Phosphorylation-independent activation of the atypical response regulator NblR

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Summary

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 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 weight phosphate donor acetyl phosphate and mutation of Asp57 to Ala had no impact on previously characterised 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 dimerisation 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.

Introduction

Cyanobacteria, photosynthetic prokaryotes that perform plant-type oxygenic photosynthesis, have developed mechanisms to modify the composition of the 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 absortion 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 Dimerisation 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 (OD). 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, homologs being present in all cyanobacterial genomes available to date. Sequence analysis predict 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 (DHp) and an ATP-binding domain (HATPase_c). 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 on *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 down regulation of photosynthetic electron transport under stress conditions (Schwarz & Grossman, 1998). Both NblS and NblR seem to be regulated by general stress signals and they play antagonistic roles at the *nblA* gene. 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 recognise 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 *E. coli* DH5α, using standard techniques.

Synechococcus strains were routinely grown photoautotrophically at 30ºC while shaking under constant illumination (40 μmol photons m^{-2} s^{-1}) provided by cool white fluorescent lights. Media used were $BG11_0$ (no added nitrogen), BG11 ($BG11_0$ plus 17.5 mM $NaNO_3$ and 10 mM Hepes/NaOH pH 7.8) or BG11-NH_4 ($BG11_0$ plus 10 mM NH_4Cl 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 30ºC under constant illumination. *Synechococcus* strains were transformed as described (Golden & Sherman, 1984), incubated for 48 hours at 30ºC 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 BG11-NH_4 cultures grown with the apropiate antibiotics (OD_{750nm} of 0.5) were harvested by centrifugation, washed twice with $BG11_0$ and finally resuspended in $BG11_0$ without antibiotics. For initiation of high light (HL) stress, BG11 cultures were grown with the appropriate antibiotics to mid exponential phase (OD_{750nm} of 0.5) under standard illumination conditions, washed twice with BG11 and finally resuspended in BG11 without antibiotics and then transferred to high light conditions (500 μmol 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. Antibiotics concentrations used for *Synechococcus* were 10 μg ml^{-1} (kanamycin) and 5 μg ml^{-1} (chloramphenicol and streptomycin).

Yeast culture and transformation procedures were as described (Ausubel, 1999). To perform yeast two-hybrid screenings, previously obtained *Synechococcus Sau*3AI (Burillo *et al.*, 2004) or *Tsp*509I 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 harboring appropriated plasmid derivatives was grown at 25-28ºC with ampiciline (50 μg ml^{-1}) and chloramphenicol (30 μg ml^{-1}). Complementation was tested on M63 containing maltose (0.3%), thiamin (0.0001%), IPTG (0.5mM) and X-gal (80 μg 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 *Escherichia coli* (RCSB code:1ZES) (Bachhawat *et al.*, 2005), FrzS from *Myxococcus xanthus* (RCSB code:2GKG) (Fraser *et al.*, 2007), beryllium fluoride activated CheY from *Escherichia coli* (RCSB code: 1FQW) (Lee *et al.*, 2001), HP1043 from *Helicobacter pylori* (RCSB code: 2PLN) as well as the NblR model were structural alignment using the LSQKAB program as implemented in the CCP4 suite .

Construction of an *nblR*^{D57A} mutant and derivative strains

An *Eco*RI-*Sal*I 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}. To generate appropriated flanking sites, the CS3 cassette (Sm^{R}) from pRL453 was re-cloned into *Hind*III pBluescriptII SK+, giving plasmid pUAGC453. A *Hinc*II-*Eco*RV containing the CS3 cassette from pUAGC453 was then cloned into the Klenow-treated *Sty*I 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 *Hind*III and *Hinc*II and Klenow filled. This blunt fragment was then cloned into *Hinc*II-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 *Pvu*I, giving 309, 186 and 51 pb fragments for wild-type *nblR* and 309 and 237 pb fragments for *nblR*^{D57A}. A clone of each type was selected for further analysis (strains WT-RCS3 and NblR^{D57A}-RCS3, respectively). 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 *Eco*RI and *Sal*I and cloned into pGAD424(+1) and pGBT9(+1), giving plasmids pUAGC300 (GAL4AD:NblR$^{1-171}$) and pUAGC301 (GAL4BD:NblR$^{1-171}$), 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 *Nru*I-open 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 BACTH derivatives, *nblR* was PCR amplified with primers NblR bth1-F and NblR bth1-R, cut with *Bam*HI and *Kpn*I, and cloned into pT25 or pUT18c, resulting in plasmids pUAGC600 (T25:NblR) and pUAGC601 (T18:NblR), respectively. The same procedure but using primers NblR bth1-F and NblR bth2-R resulted in plasmids pUAGC604 (T25:NblR$^{125-229}$) and pUAGC605 (T18:NblR$^{125-229}$).

