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Abstract:	In almost all cyanobacteria, a small gene family encodes the photosystem II reaction center D1 protein. This group of identical proteins play vital role in adaptation towards various environmental conditions, which seems to be critical in expanding into new habitats. Over the years several types of these protein forms were functionally characterised and the respective conditions for their induction or repression were identified. Several cyanobacterial D1 forms were considered being silent for a long time. With the advent of sensitive investigation methods these genes were identified to be induced under anaerobic or microaerobic conditions, however its functional role is still poorly understood. This gene was first described in Synechocystis sp. PCC 6803 as psbA1 (D1'). Our data shows induction of this gene when Synechocystis cells are shifted from high to ambient level CO2, similar to microaerobic conditions as previously shown. It is for the first time that psbA gene has been shown to be regulated by the inorganic carbon status of the cell. Hence, it is crucial to understand the mechanisms of regulation and the functional characteristics of D1 proteins as it could be used for future bio-technological applications as a virtual tool-box for modulating the function of PSII.
Suggested Reviewers:	Paula Mulo, PhD, Docent Academy Research Fellow, University of Turku pmulo@utu.fi Dr. Mulo has extensive experience in characterizing the expression of psbA gene family in cyanobacteria.
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	Yvonne Zilliges, PhD Senior Researcher, Freie Universitat Berlin yvonne.zilliges@fu-berlin.de Dr. Zilliges is one of the leading scientists in the field of cyanobacterial photosyntesis with experience in the regulation of expression of photosynthetic genes.
Opposed Reviewers:	

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Expression of *psbA1* gene in *Synechocystis* sp. PCC 6803 is influenced by CO_2

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ABSTRACT

In almost all cyanobacteria, a small gene family encodes the photosystem II reaction center D1 protein. This group of identical proteins play vital role in adaptation towards various environmental conditions, which seems to be critical in expanding into new habitats. Over the years several types of these protein forms were functionally characterised and the respective conditions for their induction or repression were identified. Several cyanobacterial D1 forms were considered being silent for a long time. With the advent of sensitive investigation methods these genes were identified to be induced under anaerobic or microaerobic conditions, however its functional role is still poorly understood. This gene was first described in *Synechocystis* sp. PCC 6803 as *psbA1* (D1'). Our data shows induction of this gene when *Synechocystis* cells are shifted from high to ambient level CO_2, similar to microaerobic conditions as previously shown. It is for the first time that *psbA* gene has been shown to be regulated by the inorganic carbon status of the cell. Hence, it is crucial to understand the mechanisms of regulation and the functional characteristics of D1 proteins as it could be used for future bio-technological applications as a virtual tool-box for modulating the function of PSII.

KEYWORDS

Synechocystis, photosystem II, *psbA1*, D1, photosynthesis, carbon dioxide, microaerobic, qRT-RT-PCR, chlorophyll, fluorescence

INTRODUCTION

The "Great Oxidation Event" arguably due to the oxygenic photosynthesis by cyanobacteria not only sculptured the early biosphere but also allowed the evolution of higher and complex life forms [1]. During this evolutionary process cyanobacteria have learned many artifices to evade all environmental conditions, which threatened their survival [2]. Deployment of different isoforms is one such adaptive strategy that supported its endurance to numerous circumstances. The *psbA* gene encoding for D1 proteins of photosystem II complex in cyanobacteria remains as a perceptible evidence [3].

Cyanobacteria genome contains multiple copies of genes encoding D1 proteins. In *Synechocystis sp.* PCC 6803, *psbA* gene family includes three genes, *psbA1* (*slr1181*), *psbA2* (*slr1311*) and *psbA3* (*sll1867*) (http://www.kazusa.or.jp/cyano/, http://genome.jgi-psf.org/mic_home.html). The *psbA2* and *psbA3* coding region exhibits 99% identity in their nucleotide sequence and encode identical D1 proteins. However, the PsbA2 is the predominant protein accounting for 90% of the total PsbA protein produced [4]. Hardly ever was the expression of *psbA1* noticed and the condition, which caused the induction of the gene, remained enigmatic until [5] distinguished the expressions *psbA1* from other isoforms of *psbA* gene under cells grown under low O_2 environment. Concurrently, [6] showed the dramatic expression of *psbA1* gene in *Synechocystis* under micro-aerobic condition. Presumably, different family of *psbA* genes and their multiplicity appears to be not arbitrary, but merely based on environmental factors [7]. Furthermore this widens the possibility of additional environmentally related parameters to be involved in operating the regulation of these families of genes.

