In vivo features of signal transduction by the essential response regulator RpaB from Synechococcus elongatus PCC 7942

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The NblS-RpaB signalling pathway, the most conserved two-component system in cyanobacteria, regulates photosynthesis and acclimatization to a variety of environmental conditions and is involved in negative regulation of high-light-induced genes. However, relevant regulatory details of the NblS-RpaB signalling pathway remain to be elucidated. We recently showed that the response regulator RpaB is regulated by specific (de)phosphorylation from the histidine kinase NblS and that RpaB and its phosphorylatable residue Asp56 are both required for viability of Synechococcus elongatus PCC 7942. We show here that the phosphorylated form of RpaB is present in cells growing under standard laboratory conditions and that high light stress affected the ratio of phosphorylated to non-phosphorylated RpaB. It also decreased the amount of rpaB transcripts without appreciably changing the total levels of RpaB. Quantitative Western blotting and confocal microscopy analyses were consistent with RpaB being a very abundant regulator, with nucleoid localization. A genetically engineered RpaB-GFP (green fluorescent protein) fusion protein rescued lethality of the rpaB null mutant, indicating that it was functional. This is, to our knowledge, the first study demonstrating in a cyanobacterium, and for a two-component response regulator, that the in vivo ratio of phosphorylated to non-phosphorylated protein changes in response to environmental conditions.

INTRODUCTION

Cyanobacteria, phototrophic organisms that perform oxygenic photosynthesis using light as an energy source, have colonized a vast number of habitats. Not surprisingly, they contain more genes encoding two-component systems per unit genome size than other bacteria (Ashby & Houmard, 2006). A high proportion of them are orphan response regulators (RRs) that outnumber histidine kinases (HKs) by almost 2:1 (24:13 in the model system Synechococcus elongatus PCC 7942, hereafter S. elongatus). Amongst cyanobacterial RR s, the OmpR/PhoB family is predominant (Galperin, 2010).

The HK NblS, called Hik33 in Synechocystis PCC 6803 (hereafter Synechocystis), and the RR RpaB are both conserved in cyanobacteria. These two proteins form one of a very limited group of signalling systems that are essential in bacteria. Their HKs share structural characteristics and are thought to respond to cell-generated signals (Dubrac et al., 2008; López-Redondo et al., 2010b). We previously demonstrated that NblS, RpaB and, importantly, key residues for phosphorylation of these proteins are all essential for cell viability in the obligated photoautotroph S. elongatus (López-Redondo et al., 2010b), suggesting that the phosphorylated form of RpaB mediates functions that are required for growth under standard culture conditions.

NblS/Hik33 was first identified in S. elongatus (van Waasbergen et al., 2002). Genetic and transcriptomic approaches suggest strongly that a particularly large number of genes are controlled by the NblS-RpaB system under a variety of stress conditions, including high light, cold-shock, osmotic, salt and oxidative stresses (Bartsevich & Shestakov, 1995; Hsiao et al., 2004; Marin et al., 2003; Mikami et al., 2002; Murata & Los, 2006; Tu et al., 2004). In S. elongatus and related cyanobacteria NblS and two paralogous RRs from the OmpR/PhoB family, RpaB and SrrA, constitute a branched pathway in which complex regulatory interactions are just emerging. Although SrrA is preferentially phosphorylated by NblS in vitro, environmental regulation of srrA prevents deleterious interference with the essential RpaB pathway (López-Redondo et al., 2010b).

In spite of the importance of the NblS-RpaB signalling pathway in cyanobacteria, the actual input signals and output responses remain largely unknown. SipA, a non-essential
factor with an SH3 fold, binds to the ATP binding domain (HATPase_c) of NblS/Hik33 and stimulates its autophosphorylation activity (Espinosa et al., 2006; López-Redondo et al., 2010a; Sakayori et al., 2009; Salinas et al., 2007). As is the case with NblS/Hik33 and RpaB, SipA is also conserved in cyanobacteria. However, little is known regarding the physiological conditions in which SipA modulates NblS activity.