The *narB* sequence was PCR amplified using primers NarB bth1-F and NarB bth1-R, cut with *Bam*HI and *Kpn*I, 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 *Eco*RI and *Sal*I and cloned into pGAD424(+1) and pGBT9(+1).

The insert present in prey plasmids pUAGC311 (GAL4AD:NarB$^{154-344}$), pUAGC313 (GAL4AD:NarB$^{154-255}$) and pUAGC315 (GAL4AD:NarB$^{112-382}$), obtained in yeast two-hybrid screenings using NblR as bait, were PCR amplified using primers transgagbt-1F and transgadgbt-1R. The PCR product was then transformed into *S.*

cerevisiae PJ696 yeast strain together with *Eco*RI-*Sal*I open pGAD424 giving plasmids pUAGC310 (GAL4BD:NarB$^{154-344}$), pUAGC312 (GAL4BD:NarB$^{154-255}$) and pUAGC314 (GAL4BD:NarB$^{112-382}$). The same strategy was used to obtain plasmid pUAGC324 (GAL4BD:NdhH$^{54-283}$) using as template the prey plasmid pUAGC325 (GAL4AD:NdhH$^{54-283}$), obtained in screenings using NblR$^{1-171}$ as bait.

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 *Nco*I and *Hind*III sites of pPROEX-HTb plasmid (Invitrogen), creating an in-frame fusion to the N-terminal His_{6}-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 μg ml^{-1}), and chloramphenicol (33 μg 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 (Marcherey-Nagel). Elution of the protein was carried out using 250 mM of 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 acetylphosphate 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 1h at RT. 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 2h at 4 ºC.

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 of 6His-NblR-RD or 6 µg of RR468. The phosphorylation reaction were stopped by the addition of 90 µl of lysis buffer (8 M urea, 2% CHAPS), 1 µl of 1M DTT and 0.5 µl of ampholytes pH 4-7. Control samples without acetyl phosphate were carried out in parallel. Samples consisting in the unphosphorylated proteins or a mix of equal amounts of unphosphorylated and phosphorylated proteins, where the unphosphorylated proteins is used as internal control, were isoelectric 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 (Amershan Biosciences) system following the indications of the manufactures. The samples were focussed at 500 V for 0.25 kVh, 1000 V for 0.5 kVh and 6000 V for 7.5 kVh at 20 ºC. Proteins were resolved in the second dimension by SDS-PAGE using 15 % acrylamide gels.

Both types of gels were stained with Coomasie blue and images analyzed 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 of protein in running buffer were individually applied to the column and eluted with running buffer at a flow rate of 0.5 ml $min^{-1}$and collected in 1 ml fractions. The fractions were analyzed by SDS-PAGE. Protein elution profiles were monitored by measuring the absorbance to 280 nm. For evaluation of the acetyl phosphate effect, the samples were incubated for 1h at RT with acetyl phosphate and $MgCl_2$ (12.5 mM) before the chromatography. Sample oxidation was carried out by treatment with 0.3 mM Cu (II)-(o-phenanthroline)$_3$ (Lee *et al.*, 1995) for 30 min at 37 ºC prior to gel filtration. Sample reduction was carried out by incubation with 10 mM DTT for 30 min at RT, and the chromatography running buffer was supplemented with 1m DTT.

Determination of luciferase activity

To determine bioluminescence, 1 ml of cultures grown to mid exponential phase were adjusted at an OD_{750nm} 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% dimethyl sulfoxide. Light emission was recorded in a Berthold LB9509 luminometer. Bioluminescence was recorded every 20 seconds for 10 minutes. 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 pigments contents spectrophotometrically

Whole-cell absorbance spectra were carried out in order to estimate pigment contents. 1ml of cultures was taken at indicated times and conditions, diluted with fresh

medium to an OD_{750nm} of 0.5 and absorbance spectra (500-800nm) 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 (Myers *et al.*, 1980).

