Environmental control of phosphorylation pathways in a branched two-component system

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Summary

NblS, the most conserved histidine kinase in cyanobacteria, regulates photosynthesis and acclimatization to a variety of environmental conditions. We used in silico, in vivo and in vitro approaches to identify RpaB and SrrA as the cognate response regulators of NblS and to characterize relevant interactions between components of this signalling system. While genetic analysis showed the importance of the NblS to RpaB phosphorylation branch for culture viability in Synechococcus elongatus PCC 7942, in vitro assays indicated a strong preference for NblS to phosphorylate SrrA. This apparent discrepancy can be explained by environmental insulination of the RpaB pathway, achieved by RpaB-dependent repression of srrA under standard, low light culture conditions. After a strong but transient increase in srrA expression upon high light exposure, negative regulation of srrA and other high light inducible genes takes place, suggesting cooperation between pathways under environmental conditions in which both RpaB and SrrA are present. Complex regulatory interactions between RpaB and SrrA, two response regulators with a common evolutionary origin that are controlled by a single histidine kinase, are thus emerging. Our results provide a paradigm for regulatory interactions between response regulators in a branched two-component system.

Introduction

Two-component systems (TCSs) are widely used in signal transduction and adaptation to environmental changes in bacteria and archa (Gao et al., 2007). They are also found in lower eukaryota and higher plants, but not in animals (Gao et al., 2007). In the prototype system, the sensor histidine kinase (HK), bearing HisKA and HATPase_c domains autophosphorylates on a conserved histidine residue and this phosphoryl group is transferred to an aspartate of the N-terminal phosphoacceptor receiver domain (Res_reg) of the cognate response regulator (RR). Phosphorylation of the RR leads to conformational changes of the C-terminal effector domain (Trans_reg), modifying its affinity for its targets. Finally, the RR is dephosphorylated by its own phosphatase activity or, in several cases, by the cognate HK. While the domains involved in the different phosphorylation steps are structurally conserved, both sensor and output domains are functionally and structurally diverse (reviewed in depth by Stock et al., 2000). This basic scenario can increase in complexity by the existence of phosphoacceptor intermediaries, ‘branched’ pathways where one HK recognizes several RR or vice versa, ‘orphan’ components with unknown partner or ancillary proteins that modulate the activity of HK or RR, allowing integration of multiple signals and generating sophisticated signalling networks similar to those founded in eukaryotes (Laub and Goulian, 2007; Mitrophanov and Groisman, 2008).

Cyanobacteria contain more genes encoding TCSs per genome size than other bacteria (Ashby and Houmard, 2006), a feature that probably reflects the vast number of habitats colonized by these organisms and the regulatory cost of using light as energy source. Additional hallmarks of cyanobacterial TCSs are the predominance of the OmpR/PhoB family amongst RR (Galperin, 2010) and the high proportion of orphan RR, that outnumber HKs by almost 2:1 (24:13 in the model system Synechococcus elongatus PCC 7942, hereafter referred to as S. elongatus). The possible significance of disperse gene organization to the evolution and function of TCSs remains largely unexplored.

The sensor NblS, the most conserved HK in cyanobacteria (Ashby and Houmard, 2006), is a key player in the perception of multiple environmental parameters and...
subsequent adaptation of the metabolic and photosynthetic machineries to changing environmental conditions. NblS was first identified in *S. elongatus* by its implication in the process called chlorosis or bleaching (van Waasbergen *et al.*, 2002), which is triggered by the NblA protein under a variety of stress conditions. Genetic inactivation of nblS has not been achieved in the obligate photoautotroph *S. elongatus*. However, null mutants of the orthologous gene from *Synechocystis* sp. PCC6803, called DspA/Hik33 could be maintained in heterotrophic conditions (Suzuki *et al.*, 2000) and have been the subject of extensive transcriptional analyses. DspA/Hik33 has been shown to regulate a large number of genes under a variety of stress conditions, including high light (HL), cold shock, osmotic, salt and oxidative stress (Bartsevich and Shrestakov, 1995; Mikami *et al.*, 2002; Marin *et al.*, 2003; Hsiao *et al.*, 2004; Tu *et al.*, 2004). The accepted view is that these different incoming signals would reduce anabolism and cause hyper-reduction of photosynthetic electron transport, leading to redox stress. Thus, NblS (Hik33/DspA) may act as a sensor of photosynthetic redox stress under a variety of environmental circumstances. It may also respond to signals that influence membrane fluidity under certain stress conditions such as cold, osmotic and salt stress (Murata and Los, 2006).

The finding that point mutations in nblS resulted in a non-bleaching phenotype (van Waasbergen *et al.*, 2002), prompted the suggestion that NblS was involved in the activation of the RR NblR, which is required for the strong increase in nblA gene expression observed during nutrient stress in *S. elongatus* (Schwarz and Grossman, 1998; Luque *et al.*, 2001; Sendersky *et al.*, 2005; Salinas *et al.*, 2007). However, NblR is an atypical RR with homology to the OmpR/PhoB family that does not interact with NblS and is not phosphorylated (Kato *et al.*, 2008; Ruiz *et al.*, 2008), thus belonging to a new class of Phosporylation-Independent Activation of Response Regulators (Ruiz *et al.*, 2008). The only protein for which a direct physical interaction with NblS has been reported so far, is SipA, a small regulatory factor conserved in cyanobacteria that binds to the ATP binding domain (HATPase_c) of NblS (Espinosa *et al.*, 2006; Salinas *et al.*, 2007; Lopez-Redondo *et al.*, 2010), stimulating its autophosphorylation activity (Sakayori *et al.*, 2009).