RpaB has been shown to bind to HLR1 (High Light Regulatory 1) sequences in *S. elongatus* and *Synechocystis* (Hanaoka & Tanaka, 2008; Kappell & van Waasbergen, 2007; Seino et al., 2009; Seki et al., 2007). The HLR1 motif consists of two direct repeats of the sequence (G/T) TTACA (T/A) (T/A), separated by two nucleotides. The current model for regulation of RpaB target genes assumes that transfer from standard or low light (LL) to high light (HL) conditions leads to NblS-mediated dephosphorylation of RpaB~P, promoting dissociation of RpaB from its (HL) target sequences and resulting in derepression of hliA and other negatively controlled genes.

As a step towards elucidating the complex regulatory interactions mediated by the essential NblS-RpaB system in *S. elongatus* we analysed the effect of HL stress on *rpaB* gene expression, RpaB abundance and phosphorylation, and investigated the functionality of a fusion protein between RpaB and the green fluorescent protein (GFP).

**METHODS**

**Culture and growth conditions.** *S. elongatus* strains were routinely grown photoautotrophically at 30°C with shaking under constant illumination (30 μE m⁻² s⁻¹) provided by cool white fluorescent lights (denoted as LL conditions). The level of HL was 1000 μE m⁻² s⁻¹, provided by SON-T PIA PLUS Philips bulbs. Photon flux densities were measured with an LI-250 quantum meter (LI-COR Bioscience). The growth medium used was BG11 (BG11o, plus 17.5 mM NaNO₃ and 10 mM HEPES/NaOH, pH 7.8). For growth on plates, the medium was solidified by addition of 1% (w/v) agar. Plates were incubated at 30°C under constant illumination. *S. elongatus* strains were transformed as described by Golden & Sherman (1984), incubated for 48 h at 30°C under illumination on nitrocellulose filters (Millipore), and transformants were selected on kanamycin- or streptomycin-containing BG11 plates. Antibiotic concentrations used were 10 μg kanamycin ml⁻¹ and 5 μg streptomycin ml⁻¹.

**Construction of plasmids and strains.** All generated constructs were analysed by automated dideoxy DNA sequencing. All cloning procedures were carried out in *Escherichia coli* DH5α using standard techniques. Strains and plasmids are listed in Table 1. Oligonucleotides are listed in Table S1 (available in the online version of this paper).

A 750 bp *gfp*-containing fragment was cut with EcoRI and *PstI* from plasmid pDS439 and cloned into pBluescript SK+, giving plasmid pUAGC107. The EcoRI–NotI fragment from pUAGC107 was cloned into pUAGC623 to generate pUAGC814. The CS3 cassette from pUAGC453 was cut with *EcoRV* and HincII and cloned into the *AclI* site of pUAGC814, giving pUAGC815. A multiple cloning site was then incorporated upstream of *gfp* in pUAGC815 to create in-frame fusions to *gfp*. To this end, pUAGC815 sequences were amplified with primer pairs NSI-F/Add-MCS-R and Add-MCS-F/CS3-2F, and the PCR products were then annealed and used as templates in a new PCR with primers NSI-F and CS3-2F. The resulting product was cut with *XhoI* and *SpeI* and cloned into pUAGC815. The new plasmid, designated pUAGC816, was used to generate strain 1GFP.

A 1004 bp DNA fragment containing *rpaB* from pUAGC819 was amplified by PCR with primers RpaB-GFP-1F and RpaB-GFP-1R, cut

