The Antisense RNA As1_flv4 in the Cyanobacterium Synechocystis sp. PCC 6803 Prevents Premature Expression of the flv4-2 Operon upon Shift in Inorganic Carbon Supply*§

Received for publication, June 14, 2012, and in revised form, July 18, 2012. Published, JBC Papers in Press, August 1, 2012, DOI 10.1074/jbc.M112.391755

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The functional relevance of natural cis-antisense transcripts is mostly unknown. Here we have characterized the association of three antisense RNAs and one intergenically encoded noncoding RNA with an operon that plays a crucial role in photoprotection of photosystem II under low carbon conditions in the cyanobacterium Synechocystis sp. PCC 6803. Cyanobacteria show strong gene expression dynamics in response to a shift of cells from high carbon to low levels of inorganic carbon (Ci), but the regulatory mechanisms are poorly understood. Among the most up-regulated genes in Synechocystis are flv4, sll0218, and flv2, which are organized in the flv4-2 operon. The flavodiiron proteins encoded by this operon open an alternative electron transfer route, likely starting from the QB site in photosystem II, under photoxidative stress conditions. Our expression analysis of cells shifted from high carbon to low carbon demonstrated an inversely correlated transcript accumulation of the flv4-2 operon mRNA and one antisense RNA to flv4, designated as1_flv4. Overexpression of As1_flv4 led to a decrease in flv4-2 mRNA. The promoter activity of as1_flv4 was transiently stimulated by Ci limitation and negatively regulated by the AbrB-like transcription regulator Sll0822, whereas the flv4-2 operon was positively regulated by the transcription factor NdhR. The results indicate that the tightly regulated antisense RNA As1_flv4 establishes a transient threshold for flv4-2 expression in the early phase after a change in Ci conditions. Thus, it prevents unfavorable synthesis of the proteins from the flv4-2 operon.

Regulatory RNAs are key transcriptional and post-transcriptional regulators of gene expression in all domains of life. In plants, small RNA-based mechanisms control almost all aspects of plant biology, including chromatin structure, genome stability, gene expression, and defense. The functions and phylogenetic distribution of plant microRNAs, one particular class of RNA regulators, have been studied comparatively well (for a review, see Ref. 1), whereas the functions of longer noncoding RNAs are only beginning to emerge (2). Transcripts, which originate from the reverse complementary strand of an annotated gene and hence fully or partially overlap with their respective mRNAs, are known as cis-natural antisense transcripts or antisense RNAs (asRNAs).3 Natural asRNAs are abundant in the plant nuclear genome (3, 4). Plant asRNAs have been more frequently observed to be associated with mRNAs of nucleus-encoded plastid and mitochondrial proteins than with other eukaryotic mRNAs (5), a fact that may be taken as a hint for the possible role of bacterial asRNAs during evolution and even after endosymbiosis. Indeed, recent observations for plant chloroplasts indicated asRNAs to be associated to 35% of all genes (6).

In cyanobacteria, the evolutionary ancestors of plant chloroplasts, asRNAs summing up to 26% of all genes for the unicellular Synechocystis sp. PCC 6803 (hereafter Synechocystis) (7, 8) and to 39% of all genes in the nitrogen-fixing Anabaena sp. PCC 7120 (hereafter Anabaena) (9) were reported. However, the functional relevance of specific antisense transcripts in plants, chloroplasts, and bacteria has barely been addressed. In cyanobacteria, the functions of only two asRNAs have been studied in

* This work was supported by Academy of Finland Projects 118637 and 132399 (to E.-M. A. and M. E.), European Union Project Solar-H2 (FP7 Contract 212508) (to E.-M. A.), the Deutsche Forschungsgemeinschaft (DFG) Focus Program “Sensory and Regulatory RNAs in Prokaryotes” SPP 1258 (hereafter, 39% of all genes in the nitrogen-fixing Anabaena sp. PCC 7120 (hereafter Anabaena) (9) were reported. However, the functional relevance of specific antisense transcripts in plants, chloroplasts, and bacteria has barely been addressed. In cyanobacteria, the functions of only two asRNAs have been studied in

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‡1 This article contains supplemental Figs. S1–S3 and Table S1.

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3 The abbreviations used are: asRNA, antisense RNA; ncRNA, noncoding RNA; PsII, photosystem II; LC, low carbon (0.038% CO2 in air); HC, high carbon (3% CO2 in air); Ci, inorganic carbon; nt, nucleotide(s); CCM, CO2-concentrating mechanism; Flv, flavodiiron; Rubisco, ribulose-bisphosphate carboxylase/oxygenase.

Background: Flavodiiron proteins encoded by the flv4-2 operon are photoprotective for photosystem II, but their regulation of expression has remained enigmatic.

Results: Expression of flv4-2 is controlled jointly by NdhR and the antisense RNA As1_flv4, whereas As1_flv4 is controlled by an AbrB-like factor.

Conclusion: As1_flv4 provides a safety threshold preventing premature expression.

Significance: Regulatory networks controlling photosynthetic photoprotection are highly complex.
molecular detail thus far. In *Anabaena*, *furA*, the gene for the ferric uptake regulator, is covered by a long asRNA (10) whose knock-out mutation results in an iron deficiency phenotype (11). In *Synechocystis*, the 177-nt asRNA *IsrR* controls the expression of *isiA*, which encodes the iron stress-induced protein A, in a co-degradation mechanism (12).

Cyanobacteria are often challenged by changes in biotic and abiotic factors in their natural environments. In particular, changes in cellular functions triggered by fluctuations in the availability of inorganic carbon (Ci) have been a subject of studies for years (13–23). Most striking is the induction of the CO2-concentrating mechanism (CCM) in cyanobacterial cells after a shift from high (>1% CO2 in air) to low (atmospheric 0.038% CO2 in air) levels of Ci. By coordinated action of different CCM components, comprising specialized Ci uptake mechanisms and the Rubisco-containing carboxysomes, cyanobacteria manage to lower the CO2 compensation point and thus overcome the otherwise limiting Ci availability (for reviews, see Refs. 24–27). Furthermore, flavodiiron (Flv) proteins have recently been shown to be involved in the low carbon (0.038% CO2 in air; LC) acclimation process (28–30). The fully sequenced (31) cyanobacterial model organism *Synechocystis* contains four genes encoding the proteins Flv1, Flv2, Flv3, and Flv4. The expression of the *flv2*, *flv3*, and *flv4* genes becomes up-regulated under LC conditions with *flv2* and *flv4* showing the strongest induction (16, 20, 28). Although the Flv1 and Flv3 proteins participate in the Mehler-like reaction (29, 32, 33), the Flv2 and Flv4 proteins were demonstrated to have a crucial role in photoprotection of photosystem II (PSII) (32, 33). We have shown recently (30) that under these conditions the small membrane protein Sll0218, which is also encoded by the *flv4-2* operon, stabilizes the PSII dimer and enables the Flv2/Flv4 heterodimer to accept electrons from PSII. Thus, the products of the *flv4-2* operon provide β-cyanobacteria with a unique and novel photoprotection mechanism. Despite numerous studies and continuous progress (15, 16, 18, 20, 23, 34–36), the understanding of the C4-controlled gene expression dynamics is still incomplete.