Recent indirect evidence has suggested that RpaB is the cognate RR of NblS. Genes containing HLR1 sequences (High Light Regulatory 1) were shown to be controlled by NblS in *Synechocystis* (Kappell *et al.*, 2006). Subsequently, it was determined that RpaB proteins from both *S. elongatus* and *Synechocystis* bind to HLR1 sequences (Kappell and van Waasbergen, 2007; Seki *et al.*, 2007; Hanaoka and Tanaka, 2008; Seino *et al.*, 2009). In addition, reports of failed attempts to inactivate nblS in *S. elongatus* (van Waasbergen *et al.*, 2002; Kappell *et al.*, 2006), and rpaB in *Synechocystis* (Ashby and Mullineaux, 1999) suggested that these two proteins might comprise a TCS essential for cell viability, but no direct proof of RpaB–NblS interaction was provided. On the other hand, genome-wide analysis of cold and hyperosmotic stress-inducible genes suggested that at least two different RRs, Rre26/RpaB and Rre31/RpaA, transduce stress signals in *Synechocystis* (Murata and Los, 2006). However, RpaA does not bind to HLR1 sequences (Kappell and van Waasbergen, 2007) and it turned out to be the cognate RR of SasA, which is involved in circadian cycle regulation (Takai *et al.*, 2006).

In this work we have established that the HK NblS and two RRs with a common evolutionary origin, RpaB (Synpcc7942_1453) and SrrA (Synpcc7942_2416), constitute a branched pathway in which the similarities between the two RRs extend to their output DNA binding domains, thus providing additional opportunities for overlap in their regulatory functions. Remarkably, the NblS system is also unique in that one of the phosphorylation pathways is essential for survival, since *in vivo* phosphorylation of RpaB is required for cell viability and SrrA over production could not substitute for RpaB in regulation of essential gene targets. Paradoxically, *in vitro* assays indicate that SrrA is preferentially phosphorylated by NblS, raising the question of how interference of SrrA with the essential RpaB phosphorylation pathway is avoided. We provide evidence for environmental insulation of the RpaB pathway by means of RpaB-dependent repression of srrA under low light (LL) conditions. The fact that expression of srrA and other HL inducible genes can be down regulated by over expression of RpaB or SrrA also suggests cooperation between pathways under environmental conditions in which both RRs are present. In summary, the results reported here contribute to the repertoire of regulatory mechanisms used by TCSs to adapt to the changing environment and call attention to the importance of branched TCSs in cyanobacteria.

**Results**

*Two parologue RRs, RpaB and SrrA, specifically interact with the HK NblS*

To search for cognate partners of NblS, we used the web accessible tool for prediction of kinase/regulator interactions (http://www.swissregulon.unibas.ch/cgi-bin/TCS.pl) developed by Burger and van Nimwegen (Burger & van Nimwegen, 2008) and based on Bayesian network methods applied to multiple sequence alignment of genomes. According to this method, RpaB is the highest probable NblS RR partner (81% probability), with SrrA as the second best candidate (18% probability). A CheY-like RR (Synpcc7942_0856) was predicted with extremely low
probability (2 e–5 %). Interestingly, RpaB and SrrA are both members of the large OmpR/PhoB family of RRs and show a high degree of similarity between them (61% identity). This resemblance and the phylogenetic distribution of rpaB and srrA genes agree with srrA originating from duplication of rpaB in a S. elongatus ancestor. The srrA gene is restricted to species branching with S. elongatus (Fig. S1), while rpaB is found in all available cyanobacterial genomes.

Previously we used yeast two-hybrid approaches to detect specific interactions between cognate two-component proteins (Martinez-Argudo et al., 2001). Since attempts to retrieve the cognate RR(s) of NblS by screening S. elongatus yeast two-hybrid libraries with a truncated NblS derivative as bait (NblS272–664) were unsuccessful (Espinosa et al., 2006), we made additional constructs to verify and further investigate the specificity and determinants of yeast two-hybrid interactions with RpaB and SrrA. The SMART (Letunic et al., 2009) and tGG predictor (Hessa et al., 2007) programs indicate that NblS is a transmembrane protein with the cytoplasmic portion containing HAMP and PAS domains preceding the conserved HisKA and HATPase_c catalytic domains. For yeast two-hybrid assays we made fusion proteins of GAL4AD and GAL4BD domains to RpaB, SrrA, RpaA and additional NblS derivatives. These included full-length NblS (NblS1–664) and subsequently smaller truncated derivatives (NblS219–664, NblS377–664, NblS272–664, NblS264–664, NblS272–664, NblS377–664) containing at least the conserved HisKA and HATPase domains. C-terminal deletion derivatives NblS1–219 and NblS1–264 were also used as negative controls. The NblS fragments present in the yeast two-hybrid fusion proteins are schematically represented in Fig. 1A (left), alongside a table with a summary of the interaction signals (Fig. 1A right) and a representative YTH assay (Fig. 1B).

In agreement with the in silico predictions, only RpaB and SrrA interacted with NblS derivatives. The interactions were specific, since none of these proteins gave signals with other HKs or RRs used as controls. Interestingly, only full-length NblS and the derivative retaining the HAMP region (NblS219–664) interacted with both RpaB and SrrA, while the shorter fragments, NblS264–664 and NblS377–664 gave interaction signals with SrrA. Control C-terminal deletion derivatives NblS1–219 and NblS1–264 did not interact with either RR. The fact that NblS377–664 gave no interaction with the RRs may be due to occlusion of the HisKA domain by the immediately adjacent N-terminal GAL4 domains. Taken together, the results support the specificity of interactions between NblS and RpaB or SrrA and suggest that, in addition to the main RR–HK interaction determinants located in the HisKA domain of the transmitter module, the HAMP–PAS region of NblS specifically contributes to RpaB recognition or binding affinity.

