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Differential Expression of Photosynthesis and Nitrogen Fixation Genes in the Cyanobacterium *Plectonema boryanum*

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The filamentous non-heterocystous cyanobacterium *Plectonema boryanum* fixes dinitrogen at a high rate during microaerobic growth in continuous illumination by temporal separation of oxygen-evolving photosynthesis and oxygen-sensitive dinitrogen fixation. The onset of nitrogen fixation is preceded by a depression in photosynthesis that establishes a sufficiently low level of dissolved oxygen in the growth medium. A several-fold reduction in the level of transcripts coding for phycocyanin (*cpc*BA) and the chlorophyll *a* binding protein of photosystem II (*psb*C) and *psb*A accompanied the depression in photosynthetic oxygen evolution. Unlike most of the other organisms examined to date, in *P. boryanum*, *psbC* and *psb*D do not appear to be co-transcribed. The *psb*C transcripts were down-regulated several fold, while the *psb*D transcript declined marginally during the nitrogen fixation phase. A decrease in dissolved oxygen and a dramatic increase in the level of *nif*H transcripts and the enzyme activity of nitrogenase were characteristic of the nitrogen fixation phase. The level of transcript for *gln*A, which encodes glutamine synthetase, was not altered. Reciprocal regulation of gene expression was well orchestrated with the alternating cycles of photosynthesis and nitrogen fixation in *P. boryanum*.

Several cyanobacteria have the unique ability to conduct both oxygenic photosynthesis and oxygen-sensitive dinitrogen fixation. Heterocystous cyanobacteria protect nitrogenase and the related redox machinery from damage by intracellular oxygen by carrying out nitrogen fixation in morphologically and physiologically differentiated cells called heterocysts (Haselkorn, 1978; Wolk et al., 1994; Böhme, 1998). Photosynthesis takes place simultaneously in spatially separated vegetative cells. Unicellular and filamentous, non-heterocystous diazotrophic cyanobacteria do not differentiate morphologically distinguishable cells. They overcome the problem of oxygen by fixing dinitrogen either during the dark phase of growth or in light at a time when photosynthesis is inhibited (Mitsui et al., 1987; Bergman et al., 1997).

The regulation of gene expression during temporal separation of photosynthesis and nitrogen fixation under diazotrophic growth of non-heterocystous cyanobacteria has not been studied in detail. The reciprocal transcription of *pet*F and *fdx*H genes in response to nitrogen status under non-diazotrophic conditions has been reported in *P. boryanum*. (Schrautemeier et al., 1994). The rhythmic expression of *nif*H and other *nif* genes during diazotrophic growth has been reported in a unicellular N_2-fixing cyanobacterium, *Synechococcus* sp. RF-1, and was shown to be regulated by the circadian clock (Huang and Chow, 1990; Huang et al., 1999).

The filamentous non-heterocystous cyanobacterium *P. boryanum* can be grown with doubling time of around 60 h in nitrogen starvation conditions by inducing diazotrophy by bubbling an anaerobic gas mixture in the presence of continuous light (Rai et al., 1992; Misra and Tuli, 1993). Under such growth conditions, the cyanobacterium shows reciprocal, alternating cycles of nitrogen fixation (N-phase) and photosynthesis (P-phase) (Stewart and Lex, 1970; Misra and Tuli, 1993). In this report, we examine the organization and expression of some of the important genes involved in nitrogen fixation and photosynthesis during the alternating cycles of carbon dioxide fixation and dinitrogen fixation in *P. boryanum*.

MATERIALS AND METHODS

Analytical and Molecular Biology Reagents

The analytical reagents used in this study were obtained from Merck (Mumbai, India) and Sigma-Aldrich (St. Louis). Molecular biology reagents were obtained from Sigma-Aldrich, Life Technologies/Gibco-BRL (Cleveland), and Sisco Research Laboratory (Mumbai, India). All enzymes and kits were obtained from Amersham-Pharmacia Biotech (Uppsala), Boehringer Mannheim (Mannheim, Germany), New England Biolabs (Beverly, MA), and Bangalore Genei Pvt. Ltd. (Bangalore, India). Radionucleotides were obtained from the Board of Radiation and Isotope Technology (Mumbai, India). Positively charged nylon membranes from Amersham-Pharmacia Biotech was used in capillary transfer of DNA and RNA.