Here we report the identification of three asRNAs and one noncoding RNA (ncRNA) associated with the *flv4-2* operon. These transcripts were primarily detected by microarray analysis (7) and 454 sequencing (8). We verified the existence of these ncRNAs by Northern blotting and characterized the asRNA *As1_flv4* in more detail. The inversely correlated accumulation of *As1_flv4* transcript with the transcripts and proteins from the *flv4-2* operon and the results obtained from artificial modulation of *As1_flv4* levels suggest a stoichiometric function of *As1_flv4* to control the expression of the *flv4-2* operon according to the environmental Ci availability. Furthermore, the direct or indirect repression by the AbrB-like transcriptional regulator Sll0822 and the control of the promoter activity by the C4 level support the assumption that ncRNAs play a significant role in the C4-regulatory network in *Synechocystis*.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—The glucose-tolerant strain of cyanobacterium, *Synechocystis* sp. PCC 6803, served as the WT. Cultivation of mutants was performed at 50 μg ml⁻¹ kanamycin and 20 μg ml⁻¹ spectinomycin, respectively. For the experiments, axenic cultures of the cyanobacteria were grown photoautotrophically under 50 μmol photons m⁻² s⁻¹ (white light) at 30 °C. Cells were cultivated in BG-11 medium (pH 7.5) and aerated by shaking in the presence of CO2-enriched air (3% CO2 in air; high carbon (HC)) or ambient air CO2 (LC). In the case of the LC shift experiment, the cells were collected by centrifugation (2 min at 1730 × g at room temperature) and resuspended in fresh BG-11, and the OD750 measured with a Spectronic Genesys 2 spectrophotometer (Thermo Fisher Scientific, Madison, WI) was adjusted to 0.8. After precultivation at HC conditions for 1 h, cultures were transferred to LC conditions. In analogous experiments, cells were aerated directly by continuous bubbling with LC or HC.

For the asRNA overexpression experiments, the two overexpression mutants *As1_flv4(+)/2* and *As1_flv4(+)/3* and a control strain (mutant in *spkA*) were precultivated in Cu²⁺-containing BG-11 medium and bubbled with HC. For induction of *petl* promoter activity by Cu²⁺ depletion (43), the cells were spun down and washed with and resuspended in Cu²⁺-free BG-11 medium. Subsequently, cultures were treated as described above.

**Generation of Promoter Probe Strains**—300- and 700-nt promoter regions of the genes encoding the asRNA *As1_flv4* and the *flv4-2* operon, respectively, were amplified by PCR using chromosomal DNA and specific primers (supplemental Table S1). After digestion with KpnI, the respective promoter fragment was ligated into the unique KpnI site of the promoter test vector pLA (37). The vector pLA allows transcriptional fusion of the promoter sequence with the luxAB genes and its stable integration into the chromosome at a neutral site (37). Plasmids with correct promoter insertion direction relative to the reporter genes were selected for subsequent transformation of *Synechocystis*. Completely segregated clones were checked by PCR analysis as described (19) and subsequently used for promoter activity measurements.

**Promoter Activity Measurements**—1-ml cell aliquots were taken at selected time points from shaking cultures with an OD750 of 0.5. Subsamples of 100 μl were transferred in triplicate to white 96-well microtiter plates (Thermo Fisher Scientific). To start the measurement, 100 μl of 2 mM decanal ready-to-use solution was added, and the plate was immediately placed into the plate reader (Wallac Victor 2 1420 multilabel counter, PerkinElmer Life Sciences). 100 mM decanal stock solution was prepared in methanol and freshly diluted with BG-11 for 2 mM decanal ready-to-use solution. Bioluminescence was measured for 30 min at 25 °C. The maximum light emission (around 10 min after the start) was used as the bioluminescence value and related to the OD750. Results are presented in relative bioluminescence units. Experiments were repeated three times.

**Generation of As1_flv4 Overexpression Strain**—All primers used for plasmid preparation in this work are listed in supplemental Table S1. To generate the overexpression construct, a 563-nt DNA fragment beginning from the mapped *as1_flv4* transcriptional start site (nucleotide 166849 according to Ref. 7) was fused with the *petl* promoter and integrated with a kana-
mycin resistance cassette in the spkA gene. The spkA gene can be used as an uncommitted integration site because this gene is disrupted by a frameshift mutation in the Synechocystis strain used (44). The DNA fragment is longer than the asRNA transcript to allow for transcription termination at its own terminator. To prevent eventual read-through, the λ phage oop terminator was fused to the 3’-end of the fragment.

First a platform for the integration of a ncRNA between the pet/p promoter and the oop terminator was constructed. The primers “5’Apal_pet/P” and “3’petl_AusII_oop_Sall” were used to amplify the pet/p promoter fragment. The construct contained Apal and Sall restriction sites for integration in the pJet-spl plasmid and an AusII restriction site for the integration of an ncRNA between pet/p promoter and oop terminator. The new pJet-spl-petlp plasmid was AusII-digested for the integration of the AusII-digested as1_flv4 fragment generated with the as_flv4_asuII_for and as_flv4_asuII_rev primers. The sense orientation of the fragment was tested with the as_flv4_asuII_rev primer and the spK_seg_for primer. The segregation of the construct in the genome was tested with the spK_seg_for and spK_Seg_rev primers. A schematic presentation of the cloning strategy is shown in supplemental Fig. S1A.

RNA Extraction and Northern Blot Analysis—Total RNA was isolated with TRIzol reagent (Invitrogen) and treated with a TURBO DNase kit (Invitrogen) to remove genomic DNA. To characterize small RNAs, RNA samples (5 μg) were mixed with RNA loading dye (Fermentas, St. Leon-Rot, Germany), denatured for 10 min at 70 °C, separated in 10% polyacrylamide-urea gel, and transferred to Hybond-N nylon membranes (GE Healthcare) by electro blotting for 1 h. For the mRNA studies, RNA samples were mixed with denaturation solution, incubated for 10 min at 70 °C, separated in 1.3% agarose gels containing 7% formaldehyde in MOPS, and transferred to Hybond-N nylon membranes by capillary blotting with 10× SSC overnight (38). After UV cross-linking, even loading and blotting were checked by methylene blue staining (0.5M sodium acetate, pH 5.2, 0.04% methylene blue). The membranes were hybridized with [α-32P]UTP-incorporated transcripts or [α-32P]CTP-labeled DNA probes. In vitro transcription was performed with the MAXiScribe kit (Invitrogen) as described (39) and labeled DNA probes obtained with the PrimeGene Labeling System (Promega, Madison, WI). Hybridization with the specific probes was performed in hybridization buffer (6× SSC, 5× Denhardt, 0.5% SDS, 1 mg ml−1 herring sperm DNA) overnight at 62 °C. The next day, membranes were washed three times with washing buffer (2× SSC, 0.1% SDS) for 10 min at 56 °C. Signals were visualized either by autoradiography on x-ray films or using a Personal Molecular Imager FX system with Quantity One software (Bio-Rad). The sequences of the primers used for the preparation of transcript probes are listed in supplemental Table S1.

