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Nitrogen status modulates the expression of RNA-binding proteins in cyanobacteria

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Abstract

Biochemical responses to cold and osmotic stresses overlap because each decreases the availability of free water. Since RNA-binding proteins are known to accumulate following cold stress and play key roles in regulating transcription termination, the effect of osmotic stress on expression of RNA-binding proteins was examined. The transcript levels of four genes encoding RNA-binding proteins (*rbpA*, *rbpB*, *rbpC* and *rbpD*) were monitored in *Anabaena* sp. PCC 7120 cultures supplemented with ammonium ions or growing under nitrogen-fixing conditions. Steady-state transcript levels of all four genes increased transiently in response to a temperature shift from 30 to 20°C under both nitrogen regimes. Osmotic stress also enhanced *rbpB*, *rbpC* and *rbpD* gene expression in ammonium grown cultures. In the absence of a combined nitrogen source, osmotic stress repressed the short-term induction of *rbp* gene expression. The accumulation of RNA-binding proteins did not follow transcript levels, but remained high 24 h after stress initiation. It is concluded that nitrogen nutrition modulates the stress-responsive regulation of RNA-binding proteins in cyanobacteria, providing a potential mechanism to integrate environmental and developmental signals.

Keywords: RNA-binding protein; *rbp*; Cold stress; Osmotic stress; Salt stress; Cyanobacterium; *Anabaena* 7120; Nitrogen fixation; Transcription regulation

1. Introduction

Cyanobacteria, like other living organisms, respond to changes in their physical and chemical environment with genetically programmed physiological adaptations. Environmental effects that produce similar biochemical consequences will induce similar physiological adaptations. For example, low temperature, increased salinity, osmotic stress and desiccation all result in a drop in water potential and outflow of water across the membranes [1–3]. Many of the adaptive responses to these stresses are well documented in the cyanobacteria. For osmotic stress the responses include: (i) exclusion of Na^+ ions, (ii) accumulation of K^+ ions and compatible solutes such as glucosylglycerol, trehalose, and betaines, and (iii) synthesis of stress-responsive proteins [4–7].

Cyanobacteria adapt to cold stress by (i) modification of cell membrane fatty acids [8–10], (ii) accumulation of osmoprotectants [11], and (iii) synthesis of stress-responsive proteins including members of a large family of RNA-binding proteins (Rbps) [12]. A relationship between cold-stress response and RNA metabolism is neither surprising nor unprecedented. First, in *Anabaena* sp. PCC7120 (*Anabaena* 7120), the RNA helicase genes, *crhB* and *crhC* are differentially upregulated in response to cold shock conditions [13]. A detailed analysis of this response has indicated that the levels of the CrhC protein are tightly regulated at the levels of transcription, mRNA stabilization, and translation [14]. Second, it has been shown that most of the RNP-type Rbps in *Anabaena var-*

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Abbreviations: Rbp, RNA-binding protein

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iabilis M3 are regulated by cold [15–16]. Taken together, the above observations begin to enumerate the elements of a larger osmotic/cold-stress response in cyanobacteria, and also suggest osmotic stresses may result in elevated expression of some or all of the Rbps that accumulate after cold stress.

The existence of Rbps in cyanobacteria, first shown by precipitation from total protein extracts of *Synechococcus leopoliensis* by anti-RNP or by anti-Sm sera [17], is now well established and supported by the results of genome sequencing projects as they become available. Approximately 30 *rbp* genes have been described in *Anabaena*/*Nostoc* [15,16,18,19], *Synechococcus* [20], *Synechocystis* [21], *Chlorogloeopsis* [18] and *Prochlorococcus*. All of the encoded polypeptides contain the highly conserved RNP1 and RNP2 sequences in a single RNA recognition motif (80 amino acids). In addition, all contain a C-terminal region or auxiliary domain that, in many cases, contains a high number of glycine residues.

