Phosphorylation-independent activation of the atypical response regulator NblR

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Cyanobacteria respond to environmental stress conditions by adjusting their photosynthesis machinery. In *Synechococcus* sp. PCC 7942, phycobilisome degradation and other acclimation responses after nutrient or high-light stress require activation by the orphan response regulator NblR, a member of the OmpR/PhoB family. Although NblR contains a putative phosphorylatable residue (Asp57), it lacks other conserved residues required to chelate the Mg²⁺ necessary for aspartic acid phosphorylation or to transduce the phosphorylation signal. In close agreement with these features, NblR was not phosphorylated *in vitro* by the low-molecular-mass phosphate donor acetyl phosphate and mutation of Asp57 to Ala had no impact on previously characterized NblR functions in *Synechococcus*. On the other hand, *in vitro* and *in vivo* assays show that the default state of NblR is monomeric, suggesting that, despite input differences, NblR activation could involve the same general mechanism of activation by dimerization present in known members of the OmpR/PhoB family. Structural and functional data indicate that the receiver domain of NblR shares similarities with other phosphorylation-independent response regulators such as FrzS and HP1043. To acknowledge the peculiarities of these atypical 'two-component' regulators with phosphorylation-independent signal transduction mechanisms, we propose the term PIARR, standing for phosphorylation-independent activation of response regulator.

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INTRODUCTION

Cyanobacteria, photosynthetic prokaryotes that perform plant-type oxygenic photosynthesis, have developed mechanisms to modify the composition of their photosynthetic machinery in response to environmental changes (Grossman *et al.*, 1993). One dramatic example of this adaptation is the process of chlorosis or bleaching, by which non-diazotrophic cyanobacteria degrade their light-harvesting antennae, the phycobilisomes, when exposed to stress conditions such as nutrient starvation (Collier & Grossman, 1992). The small protein NblA (non-bleaching protein A) is required for phycobilisome degradation (Collier & Grossman, 1994). The loss of phycobilisomes and a reduction of the chlorophyll content during stress conditions are responsible for the yellow appearance of chlorotic cultures. Degradation of phycobilisomes avoids excessive absorption of excitation energy and supplies the cell with amino acids for the synthesis of proteins required for acclimation and cell survival (Grossman *et al.*, 2001).

Two-component regulatory systems are widely used in signal transduction and adaptation to environmental changes in bacteria (Gao *et al.*, 2007). In the prototype system a phosphate is transferred from a histidine in the dimerization and histidine phosphotransfer (DHP) domain of a sensor histidine kinase (HK) to an aspartate in the receiver domain (RD) of a cognate response regulator (RR). Phosphorylation of the RD leads to conformational changes of the adjacent output domains. While the latter domain is structurally and functionally diverse, phosphorylation by cognate HKs requires structural conservation of RDs (reviewed in depth by Stock *et al.*, 2000).

Consistent with their role in adaptation to environmental changes, a HK, NblS (van Waasbergen *et al.*, 2002) and NblR (Schwarz & Grossman, 1998), a RR from the OmpR/PhoB subfamily, have been implicated in general adaptation to stress in *Synechococcus* sp. PCC 7942 (hereafter *Synechococcus*). NblS, an essential protein in *Synechococcus*, is the most conserved sensor HK in cyanobacteria, homologues being present in all cyanobacterial genomes available to date. Sequence analysis predicts the following domain organization for NblS: two N-terminal membrane-spanning regions (TM1 and TM2), a HAMP linker, a PAS domain and a conserved transmitter module, made up of a DHP domain and an ATP-binding domain (HATPase_c).

Abbreviations: BACTH, bacterial adenylate cyclase two-hybrid; HK, histidine kinase; HL, high light; PIARR, phosphorylation-independent activation of response regulator; RD, receiver domain; RR, response regulator; RLU, relative light units.

A supplementary figure is available with the online version of this paper.

Also conserved in cyanobacteria is SipA, a non-essential small regulatory factor that binds to the NblS HATPase domain and seems to cooperate with NblS in negative regulation of the *nblA* gene (Espinosa *et al.*, 2006; Salinas *et al.*, 2007). NblR is required for the strong increase in *nblA* gene expression observed during nutrient stress in *Synechococcus* (Luque *et al.*, 2001; Salinas *et al.*, 2007; Schwarz & Grossman, 1998; Sendersky *et al.*, 2005). The finding that point mutations in *nblS* also resulted in a non-bleaching phenotype (van Waasbergen *et al.*, 2002) prompted suggestions of NblS being involved in the activation of NblR. However, NblR is required for induction of the chlorosis process, whereas the reported NblS mutant *nblS-1*, probably a gain-of-function mutant (Kappell *et al.*, 2006), prevents chlorosis. NblR is also required for stress survival, and here NblS-SipA and NblR also seem to play opposite roles, as inferred from suppression of the NblR⁻ phenotype by *sipA* inactivation (Salinas *et al.*, 2007). Although the putative gene targets remain unknown, there are indications of a role for NblR in downregulation of photosynthetic electron transport under stress conditions (Schwarz & Grossman, 1998). However, the mechanism and components involved in NblR activation remain to be elucidated.

In this work we provide and discuss structural and functional evidence indicating that activation of the NblR RR by stress signals does not rely on RD phosphorylation and it is therefore at odds with the paradigm for signal transduction by two-component RRs. To recognize the existence of alternative signal transduction mechanisms of atypical proteins whose RDs maintain most of the structural features of canonical RRs, but differ at residues directly involved in RD phosphorylation, we propose the term PIARR (phosphorylation-independent activation of response regulator).

METHODS

Molecular genetic techniques, culture conditions and two-hybrid methods. Strains used in this work are listed in Table 1. Cloning procedures were carried out with *Escherichia coli* DH5 α , using standard techniques.

Synechococcus strains were routinely grown photoautotrophically at 30 °C while shaking under constant illumination (40 μ mol photons m⁻² s⁻¹) provided by cool white fluorescent lights. Media used were BG11₀ (no added nitrogen), BG11 (BG11₀ plus 17.5 mM NaNO₃ and 10 mM HEPES/NaOH pH 7.8) or BG11-NH₄ (BG11₀ plus 10 mM NH₄Cl 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. *Synechococcus* strains were transformed as described by Golden & Sherman (1984), incubated for 48 h at 30 °C under illumination on nitro-cellulose filters (Millipore), and transformants were selected on kanamycin-, chloramphenicol- or streptomycin-containing BG11 plates. For initiation of nitrogen deprivation, mid-exponential-phase BG11-NH₄ cultures grown with the appropriate antibiotics (OD₇₅₀ 0.5) were harvested by centrifugation, washed twice with BG11₀ and finally resuspended in BG11₀ without antibiotics. For initiation of high-light (HL) stress, BG11 cultures were grown with the appropriate

antibiotics to mid-exponential phase (OD₇₅₀ 0.5) under standard illumination conditions, washed twice with BG11 and finally resuspended in BG11 without antibiotics and then transferred to HL conditions (500 μ mol photons m⁻² s⁻¹). To check the ability to resume growth, drops of the different strains previously subjected to stress were spotted at different times on BG11 plates without antibiotics. Antibiotic concentrations used for *Synechococcus* were 10 μ g ml⁻¹ (kanamycin) and 5 μ g ml⁻¹ (chloramphenicol and streptomycin).