5’-Rapid Amplification of cDNA Ends—The rapid amplification of cDNA 5’-ends was performed as described (40) using RNA from WT cells grown under LC conditions. The primers used are listed in supplemental Table S1.

Protein Isolation and Western Blot Analysis—Membrane and soluble fractions from Synechocystis cells were isolated as described (28). The protein samples were solubilized in Laemmli buffer (5% β-mercaptoethanol, 6 M urea) at room temperature for 2 h and separated by 12% SDS-PAGE. Then the proteins were transferred by semidyblotting to a polyvinylidene fluoride (PVDF) membrane and immunodetected with antibodies specific for Flv4, Flv2 (28), and Sll0218 (30).

RESULTS

High Abundance of ncRNAs Connected with the flv4-2 Operon—In the cyanobacterium Synechocystis, the flv4-2 operon comprises the three genes flv4, sll0218, and flv2 (Fig. 1A). A previous tiling microarray-based screening of the Synechocystis genome for naturally occurring ncRNAs led to the discovery of 60 intergenically encoded ncRNAs and 73 cis-encoded (transcription in the antisense direction to the protein-coding region) asRNAs (7). Interestingly, an asRNA to flv4 was identified among the top scoring small regulatory RNAs in the screen (ranked 12 in the detected asRNAs with regard to the normalized mean signal intensity) and is designated here as As1_flv4. The existence of As1_flv4 transcript was verified by Northern blotting (Fig. 1B) using a single-stranded RNA probe. According to the Northern blots, main transcript lengths are 500 and ~280 nt. A more recent differential RNA sequencing analysis yielded three more candidate ncRNAs associated with the flv4-2 operon (8). These are a second asRNA to flv4 named As2_flv4, an asRNA to flv2 named As_flv2, and one putative ncRNA originating from the flv4-ctpB intergenic spacer designated as Ncr0080 (Fig. 1A). The 5’-ends of all relevant genes and ncRNAs were mapped: nucleotide positions 166849, 167538, and 168233 on the forward strand are the transcriptional start sites of the flv4-2 operon. The existence of these candidates was also verified by Northern blot analysis (Fig. 1B). Here, the main transcript lengths are about 85 and 78 nt for As2_flv4, about 80 nt for As_flv2, and about 180 nt for the ncRNA Ncr0080, respectively (chromosomal positions according to Cyanobase). The transcription start site of the ctpB gene is at position 168421. It has to be mentioned that there is no functional connection known between ctpB encoding a carboxyl-terminal protease (41, 42) and the flv4-2 operon. The existence of these candidates also was verified by Northern blot analysis (Fig. 1B). Here, the main transcript lengths are about 85 and 78 nt for As2_flv4, about 80 nt for As_flv2, and about 180 nt for the ncRNA Ncr0080, suggesting that its major 3’-end is located ~10 nt upstream of the transcription start site of the ctpB gene. For all RNAs, extra bands were also detected, most probably resulting from degradation, processing, or read-through processes. In the following experiments, we focused on the regions that correspond to these main signals and to the data obtained from deep sequencing of the transcriptome (8).

Because of the origin of Ncr0080 from an intergenic spacer in between two open reading frames (Fig. 1A), it was not clear whether the RNA was a trans-encoded ncRNA transcribed from the intergenic region between ctpB and flv4, was a cis-encoded asRNA overlapping with the 5’-UTR of the antisense encoded gene flv4, or constituted the 5’-UTR of ctpB. The latter possibility appeared to the least likely because in microarray experiments ncr0080 showed a regulation different from ctpB with maxima under high light, whereas ctpB did not respond to high light but was induced under LC (8). Because expression of flv4 had not been detected in the transcriptome analysis (8), we determined its transcription start site separately using RNA from LC-grown cells. 5’-Rapid amplification of cDNA end
analysis (Fig. 1C) indicated that flv4 transcription starts from position 168188 on the reverse strand. Additionally, −10 and −35 elements were predicted with high scores according to the evaluation of more than 3,000 transcriptional start sites in *Synechocystis* (8). Deduced from these results, the RNA Ncr0080 (transcription start site at 168233) does not overlap with the 5'-UTR of flv4 and was therefore categorized as an ncRNA.

Expression of ncRNAs, mRNAs, and respective proteins upon Shift of *Synechocystis* from HC to LC Conditions—After identification and verification of the ncRNAs, we aimed to find out their biological role. Because it is well documented that the expression of the flv4-2 operon is strongly induced under LC conditions (16, 20) and the encoded proteins have a crucial function under those conditions (28, 30), we compared the time course of accumulation of ncRNAs, mRNA transcripts, and proteins after a shift of cells from HC to LC conditions (Fig. 2).

As expected, both flv4-2 mRNA and respective proteins accumulated under LC conditions. Following the LC shift, the tricistronic flv4-2 mRNA and the encoded proteins Flv4, Sll0218, and Flv2 first appeared after 3 h (Fig. 2A). The flv4-2 mRNA appears in the form of three bands representing the three possible mRNA fragments originating from the tripartite operon. Its diffuse character is likely due to a short half-life of the mRNA. Furthermore, we also sometimes observed sense-antisense RNA interactions in gels under denaturing conditions, which may distort band appearance. High protein levels were reached after 12 (Flv4 and Flv2) and 24 h (Sll0218), respectively. The mRNA was most abundant after 24 h (Fig. 2B) under these conditions. The transcript of asRNA As1_flv4, however, accumulated in a reverse manner. The transcript was present already under HC conditions and increased strongly in abundance shortly after the LC shift (1- and 3-h time points). Subsequently, the amount of asRNA As1_flv4 declined (Fig. 2). Accumulation of As2_flv4 transcript seemed to follow similar kinetics but with lower relative abundance, whereas the As_flv2 RNA levels increased over time (Fig. 2A). 24 h after the LC shift, As_flv2 levels were about 3-fold increased compared with HC conditions. Expression of Ncr0080 was strongly affected by the LC shift. The RNA amount was transiently down-regulated for the first 6 h after LC shift and recovered to HC levels after 12 h (Fig. 2A).

