapped transcripts were classified into 4 groups: group I contains sRNAs that were partially overlapped to mRNA (PM, 6.68%); group II comprises intergenic sRNAs that were mapped to an intergenic region (IGR, 24.24%); group III were referred to as antisense sRNAs which

Table 1 Primers used in this study

sRNA/gene	Primers	Sequences(5' to 3')
sRNA0246	HEP271	CTATTTAGGCAGACTGAACGACA
	HEP272	ATTGCTGCTGCTTGCGTCTA
sRNA0320	HEP299	ATCGAAACATCCGTTGTACAT
	HEP300	AGTAAAGCTTAAGCATTGCTCAGA
sRNA0411	HEP303	TTGTAGAATCCGATGAACAATAG
	HEP304	GTTAAGATTTAACCTCACATAGCTG
sRNA0423	HEP257	TTGCAGCGAGTGTTATAAGC
	HEP258	GGTATGGAGTCGCTACGAGTTC
sRNA0468	HEP305	CTTCGCCATAACTTAAGAATTTT
	HEP306	TAGAAAACCTGATGTGTTTATGTC
sRNA0530	HEP265	TTTTGTGTTCCCTGTAGTTCA
	HEP266	GGGCATGAGGGAGAGTAATC
sRNA0580	HEP267	AGGATTACGCAGAGATTC
	HEP268	AAGAAGTCCCCCTAGTT
sRNA0645	HEP261	ATTTTCCGCTTAATCAACACC
	HEP262	AATAAATCACCAGGGCGGAG
sRNA0703	HEP301	AGTTGCCAATTAAGGGACTGTAGTT
	HEP302	GAACGGGACTGACGGGGC
sRNA1133	HEP259	AACGCGCGTGACAACAGG
	HEP260	GGTACTCCCGATAAATCCCA
sRNA0092	HEP313	TTTCGTTTTTACAACCTTTTCCA
	HEP314	GGGGCAGTTTAGCACGGT
sRNA0188	HEP307	CATTTAACAGTTTGCATCACA
	HEP308	TAGTCTGTAGTTGGTAGCCC
sRNA0410	HEP283	TCGCTCAAATACTCAAACCTGG
	HEP284	GTTTTGGGTGTGTTTCGTAGAC
sRNA0699	HEP311	AATGTTTGATAGAGATGATATAGAT
	HEP312	GCAAGAAATCAAAGGGATCA
sRNA0987	HEP309	TCTTGGAACCACTAGGATTCTG
	HEP310	GGAAGATACTCATGTTCAACG
sRNA1073	HEP287	CCGAGACTGAGATACAAAAATTC
	HEP288	AATCATTTCAACTCGCTCTTTTAG
sRNA1153	HEP295	T ATATTAACGAGGTAGTGCTGT
	HEP296	CCCCTACGGTACATCCTTA
sRNA1169	HEP285	ATTGGGGCTAGGGATTGG
	HEP286	GGGACTGGGGATTGGGAA
<i>rrn16sa</i>	HEP319	ACAATGCTACGGACAGAGGGC
	HEP320	CCTTCACGCAGGCGAGTT

were located antisense to known genes (AM, 29.76%); and group IV were those located within mRNAs (IM, 39.32%) (Fig. 2). Transcripts that were placed into either group II or III, with a total expression both in N+ and N- media bigger than 20 were kept as candidate sRNAs; thus, a total of 1219 candidate sRNAs were selected for further analysis

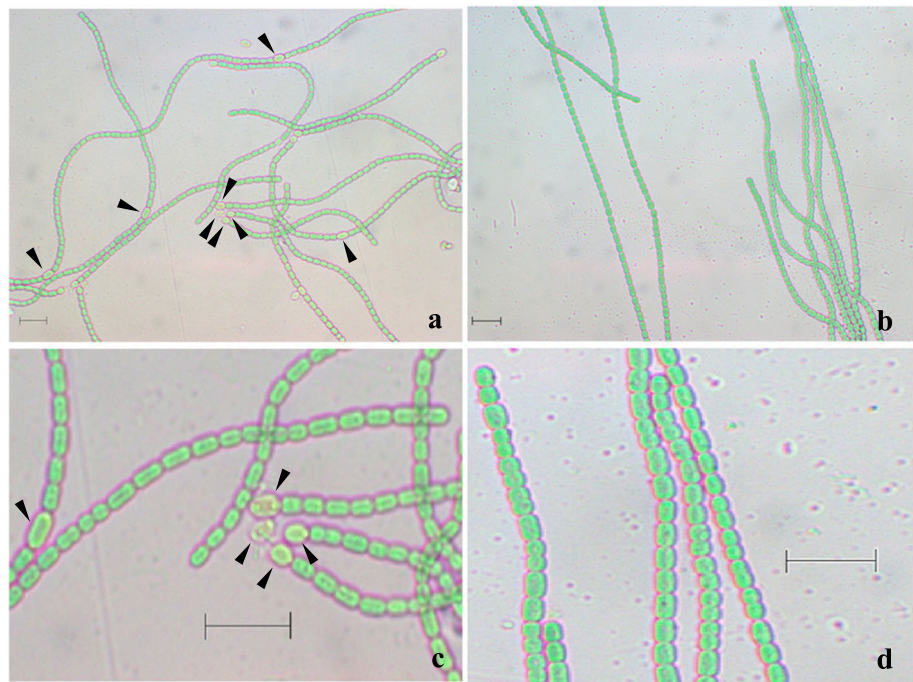


Fig. 1 Filamentous cells of *Anabaena* sp. PCC 7120 on different media after 3 d culturing. **a** and **c** Show the cells on AA/8(N[−]) medium, arrow heads indicate the heterocysts. **b** and **d** Show the vegetative cells on AA/8(N⁺) medium. Bars = 10 μm