Speculations about the functions of cyanobacterial Rbps currently rely on patterns of *rbp* gene expression and a small number of phenotypic observations. In the *A. variabilis* strain M3, eight *rbp* genes – *rbpA1* [22], *A2* [15], *A3* [16], *B*, *C*, *D* [15], *E* and *F*[12] – have been identified. Equivalents to all of these except *rbpA3* can be found in the whole genome sequence of *Anabaena* 7120 [21]. While the expression of all was induced transiently by temperature shifts from 38°C to 22°C, little else is know about their biochemical activities. However, mutational studies of the *rbpA1* gene in the M3 strain have implicated a role for the RbpA1 protein in repression of heterocyst initiation at low temperatures in the presence of nitrate [23]. This result suggests that there is a functional link between cold-stress and global nitrogen metabolism in cyanobacteria. An *rbpA2* mutation was identified as a transposon insertion that blocked growth of *Anabaena* 7120 at low temperatures, however nitrogen-dependent regulation of this locus was never examined [24–25]. Additional evidence is found in independent studies demonstrating connections between nitrogen nutrition and either cold stress or osmotic stress. Sakamoto and Bryant [9] reported that growth of *Synechococcus sp.* PCC 7002 at low temperature causes nitrogen limitation in medium containing nitrate as the sole nitrogen source. Reddy et al. [26] demonstrated that nitrate or ammonium enhanced the salt tolerance of *Anabaena torulosa* relative to growth under nitrogen-fixing conditions. Similar findings were reported for *Anabaena* 7120 [27].

Based on all the above evidence, we hypothesized (i) that *rbp* gene expression will be induced by osmotic stress, and (ii) that the nutritional state of the cells with respect to nitrogen will directly or indirectly influence *rbp* expression and function. We present evidence that the expression of four *rbp* genes is differentially regulated by osmotic and salt stress in ammonium-supplemented cultures. Non-stressed diazotrophic growth conditions also resulted in enhanced steady-state levels of some *rbp* mRNAs, but inhibited short-term osmotic-stress induction of *rbp* gene expression.

2. Materials and methods

2.1. Organisms, growth conditions and plasmids

Anabaena 7120 was grown photoautotrophically in BG-11 medium [28] either in the presence or absence of a fixed nitrogen source (2.5 mM ammonium chloride). In cold-stress experiments, cells grown at 30°C were adjusted to 20°C by swirling rapidly in an ice-water bath while monitoring with a sterile thermometer to prevent the bulk temperature of the culture from dropping below 20°C. The temperature of the cultures was then maintained at 20°C in the temperature-adjustable incubator. For osmotic-stress experiments, solid sucrose (140 or 250 mM final concentration) or solid NaCl (60 mM final concentration) was added to separate cultures growing under each of the two nitrogen regimes and mixed rapidly. *Escherichia coli* strains DH5·MCR and JM101 (Promega, Madison, WI, USA) were grown under aerobic conditions in the presence of 50 g ml^{-1} ampicillin (Sigma, St. Louis, MO, USA).

2.2. DNA cloning

The *rbp*-containing restriction fragments used in the Northern hybridization experiments along with the genome coordinates are listed in Table 1. The source of the probe fragments for *rbpA* and *rbpB* was plasmid clones described previously [19]. A 3.2-kb *Hin*dIII fragment containing *rbpC* was cloned in pBR322 (Y.Y. Grace Wu and M.E. Mulligan, unpublished). *rbpD* was initially cloned on a 4.4-kb *Hin*dIII fragment [29]; it was subcloned as a ~400-bp *Eco*RI/*Hin*dIII fragment in pGEM7ZF+ (Promega) for transcription of an *rbpD* probe. All DNA manipulations were performed using standard molecular biological techniques [30]. Plasmid DNA was isolated by CsCl density gradient centrifugation.