Because of the observed strong alterations in transcript levels of flv4-2 mRNA and As1_flv4 asRNA, we chose As1_flv4 as a target for more detailed analysis. To underpin our observation that As1_flv4 levels and flv4-2 operon products are inversely correlated with dependence on C<sub>i</sub> levels, we repeated the original time course experiment (Fig. 2) by an additional shift of the culture back to HC conditions and monitored the accumulation of both asRNA and proteins encoded by the flv4-2 operon (supplemental Fig. S2). As in Fig. 2, a shift from HC to LC resulted in strong accumulation of the proteins Flv4, Sll0218, and Flv2, whereas the concentration of As1_flv4 transcript
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FIGURE 2. Accumulation of flv4-2 operon-related ncRNAs, mRNA, and proteins after shift of Synechocystis WT cells from HC to LC conditions. Cells were precultivated under HC conditions and then shifted to LC. Samples were collected 0, 1, 3, 4.5, 6, 12, and 24 h after the LC shift and analyzed as described under “Experimental Procedures.” A, results of blotting experiments. 5 S rRNA and AtpB were used as loading controls for RNA and protein, respectively. B, quantification of signal intensities. The strongest signal intensity for each probe was set to 100%, and the other signal intensities were related accordingly. Shown are the results of one representative experiment.

diminished. Importantly, the shift back to HC conditions had an opposite effect. The proteins gradually disappeared after the HC shift and were no longer detected after 6 h. In contrast, the amounts of As1_flv4 transcript recovered rapidly and were at the highest level 12 h after shifting back to HC. In summary, we saw direct and polar responses of the asRNA and the target proteins to changing Ci levels under the cultivation conditions used here.

Modulation of asRNA As1_flv4 Abundance Effects flv4-2 Expression—The observed tightly controlled and inversely correlated accumulation of As1_flv4 transcript and its target mRNA strongly suggested a regulatory impact of As1_flv4 on the expression of the flv4-2 operon. To verify this, the abundance of the As1_flv4 transcript was artificially modulated in Synechocystis cells. To generate a mutant with elevated As1_flv4 levels, the sequence was fused with the promoter of the petJ gene, which is strongly induced in the absence of Cu²⁺ (43). The fusion construct was integrated by homologous recombination into the spkA gene. In the commonly used glucose-tolerant Synechocystis WT strain, this gene is disrupted by a frameshift mutation (44) and could therefore be regarded as a neutral integration site. After genotypic verification of the obtained overexpression mutant As1_flv4(+), two independent clones of the strain were initially characterized with regard to the expression strength of As1_flv4. Northern blot analysis (supplemental Fig. S1B) revealed successful overexpression of the asRNA As1_flv4 in the two overexpression mutants As1_flv4(+)/2 and As1_flv4(+)/3 when the cells were cultivated in Cu²⁺-free BG-11 medium for 46 h. We also tried to reduce internal As1_flv4 concentrations by expressing an antisense construct for As1_flv4, but the target asRNA levels did not change significantly for an unknown reason.

To analyze the impact of increased As1_flv4 transcript levels, we performed another time course experiment after shifting cells to LC. As expected, the control strain showed a similar expression pattern for the asRNA and the flv4-2 operon compared with WT cells in the previous experiments (Fig. 3). However, it was striking to find that compared with the control strain the overexpression mutants showed at 4.5 h after LC shift fully suppressed and at 6 h reduced (−15%) transcript levels of the flv4-2 operon (Fig. 3).

The as1_flv4 Promoter Is Transiently Induced after LC Shift—It has been well established that bacterial ncRNAs act by pairing with the target mRNA and thus modify the mRNA stability or translation in a positive or negative manner, respectively (45–48). Hence, the results of the Northern blotting experiments only demonstrate momentary RNA levels; therefore, it was of importance to unravel the true effects of changing Ci levels on transcription activity. For this purpose, we performed promoter activity analysis using a 300-nt fragment containing hypothetical promoter elements upstream of as1_flv4 and a 700-nt fragment containing the putative flv4-2 operon promoter fused with the reporter genes luxAB encoding the bioluminescent luciferase enzyme. The bioluminescence of
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FIGURE 4. Activities of \( P_{\text{as}1\_\text{flv4}} \) and \( P_{\text{flv4-2}} \) operon promoters after shift from HC to LC conditions. Promoter sequences of the \( as1\_\text{flv4} \) gene and the \( flv4-2 \) operon were fused with the luxAB genes. The generated mutant strains MpiLA-as1\_flv4 and MpiLA-flv4-2 were verified by PCR and used for luminescence measurement. The promoterless construct MpiLA served as a negative control. Cells were cultivated under HC conditions and shifted to LC. At the given time points, samples were taken, and bioluminescence was measured as described under "Experimental Procedures." The bioluminescence was normalized to the OD750 and corrected by subtraction of luminol auto-luminescence and MpiLA luminescence. Each sample was measured in triplicates. Given are the means and S.D. (error bars) of triplicates of a representative time course. \( P_{\text{as}1\_\text{flv4}} \), promoter of the \( as1\_\text{flv4} \) operon; \( P_{\text{flv4-2}} \), promoter of the \( flv4-2 \) operon; A.U., arbitrary units.

FIGURE 5. Expression of \( as1\_\text{flv4} \) and the \( flv4-2 \) operon in the mutant \( \Delta\text{ndhR} \) and the WT. Shown is a representative time course of transcript abundances of the asRNA \( as1\_\text{flv4} \) and the mRNA of the \( flv4-2 \) operon after LC shift. 5 S rRNA was used as a loading control.

FIGURE 6. Expression of \( as1\_\text{flv4} \) and the \( flv4-2 \) operon in the mutant \( \Delta\text{sll0822} \) and the WT. Shown is a representative time course of transcript abundances of the asRNA \( as1\_\text{flv4} \) and the mRNA of the \( flv4-2 \) operon after LC shift. 16 S rRNA was used as a loading control.
the Flv2/Flv4 heterodimer opens up an alternative electron transfer route, likely starting from the Q₈ site in PSII (30), thus acting as an electron valve under photooxidative stress conditions.

Apparentely related to the delicate function of the Flv2 and Flv4 proteins, the expression of the flv4-2 operon is strictly controlled. Here, for the first time, we provide evidence indicating that ncRNAs are involved in the Cᵢ-dependent control mechanisms. Four ncRNAs, three asRNAs, and one intergenically encoded ncRNA were verified to be associated with the flv4-2 operon (Fig. 1). The most pronounced response in regard to the dependence on changing Cᵢ levels was found for the asRNA As1_flv4, which accumulated strictly inversely with the mRNA and proteins derived from the target flv4-2 operon (Fig. 2 and supplemental Fig. S2).

