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

<table>
<thead>
<tr>
<th>Journal:</th>
<th>Environmental Microbiology and Environmental Microbiology Reports</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuscript ID:</td>
<td>EMI-2014-1511.R1</td>
</tr>
<tr>
<td>Manuscript Type:</td>
<td>EMI - Research article</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>22-Jan-2015</td>
</tr>
<tr>
<td>Complete List of Authors:</td>
<td>Picossi, Silvia; Consejo Superior de Investigaciones Científicas, Instituto de Bioquímica Vegetal y Fotosíntesis Flores, Enrique; Consejo Superior de Investigaciones Científicas, Instituto de Bioquímica Vegetal y Fotosíntesis Herrero, Antonia; Consejo Superior de Investigaciones Científicas, Instituto de Bioquímica Vegetal y Fotosíntesis</td>
</tr>
<tr>
<td>Keywords:</td>
<td>bacteria, environmental signal/stress responses, gene expression/regulation</td>
</tr>
</tbody>
</table>
The LysR-type transcription factor PacR is a global regulator of photosynthetic carbon assimilation in \textit{Anabaena}

Silvia Picossi, Enrique Flores and Antonia Herrero*

Instituto de Bioquímica Vegetal y Fotosíntesis, Consejo Superior de Investigaciones Científicas and Universidad de Sevilla, Américo Vespucio 49, E-41092, Seville, Spain.

*Corresponding author. Tel.: +34 954489522. Fax: +34 954460165. E-mail address: herrero@ibvf.csic.es

Keywords: ChIP; Cyanobacteria; Oxygenic phototrophy; Photoprotection; RuBisCo

Running title: Photosynthetic carbon assimilation regulator

Accession link to data:
Summary

Cyanobacteria perform water-splitting photosynthesis and are important primary producers impacting the carbon and nitrogen cycles at global scale. They fix CO₂ through ribulose bisphosphate carboxylase/oxygenase (RuBisCo) and have evolved a distinct CO₂ concentrating mechanism (CCM) that builds high CO₂ concentrations in the vicinity of RuBisCo favoring its carboxylase activity. Filamentous cyanobacteria such as *Anabaena* fix CO₂ in photosynthetic vegetative cells, which donate photosynthate to heterocysts that rely on a heterotrophic metabolism to fix N₂. 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$_2$O-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-affinity 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, C$_i$ 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\textsubscript{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\textsubscript{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\textsubscript{i} assimilation genes and genes involved in protection of the photosynthetic apparatus against oxidative damage that are regulated by C\textsubscript{i} 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 C\textsubscript{i} 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 supplied 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 µE·m$^{-2}$·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* (*psbAllI*) increased about 5-fold upon C\textsubscript{i} deprivation in CSS77, but did not appreciably change in CSS74. The *alr0223* (NdhA homolog) gene was about 2-fold induced under C\textsubscript{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 CSS77 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\textsubscript{i} limitation and to repress it when C\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{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 All3953-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\textsubscript{i} transport (*all1304*, encoding an homolog of the BicA bicarbonate transporter; *alr4156* encoding a homolog of the NdhF3 subunit of the NDH-1\textsubscript{3} CO\textsubscript{2} uptake system; and *alr0869* encoding a homolog of the NdhF4 subunit of the NDH-1\textsubscript{4} CO\textsubscript{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
For Peer Review Only

