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The LysR-type transcription factor PacR is a global regulator of photosynthetic carbon assimilation in *Anabaena*

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Summary

Cyanobacteria perform water-splitting photosynthesis and are important primary producers impacting the carbon and nitrogen cycles at global scale. They fix CO_2 through ribulose bisphosphate carboxylase/oxygenase (RuBisCo) and have evolved a distinct CO_2 concentrating mechanism (CCM) that builds high CO_2 concentrations in the vicinity of RuBisCo favoring its carboxylase activity. Filamentous cyanobacteria such as *Anabaena* fix CO_2 in photosynthetic vegetative cells, which donate photosynthate to heterocysts that rely on a heterotrophic metabolism to fix N_2. CCM elements are induced in response to inorganic carbon limitation, a cue that exposes the photosynthetic apparatus to photodamage by over-reduction. An *Anabaena* mutant lacking the LysR-type transcription factor All3953 grows poorly and dies under high light. The *rbcL* operon encoding RuBisCo is induced upon carbon limitation in the wild type but not in the mutant. ChIP-Seq analysis was used to globally identify All3953 targets under carbon limitation. Targets include, besides *rbcL*, genes encoding CCM elements, photorespiratory pathway, photosystem- and electron transport-related components, and factors, including flavodiiron proteins, with a demonstrated or putative function in photoprotection. qRT-PCR analysis of selected All3953 targets showed regulation in the wild type but not in the mutant. All3953 (PacR) is a global regulator of carbon assimilation in an oxygenic photoautotroph.

Introduction

As the organisms that developed oxygenic photosynthesis, cyanobacteria have played a crucial role in Earth's history and the evolution of life in our planet. Indeed, the production of O_2 as a result of cyanobacterial activity was responsible for the oxidation of the Earth's atmosphere about 2.5-2.3 billion years ago (Lyons *et al*., 2014). Furthermore, all the extant plastids of eukaryotic algae and plants are of cyanobacterial origin. Cyanobacteria were the first organisms to link the activity of the two types of photosystems (PSI and PSII), which allowed the generation of high electrochemical potential, and to combine them with a H_2O-splitting complex. Nowadays, most cyanobacteria are phototrophs relying on oxygenic photosynthesis to generate ATP and reducing equivalents for the fixation of CO_2 and the assimilation of inorganic nitrogen. Indeed, they are responsible for an important fraction of the primary productivity in the Earth's oceans, where they are important CO_2 and N_2 fixers, thus impacting the C and N cycles at a global scale (Knoll 2008; Price *et al*., 2008).

The enzyme responsible for the bulk of CO_2 fixation in the biosphere is ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo), which has a relatively low affinity for CO_2 and, moreover, it can also accept O_2 as a substrate. Compensating for this relatively low performance, RuBisCo is considered the most abundant enzyme on Earth. As a carboxylase, RuBisCo catalyzes the first step of the Calvin-Benson-Bassham (CBB) cycle, i.e., the incorporation of atmospheric CO_2 into ribulose-1,5-bisphosphate to give two molecules of 3-phosphoglycerate. As an oxygenase, it catalyzes the incorporation of O_2 into ribulose-bisphosphate, which produces 2-phosphoglycolate that leads to photorespiration with a subsequent loss of fixed C and energy. To increase the efficiency of CO_2 fixation, cyanobacteria have developed a distinct CO_2 concentrating mechanism (CCM) constituted by inorganic carbon (C_i) transporters that incorporate bicarbonate and CO_2 into the cell, and a proteinaceous compartment, the carboxysome, where RuBisCo, together with carbonic anhydrase, is

confined (Price *et al*., 2008; Cameron *et al*., 2014). Three high-affinitiy bicarbonate transporters (the ABC-type Cmp, also called BCT1, and the Na^+-dependent BicA and SbtA), and two CO_2 transporters (the high-affinity NDH-I_3 and the low-affinity NDH-I_4) have been identified in different cyanobacteria (see Price, 2011). Through CCM, Ci in the form of CO_2 can concentrate in the vicinity of cyanobacterial RuBisCo, to allow high specific activity for production of 3-phosphoglycerate to levels much higher than in plants (Cameron *et al*., 2014). Indeed, components of cyanobacterial CCM have been transformed in tobacco, with the result of improved specific activity of CO_2 fixation, which represents a step towards improved photosynthesis in plants (Lin *et al*., 2014).

In unicellular cyanobacteria, CCM elements are regulated by C_i availability. Especially genes encoding C_i transporters are induced, whereas the structural genes for carboxysome components and the *rbcL/S* genes encoding RuBisCo are only moderately responsive (see Price *et al*., 2008; Cameron *et al*., 2014). In chemotrophic bacteria the process of CO_2 fixation and the response to C_i limitation are usually controlled by LysR-type transcriptional regulators (LTTRs). The genes encoding the enzymes of the CBB cycle are usually found in clusters regulated by CbbR factors, which constitute a sub-family of LTTRs (Gibson and Tabita, 1996). In unicellular cyanobacteria, a number of CbbR homologs have been characterized. CmpR is an activator of the *cmp* genes (Nishimura *et al*., 2008), whereas CcmR (*aka* NdhR) acts as a transcriptional repressor of multiple genes encoding other C_i transporters (e.g., Figge *et al*., 2001; Wang *et al*., 2004). A third type of CbbR-like protein, the activator of the RuBisCo genes, has not yet been identified in cyanobacteria.

Filamentous heterocyst-forming cyanobacteria, of which *Anabaena* sp. PCC 7120 (hereafter *Anabaena*) is a model organism, additionally have the capacity for cell differentiation to turn some O_2-evolving photosynthetic cells of the filament into heterocysts, which are heterotrophic cells especialized in the fixation of atmospheric N_2. Thus, *Anabaena* is a truly pluricellular bacterium with different cell types specialized in different nutritional tasks that exchange nutrients and regulators and contribute to

the performance of the filament as the organism unit (see Flores and Herrero, 2010). Nitrogen assimilation and heterocyst differentiation in *Anabaena* are regulated by the global transcription factor NtcA, which responds to the cellular C-to-N ratio, and the heterocyst-specific transcription factor HetR (see Herrero *et al*., 2013). The *Anabaena* genomic sequence includes three genes annotated as CbbR-like LTTRs (Kaneko *et al*., 2001). Of these, *all0862* has been identified as *cmpR*, and its product activates the expression of the *cmp* operon (*alr2877-alr2880*) and the *cmpR* gene itself in response to C_i limitation (López-Igual *et al*., 2012). Notably, this regulation is effected in combination with NtcA, thus revealing a mode of co-regulation by C and N availability (López-Igual *et al*., 2012). Furthermore, in *Anabaena* the *rbcLXS* operon encoding RuBisCo, which is moderately induced under C_i limitation (López-Igual *et al*., 2012), is repressed in the heterocysts (Madan and Nierzwicki-Bauer, 1993), a regulation likely exerted by NtcA (Ramasubramanian *et al*., 1994; Picossi *et al*., 2014).

Here we have identified the CbbR-homolog All3953 as the activator of the RuBisCo-encoding operon in *Anabaena*. We have determined the All3953 regulon by ChIP-Seq, which has revealed that All3953 is a global regulator for C_i assimilation genes and genes involved in protection of the photosynthetic apparatus against oxidative damage that are regulated by Ci availability.

Results

All3953, an RbcR-like factor in Anabaena sp. PCC 7120

To gain insight into the LTTR All3953 in *Anabaena*, the expression of the *all3953* gene under different growth conditions was analyzed by northern blot. Three bands of hybridization corresponding to transcripts of ca. 1.9, 1.6 and 1.3 kb, respectively, which appeared similarly represented before and after Ci limitation could be observed (Supp. Fig. 1A). On the other hand, *all3953* expression did not significantly respond to N depletion (Supp. Fig. 1B). The latter result was consistent with previous global

transcriptional studies (Ehira and Ohmori, 2006; Flaherthy *et al*., 2011) and with the lack of binding associated to *all3953* of the N-control transcriptional regulator NtcA upon combined nitrogen depletion (Picossi *et al*., 2014).

Isolation and characterization of an all3953 mutant

To study the role of All3953, a mutant strain bearing an inactivated version of *all3953* was constructed (see Experimental procedures). In strain CSS74 most of the *all3953* gene was deleted and the C.S3 gene cassette (encoding Sm/Sp resistance) was introduced to facilitate segregation and maintenance of the mutation in *Anabaena* (Supp. Fig. 2A). As a control, strain CSS77 bearing the C.S3 gene cassette in the *Anabaena* plasmid alpha was also constructed. For *cis* complementation, a wild-type version of *all3953* was transferred to strain CSS74 in an integrative plasmid, generating strain CSS74C (Supp. Fig. 2A).

Strain CSS74 exhibited poor growth under standard growth conditions with ammonium as a nitrogen source, and it formed short filaments in liquid medium, whereas strain CSS74C behaved similarly to CSS77 (not shown). To quantify the deleterious effect of the *all3953* mutation, growth rates were calculated in liquid medium under different illumination and C_i-supply conditions. Growth rate of the control strain CSS77 was highest (0.892 $days^{-1}$) under high light (HL) and high carbon (HC), and was about 30% lower under the other tested conditions (Fig. 1A). In contrast, growth of strain CSS74 was severely affected under HL HC conditions (growth rate ca. 70% lower than that of the control) (Fig. 1A), under which it ended up dying after about 5 days (Fig. 1B). Under HL and low carbon (LC) or low light (LL) HC, the defect was close to 30% with regard to the control, although after prolonged incubation the mutant was more severely affected under HL LC. The defect was the smallest (ca. 15%) under LL LC conditions (Fig. 1A,B). In solid medium, growth of strain CSS74 was similar, and similar to that of the control, in the presence of ammonium, nitrate or no combined

nitrogen under LL LC conditions, whereas under HL LC a severe growth defect was observed with regard to the control with any of the tested nitrogen sources (not shown). In summary, the lack of All3953 had a deleterious effect on growth, especially under HL (and HC) conditions.