Organism and Growth Conditions

Plectonema boryanum strain UTEX 594 was obtained from the University of Texas collection. A dinitrogen-fixing, photoautotrophic culture was established by continuous bubbling of a N_2 and CO_2 mixture (19:1) in nitrogen-deficient $BG11_0$ (Rippka et al., 1979) medium. The inoculum was grown under aerobic conditions in BG11 medium and used to induce diazotrophic growth in a 15-L fermenter vessel under continuous illumination (light intensity 8,000 lux), as previously described (Misra and Tuli,

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1994). On-line dissolved oxygen was monitored with a Clark-type oxygen electrode installed in the fermenter vessel as previously described (Misra and Tuli, 1993; Misra, 1999). Nitrogenase was estimated by the acetylene reduction assay in the anaerobic gas phase as previously described (Misra and Tuli, 1993). Chlorophyll was extracted in methanol and estimated (Mackiney, 1941).

Isolation of DNA and Southern Hybridization

Total DNA from *P. boryanum* was prepared according to a modified protocol of Felkner and Barnum (1988). The cyanobacterial culture grown to mid-exponential phase in BG11 medium was harvested and washed in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. One gram of cell pellet was suspended in 4 mL of Suc buffer (50 mM Tris-HCl, pH 8.0, 100 mM EDTA, and 25% [w/v] Suc). Lysozyme was added to a final concentration of 5 mg/mL and incubated for 1 h at 37°C .The suspension was treated with proteinase K (100 μg/mL) and incubated at 37°C in the presence of 1.0% (w/v) SDS until it become clear. The cleared lysate was extracted with phenol and chloroform. The subsequent steps were as described previously (Vachhani et al., 1993). Total DNA (5.0 μg) was digested with restriction enzymes, treated for 30 min at 37°C with DNase-free RNase (50 μg/mL), and resolved on 1.0% (w/v) agarose. DNA fragments were transferred to positively charged nylon membranes. The DNA probes used in this study are given in Table I. The appropriate restriction fragments were cut from respective plasmids and eluted from low-melting-point agarose. Probes were labeled using a kit from Boehringer Mannheim/Roche and following the manufacturer's protocol. Prehybridization and hybridization were carried out as described by Sambrook et al. (1989).

Isolation of RNA and Hybridization

Total RNA was isolated from aliquots of the cyanobacterial culture, as previously described (Chomczynski and Sacchi, 1987), with some modifications. The cells were harvested at 4°C, and washed pellets were immediately frozen at −70°C and then thawed. A high concentration of guanidinium thiocyanate (5 M) was used and cells were broken by vortexing with acid-washed sterile glass beads (0.45–0.60 mm diameter) as described previously (Mann et al., 1991). The RNA pellet was washed with 3 M sodium acetate (pH 5.6–6.3) to reduce DNA contamination in the preparation. The RNA concentration was determined spectrophotometrically and used in northern- and dot-blot hybridization (Sambrook et al., 1989). Detection of signal and quantitation of probes hybridized to specific RNA on dot-blot filters were done using a phosphor imager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Temporal Separation of Photosynthesis and Dinitrogen Fixation

As described in our earlier studies (Misra and Tuli, 1993, 1994), a rapid photoautotrophic growth of *P. boryanum* under nitrogen starvation conditions could be achieved by bubbling the culture with an anaerobic gas mixture in continuous light. A fall in photosynthesis (monitored as light-dependent oxygen evolution) and a concomitant rise in dinitrogen fixation (monitored by acetylene reduction activity) commenced about 15 h after the onset of anaerobic bubbling (Fig. 1). At the onset of nitrogenase activity, the steady-state dissolved oxygen in the medium fell below 15 μM due to a decline in photosynthetic oxygen evolution. After the peak of the N-phase, photosynthetic oxygen evolution (P-phase) was switched on and the steady-state dissolved oxygen in the medium increased rapidly to 35 to 70 μM (Misra and Tuli, 1993). The cycle of the two reciprocal phases was repeated throughout diazotrophic growth of the organism. The aliquots were withdrawn anaerobically at the peak of the N-phase and the P-phase to prepare RNA for northern- and dot-blot hybridization experiments.

