Expression of \textit{psbA1} gene in \textit{Synechocystis} sp. PCC 6803 is influenced by CO$_2$

\textbf{1 Introduction}

The “Great Oxidation Event” arguably due to the oxygenic photosynthesis by cyanobacteria not only sculpted the early biosphere but also allowed the evolution of higher and complex life forms [1]. During this evolutionary process cyanobacteria have developed specific mechanisms in response to changing environmental conditions to ensure their survival [2]. Expression of different isoforms of proteins beneficial to cell survival under different environmental conditions is one such adaptive strategy. The \textit{psbA} gene family, encoding multiple forms of D1 proteins of the photosystem II complex in cyanobacteria [3] is such an example.

Many cyanobacterial genomes contain a small family of genes encoding D1 proteins. These different D1 isoforms are employed by cyanobacteria in various response strategies to changing environmental conditions. So far, two major strategies were described, one increasing the amount of transcript for the constitutively expressed D1 isoform and the other exchanging between two different isoforms in response to environmental stress [4-7]. In \textit{Synechocystis} sp. PCC 6803, the \textit{psbA1} coding regions exhibit 99% identity in their nucleotide sequence and encode identical proteins. However, the \textit{psbA2} transcript is predominant accounting for 90% of the total D1 protein produced [4]. Hardly ever was the expression of \textit{psbA1} noticed and the condition, which caused the induction of the gene, remained enigmatic until [5] distinguished the expressions of \textit{psbA1} from other isoforms of \textit{psbA} gene under cells grown under low O$_2$ environment. Concurrently, [6] showed the dramatic expression of \textit{psbA1} gene in \textit{Synechocystis} under microaerobic condition. Presumably, different families of \textit{psbA} genes and their multiplicity appears to be not arbitrary, but merely based on environmental factors [7]. Furthermore this widens the possibility of additional environmental parameters involved in the regulation of these families of genes.
Cyanobacteria absorb inorganic carbon in the form of carbon dioxide (CO\(_2\)) and bicarbonate and the availability of these inorganic carbon sources limits the photosynthetic rate in cyanobacteria [8]. Cyanobacteria have developed various adaptive methods to accommodate a wide range of atmospheric CO\(_2\) levels. This intricate process of acclimation follows changes at various cellular levels, including expression regulation of genes involved in the carbon concentrating mechanism (CCM) [9].

In this study, the differential expression of the psbA1 gene under various levels of CO\(_2\) was analysed in order to correlate the involvement of various proteins participating in cellular adaptation.

2 Materials and methods

2.1 Culture and growth conditions

*Synechocystis* sp. PCC 6803 was obtained from the Pasteur Culture Collection, France. Cells were grown in cylindrical glass tubes containing 200 ml of BG-11 medium. The cultures were continuously illuminated at 50 μmol photons m\(^{-2}\)s\(^{-1}\) and the temperature was maintained at 30 °C [10]. To ensure thorough nutrient and O\(_2\) availability, the cultures were bubbled with air unless stated otherwise. When cells were grown under low CO\(_2\) condition, Na\(_2\)CO\(_3\) 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) for the next 120 min. Samples were collected at 0 min, 30 min, 60 min and 120 min. In another experiment the cultures were bubbled with synthetic air, of similar composition to atmospheric air, but completely lacking CO\(_2\). Sampling was done at 0 min, 15 min, 60 min, 120 min, 1500 min and after 3000 min. Microaerobic conditions were achieved in the cultures by bubbling with pure N\(_2\) gas. Cells were harvested at 0 min, 30 min, 60 min and 120 min. Treatments were run on two separate cultures grown under the same conditions and the experiments were repeated three times to avoid culture-dependent expression variations.

2.2 RNA isolation and cDNA synthesis

The cells were harvested from 10 mL culture (OD\(_{730}\) = 0.8) by centrifugation at 8000 rpm, 4 °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™ cDNA Synthesis Kit (BioRad, California) using 1.0 μg of purified RNA as template and random hexamer primers.

2.3 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™ 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. Specific primers were designed for the detection of psbA1 transcript: forward 5’-3’-AGC-TTA-AAC-CCA-AAA-TCT-TAC-TTC-GT and reverse 5’-3’-CCA-TCA-GAG-AAG-GAG-CCT-TGA-CCA. The expression data were normalized using the level of expression of rnpB as a reference gene using specific primers: forward 5’-3’-CTC-CCG-AAA-GAC-CAG-ACT-TG and reverse 5’-3’ CCG-GGT-TCT-GTT-CTC-TGT-GT, as previously described [6].