To further characterize the growth defect of the *all3953* mutant, the rate of oxygen evolution using CO_2 as a final electron acceptor was measured under different illumination conditions in the CSS74 mutant in relation to the control strain CSS77. When exponentially-growing cells were incubated for 24 h under LL HC, the oxygen evolution rate was slightly lower in strain CSS74 in comparison to CSS77 (88 and 105 μmol O_2·[mg Chl]$^{-1}$·h^{-1}, respectively). Under HL the difference between the two strains was larger (167, for CSS77, and 110, for CSS74, μmol O_2·[mg Chl]$^{-1}$·h^{-1}).

Effect of all3953 mutation on rbcLXS expression

To test the effect of the *all3953* mutation on the expression of the *rbcLXS* operon, northern blot analysis was performed with RNA isolated from cells of the control and mutant strains grown with HC and transferred to LC conditions. After 1 h incubation with LC, a ca. 2-fold increase in the amount of the *rbcLXS* transcripts could be observed in the control strain. No induction could be detected in the mutant (Fig. 2). In the complemented strain CSS74C the expression of *rbcLXS* increased in LC similarly to the control (Fig. 2). These results indicated that the induction of the *rbcLXS* operon upon C_i deficiency was dependent on All3953 and that the defect in strain CSS74 was exclusively due to the lack of All3953.

ChIP-Seq analysis of the All3953 targets

To determine the DNA targets of All3953 at a genomic level, we used chromatin immunoprecipitation followed by high-throughput sequencing analysis. To this end, we constructed a strain (CSS57) expressing from the *all3953* promoter a version of

All3953 C-terminally fussed to TAP-tag (Rigaut *et al*., 1999), as well as a control strain (CSS68) expressing the TAP-tag alone under the control of the *all3953* promoter (Supp. Fig. 2B, see Experimental procedures). Immunoprecipitation was carried out using cells of strains CSS57 and CSS68 grown with ammonium as the nitrogen source under HC conditions and incubated for 3 h with ammonium under LC conditions.

The analysis of the sequences resulted in a total of 142 All3953 binding regions, of which 127 were located in the chromosome, 10 in plasmid alpha, three in plasmid beta and two in plasmid gamma. Each binding region was ascribed to one or two genes according to the location (midpoint) of the region, and the relative location with respect to the assigned gene was also indicated (Table 1 and Supp. Table 1). A total of 175 genes were ascribed to the 142 binding regions. The binding regions were mostly located upstream of the ascribed genes (72%), whereas 21% were intragenic and 7% were located downstream of genes. The results of the ChIP-Seq analysis are available at GEO accession number GSE58861 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSE58861).

The 175 ascribed genes were classified according to their functional category (Table 2). Remarkably, there were 19 genes encoding proteins related to photosynthesis and respiration, and 21 genes encoding regulatory proteins, including All7179, a SigB homolog. The rest were mostly genes encoding hypothetical or unknown proteins (42%), but also genes encoding proteins involved in translation, in biosynthesis of amino acids and cofactors, prosthetic groups and carriers, in transport, and in other cellular processes. Table 3 highlights All3953-binding regions related to photosynthesis and respiration among which, confirming our results of gene inactivation, the gene encoding the large subunit of the RuBisCo (*rbcL*; binding region #37) is included. The fact that a high number of genes involved in photosynthesis and C fixation, including *rbcL*, were identified as targets of RbcR suggests that this protein is a global transcription factor for photosynthetic C assimilation.

A CisFinder analysis of the primary consensus motif was carried out based on 142 high-confidence ChIP-Seq peak sequences (Fig. 3A). The consensus motif found has a dyad-symmetry architecture and matches the consensus of LysR-recognition binding sites (RBS) (T-N_{11}-A) (Maddocks and Oyston, 2008), as well as the consensus binding sites proposed for CbbR factors (TNA-$N_{7/8}$-TNA). The primary motifs identified in the central 100 nt of the binding regions are indicated in Supp. Table 1 (for some binding regions more than one motif have been identified).

Expression analysis of some All3953 targets in Anabaena

To corroborate our ChIP-Seq analysis and to support the notion that All3953 is indeed a global regulator for C fixation genes, the expression levels of some of the photosynthesis- and C-fixation-related target genes, its response to C_i limitation and its dependence on All3953 was further analyzed by qRT-PCR (Fig. 4). Strains CSS77 and CSS74 were grown in ammonium and HC under standard light conditions (80 $\mu E \cdot m^{-2} \cdot s^{-1}$) at 30ºC up to the exponential phase. They were then transferred to medium with ammonium under LC conditions. As previously shown by northern analysis, transcription levels of the *all3953* gene did not significantly change after C_i deprivation in the control strain CSS77. As expected, *all3953* transcript levels were not detectable in CSS74, corroborating that the *all3953* mutation was segregated in this strain. The *rbcL* gene (*alr1524*) was 4.6-fold induced at 3 h after transfer to LC in strain CSS77, whereas no induction was observed in CSS74, thus corroborating the dependence on All3953. Interestingly, under HC the expression of *rbcL* in the mutant was higher than in the control strain, suggesting that besides as an activator under LC, All3953 could be acting as a repressor of *rbcL* under HC conditions.

ORF *all4446* (*flv4*) was highly induced (about 100-fold), and its level was maximum 1 h after the shift to LC (Fig. 4). In the *all3953* mutant, the basal transcription of *all4446* in HC was about 8-fold lower than in the control strain, and it was only

slightly induced (2-fold) upon the shift to LC. *all3891* (*flv1A*) was induced about 6-fold 3 h after the shift to LC in the control strain, whereas only a 2-fold induction was observed in the *all3953* mutant. *all1304* (bicarbonate transporter homolog) and *alr4156* (NdhF homolog) were both highly induced (up to 30- and 20-fold, respectively) upon transfer of strain CSS77 to LC. In contrast, no induction of *alr4156* and only a small induction of *all1304* took place in strain CSS74. Expression of *alr4592* (*psbAIII*) increased about 5-fold upon C_i deprivation in CSS77, but did not appreciably change in CSS74. The *alr0223* (NdhA homolog) gene was about 2-fold induced under C_i deprivation in CSS77 but not in CSS74. Finally, the expression of *alr1004* (alanine-glyoxylate aminotransferase) was repressed by 5-fold under LC conditions in CSSL77 but not in CSS74. These results confirm that expression of the above studied genes is regulated, either positively or negatively, by All3953.

Discussion

We have identified the LTTR All3953 as the activator of the RuBisCo-encoding genes in the cyanobacterium *Anabaena* sp. PCC 7120. All3953 appears to activate the *rbcL* operon under C_i limitation and to repress it when C_i is abundant. An LTTR factor regulating the expression of the *rbcL* operon has not, to our knowledge, been described in any cyanobacterium. The expression of *all3953* does not respond to C_i limitation (Supp. Fig. 1) and, indeed, no binding region of All3953 was found ascribed to *all3953*. Thus, the *all3953* gene seems to belong to the non-autoregulated LTTRs. All3953 shares 28 and 26% identical residues with NdhR from *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002 (CcmR), respectively.

By ChIP-Seq analysis of a strain bearing a TAP-tagged version of All3953, we have determined 142 All3953-bound regions at 3 h after transfer from high to low C_i conditions, which have been assigned to 177 genes. Apart from genes encoding unknown or hypothetical proteins, the larger category is of genes encoding regulatory

proteins, including the transcriptional regulators Alr0353 (a LTTR) and All4500 (CRP-like), the two-component response regulator All3348, the two-component sensor histidine-kinase All1145 and the group 2-sigma 70-type sigma factor All7179 (Supp. Table 1). Interestingly, when comparing to *Synechocystis* sigma factors, All7179 (SigB4) is more similar to SigB, which along with SigD has been shown to be important for PSII recovery in this unicellular cyanobacterium (Pollari *et al*., 2009). All3953 also binds upstream of genes *patS* and *hetN*, whose products regulate heterocyst differentiation (Supp. Table 1). The fact that All3953 binds to the promoter region of genes encoding other regulatory proteins suggests a wide role of this protein in the physiology of the organism.

In the promoter region of the *rbcL* operon we have found three putative binding sites for All3953, Box I, Box II, and Box III (Fig. 3B) that resemble the consensus recognition sequence found by Cisfinder analysis (Fig. 3A). It is conceivable that, like in some other LTTRs these boxes combine repression and activation sites. In this regard, binding of All3953 to Box III, overlapping gene promoter elements, could be related to the repression of *rbcL* observed under high C_i (Fig. 4). On the other hand, as mentioned above, the *rbcL* operon is repressed in heterocysts by the global transcriptional regulator NtcA, for which a binding site is found overlapping the operon TSP (Ramasubramanian *et al*., 1994; Picossi *et al*., 2014) (Figure 3B). It is conceivable that NtcA binding in these differentiated cells interfere with All3952-mediated activation.

Besides being the activator of the RuBisCo genes, All3953 targets include genes involved in other processes related to carbon assimilation, such as C_i transport (*all1304*, encoding an homolog of the BicA bicarbonate transporter; *alr4156* encoding a homolog of the NdhF3 subunit of the NDH-1_3 CO_2 uptake system; and *alr0869* encoding a homolog of the NdhF4 subunit of the NDH-1_4 CO_2 uptake system); components of the carboxysome shell (*all0868,* putative *ccmK*) and 2-phosphoglycolate metabolism (*alr1004* and *alr2873*, possibly related to photorespiration [Eisenhut *et al*., 2008]). Notably, All3953 targets include also a

number of genes encoding photosystem components, such as *alr5154* (*psaA*, encoding the PSI core protein PsaA), *alr3727* (*psbAII*, encoding a component of form II of the PSII core protein PsbA [D1]), *alr4592* (*psbAIII*, encoding another component of form II of PsbA) and *alr1216* (PSII 12 kD extrinsic protein PsbU), and genes related to PS activity. In the latter group are *alr4149* (biliverdin reductase, putatively involved in phycobilisome -PSII antenna- synthesis), and genes that can participate in photosynthetic electron transfer, such as *alr0223* and *alr0348* (*ndhA* and *ndhD*, subunits of other putative NADH dehydrogenases), *alr1576* (dehydrogenase subunit), *all0737* (thioredoxin reductase), *all1365* (CytM cytochrome), *all4148* (ferredoxin I), *all3891* and *all4446* (flavodiiron proteins Flv1A and Flv4, respectively). (Besides in CO_2 uptake, NdhF3 and NdhF4 can also participate in electron transfer.)