Cyanobacteria intake inorganic carbon in the form of carbon dioxide and bicarbonate and the unavailability of these inorganic carbon limits the photosynthetic rate in cyanobacteria [8].

In this study, the differential expression of the *psbA1* gene under various levels of CO_2 were analysed which could possibly correlate the involvement of various proteins participating in cellular adaptation.

MATERIALS AND METHODS

Culture and growth conditions

Synechocystis sp. PCC 6803 was obtained from Pasteur Culture Collections, France. Cells were grown in cylindrical glass tube containing BG-11 medium. The cultures were continuously illuminated at 50 μmol photons $m^{-1}s^{-1}$ and the temperature was maintained at $30^{o}C$ [9]. To ensure thorough nutrient availability, the cultures were bubbled with air unless stated otherwise. Exceptionally, when cells were grown under low CO_2 condition, Na_2CO_3 was omitted from BG-11 medium.

High CO_2 to low CO_2 shift assay was carried out by bubbling the culture initially with 5% CO_2 (High CO_2 condition) until the optical density at 730 nm corresponds to 0.8 and the bubbling gas was changed to ambient level CO_2 (low CO_2 conditions) until 120 h. Samples were collected at 0 h, 30 min, 1 h and 2 h. In another experiment the cultures were bubbled with synthetic air, which have similar gas composition like atmospheric air, but completely lacks CO_2. Sampling was done at 0 h, 30 min, 1 h and 2 h, 24 h and after 48 h. The microaerobic conditions were achieved in the cultures by bubbling with pure N_2 gas. Cells were harvested at 0 h, 30 min, 1 h and 2 h.

RNA isolation and cDNA synthesis

The cells were harvested from 10 mL culture (OD_{730} = 0.8) by centrifugation at 8000 rpm, 4^{o}C, 5 min at previously mentioned sampling point. The cell pellets were immediately suspended in Trizol reagent (Ambion, Life Technologies, California) to prepare total RNA. The concentration of total RNA was measured with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA). RNA samples were treated with 1 U DNase (Turbo DNA free kit, Ambion, Austin, TX) to remove any trace of genomic DNA. Synthesis of cDNA was performed using iScript^{TM} cDNA Synthesis Kit (BioRad, California) using 1.0 µg of purified RNA as template and random hexamer primers.

Real Time Quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-qRT-PCR)

RT-qRT-PCR was performed in triplicate with a BioRad iQ5 System (BioRad, Berkeley, CA) using Sso Advanced^{TM} SYBR Green Supermix (Bio-Rad, Berkeley, California) in domed cap PCR tubes. Both forward and reverse primers were added at a concentration of 250 nM each in the reaction mix. The PCR protocol is as follows: one cycle of 95°C for 3 min, followed by 45 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. Negative controls (reactions without cDNA template and minus RT) were included to detect the presence of any DNA contamination. All the reactions were performed in triplicate for each cDNA sample.

Flash induced chlorophyll fluorescence

Flash-induced chlorophyll fluorescence and subsequent decay was measured using fluorometer (FL-3500, Photon System Instruments, Czech Republic) [10]. Saturating actinic flashes (20 ms) and measuring flashes (8 ms) was provided by instrument's LED system. The cyanobacterial cells were incubated in dark for 5 min prior to the measurement and then the fluorescence decay was recorded over time range on logarithmic scale both in the presence and in the absence of DCMU ((3-(3,4-dichlorophenyl)-1,1-dimethylurea)) [11].