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**Table 1. Strains and plasmids used in this work**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or relevant characteristics</th>
<th>Reference or source</th>
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</thead>
<tbody>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>F⁻ phoB lacZ (ΔM15A (lacZYA-argF) U169 endA1 recA1 hsdR17 (rK- m−) deoR thi-1 supE44 gyrA96 relA1 Δ(lacIqZM15T) 2 sΔm1598ΔTn10 Δ(proA proC) DsU169) supE44 ΔlacIΔproAΔproC Δ (ΔlacIqZM15T) 2 sΔm1598ΔTn10 Δ(proA proC) DsU169</td>
<td>Hanahan (1985)</td>
</tr>
<tr>
<td><em>S. elongatus</em></td>
<td>Wild-type <em>S. elongatus</em> PCC 7942</td>
<td>Pasteur Culture Collection</td>
</tr>
<tr>
<td><em>S. elongatus</em></td>
<td>Promoterless <em>gfp</em>, Sm³</td>
<td>This work</td>
</tr>
<tr>
<td><em>S. elongatus</em></td>
<td><em>rpaB</em>-gfp, Sm³</td>
<td>This work</td>
</tr>
<tr>
<td><em>S. elongatus</em></td>
<td><em>rpaB</em>-gfp, <em>rpaB</em>: C.K1(−), Sm³ Km³</td>
<td>This work</td>
</tr>
<tr>
<td><em>S. elongatus</em></td>
<td><em>rpaB-D56A</em>-gfp, Sm³</td>
<td>This work</td>
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<tr>
<td>pUAGC589</td>
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<td>López-Redondo et al. (2010b)</td>
</tr>
<tr>
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<td><em>rpaB</em>: C.K1(−), Ap⁰ Km³</td>
<td>López-Redondo et al. (2010b)</td>
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<tr>
<td>pUAGC623</td>
<td>pBluescript II SK + with NSI</td>
<td>Espinosa et al. (2010)</td>
</tr>
<tr>
<td>pUAGC107</td>
<td>pBluescript II SK + with <em>gfp</em>, Ap⁰</td>
<td>This work</td>
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<td>This work</td>
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<td>pUAGC814 with C.S3, Ap⁰ Sm³</td>
<td>This work</td>
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<td>pUAGC816</td>
<td>pUAGC815 with MCS upstream <em>gfp</em>, Ap⁰ Sm³</td>
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<td>This work</td>
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<tr>
<td>pUAGC692</td>
<td>pUAGC816 with <em>rpaB-D56A</em>-gfp, Ap⁰ Sm³</td>
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<td>pUAGC693</td>
<td>pUAGC816 with Φ(<em>rpaB-D56A</em>-gfp), Ap⁰ Sm³</td>
<td>This work</td>
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with XhoI and AvrII, and cloned into pUAG816. The resulting plasmid, pUAGC690, expresses an RpaB-GFP fusion protein with the linker sequence ASSGIIH between the two proteins. Transformation of S. elongatus with pUAGC690 gave rise to strain 1BGFP. Transformation of 1BGFP with pUAGC594 (Lopez-Redondo et al., 2010b) produced strain 1BGFP/BK. To generate pUAGC692, plasmid pUAGC859 was used as template in a QuikChange Mutagenesis kit (Roche) using RpaB-D56A-F and RpaB-D56A-R. Next, rpaB<sup>5661</sup> was fused to <i>gfp</i> from pUAGC16 as described above for <i>rpaB-gfp</i>. Transformation of S. elongatus with the resulting plasmid (pUAGC693) produced strain 1B<sup>5661</sup>GFP.

**Immunodetection methods.** Antiserum against purified recombinant histagged RpaB (H<sub>6</sub>-RpaB) was produced in rabbits (Pineda Antikörper Service, Berlin, Germany). An anti-GFP, N-terminal antibody produced in rabbit (Sigma-Aldrich) was used for detection of RpaB-GFP according to the manufacturer’s instructions. For immunodetection, 15 ml samples of S. elongatus cells were harvested by centrifugation at 7300 g for 5 min. The pellet was resuspended in 100 µl lysis buffer (50 mM Tris/HCl, pH 7.4, 4 mM EDTA, 0.5 mM PMSF, 0.5 mM benzamidine, 1 mM DTT), and 50 µl of 100 µm glass beads was added. The mixture was homogenized with four cycles of 30 s in a Minibeadbeater. After brief centrifugation (1600 g for 5 min), the supernatant fraction (crude protein extract) was transferred to a new tube. Protein concentrations were estimated according to Lowry (Bio-Rad RC DC) and Bradford (Bio-Rad) reagents. Both reagents yielded similar protein estimates.