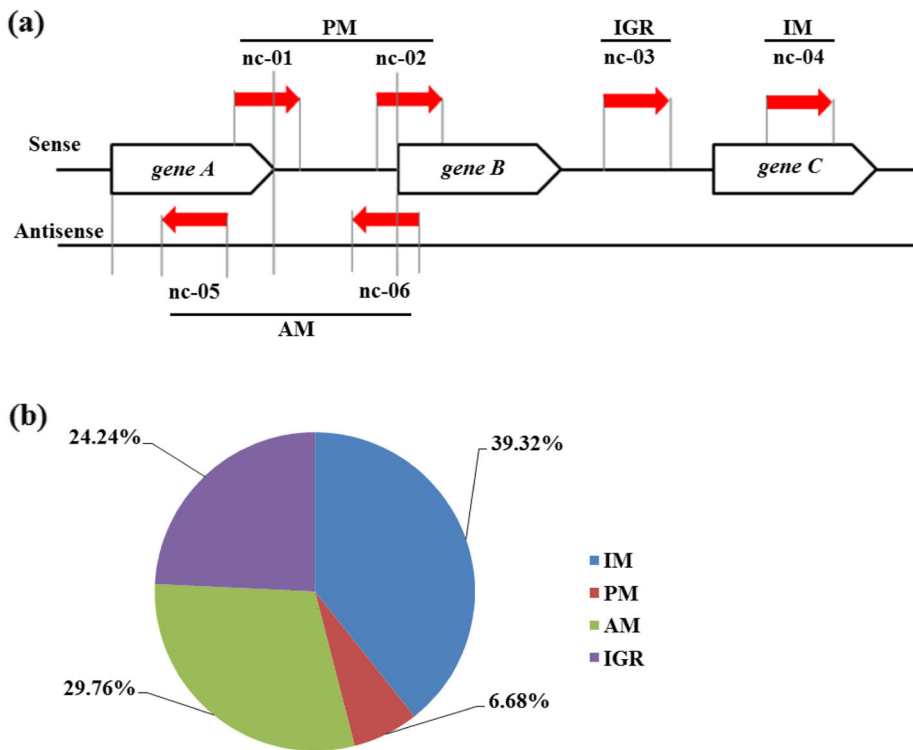


Fig. 2 **a** Illustration diagram of the four classes of assembled transcripts. *PM* partially overlap to mRNA, represented by nc-01, nc-02; *IGR* intergenic region, represented by nc-03; *IM* included in mRNA, represented by nc-04; *AM* antisense to mRNA, represented by nc-05, nc-06. **b** Pie chart clarifying the sRNA transcripts. The percentages of all the deep sequencing transcripts mapped to the previously annotated or non-annotated regions of the *Anabaena* sp. PCC 7120 genome

(supplementary Table 1). It should be noted that 157 candidate sRNAs were matched to the registered sRNAs by searching the databases of sRNAMap (Huang et al. 2009), sRNATarBase (Cao et al. 2010), SIPHI (<http://newbio.cs.wisc.edu/sRNA/>), and BSRD (Li et al. 2012), indicating the reliability of our method. As more as 83.1% of the candidate sRNAs was 20–80 nt; the size distribution of the candidate sRNAs is shown in Supplementary Fig. 1. Among all the sRNA candidates, 1124 were shared by the nitrogen-depleted group and the control group; 46 were unique in the nitrogen-depleted group and 49 in the control group.

sRNA differential expression

We identified differentially expressed sRNAs between the control (N+) and nitrogen depletion treatment (N-) groups by comparing the relative transcript abundance using the RPKM-based method (supplementary Table 2). Principally, any sRNA that has a value of ($|\log_2\text{Ratio}| \geq 1$ and $\text{FDR} \leq 0.001$) was kept as significantly differentially expressed sRNA. Totally, 418 sRNA were found to be differentially expressed between the control (N+) and nitrogen-depleted (N-) samples (supplementary Table 3), with 303 (72.49%) being significantly upregulated and 115 (27.51%) downregulated. Among them, 323 sRNA were differentially expressed in both samples; 46 sRNA were only expressed in control (N+) sample; and 49 sRNA were only expressed in nitrogen-depleted (N-) sample.

By adding different adaptors to the 3' and 5' ends of each RNA molecule in the sample prior to cDNA synthesis, RNA-seq analysis here enabled us to distinguish the direction of each transcription. In total, 110 antisense RNAs were found and 29 of them were significantly differentially expressed between the (N-) and (N+) groups (Table 2). In addition, 1109 intergenic region sRNAs were found and 389 were significantly differentially expressed (Table 3). We further characterized these antisense sRNAs and intergenic region sRNAs by retrieving putative target gene function information from the cyanobase (Table 2 and Table 3).