### Autophosphorylation activity of truncated NblS proteins

Affinity-tagged (N-terminal 6His) versions of the truncated proteins NblS377–664 and NblS219–664 were generated for in vitro studies. Most of the recombinant NblS219–664 protein was expressed in a soluble form, while NblS377–664 could be solubilized from the pellet with low amounts (2 M) of urea, suggesting that it formed weak aggregates rather than inclusion bodies. Attempts to produce intermediary constructs with truncations that extended further into the HAMP and PAS domains were not successful, generating insoluble or inactive proteins (data not shown).

We first evaluated the autophosphorylation activity of the two NblS forms using [γ-32P]-ATP. Time-dependent autophosphorylation assays of NblS219–664 and NblS377–664 showed similar profiles (Fig. 2A and B), suggesting that the presence of HAMP and PAS domains did not significantly affect the autophosphorylation activity of NblS.

Similar results were obtained after N-terminal affinity tag removal by TEV protease treatment of the same proteins (data not shown). Finally, we analyzed the kinetics for the NblS219–664 autophosphorylation reaction, which displayed Km (ATP) and kcat values of 375 μM and 0.0016 min–1 respectively (Fig. 2C). Comparison with the kinetic parameters reported for several HKS (Marina et al., 2001; Noriega et al., 2008; Gutu et al., 2010), including the structural homolog WalK, which has a Km (ATP) of 42 μM and a kcat of 0.084 min–1 (Gutu et al., 2010), indicates that NblS has a low catalytic activity.

### RpaB and SrrA are phosphorylated in vitro by NblS with different preferences; contributions of the HAMP and PAS domains to phosphatase activity

To characterize the NblS signalling pathway in vitro, we produced 6His affinity-tagged versions of RpaB and SrrA and of their receiver domains (RpaB1–122 and SrrA1–130), which include the complete catalytic machinery. The receiver domain of RpaA (RpaA1–125) was similarly produced. Because preparations of full-length versions, particularly RpaB, presented reduced solubility and yielded high batch to batch discrepancies in purity and activity, only results obtained with the receiver domains are reported here.

To evaluate phosphotransfer activity, NblS derivatives were autophosphorylated with [γ-32P]-ATP for 1 h and free ATP was removed by size exclusion chromatography prior to independent addition of RpaB1–122, SrrA1–130 and RpaA1–122. In close agreement with our in silico and yeast two-hybrid data, dephosphorylation of the two P-NblS derivatives was associated with detection of the phosphorylated forms of RpaB and SrrA, but not of RpaA (Fig. 2D). The increase in P–RpaB and P–SrrA paralleled the decrease of P–NblS377–664 during the 15 min of the assay.
In contrast, the reduction in the level of P\textsubscript{~}NblS\textsubscript{219–664} did not correlate with the increase of either P\textsubscript{~}RpaB or P\textsubscript{~}SrrA (Fig. 2D, left panel). Both RRs reached a maximum phosphorylation level in \textasciitilde 1 min that declined in the reaction time-course. Simultaneously with the decrease of the P–RR signal (Fig. 2D), NblS\textsubscript{219–664} phosphotransfer activity quickly decayed (Fig. 2E), suggesting the presence of NblS phosphatase activity which would be in equilibrium with the phosphotransfer activity as a result of P–RR accumulation. SrrA seems to be a better phosphoacceptor than RpaB for NblS\textsubscript{219–664} and, especially for NblS\textsubscript{377–664} (Fig. 2D). In summary, the results indicate that phosphotransfer rates were low in comparison with other TCSs (Skerker \textit{et al.}, 2005) and that the HAMP and/or PAS domains of NblS affect phosphotransfer and are essential for phosphatase activities.

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**Fig. 2D, right panel.** In contrast, the reduction in the level of P–NblS\textsubscript{219–664} did not correlate with the increase of either P–RpaB or P–SrrA (Fig. 2D, left panel). Both RRs reached a maximum phosphorylation level in \textasciitilde 1 min that declined in the reaction time-course. Simultaneously with the decrease of the P–RR signal (Fig. 2D), NblS\textsubscript{219–664} phosphotransfer activity quickly decayed (Fig. 2E), suggesting the presence of NblS phosphatase activity which would be in equilibrium with the phosphotransfer activity as a result of P–RR accumulation. SrrA seems to be a better phosphoacceptor than RpaB for NblS\textsubscript{219–664} and, especially for NblS\textsubscript{377–664} (Fig. 2D). In summary, the results indicate that phosphotransfer rates were low in comparison with other TCSs (Skerker \textit{et al.}, 2005) and that the HAMP and/or PAS domains of NblS affect phosphotransfer and are essential for phosphatase activities.

\[ P\textsubscript{~}RpaB \text{ and } P\textsubscript{~}SrrA \text{ are very stable but can be dephosphorylated by NblS in a nucleotide-dependent manner} \]

To determine the intrinsic stability of P–RpaB and P–SrrA, RpaB\textsubscript{1–123} and SrrA\textsubscript{1–130} were phosphorylated using the small phosphodonor [\textsuperscript{32P}] acetyl phosphate. After removing the free acetylphosphate by gel filtration chromatography, the relative intensities of P–RpaB and P–SrrA were determined.
labelled bands were determined (Fig. 2F), giving half-lives (t1/2 values) of 11.5 and 30.5 h respectively.