Yeast culture and transformation procedures were as described by Ausubel *et al.* (1999). To perform yeast two-hybrid screenings, previously obtained *Synechococcus* Sau3AI (Burillo *et al.*, 2004) or *Tsp509I* libraries (Burillo, 2006) were transformed into *Saccharomyces cerevisiae* PJ696 and mated to strain Y187 carrying either pUAGC3 or pUAGC301. To determine interaction patterns amongst selected proteins, expression from the three reporters present in PJ696/Y187 diploids was determined as previously described (Burillo *et al.*, 2004).

For bacterial adenylate cyclase two-hybrid assays, *E. coli* DHM1 harbouring appropriate plasmid derivatives was grown at 25–28 °C with ampicillin (50 μ g ml⁻¹) and chloramphenicol (30 μ g ml⁻¹). Complementation was tested on M63 containing maltose (0.3%), thiamin (0.0001%), IPTG (0.5 mM) and X-Gal (80 μ g ml⁻¹).

Modelling and structural alignment. The 3D structure model of the NblR receiver domain was produced by the combination of the structural models proposed by the Swiss-model repository (Kopp & Schwede, 2004) and MODBASE (Pieper *et al.*, 2004). This initial model was subsequently subjected to energy minimization using the program CNS (Brunger *et al.*, 1998). The quality of the final model was assessed using the PROCHECK suite of programs (Morris *et al.*, 1992). The receiver domain structures of phosphorylated Spo0A from *Bacillus stearothermophilus* (RCSB code 1QMP) (Lewis *et al.*, 1999), beryllium fluoride-activated PhoB from *E. coli* (RCSB code 1ZES) (Bachhawat *et al.*, 2005), FrzS from *Myxococcus xanthus* (RCSB code 2GKG) (Fraser *et al.*, 2007), beryllium fluoride-activated CheY from *E. coli* (RCSB code 1FQW) (Lee *et al.*, 2001), HP1043 from *Helicobacter pylori* (RCSB code 2PLN) as well as the NblR model were structurally aligned using the LSQKAB program as implemented in the CCP4 suite (Collaborative Computational Project, Number 4, 1994).

Construction of an *nblR*^{D57A} mutant and derivative strains. An *EcoRI*–*SalI* fragment from plasmid pENS43 carrying the *nblR* gene was cloned into pBluescriptII SK(+), giving plasmid pUAGC235. QuickChange Mutagenesis with primers NblR-D57A-F and NblR-D57A-R and plasmid pUAGC235 as template resulted in plasmid pUAGC238, carrying *nblR*^{D57A} (see Table 2 for primers). To generate appropriate flanking sites, the CS3 cassette (Sm^R) from pRL453 was recombined into *HindIII* pBluescriptII SK(+), giving plasmid pUAGC453. A *HincII*–*EcoRV* fragment containing the CS3 cassette from pUAGC453 was then cloned into the Klenow-treated *Styl* site of pUAGC238 (downstream *nblR*^{D57A}), giving plasmid pUAGC240. Downstream sequences of *nblR* (670 bp) were amplified from genomic *Synechococcus* DNA using primers NblR-down-4F and NblR-down-4R. The PCR product was then cut with *HindIII* and *HincII* and Klenow filled. This blunt fragment was then cloned into *HincII*-cut pUAGC240, giving plasmid pUAGC239, carrying the CS3 cassette between *nblR* and the ORF Synpcc7942_2306. Transformation of pUAGC239 into *Synechococcus* resulted in stable chromosomal integration of the CS3 cassette and adjacent sequences, as confirmed by PCR with primers NblR-1F and CS3-2R. The presence of *nblR*^{D57} or *nblR* alleles was checked by a second PCR with primers NblR-1F and NblR-1R followed by digestion with *PvuII*, giving 309, 186 and 51 bp fragments for wild-type *nblR* and 309 and 237 bp fragments for *nblR*^{D57A}. A clone of each type was selected for further analysis (strains WT-RCS3 and NblR^{D57A}-RCS3, respectively).