number of genes encoding photosystem components, such as \textit{alr5154} (\textit{psaA}, encoding the PSI core protein PsaA), \textit{alr3727} (\textit{psbAII}, encoding a component of form II of the PSII core protein PsbA [D1]), \textit{alr4592} (\textit{psbAIII}, encoding another component of form II of PsbA) and \textit{alr1216} (PSII 12 kD extrinsic protein PsbU), and genes related to PS activity. In the latter group are \textit{alr4149} (biliverdin reductase, putatively involved in phycobilisome -PSII antenna- synthesis), and genes that can participate in photosynthetic electron transfer, such as \textit{alr0223} and \textit{alr0348} (\textit{ndhA} and \textit{ndhD}, subunits of other putative NADH dehydrogenases), \textit{alr1576} (dehydrogenase subunit), \textit{all0737} (thioredoxin reductase), \textit{all1365} (CytM cytochrome), \textit{all4148} (ferredoxin I), \textit{all3891} and \textit{all4446} (flavodiiron proteins Flv1A and Flv4, respectively). (Besides in CO\textsubscript{2} uptake, NdhF3 and NdhF4 can also participate in electron transfer.)

Reports on gene expression regulation by C\textsubscript{i} are scarce for \textit{Anabaena}. However, in the unicellular cyanobacterium \textit{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\textsubscript{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 \textit{et al.}, 2004). Our analysis extends the array of photosynthetic genes responding to C\textsubscript{i} limitation, remarkably to include the core PSI reaction center \textit{psaA} gene. Moreover, except for \textit{alr1004} that responds negatively, all the \textit{Anabaena} photosynthetic genes mentioned above increase expression upon the shift to C\textsubscript{i} limitation.

Noteworthy, for the majority of photosynthetic gene targets of All3953 in \textit{Anabaena}, a function in protection against reactive oxygen species, which can be generated by C\textsubscript{i} limitation or exposure to HL, has either been described or could be predicted. Thus, the \textit{psbAII} and \textit{psbAIII} genes are induced under HL in the unicellular \textit{Synechococcus} sp. PCC 7942, and cells cultured under HL showed more form II, and
less form I (encoded by psbA1), 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 O2 (Eisenhut et al., 2008). To the best of our knowledge, the regulator responsible for the response to Ci 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 Ci 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 Ci 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 µE·m⁻²·s⁻¹) in liquid BG11₀ medium (Rippka et al., 1979) supplemented with 3 mM
NH₄Cl, 6 mM TES buffer and 10 mM NaHCO₃ and bubbled with a mixture of CO₂ and
air (1% v/v) (HC). Other conditions used were no NaHCO₃ supplement and bubbling
with air (LC); 12 µE·m⁻²·s⁻¹ (LL); 175 µE·m⁻²·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 (BglIII) and all3953-15 (Sall), and primers all3953-16
(Sall) and all3953-17 (PstI), respectively (all primers are specified in Supp. Table 2).
The PCR products were digested with Sall 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 BglIII and PstI and ligated to the BglIII-PstI-digested
pRL271 (Black et al., 1993), obtaining plasmid pCSS161. Plasmid pCSS161 was
digested with SpeI and the 2-kb Sm’ Sp’ 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 ∆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′ and Sp′ 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′ encoding vector pRL424 (Elhai and Wolk, 1988) producing plasmid pCSS164, which was transferred to strain CSS74 by conjugation followed by selection for Nm′. 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 \textit{all3953} promoter, a 0.4-kb region upstream of \textit{all3953} was PCR-amplified using primers all3953-11 and all3953-18 and DNA of pCSS107 as the template. The PCR product was digested with Sall and ligated to the PCR-amplified TAP-tag digested with Sall, 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 \(\mu \text{g Chl.ml}^{-1}\)) in the light (80 \(\mu \text{E.m}^{-2}.\text{s}^{-1}\)) in medium supplemented with 2 \(\mu \text{g.ml}^{-1}\) 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 \(-20^\circ \text{C}\) until used. Pellets corresponding to about 25 ml of culture were resuspended in 500 \(\mu \text{l}\) 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 \(\mu \text{l}\) of glass beads (acid-washed, 425-600 \(\mu \text{m}\) [Sigma]), the cells were broken in a multivortexer at 2,000 rpm for 1 h at 4\(^{\circ}\)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·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 4°C 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 (All39530TAP), and thus to determine the specific binding regions of All39530-TAP in the genome of *Anabaena*.
For Peer Review Only