When P–RpaB1–123 and P–SrrA1–130 were incubated with ADP (data not shown) or with equimolecular amounts of NbsI$5^{19}$–644 and NbsI$37^{7}$–644 (compare control and +NbsI lanes in Fig. 2G), the stability of P–RpaB or P–SrrA was not affected. However, ADP promoted NbsI$5^{19}$–644 (but not NbsI$37^{7}$–644) mediated dephosphorylation of both P–RRs (compare −ADP and +ADP lanes in Fig. 2G) with rather similar kinetics (approximately 35 and approximately 20% of each P–RR is detected after 5 and 15 min of incubation with NbsI), confirming the requirement of the HAMP–PAS region for NbsI phosphatase activity and the reported effect of ADP on certain HKs (Zhu et al., 2000; Jagadeesan et al., 2009).

**NbsI has preference for SrrA versus RpaB in the phosphotransfer but not in the phosphatase reaction**

To investigate the preference of NbsI phosphotransfer, NbsI$5^{19}$–644 and NbsI$37^{7}$–644 were autophosphorylated as above, incubated with different molar ratios of RpaB1–123 and SrrA1–130 and the reaction allowed to proceed for 2 min (Fig. 3A). P–SrrA1–130 was the most abundant phosphorylated protein, observed even with a RpaB/SrrA ratio of 9:1. P–RpaB1–123 could only be detected when the phosphatase-deficient NbsI$37^{7}$–644 protein was used and there was at least as much RpaB as SrrA. This preference of NbsI towards SrrA1–130 was further confirmed with NbsI$37^{7}$–644 in a time-course reaction. P–NbsI$37^{7}$–644 was incubated with equimolecular amounts of both RpaB1–123 and SrrA1–130 and samples taken at different intervals, up to 30 min (Fig. 3B). P–SrrA1–130 was already detectable after 30 s and reached the steady-state after 10 min of reaction, while P–RpaB1–123 needed 2 min for detection and increased with different kinetics. In the same time-course, phosphorylation of RpaB1–123 amounted to approximately 12–17% of P–SrrA1–130, thus confirming the phosphotransfer preference of NbsI for SrrA.

To investigate the preference for the RR in the NbsI-dependent dephosphorylation reaction, competition assays were performed with NbsI$5^{19}$–644, RpaB1–123 and SrrA1–130 were phosphorylated with [32P] acetyl phosphate and mixed in equimolecular amounts with substoichiometric amounts of NbsI$5^{19}$–644 in the presence of ADP. As shown in Fig. 3C, ADP–NbsI$5^{19}$–644 induced dephosphorylation of P–RpaB1–123 and P–SrrA1–130 with similar rates, showing no greater preference for P–SrrA1–130 than for P–RpaB1–123.

**Only the RpaB branch of the NbsI signalling pathway is essential for cell viability in S. elongatus**

Reports of failed attempts to inactivate nbsI or rpaB in S. elongatus (van Waasbergen et al., 2002; Kappell et al., 2006), suggested that the corresponding proteins may be essential. To further investigate the essentiality of the NbsI system, we first tried to independently inactivate nbsI, rpaB and srrA using different marker cassettes and routine procedures for allelic replacement (Figs S2–S4). Wild-type nbsI or rpaB alleles persisted in all transformant clones, supporting previous inferences regarding the essential nature of these genes (Figs S2 and S3). In contrast, complete segregation of srrA null alleles from transformant clones was readily achieved (Fig. S4), a result confirming that srrA is not essential for cell viability under laboratory conditions (Anandan et al., 1996).

To further confirm that the NbsI–RpaB signalling pathway is essential, we introduced an extra copy of the rpaB gene into neutral site 1 under the control of a constitutive promoter present in the C.K1 cassette (ΦC.K1–rpaB). Since rpaB was readily inactivated in the presence of the ectopic gene fusion, we concluded that RpaB is indeed an essential RR.

**Point mutations in key residues required for phosphotransfer confer lethality**

To explore whether phosphorylation was required for essential functions inferred for these proteins, we targeted key phosphorylation sites in both NbsI and RpaB. We first constructed NbsI variants with point mutations at the predicted site of phosphorylation (H407A) or at a key residue predicted to be required for ATP binding and autophosphorylation (N524A). The nbsI$H407A$ mutation was linked to a marker cassette (C.S3) and introduced into S. elongatus by allelic replacement. After transformation, the Φ(nbsI$H407A$–C.S3) allele was detected in a few clones during early restriction fragment length polymorphism (RFLP) analyses but not after additional transfers onto streptomycin-containing plates (Fig. S5). Equivalent results were obtained when the same approach was used to generate the N524A substitution in NbsI, that is, nbsI could not be replaced with the Φ(nbsI$S524A$–C.S3) derivative. Instead, clones containing wild-type Φ(nbsI–C.S3) were obtained in all cases, strongly suggesting that phosphorylation of NbsI at H407 is essential for survival of S. elongatus.

Next we attempted to mutate the phosphoacceptor Asp and introduce rpaB$D56E$ or rpaB$S66E$ mutations into S. elongatus. Although it is highly unlikely that the corresponding proteins could be phosphorylated in vivo, it is possible that they may confer different phenotypes, since in some RRs, a large negatively charged residue at this position can partially mimic the effect of phosphorylation (Klose et al., 1993; Siam and Marczynski, 2003). To infer with confidence that point mutations in rpaB could be efficiently introduced by homologous recombination into
Fig. 2. In vitro characterization of autophosphorylation, phosphotransfer and phosphatase reactions of NblS, RpaB and SrrA TCS.

A. Autophosphorylation of NblS. Recombinant NblS219–664 (left) or NblS377–664 (right) (3.4 μM) were incubated with [γ-32P]ATP (0.3 mM) and aliquots were withdrawn at the indicated time points, separated by SDS-PAGE, visualized by autoradiography and quantified by Phospho-Imager. The values obtained for a representative experiment are plotted in B.