Table 1. Strains and plasmids

Strain or plasmid	Genotype or relevant characteristics	Source or reference
<i>E. coli</i> DH5 α	F ⁻ ϕ 80dlacZAM15(<i>lacZYA-argF</i>)U169 <i>endA1 recA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>deoR thi-1 supE44 gyrA96 relA1 λ⁻</i>	Hanahan (1985)
<i>E. coli</i> DHM1	F ⁻ <i>glnV44</i> (AS) <i>recA1 endA gyrA46 thi-1 hsdR17 spoT1 frbD1 cya-854</i>	Karimova <i>et al.</i> (2005)
<i>E. coli</i> BL21-CodonPlus(DE3)-RIL	<i>E. coli</i> B F ⁻ <i>ompT hsdS</i> (r _B ⁻ m _B ⁻) dcm ⁺ Tet ^r <i>gal λ</i> (DE3) <i>endA Hte</i> [<i>argU ileY leuW Cam</i> ^r]	Stratagene
<i>S. cerevisiae</i> Y187	<i>MATα ura3-52 his3-200 ade2-101 trp1-901 leu2-3,112 gal4Δ met⁻</i> <i>gal80Δ URA::GAL1_{UAS}-GAL1_{TATA}-lacZ</i>	Harper <i>et al.</i> (1993)
<i>S. cerevisiae</i> PJ696	<i>MATα ade2Δ trp1-901 leu2-3,112 ura3-52 his3-200 cyh^R can^R gal4Δ</i> <i>gal80Δ met2⁻ GAL2::ADE2 GAL1::HIS3 GAL7:lacZ</i>	James <i>et al.</i> (1996)
<i>Synechococcus</i> sp. PCC 7942	Wild-type <i>Synechococcus</i> sp. PCC 7942	Pasteur culture collection
<i>Synechococcus</i> NblR45	NblR ⁻ , Km ^r	Luque <i>et al.</i> (2001)
<i>Synechococcus</i> WT-C103	P _{nblA} :: <i>luxAB</i> into NSII, Cm ^r	Espinosa <i>et al.</i> (2007)
<i>Synechococcus</i> NblR45-C103	NblR ⁻ , P _{nblA} :: <i>luxAB</i> , Cm ^r Km ^r	Salinas <i>et al.</i> (2007)
<i>Synechococcus</i> WT-RCS3	CS3 downstream <i>nblR</i> , Sm ^r	This work
<i>Synechococcus</i> NblR ^{D57A} -RCS3	NblR ^{D57A} , Sm ^r	This work
<i>Synechococcus</i> WT-RCS3-C103	P _{nblA} :: <i>luxAB</i> , Cm ^r Sm ^r	This work
<i>Synechococcus</i> NblR ^{D57A} -RCS3-C103	NblR ^{D57A} , P _{nblA} :: <i>luxAB</i> , Cm ^r Sm ^r	This work
pBluescriptII SK(+)	Cloning vector, Ap ^r	Stratagene
pENS43	1.8 kb genomic <i>nblR</i> fragment cloned into pRL519, Ap ^r	Luque <i>et al.</i> (2001)
pENS38	pPROEX-HTb encoding 6His-NblR, Ap ^r	Luque <i>et al.</i> (2001)
pNblR-RD	pPROEX-HTb encoding 6His-NblR ¹⁻¹²⁴ , Ap ^r	This work
pTM0468	pET22b encoding full-length RR468, Ap ^r	Casino <i>et al.</i> (2007)
pUAGC235	pBluescriptII SK(+) with <i>nblR</i> , Ap ^r	This work
pUAGC238	pBluescriptII SK(+) with <i>nblR</i> ^{D57A} , Ap ^r	This work
pUAGC240	pUAGC238 with CS3, Ap ^r Sm ^r	This work
pUAGC239	pUAGC240 with <i>nblR</i> downstream sequences, Ap ^r Sm ^r	This work
pUAGC453	pBluescriptII SK(+) with CS3, Ap ^r Sm ^r	This work
pGAD424	<i>LEU2</i> , GAL4(768-881)AD, Ap ^r	Bartel <i>et al.</i> (1993)
pGBT9	<i>TRP1</i> , GAL4(1-147)BD, Ap ^r	Bartel <i>et al.</i> (1993)
pGAD424(+ 1)	As pGAD424 with a different frame (+ 1), Ap ^r	Roder <i>et al.</i> (1996)
pGBT9(+ 1)	As pGBT9 with a different frame (+ 1), Ap ^r	Roder <i>et al.</i> (1996)
pADC	<i>LEU2</i> , GAL4-AD for C-terminal cloning, Ap ^r	Millson <i>et al.</i> (2003)
pBDC	<i>TRP1</i> , GAL4-BD for C-terminal cloning, Ap ^r	Millson <i>et al.</i> (2003)
pUAGC51	GAL4AD:NblS ²⁷²⁻⁶⁶⁴ , Ap ^r	Espinosa <i>et al.</i> (2006)
pUAGC52	GAL4BD:NblS ²⁷²⁻⁶⁶⁴ , Ap ^r	Espinosa <i>et al.</i> (2006)
pUAGC53	GAL4AD:NblS, Ap ^r	Espinosa <i>et al.</i> (2006)
pUAGC54	GAL4BD:NblS, Ap ^r	Espinosa <i>et al.</i> (2006)
pUAGC1	GAL4AD:NblR, Ap ^r	Burillo (2006)
pUAGC3	GAL4BD:NblR, Ap ^r	Burillo (2006)
pUAGC300	GAL4AD:NblR ¹⁻¹⁷¹ , Ap ^r	This work
pUAGC301	GAL4BD:NblR ¹⁻¹⁷¹ , Ap ^r	This work
pUAGC597	NblR: GAL4AD, Ap ^r	This work
pUAGC598	NblR: GAL4BD, Ap ^r	This work
pUAGC615	GAL4AD:NarB, Ap ^r	This work
pUAGC616	GAL4BD:NarB, Ap ^r	This work
pUAGC310	GAL4BD:NarB ¹⁵⁴⁻³⁴⁴ , Ap ^r	This work
pUAGC311	GAL4AD:NarB ¹⁵⁴⁻³⁴⁴ , Ap ^r	This work
pUAGC312	GAL4BD:NarB ¹⁵⁴⁻²⁵⁵ , Ap ^r	This work
pUAGC313	GAL4AD:NarB ¹⁵⁴⁻²⁵⁵ , Ap ^r	This work
pUAGC314	GAL4BD:NarB ¹¹²⁻³⁸² , Ap ^r	This work
pUAGC315	GAL4AD:NarB ¹¹²⁻³⁸² , Ap ^r	This work
pUAGC324	GAL4BD:NdhH ⁵⁴⁻²⁸³ , Ap ^r	This work
pUAGC325	GAL4AD:NdhH ⁵⁴⁻²⁸³ , Ap ^r	This work
pT25	CyaA(1-224)T25, Cm ^r	Karimova <i>et al.</i> (1998)
pUT18c	CyaA(225-399)T18, Ap ^r	Karimova <i>et al.</i> (1998)
pUAGC439	T25:NblS, Cm ^r	Espinosa <i>et al.</i> (2006)
pUAGC600	T25:NblR, Cm ^r	This work
pUAGC601	T18:NblR, Ap ^r	This work
pUAGC604	T25:NblR ¹²⁵⁻²²⁹ , Cm ^r	This work
pUAGC605	T18:NblR ¹²⁵⁻²²⁹ , Ap ^r	This work
pUAGC602	T25:NarB, Cm ^r	This work
pUAGC603	T18:NarB, Ap ^r	This work

Table 2. Oligonucleotides

Name	Sequence
ADC-F	5'-CAAGCTATACCAAGCATACAATCCAAGATG-3'
ADC-R	5'-GGAATTAATTCCGCTTTATCCATCTTTGCAAAGGC-3'
BDC-F	5'-CAACTCCAAGCTTGAAGCAAGCCTCGATG-3'
BDC-R	5'-CGATAGAAGACAGTAGCTTCATCTTTTCG-3'
CS3-2R	5'-ACAAAACGGTTTACCAGCAT-3'
NblR-1F	5'-GAGTGAGGAAGAATTCTGATCGCGCCAGCCTC-3'
NblR-1R	5'-CTCTCGCGTCGACACTTAGCGCGGATGCTC-3'
NblR-3	5'-GGCAGCGCAGTCGACTTTGAAGCCATG-3'
NarB-1F	5'-GAGGGAATTCTCGATCTCGATCTCTCGAAGTTTC-3'
NarB-1R	5'-GCCTAGAGGTCGACTTAGCGATCG-3'
NblR ADC-F	5'-AGCATACAATCCAAGATGATCGCGCCAGCCTCGCCACACA-3'
NblR ADC-R	5'-CGCTTTATCCATCTTTGCAAAGGCGCTTTCGCGTAGGACATAACCG-3'
NblR BDC-F	5'-GCTTGAAGCAAGCCTCGATGATCGCGCCAGCCTCGCCACAC-3'
NblR BDC-R	5'-CAGTAGCTTCATCTTTTCGGCTTTCGCGTAGGACATAACC-3'
Transgadgbt-1F	5'-CGCACATCATCATCGGAAGAGAGTAGTAACAAAGGTCAAAGAC AGTTGACTGTATCGCCGAACCCAAAAAAGAGATCG-3'
Transgadgbt-1R	5'-ATAACTTATTTAATAATAAAAAATCATAAATCATAAGAAATTCGCCCGGAATT AGCTTGGCGTTTTTCAGTATCTACGATTC-3'
NblR bth1-F	5'-TGAGGGGATCCTATGATCGCGC-3'
NblR bth1-R	5'-ATCGCGGTACCTTAGCTTTCGC-3'
NblR bth2-F	5'-TACAGGATCCACCTGCTGCTCA-3'
NarB bth-1F	5'-GCGCGGATCCAATGTTTCGATC-3'
NarB bth-1R	5'-TTGCGGTACCTTAGATTGTGAAG-3'
NblR-D57A-F	5'-GATTTAGTCATCGTCGCTCGCATGGCGGGCGG-3'
NblR-D57A-R	5'-CCGCCCGCCATGCGAGCGACGATGACTAAATC-3'
NblR-down-4F	5'-TGTAAGAGCTTGC GGCTC-3'
NblR-down-4R	5'-CTTTAGGTCGACTGCAGTCTCGTCATC-3'
NblR-RD-F	5'-GGCGCCATGGTCGCGCCAGCCTCGCC-3'
NblR-RD-R	5'-GCAGAAGCTTATCGTTGTAATAGAG-3'

To obtain wild-type and *nblR*^{D57} derivatives carrying the *P_{nblA}::luxAB* reporter fusion, plasmid pUAGC239 was transformed into strain WT-C103 (Espinosa *et al.*, 2007) and transformant clones analysed as above to independently recover each type of *nblR* alleles (strains WT-RCS3-C103 and NblR^{D57A}-RCS3-C103).