Specificity of Gene Probes and Copy Number

The DNA probes (Table I) used in this study were from heterologous cyanobacterial species and showed fairly high specificity of hybridization with the DNA from *P. boryanum*. As shown in Figure 2, all bands were clear and distinct when hybridization was carried out at 65°C, except *cpc*, which was hybridized at 55°C.

Among the genes encoding some of the major proteins of photosystem II (PSII), the results presented in Figure 2 suggest the presence of at least two copies of *psb*A and *psb*D each in *P. boryanum* and a single copy of *psb*C. The probe for *cpc*BA hybridized strongly to a (3.2-kb) *Hind*III and an (2.4-kb) *Eco*RI fragment and weakly to a few additional fragments. The multiple bands obtained with the *cpc* gene probe could be due to partial homology among phycobiliprotein genes and/or multiple copies of the phycocyanin

Table I. *DNA probes used in this study*

Source Organism	Plasmid	Gene	Gene Product	Fragment Used as Probe	Reference
Synechocystis 6803	pKW1266	*psb*A	D1	0.5-kb *Kpn*I-*Hind*III	Jansson et al. (1987)
Synechococcus 7942	pAM011	*psb*D	D2	1.3-kb *Bam*HI	Golden and Stearn (1988)
	pGS102	*psb*C	CP43	0.4 kb *Pst*I-*Hind*III	Golden and Stearn (1988)
F. diplosiphon	pFD126	*cpc*BA	Phycocyanin	0.9-kb *Dde*I	Conley et al. (1985)
Anabaena 7120	pAN154.3	*nif*H	Nitrogenase reductase	1.8-kb *Hind*III	Mavarech et al. (1980)
	pAN503	*gln*A	Glutamine synthetase	1.4-kb *Eco*RI	Tumer et al. (1983)
	pAN621	*rm*	23SrRNA	4.0-kb *Eco*RI	Nierzwicki-Bauer and Haselkorn (1986)

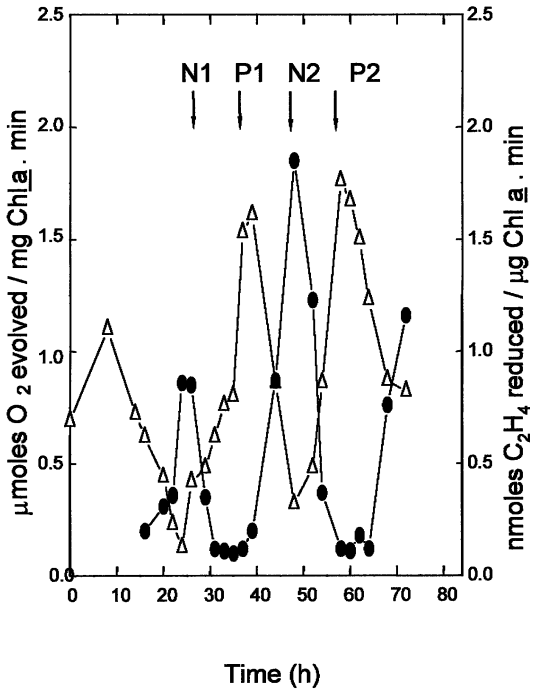

Figure 1. Alternating cycles of nitrogen fixation (N1 and N2) and photosynthesis (P1 and P2) in *P. boryanum* strain UTEX 594 growing in continuous light under nitrogen-fixing conditions. Acetylene reduction activity (●) and light-dependent oxygen evolution (△) were monitored in the medium during the first two cycles.