2.3. Preparation, labeling and detection of gene-specific probes

The *rbpA* and *rbpB* probes were prepared following gel extraction of the respective 1.0- and 1.3-kb *Hin*dIII cloned restriction fragments; for the *rbpC* probe, a 1.2-kb *Eco*RV/*Hin*dIII fragment was prepared from the 3.2-kb cloned *Hin*dIII fragment. All three fragments were digoxigenin labeled using a DIG-High-prime labeling kit (Roche, Indianapolis, IN, USA). These probes were detected using the CDP*TM chemiluminescent substrate (Roche) according to the manufacturer's directions and recorded on an autoradiographic film (BioMax, Kodak,

Rochester, NY, USA). The *rbpD*-specific probe was generated by in vitro transcription of the *rbpD* plasmid (Table 1) using the STRIP-EZ kit (Ambion, Austin, TX, USA). Hybridized molecules were identified using the Brightstar psoralen/biotin non-isotopic DNA labeling kit as outlined in the manufacturer's protocol (Ambion, Austin, TX, USA).

2.4. RNA isolation and Northern hybridizations

Exponential phase cultures of *Anabaena* 7120 were subjected to cold (20°C), sucrose (140 or 250 mM) or salt stress (60 mM NaCl) for 0–24 h. The cells were harvested by centrifugation and the pellets were immediately frozen in liquid nitrogen. Frozen *Anabaena* 7120 pellets were ground in liquid nitrogen in a mortar and pestle and resuspended in 10 ml of RNA lysis buffer (100 mM Tris–HCl, pH 8.0; 100 mM LiCl; 5 mM EDTA pH7.6; 100 mM NaCl; 100 mM sodium acetate, pH 5.2; 1% (w/v) sodium dodecyl sulfate (SDS) and 250 μl β-mercaptoethanol). After several extractions with phenol:chloroform (24:1 v/v), RNA was precipitated in 2 M LiCl. The RNA pellet was washed with 70% ethanol, resuspended in diethylpyrocarbonate-treated water, and quantified using a GeneQuant automated machine (BioRad, Hercules, CA, USA). Total *Anabaena* 7120 RNA (7 g) was electrophoresed on a 1.2% denaturing (formaldehyde) agarose gel, transferred to positively charged nylon membranes, and cross-linked by baking at 120°C for 2 h in a vacuum oven (Fisher, Pittsburgh, PA, USA). Hybridizations were performed either in 50% formamide at 42°C (for *rbpA*, *rbpB* and *rbpC*) or at 68°C (for *rbpD*); all hybridizations used 2 pM of the denatured labeled probes. Following hybridization, blots were washed twice at 65°C in 2× saline sodium citrate (SSC)/0.1% SDS for 15 min per wash followed by two washes in 0.1× SSC/0.1% SDS for 20 min at 65°C. Probes were detected as described above. Data shown in the figures are representative of at least two independent experiments.

2.5. Protein analysis

Frozen *Anabaena* 7120 cell pellets were resuspended in 5 ml of protein lysis buffer (20 mM MOPS, pH 7.4; 20 mM KCl; 2 mM dithiothreitol; 10% glycerol; 1 mM phenylmethylsulfonyl fluoride) and lysed by passage through a French pressure cell (Spectronic, Rochester, NY, USA). Protein concentrations were determined using the BCA protein assay (BioRad). Proteins were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) using the Laemmli protocol [31]. Western blot analysis was performed using standard protocols [32] with PVDF membranes (BioRad) and probed with a 1:500 dilution of *A. variabilis* M3 anti-RbpA1 primary antibody (a generous gift from N. Sato, Saitama University, Japan). Alkaline phosphatase-conjugated rabbit anti-guinea pig IgG (Sigma) was used as the second step antibody and detected with NBT/BCIP (Sigma).