Construction of yeast and bacterial two-hybrid plasmids. A 485 bp fragment from the *nblR* gene was PCR amplified with primers NblR-1F and NblR-1R, cut with *EcoRI* and *SalI* and cloned into pGAD424(+1) and pGBT9(+1), giving plasmids pUAGC300 (GAL4AD:NblR¹⁻¹⁷¹) and pUAGC301 (GAL4BD:NblR¹⁻¹⁷¹), respectively. To produce a NblR:GAL4AD protein fusion, PCR amplification of *nblR* sequences with primers NblR ADC-F and NblR ADC-R was followed by a second PCR with primers ADC-F and ADC-R. This product was mixed with *NruI*-opened pADC and transformed into *S. cerevisiae* PJ696, to produce, by homologous recombination, plasmid pUAGC597. Similarly, to obtain a NblR:GAL4BD protein fusion, primers NblR BDC-F and NblR BDC-R and then BDC-F and BDC-R were used in consecutive PCRs, the product mixed with linear pBDC and transformed into *S. cerevisiae* Y187, giving plasmid pUAGC598. To obtain bacterial adenylate cyclase two-hybrid (BACTH) derivatives, *nblR* was PCR amplified with primers NblR bth1-F and NblR bth1-R, cut with *BamHI* and *KpnI*, and cloned into pT25 or pUT18c, resulting in plasmids pUAGC600 (T25:NblR) and pUAGC601 (T18:NblR), respectively. The same procedure but using primers NblR bth2-F and NblR bth1-R resulted in plasmids pUAGC604 (T25:NblR¹²⁵⁻²²⁹) and pUAGC605 (T18:NblR¹²⁵⁻²²⁹).

The *narB* sequence was PCR amplified using primers NarB bth1-F and NarB bth1-R, cut with *BamHI* and *KpnI*, and cloned into pT25 or pUT18c, resulting in plasmids pUAGC602 (T25:NarB) and pUAGC603 (T18:NarB), respectively. To obtain plasmids pUAGC615 (GAL4AD:NarB) and pUAGC616 (GAL4BD:NarB), the *narB* sequence was amplified with primers NarB-1F and NarB-1R, cut with *EcoRI* and *SalI* and cloned into pGAD424(+1) and pGBT9(+1).

Inserts from prey plasmids pUAGC311 (GAL4AD:NarB¹⁵⁴⁻³⁴⁴), pUAGC313 (GAL4AD:NarB¹⁵⁴⁻²⁵⁵) and pUAGC315 (GAL4AD:NarB¹¹²⁻³⁸²), obtained in yeast two-hybrid screenings using NblR as bait, were PCR amplified using primers transgadgbt-1F and transgadgbt-1R. The PCR together with linearized (*EcoRI*-*SalI*) pGBT9 was then transformed into *S. cerevisiae* PJ696, giving plasmids pUAGC310 (GAL4BD:NarB¹⁵⁴⁻³⁴⁴), pUAGC312 (GAL4BD:NarB¹⁵⁴⁻²⁵⁵) and pUAGC314 (GAL4BD:NarB¹¹²⁻³⁸²). The same strategy was used to obtain plasmid pUAGC324 (GAL4BD:NdhH⁵⁴⁻²⁸³) from pUAGC325 (GAL4AD:NdhH⁵⁴⁻²⁸³).

Protein cloning, expression and purification. DNA sequences encoding residues 1-124 of NblR (6His-NblR-RD) were PCR amplified from *Synechococcus* genomic DNA using primers NblR-RD-F and NblR-RD-R. The PCR product was cloned into the *NcoI* and *HindIII* sites of plasmid pPROEX-HTb (Invitrogen), creating an in-frame fusion to the N-terminal His₆-tag. The resulting plasmid was named pNblR-RD.

The RR468 protein was expressed and purified as previously described (Casino *et al.*, 2007). For expression of 6His-NblR and 6His-NblR-RD

proteins, plasmids pENS38 and pNblR-RD were transformed into the *E. coli* strain BL21-codonPlus (DE3)-RIL (Stratagene). Cells were grown in the autoinductive ZYP-5052 medium supplemented with ampicillin ($100 \mu\text{g ml}^{-1}$) and chloramphenicol ($33 \mu\text{g ml}^{-1}$), and the expression of proteins was carried out following an autoinduction method (Studier, 2005). Cells were harvested, disrupted by sonication, and the soluble fraction was purified by Ni-affinity chromatography using Protino Ni-TED 1000 (Macherey-Nagel). Elution of the protein was carried out using 250 mM imidazol. The purest fractions (as determined by SDS-PAGE and Coomassie blue staining) were pooled, washed with storage buffer (25 mM Tris/HCl pH 8.0, 100 mM NaCl), concentrated using Amicon Ultra (Millipore) and stored at -80°C . Proteins were quantified spectrophotometrically using the method of Bradford (Bio-Rad).

Phosphorylation assay with acetyl phosphate and resolution by native PAGE and 2D gel electrophoresis. Purified 6His-NblR, 6His-NblR-RD and RR468 were autophosphorylated in kinase buffer (50 mM Tris pH 8.0, 100 mM KCl, 10 mM MgCl_2) containing 12.5 mM acetyl phosphate for 1 h at room temperature. After phosphorylation, loading buffer (62.5 mM Tris pH 6.8, 30 % glycerol, 0.01 % bromophenol blue) was added in a 1:4 ratio to the samples and these were subsequently subjected to Native PAGE on a 10 % gel at 100 V for 2 h at 4°C .

To evaluate phosphorylation by 2D gel electrophoresis, the reactions with acetyl phosphate were carried out in a final volume of 10 μl , including 15 μg 6His-NblR-RD or 6 μg RR468. The phosphorylation reactions were stopped by the addition of 90 μl lysis buffer (8 M urea, 2 % CHAPS), 1 μl 1 M DTT and 0.5 μl ampholytes pH 4-7. Control samples without acetyl phosphate were run in parallel. Samples consisting of the unphosphorylated proteins or a mix of equal amounts of unphosphorylated and phosphorylated proteins, where the unphosphorylated proteins were used as internal control, were isoelectrically focused for each protein in the first dimension using a 7 cm Immobiline Drystrip (pH 4.0–7.0; GE Healthcare) and an Ettan IPGphor (Amersham Biosciences) system following the indications of the manufacturers. The samples were focused at 500 V for 0.25 kV h, 1000 V for 0.5 kV h and 6000 V for 7.5 kV h at 20°C . Proteins were resolved in the second dimension by SDS-PAGE using 15 % acrylamide gels.

Both types of gels were stained with Coomassie blue and images analysed using a Fuji LAS-3000 imaging system and the MultiGauge Fujifilm program.