RT-PCR validation

For validation of the Illumina sequencing results, a number of 18 sRNA were randomly chosen for qRT-PCR analysis. The qRT-PCR results showed in Fig. 3, 11 sRNA showed significant differences in the relative expression between N+ and N- groups, and 7 sRNAs were in a significantly upregulated trend in N- group compared with N+ group, while 4 sRNAs were significantly downregulated in N- group. Similar trends were observed for the expression of these 18 sRNA analyzed by the 2 methods, the biggest discrepancy was around 7-fold difference in sRNA1133, and the lowest was around

0.5-fold difference in sRNA0703 (Fig. 3). However, the relative expression results from these 2 analyses did not match perfectly, perhaps due to sequencing biases or different normalization controls.

sRNA target gene prediction

To better understand the role of sRNAs in different biological modules, sRNA-regulating target genes were predicted using IntaRNA (Busch et al. 2008; Wright et al. 2014). Totally, 6043 target genes, which covering 97.11% of ORFs in *Anabaena* 7120, were found from the prediction using the 418 differentially expressed sRNAs as queries (supplementary Table 3). This indicates that single sRNA could target multiple genes. To further explore the differences of biological modules between the (N+) and (N-) groups, target genes that were predicted using the 49 (N+) unique sRNAs and 46 (N-) unique sRNAs as queries were subjected to functional categorization using the Gene ontology (GO) database (Consortium 2015). In total, 1674 target genes were predicted from the (N+) unique sRNAs, and 1811 were predicted from the (N-) unique sRNAs, while 630 were predicted in both. Functional annotation tool WEIGO (Ye et al. 2006) was used to analyze the target GO annotation terms. As shown in Fig. 4, there is no major difference for the GO analysis between these 2 groups. The GO terms were distributed widely with regard to their respective biological processes, from metabolic process to translation or transcription regulation and signal transducing.

Discussion

With the development of high-throughput sequencing methods such as RNA-seq, small RNAs have been increasingly recognized as major modulators of gene expression in bacteria (Prévost et al. 2011; Eisenhut et al. 2012; Sakurai et al. 2012; Yan et al. 2013; Behrens et al. 2014; Papenfort et al. 2015; Luo et al. 2019). However, only a few studies have sought to identify the global profile of sRNAs in cyanobacteria (Voß et al. 2007; Georg et al. 2009; Voß et al. 2009; Mitschke et al. 2011b; Bi et al. 2018). Flaherty et al. (2011) used the directional RNA-seq to analyze the *Anabaena* transcriptome during nitrogen step-down. Their RNA-seq data provided information on transcript abundance and boundaries, including detection of operons and the length of the untranslated region (UTR) of each transcript. Totally, they found that 434 and 396 genes were substantially upregulated at 12 h and 21 h, respectively. In contrast, only 32 and 35 genes were downregulated at 12 h and 21 h, respectively (Flaherty et al. 2011). Using the differential RNA sequencing method, Mitschke et al. (2011a, 2011b) addressed the differential use of transcriptional start sites (TSS) in *Anabaena* PCC 7120 after nitrogen depletion in the wild type strain and a *hetR* mutant which was unable to

Table 2 Antisense RNAs transcribed within the ORF or UTR of genes with more than 1-fold expression change after nitrogen step-down

Gene	Gene function	sRNA ID	RNA location	RPKM log2 ratio
<i>all0136</i>	30S ribosomal protein S1	sRNA0120	<i>all0136</i>	1.92
<i>all0270</i>	Diacylglycerol kinase	sRNA0158	<i>all0270</i> ORF	3.56
<i>all0271</i>	Hypothetical protein	sRNA0161	<i>all0271</i> 3' UTR	1.28
<i>all1290</i>	PvuII DNA methyltransferase	sRNA0332	<i>all1290</i> 3' UTR	1.36
<i>all2096</i>	Oxidase	sRNA0499	<i>all2096</i> 5' UTR	1.19
<i>all2457</i>	Hypothetical protein	sRNA0573	<i>all2457</i>	1.08
<i>all3226</i>	Hypothetical protein	sRNA0711	<i>all3226</i> 3' UTR	2.45
<i>all5062</i>	Glyceraldehyde-3-phosphate dehydrogenase	sRNA1145	<i>all5062</i>	– 1.31
<i>all5098</i>	Hypothetical protein	sRNA1153	<i>all5098</i>	– 10.42
<i>all7592</i>	Cation transporting ATPase	sRNA0055	<i>all7592-asl7593</i> intergenic region	11.38
<i>alr0821</i>	Hypothetical protein	sRNA0259	<i>alr0821</i> ORF	3.43
<i>alr0877</i>	Probable acylphosphatase	sRNA0272	<i>alr0877</i>	1.64
<i>alr1044</i>	Transcriptional regulator	sRNA0295	<i>alr1044</i> ORF and 5' UTR	– 12.39
<i>alr1139</i>	Hypothetical protein	sRNA0305	<i>alr1139</i> ORF	– 11.35
<i>alr1231</i>	Two-component hybrid sensor and regulator	sRNA0320	<i>alr1231-alr1232</i> intergenic region	3.94
<i>alr1669</i>	Similar to chitooligosaccharide deacetylase NodB	sRNA0411	<i>alr1669</i>	2.39
<i>alr2125</i>	Unknown protein	sRNA0503	<i>alr2125</i>	1.01
<i>alr2887</i>	Outer-membrane protein TolC	sRNA0636	<i>alr2887-alr2888</i> intergenic region	1.54
<i>alr2895</i>	Imidazoleglycerol-phosphate synthase, cyclase subunit HisF	sRNA0639	<i>alr2895-asr2896</i> intergenic region	– 2.78
<i>alr3018</i>	Probable DNA polymerase, bacteriophage-type	sRNA0668	<i>alr3018</i>	– 2.51
<i>alr3415</i>	Hypothetical protein	sRNA0755	<i>alr3415</i> 5' UTR	– 1.24
<i>alr3479</i>	Similar to nitrogen regulation protein NtrR	sRNA0768	<i>alr3479</i> ORF	– 11.32
<i>alr4156</i>	NADH dehydrogenase subunit 5	sRNA0956	<i>alr4156</i> 5' UTR	1.84
<i>alr4577</i>	Lipoprotein signal peptidase	sRNA1042	<i>alr4577-all4578</i> intergenic region	– 10.55
<i>alr4718</i>	ATP-dependent RNA helicase	sRNA1072	<i>alr4718</i> 5' UTR	– 1.25
<i>asl0822</i>	Hypothetical protein	sRNA0260	<i>asl0822</i> 3' UTR	11.11
<i>asr0098</i>	Unknown protein	sRNA0103	<i>asr0098</i> 5' UTR	– 2.12
<i>asr2016</i>	Unknown protein	sRNA0468	<i>asr2016</i>	2.26
<i>asr4319</i>	Photosystem I protein E	sRNA0987	<i>asr4319</i>	– 3.2

