Effect of nitrate availability on expression of multi sensor histidine kinase gene in *Spirulina platensis* PCC 7345

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Abstract Addition of nitrate (NO_3^-) to a suspension of NO_3^- -depleted *Spirulina platensis* PCC 7345 restored back the PSII reaction centre efficiency. Using RAPD/RT-PCR differential display, we have detected the upregulation of a novel gene in filaments starved of nitrogen when enriched with NO_3^- . Semiquantitative RT-PCR clearly shows that NO_3^- repletion induces the expression of this gene. Its nucleotide sequence is homologous to multi sensor histidine kinase (*hstk*) from *Spirulina platensis* NIES-39 and *Spirulina maxima* CS-328 genes. The open reading frame of this gene encodes a protein of 98 amino acids contains sensory domain of PAS domain.

Keyword *Spirulina* · PSII reaction centre efficiency · Differential display · Histidine kinase

Introduction

Nitrate assimilation is the major process of nitrogen acquisition in non-nitrogen fixing cyanobacteria, *Spirulina platensis* (Guerrero et al. 1981). Nitrate uptake and reduction to ammonia operates with the use of the photosynthetically generated assimilatory power (Arnon 1984), where photolysis of water provides the reducing power required for nitrate reduction (Flores et al. 2005). Thus, an imbalance in intracellular C:N ratio indicates that the cells are under nitrogen stress while there is adequate supply of carbon (Muro-Pastor et al. 2001). In cyanobacteria, nitrogen

depletion leads to the loss of Chl *a* and increase in nonphotochemically active carotenoids (Herzig and Falkowski 1989; Geider et al. 1993). This leads to modulation in the organization and the function of photosynthetic apparatus.

Nitrogen fixing cyanobacteria reduces molecular nitrogen to cope up with nitrogen depletion. Whereas, in non-nitrogen fixing cyanobacteria, nitrogen depletion causes ‘chlorosis’ (Allen and Smith 1969), converting them to non-pigmented resting cells, which are able to survive prolonged periods of starvation (Sauer et al. 2001). In *S. platensis*, the immediate effect of nitrate starvation is the degradation of light harvesting antenna complex (Peter et al. 2010a; Sauer et al. 1999). Photosystem II (PSII) catalyzed electron transport is reported more susceptible to nitrate starvation (Peter et al. 2010b). These changes in photochemical energy conservation can be monitored by recording their fluorescence emission spectra.

The organism has evolved with diverse signal transduction system to respond to external or internal changes to cope with these drastic environmental conditions (Tandeau de Marsac and Houmard 1993; Zhang et al. 2007). The genes involved in such signal transduction are not constitutively expressed, but are carefully regulated by environmental signals (Lam et al. 1996). Differential display of mRNA is the most sensitive procedure used to analyze such differential gene expression in cyanobacteria and higher plants (Liu and Xiong 2007; Bhaya et al. 2000; Wang and Feuerstein 1995).

Thus the aim of this study is to understand the effect of nitrate on PSII reaction centre efficiency after nitrate starvation and to identify nitrate response genes involved in nitrate signaling in *Spirulina platensis* PCC 7345. In plants, if the complete genome sequence is not available, differential expression analysis is done using RAPD/RT-PCR differential display (Yoshida et al. 1994; Liu and Xiong 2007). We have attempted to use this technique for

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the first time in cyanobacteria, to look for the nitrate response genes. We observed that nitrogen depletion decreases PSII reaction centre efficiency, which can be restored back by the addition of nitrate and a multi-sensor histidine kinases (*hstk*) might be involved in the process.

Materials and methods

Growth conditions

Pure cultures of *S. platensis* (strain PCC 7345) were grown in Zarrouk's medium at pH 11.5 (Zarrouk 1966), at 30 ± 2 °C under continuous white light illumination (4 Klux) for 8–9 days, till they have reached the exponential phase.