RESULTS

123 Cyanobacteria exploit light energy to fuel the accumulation of inorganic carbon (C_i)
124 within the cells. They have developed various adaptive methods to acclimate a wide range of
125 atmospheric CO_2 levels. This intricate process of acclimation follows a syndrome of changes
126 at various cellular levels, including articulation of gene expressions involved in the
127 carbon concentrating mechanism (CCM) [12].

128 In our experiments, the culture exposed to microaerobic conditions, displays a strong
129 induction of the *psbA1*gene, in direct agreement with previous studies (Fig. 1a) [5,6]. When
130 the *Synechocystis* cells were shifted from high CO_2 to ambient CO_2 conditions, the *psbA1*
131 gene showed an induction in the expression up to 13-fold within 15 min and remained up-
132 regulated until 120 h under atmospheric CO_2 level (Fig. 1b). Nevertheless, a negligible
133 decrease in the expression of *psbA1* gene was observed commencing 1 h after treatment
134 while the expression remained well above the control level (Fig. 1b). Interestingly, when the
135 cells are recovered from low CO_2 shift, the expression of *psbA1* continues to increase higher
136 compared to the cells subjected to microaerobic conditions where the level of *psbA1*
137 decreases and returns to the original level. During microaerobic treatments, when nitrogen is
138 introduced into the medium in order to remove oxygen, CO_2 is lost as well and could be the
139 reason for *psbA* gene induction. If this is the case we should see an induction independent of
140 the oxygen concentration. To test this hypothesis we applied a treatment with synthetic air
141 composed solely of oxygen and nitrogen expecting an induction of the gene expression. On
142 the contrary, when the *Synechocystis* cells were grown under CO_2 free synthetic air, the
143 induction of *psbA1* is imperceptible (Fig. 2). The chlorophyll fluorescence decay kinetics
144 measured in cells shifted from high CO_2 to ambient CO_2 after 30 min, showed no significant
145 changes in PSII function of both donor and acceptor side of the photosystem with an electron
146 transfer similar to the cells subjected to microaerobic conditions (Fig. 3).

In cyanobacteria, the *psbA* gene expression is regulated at the various levels of transcription initiation, elongation, and termination, mRNA stability as well as translation [13]. As an attempt to investigate any CO_2 responsive regulator controlling the expression of *psbA1* gene, we analyzed the upstream sequence of *psbA1* gene. NdhR, a LysR type regulator, has been shown to control the expression of most of the Ci responsive gene in *Synechocystis* by binding to a unique signature sequence denoted by a pattern T-N_{11}-A [14,15]. Investigation of *psbA1* gene sequence also revealed similar palindromic sequence pattern at 146 nucleotide upstream the start codon (Fig. 4) indicating the conceivable regulation by NdhR under low CO_2 condition.

DISCUSSIONS

Cyanobacteria redefine their metabolic processes at various level based on the changing environment, which helped their survival during the course of evolution. Establishment of flexible photosynthetic process remained essential in cyanobacteria as the early photosynthetic machinery became useless due to the formation of modern oxidative atmosphere [16]. Ancient oxygen-less atmosphere exhibited carbon dioxide concentration, several orders higher than present atmospheric level. The gradual decline in CO_2 concentration and increase in oxygen level promoted the formation of diverse genes needed for its adaptation and supressed genes, which are unable to cope with the modern oxidative environment [17].

The D1' protein encoded by *psbA1* gene in *Synechocystis* remains unexpressed during the growth conditions but induced when the cells were shifted to microaerobic conditions [5,6]. In both aforementioned studies the microaerobic environment was achieved by bubbling either argon or nitrogen gas into the culture. Our results with high CO_2 to low CO_2 shift experiments show a clear induction of the *psbA1* gene from *Synechocystis sp.* PCC 6803 in conditions where the oxygen concentration is maintained constant. A smaller induction