Reports on gene expression regulation by C_i are scarce for *Anabaena*. However, in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 transcriptomic analysis has already shown down-regulation of some genes encoding polypeptides of PSI and PSII complexes as well as of phycobilisome components, upon transfer to C_i limitation conditions, likely as an adaptation to lower assimilatory power demand, and up-regulation of some PSII core polypeptides, interpreted as adaptation to conditions of shortage of electron acceptors that could lead to photodamage and increased turnover of PS core components (Wang *et al*., 2004). Our analysis extends the array of photosynthetic genes responding to C_i limitation, remarkably to include the core PSI reaction center *psaA* gene. Moreover, except for *alr1004* that responds negatively, all the *Anabaena* photosynthetic genes mentioned above increase expression upon the shift to C_i limitation.

Noteworthy, for the majority of photosynthetic gene targets of All3953 in *Anabaena*, a function in protection against reactive oxygen species, which can be generated by C_i limitation or exposure to HL, has either been described or could be predicted. Thus, the *psbAII* and *psbAIII* genes are induced under HL in the unicellular *Synechococcus* sp. PCC 7942, and cells cultured under HL showed more form II, and

less form I (encoded by *psbAI*), of D1 compared to cells under LL (Schaefer and Golden, 1989). Regarding flavodiiron proteins genes, *flv1A, flv3A*, and specially *flv2* and *flv4* of *Anabaena* have been shown up-regulated in vegetative cells in low Ci, and *flv1A* and *flv3A* also in high light, whereas *flv1B* and *flv3B* are expressed exclusively in heterocysts (Ermakova *et al*., 2013). Whereas Flv1A and Flv3A appear involved in photoreduction of oxygen to water by removing excess electrons from PSI through NAD(P)H dehydrogenases, Flv2 and Flv4 could have a role in photoprotection of PSII under low Ci (Ermakova *et al*., 2013). Regarding photorespiration, it has also been considered to have a role in removal of excess O_2 (Eisenhut *et al*., 2008). To the best of our knowledge, the regulator responsible for the response to C_i availability of any photosynthetic gene has not been identified in cyanobacteria (oxygenic phototrophs). Our ChIP-Seq and expression analysis indicate that All3953 is a regulator of photosynthetic genes in *Anabaena.*

The growth rate of *Anabaena* is highest under HL HC conditions (Fig. 1), implying that this cyanobacterium has mechanisms to get profit of HC while counteracting HL stress. The *all3953* mutant strain CSS74 exhibited a growth defect in all the conditions tested, but especially under HL, where it ends-up dying (Fig. 1). This shares the idea that in *Anabaena* All3953 is required to cope with HL stress. The effect of the lack of All3953 seems more detrimental in relation to impaired photoprotection than to impaired C_i scavenging (preference of the mutant for LL LC over HL HC conditions). Indeed, even in LL, the mutant seems to perform slightly better with LC than with HC (Fig. 1). Although LC could suppose a limitation of electron acceptors, an increased rate of photorespiration under LC together with the fact that some of the All3953 targets that could cope with excess oxygen are residually induced upon the transfer from HC to LC in the CSS74 mutant (Fig. 4) could contribute to the preference of this strain for LC over HC, especially under HL.

Our results show that in *Anabaena* the responses to C_i availability include regulation of genes encoding elements of CCM and RuBisCo, but also of

photosynthetic genes to adjust generation of assimilatory power while preserving the photosynthetic apparatus from oxidative damage, which is specially relevant in oxygenic phototrophs. Because All3953 is a transcriptional regulator globally coordinating these responses, we have named it PacR (Photosynthetic assimilation of carbon Regulator).

Experimental procedures

Strains

Anabaena sp. strain PCC 7120 was grown photoautrophically at 30°C with illumination (80 $\mu E \cdot m^{-2} \cdot s^{-1}$) in liquid $BG11_0$ medium (Rippka *et al*., 1979) supplemented with 3 mM NH_4Cl, 6 mM TES buffer and 10 mM $NaHCO_3$ and bubbled with a mixture of CO_2 and air (1% v/v) (HC). Other conditions used were no $NaHCO_3$ supplement and bubbling with air (LC); 12 $\mu E \cdot m^{-2} \cdot s^{-1}$ (LL); 175 $\mu E \cdot m^{-2} \cdot s^{-1}$ (HL). For the mutants generated in this work, antibiotics were used at the following concentrations: Sm, 2 $\mu g\ ml^{-1}$; Sp, 2 $\mu g\ ml^{-1}$; and Nm, 25 $\mu g\ ml^{-1}$ for bubbled cultures; and Sm, 5 $\mu g\ ml^{-1}$; Sp, 5 $\mu g\ ml^{-1}$; and Nm, 40 $\mu g\ ml^{-1}$ for cultures in solid medium.

Strain construction

To construct a mutant of the *all3953* gene, the 5' and 3' end of the gene, along with the flanking regions, were PCR amplified using chromosomal DNA of PCC 7120 as the template and primers all3953-14 (BglII) and all3953-15 (SalI), and primers all3953-16 (SalI) and all3953-17 (PstI), respectively (all primers are specified in Supp. Table 2). The PCR products were digested with SalI and ligated. The resulting mixture was used as a template for overlapping PCR with primers all3953-14 and all3953-17. The new PCR product was digested with BglII and PstI and ligated to the BglII-PstI-digested pRL271 (Black *et al*., 1993), obtaining plasmid pCSS161. Plasmid pCSS161 was digested with SpeI and the 2-kb Sm^r Sp^r gene cassette C.S3, excised with XbaI from

pCSE120 [S.K3/L.HEH2 (BamHI)/C.S3 (BamHI); nomenclature as in (Elhai and Wolk, 1988)], was inserted obtaining plasmid pCSS162. Plasmid pCSS162 was transferred to strain PCC 7120 by conjugation (Elhai *et al*., 1997). Exconjugants resistant to Sm and Sp, which had the Δ*all3953*::C.S3 construct integrated by double recombination were selected, obtaining strain CSS74. The segregation of the mutation was tested by PCR (Supp. Fig. 2) with primers all3953-14, all3953-15, all3953-17 and all3953-20.

To construct a control strain expressing Sm^r and Sp^r plasmid pCSS163, a derivative of plasmid pCSEL24 (Olmedo-Verd *et al*., 2006) containing the C.S3 gene cassette, was transferred to *Anabaena* by conjugation. Exconjugants that had the pCSS163 integrated in the alpha plasmid of *Anabaena* were selected, obtaining the strain CSS77 (Supp. Fig. 2).

To complement the *all3953* mutation of strain CSS74, a DNA fragment encompassing the whole *all3953* gene and sequences upstream from it was amplified by PCR using the primer pair all3953-24/all3953-25, both including EcoRI sites, and strain PCC 7120 DNA as the template. This fragment was cloned in the EcoRI site of the mobilizable Nm^r encoding vector pRL424 (Elhai and Wolk, 1988) producing plasmid pCSS164, which was transferred to strain CSS74 by conjugation followed by selection for Nm^r. The genomic structure of the exconjugants in the *all3953* region (Supp. Fig. 2) was corroborated by PCR.

To construct a strain expressing All3953-C-TAP, the *all3953* gene (including the upstream region) was PCR-amplified with primers all3953-11 and all3953-12 and DNA of PCC 7120 as the template. The TAP-tag was PCR-amplified with primers TAPtag-1 and TAPtag-2 using DNA of plasmid pBS1479 as the template (Puig *et al*., 2001). The two PCR products were digested with SalI and ligated, after which the ligation product was used as the template for an overlapping PCR using primers all3953-11 and TAPtag-2. The PCR product was digested with PstI and ligated to the mobilizable vector pCSV3 (Valladares *et al*., 2011) digested with PstI, rendering plasmid pCSS107.

To construct a control strain with the TAP-tag under the control of the *all3953* promoter, a 0.4-kb region upstream of *all3953* was PCR-amplified using primers all3953-11 and all3953-18 and DNA of pCSS107 as the template. The PCR product was digested with SalI and ligated to the PCR-amplified TAP-tag digested with SalI, after which the ligation product was used as the template for an overlapping PCR using primers all3953-11 and TAPtag-2. The PCR product was digested with PstI and ligated to PstI-digested pCSV3 to give plasmid pCSS157. Plasmids pCSS107 and pCSS157 were transferred by conjugation to strain PCC 7120 and single Sm^r Sp^r recombinants were selected, obtaining strain CSS57 and CSS68, respectively. Western blots using Peroxidase-Anti-Peroxidase Soluble Complex (PAP, Sigma-Aldrich) were performed to ensure that the two strains expressed the TAP-tag (Supp. Fig. 2B).

Chromatin immunoprecipitation

Cells of strains CSS57 growing exponentially (3-5 µg Chl·ml^{-1}) in the light (80 µE·m^{-2}·s^{-1}) in medium supplemented with 2 µg·ml^{-1} Sm and Sp, in HC conditions were incubated with LC for 3 h. Formaldehyde was then added to the cultures to a final concentration of 1%, and the cultures were incubated for 15 min. Glycine was added at 125 mM final concentration and the incubation was continued for 5 min to stop the fixing reaction. The cells were then filtered, washed with cold TBS (20 mM Tris-HCl, pH 7.4, 140 mM NaCl) and collected in tubes (25 ml of culture per tube). The pellets were frozen in liquid nitrogen and stored at -20°C until used. Pellets corresponding to about 25 ml of culture were resuspended in 500 µl of lysis buffer (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, supplemented with Mini EDTA-free protease inhibitor cocktail [Roche]) and, after addition of 150 µl of glass beads (acid-washed, 425-600 µm [Sigma]), the cells were broken in a multivortexer at 2,000 rpm for 1 h at 4°C. The cell lysates were collected by centrifugation and the extracts were subjected to sonication to shear the DNA to about 300-bp fragments (60 cycles of 10 s, 20 s ice, 15% amplitude, in a Branson Digital

Sonifier). After centrifugation to eliminate cell debris, the whole-cell extracts were stored at −20°C or immediately used for immunoprecipitation.