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= $2^{\Delta Ct(sample) - Ct(control)}$, 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$_2$S$_2$O$_4$ as the reducing agent. O$_2$ production was measured in the light (400 µE·m$^{-2}$·s$^{-1}$) after a seven-minute incubation in the dark.
Acknowledgments

This work was supported by grants BFU2010-17980 and BFU2013-44686-P from the Spanish government, co-financed by FEDER. The authors are grateful to K. Kornacker for carrying out the Triform and CisFinder analyses, and to H. Auer and I. Pons, from the Functional Genomics Core Facility of the IRB, Barcelona (Spain); to F. Monje-Casas and Yagut Allahverdiyeva-Rinne for critical reading of the manuscript and to M. J. Huertas and A. Torrado for help with O₂ production measurements.

REFERENCES


Figure legends:

Fig. 1. Growth of the all3953 mutant. A, The growth rate constant ($\mu = \ln 2 / t_d$, 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. $\Delta$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 $\Delta$all3953 mutant and complemented strain. Northern analysis carried out with RNA from strains CSS77 (control) CSS74 ($\Delta$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$_8$TAC) is indicated in green, and the three putative binding sites for All3953 (Box I, Box II and Box III) are indicated in blue.
Fig. 4. qRT-PCR analysis of the expression of selected photosynthesis and respiration-related All3953 gene targets. Transcriptional response of the indicated genes to \( C_i \) limitation in the control (CSS77) and \( \Delta all3953 \) mutant (CSS74) strains was investigated. RNA was isolated from cells grown with 10 mM NaHCO\(_3\)-supplemented medium bubbled with 1\%CO\(_2\) in air (0) incubated for 1 h (1) or 3 h (3) in NaHCO\(_3\)-free medium bubbled with air. Bars represent the mean transcript levels (± standard deviation) in three independent experiments.
The LysR-type transcription factor PacR is a global regulator of photosynthetic carbon assimilation in Anabaena

Silvia Picossi, Enrique Flores and Antonia Herrero*

Instituto de Bioquímica Vegetal y Fotosíntesis, Consejo Superior de Investigaciones Científicas and Universidad de Sevilla, Américo Vespucio 49, E-41092, Seville, Spain.

*Corresponding author. Tel.: +34 954489522. Fax: +34 954460165. E-mail address: herrero@ibvf.csic.es

Keywords: ChIP; Cyanobacteria; Oxygenic phototrophy; Photoprotection; RuBisCo

Running title: Photosynthetic carbon assimilation regulator

Accession link to data:
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$_2$O-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 lead 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-affinity bicarbonate transporters (the ABC-type Cmp, also called BCT1, and the Na⁺-dependent BicA and SbtA), and two CO₂ transporters (the high-affinity NDH-I₅ and the low-affinity NDH-I₆) have been identified in different cyanobacteria (see Price, 2011). Through CCM, Ci in the form of CO₂ 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 CO₂ fixation, which represents a step towards improved photosynthesis in plants (Lin et al., 2014).

In unicellular cyanobacteria, CCM elements are regulated by Ci availability. Especially genes encoding Ci 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 CO₂ fixation and the response to Ci 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 Ci 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 O₂-evolving photosynthetic cells of the filament into heterocysts, which are heterotrophic cells specialized in the fixation of atmospheric
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 C\(_i\) 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 C\(_i\) 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; Flaherty 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-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 medium Finally, 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 shown Fig. 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 CO₂ 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 O₂·[mg Chl]⁻¹·h⁻¹, respectively). Under HL the difference between the two strains was larger (167, for CSS77, and 110, for CSS74, µmol O₂·[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\textsubscript{i} deficiency was dependent on All3953 and that the defect in strain CSS74 was exclusively due to the lack of All3953.