**asRNA As1_flv4 Prevents Premature Expression of the flv4-2 Operon**—Based on the expression kinetics of the asRNA As1_flv4 and its target flv4-2 operon mRNA (Fig. 2) on the promoter activity measurements (Fig. 4) as well as on the characterization of the As1_flv4[+] overexpression mutants (Fig. 3), we suggest the model presented in Fig. 7A to explain the regulation of the flv4-2 operon by the asRNA As1_flv4 in response to changes in Cᵢ conditions.

As1_flv4 originates from the antisense strand to flv4. Both the asRNA and its target mRNA, flv4-2, are transcribed under HC conditions according to the promoter activity studies (Fig. 4). Most likely, the asRNA As1_flv4 pairs with its target, the flv4-2 mRNA, and the RNA duplexes are directed to co-degradation. Because the asRNA transcript is more abundant, the target mRNA is outcompeted, and no free flv4-2 mRNA occurs inside the cells. Indeed, neither the flv4-2 mRNA nor the respective proteins but only the asRNA As1_flv4 can be detected under HC conditions. The mechanisms of asRNA-mRNA interaction and subsequent degradation are poorly understood in cyanobacteria. In regard to the degradation of RNA duplexes, it was shown recently (53) that this process is mediated by the double-stranded RNA-specific RNase, RNase III, in Staphylococcus aureus. The involvement of RNase III has been postulated also for cyanobacteria (7, 10, 12, 48), and in this context, it is interesting to note that in contrast to most other bacteria Synechocystis has two different RNase III genes (slr0346 and slr1646).

When Synechocystis cells were exposed to LC conditions, the promoter of as1_flv4 was induced (Fig. 4). Intriguingly, the induction of the asRNA promoter was only transient but sufficient to prevent premature biosynthesis of the flv4-2-encoded proteins. Only if the LC signal persisted did the increasing flv4-2 promoter activity (Fig. 4) lead to high production of flv4-2 mRNA. At the time point when the mRNA level exceeds the asRNA abundance, the threshold is overcome, and flv4-2 mRNA becomes available for ribosome binding and subsequent protein biosynthesis (Fig. 7A). Finally, Flv2 and Flv4 assemble as heterodimers, Slr0218 stabilizes the PSII dimer, and thus the proteins protect the cell against photoinhibition under LC conditions (30). In general, the strong transcriptional response to a shift from HC to LC seems to be transient. Deduced from our previous results with steadily LC-grown cells (22, 28, 30) and prolonged promoter activity measurements (supplemental Fig. S3B), we hypothesize that after a peak in the accumulation of the flv4-2 mRNA the transcript amounts decline but remain at levels higher than those at prestress conditions, allowing for sufficient Flv2, Flv4, and Slr0218 synthesis under prolonged or even permanent LC treatment. This behavior is probably inversely mirrored by the asRNA As1_flv4 (Fig. 7A).

A mechanism similar to that for As1_flv4 was previously postulated for the asRNA IsrR (12). It was demonstrated that IsrR controls the expression of the gene isiA encoding the iron stress-induced protein IsiA in a stoichiometric manner. Artificial modulation of the internal IsrR levels was used to success...
fully prove such a regulatory function. Likewise, the artificial overexpression of the asRNA As1_flv4 resulted in decreased and delayed expression of the flv4-2 operon (Fig. 3).

In summary, the inverse correlation of asRNA As1_flv4 levels with flv4-2 transcripts and the results from the As1_flv4 overexpression experiments after a shift from HC to LC conditions are consistent with a major function of As1_flv4 in the early phase of the acclimation process to changed C_l levels. The amount of As1_flv4 transcripts sets a threshold for flv4-2 mRNA accumulation and thus delays the protein synthesis from the flv4-2 operon. It is also valid to assume that the asRNA As1_flv4 ensures rapid degradation of the flv4-2 mRNA and thus shuts down the Flv4-2-mediated electron valve promptly as discussed for IsrR in the shutdown of IsiA expression (54). The rapid depletion of the proteins Flv2, Flv4, and Sll0218 after a shift from LC to HC conditions is illustrated in supplemental Fig. S2.

**AbrB-like Transcriptional Regulator Sll0822 Controls the Expression of as1_flv4**—Importantly, we demonstrate here that the promoter activity of the asRNA encoding gene as1_flv4 after an LC shift is transiently induced (Fig. 4). To our knowledge, such transient induction is so far the first demonstration for a stress-responsive asRNA promoter in cyanobacteria. For comparison, the *isrR* promoter is to present knowledge constitutively active (48). The fact that the C_l levels control the promoter activity of the as1_flv4 prompted us to search for a transcriptional regulator protein exerting this control. We selected the NdhR protein and the AbrB-like protein Sll0822 as reasonable candidates and studied their impact on the expression of as1_flv4 and the flv4-2 operon, respectively. NdhR, which is also known as CcmR (34), belongs to the LysR family of transcriptional regulators. This large family of prokaryotic regulators typically activates genes and responds to coinducers (55). One of the best characterized examples is BenM, which controls genes involved in aromatic compound degradation in *Acinetobacter* sp. ADP1 (56). In cyanobacteria, besides NdhR, also the LysR-type transcriptional regulator CmpR was demonstrated to be involved in LC-induced gene expression. CmpR acts as activator of the *cmp* operon encoding the ATP-binding cassette-type bicarbonate transporter BCT1 (35). By contrast, NdhR has been characterized as a repressor of a regulon that includes genes encoding components of the inducible C_l uptake system, such as *sbtA*, *ndhF3*, *ndhD3*, and *cupA* (16). Sll0822, an AbrB-like protein, is suggested to serve as a repressor of LC-induced genes such as *sbtA* and *ndhF3* (36) and is central in the regulation of carbon and nitrogen metabolism (49, 50). The results of our studies with mutants in either *ndhR* or *sll0822* are the basis for a hypothetical model (Fig. 7B) on the integrated function of the transcriptional regulators NdhR and Sll0822 and the asRNA As1_flv4 in C_l-controlled expression of the flv4-2 operon. Changes in CO_2_ partial pressure and alterations in the level of HCO_3_ (18), cAMP (57), or 2-phosphoglycerolate (20, 35) act as signals and trigger the cellular response toward LC stress. The transcriptional regulator NdhR is directly or indirectly involved in the activation of the promoter of the flv4-2 operon but has no effect on the accumulation of the asRNA (Fig. 5 and supplemental Fig. S3). Because we could not identify NdhR binding sites in the promoter regions of the flv4-2 operon, it is likely that NdhR has secondary impact on the expression of this operon as postulated previously (16). In contrast, the AbrB-like protein Sll0822 controls primarily or solely the expression of the asRNA As1_flv4 as demonstrated by accumulation of high amounts of As1_flv4 transcript in the mutant Δsll0822 (Fig. 6). Again, the control can be either direct or indirect. Predictions, however, are not possible because the binding sites of Sll0822 have not yet been identified. The tightly regulated amount of asRNA As1_flv4 functions as a tool to “fine-tune” the post-transcriptional level to assure timed biosynthesis of the proteins encoded by the flv4-2 operon. Furthermore, it allows the integration of different signal sources, which are transduced by NdhR or Sll0822, respectively. As a result, the production of the Flv2, Flv4, and Sll0218 proteins provides PSII with a photoprotection mechanism under LC conditions when the terminal electron acceptors are scarce.