under that same conditions was previously recorded in a microarray study by [18] but dismissed at the time by the scientific community as being unreliable and never verified by other methods of investigation until now. Further, we explored the possibility that oxygen does not play any role at all in the induction of *psbA* gene given the fact that the previously employed methods for obtaining microaerobic conditions are depleting not only oxygen but also other atmospheric gases including CO_2 and the induction of *psbA1* gene might be a result from cumulative effect of O_2 and CO_2 depletion or specifically to CO_2 scarcity. Our experiments with synthetic air, containing only oxygen and nitrogen, where the CO_2 is completely missing, showed no change in the expression level of *psbA1*. Hence, the lack of CO_2 alone is not sufficient to induce the expression of the gene (Fig. 2). These results allow us to conclude that both O_2 and CO_2 act as trigger in inducing *psbA1* gene expression. The induction by CO_2 is triggered by the shift from a higher concentration to atmospheric CO_2 levels by unknown mechanisms, which needs to be unravelled. This shows for the first time that one member of the cyanobacterial *psbA* gene family is under control of CO_2 concentration, fact supported also by the presence in the promoter region of the gene of regulatory elements responsible for carbon regulation (Fig. 4). In both cases of induction, CO_2 and microaerobically triggered no significant functional changes were recorded on both the donor or the acceptor side of photosystem II allowing us to conclude that the D1' isoform, while induced, is still very scares and does not affect the general reaction center function or its photochemistry is similar to that of regularly expressed D1.

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FIGURE LEGENDS

Figure 1 **RT-qRT-PCR Analysis:** Expression level of *psbA1* gene quantified from cells subjected to high CO_2 to low CO_2 shift (a) and under microaerobic conditions (b). Measurements were carried out from the cells harvested from control (0 h) and treatments (15 min, 30 min, 1 h and 2 h). Recovery measurements were taken after the cells were shifted to control conditions until 2 h at 120 min and 180 min.

Figure 2 **RT-qRT-PCR Analysis:** Expression level of *psbA1* gene quantified from cells subjected to synthetic air. Measurements were carried out from the cells harvested from control (0 h) and treatments (15 min, 1 h, 2 h, 25 h and 50 h).

Figure 3 **Influence of microaerobiosis and low CO_2 on PSII function:** Chlorophyll fluorescence decay kinetics measured from cells grown under microaerobic without DCMU (a) and with DCMU (c) and high CO_2 to low CO_2 shift without DCMU (b) and with DCMU (d). Measurements were taken for control and treated cells (2h after shift to microaerobic condition and 48h after shift to low CO_2).

Figure 4 **Sequence alignment:** The probable NdhR-binding site T (N) $_{11}$A in the upstream sequence of *psbA1* gene. The LysR-type T (N) $_{11}$ A motif of *psbA1* was compared to *Synechocystis slr1512* (*sbtA*) and *sll1732* (*ndhF3*)