Results and Discussion

NblR lacks residues conserved in canonical response regulators and is not phosphorylated *in vitro* by acetylphosphate

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 (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 and 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 residues disposition in the alignment, we chose the CheY and PhoB beryllium fluoride activated structures (Lee *et al.*, 2001) as prototypical RD domains, as well as the phosphorylated Spo0A structure (Lewis *et al.*, 1999), the first canonical RD crystallized in the phosphorylated form. The structures of *Myxococcus xantus* 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. 1A, NblR apparently retains the overall folding of RDs, as it 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 playing a critical role in activation of RRs. Upon phosphorylation, this conserved Thr/Ser residue is hydrogen bonded with the phosphoryl group, inducing the reposition of the loop that connects the β4 strand with the α4 helix and the concomitant orientation inwards of an exposed aromatic residue in β5 (Tyr106 in CheY). This orchestrated movement is called the "T-loop-Y" coupling switch and its ultimate output is the reorganization of the α4-β5-α5 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 a 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.

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 over produced and purified. Phosphorylation was compared with RR468, a canonical RR protein from *Thermotoga maritima,* on a nondenaturing gel (Fig. 2A). Consequent 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 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 shown 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 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.1. Strain NblR^{D57A}-RCS3 contains the mutation at its chromosomal emplacement (*nblR* ^{D57A} allele) and the streptomycin-resistance cassette CS3 located downstream. To exclude polar effects and minimise possible artefacts due to 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 is shown as control and referred 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 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. 3A, when wild type and mutant derivatives were subjected to high light 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 four days), drops of all cultures were plated and incubated in various conditions to visually determine their ability to resume growth. As shown in Fig. 3B, 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 high light 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).

To determine the impact of the D57A substitution on *nblA* gene induction and chlorosis, we analyzed the absorbance spectra of mutant and control cultures subjected to nitrogen and high light 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. 3C, 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 bioluminiscence 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 characterised. 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 α4-β5-α5 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 recognise 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 α4-β5-α5 surface and it has been proposed that they all share a common mechanism of activation that involves dimerisation of RDs using the α4-β5-α5 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 characterised OmpR/PhoB family members in particular bind to DNA as dimers, it was important to address the oligomerisation 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 not appreciable effects on the elution volume of both proteins (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 α4-β5-α5 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 dithiothreitol (DTT) and subsequently 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-phenantroline for 30 min previously 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 6His-tag impact in the quaternary structure, analogous filtration assays were carried out with both proteins after tag removal, obtaining similar results (data not shown).

The *in vitro* data, indicating that 6His-NblR and 6His-NblR-RD are monomeric, is 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 interactions 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 reconstitution of GAL4 transcriptional activity (Fields & Song, 1989) and the bacterial adenylate cyclase two-hybrid (BACTH) system, based on reconstitution of a cyclic AMP signaling cascade in an *E. coli cya* strain (Karimova *et al.*, 1998). For yeast two-hybrid analyses, constructs included 2 full length (NblR) and 2 C-terminally truncated derivatives of NblR ($NblR^{1-171}$) each one fused to upstream GAL4 domains (GAL4BD and GAL4AD) and 2 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 is aimed to minimise 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 6 protein fusions gave appropriate expression in yeast, but none of the 9 pair combinations of these proteins used to test NblR-NblR interactions gave interaction signals (Table 3). The same result, no signal interaction between NblR proteins, 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 (Martinez-Argudo *et al.*, 2002; Salinas & Contreras, 2003, Espinosa *et al.*, 2006). 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 HK, that are 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-NblR$^{1\text{-}171}$ as baits in

*Sau*3AI and *Tsp*509I-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 appropriated combinations with NblS and $NblS^{272-664}$ (see Table 1 and Fig.5 for plasmid details). For all 10 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 confirmed lack of interaction between NblS and NblR for all 6 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 *Bortedella pertussis* adenylate cyclase to NblS, and analyzed 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-NblR$^{1-171}$. From the size of the clones, it can be inferred that NarB$^{154-255}$, encoding part of the molybdopterin domain, and NdhH$^{54-283}$ contain determinants for interaction with NblR. Additional yeast two-hybrid assays validated these interactions and localised the interaction with NarB$^{154-255}$ 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 found 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 appropriated expression of the *Synechococcus narB* gene leads to nitrate reductase activity in *E. coli* (Rubio *et al.*, 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 NblR$^{125-229}$ 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 histidine kinases and does not fit the two-component paradigm. It should be noted that, Kato et al 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. While they did not detect NdhH as prey protein in their yeast two-hybrid screenings with NblR

as bait, they detected a protein not found by us, MreC. This partial overlap in prey fishing is not surprising given that the strategies for library construction differed between the two laboratories. The challenge is now to determine whether these three proteins could also play a role in regulation of NblR activity.

Since 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), 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 manner to achieve activation by dimerisation 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.