3. Results

The major objective of this study was to assess of the effect of global nitrogen status on *rbp* gene expression, first with respect to cold stress and then in response to osmotic stresses. Four *rbp* genes were selected for study and changes in gene expression were followed by Northern hybridization. The gene designation and genome map coordinates for each hybridization probe used are listed in Table 1. None of these probes cross-hybridizes to other *rbp* genes (data not shown). In preliminary Northern hybridization experiments, the expression of *rbpA*, *rbpB*, *rbpC*, and *rbpD* in ammonium-grown cultures of *Anabaena* 7120 was transiently induced by shifting the growth temperature from 30°C to 20°C. We observed higher levels of expression 2 h after the temperature shift. The signals declined thereafter but were still observable at 24 h post-shift (Fig. 1A). This result was essential to establish that *Anabaena* 7120 *rpb* genes are cold-inducible like those of the non-diazotrophic *A. variabilis* M3 [15] and set the stage for investigation of the interaction between low temperature and diazotrophic growth conditions which is not possible in the M3 strain. In addition, the transcript identified by the *Anabaena* 7120 *rbpC* probe was 820 nucleotides in length, similar to that of the *rbpA1-rpsU* transcript in *A. variabilis* M3. We next examined cold-shock induction of *rbp* gene expression in nitrogen-fixing and ammonium ion-supplemented cultures. As shown in Fig. 1B (lanes 1 and 2), at normal temperature, nitrogen-fixing cultures expressed higher levels of *rbpB*, *rbpC* and *rbpD* than did ammonium-grown cultures. Control lanes from Fig. 1A are reproduced in Fig. 1B to facilitate comparison of signal intensity. All lanes in Fig. 1A,B derive from the same blot. Temperature shift to induce the cold-shock

Table 1
Anabaena 7120 *rbp* probes, genome coordinates, and alternate gene designations

Anabaena 7120 gene	Vector	Cloning site(s)	Kazusa map coordinates	Kazusa designation	*A. variabilis* M3 designation
rbpA	pUC18	*Hin*dIII	2 494 620–2 495 629	*rbpA2*, alr2087	*rbpA2*
rbpB	pUC18	*Hin*dIII	3 564 821–3 566 121	*rbpB*, all2928	*rbpB*
rbpC	pBR322	*Hin*dIII	862 829–863 887	*rbpA1*, alr0741	*rbpA1*
rbpD	pGEMZf+	*Eco*RI/*Hin*dIII	5 583 563–5 583 931	*rbpD*, alr4683	*rbpC*