B. Curve of autophosphorylation reaction. Velocity versus [ATP] curve used to calculate Km(ATP) and kcat for NblS219–664 autophosphorylation. The inset represents Lineweaver–Burk plots of the corresponding reaction velocities as 1/V versus 1/ATP.

C. Derepression of srrA by HL and transcriptional control of RpaB deficiency.

Visual inspection revealed the presence of HLR1 motifs of these sequences, a 22 bp DNA fragment containing the HLR1 motifs of srrA (~52 to ~30) was used as a probe in gel retardation assays. As expected, a shifted complex was observed in the presence of RpaB and the complex was dissociated when the unlabelled probe was used as competitor (Fig. 4B). A control assay carried out with a hisA promoter fragment that included the HLR1 sequences (~61 to ~31) required higher amounts of RpaB to generate a similar complex, suggesting higher affinity of RpaB for srrA than for hisA target sequences.

Next, we provided in vivo evidence of the functionality of HLR1 sequences upstream of srrA by reverse transcription polymerase chain reaction (RT-PCR) analyses in which we included the HL induced genes hisA and nblA as positive controls, and the rpaB gene, expected to be constitutive, as a negative and loading control (Fig. 4C and D). The sipA gene, encoding the NblS auxiliary protein SipA was also included in the analyses in order to explore the possibility of its regulation by light. Consistent with reported expression patterns for HLR1-containing genes (Kappell and van Waasbergen, 2007; Seki et al., 2007; Hanaoka and Tanaka, 2008; Seino et al., 2009), srrA transcripts were hardly detectable from S. elongatus cultures grown under standard light conditions, but became very abundant upon transfer of cultures to HL. In agreement with the lack of an HLR1 motif, sipA transcripts did not change in the course of the experiment.

To further support the involvement of RpaB in srrA regulation we analyzed the effect of increasing rpaB expression on HLR1-regulated genes. To this end, a strain carrying the fusion Φ(C.K1–rpaB) was obtained and expression of selected genes was compared by RT-PCR under standard growth conditions. The increase in rpaB gene dosage resulted in a significant decrease in transcript levels of all the HL-regulated genes tested, including srrA (Fig. 4E and F), thus supporting the inferred repression of srrA by RpaB. Western analysis indicates that the CK11RpaB1 strain contains just slightly increased levels of RpaB protein (data not shown), suggesting that posttranscriptional regulation prevents over accumulation of RpaB protein.
The structure of the RR PhoB in complex with its DNA targets has identified four positions that would confer DNA binding specificity (Blanco et al., 2002). RpaB and SrrA both contain the same residues at these positions (Fig. S6), thus raising the question of whether SrrA can also regulate RpaB target genes. To explore this issue, a strain expressing higher levels of srrA transcripts, CK1SrrAI, was constructed using the same approach used for rpaB and analyzed in parallel with relevant control strains under standard culture conditions. Interestingly, the Φ(C.K1–srrA) construct also had a negative impact on the transcript levels from the HLR1-controlled genes hliA and nblA, although less pronounced that the observed with Φ(C.K1–rpaB) (Fig. 4E and F). The apparently healthy phenotype of S. elongatus carrying Φ(C.K1–srrA) suggests that increasing the levels of srrA

**Fig. 3.** NblS has preference for SrrA versus RpaB in the phosphotransfer but not in the phosphatase reaction. (A, B) NblS phosphotransfers preferentially to SrrA rather than RpaB.

A. NblS219–664 (upper) or NblS377–664 (lower) were phosphorylated with [γ-32P]-ATP (0.3 mM) for 30 min and independent aliquots were incubated with different molar ratios of RpaB: SrrA. The phosphotransfer reactions were carried out for 2 min and phosphorylated species, indicated by arrows and labelled, were visualized by autoradiography after SDS-PAGE.

B. NblS377–664 was phosphorylated as in A and mixed with equimolar amounts of RpaB and SrrA (final concentration of RR is two times the concentration of HK) and the time-course of the phosphotransfer reaction was followed by autoradiography (upper panel) and the bands corresponding to the P–RR (indicates by arrows and labelled) were quantified and plotted (lower panel).

C. NblS did not exhibit a preference for either P–RpaB or P–SrrA in the phosphatase reaction. RpaB and SrrA were phosphorylated with [32P] acetyl phosphate and incubated with NblS219–664 in 1/10 relationship (RR/NblS) in the presence of 1 mM ADP. Time-course of P–RR dephosphorylation induced by NblS was monitored by autoradiography (inset), P–RRs were quantified and represented as the Ln of average signal intensity of bands versus incubation time.
transcripts is not enough to compromise RpaB phosphorylation. In addition, we can not exclude that srrA is also posttranscriptionally regulated.

The similarities between RpaB and SrrA and the low level of expression of srrA under LL (standard growth conditions) made us question whether increased srrA expression from a constitutive promoter could substitute for RpaB essential functions. To explore this issue, S. elongatus was transformed with rpaB::C.S3 and rpaB::C.K1–srrA constructs (Figs S3 and S4), and multiple clones corresponding to double transformants (kanamycin- and streptomycin-resistant) were analysed by PCR. However, complete segregation of the rpaB::C.S3 alleles could not be detected after several transfers onto selective media (data not shown), suggesting that SrrA can not regulate essential RpaB target genes.