Figure Legends

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 with 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 aspartic, the Mg^{2+} chelating and conformational switch positions are represented in ball-and-stick with carbon atoms in the same colour that the corresponding ribbon structure, oxygen in red, sulphur in blue and phosphorous 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 isolated RD structures 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(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) has been taken form SMART server (Letunic *et al.*, 2006) and appears below the sequence alignment (CONS/80% line) printed following the SMART abbreviation code [uppercase letters indicate the corresponding aminoacid; lowercase letters correspond to the following aminoacid groups: o (S,T), - (D,E), l (I,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,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 ligthblue and

the switch residue positions in green. The alternative phosphoaccepting aspartic in FrzS and HP1043 are highlighted in red and the NblR cysteine residues in magenta.

Fig. 2. Phosphorylation with acetyl phosphate and analysis by Native and 2D gel eletrophoresis. (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 in the unphosphorylated proteins (-AcP) or a mix of equal amounts of unphosphorylated and phosphorylated proteins (+AcP), where the unphosphorylated proteins was used as internal control. The unphosphorylated and phosphorylated forms in + AcP gels are indicated with closed and open arrows, respectively.

Fig. 3. Effect of the D57A substitution at NblR on *Synechococcus* cultures subjected to stress. (A) Growth, as measured by OD_{750nm}, of WT-RCS3 (▲), $NblR^{D57A}$-RCS3 (♦) and NblR45 (●) at 500 μmol photons $m^{-2}s^{-1}$. Mean values and standard deviations of three independent experiment are shown. (B) 5 μl drops of cultures from $NblR^{D57A}$-RCS3 (R^{D57A}), WT-RCS3 (WT) and NblR45 (R^-) were spotted in BG11 solid medium after nitrogen deprivation (-N) or high light (HL) stress conditions up to four days, incubated in standard light conditions and photographed five days later. 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 (time 0) containing to nitrogen deprived medium (-N). Mean values from three to four experiments are shown. (D) Time

course of P_{nblA}::*luxAB* induction in *Synechococcus* strains. Cells were grown in the presence of NH_4^+ 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 (NH_4^+) and 24 hours after nitrogen deprivation (-N) from three independent experiments are given in the inset.

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 acetylphosphate, (C) reduced with DTT and (D) oxidized with Cu-Phenanthroline. The elution volumes for each peak are indicated.

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, Molybdop_Fe4S4 domain; MO, Molybdopterin domain; MoB, Molybdop_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 $NblR^{1-171}$ as bait, respectively.

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TABLE 1. Strains and plasmids.