Immunoprecipitation of DNA was carried out as described (Picossi *et al*., 2014), with some modifications. Whole-cell extracts were prepared at 4 $mg \cdot ml^{-1}$ of total protein with lysis buffer (in 500 µl total volume). A 50-µl sample was taken as the input sample, and the extracts were incubated with 15 µl IgG-conjugated Dynabeads (about 6 µg IgG) at 4ºC with rotation for 12-14h. The washes of the Dynabeads, as well as the elution of the immunoprecipitated material, the crosslinking reversion and the isolation of the DNA were performed as in (Picossi *et al*., 2014).

Massive sequencing of the immunoprecipitated DNA

Input and ChIP DNA samples were sent for sequencing to the Functional Genomics Core Facility of the Institute for Research in Biomedicine, Barcelona (Spain). Next generation sequencing was carried out using Illumina's sequencing technology. ChIP DNA Sample Prep Kit (Illumina) was used for library preparation. Libraries were loaded at 8 pM concentration into the flow cell using the Cluster Station running recipe V7 with the Single-Read Cluster Generation Kit v4 (all Illumina). The flow cell was loaded into the Genome Analyzer II and samples were sequenced for 120 nucleotides from a single end using the Sequencing Kit v5 and recipe v8 (all Illumina). Manufacturer's recommendations were strictly followed. Illumina sequencing data were pre-processed with the standard Illumina pipeline version 1.5 and sequences were aligned to the PCC 7120 genome (http://genome.microbedb.jp/cyanobase/Anabaena) with the Bowtie software 0.12.5 (Langmead *et al*., 2009). The analysis of the results were carried out using the Triform algorithm (Kornacker *et al*., 2012) as in (Picossi *et al*., 2014). The sequences in the ChIP samples of strain CSS68 (TAP control) were used as the background of the sequences found for strain CSS57 (All3953-TAP), and thus to determine the specific binding regions of All3953-TAP in the genome of *Anabaena*.

The binding regions were visualized and analyzed using the UCSC Microbial Genome Browser (Scheneider *et al*., 2006). They were ascribed to one or two genes, in case it was not possible to ascribe them to only one, and classified as upstream of the gene, if the midpoint of the binding region was located upstream of the start of the gene, internal, if the midpoint of the binding region was inside the gene, or downstream, if the midpoint of the binding region was located downstream of the end of the gene to which it had been ascribed.

Northern and qRT-PCR analyses

Isolation of total RNA from *Anabaena* was done as described previously (Mohamed and Jansson, 1989). Northern analysis was performed as described previously (López-Igual *et al*., 2012).

For qRT-PCR, 750 ng of DNA-free RNA samples were used for all the PCR primer pairs. For the RT reaction, the Quantitech Reverse transcription kit (Qiagen), with the Random Hexamer Primer mix (100 ng per sample) (Bioline) was used. The cDNA produced was diluted 7.5 times to use 2 µl of cDNA per PCR reaction. PCR was done using the Quantimix Easy SYG Kit (Biotools) (SYBR green I) in a iCycler iQ Multi-Color Real Time PCR Detection System (Bio-Rad). The abundance of a transcript in the RNA sample was calculated as: abundance= 2^[Ct(sample)-Ct(control)], where the RNA sample of the control strain CSS77 in HC condition (0) was used as the control.

Oxygen evolution

2-ml samples of exponentially grown cultures in HC LL or HC HL conditions were used to measure O_2 evolution with an O_2 electrode calibrated with culture medium and $Na_2S_2O_4$ as the reducing agent. O_2 production was measured in the light (400 $\mu E \cdot m^{-2} \cdot s^{-1}$) after a seven-minute incubation in the dark.

Acknowledgments

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Figure legends:

Fig. 1. Growth of the *all3953* mutant. A, The growth rate constant (μ = ln2/t_d, where t_d is the doubling time) was calculated from the increase of protein content determined in 0.2 ml samples of cultures. The table shows the mean and standard deviation from 3 independent cultures of each strain and condition. Δ*all3953* is strain CSS74; CSS77 is a control strain that carries the Sm/Sp-resistant determinant in a wild-type background. B, Samples of cultures were photographed after 5 days of incubation under the indicated conditions. HL, high light; LL, low light; HC, high carbon; LC, low carbon.

Fig. 2. Expression of *rbcLXS* in the Δ*all3953* mutant and complemented strain. Northern analysis carried out with RNA from strains CSS77 (control) CSS74 (Δ*all3953*) and CSS74C (CSS74 complemented) was isolated from cells grown with HC (0) and incubated for 1h (1) with LC. The membranes were hybridized with an internal fragment of the *rbcL* gene (upper panels) and, as a loading and transfer control, of the *rnpB* gene (lower panels). Arrowheads point to the main transcripts detected with the *rbcL* gene probe (approximate sizes are indicated).

Fig. 3. Consensus All3953 binding sequence and *rbcL* promoter. A, The primary consensus motif based on 142 high confidence CSS57 ChIP-Seq peak sequences is shown with indication of the probability of occurrence of each base along the 22-nt sequence. W is A or T; Y is C or T; B is C, G or T. B, Structure of the *rbcLXS* promoter region. The transcription initiation point of the operon (+1) and the -10 and -35 boxes (from Nierzwicki-Bauer *et al*., 1984) are indicated in red. The NtcA-binding site ($GTAN_8TAC$) is indicated in green, and the three putative binding sites for All3953 (Box I, Box II and Box III) are indicated in blue.

633 **Fig. 4.** qRT-PCR analysis of the expression of selected photosynthesis and respiration-
634 related All3953 gene targets. Transcriptional response of the indicated genes to C_i
635 limitation in the control (CSS77) and Δ*all3953* mutant (CSS74) strains was
636 investigated. RNA was isolated from cells grown with 10 mM $NaHCO_3$-supplemented
637 medium bubbled with 1%CO_2 in air (0) incubated for 1 h (1) or 3 h (3) in $NaHCO_3$-free
638 medium bubbled with air. Bars represent the mean transcript levels (± standard
639 deviation) in three independent experiments.

640

The LysR-type transcription factor PacR is a global regulator of photosynthetic carbon assimilation in *Anabaena*

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Summary

Cyanobacteria perform water-splitting photosynthesis and are important primary producers impacting the carbon and nitrogen cycles at global scale. They fix CO_2 through ribulose bisphosphate carboxylase/oxygenase (RuBisCo) and have evolved a distinct CO_2 concentrating mechanism (CCM) that builds high CO_2 concentrations in the vicinity of RuBisCo favoring its carboxylase activity. Filamentous cyanobacteria such as *Anabaena* fix CO_2 in photosynthetic vegetative cells, which donate photosynthate to heterocysts that rely on a heterotrophic metabolism to fix N_2. CCM elements are induced in response to inorganic carbon limitation, a cue that exposes the photosynthetic apparatus to photodamage by over-reduction. An *Anabaena* mutant lacking the LysR-type transcription factor All3953 grows poorly and dies under high light. The *rbcL* operon encoding RuBisCo is induced upon carbon limitation in the wild type but not in the mutant. ChIP-Seq analysis was used to globally identify All3953 targets under carbon limitation. Targets include, besides *rbcL*, genes encoding CCM elements, photorespiratory pathway, photosystem- and electron transport-related components, and factors, including flavodiiron proteins, with a demonstrated or putative function in photoprotection. qRT-PCR analysis of selected All3953 targets showed regulation in the wild type but not in the mutant. All3953 (PacR) is a global regulator of carbon assimilation in an oxygenic photoautotroph.

Introduction

As the organisms that developed oxygenic photosynthesis, cyanobacteria have played a crucial role in Earth's history and the evolution of life in our planet. Indeed, the production of O_2 as a result of cyanobacterial activity was responsible for the oxidation of the Earth's atmosphere about 2.5-2.3 billion years ago (Lyons *et al*., 2014). Furthermore, all the extant plastids of eukaryotic algae and plants are of cyanobacterial origin. Cyanobacteria were the first organisms to link the activity of the two types of photosystems (PSI and PSII), which allowed the generation of high electrochemical potential, and to combine them with a H_2O-splitting complex. Nowadays, most cyanobacteria are phototrophs relying on oxygenic photosynthesis to generate ATP and reducing equivalents for the fixation of CO_2 and the assimilation of inorganic nitrogen. Indeed, they are responsible for an important fraction of the primary productivity in the Earth's oceans, where they are important CO_2 and N_2 fixers, thus impacting the C and N cycles at a global scale (Knoll 2008; Price *et al*., 2008).

The enzyme responsible for the bulk of CO_2 fixation in the biosphere is ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo), which has a relatively low affinity for CO_2 and, moreover, it can also accept O_2 as a substrate. Compensating for this relatively low performance, RuBisCo is considered the most abundant enzyme on Earth. As a carboxylase, RuBisCo catalyzes the first step of the Calvin-Benson-Bassham (CBB) cycle, i.e., the incorporation of atmospheric CO_2 into ribulose-1,5-bisphosphate to give two molecules of 3-phosphoglycerate. As an oxygenase, it catalyzes the incorporation of O_2 into ribulose-bisphosphate, which produces 2-phosphoglycolate that ~~can~~ leads to photorespiration with a subsequent loss of fixed C and energy. To increase the efficiency of CO_2 fixation, cyanobacteria have developed a distinct CO_2 concentrating mechanism (CCM) constituted by inorganic carbon (C_i) transporters that incorporate bicarbonate and CO_2 into the cell, and a proteinaceous compartment, the carboxysome, where RuBisCo, together with carbonic anhydrase, is

confined (Price *et al*., 2008; Cameron *et al*., 2014). Three high-affinitiy bicarbonate transporters (the ABC-type Cmp, also called BCT1, and the Na^+-dependent BicA and SbtA), and two CO_2 transporters (the high-affinity NDH-I_3 and the low-affinity NDH-I_4) have been identified in different cyanobacteria (see Price, 2011). Through CCM, Ci in the form of CO_2 can concentrate in the vicinity of cyanobacterial RuBisCo, to allow high specific activity for production of 3-phosphoglycerate to levels much higher than in plants (Cameron *et al*., 2014). Indeed, components of cyanobacterial CCM have been transformed in tobacco, with the result of improved specific activity of CO_2 fixation, which represents a step towards improved photosynthesis in plants (Lin *et al*., 2014).