FIGURES

Figure 1

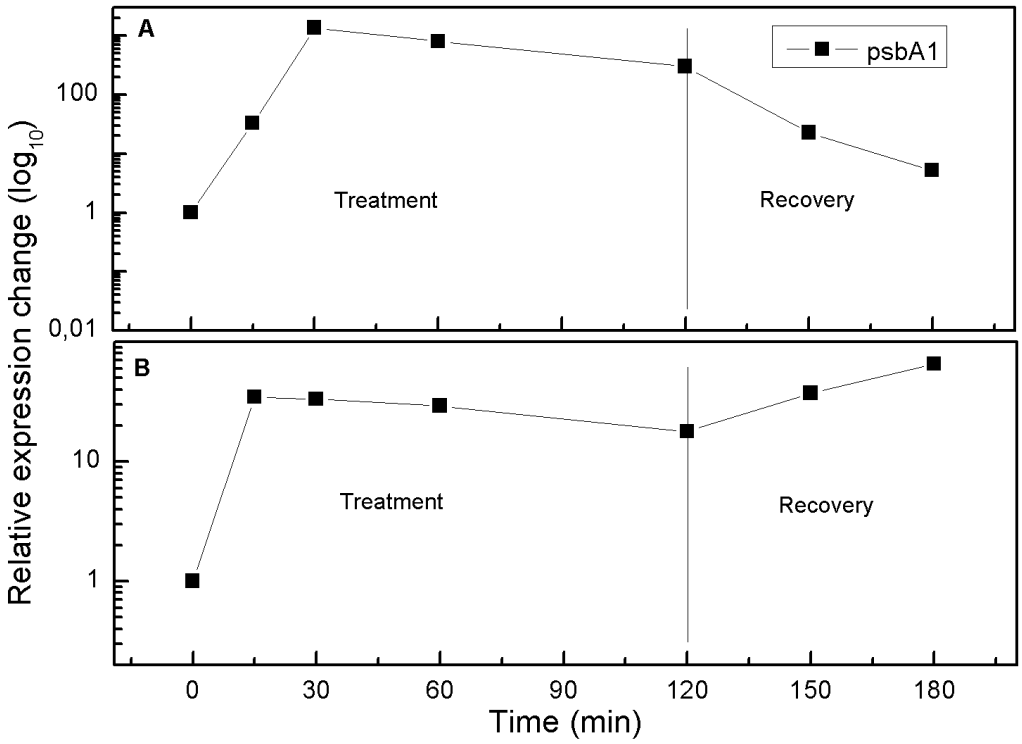
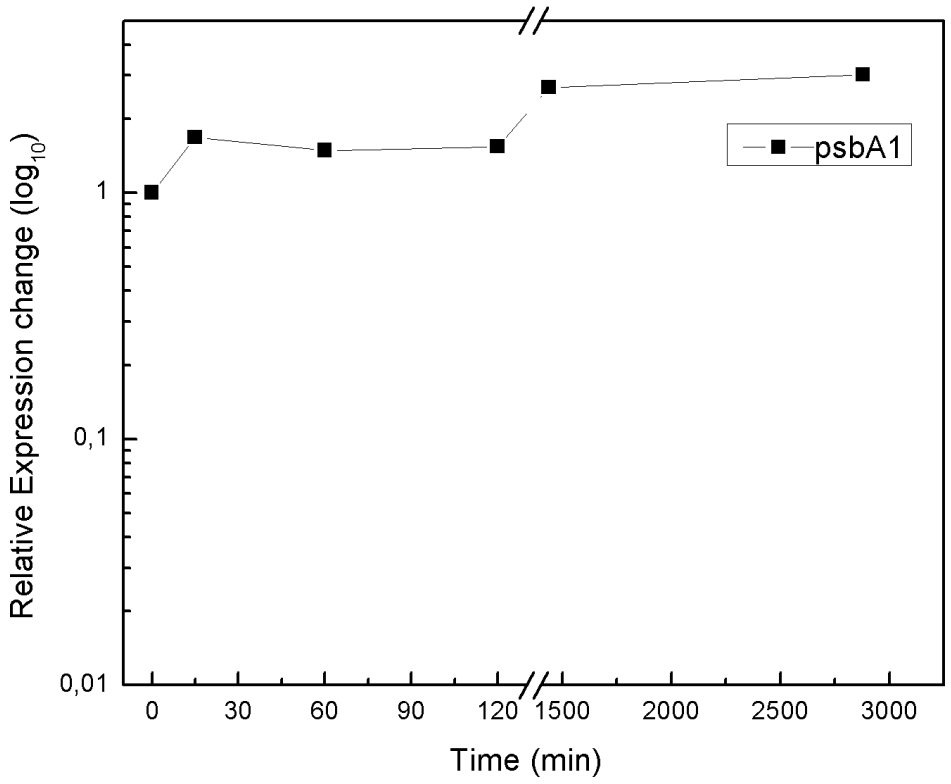
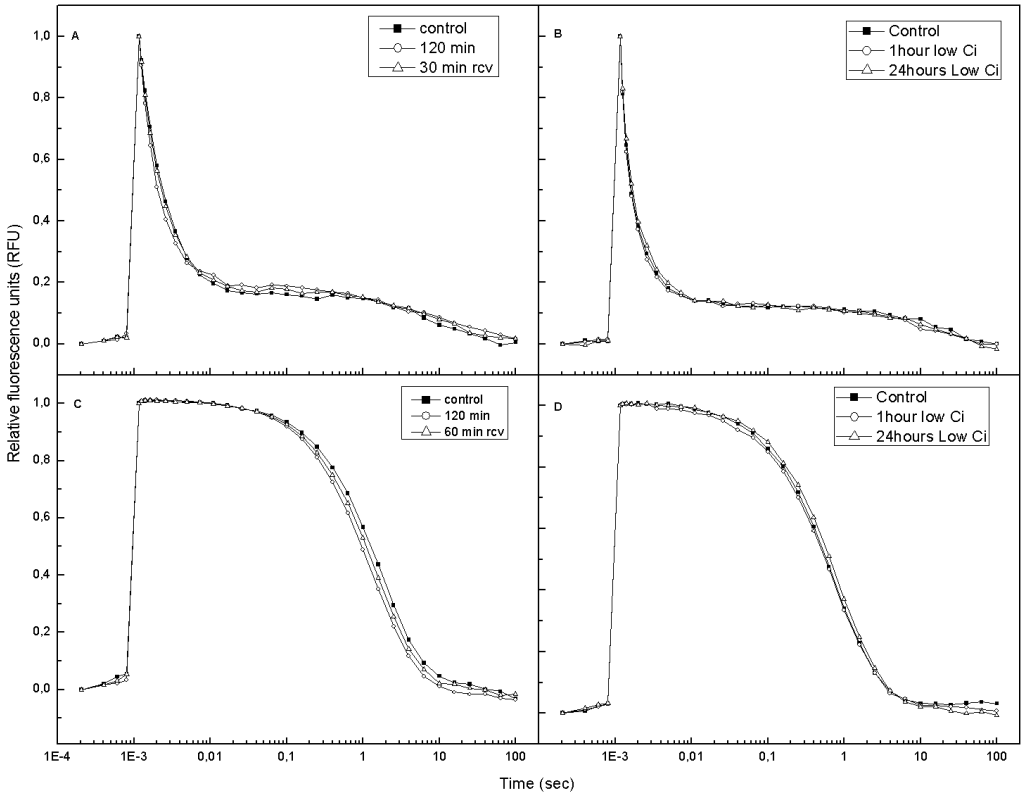