Gene functions were retrieved from cyanobase (<http://genome.microbedb.jp/CyanoBase>). Positive RPKM log2 ratio indicates upregulation and negative values indicate downregulation

differentiate heterocysts (Buikema and Haselkorn 1991; Mitschke et al. 2011a). They compared RNA profiles between these 2 strains grown in medium with or without combined nitrogen for 8 h. They identified > 900 putative TSS with a minimal 8-fold change in response to nitrogen deficiency. Among them, 209 were not induced in the *hetR* mutant indicating their involvement in heterocyst differentiation (Mitschke et al. 2011a). No study has been carried out to characterize the transcriptome in *Anabaena* PCC 7120 at later steady stage after combined nitrogen deprivation. In this study, we utilized the deep RNA-seq method for the identification of sRNAs in the model cyanobacterium *Anabaena* sp. strain PCC 7120 under steady state after 12-day nitrogen step-down. We identified 14,132

transcripts in total, which was comparable to transcripts (13,705) identified by Mitschke et al. (2011a, 2011b). Generally, sRNAs are transcribed from the non-coding regions, such as the antisense region and 5' or 3' UTR region (Gottesman and Storz 2011; Tsai et al. 2015). So we kept 1219 sRNAs that were located either on the antisense strand or intergenic region for further analysis (supplementary Table 2). Among them, 29 antisense sRNAs and 389 intergenic sRNAs were significantly differently expressed between the (N–) and (N+) samples (supplementary Table 3).

The 2 studies based on deep sequencing mentioned above have shown the presence of multiple non-coding RNA and antisense transcripts (Flaherty et al. 2011; Mitschke et al. 2011a). For example, Mitschke et al.

Table 3 Intergenic region sRNA with more than 10-fold expression change after nitrogen step-down