The biological functions of the other two asRNAs, As2_flv4 and As_flv2, are not yet clear. The As2_flv4 transcript accumulation is similar to that of As1_flv4 and hence might back up the As1_flv4-dependent delay in target protein synthesis. The Northern blot experiments (Fig. 2) did not show an inverse correlation for As_flv2 and its target mRNA as is the case with As1_flv4. It might be that the asRNA As_flv2 rather acts as a stabilizing element on the flv4-2 operon or solely on the flv2 transcript. The biological function of the noncoding RNA Ncr0080 and the identity of its target mRNA in particular remain completely unknown, but the C_l-dependent accumulation points to a possible role in LC acclimation as well.

**Ecological Implications of asRNA-regulated Control of flv4-2 Operon Expression and Beyond**—The postulated function of some bacterial asRNAs is the establishment of a threshold for expression of the target gene. This threshold is thought to provide a safety mechanism against transcriptional noise or transient stress signals (48, 53, 54, 58). For *Synechocystis*, it is mandatory to avoid the premature expression of the flv4-2 operon after a shift from HC to LC conditions for the following reasons. First, each flavodiiron protein contains two iron molecules. The iron quota of *Synechocystis* is 1 order of magnitude higher than that of the similarly sized *Escherichia coli* (59), and its bioavailability in aquatic environments is frequently limited because Fe^{3+} forms insoluble crystals. Therefore, iron is often a limiting factor in the cyanobacterial environment (60) and should rather be available for essential enzymes involved in e.g. photosynthesis than wasted without a crucial reason like transient C_l limitation. Second, Sll0218, Flv2-, and Flv4-catalyzed reactions consume electrons from PSII (30). Under HC conditions, it is favorable for the cyanobacterium to direct the photosynthetically produced energy toward CO_2_ fixation and rather than to waste the energy in Flv4-2-mediated processes. The tight control of the flv4-2 operon is thought to ensure maximal photosynthetic efficiency in unstressed cells in HC and conversely to provide photoprotection upon stressful conditions in LC. However, it has to be noted that the growth conditions (e.g. low and continuous light) used here do not essentially reflect natural conditions. Hence, our suggested ecological implications have to be considered as potentially limited. The real environmental growth conditions provide substantially stronger, more diverse, and more frequent fluctuations in irradiance than our labora-
tory conditions, including day/night cycles and the combination with other stress conditions. Therefore, we assume that in natural environments it is much more important to buffer against short term environmental changes, e.g. to prevent the initiation of long term acclimation processes upon only a short term stress period. It also seems favorable to integrate different signals to prevent responses that are beneficial at an isolated stress condition but may be detrimental in a more complex superposition of different environmental and metabolic cues.

This biological important fine-tuning is likely to be a main function of many regulatory RNAs and especially cis-asRNAs (48).

To our knowledge, this is the first time the involvement of an asRNA (As1_flv4) in the bacterial C4-regulatory network has been demonstrated. The asRNA As1_flv4 contributes at least partially to the C4-dependent regulation of the Flv4-2 proteins by establishing the safety threshold and timely shifted expression during the early phase of LC acclimation. It is also of note that altogether 16 asRNAs and 29 ncRNAs respond to C4 limitation in Synechocystis (8). The observations made here for the strict control of the flv4-2 operon are also possibly applicable for other elements of the C4 regulon in Synechocystis.

Acknowledgments—We thank Annegret Wilde for helpful discussion and Tuomas Huovinen for support with the bioluminescence measurements. The mutant ∆ndhR was kindly provided by Robert Burnap, and the mutant ∆sl0822, originally created by Yukako Hihara, was provided by Aaron Kaplan. Martin Hagemann kindly provided the promoter test vector pILA. The plasmid pJet-spk was kindly provided by Ekaterina Kuchmina (A. Wilde laboratory). We thank Doreen Schwarz for support with ∆sl0822. We acknowledge technical support by Maija Holmström, Annina Leppäniemi, and Gudrun Krüger.