Gel filtration chromatography. Gel filtration chromatography was carried out in a Superdex 200 HR 10/30 column (Amersham Biosciences) equilibrated with running buffer (50 mM Tris pH 7.5, 150 mM NaCl) and calibrated with a cocktail of molecular mass standards, containing Blue Dextran 2000 (~ 2000 kDa), β -amylase (200 kDa), alcohol dehydrogenase (150 kDa), BSA (66 kDa), carbonic anhydrase (29 kDa), cytochrome *c* (12.4 kDa) and ATP (0.57 kDa). Samples (100 μl) of 6His-NblR or 6His-NblR-RD containing 150 μg protein in running buffer were individually applied to the column, eluted with running buffer at a flow rate of 0.5 ml min^{-1} and collected in 1 ml fractions. The fractions were analysed by SDS-PAGE. Protein elution profiles were monitored by measuring the absorbance at 280 nm. For evaluation of the acetyl phosphate effect, the samples were incubated for 1 h at room temperature with acetyl phosphate and MgCl_2 (12.5 mM) before chromatography. Sample oxidation was carried out by treatment with 0.3 mM $\text{Cu(II)-(}o\text{-phenanthroline)}_3$ (Lee *et al.*, 1995) for 30 min at 37°C prior to gel filtration. Sample reduction was carried out by incubation with 10 mM DTT for 30 min at room temperature, and the chromatography running buffer was supplemented with 1 mM DTT.

Determination of luciferase activity. To determine bioluminescence, 1 ml of cultures grown to mid-exponential phase were adjusted to an OD_{750} of 0.5 and supplemented with decanal to a final concentration of 0.25 mM from a 50 mM stock solution made up in 10 % DMSO. Light emission was recorded in a Berthold LB9509 luminometer. Bioluminescence was recorded every 20 s for 10 min. Light emission increased to a maximum and then declined. Maximum luminescence at the peak, presented as RLU (relative light units) by the instrument, is the value used at each selected time point.

Determination of pigment contents spectrophotometrically. Whole-cell absorbance spectra were obtained in order to estimate pigment contents. Samples (1 ml) of cultures were taken at the indicated times, diluted with fresh medium to an OD_{750} 0.5, and absorbance spectra (500–800 nm) were recorded on a UV/Visible Ultrospec 2100 pro (GE Healthcare Life Sciences). Pigment content was calculated based on absorbance maxima at 631 nm for phycocyanin and 684 nm for chlorophyll *a*, essentially as described by Myers *et al.* (1980).

RESULTS AND DISCUSSION

NblR lacks residues conserved in canonical response regulators

Three groups of amino acids are essential for phosphorylation of RDs and the consequent response: the phosphoaccepting aspartate (Asp57 in CheY numeration), the catalytic residues (Asp12, Asp13 and Lys109) and the conformational switch residues (Thr87 and Tyr106) (Dyer & Dahlquist, 2006). However, NblR lacks two of these key amino acids (see Fig. 1b). In this context, it is worth noting recent reports of atypical RRs with striking deviations from canonical RDs (Fraser *et al.*, 2007; Schar *et al.*, 2005). To illustrate key discrepancies between canonical RDs, NblR RD and other atypical RDs, we produced a 3D structure model of the NblR RD and used it to generate a structural alignment with selected canonical and non-canonical RDs. In order to emphasize the catalytic residue disposition in the alignment, we chose the CheY and PhoB beryllium fluoride-activated structures (Lee *et al.*, 2001) as prototypical RDs, as well as the phosphorylated Spo0A structure (Lewis *et al.*, 1999), the first canonical RD crystallized in the phosphorylated form. The structures of *Myxococcus xanthus* FrzS (Fraser *et al.*, 2007) and *Helicobacter pylori* HP1043 (Schar *et al.*, 2005) were used as a model of atypical RDs. As shown in Fig. 1(a), NblR apparently retains the overall folding of RDs, as is the case with the RDs from FrzS and HP1043. In NblR, the highly conserved aspartic acid and threonine residues (Asp13 and Thr87 in CheY) are substituted by serine and methionine (Ser14 and Met85), respectively. Asp13, probably the main residue involved in Mg^{2+} binding (Hubbard *et al.*, 2003; Lee *et al.*, 2001), is substituted for Ser and Lys, respectively, in FrzS and HP1043. Consistent with these changes, FrzS is insensitive to Mg^{2+} and HP1043 cannot be phosphorylated *in vitro* (Fraser *et al.*, 2007; Schar *et al.*, 2005).

Met85 of NblR is at the conserved Thr/Ser position that plays a critical role in activation of RRs. Upon

Fig. 1. Structure and sequence comparisons of NblR RD with canonical and non-canonical RR RDs. (a) Ribbon representation of the 3D model of NblR RD (magenta) superimposed on the RD structures of the phosphorylated canonical RR Spo0A from *B. stearothermophilus* (yellow; 1QMP) and the non-canonical RR FrzS from *M. xanthus* (green; 2GkG). The residues at the phosphoaccepting aspartate, the Mg^{2+} -chelating and conformational switch positions are represented in ball-and-stick with carbon atoms in the same colour as the corresponding ribbon structure: oxygen in red, sulphur in blue and phosphorus in orange. Residues are labelled and numbered for NblR. The two cysteine residues of NblR are shown as ball-and-stick and the possible disulphide bridge is denoted by a blue broken line. Black broken lines denote contacts with the phosphoryl group and the Mg^{2+} ion (cyan sphere) in the phosphorylated Spo0A structure. (b) Structure-based sequence alignment for the NblR model with the structure of atypical RRs FrzS (2GKG) from *M. xanthus* and HP1043 (2PLN) from *H. pylori*, and canonical RRs Spo0A (1QMP) from *B. stearothermophilus*, CheY (1FQW) and PhoB (1ZES) from *E. coli* was performed using LSQKAB (Collaborative Computational Project, Number 4, 1994). Strand and helices of NblR are indicated above the sequence alignment as labelled yellow and blue boxes, respectively. Consensus sequence for each position (>80% occurrence) was taken from the SMART server (Letunic *et al.*, 2006) and appears below the sequence alignment (CONS/80% line) printed following the SMART abbreviation code [upper-case letters indicate the corresponding amino acid; lower-case letters correspond to the following amino acid groups: o (S,T), - (D,E), l (L,V), a (F,H,W,Y), s (A,C,D,G,N,P,S,T,V), p (C,D,E,H,K,N,Q,R,S,T), t (A,C,D,E,G,H,K,N,Q,R,S,T), h (A,C,F,G,H,I,L,K,L,M,R,T,V,W,Y), . (any)]. The conserved phosphoaccepting aspartic acid position is shaded in red, the conserved catalytic residue positions in light blue and the switch residue positions in green. The alternative phosphoaccepting aspartate residues in FrzS and HP1043 are highlighted in red and the NblR cysteine residues in magenta.

phosphorylation, this conserved Thr/Ser residue is hydrogen bonded with the phosphoryl group, inducing the repositioning of the loop that connects the $\beta 4$ strand with the $\alpha 4$ helix and the concomitant orientation inwards of an

exposed aromatic residue in $\beta 5$ (Tyr106 in CheY). This orchestrated movement is called the 'T-loop-Y' coupling switch and its ultimate output is the reorganization of the $\alpha 4$ - $\beta 5$ - $\alpha 5$ surface, leading to changes in the affinity for the

effector domain, downstream protein targets, or, in the OmpR-PhoB family, receiver dimerization (Dyer & Dahlquist, 2006). The presence of methionine in this position, which precludes the hydrogen bond with the phosphoryl group, would prevent this activation switch. Thus, the Ser14 and Met85 substitutions would impair phosphorylation and the consequent activation of NblR.

NblR is not phosphorylated *in vitro* by acetyl phosphate

Response regulators can be phosphorylated *in vitro* and *in vivo* by the small phosphate donor acetyl phosphate (McCleary & Stock, 1994). The *pta* gene, encoding phosphotransacetylase, has not been identified in the genome of *Synechococcus* and therefore phosphorylation of response regulators by acetyl phosphate is unlikely to be of physiological significance in this cyanobacterium. However, the ability of acetyl phosphate to phosphorylate a given response regulator *in vitro* would indicate that the protein can be phosphorylated *in vivo*.