genes, as reported in other cyanobacteria (Grossman et al., 1993). Southern-blot analysis with *nif*H (Fig. 2), *nif*D, and *nif*K (data not included) gene probes suggested the presence of these genes in single copy in *P. boryanum*, which agrees with earlier results for this organism (Apte and Thomas, 1987). That study showed that, unlike heterocystous cyanobacteria, the *nif*HDK operon is contiguous and

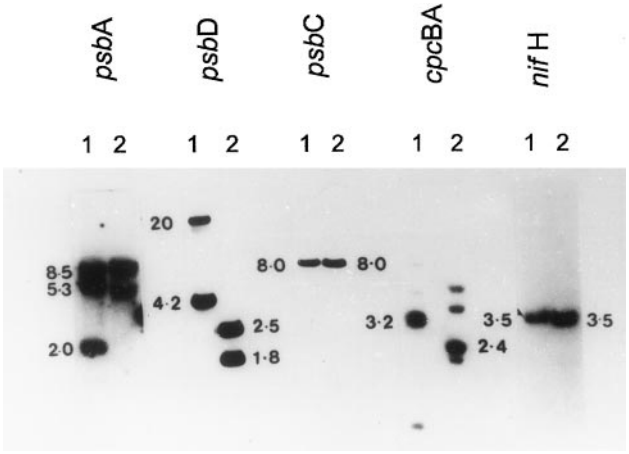

Figure 2. Southern-blot analysis of *P. boryanum* strain UTEX 594 DNA. Total DNA was digested with *Hind*III (lanes 1) and *Eco*RI (lanes 2) and hybridized with genes probe for *psb*A, *psb*D, *psb*C, *cpc*BA, and *nif*H. Sizes of the major hybridizing fragments are given on the sides of the lane (in kb). All hybridizations were carried out at 65°C, except for *cpc* at 55°C.

present as a 3.6-kb *Eco*RI fragment on the chromosome of *P. boryanum* strain 594. Nucleotide sequence and differential expression of *nif*H have also been shown in a different strain of *P. boryanum* strain M101 (Fujita et al., 1991). A gene representative of nitrogen metabolism, *gln*A, was also present in single copy in *P. boryanum* (data not included).

Level of Transcripts during Temporal Separation of Photosynthesis and Nitrogen Fixation

nif Genes

The abundance of transcripts for the above genes was determined by dot-blot hybridization of total RNA prepared from the aliquots drawn during two successive N- and P-phases (i.e. N1, P1, N2, and P2) as shown in Figure 1. The blots were hybridized to a 23S r DNA (*rrn*) gene probe to ascertain that equal amounts of total RNA were blotted uniformly (Fig. 3). Hybridization of the dot blots with the *nif*H gene probe showed that the *nif* transcripts were present at a high level during the N-phase and nearly disappeared during the P-phase (Fig. 3). On northern blots (Fig. 4), the predominant *nif*H transcript was the 1.4-kb form, while less-intense bands, apparently representing the multicistronic transcripts *nif*HD and *nif*HDK, were seen at approximately 2.6 and 4 kb, respectively. Multiple *nif* transcripts produced by termination events at the ends of the *nif*H and *nif*D genes have been previously reported in *P. boryanum* (Fujita et al., 1991) and heterocystous cyanobacteria (Haselkorn et al., 1986). All three transcripts in *P. boryanum* disappeared nearly completely during the

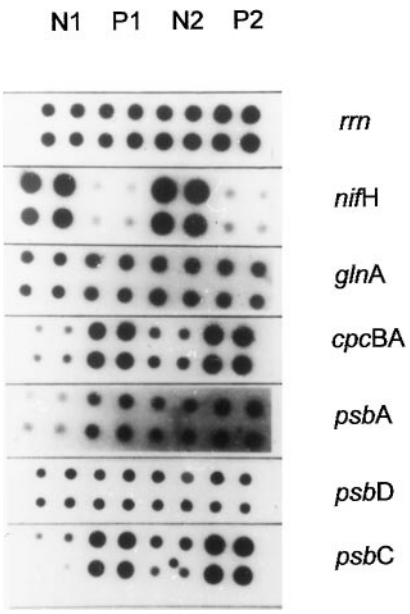

Figure 3. Dot-blot analysis of the total RNA isolated from a *P. boryanum* culture. Aliquots were drawn during the first two nitrogen fixation (N1 and N2) and photosynthesis (P1 and P2) phases, as shown in Figure 1. The RNA samples blotted in quadruplicate from each phase were hybridized with *rrn*, *nif*H, *gln*A, *cpc*BA, *psb*A, *psb*D, and *psb*C gene probes. All hybridizations were carried out at 65°C except that for *cpc*, which was at 60°C.