Pre-gene	Function	Next-gene	Function	sRNA ID	Strand	RPKM log2 Ratio
<i>all0071</i>	Unknown protein	<i>alr0072</i>	Two-component response regulator	sRNA0096	-	12.11
<i>all0138</i>	Photosystem II CP47 protein	<i>all0139</i>	Unknown protein	sRNA0125	-	12.53
<i>all0323</i>	Serine/threonine kinase with two-component sensor domain	<i>all0324</i>	Hypothetical protein	sRNA0170	-	-10.99
<i>all0443</i>	Hypothetical protein	<i>alr0444</i>	Unknown protein	sRNA0187	+	-13.03
<i>all0626</i>	H ⁺ /Ca ²⁺ exchanger	<i>alr0627</i>	Unknown protein	sRNA0227	+	-10.42
<i>all0638</i>	Two-component hybrid sensor and regulator	<i>asl0639</i>	Hypothetical protein	sRNA0230	-	11.53
<i>all0640</i>	ABC transporter	<i>all0641</i>	Hypothetical protein	sRNA0232	-	11.57
<i>all0772</i>	Hypothetical protein	<i>asr0773</i>	Hypothetical protein	sRNA0251	+	11.33
<i>all0945</i>	Succinate dehydrogenase iron-sulfur protein subunit	<i>alr0946</i>	Hypothetical protein	sRNA0281	+	-10.79
<i>all0945</i>	Succinate dehydrogenase iron-sulfur protein subunit	<i>alr0946</i>	Hypothetical protein	sRNA0283	-	10.92
<i>all0978</i>	Two-component hybrid sensor and regulator	<i>all0979</i>	Unknown protein	sRNA0289	+	-11.73
<i>all1172</i>	Hypothetical protein	<i>all1173</i>	DNA-binding protein, starvation-inducible	sRNA0314	-	11.23
<i>all1221</i>	Biogenesis of thylakoid protein A	<i>alr1222</i>	Hypothetical protein	sRNA0316	+	11.23
<i>all1280</i>	Two-component sensor histidine kinase	<i>all1281</i>	Two-component response regulator	sRNA0331	-	-11.81
<i>all1455</i>	Nitrogenase iron protein NifH	<i>all1456</i>	Nitrogen fixation protein NifU	sRNA0361	-	10.92
<i>all1517</i>	Nitrogen fixation protein NifB	<i>asl1518</i>	Unknown protein	sRNA0371	-	11.98
<i>all1651</i>	Transcriptional regulator	<i>alr1652</i>	Unknown protein	sRNA0406	+	11.23
<i>all2063</i>	Unknown protein	<i>alr2064</i>	Hypothetical protein	sRNA0479	-	-10.49
<i>all2124</i>	WD-40 repeat protein	<i>alr2125</i>	Unknown protein	sRNA0502	+	11.049
<i>all2152</i>	Transposase	<i>alr2153</i>	Outer membrane heme receptor	sRNA0514	+	-11.59
<i>all2509</i>	Similar to phosphoenolpyruvate synthase	<i>alr2510</i>	Hypothetical protein	sRNA0579	-	-10.49
<i>all2521</i>	Cysteine synthase	<i>alr2522</i>	Unknown protein	sRNA0580	+	12.05
<i>all2567</i>	Phosphoketolase	<i>all2568</i>	Hypothetical protein	sRNA0586	-	-10.73
<i>all2661</i>	Unknown protein	<i>alr2662</i>	Unknown protein	sRNA0595	-	-10.79
<i>all2908</i>	Cation transport ATPase	<i>all2909</i>	Hypothetical protein	sRNA0642	+	-10.67
<i>all2962</i>	Transcriptional regulator	<i>all2963</i>	Oxidoreductase	sRNA0660	-	11.28
<i>all3022</i>	Unknown protein	<i>all3023</i>	Hypothetical protein	sRNA0669	-	-10.49
<i>all3545</i>	Unknown protein	<i>alr3546</i>	HetF	sRNA0786	+	-12.51
<i>all3545</i>	Unknown protein	<i>alr3546</i>	HetF	sRNA0787	-	11.88
<i>all3677</i>	Hypothetical protein	<i>all3678</i>	Hypothetical protein	sRNA0830	+	-10.55
<i>all4289</i>	Hypothetical protein (ycf4)	<i>alr4290</i>	Photosystem II protein D2	sRNA0976	+	12.59
<i>all4289</i>	Hypothetical protein (ycf4)	<i>alr4290</i>	Photosystem II protein D2	sRNA0977	+	10.92
<i>all4376</i>	Probable glycosyl transferase	<i>all4377</i>	RNA-binding protein, rbpG	sRNA0995	-	11.28
<i>all4450</i>	Ribonuclease II	<i>asl4451</i>	30S ribosomal protein S18	sRNA1004	+	11.05
<i>all4636</i>	Similar to two-component sensor histidine kinase	<i>alr4637</i>	Unknown protein	sRNA1052	-	-11.76
<i>all4963</i>	Adenylyl cyclase CyaC	<i>alr4964</i>	Hypothetical protein	sRNA1117	-	-10.42
<i>all4963</i>	Adenylyl cyclase CyaC	<i>alr4964</i>	Hypothetical protein	sRNA1122	+	-10.49
<i>all5013</i>	Unknown protein	<i>all5014</i>	Hypothetical protein	sRNA1133	-	12.88
<i>all5274</i>	Hypothetical protein	<i>alr5275</i>	6-phosphogluconate dehydrogenase	sRNA1169	-	-10.62
<i>all5309</i>	Two-component hybrid sensor and regulator	<i>all5310</i>	Unknown protein	sRNA1189	+	10.92
<i>all5337</i>	Similar to TRK system potassium uptake	<i>alr5338</i>	3',5'-cyclic-nucleotide	sRNA1191	-	-10.42

Table 3 Intergenic region sRNA with more than 10-fold expression change after nitrogen step-down (*Continued*)