Discussion

Given the transient nature of most signals regulating TCSs, the great majority of HKs and RRs are not required for survival of laboratory cultures. However, we have shown that phosphorylation of both Nbis and RpaB are essential for cell viability and thus these two proteins conform to an essential TCS, or rather the essential branch of a more complex TCS. In the few cases of TCSs where only the RR is essential (Winkler and Hoch, 2008), acetyl phosphate appears to substitute for the HK (Wolfe, 2010). The fact we were not able to obtain viable cultures of S. elongatus when Nbis was inactivated or replaced with non-phosphorylatable substitutions agrees with the apparent absence of pta genes (encoding phosphotransacetylase) in this cyanobacterium.

A paradigm for essential TCSs is provided by YycGF, WalKR or VicKR, which are involved in cell-wall metabolism in low G + C Gram-positive bacteria (Dubrac et al., 2008). Interestingly, the structural organization of these essential HKs is remarkably similar, with N-terminally anchored membrane regions and HAMP and PAS domains preceding the HisKA and HATPase_c domains. With the exception of Nbis, this particular domain organization has not been found in other cyanobacterial HKs (Ashby and Houmard, 2006). It has been proposed that YycG activity is triggered by detection of the levels of available Lipid II in the extracytoplasmic compartment (Dubrac et al., 2008). Given the involvement of Nbis in detection of changes in membrane fluidity associated with stress conditions such as chilling, osmotic and salt stress (Murata and Los, 2006), it is tempting to propose that Nbis could detect signals emanating from membrane activity.

In response to unknown signals, non-essential auxiliary proteins interact with the HKs YycG and Nbis to modulate their activity. In the former system they are membrane anchored and have a negative role (Szmurant et al., 2007; Szmurant et al., 2008), while the auxiliary protein SipA stimulates autophosphorylation by interacting with the HATPase_c domain of Nbis. This stimulatory role is consistent with the slow autophosphorylation and phosphotransfer rates of Nbis proteins observed in vitro (Fig. 2).

Sequence analysis of RpaB and SrrA receiver domains guided by the structures of HK853/RR468 and ThkA/TrrA complexes from Thermotoga maritima (Casino et al., 2009; Yamada et al., 2009) allows prediction of RR residues likely to interact with Nbis (Fig. S6) and can be used to rationalize the results reported here. Current data indicate that HK–RR recognition is mediated mainly by the interaction of the HK HisKA domain with the α1-helix and the three loops (L3, L4 and L5) surrounding the Asp phosphoacceptor in the RR. In spite of the high degree of identity between the receiver domains of RpaB and SrrA (73%), four out of the seven residues in the α1-helix, that are predicted to interact with Nbis, differ between them (three conservative substitutions and the change of RpaB Thr23 for SrrA Met31). These substitutions may explain the differences between the two RRs found in our interaction and phosphorylation analyses. Since NbisS (T77–T64) showed a higher phosphotransfer rate than NbisS (T77–T64) for both RRs, interactions of the PAS and/or HAMP domains with the RRs may occur during the phosphotransfer reaction. However, the higher preference of both Nbis derivatives for SrrA suggests that contacts with PAS and/or HAMP domains are related to the formation of a productive HK–RR complex rather than discriminating between the RRs.

In the ThkA–TrrA complex the PAS domain of ThkA interacts with the α4-helix of TrrA. The fact that residues of α4 in RpaB and SrrA are quasi-identical (only a Glu/Asp conservative change) agrees with the similar susceptibility of the two RRs for Nbis phosphatase activity. The strict PAS domain requirement for the phosphatase activity supports the current vision that both reactions are not the reverse of each other (Gao and Stock, 2009) and agrees with recent reports on YycG and ThkA (Yamada et al., 2009; Gutu et al., 2010). The gain of function mutation (G379D, within the PAS domain) presents in the nblS-1 strain (van Waasbergen et al., 2002) further supports the regulatory importance of the PAS domain.

The strong preference of Nbis to phosphorylate SrrA and the importance of the Nbis to RpaB phosphorylation branch for culture viability can be reconciled with the presence of a negative regulatory circuit working at two different levels. SrrA protein would interfere with Nbis-mediated phosphorylation of RpaB, while RpaB-P would be involved in transcriptional down regulation of the srrA gene. Environmental cues would impact on this circuit to switch between two gene expression programs, one that is implemented exclusively by RpaB and a second, trig-

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Fig. 4. Analysis of putative HLR1 sequences and HL inducible transcripts.

A. Alignment of putative HLR1 sequences upstream of srrA with those of hliA and nblA indicating positions relative to the start codon. Arrows indicate each repeating unit within the HLR1 motif. A consensus sequence is shown underneath.

B. Competitive gel mobility shift assay to detect specific RpaB binding to HLR1 motifs. Fluorescein labelled DNA fragments (50 fmol) containing the HLR1 motifs of srrA or hliA were incubated with increasing amounts of full-length RpaB in the presence or absence of either the same unlabelled competitor probe (self) or an unlabelled non-specific competitor fragment (non-self) at the indicated molar fold excesses.

C. Expression levels of srrA, hliA, nblA and sipA during HL stress. RT-PCR assays were carried out with wild-type S. elongatus grown under LL conditions (lane 0), then shifted to HL conditions to further extract RNA at indicated time points in minutes (lanes 10, 30 and 180).

D. Quantification of the band intensity of transcript in RT-PCR of C by ImageJ software. Values are presented relative to that in LL-grown cells.

E. Expression levels of rpaB, srrA, hliA, nblA and sipA in over expression strains. RT-PCR assays from WTK (wild-type control, lane W), CK1RpaBI (lane B’) and CK1SrRAI (lane A’) strains grown under standard light.