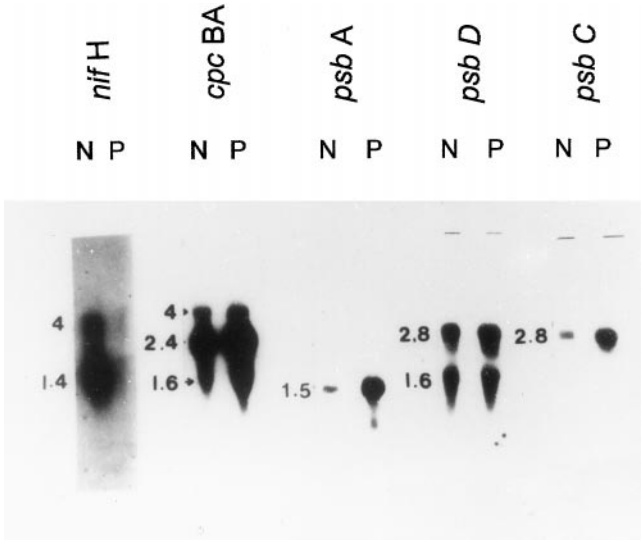

Figure 4. Northern-blot analysis of total RNA isolated from diazotrophic cultures of *P. boryanum* during the first nitrogen fixation (N) and photosynthesis (P) phases. The probes were *nif*H, *cpc*BA, *psb*A, *psb*D, and *psb*C. Sizes of the hybridizing transcripts are given on the lanes in kb. All hybridizations were carried out at 65°C, except that for *cpc*, which was at 60°C.

P-phase. On dot-blots, the level of *nif*H transcripts showed rhythmic changes that were completely in agreement with the appearance and disappearance of the acetylene reduction activity of nitrogenase (Fig. 1). Such a pattern was not followed by the *gln*A transcripts encoding Gln synthetase, the key enzyme involved in the assimilation of newly fixed nitrogen (Fig. 3). In agreement with the unchanged level of the transcript, the level of Gln synthetase enzyme activity in crude extracts remained unaltered during the N- and P-phases (data not shown).

cpcBA Genes

Among the genes related to photosynthesis, the transcripts for α- and β-subunits of phycocyanins showed a remarkable decline in the N-phase and a rapid 5- to 8-fold increase in the subsequent P-phase in dot-blot hybridization (Fig. 3). The results of northern hybridization (Fig. 4) agreed with those of dot-blots in showing stronger signals of hybridization with RNA prepared from the culture in the P-phase. Transcripts of all three sizes, i.e. 4, 2.4, and 1.6 kb, proportionately declined during the N1-phase. The 2.4-kb transcript was most prominent during both the growth phases. The relative depression in the level of all three transcripts was higher during the N-phase, i.e. immediately after transfer of the culture to the nutrient-deficient medium, compared with that during the subsequent N-phases.

Photosystem II Reaction Center Component Genes

The level of *psb*A transcript in the P-phase was distinctly higher than that in the preceding N-phase (Fig. 3). Since there are multiple genes for *psb*A (Fig. 2) in *P. boryanum*, northern hybridization was conducted to examine the sizes of the transcripts. An RNA band of 1.5 kb in the N-phase and two RNA bands of 1.5 and 0.6 kb in the P-phase hybridized with the *psb*A gene probe (Fig. 4). The intensity of the 1.5-kb band was higher in the P-phase than in the N-phase and agrees with the results of dot-blot hybridization. The *psb*A transcript declined to a very low level during the N1-phase, i.e. immediately after the culture was inoculated into nitrogen-deficient medium. During the next N-phase, i.e. N2, the decrease in the level of *psb*A was not as much as that in N1, and was insignificant compared with that in the P2-phase (Fig. 3).