2.4 Flash induced chlorophyll fluorescence

Flash-induced chlorophyll fluorescence and subsequent decay was measured using fluorometer (FL-3500, Photon System Instruments, Czech Republic) [11]. Saturating actinic flashes (20 μs) and measuring flashes (8 μs) 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)) [12].
3 Results

In our experiments, the culture exposed to microaerobic conditions displays a strong induction of the psbA1 gene, in direct agreement with previous studies (Fig. 1a) [5, 6]. When the Synechocystis cells were shifted from high CO2 to ambient CO2 conditions, the psbA1 gene showed increased expression up to 30-fold within 15 min and remained up-regulated until 120 min under atmospheric CO2 level (Fig. 1b). Nevertheless, a negligible decrease in the expression of psbA1 gene was observed commencing 60 min after treatment while the expression remained well above the control level (Fig. 1b). Interestingly, when the cells are recovered from low CO2 shift, the expression of psbA1 continues to increase compared to the cells subjected to microaerobic conditions where the level of psbA1 decreases and returns to the original level (Fig. 1a,b). During microaerobic treatments, when nitrogen is introduced into the medium in order to remove oxygen, CO2 is lost as well and could be the reason for psbA1 gene induction. If this is the case we should see an induction independent of the oxygen concentration. To test this hypothesis, cells grown on 5% CO2 conditions were exposed to synthetic air composed solely of oxygen and nitrogen expecting an induction of the gene expression. On the contrary, when the Synechocystis cells were subjected to CO2 free synthetic air, the induction of psbA1 is imperceptible (Fig. 2). The chlorophyll fluorescence decay kinetics in the cells shifted from high CO2 to ambient CO2 after 30 min, showed no significant changes in PSII function of both donor and acceptor side of the photosystem with an electron transfer similar to the cells subjected to microaerobic conditions (Fig. 3).

In cyanobacteria, 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 CO2 responsive regulator controlling the expression of the psbA1 gene, we analyzed the upstream sequence of the 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-N11-A [14,15] presented in Figure 4 as the consensus sequence. 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 CO2 condition.

Figure 1. RT-qRT-PCR Analysis: Expression level of psbA1 gene quantified from cells subjected to microaerobic conditions (a) and under high CO2 to low CO2 shift (b). Measurements were carried out from the cells harvested from control (0 min) and treatments (15 min, 30 min, 60 min and 120 min). Recovery measurements were taken after the cells were shifted back to control conditions at 120 min and 180 min from the start of the experiment.
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**Figure 2.** RT-qRT-PCR Analysis: Expression level of psbA1 gene quantified from cells grown on 5% CO2 and subjected to synthetic air. Measurements were carried out from the cells harvested from control (0 min) and treatments (15 min, 60 min, 120 min, 1500 min and 3000 min).

**Figure 3.** Influence of microaerobiosis and low CO2 on PSII function: Chlorophyll fluorescence decay kinetics measured from cells grown under microaerobic without DCMU (a) and with DCMU (c) and high CO2 to low CO2 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 CO2).
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, a fact supported also by the presence in the promoter region of the gene of regulatory elements responsible for carbon regulation (Fig. 4). No such regulatory elements were found in the promoter regions of similar genes from Anabaena sp. PCC 7120 or Thermosynechococcus elongatus BP-1 being an indication that in Synechocystis sp. PCC 6803 we have a specific form of expression regulation (data not shown). In both cases of induction, CO$_2$ or microaerobically triggered, no significant functional changes were recorded on either the donor or the acceptor side of photosystem II. Hence, we conclude that the induction of psbA1 gene in Synechocystis does not generate a modification of the general reaction center function or its photochemistry. This remains similar to normal growth conditions where regularly expressed D1 occupies the PSII reaction centres.

4 Discussion

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 processes remained essential in cyanobacteria as the early photosynthetic machinery became useless due to the formation of the modern oxidative atmosphere [16]. The ancient anoxic environment exhibited a carbon dioxide concentration several orders of magnitude higher than the present atmospheric level. The subsequent modification of atmospheric gas concentrations prompted the development of response mechanisms able to cope with the modern oxidative environment [17].

The D1’ protein encoded by the psbA1 gene in Synechocystis remains unexpressed during growth conditions [10] but is 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 99.9% N$_2$ and 0.1% CO$_2$ gas into the culture. Our results with high CO$_2$ (5%) to low CO$_2$ (atmospheric level) shift experiments show a clear induction of the psbA1 gene from Synechocystis sp. PCC 6803 in conditions where the oxygen concentration is constant. A smaller induction under the same conditions was previously recorded in a microarray study [18] but 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 psbA1 gene given the fact that the previously employed methods for obtaining microaerobic conditions [6] were depleting not only oxygen but also other atmospheric gases including CO$_2$ and the induction of psbA1 gene might result from the cumulative effect of O$_2$ and CO$_2$ depletion. Our experiments with synthetic air, containing only oxygen

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