F. Quantification of the band intensity of transcripts in the RT-PCR of E by ImageJ software. Values are presented relative to that in wild-type strain. In C and E a representative experiment from one of two independent RNA extractions is shown and mmpB is used as a loading control. Amplification cycles used on PCR reactions are indicated at the right of each picture.

gerated by RpaB dephosphorylation, that requires both RpaB and SrrA. The key contributions of this work to the function of NbiIS in the context of HL acclimatization are incorporated in Fig. 5. A common feature with the precedent model, supported from work on different laboratories (see Hanaoka & Tanaka, 2008 for an extended discussion), is the induction of RpaB dephosphorylation upon transfer of cultures to HL.

During standard growth conditions, simplified here as LL, NbiIS phosphorylates RpaB, which in turn would regulate (positively and/or negatively) unknown essential target genes as well as repress ‘HL stress genes’, including hliA, nblA and srrA. This latter repression would prevent interference of SrrA with the RpaB phosphorylation branch. Since the SrrA-R: RpaB-P ratio would be very low, RpaB-P would be the main controller of the HLR1 regulon. In the context of the NbiIS pathway, this situation can be referred as pathway insulation for the essential RpaB branch.

Upon exposure to HL, RpaB-P would be rapidly dephosphorylated, resulting in derepression of ‘stress genes’ hliA, nblA and srrA. Because of the essential role inferred for NbiIS-dependent phosphorylation of RpaB, we presume that RpaB dephosphorylation is a transient event, during which SrrA would be synthesized and preferentially phosphorylated by NbiIS to the detriment of RpaB-P levels, thus amplifying the stress signal.

During HL stress SrrA would implement stress-specific programs to adapt the photosynthesis machinery to HL and down regulate previously derepressed genes. Two independent lines of evidence support the idea that SrrA represses at least some RpaB targets during prolonged HL stress: the RpaB-independent downregulation of hliA (Hanaoka and Tanaka, 2008) and the faster bleaching of SrrA-deficient strains (data not shown), which agrees with the negative impact that increased srrA gene dosage has on nbI/transcript levels (Fig. 4). In addition, mechanisms to prevent the RpaB-P: RpaB ratio from reaching irreversibly low levels and compromise cell viability must be operative during prolonged HL stress, amongst them down regulation of srrA gene expression. During prolonged stress, the SrrA-P: RpaB-P ratio and the relative affinity of each of the RRs for their respective gene targets would determine the expression pattern of the partially overlapping RpaB and SrrA regulons and lead to implementation of specific programs for photosynthesis acclimatization and survival.

Branched pathways with multiple HKs phosphorylating a RR (many-to-one) or one HK phosphorylating multiple RRs (one-to-many) emphasize the complexity of interactions involving two-component regulators. So far, known examples of one-to-many pathways are from systems where regulators display atypical domain organization or are part of phosphorelays (Laub and Goulian, 2007). The paradigmatic TCS for chemotaxis is also a branched system where the HK (CheA) presents a non-orthodox domain organization and the two RRs (CheY and CheB) differ in structural organization and cellular functions (Kirby, 2009). In contrast, in the NbiIS system, the HK displays an orthodox HisKA/HATPase domain arrangement and the RRs (RpaB and SrrA) are paralagous proteins with the same domain organization suited for transcriptional regulation, a feature that increases the potential for complex regulatory interactions.

The interplay of negative regulatory interactions between RpaB and SrrA is reminiscent of the classic lysis–lysogen switch of phage λ in which the two related regulators CI and Cro repress each others genes (Oppenheim et al., 2005), and of the recently reported SinR–SlrR switch for cell separation in Bacillus subtilis (Chai et al., 2010) where protein–protein, as well as DNA–protein interactions take place. In the NbiIS system interactions take place at the level of DNA–protein interactions and at the level of protein activity, by means of the inferred competition between the phosphorylation branches of the NbiIS system.

In summary, the NbiIS system provides a paradigm for a branched TCS made up of orthodox HK and RR components, where multiple regulatory interactions between closely related RRs take place to ensure acclimatization and survival. Under non-stress conditions these interactions result in pathway insulation of the essential RpaB branch, while stress lead to a dynamic situation where both
pathways are operative. Further work aiming to identify relationships amongst orphan HKs and RRs is required to understand the regulatory potential of branched TCSs.

**Experimental procedures**

**Strains, plasmids and oligonucleotides**

Cloning procedures were carried out with *Escherichia coli* DH5α. See Table S1 for relevant genotypes of strains, Tables S2–S4 for plasmids and Table S5 for primers.

**Molecular genetic techniques, culture conditions and protein purification**

*Synechococcus elongatus* strains were routinely grown photoautotrophically at 30°C while shaking under constant illumination (50 μmol photons m⁻² s⁻¹) provided by cool white fluorescent lights (denoted as standard light or LL conditions). The level of HL was 1000 μmol photons m⁻² s⁻¹ (provided by SON-T PIA PLUS Philips bulbs). Photon flux densities were measured with LI-250 quantum-meter (LI-COR Bioscience). Media used was BG11 (BG11 0 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.

**Fig. 5.** Model for light acclimatization in the branched NblS system. Only regulatory interactions between the two RRs and the HL inducible *srrA* gene are illustrated. Left panel, under LL RpaB (pink ovals) is phosphorylated by NblS and *srrA* gene expression repressed by RpaB-P. The RpaB-P/RpaB ratio is high. Central panel, upon HL exposure NblS is transiently switched to the phosphatase conformation, leading to RpaB dephosphorylation and subsequent derepression of *srrA*. The RpaB-P/RpaB ratio is low. Right panel, NblS regains kinase activity (mechanism unknown) in spite of HL persistence. The phosphotransfer preference, that results in SrrA (blue ovals) interference with RpaB phosphorylation, is counterbalanced by RpaB-mediated down regulation of the *srrA* gene. The RpaB–P–RpaB ratio would reach equilibrium at intermediate levels. Lines with blunted ends indicate a negative effect on gene expression, which is smaller when the line is dashed. Waved arrows indicate *srrA* transcripts. Black blocks represent repressible HLR1 sites. Although RpaB protein levels would remain constant is represented smaller than SrrA in the right panel to emphasize competition for NblS phosphorylation.
reporters present in PJ696/Y187 diploids was determined as previously described (Burillo et al., 2004). Protein purification is described in Experimental procedures.