Unlike *psb*A, the steady-state level of transcripts for *psb*D declined marginally (10%–20%) as the culture shifted from the P-phase to the N-phase (Fig. 3). Northern hybridization with the *psb*D gene probe showed the presence of two transcripts of 2.8 and 1.6 kb (Fig. 4) during both the P-phase and the N-phase. The level of both transcripts in the N1 phase was about 80% of that in the P1 phase. Unlike *cpc*BA and *psb*A, the decline in *psb*D transcript in N1 was not significantly more than that in the N2 phase. Northern hybridization with an internal *psb*C gene probe showed its hybridization with the 2.8-kb RNA, as did *psb*D (Fig. 4). However, results from dot-blot hybridization (Fig. 3) and northern hybridization (Fig. 4) demonstrated a cyclic 70% to 90% reduction in the level of 2.8-kb transcript hybridized with *psb*C probe in the N-phase compared with the P-phase. The smaller 1.6-kb transcript homologous to *psb*D did not hybridize with *psb*C. A several-fold decline in hybridization of the 2.8-kb transcript to the *psb*C gene probe and only a marginal decline in its hybridization to the *psb*D gene probe in the N-phase was observed even when the same blot was hybridized after stripping the previous probe. As in the case of *cpc*BA and *psb*A, the transcript of *psb*C declined to a much lower level during the N-phase immediately after the culture was transferred to conditions that permitted nitrogen fixation compared with the subsequent N-phases.

DISCUSSION

Temporal separation of photosynthesis and dinitrogen fixation during growth in continuous light has been reported in *P. boryanum* (Rai et al., 1992; Misra and Tuli, 1994) and in a few other filamentous and unicellular non-heterocystous cyanobacteria (Bergman et al., 1997). Most of the non-heterocystous cyanobacteria accumulate a high level of carbon reserves such as glycogen (Mullineaux et al., 1980; Schneegurt et al., 1994) during the light phase, which are mobilized to provide reductant and ATP to fix dinitrogen during the dark phase when grown in a diurnal light-dark regime. A depression in photosynthesis has been attributed to the absence of light (as in the dark phase), low light intensity (as at dawn and dusk), and circadian rhythm (Kondo et al., 1993). The molecular basis of temporal separation is not known in any of these cyanobacteria.

The developmentally simple, non-heterocystous cyanobacterium *P. boryanum* has a versatile physiology that allows it to reversibly modulate uncoupling of the activity of the two photosystems in response to intracellular nitro-

gen status (Misra and Tuli, 1993). Under the experimental conditions of anaerobic bubbling, nitrogenase activity appeared in a culture of *P. boryanum* when dissolved oxygen decreased to about 15 μM following the depression in photosynthetic oxygen evolution (Fig. 1). We earlier reported that metabolic changes such as changes in the C-N ratio, the appearance of acetylene reduction activity, and PSII-independent CO_2 fixation (Misra and Tuli, 1993) are associated with photoautotrophic growth under diazotrophic conditions. Under such growth conditions, the uncoupling of photosystem activity (Misra and Tuli, 1994) and impairment of electron transport between Q_A and Q_B (Misra and Desai, 1993) were also linked with the altered excitation energy transfer from phycobilisome to the photosystems (H.S. Misra and S.K. Mahajan, unpublished data).