\textit{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 \textit{all3953} promoter a version of All3953 C-terminally fused to TAP\textsuperscript{Tag} (Rigaut \textit{et al}., 1999), as well as a control strain (CSS68) expressing the TAP\textsuperscript{Tag} alone under the control of the \textit{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 µE·m$^{-2}$·s$^{-1}$) at 30°C 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\textsubscript{i} deprivation in CSS77, but did not appreciably change in CSS74. The alr0223 (NdhA homolog) gene was about 2-fold induced under C\textsubscript{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\textsubscript{i} limitation and to repress it when C\textsubscript{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\textsubscript{i} limitation (Supp. Fig. 1) and, indeed, no binding region of All3953 was found described
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 rubL 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 rubL observed under high C\(_i\) (Fig. 4). On the other hand, as mentioned above, the rubL 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\textsubscript{i} transport (\textit{all1304}, encoding an homolog of the BicA bicarbonate transporter; \textit{alr4156} encoding a homolog of the NdhF3 subunit of the NDH-1\textsubscript{3} CO\textsubscript{2} uptake system; and \textit{alr0869} encoding a homolog of the NdhF4 subunit of the NDH-1\textsubscript{4} CO\textsubscript{2} uptake system); components of the carboxysome shell (\textit{all0868}, putative \textit{ccmK}) and 2-phosphoglycolate metabolism (\textit{alr1004} and \textit{alr2873}, possibly related to photorespiration \cite{Eisenhut et al., 2008}). Notably, All3953 targets include also a number of genes encoding photosystem components, such as \textit{alr5154} (\textit{psaA}, encoding the PSI core protein PsaA), \textit{alr3727} (\textit{psbAll}, encoding a component of form II of the PSII core protein PsbA [D1]), \textit{alr4592} (\textit{psbAll}, encoding another component of form II of PsbA) and \textit{alr1216} (PSII 12 kD extrinsic protein PsbU), and genes related to PS activity. In the latter group are \textit{alr4149} (biliverdin reductase, putatively involved in phycobilisome -PSI antenna- synthesis), and genes that can participate in photosynthetic electron transfer, such as \textit{alr0223} and \textit{alr0348} (\textit{ndhA} and \textit{ndhD}, subunits of other putative NADH dehydrogenases), \textit{alr1576} (dehydrogenase subunit), \textit{all0737} (thioredoxin reductase), \textit{all1365} (CytM cytochrome), \textit{alr4148} (ferredoxin I), \textit{all3891} and \textit{all4446} (flavodiiron proteins Flv1A and Flv4, respectively). (Besides in CO\textsubscript{2} uptake, NdhF3 and NdhF4 can also participate in electron transfer.)

Reports on gene expression regulation by C\textsubscript{i} are scarce for \textit{Anabaena}. However, in the unicellular cyanobacterium \textit{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\textsubscript{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 \cite{Wang et al., 2004}. Our analysis extends the array of photosynthetic genes responding to C\textsubscript{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 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 limitation or exposure to HL, has either been described or could be
predicted. Thus, the psbAll and psbAll 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 O2 (Eisenhut et al., 2008). To the best
of our knowledge, the regulator responsible for the response to Ci 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
data 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 Ci 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*<sub>j</sub> 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 photoautotrophically at 30°C with illumination (80 µE·m<sup>−2·s</sup><sup>−1</sup>) in liquid BG11 medium (Rippka et al., 1979) supplemented with 3 mM NH₄Cl, 6 mM TES buffer and 10 mM NaHCO₃ and bubbled with a mixture of CO₂ and air (1% v/v) (HC). Other conditions used were no NaHCO₃ supplement and bubbling with air (LC); 12 µE·m<sup>−2·s</sup><sup>−1</sup> (LL); 175 µE·m<sup>−2·s</sup><sup>−1</sup> (HL). For growth on plates illumination was 9 µE·m<sup>−2·s</sup><sup>−1</sup> (LL) or 34 µE·m<sup>−2·s</sup><sup>−1</sup> (HL). For the mutants generated in this work, antibiotics were used at the following concentrations: Sm, 2 µg ml<sup>−1</sup>; Sp, 2 µg ml<sup>−1</sup>; and Nm, 25 µg ml<sup>−1</sup> for bubbled cultures; and Sm, 5 µg ml<sup>−1</sup>; Sp, 5 µg ml<sup>−1</sup>; and Nm, 40 µg ml<sup>−1</sup> for cultures in solid medium.