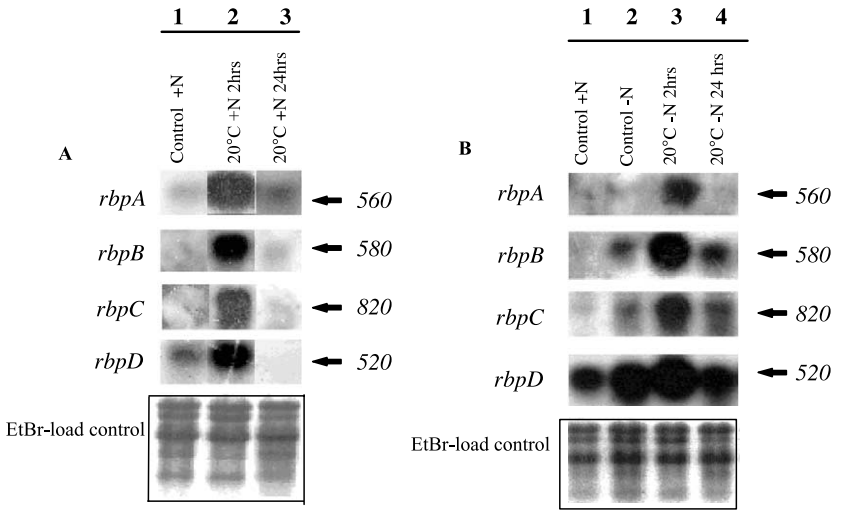

Fig. 1. Expression of *rbp* genes in response to cold shock. Total RNA was isolated from *Anabaena* 7120 cells grown at 30°C (control) or following incubation at 20°C for 2 h or 24 h as indicated. Seven μg of each RNA sample was separated on a denaturing 1.2% agarose gel, transferred to nylon membranes, and hybridized with probes specific for *rbpA*, *rbpB*, *rbpC* or *rbpD* as indicated. A: RNA from cells grown in ammonium-supplemented media. B: RNA from cells grown under nitrogen-fixing conditions (lanes 2–4); a sample of RNA from ammonium-grown cells was included for comparison (lane 1). The estimated size of each transcript is shown on the right. A representative photo of the ethidium bromide-stained RNA gel is shown below each panel to demonstrate that approximately equal amounts of RNA were loaded in each lane.

response also resulted in transiently elevated expression of all four *rbp* genes in nitrogen-fixing cultures (Fig. 1B, lanes 3 and 4). Again, high levels of expression were observed 2 h after the temperature shift, then declined again by 24 h. These results link the regulation of *rbpB*, *rbpC*, and *rbpD* gene expression to the global nitrogen status.

We next examined the effect of osmotic stresses on *rbp* gene expression to test the hypothesis that genes responsive to cold stress will be induced by osmotic stresses as well. RNA was isolated 2 h after treatments to coincide with the time point of maximal *rbp* expression after cold-stress induction. As shown in Fig. 2A, expression of the *rbpB*, *rbpC*, and *rbpD* genes was elevated in response to osmotic and salt stress relative to controls in ammonium-grown cultures (compare lane 1 with lanes 5 and 7). However, the levels of *rbpA*, *rbpB*, and *rbpC* transcripts in-

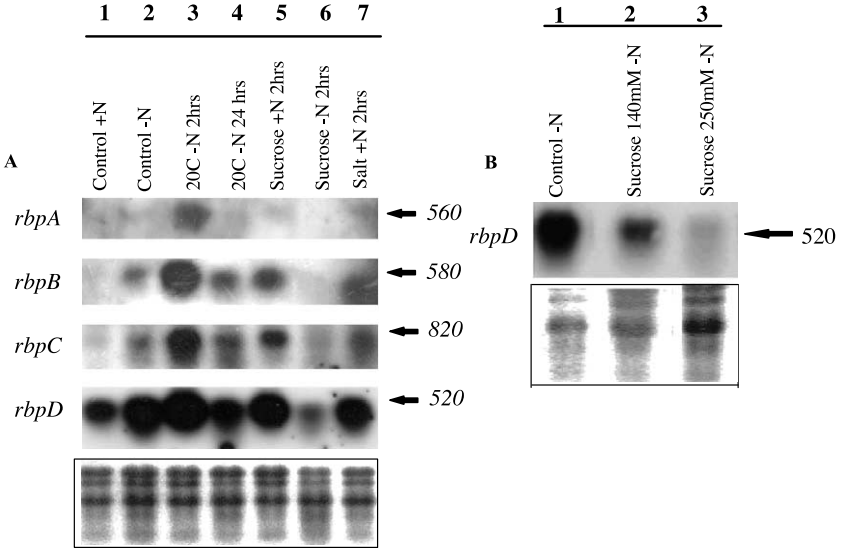

Fig. 2. Stress-induced activation of *rbp* genes in *Anabaena* 7120 is modulated by nitrogen status. A: Total RNA was isolated from *Anabaena* 7120 cells grown to exponential phase at 30°C (control) in ammonium-supplemented media (+N) or under nitrogen-fixing conditions (−N) and then subjected to either cold (lanes 3 and 4), 250 mM sucrose (lanes 5 and 6), or 60 mM NaCl (lane 7) stress as indicated. RNA samples were separated and probed, and estimated transcript sizes are the same as in Fig. 1. B: Total RNA was isolated from *Anabaena* 7120 cells grown under nitrogen-fixing (−N) conditions and subjected to osmotic shock with either 140 mM (lane 2) or 250 mM sucrose (lane 3). In this case, RNA was probed only with the *rbpD* probe. In both panels, a representative photo of the ethidium bromide-stained RNA gel is shown below each panel to demonstrate that approximately equal amounts of RNA were loaded in each lane.