In unicellular cyanobacteria, CCM elements are regulated by C_i availability. Especially genes encoding C_i transporters are induced, whereas the structural genes for carboxysome components and the *rbcL/S* genes encoding RuBisCo are only moderately responsive (see Price *et al*., 2008; Cameron *et al*., 2014). In chemotrophic bacteria the process of CO_2 fixation and the response to C_i limitation are usually controlled by LysR-type transcriptional regulators (LTTRs). The genes encoding the enzymes of the CBB cycle are usually found in clusters regulated by CbbR factors, which constitute a sub-family of LTTRs (Gibson and Tabita, 1996). In unicellular cyanobacteria, a number of CbbR homologs have been characterized. CmpR is an activator of the *cmp* genes (Nishimura *et al*., 2008), whereas CcmR (*aka* NdhR) acts as a transcriptional repressor of multiple genes encoding other C_i transporters (e.g., Figge *et al*., 2001; Wang *et al*., 2004). A third type of CbbR-like protein, the activator of the RuBisCo genes, has not yet been identified in cyanobacteria.

~~The identification of a third type of CbbR-like protein, the activator of the RuBisCo genes, RbcR, has remained elusive in cyanobacteria.~~

Filamentous heterocyst-forming cyanobacteria, of which *Anabaena* sp. PCC 7120 (hereafter *Anabaena*) is a model organism, additionally have the capacity for cell differentiation to turn some O_2-evolving photosynthetic cells of the filament into heterocysts, which are heterotrophic cells especialized in the fixation of atmospheric

N_2. Thus, *Anabaena* is a truly pluricellular bacterium with different cell types specialized in different nutritional tasks that exchange nutrients and regulators and contribute to the performance of the filament as the organism unit (see Flores and Herrero, 2010). Nitrogen assimilation and heterocyst differentiation in *Anabaena* are regulated by the global transcription factor NtcA, which responds to the cellular C-to-N ratio, and the heterocyst-specific transcription factor HetR (see Herrero *et al*., 2013). The *Anabaena* genomic sequence includes three genes annotated as CbbR-like LTTRs (Kaneko *et al*., 2001). Of these, *all0862* has been identified as *cmpR*, and its product activates the expression of the *cmp* operon (*alr2877-alr2880*) and the *cmpR* gene itself in response to C_i limitation (López-Igual *et al*., 2012). Notably, this regulation is effected in combination with NtcA, thus revealing a mode of co-regulation by C and N availability (López-Igual *et al*., 2012). Furthermore, in *Anabaena* the *rbcLXS* operon encoding RuBisCo, which is moderately induced under C_i limitation (López-Igual *et al*., 2012), is repressed in the heterocysts (Madan and Nierzwicki-Bauer, 1993), a regulation likely exerted by NtcA (Ramasubramanian *et al*., 1994; Picossi *et al*., 2014).

Here we have identified the CbbR-homolog All3953 as the activator of the RuBisCo-encoding operon in *Anabaena*. We have determined the All3953 regulon by ChIP-Seq, which has revealed that All3953 is a global regulator for C_i assimilation genes and genes involved in protection of the photosynthetic apparatus against oxidative damage that are regulated by Ci availability.

Results

All3953, an RbcR-like factor in Anabaena sp. PCC 7120

To gain insight into the LTTR All3953 in *Anabaena*, the expression of the *all3953* gene under different growth conditions was analyzed by northern blot. Three bands of hybridization corresponding to transcripts of ca. 1.9, 1.6 and 1.3 kb, respectively, which appeared similarly represented before and after Ci limitation could be observed (Supp.

Fig. 1A). On the other hand, *all3953* expression did not significantly respond to N depletion (Supp. Fig. 1B). The latter result was consistent with previous global transcriptional studies (Ehira and Ohmori, 2006; Flaherthy *et al*., 2011) and with the lack of binding associated to *all3953* of the N-control transcriptional regulator NtcA upon combined nitrogen depletion (Picossi *et al*., 2014).

Isolation and characterization of an all3953 mutant

To study the role of All3953, a mutant strain bearing an inactivated version of *all3953* was constructed (see Experimental procedures). In strain CSS74 most of the *all3953* gene was deleted and the C.S3 gene cassette (encoding Sm/Sp resistance) was introduced to facilitate segregation and maintenance of the mutation in *Anabaena* (Supp. Fig. 2A). As a control, strain CSS77 bearing the C.S3 gene cassette in the *Anabaena* plasmid alpha was also constructed. For *cis* complementation, a wild-type version of *all3953* was transferred to strain CSS74 in an integrative plasmid, generating strain CSS74C (Supp. Fig. 2A).

Strain CSS74 exhibited poor growth under standard growth conditions with ammonium as a nitrogen source, and it formed short filaments in liquid medium, whereas strain CSS74C behaved similarly to CSS77 (not shown). To quantify the deleterious effect of the *all3953* mutation, growth rates were calculated in liquid medium under different illumination and C_i-supply conditions ~~(Fig. 1)~~. Growth rate of the control strain CSS77 was highest (0.892 $days^{-1}$) under high light (HL) and high carbon (HC), and was about 30% lower under the other tested conditions (Fig. 1A). In contrast, growth of strain CSS74 was severely affected under HL HC conditions (growth rate ca. 70% lower than that of the control) (Fig. 1A), under which it ended up dying after about 5 days (Fig. 1B). Under HL and low carbon (LC) or low light (LL) HC, the defect was close to 30% with regard to the control, although after prolonged incubation the mutant was more severely affected under HL LC. The defect was the

smallest (ca. 15%) under LL LC conditions (Fig. 1A,B). In solid mediumFinally, growth of strain CSS74 was similar, and similar to that of the control, in the presence of ammonium, nitrate or no combined nitrogen the effect of the N source on the growth of strain CSS74 was tested in solid medium under LL LC and HL LC conditions, whereas. under HL LC In LL LC growth of strain CSS74 was similar, and similar to that of the control, in the presence of ammonium, nitrate or no combined nitrogen (Figure 1C). However, under HL LC a severe growth defect was observed with regard to the control with any of the tested nitrogen sources (not shownFig. 1C). It is worth noting that, because the CSS74 strain formed short filaments, colonies were observed at a lower chlorophyll concentration than in the control strain. In summary, the lack of All3953 had a deleterious effect on growth, especially under HL (and HC) conditions.

To further characterize the growth defect of the *all3953* mutant, the rate of oxygen evolution using CO_2 as a final electron acceptor was measured under different illumination conditions in the CSS74 mutant in relation to the control strain CSS77. When exponentially-growing cells were incubated for 24 h under LL HC, the oxygen evolution rate was slightly lower in strain CSS74 in comparison to CSS77 (88 and 105 μmol O_2·[mg Chl]$^{-1}$·h^{-1}, respectively). Under HL the difference between the two strains was larger (167, for CSS77, and 110, for CSS74, μmol O_2·[mg Chl]$^{-1}$·h^{-1}).

Effect of all3953 mutation on rbcLXS expression

To test the effect of the *all3953* mutation on the expression of the *rbcLXS* operon, northern blot analysis was performed with RNA isolated from cells of the control and mutant strains grown with HC and transferred to LC conditions. After 1 h incubation with LC, a ca. 2-fold increase in the amount of the *rbcLXS* transcripts could be observed in the control strain. No induction could be detected in the mutant (Fig. 2). In the complemented strain CSS74C the expression of *rbcLXS* increased in LC similarly to the control (Fig. 2). These results indicated that the induction of the *rbcLXS* operon

upon C_i deficiency was dependent on All3953 and that the defect in strain CSS74 was exclusively due to the lack of All3953.

ChIP-Seq analysis of the All3953 targets

To determine the DNA targets of All3953 at a genomic level, we used chromatin immunoprecipitation followed by high-throughput sequencing analysis. To this end, we constructed a strain (CSS57) expressing from the *all3953* promoter a version of All3953 C-terminally fussed to TAP-tag (Rigaut *et al*., 1999), as well as a control strain (CSS68) expressing the TAP-tag alone under the control of the *all3953* promoter (Supp. Fig. 2B, see Experimental procedures). Immunoprecipitation was carried out using cells of strains CSS57 and CSS68 grown with ammonium as the nitrogen source under HC conditions and incubated for 3 h with ammonium under LC conditions.

The analysis of the sequences resulted in a total of 142 All3953 binding regions, of which 127 were located in the chromosome, 10 in plasmid alpha, three in plasmid beta and two in plasmid gamma. Each binding region was ascribed to one or two genes according to the location (midpoint) of the region, and the relative location with respect to the assigned gene was also indicated (Table 1 and Supp. Table 1). A total of 175 genes were ascribed to the 142 binding regions. The binding regions were mostly located upstream of the ascribed genes (72%), whereas 21% were intragenic and 7% were located downstream of genes. The results of the ChIP-Seq analysis are available at GEO accession number GSE58861 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSE58861).

The 175 ascribed genes were classified according to their functional category (Table 2). Remarkably, there were 19 genes encoding proteins related to photosynthesis and respiration, and 21 genes encoding regulatory proteins, including All7179, a SigB homolog. The rest were mostly genes encoding hypothetical or unknown proteins (42%), but also genes encoding proteins involved in translation, in

biosynthesis of amino acids and cofactors, prosthetic groups and carriers, in transport, and in other cellular processes. Table 3 highlights All3953-binding regions related to photosynthesis and respiration among which, confirming our results of gene inactivation, the gene encoding the large subunit of the RuBisCo (*rbcL*; binding region #37) is included. The fact that a high number of genes involved in photosynthesis and C fixation, including *rbcL*, were identified as targets of RbcR suggests that this protein is a global transcription factor for photosynthetic C assimilation.

A CisFinder analysis of the primary consensus motif was carried out based on 142 high-confidence ChIP-Seq peak sequences (Fig. 3A). The consensus motif found has a dyad-symmetry architecture and matches the consensus of LysR-recognition binding sites (RBS) (T-N_{11}-A) (Maddocks and Oyston, 2008), as well as the consensus binding sites proposed for CbbR factors (TNA-$N_{7/8}$-TNA). The primary motifs identified in the central 100 nt of the binding regions are indicated in Supp. Table 1 (for some binding regions more than one motif have been identified).