The differences, at the level of primary structure, between the NblR RD and canonical regulators strongly suggested that phosphorylation at the conserved Asp57 is not the mechanism of NblR activation. To investigate this issue, we first performed *in vitro* phosphorylation assays with acetyl phosphate. Both full-length NblR (6His-NblR) and the NblR RD (6His-NblR-RD) were overproduced and purified. Phosphorylation was compared with RR468, a canonical RR protein from *Thermotoga maritima*, on a non-denaturing gel (Fig. 2a). Consistent with phosphorylation-induced dimerization in RRs of the OmpR/PhoB family (Bachhawat *et al.*, 2005), incubation of RR468 with acetyl phosphate induces a band shift. In contrast,

6His-NblR-RD and 6His-NblR did not change in mobility, suggesting that these proteins were not phosphorylated. Experiments carried out in parallel with NblR and NblR-RD proteins after 6His-tag removal with TEV protease showed equivalent results (data not shown).

To exclude possible artefacts interfering with detection of phosphorylated forms of 6His-NblR-RD and RR468 in native gels, we used additional experimental approaches. Since the addition of a phosphoryl group to proteins reduces their pI by around 0.2 units (Jeon *et al.*, 2001), we performed 2D gel electrophoresis before and after addition of the phosphodonor. In full agreement with previous results, incubation with acetyl phosphate shifted RR468 towards the acidic part of the gel and had no effect on 6His-NblR-RD (Fig. 2b). Finally, the samples were subjected to mass spectroscopy, confirming that the incubation with acetyl phosphate introduces a single phosphoryl group in RR468 and that 6His-NblR-RD did not change its mass (data not shown), confirming the lack of phosphate incorporation to NblR proteins.

Phosphorylation of Asp57 is not required for NblR functions

The structural features of the NblR RD noted above and its lack of phosphorylation by acetyl phosphate strongly suggested that, in spite of conservation, Asp57 was not involved in NblR activation. To determine whether this residue still plays a regulatory role during acclimation to stress, we constructed *Synechococcus* strains in which Asp57 was replaced by Ala. The strategy for allelic replacement is outlined in Supplementary Fig. S1 (available with the online version of this paper). Strain NblR^{D57A}-RCS3 contains the mutation at its chromosomal emplacement

Fig. 2. Phosphorylation with acetyl phosphate and analysis by native and 2D gel electrophoresis. (a) 6His-NblR, 6His-NblR-RD and RR468 were incubated in the presence (+AcP) or absence (-AcP) of acetyl phosphate and separated according to their charge/mass relation by native PAGE. Unphosphorylated and phosphorylated forms of RR468 are indicated. (b) 6His-NblR-RD and RR468 were incubated in presence or absence of acetyl phosphate for 60 min. Samples for 2D gel electrophoresis consisted of the unphosphorylated proteins (-AcP) or a mix of equal amounts of unphosphorylated and phosphorylated proteins (+AcP), where the unphosphorylated protein was used as internal control. The unphosphorylated and phosphorylated forms in +AcP gels are indicated with filled and open arrows, respectively.

(*nblR*^{D57A} allele) and the streptomycin-resistance cassette CS3 located downstream. To exclude polar effects and minimize possible artefacts due to the presence of the CS3 cassette, a streptomycin-resistant control strain retaining the wild-type *nblR* allele (WT-RCS3) was generated in parallel. Homozygosis for CS3 alleles was promptly achieved and it was confirmed that the presence of the streptomycin-resistant cassette CS3 did not confer significant phenotypic differences to the wild-type *Synechococcus* strain under standard or stress conditions (data not shown). For simplicity, only data produced with the strain WT-RCS3 are shown as control and referred to as wild-type hereafter.

To determine the impact of the D57A substitution on NblR function, we analysed the ability of the *nblR*^{D57A} mutant to respond to conditions requiring NblR activity. In particular, high light (HL) irradiation and nitrogen deficiency allow clear discrimination between wild-type *Synechococcus*

and *nblR* null derivatives (Luque *et al.*, 2001; Salinas *et al.*, 2007; Schwarz & Grossman, 1998). As shown in Fig. 3(a), when wild-type and mutant derivatives were subjected to HL irradiation only the *nblR* null mutant (strain NblR45) ceased growth. The *nblR*^{D57A} strain continued to grow, at a rate similar to that of the wild-type control. At different times (up to 4 days), drops of all cultures were plated and incubated in various conditions to visually determine their ability to resume growth. As shown in Fig. 3(b), only the null mutant failed to recover appropriately, while the ability of the *nblR*^{D57A} mutant to recover from stress was similar to that of the wild-type, indicating that Asp57 is not required to increase the resistance of *Synechococcus* to HL stress (Fig. 3b, panel HL). Equivalent results, i.e. no difference between wild-type and *nblR*^{D57A} strains in conditions in which the null mutant is clearly impaired, were obtained when cultures were subjected to nitrogen starvation (Fig. 3b, panel -N).

Fig. 3. Effect of the D57A substitution at NblR on *Synechococcus* cultures subjected to stress. (a) Growth, as measured by OD₇₅₀, of WT-RCS3 (▲), NblR^{D57A}-RCS3 (◆) and NblR45 (●) at 500 μmol photons m⁻² s⁻¹. Mean values and standard deviations of three independent experiments are shown. (b) Drops (5 μl) of cultures from NblR^{D57A}-RCS3 (R^{D57A}), WT-RCS3 (WT) and NblR45 (R⁻) were spotted on BG11 solid medium after nitrogen deprivation (-N) or high-light (HL) stress conditions up to 4 days, incubated in standard light conditions and photographed 5 days later. A picture taken from one representative experiment is shown in each case. (c) Relative amount of phycocyanin (PC) as a function of time. Strains NblR^{D57A}-RCS3, WT-RCS3 and NblR⁻ were transferred at mid-exponential phase from ammonium-containing to nitrogen-deprived medium (-N). Mean values from three or four experiments are shown. (d) Time-course of P_{nblA}::luxAB induction in *Synechococcus* strains. Cells were grown in the presence of NH₄⁺ and when they reached mid-exponential phase, they were shifted to nitrogen-free medium. After nitrogen deprivation at time 0, bioluminescence from the reporter strains was recorded. Data from one representative experiment out of three is shown. Mean values and standard deviations at time 0 (NH₄⁺) and 24 h after nitrogen deprivation (-N) from three independent experiments are given in the inset table.

To determine the impact of the D57A substitution on *nblA* gene induction and chlorosis, we analysed the absorbance spectra of mutant and control cultures subjected to nitrogen stress as well as the expression of the reporter fusion $P_{nblA}::luxAB$ (Espinosa *et al.*, 2007), which is positively regulated by NblR (Salinas *et al.*, 2007). As shown in Fig. 3(c), the kinetics of phycocyanin loss were identical in *nblR*^{D57A} and wild-type cultures subjected to nitrogen starvation, conditions in which the NblR[−] strain maintained high phycocyanin levels. In line with this, when *nblR*^{D57A} and wild-type cultures carrying the $P_{nblA}::luxAB$ reporter fusion were subjected to nitrogen deprivation, a strong and equal increase in the bioluminescence signal was observed in wild-type and *nblR*^{D57A} strains but not in NblR[−] cultures (Fig. 3d), thus indicating that Asp57 does not play a role in *nblA* gene activation under the stress conditions used here.