#### Strain construction
To construct a mutant of the \textit{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 (Sall), and primers all3953-16 (Sall) and all3953-17 (PstI), respectively (all primers are specified in Supp. Table 2). The PCR products were digested with Sall 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 \textit{et al.}, 1993), obtaining plasmid pCSS161. Plasmid pCSS161 was digested with SpeI and the 2-kb Sm\textsuperscript{r} Sp\textsuperscript{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 \textit{et al.}, 1997). Exconjugants resistant to Sm and Sp, which had the \textit{\textDelta 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\textsuperscript{r} and Sp\textsuperscript{r} plasmid pCSS163, a derivative of plasmid pCSE124 (Olmedo-Verd \textit{et al.}, 2006) containing the C.S3 gene cassette, was transferred to \textit{Anabaena} by conjugation. Exconjugants that had the pCSS163 integrated in the alpha plasmid of \textit{Anabaena} were selected, obtaining the strain CSS77 (Supp. Fig. 2).

To complement the \textit{all3953} mutation of strain CSS74, a DNA fragment encompassing the whole \textit{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\textsuperscript{r} encoding vector pRL424 (Elhai and Wolk, 1988) producing plasmid pCSS164, which was transferred to strain CSS74 by conjugation followed by selection for Nm\textsuperscript{r}. The genomic structure of the exconjugants in the \textit{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<sup>r</sup> Sp<sup>r</sup> 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<sup>-1</sup>) in the light (80 µE·m<sup>2</sup>·s<sup>-1</sup>) in medium supplemented with 2 µg·ml<sup>-1</sup> 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·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 4°C 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 (All39530TAP), and thus to determine the specific binding regions of All39530-TAP in the genome of Anabaena. The binding regions were visualized and analyzed using the UCSC Microbial Genome Browser (Schneider 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$_2$S$_2$O$_4$ as the reducing agent. O$_2$ production was measured in the light (400 µE·m$^{-2}$·s$^{-1}$) after a seven-minute incubation in the dark.

Acknowledgments

This work was supported by grants BFU2010-17980 and BFU2013-44686-P from the Spanish government, co-financed by FEDER. The authors are grateful to K. Kornacker for carrying out the Triform and CisFinder analyses, and to H. Auer and I. Pons, from the Functional Genomics Core Facility of the IRB, Barcelona (Spain); to F. Monje-Casas and Yagut Allahverdiyeva-Rinne for critical reading of the manuscript and to M. J. Huertas and A. Torrado for help with O$_2$ production measurements.

REFERENCES


Figure legends:

**Fig. 1.** Growth of the all3953 mutant. A, The growth rate constant (\( \mu = \ln 2 / t_d \), 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. \( \Delta \)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\(_4\)Cl (A), 17.6 mM NaNO\(_3\) (N), or no combined nitrogen (0N). 5-\( \mu \)l 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 \( \Delta \)all3953 mutant and complemented strain. Northern analysis carried out with RNA from strains CSS77 (control) CSS74 (\( \Delta \)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 mpB 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...
(GTAN₆TAC) is indicated in green, and the three putative binding sites for All3953 (Box I, Box II and Box III) are indicated in blue.