Expression analysis of some All3953 targets in Anabaena

To corroborate our ChIP-Seq analysis and to support the notion that All3953 is indeed a global regulator for C fixation genes, the expression levels of some of the photosynthesis- and C-fixation-related target genes, its response to C_i limitation and its dependence on All3953 was further analyzed by qRT-PCR (Fig. 4). Strains CSS77 and CSS74 were grown in ammonium and HC under standard light conditions (80 $\mu E \cdot m^{-2} \cdot s^{-1}$) at 30ºC up to the exponential phase. They were then transferred to medium with ammonium under LC conditions. As previously shown by northern analysis, transcription levels of the *all3953* gene did not significantly change after C_i deprivation in the control strain CSS77. As expected, *all3953* transcript levels were not detectable in CSS74, corroborating that the *all3953* mutation was segregated in this strain. The *rbcL* gene (*alr1524*) was 4.6-fold induced at 3 h after transfer to LC in strain CSS77,

whereas no induction was observed in CSS74, thus corroborating the dependence on All3953. Interestingly, under HC the expression of *rbcL* in the mutant was higher than in the control strain, suggesting that besides as an activator under LC, All3953 could be acting as a repressor of *rbcL* under HC conditions.

ORF *all4446* (*flv4*) was highly induced (about 100-fold), and its level was maximum 1 h after the shift to LC (Fig. 4). In the *all3953* mutant, the basal transcription of *all4446* in HC was about 8-fold lower than in the control strain, and it was only slightly induced (2-fold) upon the shift to LC. *all3891* (*flv1A*) was induced about 6-fold 3 h after the shift to LC in the control strain, whereas only a 2-fold induction was observed in the *all3953* mutant. *all1304* (bicarbonate transporter homolog) and *alr4156* (NdhF homolog) were both highly induced (up to 30- and 20-fold, respectively) upon transfer of strain CSS77 to LC. In contrast, no induction of *alr4156* and only a small induction of *all1304* took place in strain CSS74. Expression of *alr4592* (*psbAIII*) increased about 5-fold upon C_i deprivation in CSS77, but did not appreciably change in CSS74. The *alr0223* (NdhA homolog) gene was about 2-fold induced under C_i deprivation in CSS77 but not in CSS74. Finally, the expression of *alr1004* (alanine-glyoxylate aminotransferase) was repressed by 5-fold under LC conditions in CSSL77 but not in CSS74. These results confirm that expression of the above studied genes is regulated, either positively or negatively, by All3953.

Discussion

We have identified the LTTR All3953 as the activator of the RuBisCo-encoding genes in the cyanobacterium *Anabaena* sp. PCC 7120. All3953 appears to activate the *rbcL* operon under C_i limitation and to repress it when C_i is abundant. An LTTR factor regulating the expression of the *rbcL* operon has not, to our knowledge, been described in any cyanobacterium. The expression of *all3953* does not respond to C_i limitation (Supp. Fig. 1) and, indeed, no binding region of All3953 was found adscribed

to *all3953*. Thus, the *all3953* gene seems to belong to the non-autoregulated LTTRs. All3953 shares 28 and 26% identical residues with NdhR from *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002 (CcmR), respectively.

By ChIP-Seq analysis of a strain bearing a TAP-tagged version of All3953, we have determined 142 All3953-bound regions at 3 h after transfer from high to low C_i conditions, which have been assigned to 177 genes. Apart from genes encoding unknown or hypothetical proteins, the larger category is of genes encoding regulatory proteins, including the transcriptional regulators Alr0353 (a LTTR) and All4500 (CRP-like), the two-component response regulator All3348, the two-component sensor histidine-kinase All1145 and the group 2-sigma 70-type sigma factor All7179 (Supp. Table 1). Interestingly, when comparing to *Synechocystis* sigma factors, All7179 (SigB4) is more similar to ~~a homolog of *Synechocystis*~~ SigB, which~~,~~ along with SigD has been shown to be important for PSII recovery in this unicellular cyanobacterium (Pollari *et al*., 2009). All3953 also binds upstream of genes *patS* and *hetN*, whose products regulate heterocyst differentiation (Supp. Table 1). The fact that All3953 binds to the promoter region of genes encoding other regulatory proteins suggests a wide role of this protein in the physiology of the organism.

In the promoter region of the *rbcL* operon we have found three putative binding sites for All3953, Box I, Box II, and Box III (Fig. 3B) that resemble the consensus recognition sequence found by Cisfinder analysis (Fig. 3A). It is conceivable that, like in some other LTTRs these boxes combine repression and activation sites. In this regard, binding of All3953 to Box III, overlapping gene promoter elements, could be related to the repression of *rbcL* observed under high C_i (Fig. 4). On the other hand, as mentioned above, the *rbcL* operon is repressed in heterocysts by the global transcriptional regulator NtcA, for which a binding site is found overlapping the operon TSP (Ramasubramanian *et al*., 1994; Picossi *et al*., 2014) (Figure 3B). It is conceivable that NtcA binding in these differentiated cells interfere with All3952-mediated activation.

Besides being the activator of the RuBisCo genes, All3953 targets include genes involved in other processes related to carbon assimilation, such as C_i transport (*all1304*, encoding an homolog of the BicA bicarbonate transporter; *alr4156* encoding a homolog of the NdhF3 subunit of the NDH-1_3 CO_2 uptake system; and *alr0869* encoding a homolog of the NdhF4 subunit of the NDH-1_4 CO_2 uptake system); components of the carboxysome shell (*all0868,* putative *ccmK*) and 2-phosphoglycolate metabolism (*alr1004* and *alr2873*, possibly related to photorespiration [Eisenhut *et al*., 2008]). Notably, All3953 targets include also a number of genes encoding photosystem components, such as *alr5154* (*psaA*, encoding the PSI core protein PsaA), *alr3727* (*psbAII*, encoding a component of form II of the PSII core protein PsbA [D1]), *alr4592* (*psbAIII*, encoding another component of form II of PsbA) and *alr1216* (PSII 12 kD extrinsic protein PsbU), and genes related to PS activity. In the latter group are *alr4149* (biliverdin reductase, putatively involved in phycobilisome -PSII antenna- synthesis), and genes that can participate in photosynthetic electron transfer, such as *alr0223* and *alr0348* (*ndhA* and *ndhD*, subunits of other putative NADH dehydrogenases), *alr1576* (dehydrogenase subunit), *all0737* (thioredoxin reductase), *all1365* (CytM cytochrome), *all4148* (ferredoxin I), *all3891* and *all4446* (flavodiiron proteins Flv1A and Flv4, respectively). (Besides in CO_2 uptake, NdhF3 and NdhF4 can also participate in electron transfer.)

Reports on gene expression regulation by C_i are scarce for *Anabaena*. However, in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 transcriptomic analysis has already shown down-regulation of some genes encoding polypeptides of PSI and PSII complexes as well as of phycobilisome components, upon transfer to C_i limitation conditions, likely as an adaptation to lower assimilatory power demand, and up-regulation of some PSII core polypeptides, interpreted as adaptation to conditions of shortage of electron acceptors that could lead to photodamage and increased turnover of PS core components (Wang *et al*., 2004). Our analysis extends the array of photosynthetic genes responding to C_i limitation,

remarkably to include the core PSI reaction center *psaA* gene. Moreover, except for *alr1004* that responds negatively, all the *Anabaena* photosynthetic genes mentioned above increase expression upon the shift to C_i limitation.

Noteworthy, for the majority of photosynthetic gene targets of All3953 in *Anabaena*, a function in protection against reactive oxygen species, which can be generated by C_i limitation or exposure to HL, has either been described or could be predicted. Thus, the *psbAII* and *psbAIII* genes are induced under HL in the unicellular *Synechococcus* sp. PCC 7942, and cells cultured under HL showed more form II, and less form I (encoded by *psbAI*), of D1 compared to cells under LL (Schaefer and Golden, 1989). Regarding flavodiiron proteins~~,~~ genes, *flv1A, flv3A*, and specially *flv2* and *flv4* of *Anabaena* have been shown up-regulated in vegetative cells in low Ci, and *flv1A* and *flv3A* also in high light, whereas *flv1B* and *flv3B* are expressed exclusively in heterocysts (Ermakova *et al*., 2013). Whereas Flv1A and Flv3A appear involved in photoreduction of oxygen to water by removing excess electrons from PSI through NAD(P)H dehydrogenases, Flv2 and Flv4 could have a role in photoprotection of PSII under low Ci (Ermakova *et al*., 2013). Regarding photorespiration, it has also been considered to have a role in removal of excess O_2 (Eisenhut *et al*., 2008). To the best of our knowledge, the regulator responsible for the response to C_i availability of any photosynthetic gene has not been identified in cyanobacteria (oxygenic phototrophs). Our ChIP-Seq and expression analysis indicate that All3953 is a regulator of photosynthetic genes in *Anabaena.*

The growth rate of *Anabaena* is highest under HL HC conditions (Fig. 1), implying that this cyanobacterium has mechanisms to get profit of HC while counteracting HL stress. The *all3953* mutant strain CSS74 exhibited a growth defect in all the conditions tested, but especially under HL, where it ends-up dying (Fig. 1). This shares the idea that in *Anabaena* All3953 is required to cope with HL stress. The effect of the lack of All3953 seems more detrimental in relation to impaired photoprotection than to impaired C_i scavenging (preference of the mutant for LL LC over HL HC

conditions). Indeed, even in LL, the mutant seems to perform slightly better with LC than with HC (Fig. 1). Although LC could suppose a limitation of electron acceptors, an increased rate of photorespiration under LC together with the fact that some of the All3953 targets that could cope with excess oxygen are residually induced upon the transfer from HC to LC in the CSS74 mutant (Fig. 4) could contribute to the preference of this strain for LC over HC, especially under HL.