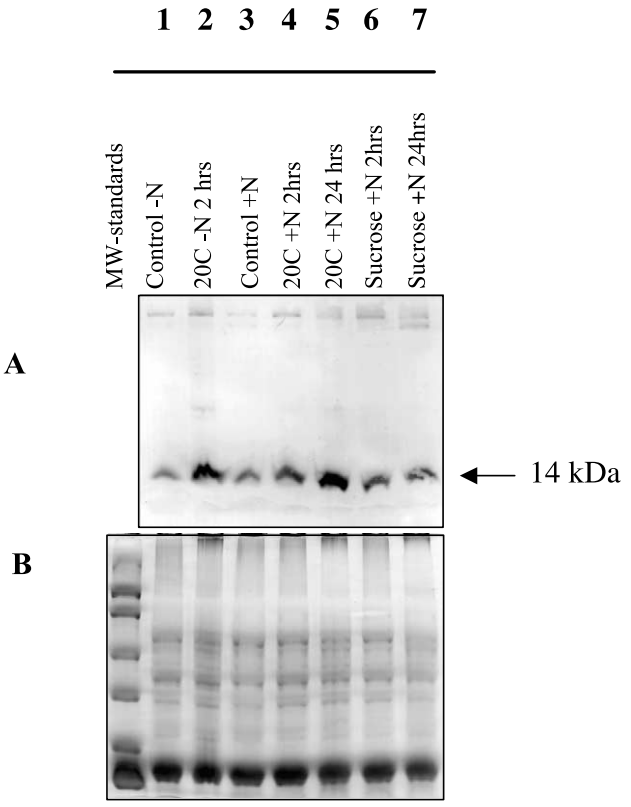

Fig. 3. Rbps accumulate in response to cold and sucrose stress. Total proteins were isolated from *Anabaena* 7120 cells grown to exponential phase at 30°C (control, lanes 1 and 3) in ammonium-supplemented media (+N) or under nitrogen-fixing conditions (−N) and then subjected to either cold (lanes 2, 4 and 5), or 250 mM sucrose (lanes 6 and 7), stress for 2 or 24 h as indicated. A: Proteins (10 μg) were separated on 15% SDS–PAGE gels, electroblotted to PVDF membrane, and probed with the *A. variabilis* M3 anti-RbpA1 antibody. A single band of estimated size 14 kDa representing total Rbps was detected. B: A representative Coomassie Blue-stained SDS–PAGE gel is shown to demonstrate that approximately equal amounts of protein were loaded in each lane. The BioRad broad range protein molecular weight standards (MW) were included in the left-hand lane.

duced after osmotic stress were lower than those induced by cold stress (compare lanes 3, 5 and 7). Expression of *rbpA* was only slightly responsive to osmotic or salt treatments, if at all. In contrast, osmotic- and salt-stress induction of the *rbpD* gene resulted in transcript levels similar to those observed after cold-stress induction (Fig. 2A, lanes 3, 5 and 7). In all of our experiments, *rbpD* was consistently expressed at higher levels than the other three *rbp* genes examined in unstressed ammonium-grown cultures.

A remarkable result was that diazotrophic growth markedly inhibited steady-state levels of *rbp* gene expression in response to osmotic stress (Fig. 2A, lane 6). This effect was observed for all four *rbp* genes, and, in the case of *rbpD* expression, was proportional to the osmolyte concentration as shown in Fig. 2B. These results clearly demonstrate that nitrogen nutrition affects stress-responsive *rbp* gene expression.