Canonical receivers, pseudo-receivers and the PIARR group

Several proteins with RD-like structural folds lacking residues involved in aspartic acid phosphorylation and signal transduction and having extended loop regions that align poorly to canonical RD have been characterized. The cyanobacterial circadian clock protein KaiA (Williams *et al.*, 2002) is one of these proteins with a pseudo-RD. However, the RDs of FrzS, HP1043 and NblR are not as divergent from canonical RDs as pseudo-RD (see Fraser *et al.*, 2007 for an extended discussion) and they still contain some of the consensus residues around the canonical phosphorylation pocket and the output face (Fig. 1). Importantly, FrzS, HP1043 and the NblR-modelled RDs maintain the essential nature of the $\alpha 4$ - $\beta 5$ - $\alpha 5$ face, suggesting its involvement in signal propagation. In the case of FrsZ, the switch Tyr and a neighbouring His residue have been shown to be essential for function. Therefore, in spite of the lack of phosphorylation, these atypical RDs conserve key features involved in signal propagation. To recognize the existence of alternative input mechanisms for signal transduction within the two-component RR superfamily, we propose the term PIARR, standing for phosphorylation-independent activation of response regulator. In this manner, in addition to the grouping into classical RR families on the basis of output domain homology, proteins differing in the mechanism of input signalling, the so-called hybrid RDs (Fraser *et al.*, 2007), can also be distinguished on the basis of receiver features.

In vitro and *in vivo* assays indicate that NblR is monomeric

OmpR/PhoB regulators are DNA-binding proteins with a high degree of conservation of the $\alpha 4$ - $\beta 5$ - $\alpha 5$ surface and it has been proposed that they all share a common mechanism of activation that involves dimerization of RDs using the $\alpha 4$ - $\beta 5$ - $\alpha 5$ surface (Gao *et al.*, 2007; Toro-Roman *et al.*, 2005). Output domain homology places

NblR and HP1043 with the abundant OmpR/PhoB family of RRs. HP1043 is constitutively active *in vivo* and purified HP1043 is a dimer whose RD structure resembles the active and phosphorylated form of PhoB. Since helix–turn–helix proteins in general and all characterized OmpR/PhoB family members in particular bind to DNA as dimers, it was important to address the oligomerization status of NblR.

To determine the oligomeric state of NblR *in vitro*, 6His-NblR and 6His-NblR-RD proteins were subjected to gel filtration chromatography on a Superdex 200 column. 6His-NblR and 6His-NblR-RD eluted as single peaks with elution volumes of 15.1 ml and 16.1 ml that corresponded on the calibrated column to masses of 28 000 and 16 000 Da, respectively (Fig. 4a). The calculated masses for the polypeptide of 6His-NblR and 6His-NblR-RD are 28 590 and 15 800 Da respectively, in agreement with a monomeric state. Although our data showed that NblR is not phosphorylated *in vitro* by small phosphodonors (see above), the effect of the acetyl phosphate in the quaternary structure of NblR was also evaluated. As expected, pre-incubation with acetyl phosphate had no appreciable effects on the elution volume of either protein (Fig. 4b).

The structural model of NblR strongly suggests the possibility of a disulphide bridge between Cys69 and Cys96 (Fig. 1a). To explore the possibility that the redox state could regulate the $\alpha 4$ - $\beta 5$ - $\alpha 5$ surface and, consequently, dimerization, we also estimated the molecular mass of 6His-NblR and 6His-NblR-RD under oxidizing and reducing conditions. The incubation of both proteins with the reducing agent DTT and subsequent chromatography in the Sephadex 200 column equilibrated with a buffer containing DTT showed that both proteins behaved as monomers (Fig. 4c). To ensure a complete oxidation state, the proteins were incubated with the oxidizing agent Cu-phenanthroline for 30 min prior to the chromatography. The oxidized proteins showed a similar elution profile to both the non-treated protein and the reduced protein (Fig. 4d). Therefore, in our experimental conditions, the redox state had no major effect on the oligomeric nature of the purified NblR proteins analysed. To assess the impact of the 6His-tag on the quaternary structure, analogous filtration assays were carried out with both proteins after tag removal; these gave similar results (data not shown).

The *in vitro* data, indicating that 6His-NblR and 6His-NblR-RD are monomeric, are still compatible with NblR activation by dimer formation in *Synechococcus* and we wondered whether we could find *in vivo* indications of associations between NblR monomers using two-hybrid interaction assays. We reasoned that, although these systems would not allow specific activation by stress signals, they could be more sensitive than *in vitro* systems and/or provide a more physiological environment for monomer association. With this in mind, we performed assays using the yeast two-hybrid system, based on

Fig. 4. Gel filtration analysis of 6His-NblR and 6His-NblR-RD. The absorbance elution profiles of 6His-NblR (left) and 6His-NblR-RD (right) proteins after chromatography on Superdex 200 HR under different conditions are shown: (a) native, (b) phosphorylated with acetyl phosphate, (c) reduced with DTT, and (d) oxidized with Cu-phenanthroline. The elution volumes for each peak are indicated.

reconstitution of GAL4 transcriptional activity (Fields & Song, 1989) and the BACTH system, based on reconstitution of a cyclic AMP signalling cascade in an *E. coli cya* strain (Karimova *et al.*, 1998). For yeast two-hybrid analyses, constructs included two full-length (NblR) and two C-terminally truncated derivatives of NblR (NblR¹⁻¹⁷¹), each one fused to upstream GAL4 domains

(GAL4BD and GAL4AD), and two full-length NblR derivatives, each one fused to C-terminally located GAL4BD and GAL4AD polypeptides (see Table 1 and Fig. 5 for plasmid details). The downstream location of GAL4 domains in fusion proteins was aimed at minimizing possible artefacts resulting from inappropriate conformations of the NblR RD contiguous to the GAL domain, i.e. false negatives. Expression of *HIS3*, *ADE2* and *lacZ* reporters in Y187/PJ696 diploids containing relevant pairs of fusion proteins was determined as previously described (Burillo *et al.*, 2004). All six protein fusions gave appropriate expression in yeast, but no signal interactions between NblR proteins were found (nine pair combinations of these proteins were tested, Table 3). The same result was found with full-length NblR derivatives using the independent BACTH system, thus providing additional evidence of the monomeric conformation of NblR *in vivo*. Taken together, both *in vitro* and *in vivo* analyses indicate that the default state of NblR is monomeric.

Interactions of NblR with *Synechococcus* proteins

The yeast two-hybrid system has been shown to detect specific interactions, between cognate two-component proteins (Martinez-Argudo *et al.*, 2001; Ohta & Newton, 2003) and between HKs and specific regulators (Espinosa *et al.*, 2006; Martinez-Argudo *et al.*, 2002). In contrast to classical genetic screens, yeast two-hybrid approaches rely on protein-protein interactions, and not on phenotype or viability, a clear advantage when dealing with phenotypes difficult to assay or essential functions. It should be noted that the relatively downstream location of interaction determinants in HKs, usually preceded by N-terminal transmembrane and sensory domains, increases the chances of identifying these proteins as yeast two-hybrid preys. To further increase the chances of detecting interactions of the relevant receiver domain of NblR, we used GAL4BD-NblR and GAL4BD-NblR¹⁻¹⁷¹ as baits in *Sau3AI* and *Tsp509I*-generated *Synechococcus* yeast two-hybrid libraries (Burillo, 2006; Burillo *et al.*, 2004). However, no HK polypeptides were found in the screenings.