Protein immunodetection was performed on protein-loaded PVDF membranes, transferred from acrylamide gels using a semi-dry system. Membrane was blocked with TBS (20 mM Tris, pH 7.5, 500 mM NaCl) solution containing 5% BSA for 1 h at room temperature, and incubated overnight in a 1:5000 anti-RpaB or 1:10 000 anti-GFP dilution in TBS solution containing 2% BSA. The membrane was incubated with ECL rabbit IgG, HRP-linked F(ab’)<sub>2</sub> fragment (from donkey) (GE Healthcare). Immunoreactive bands were detected using the ECL Plus Western blotting Detection kit (GE Healthcare) and scanning in a Typhoon 9410 fluorescence imaging system (GE Healthcare) using a 488 nm/520BP40 laser/filter. A control of total protein loading and quality transfer was obtained after membrane staining with Fast Green (FCF).

The standard curve for RpaB quantification was generated by adding 5–100 ng H<sub>6</sub>-RpaB, purified as described previously (Lopez-Redondo et al., 2010b), to 10 µg protein extract from 1BGFP/BK. Immunoblot steps were as described above and quantification of band intensity was performed with Image Quant (Amersham-Biosciences).

**Phos-tag acrylamide SDS-PAGE separation of phosphorylated and unphosphorylated RpaB proteins.** Aliquots of cultures grown in BG11 under standard light and after a shift to HL or kept in standard light were taken at different time points. Cells were quickly spun down at 4 °C and the pellet was stored at −20 °C. Protein extracts were obtained as described above. Samples were kept on ice and loaded omitting the heating step to prevent hydrolysis of phospho-Asp. Phos-tag acrylamide gels, prepared according to the manufacturer’s instructions, were loaded with 10 µg protein per lane and run under constant voltage (100 V) until the dye front ran off the gel. Proteins were transferred to PVDF and immunodetection was carried out as described above. The ratio between phosphorylated and unphosphorylated RpaB was determined after band quantification with Image Quant TL analysis software (Amersham Biosciences).

To determine the stability of the phosphoryl group of RpaB during protein extract preparation, first H<sub>6</sub>-RpaB (approx. 35 µg) was phosphorylated in <i>vitro</i> in the presence of acetyl-phosphate (25 mM) and purified as described previously (Lopez-Redondo et al., 2010b). Purified H<sub>6</sub>-RpaB, treated with or without acetyl-phosphate, was resuspended in lysis buffer (described above) and used for crude protein extract preparation from strain 1BGFP/BK following the steps described.

**Real-time RT-PCR analysis.** S. elongatus cells were routinely grown until they reached mid–maximum-phase (OD<sub>750</sub> 0.5) in LL conditions and then transferred to the HL condition. Aliquots of 50 ml were removed for RNA extractions at different time points. The samples were centrifuged and pellets were stored at −20 °C. Total RNA was isolated using a hot phenol method and total nucleic acids were quantified based on absorbance at 260 nm. Then, 10 µg total nucleic acids was treated using a TURBO DNA-free kit (Ambion) following the manufacturer’s protocol. Integrity of purified RNA was verified by electrophoresis. About 0.5 µg RNA was reverse transcribed using RevertAid H Minus M-Mul V Reverse Transcriptase (Fermentas) in a total volume of 30 µl, using primers RpaB7942-2R, PsA-A-R, NewHilA-R and RnpB-R for <i>rpaB</i>, <i>psaA</i>, <i>hliA</i> and <i>rnpB</i> cDNAs, respectively.

Quantitative PCR was performed with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using Maxima SYBR Green/ROX qPCR Master Mix reagents (Fermentas). Each 20 µl reaction mixture contained 1× buffer, 0.4 µM each primer (Table S1) and 2 µl the reverse transcription reaction. The reaction mixtures were incubated for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 30 s at 60 °C and 15 s at 72 °C. After the last cycle, the PCR products were subjected to heat denaturation over a temperature gradient from 60 to 95 °C. A complete assay consisted of three independent experiments in which each sample was analysed in triplicate. All samples were tested for the presence of residual DNA during quantitative real-time PCR with a reverse transcriptase-free control.