Figure 2

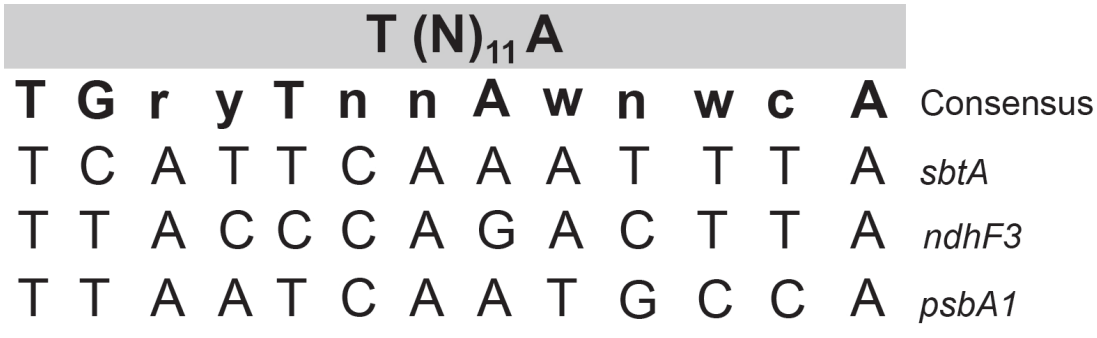

Relative Expression change (log10)
1
0,1
0,01
0
30
60
90
120
1500
2000
2500
3000
Time (min)
psbA1

304
305 Figure 3



306
307
308
309

Figure 4


T (N)11 A
T G r y T n n A w n w c A Consensus
T C A T T C A A A T T T A sbtA
T T A C C C A G A C T T A ndhF3
T T A A T C A A T G C C A psbA1