Our results show that in *Anabaena* the responses to C_i availability include regulation of genes encoding elements of CCM and RuBisCo, but also of photosynthetic genes to adjust generation of assimilatory power while preserving the photosynthetic apparatus from oxidative damage, which is specially relevant in oxygenic phototrophs. Because All3953 is a transcriptional regulator globally coordinating these responses, we have named it PacR (Photosynthetic assimilation of carbon Regulator).

Experimental procedures

Strains

Anabaena sp. strain PCC 7120 was grown photoautrophically at 30°C with illumination (80 $\mu E \cdot m^{-2} \cdot s^{-1}$) in liquid $BG11_0$ medium (Rippka *et al*., 1979) supplemented with 3 mM NH_4Cl, 6 mM TES buffer and 10 mM $NaHCO_3$ and bubbled with a mixture of CO_2 and air (1% v/v) (HC). Other conditions used were no $NaHCO_3$ supplement and bubbling with air (LC); 12 $\mu E \cdot m^{-2} \cdot s^{-1}$ (LL); 175 $\mu E \cdot m^{-2} \cdot s^{-1}$ (HL). ~~For growth on plates illumination was 9 $\mu E \cdot m^{-2} \cdot s^{-1}$ (LL) or 34 $\mu E \cdot m^{-2} \cdot s^{-1}$ (HL).~~ For the mutants generated in this work, antibiotics were used at the following concentrations: Sm, 2 μg ml^{-1}; Sp, 2 μg ml^{-1}; and Nm, 25 μg ml^{-1} for bubbled cultures; and Sm, 5 μg ml^{-1}; Sp, 5 μg ml^{-1}; and Nm, 40 μg ml^{-1} for cultures in solid medium.

Strain construction

To construct a mutant of the *all3953* gene, the 5' and 3' end of the gene, along with the flanking regions, were PCR amplified using chromosomal DNA of PCC 7120 as the template and primers all3953-14 (BglII) and all3953-15 (SalI), and primers all3953-16 (SalI) and all3953-17 (PstI), respectively (all primers are specified in Supp. Table 2). The PCR products were digested with SalI and ligated. The resulting mixture was used as a template for overlapping PCR with primers all3953-14 and all3953-17. The new PCR product was digested with BglII and PstI and ligated to the BglII-PstI-digested pRL271 (Black *et al*., 1993), obtaining plasmid pCSS161. Plasmid pCSS161 was digested with SpeI and the 2-kb Sm^r Sp^r gene cassette C.S3, excised with XbaI from pCSE120 [S.K3/L.HEH2 (BamHI)/C.S3 (BamHI); nomenclature as in (Elhai and Wolk, 1988)], was inserted obtaining plasmid pCSS162. Plasmid pCSS162 was transferred to strain PCC 7120 by conjugation (Elhai *et al*., 1997). Exconjugants resistant to Sm and Sp, which had the Δ*all3953*::C.S3 construct integrated by double recombination were selected, obtaining strain CSS74. The segregation of the mutation was tested by PCR (Supp. Fig. 2) with primers all3953-14, all3953-15, all3953-17 and all3953-20.

To construct a control strain expressing Sm^r and Sp^r plasmid pCSS163, a derivative of plasmid pCSEL24 (Olmedo-Verd *et al*., 2006) containing the C.S3 gene cassette, was transferred to *Anabaena* by conjugation. Exconjugants that had the pCSS163 integrated in the alpha plasmid of *Anabaena* were selected, obtaining the strain CSS77 (Supp. Fig. 2).

To complement the *all3953* mutation of strain CSS74, a DNA fragment encompassing the whole *all3953* gene and sequences upstream from it was amplified by PCR using the primer pair all3953-24/all3953-25, both including EcoRI sites, and strain PCC 7120 DNA as the template. This fragment was cloned in the EcoRI site of the mobilizable Nm^r encoding vector pRL424 (Elhai and Wolk, 1988) producing plasmid pCSS164, which was transferred to strain CSS74 by conjugation followed by selection for Nm^r. The genomic structure of the exconjugants in the *all3953* region (Supp. Fig. 2) was corroborated by PCR.

To construct a strain expressing All3953-C-TAP, the *all3953* gene (including the upstream region) was PCR-amplified with primers all3953-11 and all3953-12 and DNA of PCC 7120 as the template. The TAP-tag was PCR-amplified with primers TAPtag-1 and TAPtag-2 using DNA of plasmid pBS1479 as the template (Puig *et al*., 2001). The two PCR products were digested with SalI and ligated, after which the ligation product was used as the template for an overlapping PCR using primers all3953-11 and TAPtag-2. The PCR product was digested with PstI and ligated to the mobilizable vector pCSV3 (Valladares *et al*., 2011) digested with PstI, rendering plasmid pCSS107.

To construct a control strain with the TAP-tag under the control of the *all3953* promoter, a 0.4-kb region upstream of *all3953* was PCR-amplified using primers all3953-11 and all3953-18 and DNA of pCSS107 as the template. The PCR product was digested with SalI and ligated to the PCR-amplified TAP-tag digested with SalI, after which the ligation product was used as the template for an overlapping PCR using primers all3953-11 and TAPtag-2. The PCR product was digested with PstI and ligated to PstI-digested pCSV3 to give plasmid pCSS157. Plasmids pCSS107 and pCSS157 were transferred by conjugation to strain PCC 7120 and single Sm^r Sp^r recombinants were selected, obtaining strain CSS57 and CSS68, respectively. Western blots using Peroxidase-Anti-Peroxidase Soluble Complex (PAP, Sigma-Aldrich) were performed to ensure that the two strains expressed the TAP-tag (Supp. Fig. 2B).

Chromatin immunoprecipitation

Cells of strains CSS57 growing exponentially (3-5 µg Chl·ml^{-1}) in the light (80 µE·m^{-2}·s^{-1}) in medium supplemented with 2 µg·ml^{-1} Sm and Sp, in HC conditions were incubated with LC for 3 h. Formaldehyde was then added to the cultures to a final concentration of 1%, and the cultures were incubated for 15 min. Glycine was added at 125 mM final concentration and the incubation was continued for 5 min to stop the fixing reaction. The cells were then filtered, washed with cold TBS (20 mM Tris-HCl, pH 7.4, 140 mM NaCl) and collected in tubes (25 ml of culture per tube). The pellets were

frozen in liquid nitrogen and stored at -20°C until used. Pellets corresponding to about 25 ml of culture were resuspended in 500 µl of lysis buffer (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, supplemented with Mini EDTA-free protease inhibitor cocktail [Roche]) and, after addition of 150 µl of glass beads (acid-washed, 425-600 µm [Sigma]), the cells were broken in a multivortexer at 2,000 rpm for 1 h at 4°C. The cell lysates were collected by centrifugation and the extracts were subjected to sonication to shear the DNA to about 300-bp fragments (60 cycles of 10 s, 20 s ice, 15% amplitude, in a Branson Digital Sonifier). After centrifugation to eliminate cell debris, the whole-cell extracts were stored at −20°C or immediately used for immunoprecipitation.

Immunoprecipitation of DNA was carried out as described (Picossi *et al*., 2014), with some modifications. Whole-cell extracts were prepared at 4 $mg \cdot ml^{-1}$ of total protein with lysis buffer (in 500 µl total volume). A 50-µl sample was taken as the input sample, and the extracts were incubated with 15 µl IgG-conjugated Dynabeads (about 6 µg IgG) at 4ºC with rotation for 12-14h. The washes of the Dynabeads, as well as the elution of the immunoprecipitated material, the crosslinking reversion and the isolation of the DNA were performed as in (Picossi *et al*., 2014).

Massive sequencing of the immunoprecipitated DNA

Input and ChIP DNA samples were sent for sequencing to the Functional Genomics Core Facility of the Institute for Research in Biomedicine, Barcelona (Spain). Next generation sequencing was carried out using Illumina's sequencing technology. ChIP DNA Sample Prep Kit (Illumina) was used for library preparation. Libraries were loaded at 8 pM concentration into the flow cell using the Cluster Station running recipe V7 with the Single-Read Cluster Generation Kit v4 (all Illumina). The flow cell was loaded into the Genome Analyzer II and samples were sequenced for 120 nucleotides from a single end using the Sequencing Kit v5 and recipe v8 (all Illumina). Manufacturer's

recommendations were strictly followed. Illumina sequencing data were pre-processed with the standard Illumina pipeline version 1.5 and sequences were aligned to the PCC 7120 genome (http://genome.microbedb.jp/cyanobase/Anabaena) with the Bowtie software 0.12.5 (Langmead *et al*., 2009). The analysis of the results were carried out using the Triform algorithm (Kornacker *et al*., 2012) as in (Picossi *et al*., 2014). The sequences in the ChIP samples of strain CSS68 (TAP control) were used as the background of the sequences found for strain CSS57 (All3953-TAP), and thus to determine the specific binding regions of All3953-TAP in the genome of *Anabaena*. The binding regions were visualized and analyzed using the UCSC Microbial Genome Browser (Scheneider *et al*., 2006). They were ascribed to one or two genes, in case it was not possible to ascribe them to only one, and classified as upstream of the gene, if the midpoint of the binding region was located upstream of the start of the gene, internal, if the midpoint of the binding region was inside the gene, or downstream, if the midpoint of the binding region was located downstream of the end of the gene to which it had been ascribed.

Northern and qRT-PCR analyses

Isolation of total RNA from *Anabaena* was done as described previously (Mohamed and Jansson, 1989). Northern analysis was performed as described previously (López-Igual *et al*., 2012).

For qRT-PCR, 750 ng of DNA-free RNA samples were used for all the PCR primer pairs. For the RT reaction, the Quantitech Reverse transcription kit (Qiagen), with the Random Hexamer Primer mix (100 ng per sample) (Bioline) was used. The cDNA produced was diluted 7.5 times to use 2 µl of cDNA per PCR reaction. PCR was done using the Quantimix Easy SYG Kit (Biotools) (SYBR green I) in a iCycler iQ Multi-Color Real Time PCR Detection System (Bio-Rad). The abundance of a transcript in the RNA sample was calculated as: abundance= 2^[Ct(sample)-Ct(control)], where the RNA sample of the control strain CSS77 in HC condition (0) was used as the control.