The fact that the HK NblS had been identified in *nbl* screenings, and subsequently proposed as the NblR cognate partner in signal transduction, prompted us to confirm the negative results in our two-hybrid searches and further explore the possibility of a direct protein interaction between NblS and NblR by performing additional and more direct interaction assays with these proteins. To this end, all six GAL4AD or GAL4BD fusions to NblR derivatives used above were assayed in the appropriate combinations with NblS and NblS²⁷²⁻⁶⁶⁴ (see Table 1 and Fig. 5 for plasmid details). For all ten protein fusions, appropriate expression in yeast was previously verified (Espinosa *et al.*, 2006; and data not shown). In agreement with previous results, analysis of reporter expression in diploids containing relevant pairs of fusion proteins

Fig. 5. Schematic representation of the different NblR, NblS, NdhH and NarB polypeptides encoded by two-hybrid plasmids obtained and/or used in this study. Numbers indicate the amino acid boundaries of the polypeptides present in each given plasmid. Domain nomenclature for NblR and NblS as in the text. MO-Fe4S4, molybdopterin Fe4S4 domain; MO, molybdopterin domain; MoB, molybdopterin-binding domain; NADH-dehyd, NADH dehydrogenase domain. The position of yeast (GAL4AD and GAL4BD) and bacterial (T25 and T18) domains related to the polypeptides is shown. Symbols used to represent the different GAL4 or CyaA domains are listed in the inset. The short NarB and NdhH polypeptides fused to GAL4AD domains were obtained in yeast two-hybrid screenings using NblR or NblR¹⁻¹⁷¹ as bait, respectively.

confirmed lack of interaction between NblS and NblR for all six pairs of fusion proteins tested (Table 3).

The BACTH system provides an independent assay particularly appropriate for membrane-anchored bacterial proteins (Karimova *et al.*, 2005) and might therefore provide a more physiological environment for NblS function. We produced fusion proteins of the two fragments (T25 and T18) of the catalytic domain of *Bordetella pertussis* adenylate cyclase to NblS, and analysed its ability to complement the Cya⁻ phenotype when paired with NblR. Again, no interaction was found between NblS and NblR derivatives. Since appropriate expression in the host *E. coli* strain was verified for the T25-NblS protein, but not for the T18-NblS protein, the latter fusion is not included in Table 1 and Fig. 5. Taken together, the yeast and bacterial two-hybrid analyses strongly argue against protein–protein interactions between the two *nbl* regulators NblS and NblR.

Screening of *Synechococcus* libraries with NblR polypeptides as baits produced unexpected results. Instead of HK

polypeptides, multiple clones containing *narB*, encoding nitrate reductase, and *ndhH*, encoding a subunit of the NDH-1 complex, were found as preys. In particular, screening of the libraries with GAL4BD-NblR as bait rendered *narB* preys while *ndhH* clones appeared in screenings with GAL4BD-NblR¹⁻¹⁷¹. From the size of the clones, it can be inferred that NarB¹⁵⁴⁻²⁵⁵, encoding part of the molybdopterin domain, and the fragment NdhH⁵⁴⁻²⁸³ contain determinants for interaction with NblR. Additional yeast two-hybrid assays validated these interactions and localized the interaction with NarB¹⁵⁴⁻²⁵⁵ to the C-terminal DNA-binding domain of NblR.

The interaction of the output DNA-binding domain of NblR with the nitrate assimilation enzyme was particularly intriguing and prompted us to verify the interaction with the full-length NarB protein and to perform independent protein–protein interactions assays in *E. coli*. Since appropriate expression of the *Synechococcus narB* gene leads to nitrate reductase activity in *E. coli* (Rubio *et al.*,

Table 3. Yeast two-hybrid interactions

The GAL4AD and GAL4BD fusion proteins carried by diploids are indicated on the left and at the top, respectively. In the case of the NblR full-length fusions, the position of the GAL4 domain is also indicated. Asterisks refer to absence of proteins fused to GAL4 domains. Levels of interaction were classified according to the strength of the signals, as previously described (Burillo *et al.*, 2004). ND, Not determined.

	BD-NblR	NblR-BD	NblR ¹⁻¹⁷¹	NblS	NblS ²⁷²⁻⁶⁶⁴	NarB ¹⁵⁴⁻²⁵⁵	NarB ¹⁵⁴⁻³⁴⁴	NarB ¹¹²⁻³⁸²	NarB	NdhH ⁵⁴⁻²⁸³	*
AD-NblR	—	—	—	—	—	+/-	+	+	+	—	—
NblR-AD	—	—	—	—	—	+/-	+	+	+	ND	—
NblR ¹⁻¹⁷¹	—	—	—	—	—	—	—	—	—	++	—
NblS	—	—	—	—	—	—	—	—	—	—	—
NblS ²⁷²⁻⁶⁶⁴	—	—	—	—	—	—	—	—	—	—	—
NarB ¹⁵⁴⁻²⁵⁵	+/-	+/-	—	—	—	—	—	—	—	—	—
NarB ¹⁵⁴⁻³⁴⁴	+	+	—	—	—	—	—	—	—	—	—
NarB ¹¹²⁻³⁸²	+	+	—	—	—	—	—	—	—	—	—
NarB	+	+	—	—	—	—	—	—	—	—	—
NdhH ⁵⁴⁻²⁸³	—	ND	++	—	—	—	—	—	—	+	—
*	—	—	—	—	—	—	—	—	—	—	—

1996), it was important to test the NblR–NarB interaction in this heterologous system. BACTH assays confirmed the interaction between NarB and both NblR and NblR¹²⁵⁻²²⁹ derivatives, indicating that NblR and NarB also have considerable affinity to each other in a prokaryotic intracellular environment.

Integration of stress signals by NblR and PIARR proteins

We have shown here that NblR regulation seems to be independent of phosphorylation by HKs and does not fit the two-component paradigm. It should be noted that Kato *et al.* (2008) have very recently obtained some of the results presented in this work: failure to detect a regulatory phenotype for an independently constructed *nblR*^{D57A} mutant and detection of specific interactions between NblR and NarB in yeast two-hybrid screenings with NblR. While these authors did not detect NdhH polypeptides as preys, they detected a protein not found by us, MreC. Fishing of different preys is not surprising given that the strategies for library construction differed between the two laboratories. The challenge is now to determine the physiological significance of the protein–protein interactions with NblR detected by yeast two-hybrid methods.

Nitrogen and sulphur starvation stress signals did not regulate *nblR* transcripts and purified NblR had very low affinity for its target regulatory region at *nblA* (Luque *et al.*, 2001), suggesting that, as expected for a RR, the regulation is at the level of protein activity. NblR might be regulated as other OmpR/PhoB proteins, being able to switch between active dimers and inactive monomers. If that is the case, the implication is that phosphorylation would not be the only means to achieve activation by dimerization within the OmpR/PhoB family.

It is now clear that phosphorylation of canonical RD, the paradigm for signal input into RRs, must coexist with

alternative ways of communication operating in PIARR proteins. It can be anticipated that research with these atypical RRs would greatly deepen our understanding of prokaryotic signal transduction.

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