Pre-gene	Function	Next-gene	Function	sRNA ID	Strand	RPKM log2 Ratio
	protein		phosphodiesterase			
<i>all7033</i>	Unknown protein	<i>asl7034</i>	Transcriptional regulator	sRNA0004	+	11.57
<i>all7160</i>	Hypothetical protein	<i>all7161</i>	Transposase	sRNA0013	+	-10.49
<i>alr0113</i>	Hypothetical protein (ycf52)	<i>alr0114</i>	Hypothetical protein	sRNA0112	+	-10.62
<i>alr0452</i>	Hypothetical protein	<i>all0453</i>	Cobalamin biosynthesis precorrin-3 methylase	sRNA0191	-	-10.42
<i>alr0537</i>	Phycobilisome rod-core linker protein cpcG	<i>all0538</i>	Probable methyltransferase	sRNA0211	+	10.92
<i>alr0600</i>	Unknown protein	<i>all0601</i>	Hypothetical protein	sRNA0220	-	-10.49
<i>alr0738</i>	Sugar ABC transporter	<i>alr0739</i>	Hypothetical protein	sRNA0246	+	11.48
<i>alr0851</i>	Lysophospholipase	<i>all0852</i>	Hypothetical protein	sRNA0269	+	-10.99
<i>alr0965</i>	Unknown protein	<i>all0966</i>	Hypothetical protein	sRNA0288	-	-11.32
<i>alr1155</i>	Carbamoyl phosphate synthase small subunit	<i>asr1156</i>	Hypothetical protein	sRNA0308	+	12.28
<i>alr1360</i>	Unknown protein	<i>all1361</i>	Similar to cytochrome P450	sRNA0350	+	-10.49
<i>alr2054</i>	Aldo/keto reductase	<i>alr2055</i>	Unknown protein	sRNA0474	+	14.04
<i>alr2326</i>	Hypothetical protein	<i>all2327</i>	Phycobilisome core component; ApcF	sRNA0530	+	12.31
<i>alr2489</i>	Unknown protein	<i>asl2490</i>	Hypothetical protein	sRNA0577	+	11.38
<i>alr2532</i>	Sugar ABC transport system	<i>all2533</i>	Prolyl endopeptidase	sRNA0582	+	-10.89
<i>alr2709</i>	Hypothetical protein	<i>alr2710</i>	Hypothetical protein	sRNA0602	+	-10.62
<i>alr2856</i>	Hypothetical protein	<i>alr2857</i>	Unknown protein	sRNA0624	+	-11.86
<i>alr2920</i>	Putative short chain oxidoreductase	<i>alr2921</i>	Similar to tRNA-(MS[2]IO[6]A)-hydroxylase	sRNA0645	+	12.63
<i>alr2920</i>	Putative short chain oxidoreductase	<i>alr2921</i>	Similar to tRNA-(MS[2]IO[6]A)-hydroxylase	sRNA0646	-	11.28
<i>alr2936</i>	Pantothenate synthetase PanC	<i>asr2937</i>	Unknown protein	sRNA0650	-	11.43
<i>alr2938</i>	Iron superoxide dismutase; SodB	<i>asr2939</i>	Unknown protein	sRNA0653	+	12.17
<i>alr3139</i>	Hypothetical protein	<i>alr3140</i>	Hypothetical protein	sRNA0692	+	11.28
<i>alr3139</i>	Hypothetical protein	<i>alr3140</i>	Hypothetical protein	sRNA0693	-	11.85
<i>alr3139</i>	Hypothetical protein	<i>alr3140</i>	Hypothetical protein	sRNA0694	+	13.15
<i>alr3350</i>	Histone deacetylase/AcuC/AphA family protein	<i>alr3351</i>	Hypothetical protein	sRNA0735	-	-10.79
<i>alr3589</i>	Unknown protein	<i>alr3590</i>	Unknown protein	sRNA0793	+	-12.05
<i>alr3589</i>	Unknown protein	<i>alr3590</i>	Unknown protein	sRNA0794	-	-11.67
<i>alr3662</i>	Chaperonin GroEL	<i>alr3663</i>	Unknown protein	sRNA0824	+	11.53
<i>alr4104</i>	Transposase	<i>alr4105</i>	Two-component sensor histidine kinase	sRNA0945	+	12.67
<i>alr4592</i>	Photosystem II protein D1	<i>all4593</i>	Hypothetical protein	sRNA1047	-	-12.88
<i>alr4674</i>	Putative transcripton factor for heterocyst differentiation DevT	<i>all4675</i>	Transposase	sRNA1065	+	-10.73
<i>alr4808</i>	Competence-damage inducible protein CinA homolog	<i>asr4809</i>	Inorganic carbon transport; lctA	sRNA1083	-	-10.55
<i>alr4848</i>	Riboflavin kinase/FAD synthase	<i>alr4849</i>	Methanol dehydrogenase regulatory protein	sRNA1104	+	-13.05
<i>alr5357</i>	Polyketide synthase; HetM	<i>alr5358</i>	Ketoacyl reductase; HetN	sRNA1195	+	11.77
<i>asl0095</i>	Unknown protein	<i>alr0096</i>	Solaneyl diphosphate synthase	sRNA0100	+	11.23
<i>asl1778</i>	Unknown protein	<i>alr1779</i>	Similar to cyanophycin synthetase	sRNA0435	+	-11.62
<i>asl2301</i>	PatS	<i>all2302</i>	Patatin-like protein	sRNA0521	-	-10.67
<i>asl3784</i>	Hypothetical protein	<i>all3785</i>	Hypothetical protein	sRNA0853	+	14.2
<i>asl3978</i>	Unknown protein	<i>alr3979</i>	Period-extender of the circadian clock;	sRNA0905	-	10.92