The real-time PCR data were analysed using 7000 System Sequence Detection Software version 1.2.3 (Applied Biosystems). Data were plotted as normalized reporter signals, representing the levels of fluorescence detected during the PCR process after subtraction of background noise versus cycle number. A threshold was set manually in the middle of the linear phase of the amplification curve. The cycle threshold (C<sub>T</sub>) value is defined as the cycle in which the increase in the reporter signal (fluorescence) crosses the threshold. The C<sub>T</sub> difference between treated (10, 30 and 180 min) and non-treated (0 min) samples is ΔC<sub>T</sub>. The cDNA n-fold change for each gene at selected time points was determined according to 2<sup>−ΔΔC<sub>T</sub></sup>.

**Microscopy, image acquisition and analysis.** Exponentially growing cells were mounted on 1% low-melting-point agarose pads for microscopy. To stain DNA, cell samples were supplemented with DAPI (4',6-diamidino-2-phenylindole; 200 ng µl<sup>−1</sup>). Samples were observed and photographed with a Leica confocal microscope (running under Leica Confocal Software version 2.61, Leica Microsystems) equipped with HeNe/red (633 nm), HeNe/green (543 nm) and blue argon/diode lasers (458, 476, 488, 514 nm) and a DM IRBE2 inverted microscope (HCX PL APO 63× oil-immersion objective, numerical aperture 1.4). Filter specificities were as follows: ex633 (HeNe laser, 3%), TD 488/543/633, em665–700 gain 540 V (for cyano bacterial autofluorescence analysis); ex488 (argon laser, 20%), TD 488/543/633, em495–537 gain 720 V (for GFP analysis); and ex405 (argon/diode laser, 60%), RT 30/70, em434–473 gain 900 V (for DAPI analysis). The pinhole was 1.00 Airy units. Image analysis was carried out with Leica Software 2.61 and Image J 1.44p.

**RESULTS**

**Effect of HL exposure on levels of rpaB transcripts and RpaB protein**

Visual inspection revealed the presence of HLR1 sequences located at −118 to −101 from the transcription start site of
the *S. elongatus rpaB* gene (Fig. 1a). The possibility of an entry point for HL regulation prompted us to analyse *rpaB* transcripts under experimental conditions in which we observe a very high induction in levels of *hliA* transcripts (Fig. S1). The *psaA* gene, which encodes a subunit of the PSI reaction centre, was also included in the experiments because it bears an HLR1 motif at −63 to −46 from the transcription start site, and we considered it to be a good candidate to be positively regulated under LL by RpaB/HLR1 in *S. elongatus*. The *rnpB* gene, expected to be constitutive, was included as a negative and loading control.

To determine the effect of HL treatment on *rpaB* and *psaA* transcript levels, we performed quantitative RT-PCR. Cultures grown under standard light conditions (LL) were transferred to HL and the levels of transcripts were determined at intervals. As shown in Fig. 1(b), upon exposure to HL levels of both *rpaB* and *psaA* transcripts decreased. However, the kinetics of these events differed between the two genes. The lowest levels of transcripts for *rpaB* and *psaA* were recorded at 10 and 30 min, corresponding to 60 and 20% of their initial levels, respectively. Furthermore, for *psaA* this recovery was not completed at the end of the 180 min of HL treatment.

It has been reported that HL exposure did not alter the levels of recombinant RpaB-FLAG in *S. elongatus* (Hanaoka & Tanaka, 2008) and we therefore investigated whether levels of the endogenous RpaB protein would also remain constant under our HL treatment. We obtained polyclonal antibodies raised against *S. elongatus* RpaB to use them in Western blot analysis comparing crude cell protein extracts from cultures grown on LL and at different time points after transfer to HL. As shown in Fig. 1(c), the amount of RpaB was constant during the time course of the experiment, a result in very close agreement with those obtained by others with the RpaB-FLAG fusion.