3.1. *Rbps accumulate in response to cold and osmotic stress in* Anabaena *7120*

Western blot experiments were performed to monitor the accumulation of *Anabaena* Rbps. An antibody raised against *A. variabilis* M3 RbpA1 (*Anabaena* 7120 RbpC) was used which reacts with all four of the corresponding *A. variabilis* M3 *rbp* gene products under study here [15]. Rbps were identified in control cultures both under nitrogen-fixing and non-fixing conditions at nearly equal levels (Fig. 3, lanes 1 and 3) despite higher *rbpB*. *rbpC* and *rbpD* transcript levels in the nitrogen-fixing cultures. Total Rbp levels after cold stress were higher in both nitrogen-fixing and ammonium-supplemented cultures (compare Fig. 3, lanes 1–4). After cold-stress induction, maximal Rbp levels were observed 24 h following stress (Fig. 3, lanes 3 and 5) unlike *rbp* mRNA levels which peak at 2 h and decline thereafter. Rbps were higher than control values in cultures subjected to osmotic stress but did not reach the same levels observed in cold-stressed cultures (Fig. 3, lanes 3–5). The same trend was seen in comparison of cold-stress and osmotic-stress induction of *rbp* transcript levels shown in Fig. 2. The effect of nitrogen-fixing conditions on post-transcriptional regulation of Rbp levels after osmotic stress was not examined in this study.

4. Discussion

Numerous reports have illustrated the similarity of the biochemical responses to cold and osmotic stresses which decrease the availability of free water. These have been summarized in detail for bacterial systems by Potts [4]. It has also been demonstrated that suboptimal nitrogen nutrition has a negative effect on cold adaptation in single-celled cyanobacteria [9] and further that osmotic/salt-stress adaptation in filamentous strains is reduced under nitrogen-fixing conditions [26]. As a result of these considerations, we set out to determine if *rbp* gene expression, known to be inducible by cold stress, might also be responsive to osmotic stress, and whether the nitrogen nutritional status might also be a key regulator of *rbp* gene expression. To do so required use of a well-characterized diazotrophic strain to examine *rbp* regulation across the full range of nitrogen assimilation pathways. The well-studied diazotrophic *Anabaena* 7120, whose complete genome sequence is known [21], was the logical choice for these studies. The original experiments demonstrating cold-stress induction of Rbps in cyanobacteria were carried out in the filamentous strain *A. variabilis* M3 ([22]. The M3 strain is unable to fix atmospheric nitrogen due to a block in the heterocyst differentiation pathway that has never been characterized.

It is now clear that cold and osmotic stresses have similar effects on *rbp* gene expression in *Anabaena* 7120. First, we observed a very similar pattern of *rbp* gene expression

in *Anabaena* 7120 in response to cold stress as seen previously in *A. variabilis* M3. Second, osmotic and salt stresses were shown to activate *rbp* gene expression in ammonium-grown cultures. Third, Rbps were found to be present in both normal and stressed cultures while RNA profiles provided clear evidence that the composition of the Rbp pool is likely to change with stress and nitrogen source. Fourth, the levels of *rbp* mRNAs and Rbps did not correlate completely over the 24 h of observation. This result is consistent with evidence for post-transcriptional regulation observed for *rbp* genes in *A. variabilis* M3 [15] and for a cold-inducible RNA helicase gene from *Anabaena* 7120 [14]. Finally, cold- and osmotic-stress activation was not identical across the four *rbp* genes tested. For example, cold stress elevated the levels of steady-state *rbpA* transcripts more than osmotic stress, while the *rbpB*, *rbpC* and *rbpD* transcripts were more responsive to osmotic stresses. Thus, we should expect functional similarities and differences between the various Rbps in *Anabaena* 7120. While the biochemical function of the Rbps is currently unknown, the possibility that Rbps may mediate post-transcriptional regulatory effects is worth noting.

The source of nitrogen had profound effects on steady-state and stress-inducible *rbp* gene expression. Diazotrophic growth by itself resulted in higher steady-state levels of *rbpB*, *rbpC* and *rbpD* mRNA levels. However, the level of Rbps detected by the antibody was not significantly different in unstressed nitrogen-fixing versus ammonium-grown cultures. These results also strongly suggest the composition of the Rbp pool is likely to vary as a function of both cold/osmotic-stress status and nitrogen source.