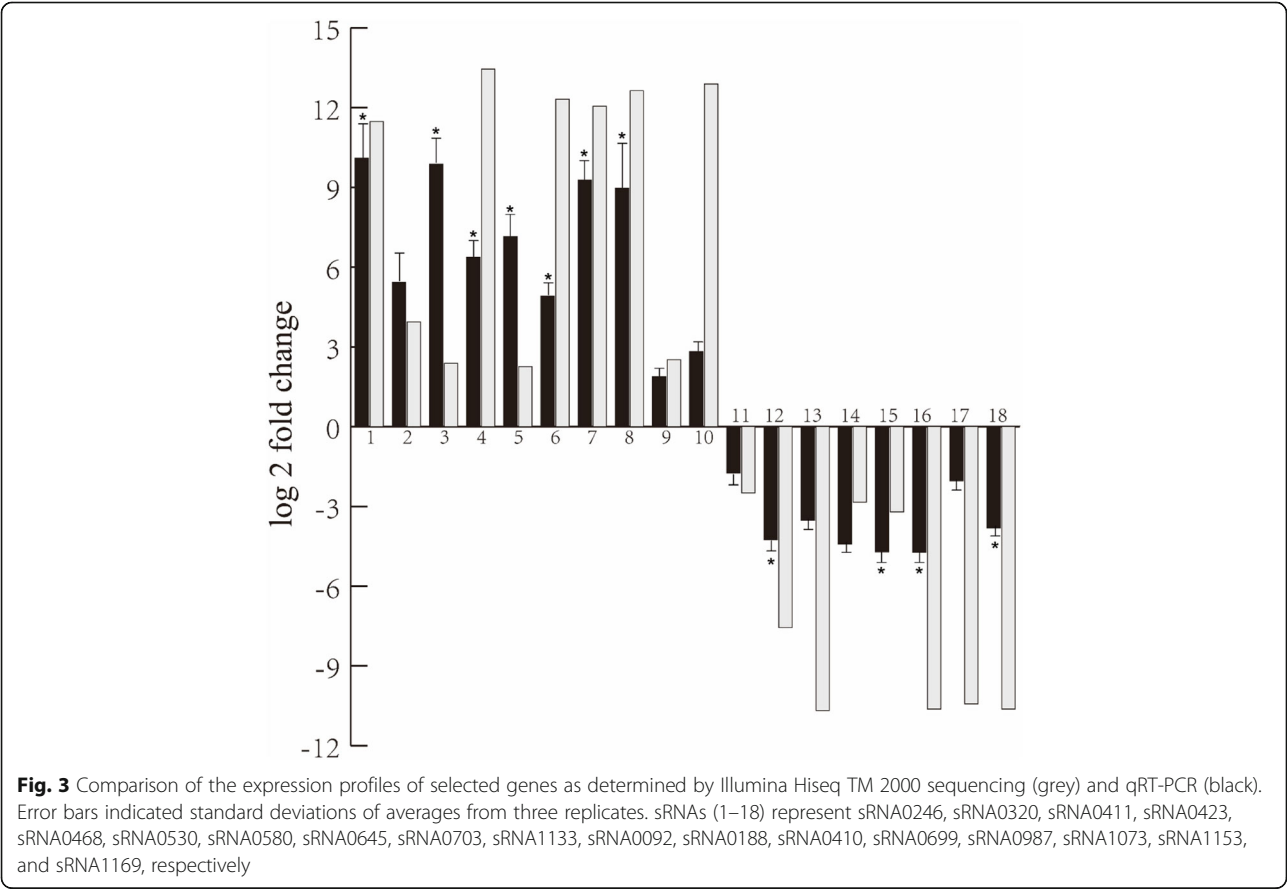
Table 3 Intergenic region sRNA with more than 10-fold expression change after nitrogen step-down (*Continued*)

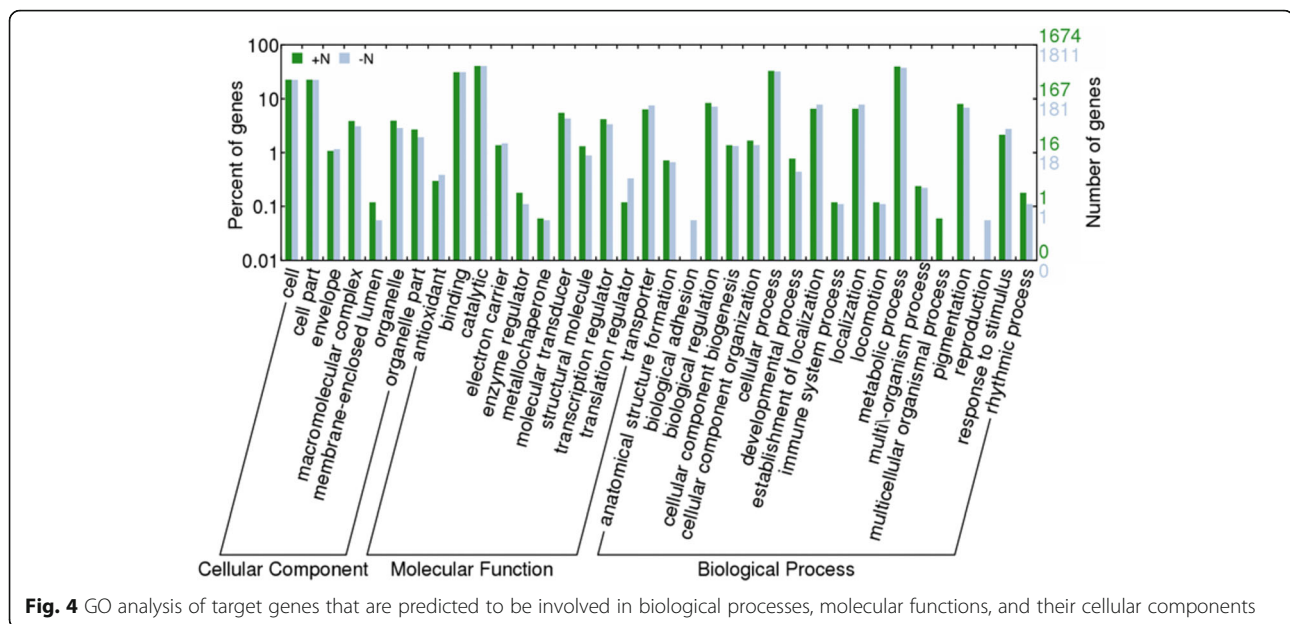
Pre-gene	Function	Next-gene	Function	sRNA ID	Strand	RPKM log2 Ratio
			Pex			
<i>asl4014</i>	Unknown protein	<i>alr4015</i>	Hypothetical protein	sRNA0923	-	11.57
<i>asl4557</i>	Rubredoxin	<i>all4558</i>	Probable dioxygenase Rieske iron-sulfur component	sRNA1038	-	10.92
<i>asr0382</i>	Hypothetical protein	<i>alr0383</i>	Unknown protein	sRNA0180	+	11.28
<i>asr1667</i>	Unknown protein	<i>alr1668</i>	Putative glycosyl transferase	sRNA0410	-	-10.67
<i>asr1714</i>	Hypothetical protein	<i>alr1715</i>	Hypothetical protein	sRNA0423	+	13.44
<i>asr3089</i>	Transglycosylase-associated protein	<i>alr3090</i>	Hypothetical protein	sRNA0673	+	12.55
<i>asr3935</i>	DNA binding protein HU	<i>alr3936</i>	Histidinol-phosphate aminotransferase	sRNA0881	+	10.92
<i>asr4747</i>	Unknown protein	<i>asl4748</i>	Hypothetical protein	sRNA1073	-	-10.62