Strain or Plasmid	Genotype or relevant characteristics	Source or reference
E. coli DH5α	F^- ϕ80d*lacZ*ΔM15Δ(*lacZYA-argF*)U169 *endA1 recA1 hsdR17*(r_K^- m_K^+) *deoR thi-1 supE44 gyrA96 relA1* λ^-	(Hanahan, 1985)
E. coli DHM1	F^- *glnV44*(AS) *recA1 endA gyrA46 thi-1 hsdR17 spoT1 frbD1 cya-854*	(Karimova *et al.*, 2005)
S. cerevisiae Y187	MATα *ura3-52 his3-200 ade2-101 trp1-901 leu2-3, 112 gal4Δ met*$^-$ *gal80Δ URA::GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*lacZ*	(Harper *et al.*, 1993)
S. cerevisiae PJ696	MATa *ade2Δ trp1-901 leu2-3,112 ura3-52 his3-200 cyh*^{R} *can*^{R} *gal4Δ gal80Δ met2*$^-$ *GAL2::ADE2 GAL1::HIS3* GAL7:*lacZ*	(James *et al.*, 1996)
Synechococcus sp. PCC7942	Wild-type *Synechococcus* sp. PCC 7942	Pasteur culture collection
Synechococcus NblR45	$NblR^-$, Km^r	(Luque *et al.*, 2001)
Synechococcus WT-C103	P_{nblA}::*luxAB* into NSII, Cm^r	(Espinosa *et al.*, 2007)
Synechococcus NblR45-C103	$NblR^-$, P_{nblA}::*luxAB*, Cm^r Km^r	(Salinas *et al.*, 2007)
Synechococcus WT-RCS3	CS3 downstream *nblR*, Sm^r	This work
Synechococcus $NblR^{D57A}$-RCS3	$NblR^{D57A}$, Sm^r	This work
Synechococcus WT-RCS3-C103	P_{nblA}::*luxAB*, Cm^r Sm^r	This work
Synechococcus $NblR^{D57A}$-RCS3-C103	$NblR^{D57A}$, P_{nblA}::*luxAB*, Cm^r Sm^r	This work
pBluescriptII SK(+)	Cloning vector, Ap^r	Stratagene
pENS43	1.8 kb genomic *nblR* fragment cloned into pRL519	(Luque *et al.*, 2001)
pENS38	pPROEX-HTb encoding 6His-NblR	(Luque *et al.*, 2001)
pNblR-RD	pPROEX-HTb encoding 6His-$NblR^{1-124}$, Ap^r	This work
pTM0468	pET22b encoding full-length RR468, Ap^r	(Casino *et al.*, 2007)
pUAGC235	pBluescriptII SK(+) with *nblR*, Ap^r	This work
pUAGC238	pBluescriptII SK(+) with $nblR^{D57A}$, Ap^r	This work
pUAGC240	pUAGC238 with CS3, Ap^r Sm^r	This work
pUAGC239	pUAGC240 with *nblR* downstream sequences, Ap^r Sm^r	This work
pUAGC453	pBluescriptII SK(+) with CS3, Ap^r Sm^r	This work
pGAD424	*LEU2*, GAL4(768-881)AD, Ap^r	(Bartel *et al.*, 1993)
pGBT9	*TRP1*, GAL4(1-147)BD, Ap^r	(Bartel *et al.*, 1993)
pGAD424(+1)	As pGAD424 with a different frame (+1), Ap^r	(Roder *et al.*, 1996)
pGBT9(+1)	As pGBT9 with a different frame (+1), Ap^r	(Roder *et al.*, 1996)
pADC	*LEU2*, GAL4-AD for C-terminal cloning, Ap^r	(Millson *et al.*, 2003)
pBDC	*TRP1*, GAL4-BD for C-terminal cloning, Ap^r	(Millson *et al.*, 2003)
pUAGC51	GAL4AD:$NblS^{272-664}$, Ap^r	(Espinosa *et al.*, 2006)

pUAGC52	GAL4BD:NblS$^{272-664}$, Ap^{r}	(Espinosa *et al.*, 2006)
pUAGC53	GAL4AD:NblS, Ap^{r}	(Espinosa *et al.*, 2006)
pUAGC54	GAL4BD:NblS, Ap^{r}	(Espinosa *et al.*, 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 *et al.*, 1998)
pUT18C	CyaA(225 to 399)T18, Ap^{r}	(Karimova *et al.*, 1998)
pUAGC439	T25:NblS, Cm^{r}	(Espinosa *et al.*, 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’ CGATAGAAGACAGTAGCTTCATCTTTCG 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’ CAGTAGCTTCATCTTTCGGCTTTCGCGTAGGACATAACC 3’
Transgadgbt 1F	5’CGCACATCATCATCGGAAGAGAGTAGTAACAAAGGTCAAAGACAGT TGACTGTATCGCCGAACCCAAAAAAAGAGATCG 3’
Transgadgbt 1R	5’ATAACTTATTTAATAATAAAAATCATAAATCATAAGAAATTCGCCCGGAATTAGCTTGGCGTT TTTCAGTATCTACGATTC 3’
NblR bth1-F	5’ TGAGGGGATCCTATGATCGCGC 3’
NblR bth1-R	5’ ATCGCGGTACCTTAGCTTTCGC 3’
NblR bth2-F	5’ TACAGGATCCACCTGCTGCTCA 3’
NarB bth-1F	5’ GCGCGGATCCAATGTTCGATC 3’
NarB bth-1R	5’ TTGCGGTACCTTAGATTGTGAAG 3’
NblR-D57A-F	5’ GATTTAGTCATCGTCGCTCGCATGGCGGGCGG 3’
NblR-D57A-R	5’ CCGCCCGCCATGCGAGCGACGATGACTAAATC 3’
NblR-down-4F	5’ TGTAGAAGCTTGCGGCTC 3’
NblR-down-4R	5’ CTTTAGGTCGACTGCAGTCTCGTCATC 3’
NblR-RD-F	5’ GGCGCCATGGTCGCGCCAGCCTCGCC 3’
NblR-RD-R	5’ GCAGAAGCTTATCGTTGTAAATAGAG 3’

TABLE 3. Yeast two-hybrid interactions.

	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	++	-	-	-	-	-	-	+	-
*	-	-	-	-	-	-	-	-	-	-	-

The GAL4AD and GAL4BD fusion proteins carried by diploids are indicated on left and top of panels, 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).