**Cellular abundance of the RR RpaB**

To estimate the cellular amount of RpaB by quantitative Western blotting with anti-RpaB, we mixed various amounts of purified H6-RpaB with 10 μg protein extract from *S. elongatus* strain 1BGFP/BK (Table 1), which expresses only RpaB-GFP, a longer (but functional, see below) version of the protein. We exploited the difference in size to prevent interference of the endogenous RpaB with the similarly sized purified protein (H6-RpaB). As RpaB-GFP migrated much more slowly (data not shown),

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**Fig. 1.** Effect of HL on *rpaB* and *psaA* transcripts and on RpaB protein levels. (a) HLR1 sequences upstream of *rpaB* and *psaA*. Numbers refer to positions relative to the transcription start points (filled arrows). (b) Impact of HL on levels of *rpaB*, *psaA* and *rnpB* transcripts determined by quantitative RT-PCR. The assays were carried out on RNA extracted from *S. elongatus* cells grown under standard light (LL, 0 min) and then shifted to HL (10, 30 and 180 min). Transcript levels are relative to those under LL (time 0), and are the means ± s.d. of three repeated measurements from three independent experiments. (c) Effect of HL on RpaB protein levels. The RpaB band is indicated with an arrowhead. A representative result of three independent experiments, with its protein loading and transfer quality control (bottom panel), is shown.
only H$_6$-RpaB was detected in the corresponding gel region (Fig. 2a). Protein extracts from the wild-type strain under LL conditions were used in parallel for estimations of RpaB. In all samples, the amount of RpaB detected fell within the linear range of the standard curve, giving $76 \pm 13$ ng per 10 mg of total cell protein extracts (Fig. 2b).

HL stress triggers partial dephosphorylation of the RpaB~P pool

To gain additional insight into HL signalling at the RpaB pathway, we took advantage of the relatively high stability of RpaB~P, with a half-life of 11.5 h at room temperature (López-Redondo et al., 2010b), to detect both RpaB and RpaB~P forms using phos-tag-based reagents in combination with Western blots using anti-RpaB. As shown in Fig. 3(a), this strategy revealed two bands, one of which (the upper band) was heat-labile, as expected for the phosphorylated form of RR. Therefore, it was possible to detect both RpaB and RpaB~P forms from S. elongatus extracts.

Next, protein extracts were obtained from S. elongatus cultures under LL and at different points after being transferred to HL, subsequently separated by phos-tag acrylamide electrophoresis and then analysed by Western blotting with anti-RpaB. As shown in Fig. 3(b), approximately 30% of the total RpaB protein detected from LL cultures of wild-type S. elongatus was RpaB~P. However, comparison of the RpaB~P to total RpaB ratio along the time points of the experiment revealed a rapid and transient HL-dependent drop in relative RpaB~P levels. In particular, during the first 2 min of the HL treatment, the RpaB~P to total RpaB ratio decreased from 30 to 15%, after which it showed a very slow recovery that was not complete at the end of the experiment (150 min of HL stress).

The experiment detailed in Fig. 3(b) provided information on the in vivo dynamics of RpaB dephosphorylation upon...
HL treatment but did not necessarily provide an accurate determination of the RpaB–P to total RpaB ratio, which might change as a consequence of the manipulation of cultures and protein extracts. To evaluate the impact of the protocol followed to obtain cell extracts on the stability of RpaB–P, recombinant H6-RpaB was first phosphorylated in vitro by addition of acetyl-phosphate, and this resulted in a mixture of H6-RpaB and H6-RpaB–P (Fig. 3c, compare lanes 1 and 2). The H6-RpaB(–P) protein mixture was then incorporated in the cell pellets to be processed for protein extraction. For this experimental control, the pellet was obtained from strain 1BGFP/BK, so we could easily distinguish H6-RpaB from H6-RpaB–P without interference by the S. elongatus RpaB protein. As shown in Fig. 3(c), incubation of H6-RpaB with S. elongatus cell extracts decreased the level of phosphorylation of the recombinant protein (compare lanes 1 and 3), indicating that the experimental manipulations did result in a decrease of the RpaB–P to RpaB ratio. In addition, no phosphorylation of recombinant H6-RpaB took place during extract preparation (Fig. 3c, compare lanes 2 and 4). On the other hand, a possible impact of culture manipulation on the observed RpaB–P to RpaB ratio was excluded by repeating the experiment under LL (Fig. S2).