**NbIS autophosphorylation assays**

NbIS autophosphorylation were assayed at room temperature in kinase buffer (50 mM Tris-HCl pH 8; 50 mM KCl; 5 mM MgCl₂, 0.1 mM EDTA, 2 mM DTT). Reaction mixtures generally contained 3.4 μM NbIS an the reaction were initiated by the addition of 0.3 mM [γ-32P]-ATP (1 Ci/mmol; Perkin Elmer). Aliquots of the reaction mixture were withdrawn at indicated times and mixed with 6x SDS-PAGE loading buffer. The phosphorylated species were separated by SDS-PAGE (17% polyacrylamide), the gels were dried and the radiolabelled bands were visualized and quantified using a Fluoro Image Analyzer FLA-5000 (Fuji Film) and the program Multi Gauge.

**Phosphotransfer assays**

For phosphotransfer reactions NbIS was phosphorylated with [γ-32P]-ATP at room temperature for 60 min and P-NbIS was purified by two consecutive steps of gel filtration using PD SpinTrap G-25 columns (GE Healthcare). Purified P-NbIS (3.4 μM) was mixed with 3.4 μM of the corresponding RR at RT for the indicated times. The reactions were quenched in 6x loading buffer and were loaded without heating in 17% polyacrylamide gels. Radiolabelled species were detected as above.

**RR phosphorylation, P-RR stability and Phosphatase assays**

[32P] acetyl phosphate was enzymatically synthesized as reported (Jagadeesan et al., 2009). To autophosphorylate RRs, [32P] acetyl phosphate was added to 2.8 μM of response regulators in kinase buffer and the mixture was incubated for 60 min at RT. P-RR were purified using PD SpinTrap G-25 columns (GE Healthcare).

To determine the stability of the phosphoryl group on the response regulators, P-RR were incubated at RT and aliquots removed at the indicated times, quenched with 6x loading buffer and stored at −20°C. The samples were loaded without heating in SDS-PAGE, resolved as above and the phosphorylated species were quantified by phosphorimaging.

NbIS-dependent RR dephosphorylation assays were carried out with 2.8 μM P-RR and 2.8 μM kinase at RT in kinase buffer, the reaction was quenched to different times with 6x sample buffer and analyzed as above.

**Electrophoretic gel mobility shift assays**

Probes containing *S. elongatus* srrA and hilA promoters (nucleotides −52 to −30 and −61 to −31 relative to the transcription initiation site respectively) were prepared by annealing equimolar amounts of 5′ fluorescent labelled complementary oligonucleotides SrrA–probe–Flc(+):SrrA–probe–Flc(−), and HilA–probe–Flc(+):HilA–probe–Flc(−) respectively.

Binding reactions were performed by mixing recombinant RpaB (from 13.3 to 166.6 pmol) with 50 fmol of fluorescent probe and 1 μg of poly(dI-dC) in a final volume of 20 μl of binding buffer (20 mM HEPES, pH 8, 50 mM NaCl, 5 mM MgCl₂, 0.1 mM EDTA, 5% glycerol and 1 mM dithiothreitol), at 4°C for 30 min. For competition assays, recombinant RpaB in binding buffer was preincubated with a 25- or 250-fold excess amount of unlabelled competitor DNA fragment at 4°C for 30 min. DNA–protein complexes were resolved on a native 6% polyacrylamide gel, run at 100 V and 4°C for approximately 1 h and visualized on the Fuji Film FLA3000 gel imaging system (Ex 473 nm; Y520 filter).

**Construction and analysis of nbIS, rpaB and srrA derivative strains**

Strategies used for construction of nbIS, rpaB and srrA derivative strains, and description of plasmids are provided in Experimental procedures, Supporting information Table S2 and Figs S2–S5.

**RT-PCR analyses**

*Synechococcus elongatus* cells were routinely grown until they reached mid-exponential phase (OD750 ~0.5) in LL conditions. For HL treatments the cultures were exposed to 1000 μmol photons m⁻² s⁻¹ white light and at the indicated times, 50 ml of aliquots were removed for RNA extractions. The samples were rapidly chilled on ice, centrifuged and pellets stored at −20°C. Total RNA was isolated using a hot phenol method and treated with DNase I (Turbo DNase, Ambion). After PCR verification that no contaminant DNA was present, 0.5 μg of total RNA was retrotranscribed in a total volume of 30 μl with RevertAid H Minus M-Mul V Reverse Transcriptase (Fermentas) using primers RnPB-R, RpaB7942-2R, SrrA-OV-1R, NewhliA-R, NblA-R and Sip1-BTH-R. Five microlitres of sample of retrotranscription reactions were subjected to different amplification cycles to optimize reactions, using Biotools DNA Polymerase (Biotools B&M Laboratories), with the following primer pairs: RpaB-BYTH-1F/RpaB7942-2R, SrrA-D64A-F/SrrA-OV-1R, NewhliA-F/NewhliA-R, NblA-F/NblA-R, Sip1-BTH-F/Sip1-BTH-R and RnPB-B/RnPB-R.

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**References**


Supporting information

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