Gene functions were retrieved from cyanobase (<http://genome.microbedb.jp/CyanoBase>). Positive RPKM log2 ratio indicates upregulation and negative values indicate downregulation

(2011a, 2011b) detected 1414 intergenic transcripts and 2412 antisense RNAs, which were 17% and 39% of all genes in the genome, respectively. However, we detected 5557 intergenic transcripts (39.32%) and 4205 antisense RNA (29.76%), the discrepancy might come from the different preparation of RNA samples (< 200 nt vs whole RNA) or different culture condition.

Our RNA-seq data showed sRNAs throughout the *Anabaena* transcriptome. For example, we identified an antisense transcript sRNA0414 covering 21 nt of 3' end of *alr1690* and 73-nt downstream sequence. It partially overlapped with a previously identified cis-acting antisense RNA, α -furA, which was co-transcribed with *alr1690* and could modulate ferric uptake regulation





protein FurA (*All1691*) expression (Hernández et al. 2006; Hernández et al. 2010). Also, we found 8 transcripts around a heterocyst regulatory gene *alr3546* (*hetF*). One of them, sRNA0787, matched the non-coding RNA NsiR1 (Ionescu et al. 2010; Muro-Pastor 2014). This indicates that the NsiR1 might reach its expression peak around 12 h (Ionescu et al. 2010; Muro-Pastor 2014), then maintains a low abundance to regulate target genes at later steady state. Regulation of nitrogen fixation genes is the culminating event of heterocyst differentiation. Our RNA-seq data showed that strong upregulation of sRNA3661 and sRNA3671 that target intergenic region of *all1517* (*nifB*)-*all1518* and *all1455* (*nifH*)-*all1456* (*nifU*) after nitrogen step-down (Table 3). These transcripts might be the degradation of long mRNA of these genes, which indicates that nitrogenase enzymes still maintain active functions at later steady state. However, other well-characterized nitrogen fixation genes, such as heterocyst exopolysaccharide synthesis (*hep*) and heterocyst glycolipid synthesis (*hgl*) were not observed to be regulated by differently expressed sRNAs here. This indicates that the mature heterocyst might not require an upregulation of these genes at later steady state or not regulated by sRNA presented in this study.

Conclusion

We have comprehensively analyzed sRNA expression profile after nitrogen step-down using *Anabaena* PCC 7120 and detected 418 differentially expressed sRNAs. Though the targets of sRNAs are currently unknown and need to be further analyzed, the antisense transcripts and intergenic RNAs perhaps play important

roles in regulation during the response to combined nitrogen deprivation. This study provides more information about the regulatory network of sRNAs and will advance our understanding of sRNA functions in cyanobacteria.

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s13213-020-01557-w>.

Additional file 1: Supplementary figure 1. Length distribution of sRNA candidates.

Additional file 2

Additional file 3

Additional file 4

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Authors' contributions

PH, KC, XF: Conceived and designed the experiments. PH, XC, KC: Performed the experiments. PH, XC, KC, XF: Analyzed the data. PH, XC, KC: Contributed reagents/materials/analysis tools. PH, KC, XF: Wrote the paper. The author(s) read and approved the final manuscript.

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Ethics approval and consent to participate

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Consent for publication

All relevant personnel had been informed.

Competing interests

The authors declare that they have no competing interests.

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