A striking result was observed when *Anabaena* 7120 cells were osmotically stressed in the absence of a combined nitrogen source. The *rbpB*, *rbpC* and *rbpD* transcripts were downregulated below levels seen in the unstressed nitrogen-fixing control cultures. If the Rbps function solely as mediators of stress responses, down regulation under nitrogen-fixing conditions is puzzling. Fernandes et al. [33] showed that nitrogen fixation in *A. torulosa* and *Anabaena* sp. Strain L-31 was very sensitive to salinity stress but relatively insensitive to osmotic stress. Their result argues against existence of an osmotically sensitive step in one or more of the many biochemical pathways supporting nitrogen fixation. An alternative explanation is that one or more of the *Anabaena* Rbps function to suppress heterocyst differentiation in vegetative cells and that, like cold stress, osmotic stress activates pathways that regulate heterocyst spacing. A similar model has been proposed based on evidence that the *rbpA1* gene product plays a role in repression of heterocyst differentiation in vegetative cells [23]. These authors suggest that the formation of a specific RNA secondary structure on target transcript(s) is stabilized by cold temperatures and destabilized by the stress-inducible RbpA1. Our results are consistent with this model and extend it by demonstrating a fundamental alteration of Rbp regulation in diazotrophic cultures. If osmotic stress results in temporary nitrogen limitation as suggested by others [9,34], perhaps nitrogen-fixing cultures are more sensitive to that signal than ammonium-grown cultures and are responding by adjusting heterocyst frequency. If true, one would expect genes like *hetA*, *patA*, *patS*, *patN* and perhaps the *rbp* genes themselves to be likely targets of Rbp-mediated post-transcriptional control [21]. Many examples of Rbp-mediated control of transcription termination in bacteria are now known [35].

If the *Anabaena* 7120 Rbps were to regulate transcription elongation/termination of one or more of the heterocyst regulatory genes, it would provide a mechanism for integration of environmental signaling pathways with developmental control of heterocyst formation. Environmental modulation of the heterocyst developmental pathway is known to occur in plant/cyanobacterial symbiosis [36]. An osmotic signaling pathway is also known in *Anabaena* 7120, the *orrA* response regulator system [37]. Thus, a detailed study of Rbp targets and function is likely to shed light on regulatory circuits unique to the heterocystous cyanobacteria.

One further point worth noting is that the solubility of molecular oxygen in water increases with decreasing temperature. As temperature drops from 30 to 20°C the solubility of O_2 in water increases approximately 15–20% depending on solute concentrations. Thus, it could be argued the temperature-dependent increases in *rbp* expression observed also correlate with increased O_2. Addition of solutes counteracts the effects of lower temperatures on O_2 solubility, although the quantitative effects are not as large as the temperature effects, on the order of a few percent [38]. Since the system under study is not a solution in contact with the atmosphere but a growing culture of oxygen-evolving cyanobacteria, changes in the rate of photosynthetic oxygen evolution are likely to be far more quantitatively important than solubility effects. Further, the nitrogen-fixing cultures were grown in continuous light, fully inducing heterocyst formation and biochemical mechanisms for protecting nitrogenase from molecular oxygen [39]. It is also well known that *Anabaena* 7120 is unable to grow photo-heterotrophically. So while sucrose was used as an osmoticum, stimulated respiration as a means of lowering oxygen levels is not a tenable hypothesis. Finally, if the *rbp* genes were responding primarily to increased molecular oxygen tensions, one would not have expected to see the decrease in all four *rbp* mRNA levels in diazotrophic cultures after sucrose addition, relative to ammonium-grown cultures (Fig. 2A, lane 6). Identification of the molecular targets of each Rbp will be necessary in order to sort out the multiple possibilities outlined here.

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