RpaB-GFP complements essential functions of RpaB in S. elongatus

To visualize RpaB cellular localization by confocal microscopy, we constructed a protein fusion between RpaB and GFP separated by a 7 aa linker and expressed from a neutral site (NSI) of the S. elongatus chromosome. To maintain physiological regulation, sequences up to 250 bp upstream of the rpaB coding region were included in the construct. To allow selection on streptomycin plates of S. elongatus recombinants obtained by allelic replacement, the C.S3 marker cassette was cloned downstream of rpaB-gfp. A similar construct without rpaB sequences was also generated for control purposes. In each case, allele replacement and complete segregation of selected transformants were verified by PCR analysis (Fig. 4a). Western blot analysis confirmed the expression of the expected proteins: RpaB-GFP and RpaB in the newly generated strain (Fig. 4b, lanes labelled 1BGFP) and just RpaB in the control strain (Fig. 4b, lanes labelled 1GFP).

Because RpaB is essential for viability (López-Redondo et al., 2010b), it was important to know whether RpaB-GFP can rescue the lethality conferred by rpaB inactivation in S. elongatus. In particular, we sought to replace rpaB by the allele C.K1::rpaB in both strains 1BGFP and 1GFP, the latter used as wild-type control. PCR analyses (with oligonucleotides hybridizing to the rpaB region, see Fig. S3 for details) of kanamycin-resistant clones from the corresponding transformations confirmed that strain 1BGFP did allow rpaB inactivation (Fig. 4a) but 1GFP did not (data not shown). The newly generated strain bearing C.K1::rpaB and rpaB-gfp was named 1BGFP/BK. As expected, a longer band (1959 bp) could be amplified by PCR from strain 1BGFP/BK instead of the shorter one (879 bp), identifying the wild-type rpaB allele in strains 1GFP and 1BGFP. Consistent with the absence of wild-type rpaB alleles, Western blot analysis confirmed that 1BGFP/BK expressed RpaB-GFP but not RpaB (Fig. 4b, lanes 1BGFP/BK). Therefore, the results indicate that RpaB-GFP provides all RpaB functions that are essential for cell viability.

To confirm the importance of the phosphorylatable Asp56 on the essential functions provided by RpaB-GFP, we next introduced a point mutation (D56A) at the corresponding position and tested the ability of the rpaB^{D56A}-gfp allele to complement the rpaB null mutant exactly as before. As expected, rpaB alleles persisted in the streptomycin-resistant transformants (Fig. S3), indicating that the integrity of Asp56 from RpaB is essential for viability. Furthermore, detection with anti-RpaB of a heat-labile band with reduced mobility compared with RpaB-GFP (Fig. S4) confirmed the presence of RpaB–P-GFP in wild-type strains carrying the rpaB-gfp gene fusion.

Confocal microscopy analysis and visualization of RpaB-GFP in S. elongatus cells

Strains expressing RpaB-GFP or just RpaB were analysed in parallel by laser scanning confocal microscopy. Schematic
representations of the relevant chromosomal regions and constructs described here are shown at the top of Fig. 5. Additional details on strain construction and subsequent PCR analysis are shown in Fig. S3. First, the specificity of the RpaB-GFP signal was confirmed by comparing strain 1BGFP, which produced an intense GFP fluorescence signal, with the control strain 1GFP, which gave just low background noise (Fig. 5c). Visualization of the different cellular compartments was aided by recording the autofluorescence (red signal) from thylakoid membranes, by staining DNA with DAPI and by merging the different signals (Fig. 5a, b, d–f). In 1BGFP, most of the GFP fluorescence signal was detected in the most inner and central regions of the cytoplasm, corresponding to the nucleoid.

**DISCUSSION**

Here, we show that the essential RR RpaB is phosphorylated in *S. elongatus* and that HL stress triggers RpaB de-phosphorylation, thus confirming predictions for the mechanism operating at a key step of the NblS-RpaB signal transduction pathway. Furthermore, the kinetics of RpaB dephosphorylation is fully consistent with the timing of other HL-induced events, including the induction of *srrA*, thought to be important for implementation of stress-specific programmes (López-Redondo *et al.*, 2010b) and the decrease in the binding of RpaB to the *hliA* and *rpoD3* promoters (Hanaoka & Tanaka, 2008).