REFERENCES

1. Cuperus, J. T., Fahlgren, N., and Carrington, J. C. (2011) Evolution and functional diversification of MIRNA genes. Plant Cell 23, 431–442
2. De Lucia, F., and Dean, C. (2011) Long non-coding RNAs and chromatin regulation. Curr. Opin. Plant Biol. 14, 168–173
3. Zhou, X., Sunkar, R., Jin, H., Zhu, J. K., and Zhang, W. (2009) Genome-wide identification and analysis of small RNAs originated from natural antisense transcripts in Oryza sativa. Genome Res. 19, 70–78
4. Zhang, X., Xia, J., Li, Y. E., Barrera-Figueroa, B. E., Zhou, X., Gao, S., Lu, L., Niu, D., Chen, Z., Leung, C., Wong, T., Zhang, H., Guo, J., Li, Y., Liu, R., Liang, W., Zhu, J. K., Zhang, W., and Jin, H. (2012) Genome-wide analysis of plant nat-siRNAs reveals insights into their distribution, biogenesis and function. Genome Biol. 13, R20
5. Jin, H., Vacic, V., Girke, T., Lonard, S., and Zhu, J. K. (2008) Small RNAs and the regulation of cis-natural antisense transcripts in Arabidopsis. BMC Mol. Biol. 9, 6
6. Zhelyazkova, P., Sharma, C. M., Förster, K. U., Liere, K., Vogel, J., and Börner, T. (2012) The primary transcriptome of barley chloroplasts: numerous non-coding RNAs and the dominating role of the plastid-encoded RNA polymerase. Plant Cell 24, 123–136
7. Georg, J., Voss, B., Scholz, I., Mitschke, J., Wilde, A., and Hess, W. R. (2009) Evidence for a major role of antisense RNAs in cyanobacterial gene regulation. Mol. Syst. Biol. 5, 305
8. Mitschke, J., Georg, J., Scholz, I., Sharma, C. M., Dienst, D., Bantscheff, J., Voss, B., Steglich, C., Wilde, A., Vogel, J., and Hess, W. R. (2011) An experimentally anchored map of transcriptional start sites in the model cyanobacterium Synechocystis sp. PCC 6803. Proc. Natl. Acad. Sci. U.S.A. 108, 2124–2129
9. Mitschke, J., Vioque A., Haas F., Hess W. R., and Muro-Pastor, A. M. (2011) Dynamics of transcriptional start site selection during nitrogen stress-induced cell differentiation in Anabaena sp. PCC7120. Proc. Natl. Acad. Sci. U.S.A. 108, 20130–20135
10. Hernández, J. A., Muro-Pastor, A. M., Flores, E., Bes, M. T., Peleato, M. L., and Fillat, M. F. (2006) Identification of a furA cis antisense RNA in the cyanobacterium Anabaena sp. PCC 7120. J. Mol. Biol. 355, 325–334
11. Hernández, J. A., Alonso, I., Pellicer, S., Luisa Peleato, M., Cases, R., Strasser, R. J., Barja, F., and Fillat, M. F. (2010) Mutants of Anabaena sp. PCC 7120 lacking alr1690 and alpha-furA antisense RNA show a pleiotropic phenotype and altered photosynthetic machinery. J. Plant Physiol. 167, 430–437
12. Dühring, U., Axmann, I. M., Hess, W. R., and Wilde, A. (2006) An internal antisense RNA regulates expression of the photosynthesis gene isiA. Proc. Natl. Acad. Sci. U.S.A. 103, 7054–7058
13. Benschop, J. J., Badger, M. R., and Dean Price, G. (2003) Characterisation of CO2 and HCO3 uptake in the cyanobacterium Synechocystis sp. PCC6803. Photosynth. Res. 77, 117–126
14. McGinn, P. J., Price, G. D., Malesza, R., and Badger, M. R. (2003) Inorganic carbon limitation and light control the expression of transcripts related to the CO2-concentrating mechanism in the cyanobacterium Synechocystis sp strain PCC6803. Plant Physiol. 132, 218–229
15. Woodger, F. J., Badger, M. R., and Price, G. D. (2003) Inorganic carbon limitation induces transcripts encoding components of the CO2-concentrating mechanism is Synechococcus sp. PCC7942 through a redox-independent pathway. Plant Physiol. 133, 2069–2080
16. Wang, H. L., Postier, B. L., and Burnap, R. L. (2004) Alterations in global patterns of gene expression in Synechocystis sp. PCC 6803 in response to inorganic carbon limitation and the inactivation of ndhR, a LysR family regulator. J. Biol. Chem. 279, 5739–5751
17. Zhang, P., Battchkiva, N., Jansen, T., Appel, J., Ogawa, T., and Aro, E. M. (2004) Expression and functional roles of the two distinct NDH-1 complexes and the carbon acquisition complex NdhD3/NdhF3/CupA/Sll1735 in Synechocystis sp. PCC 6803. Plant Cell 16, 3326–3340
18. Woodger, F. J., Badger, M. R., and Price, G. D. (2005) Sensing of inorganic carbon limitation in Synechococcus PCC7942 is correlated with the size of the internal inorganic carbon pool and involves oxygen. Plant Physiol. 139, 1959–1969
19. Eisenhut, M., Kahlen, S., Hasse, D., Ewald, R., Lienam-Hurwitz, J., Ogawa, T., Ruth, W., Bawe, H., Kaplan, A., and Hagemann, M. (2006) The plant-like C2 glycolate cycle and the bacterial-like glycerate pathway cooperate in phosphoglycolate metabolism in cyanobacteria. Plant Physiol. 142, 333–342
20. Eisenhut, M., von Wobeser, E. A., Jonas, L., Schubert, H., Ibelings, B. W., Bawe, H., Matthijs, H. C., and Hagemann, M. (2007) Long-term response toward inorganic carbon limitation in wild type and glycolate turnover mutants of the cyanobacterium Synechocystis sp. strain PCC 6803. Plant Physiol. 144, 1946–1959
21. Eisenhut, M., Huege, J., Schwarz, D., Bawe, H., Kopka, J., and Hagemann, M. (2008) Metabolome phenotyping of inorganic carbon limitation in cells of the wild type and photorespiratory mutants of the cyanobacterium Synechocystis sp. strain PCC6803. Plant Physiol. 148, 2109–2120
22. Battchkiva, N., Vainonen, J. P., Vorontsova, N., Keranen, M., Carmel, D., and Aro, E. M. (2010) Dynamic changes in the proteome of Synechocystis sp. 6803 in response to CO2 limitation revealed by quantitative proteomics. J. Proteome Res. 9, 5896–5912
23. Schwarz, D., Nodop, A., Hüge, J., Purfürst, S., Forchhammer, K., Michel, K. P., Bawe, H., Kopka, J., and Hagemann, M. (2011) Metabolic and transcriptomic phenotyping of inorganic carbon acclimation in the cyanobacterium Synechococcus elongatus PCC 7942. Plant Physiol. 155, 1640–1665
24. Kaplan, A., and Reinhold, L. (1999) CO2 concentrating mechanisms in photosynthetic microorganisms. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50, 539–570
25. Badger, M. R., and Price, G. D. (2003) CO2 concentrating mechanisms in cyanobacteria: molecular components, their diversity and evolution. J. Exp. Bot. 54, 609–622
26. Giordano, M., Beardall, I., and Raven, J. A. (2005) CO2 concentrating mechanisms in algae: mechanisms, environmental modulation, and evo-
Effects of an asRNA on Gene Expression in Synechocystis

32. Vicente, J. B., Gomes, C. M., Wasserfallen, A., and Teixeira, M. (2002) Flavodiiron proteins in oxygenic photosynthetic organisms: photoprotection of photosystem II by FvL2 and FvL4 in *Synechocystis* sp. PCC 6803. *PLoS One* **4**, e5331.

27. Price, G. D., Badger, M. R., Woodger, F. J., and Long, B. M. (2008) Advances in understanding the cyanobacterial CO2-concentrating-mechanism (CCM): functional components, C4 transporters, diversity, genetic regulation and prospects for engineering into plants. *J. Exp. Bot.* **59**, 1441–1461.

29. Allahverdiyeva, Y., Ermakova, M., Eisenhut, M., Zhang, P., Richaud, P., Aro, E. M., and Tabata, S. (1996) Sequence analysis of the genome of the unicellular cyanobacterium *Synechococcus elongatus* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. *DNA Res.* **3**, 109–136.

28. Zhang, P., Eisenhut, M., Brandt, A. M., Carmel, D., Silén, H. M., Vass, I., Allahverdiyeva, Y., Salminen, T. A., and Aro, E. M. (2012) Operon fvl-fvl4 provides cyanobacteria with a novel photoprotection mechanism. *Plant Cell* **24**, 1952–1971.