Fig. 4. qRT-PCR analysis of the expression of selected photosynthesis and respiration-related All3953 gene targets. Transcriptional response of the indicated genes to Cᵢ limitation in the control (CSS77) and ∆all3953 mutant (CSS74) strains was investigated. RNA was isolated from cells grown with 10 mM NaHCO₃-supplemented medium bubbled with 1%CO₂ in air (0) incubated for 1 h (1) or 3 h (3) in NaHCO₃-free medium bubbled with air. Bars represent the mean transcript levels (± standard deviation) in three independent experiments.
Table 1. Results of the ChIP-Seq analysis of All3953 binding to DNA at 3 h after C\textsubscript{i} limitation

<table>
<thead>
<tr>
<th></th>
<th>Binding regions found</th>
<th>Genes ascribed</th>
<th>Position of the binding region with respect to the gene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>upstream</td>
</tr>
<tr>
<td>Chromosome</td>
<td>127</td>
<td>157</td>
<td>118</td>
</tr>
<tr>
<td>Alpha</td>
<td>10</td>
<td>13</td>
<td>8</td>
</tr>
<tr>
<td>Beta</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Gamma</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>142</td>
<td>175</td>
<td>126</td>
</tr>
</tbody>
</table>

*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.

<table>
<thead>
<tr>
<th>Functional category</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acid biosynthesis</td>
<td>5</td>
</tr>
<tr>
<td>Biosynthesis of cofactors, prosthetic groups, and carriers</td>
<td>4</td>
</tr>
<tr>
<td>Cell envelope</td>
<td>2</td>
</tr>
<tr>
<td>Cellular processes</td>
<td>5</td>
</tr>
<tr>
<td>Central intermediary metabolism</td>
<td>3</td>
</tr>
<tr>
<td>DNA replication, recombination and repair</td>
<td>3</td>
</tr>
<tr>
<td>Energy metabolism</td>
<td>4</td>
</tr>
<tr>
<td>Other categories</td>
<td>19</td>
</tr>
<tr>
<td>Photosynthesis and respiration</td>
<td>19</td>
</tr>
<tr>
<td>Purines, pyrimidines, nucleosides and nucleotides</td>
<td>2</td>
</tr>
<tr>
<td>Regulatory proteins</td>
<td>21</td>
</tr>
<tr>
<td>Translation</td>
<td>9</td>
</tr>
<tr>
<td>Transport and binding proteins</td>
<td>4</td>
</tr>
<tr>
<td>Unknown and hypothetical proteins</td>
<td>75</td>
</tr>
<tr>
<td>BR</td>
<td>NLQ</td>
</tr>
<tr>
<td>----</td>
<td>------</td>
</tr>
<tr>
<td>7</td>
<td>172.84</td>
</tr>
<tr>
<td>12</td>
<td>38.58</td>
</tr>
<tr>
<td>19</td>
<td>62.81</td>
</tr>
<tr>
<td>25</td>
<td>238.45</td>
</tr>
<tr>
<td>27</td>
<td>66.90</td>
</tr>
<tr>
<td>31</td>
<td>36.15</td>
</tr>
<tr>
<td>34</td>
<td>385.11</td>
</tr>
<tr>
<td>35</td>
<td>31.96</td>
</tr>
<tr>
<td>37</td>
<td>63.26</td>
</tr>
<tr>
<td>38</td>
<td>204.06</td>
</tr>
<tr>
<td>39</td>
<td>29.78</td>
</tr>
<tr>
<td>86</td>
<td>170.25</td>
</tr>
<tr>
<td>91</td>
<td>155.06</td>
</tr>
<tr>
<td>98</td>
<td>166.48</td>
</tr>
<tr>
<td>99</td>
<td>61.47</td>
</tr>
<tr>
<td>108</td>
<td>163.25</td>
</tr>
<tr>
<td>112</td>
<td>233.71</td>
</tr>
<tr>
<td>121</td>
<td>51.04</td>
</tr>
</tbody>
</table>

*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).
Figure 1
262x350mm (300 x 300 DPI)
Figure 2
262x350mm (300 x 300 DPI)
Figure 3
297x420mm (300 x 300 DPI)
Figure 4
297x420mm (300 x 300 DPI)