The *in vivo* dynamics of RpaB (de-)phosphorylation upon HL treatment was determined by phos-tag separation followed by Western blotting (Barbieri & Stock, 2008), a method described for the well-characterized RR involved in *E. coli* phosphate assimilation PhoB. We show here that the same approach is applicable to monitoring aspartate phosphorylation from RpaB, a result in line with the relatively high stability previously determined for purified H6-RpaB~P (López-Redondo *et al.*, 2010b).

HL treatment triggered an immediate decrease in levels of RpaB~P. However, the extent of HL-induced RpaB dephosphorylation registered was relatively low, suggesting that complete dephosphorylation of the RpaB~P pool is not required for stress acclimatization. Although we cannot exclude that other experimental conditions or environmental stress might have a greater impact on RpaB~P, it is worth noting that the same HL treatment resulted in very high induction of *hliA* transcripts (Fig. S1). Given the considerable amount of RpaB protein found in *S. elongatus* cells (approx. 0.8% of total extracted protein), it would not be surprising if not all of it is susceptible to being dephosphorylated at once, or if complete dephosphorylation would require the simultaneous occurrence of more than one stress signal.

As the laboratory manipulations carried out to extract proteins from cell pellets per se favoured dephosphorylation of purified H6-RpaB~P, the value of 30% obtained for the intracellular proportion of RpaB~P to total RpaB protein under LL is not a precise estimation, and the actual proportion of RpaB~P is probably higher. However, the results still suggest that not all of the RpaB protein is in the RpaB~P form under LL conditions and, given the abundance of the RR RpaB, it would be reasonable to think...
that, in addition to RpaB~P, RpaB may also play a regulatory role in *S. elongatus* cells. In this context, Kato *et al.* (2011) have suggested that RpaB~P and RpaB may bind with similar affinity to *(nblA)* DNA fragments *in vitro*.

The role of RpaB in negative regulation of genes dramatically expressed during HL stress, such as the paradigmatic *hlIA* gene, has attracted much attention. However, the essential requirement of RpaB under standard (non-stress or LL) laboratory conditions suggests the importance of RpaB for normal growth of cultures, and recent studies support the involvement of RpaB~P and/or HLRI sequences on positive regulation (under LL) of target genes essential for viability (López-Redondo *et al.*, 2010b) and photosynthesis (Eriksson *et al.*, 2000; Muramatsu & Hihara, 2006, 2007; Seino *et al.*, 2009; Takahashi *et al.*, 2010). The abundance of RpaB~P in *S. elongatus* and the behaviour of both rpaB and paaA transcripts during HL exposure, reported here, emphasize the same idea. The actual impact of HL treatment on rpaB transcripts was quite low and did not seem to alter levels of the RpaB protein. A possible interpretation of these results is that *S. elongatus* can sense RpaB levels and keep them relatively constant in spite of changes in transcript levels.

Consistent with the role of RpaB as a DNA binding regulator and its abundance in *S. elongatus*, a strong fluorescent signal occupying the main central area of the cell was detected for the RpaB-GFP fusion by confocal microscopy. In addition to this strong signal clearly located throughout the nucleoid region, weaker GFP signals coming from rather disperse foci were also detected in the region surrounding the nucleoid. Although we could not correlate these weak signals to RpaB-GFP with confidence, their distribution was compatible with thylakoid localization. In this context, it is worth noting the identification by proteomic analysis of NblS (Pisareva *et al.*, 2011) and RpaB (Srivastava *et al.*, 2005) at the thylakoid membrane of *Synechocystis*.

Functional complementation of the rpaB null mutant by the rpaB-gfp gene fusion and detection of RpaB~P-GFP from cell extracts indicated that RpaB-GFP can provide RpaB essential functions and that the GFP tag does not prevent RpaB phosphorylation. The finding that a point mutation derivative affecting the phosphorylatable residue (D56A) of RpaB-GFP did not complement the rpaB null mutant further underlines the importance of RpaB~P for cell viability. Additional experimentation is needed to further our understanding of how this global RR contributes to cell homeostasis in cyanobacteria.

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