26. Majdalani, N., Cunning, C., Sledjeski, D., Elliott, T., and Gottesman, S. (1998) DsrA RNA regulates translation of RpoS message by an anti-antisense mechanism, independent of its action as an antisilencer of transcription. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 12462–12467.

25. Janssen, T., Kidron, H., Soitamo, A., Salminen, T., and Mäenpää, P. (2003) Biaryl-type protein kinase, SpkA, is required for normal motility of the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803. *J. Bacteriol.* **185**, 1505–1510.

24. Kamei, A., Yuasa, T., Orikawa, K., Geng, X. X., and Ikeuchi, M. (2001) An AbrB-like transcriptional regulator, SpkA, is required for normal motility of the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803. *J. Bacteriol.* **183**, 29059–29066.

23. Altuvia, S., Weinstein-Fischer, D., Zhang, A., Postow, L., and Storz, G. (1997) A small, stable RNA induced by oxidative stress: role as a pleiotropic regulator and antimutator. *Cell* **90**, 43–53.

22. Ishii, A., and Hihara, Y. (2008) An AbrB-like transcriptional regulator, Sll0822, is essential for the activation of nitrogen-regulated genes in *Synechocystis* sp. PCC 6803. *Plant Physiol.* **148**, 660–670.

21. Nishimura, T., Takahashi, Y., Yamaguchi, O., Suzuki, H., Maeda, S., and Omata, T. (2008) Mechanism of low CO2-induced activation of the cnp1 bicarbonate transporter operon by a LysR family protein in the cyanobacterium *Synechococcus elongatus* strain PCC 7942. *Mol. Microbiol.* **68**, 98–109.

20. Yamauchi, Y., Kaniya, Y., Kaneko, Y., and Hihara, Y. (2011) Physiological roles of the cyAbrB transcriptional regulator pair Sll0822 and Sll0359 in *Synechocystis* sp. strain PCC 6803. *J. Bacteriol.* **193**, 3702–3709.

19. Summerfield, T. C., Toepel, J., and Shererman, L. A. (2008) Low-oxygen induction of normally cryptic *psbA* genes in cyanobacteria. *Biochemistry* **47**, 12939–12941.

18. Summerfield, T. C., and Sherman, L. A. (2008) Global transcriptional response of the alkali-tolerant cyanobacterium *Synechocystis* sp. strain PCC 6803 to a pH 10 environment. *Appl. Environ. Microbiol.* **74**, 5276–5284.

17. Ishii, A., and Hihara, Y. (2008) An AbrB-like transcriptional regulator, Sll0822, is essential for the activation of nitrogen-regulated genes in *Synechocystis* sp. PCC 6803. *Plant Physiol.* **148**, 660–670.

16. Altuvia, S., Weinstein-Fischer, D., Zhang, A., Postow, L., and Storz, G. (1997) A small, stable RNA induced by oxidative stress: role as a pleiotropic regulator and antimutator. *Cell* **90**, 43–53.

15. Ishii, A., and Hihara, Y. (2008) An AbrB-like transcriptional regulator, Sll0822, is essential for the activation of nitrogen-regulated genes in *Synechocystis* sp. PCC 6803. *Plant Physiol.* **148**, 660–670.

14. Ishii, A., and Hihara, Y. (2008) An AbrB-like transcriptional regulator, Sll0822, is essential for the activation of nitrogen-regulated genes in *Synechocystis* sp. PCC 6803. *Plant Physiol.* **148**, 660–670.

13. Ishii, A., and Hihara, Y. (2008) An AbrB-like transcriptional regulator, Sll0822, is essential for the activation of nitrogen-regulated genes in *Synechocystis* sp. PCC 6803. *Plant Physiol.* **148**, 660–670.

12. Ishii, A., and Hihara, Y. (2008) An AbrB-like transcriptional regulator, Sll0822, is essential for the activation of nitrogen-regulated genes in *Synechocystis* sp. PCC 6803. *Plant Physiol.* **148**, 660–670.

11. Ishii, A., and Hihara, Y. (2008) An AbrB-like transcriptional regulator, Sll0822, is essential for the activation of nitrogen-regulated genes in *Synechocystis* sp. PCC 6803. *Plant Physiol.* **148**, 660–670.

10. Ishii, A., and Hihara, Y. (2008) An AbrB-like transcriptional regulator, Sll0822, is essential for the activation of nitrogen-regulated genes in *Synechocystis* sp. PCC 6803. *Plant Physiol.* **148**, 660–670.

9. Ishii, A., and Hihara, Y. (2008) An AbrB-like transcriptional regulator, Sll0822, is essential for the activation of nitrogen-regulated genes in *Synechocystis* sp. PCC 6803. *Plant Physiol.* **148**, 660–670.

8. Ishii, A., and Hihara, Y. (2008) An AbrB-like transcriptional regulator, Sll0822, is essential for the activation of nitrogen-regulated genes in *Synechocystis* sp. PCC 6803. *Plant Physiol.* **148**, 660–670.

7. Ishii, A., and Hihara, Y. (2008) An AbrB-like transcriptional regulator, Sll0822, is essential for the activation of nitrogen-regulated genes in *Synechocystis* sp. PCC 6803. *Plant Physiol.* **148**, 660–670.

6. Ishii, A., and Hihara, Y. (2008) An AbrB-like transcriptional regulator, Sll0822, is essential for the activation of nitrogen-regulated genes in *Synechocystis* sp. PCC 6803. *Plant Physiol.* **148**, 660–670.

5. Ishii, A., and Hihara, Y. (2008) An AbrB-like transcriptional regulator, Sll0822, is essential for the activation of nitrogen-regulated genes in *Synechocystis* sp. PCC 6803. *Plant Physiol.* **148**, 660–670.

4. Ishii, A., and Hihara, Y. (2008) An AbrB-like transcriptional regulator, Sll0822, is essential for the activation of nitrogen-regulated genes in *Synechocystis* sp. PCC 6803. *Plant Physiol.* **148**, 660–670.

3. Ishii, A., and Hihara, Y. (2008) An AbrB-like transcriptional regulator, Sll0822, is essential for the activation of nitrogen-regulated genes in *Synechocystis* sp. PCC 6803. *Plant Physiol.* **148**, 660–670.

2. Ishii, A., and Hihara, Y. (2008) An AbrB-like transcriptional regulator, Sll0822, is essential for the activation of nitrogen-regulated genes in *Synechocystis* sp. PCC 6803. *Plant Physiol.* **148**, 660–670.

1. Ishii, A., and Hihara, Y. (2008) An AbrB-like transcriptional regulator, Sll0822, is essential for the activation of nitrogen-regulated genes in *Synechocystis* sp. PCC 6803. *Plant Physiol.* **148**, 660–670.