Cyclic changes in PSII components and nitrogenase activity during nitrogen fixation growth of *P. boryanum* were accompanied by changes in the abundance of the transcripts for several of the relevant genes. During the N-phase, along with the increase in *nif*H transcripts, the multiple-size transcripts that hybridized with *cpc*BA, phycocyanin genes, decreased substantially (Fig. 4). This can result in the disappearance of phycobiliproteins in the N-phase (Misra and Tuli, 1993) and contribute substantially to the N-phase-associated impairment of photosynthesis due to inefficient and altered excitation energy transfer from phycobilisome to the photosystems (results not shown). The down-regulation of *cpc*BA in the N-phase agrees with the well-established disappearance of phycobiliproteins as the first symptom of nitrogen starvation in both heterocystous and non-heterocystous cyanobacteria (Stewart, 1980). In *Synechocystis* sp. strain PCC 6308, the composition of phycobilisome and its association with soluble and membrane polypeptides are regulated by the nitrogen status in the cells (Duke et al., 1989).

Our results suggest that there are at least two copies of *psb*A (D1) in *P. boryanum*, although, as in other cyanobacteria, their transcripts are not distinguishable by size on northern blots (Figs. 2 and 4). A low level of expression from the *psb*A gene(s) could be due to poor transcription and to rapid transcript turnover from one or both copies during the N-phase. Such aspects remained to be examined. However, no specific degradation product of the *psb*A transcript was visible during the N-phase in *P. boryanum* (Fig. 4), although photosynthetic electron transport is impaired (Misra and Desai, 1993), leading to uncoupling of the activity of the two photosystems (Misra and Tuli, 1994). The appearance of a highly stable degradation product of psbA-2 and psbA-3 has been reported in *Synechocystis* PCC6803 when photosynthetic electron transport was inhibited (Mohamed et al., 1993).

A differential expression level of *psb*D (D2) and *psb*C (CP43) during temporal separation of photosynthesis in *P. boryanum* is particularly interesting. *P. boryanum* makes two *psb*D transcripts of 2.8 and 1.6 kb, which are synthesized constitutively during both the N- and the P-phases (Fig. 4), while *psb*C transcripts present in 2.8-kb mRNA are regulated tightly under diazotrophic conditions. Thus, *P. boryanum* may be another exception in which *psb*C may not be co-transcribed with *psb*D or may be regulated differentially. In *Anabaena* sp. PCC 7120, though *psb*C is in operon *psb*D1-C, its transcript is formed and regulated independently of *psb*D from an internal promoter under iron stress (Leonhardt and Straus, 1994). The constitutive presence of the two *psb*D transcripts, lack of hybridization of any of these transcripts with the *psb*C probe in the N-phase, and the results of Southern hybridization suggest that *P. boryanum* may have two copies of *psb*D, neither of which is regulated tightly during diazotrophic growth. A severe decline in the level of transcript for *cpc*BA, *psb*A, and *psb*C immediately after transfer of the culture into nitrogen-deficient medium (i.e. N1 in Fig. 3) suggests a role for these genes in regulating the function of the oxygen-evolving complex of PSII. A relatively higher level of these transcripts during the subsequent N-phase (i.e. N2) correlates well with the relatively higher level of light-dependent oxygen evolution in the N2 compared with the N1 phase (Fig. 1).

Our study establishes that the accomplishment of alternating cycles of photosynthesis and dinitrogen fixation in the non-heterocystous cyanobacterium *P. boryanum* is regulated at the metabolic level (Rai et al., 1992; Misra and Tuli, 1994) as well as at the genetic level. The level of transcripts for several genes critical to the function of oxygenic photosynthesis is brought down during microaerobic growth in nitrogen-deficient medium to cause cyclic depression in oxygen evolution even during growth under continuous illumination. Concomitant with the decrease in dissolved oxygen, the level of the *nif*H transcripts required for nitrogen fixation rises to allow the fixation of dinitrogen at the expense of carbon reserves built during the preceding photosynthetic phase. The present study did not attempt to resolve if the cyclic changes in the level of transcripts were due to regulation of gene expression per se or to differential stability of a given transcript during the N- and P-phases. Several interesting questions related to the conversion of the metabolic signal of N-starvation into a genetic signal that regulates the level of transcripts central to CO_2 and N_2 fixation remains to be addressed. *P. boryanum* provides a promising system with which to study these aspects, since it is a developmentally simple organism, modulates its physiology in a highly malleable manner, and can be subjected to genetic manipulation (Vachhani et al., 1993).

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