Oxygen evolution

2-ml samples of exponentially grown cultures in HC LL or HC HL conditions were used to measure O_2 evolution with an O_2 electrode calibrated with culture medium and $Na_2S_2O_4$ as the reducing agent. O_2 production was measured in the light (400 $\mu E \cdot m^{-2} \cdot s^{-1}$) after a seven-minute incubation in the dark.

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Figure legends:

Fig. 1. Growth of the *all3953* mutant. A, The growth rate constant (μ = ln2/t_d, where t_d is the doubling time) was calculated from the increase of protein content determined in 0.2 ml samples of cultures. The table shows the mean and standard deviation from 3 independent cultures of each strain and condition. Δ*all3953* is strain CSS74; CSS77 is a control strain that carries the Sm/Sp-resistant determinant in a wild-type background. B, Samples of cultures were photographed after 5 days of incubation under the indicated conditions. ~~C, Growth in solid medium (no $NaHCO_3$) with 3 mM NH_4Cl (A), 17.6 mM $NaNO_3$ (N), or no combined nitrogen (-N). 5 µl drops, containing 50, 5, 0.5 or 0.05 ng Chl, were spotted, incubated for 14 days and photographed.~~ HL, high light; LL, low light; HC, high carbon; LC, low carbon.

Fig. 2. Expression of *rbcLXS* in the Δ*all3953* mutant and complemented strain. Northern analysis carried out with RNA from strains CSS77 (control) CSS74 (Δ*all3953*) and CSS74C (CSS74 complemented) was isolated from cells grown with HC (0) and incubated for 1h (1) with LC. The membranes were hybridized with an internal fragment of the *rbcL* gene (upper panels) and, as a loading and transfer control, of the *rnpB* gene (lower panels). Arrowheads point to the main transcripts detected with the *rbcL* gene probe (approximate sizes are indicated).

Fig. 3. Consensus All3953 binding sequence and *rbcL* promoter. A, The primary consensus motif based on 142 high confidence CSS57 ChIP-Seq peak sequences is shown with indication of the probability of occurrence of each base along the 22-nt sequence. W is A or T; Y is C or T; B is C, G or T. B, Structure of the *rbcLXS* promoter region. The transcription initiation point of the operon (+1) and the -10 and -35 boxes (from Nierzwicki-Bauer *et al*., 1984) are indicated in red. The NtcA-binding site

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641 ($GTAN_8TAC$) is indicated in green, and the three putative binding sites for All3953 (Box
642 I, Box II and Box III) are indicated in blue.
643
644 **Fig. 4.** qRT-PCR analysis of the expression of selected photosynthesis and respiration-
645 related All3953 gene targets. Transcriptional response of the indicated genes to C_i
646 limitation in the control (CSS77) and Δ*all3953* mutant (CSS74) strains was
647 investigated. RNA was isolated from cells grown with 10 mM $NaHCO_3$-supplemented
648 medium bubbled with 1%CO_2 in air (0) incubated for 1 h (1) or 3 h (3) in $NaHCO_3$-free
649 medium bubbled with air. Bars represent the mean transcript levels (± standard
650 deviation) in three independent experiments.
651

Table 1. Results of the ChIP-Seq analysis of All3953 binding to DNA at 3 h after C_i limitation

	Binding regions found	Genes ascribed	Position of the binding region with respect to the gene		
			upstream	internal	downstream
Chromosome	127	157	118	29	10
Alpha	10	13	8	4	2
Beta	3	3	0	3	0
Gamma	2	2	0	1	1
Total	142	175	126	37	13

*Some of the binding regions were ascribed to more than one gene (see Supp. Table 1).

Table 2. Functional categories of the genes ascribed to the All3953 binding regions.

Functional category	Number
Amino acid biosynthesis	5
Biosynthesis of cofactors, prosthetic groups, and carriers	4
Cell envelope	2
Cellular processes	5
Central intermediary metabolism	3
DNA replication, recombination and repair	3
Energy metabolism	4
Other categories	19
Photosynthesis and respiration	19
Purines, pyrimidines, nucleosides and nucleotides	2
Regulatory proteins	21
Translation	9
Transport and binding proteins	4
Unknown and hypothetical proteins	75

Table 3. All3953-binding regions assigned to photosynthesis and respiration-related genes.

BR	NLQ	LOC	GENE	POSITION	FUNCTION¶	ST	START	SEQ
7	172,84	239694	*alr0223**	upstream	NADH dehydrogenase subunit 1; NdhA	+	239712	AGGTATTAGTTTAACTAATGTT
12	38,58	398948	*alr0348*	upstream close	NADH dehydrogenase subunit 4; NdhD	+	398912	AACAATTCCTTAATATAATGTA
19	62,81	859596	*all0737*	upstream	thioredoxin reductase	-	859596	ATTCATAAAAGCGTTTTATATC
25	238,45	997693	*all0868*	upstream	CO_2 concentrating mechanism protein CcmK	+	997698	ATGTATAAGTTTTATTAATATG
			alr0869	upstream	NADH dehydrogenase subunit 5			
27	66,90	1175262	*alr1004**	upstream	alanine--glyoxylate aminotransferase	+	1175259	GTATATAGGCGATCATTATGGC
31	36,15	1433154	*alr1216*	upstream	photosystem II 12 kD extrinsic protein PsbU	+	1433147	AAATATTGTGAGCATTAATAAG
34	385,11	1547013	*all1304**	upstream	bicarbonate transporter	-	1546894	GTGCATTTGCAATAGTTATTAT
35	31,96	1620674	*all1365*	downstream	cytochrome CytM	+	1620645	CGTAATAAATTTTAATCATCAT
37	63,26	1785455	*alr1524**	upstream	RbcL	+	1785446	ACTTATGCCATTTCTTGATATA
38	204,06	1843221	*alr1576*	upstream far	dehydrogenase subunit	-	1843218	AGTAATAACTGCTACTTATTAC
61	29,78	3499605	*alr2873*	internal 3' end	possible glycerate kinase	-	1843218	CAAAATTAAACTGTCTAATTTC
86	170,25	4499861	*alr3727*	upstream	photosystem II protein D1 (*psbAII*)	+	4499883	GTATATATATTTTAGTAATATT
91	155,06	4693128	*all3891**	upstream	flavoprotein (*flv1A*)	+	4693099	ATTTATAAGTTTTACTTAAGCT
98	166,48	4993006	*all4148*	upstream	ferredoxin I	-	4993375	AACCATAAATTTTTCTAATAAC
99	61,47	4999503	*alr4156**	upstream	NADH dehydrogenase subunit 5; NdhF	+	4999503	AAAGATAAATTTGCCTTATTTA
108	163,25	5332512	*all4446**	upstream	flavoprotein (*flv4*)	-	5332500	AATAATAAATTTTACTAATAAA
112	233,71	5489698	*alr4592**	upstream	photosystem II protein D1 (*psbAIII*)	+	5489979	CTATATAGTTTTTACTCATATT
121	51,04	6151146	*alr5154*	upstream	photosystem I core protein A1	+	6151133	GGACATAAGTTTTACGAATTGT

*Genes whose expression has been studied by qRT-PCR

¶Functions are as specified in cyanobase (http://genome.microbedb.jp/cyanobase/Anabaena).

BR: binding region, NLQ: -logQvalue , LOC: chromosome location of the midpoint of the binding region, ST: DNA strand, START: chromosome location of the 5' end of the putative binding sequence of All3953 (SEQ).

Picossi *et al*., Fig. 1

A

Growth conditions	μ (day^{-1})	
	CSS77	CSS74
HL HC	0.892±0.072	0.287±0.074
HL LC	0.602±0.078	0.406±0.030
LL HC	0.620±0.061	0.454±0.027
LL LC	0.607±0.051	0.523±0.028

B

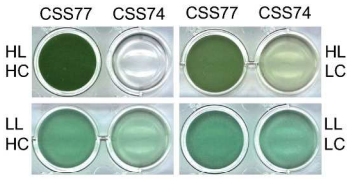

Figure 1
262x350mm (300 x 300 DPI)

Picossi *et al*., Fig. 2

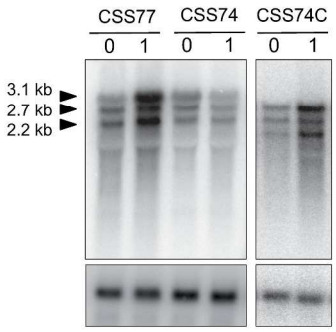

Figure 2
262x350mm (300 x 300 DPI)

Picossi *et al.*, Fig. 3

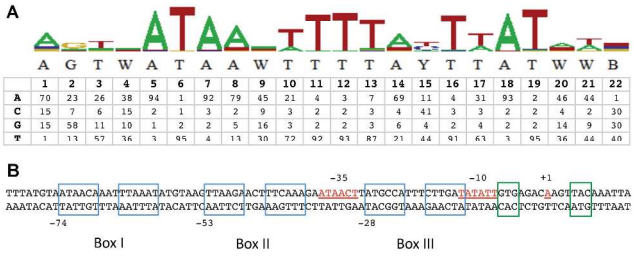

Figure 3
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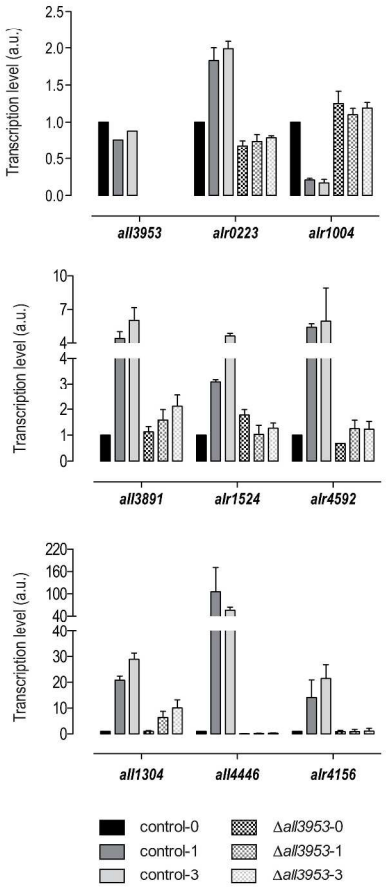

Figure 4
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