Nitrate treatment

All treatments were done using exponentially growing cultures (9-day-old culture). 100 mL cultures were washed twice with ice-cold sodium bicarbonate buffer (10 mM; pH 10.5). The pellets were re-suspended in a nitrate free Zarrouk's medium for 48 h for NO_3^- depletion (Collier and Grossman 1992) and further added 10 mM KNO_3 for NO_3^- repletion (El-Anwar et al. 1999). NO_3^- depleted filaments were used as controls. The samples were kept on a shaker under continuous white light illumination (4 Klux) at 30 °C for 2 hrs.

Isolation of thylakoid membranes

Thylakoid membranes were isolated from *Spirulina* according to the protocol reported by Sudhir et al. (2005). Filaments with a cell density at $5 \mu\text{g Chl } a \text{ mL}^{-1}$ were used. The filaments were washed with buffer A (100 ml of 25 mM HEPES –NaOH containing 30 mM CaCl_2 , pH 7.5) and suspended in 10 ml of buffer B (25 mM HEPES–NaOH, 30 mM CaCl_2 , and 0.8 M sorbitol containing 1 mM phenyl-methylsulfonyl fluoride (PMSF), pH 7.5) for lysis. Cell lysis was carried out by sonication at 4 °C at 10 w for 10 s using Microson ultrasonic cell disrupter. Thylakoids were separated from the cell debris by centrifugation at $9,000 \times g$ for 5 min. The supernatant was again centrifuged at $40,000 \times g$ for 90 min at 4 °C. The pellet was re-suspended in buffer B and used for fluorescence measurement.

Fluorescence measurements

Room temperature fluorescence was measured and the emission spectra were recorded with Horiba Jobin-Yvon Fluoromax 4 Spectrofluorometer. Excitation wavelength was set at 440 nm and slit widths were adjusted to 10 nm (excitation) and 5 nm (emission) (Sudhir et al. 2005).

Isolation of total RNA

For RNA isolation, 2 ml of cultures (from both NO_3^- depleted and repleted filaments) were used. Total RNA was isolated using RNeasy Kit (Qiagen, Melbourne, Australia). For cell lysis, 2 mL of the culture was suspended in lysis buffer (RLT buffer, Qiagen, Melbourne, Australia) containing guanidine thiocyanate. The suspension was then sonicated at 4 °C at 10 w for 10 s using Microson ultrasonic cell disrupter. Further isolation was carried out as per the manufacturer's instructions. RNase-Free DNase Set (Qiagen) was used to digest contaminating DNA in RNA solutions prior to RNA cleanup. RNA was quantified by measuring the absorbance at 260/280 nm and was analyzed on formaldehyde gel. RNA thus isolated was used for RAPD/RT-PCR differential display and semi-quantitative RT-PCR.

RAPD/RT-PCR differential display

To identify differentially expressed genes during NO_3^- repletion, we compared mRNAs of NO_3^- repleted *S. platensis* with NO_3^- depleted filaments as control, using RAPD/RT-PCR differential display (Yoshida et al. 1994; Liu and Xiong 2007). cDNA synthesis was carried out with PCR using 10-base RAPD primers (Table 1) (Ocimum biosolutions, India), that were arbitrarily chosen. RT-PCR differential display was carried out using 20 ng of total RNA by One step RT-PCR kit (Qiagen). RT-PCR was performed with different pairs of arbitrary primers in each reaction. RT-PCR condition includes initial activation at 50 °C for 30 min, denaturation at 94 °C for 15 min, primer touchdown of 35 cycles of 94 °C for 60 s, 32 °C for 60 s, and 72 °C for 60 s, with the final extension for 7 min. The PCR products were separated by 6 % non-denaturing polyacrylamide sequencing gel in 1X TBE buffer at 30 mA. The reproducibility was demonstrated by comparing replicate results from fresh RNA sample. The cDNA was then eluted from polyacrylamide gel using QIAEX II gel extraction kit (Qiagen) and used for sequencing (Ocimum Biosolution). A homology search was performed and the amino acid sequence was deduced. The resulted sequence was deposited at the GenBank (Genebank accession no: GQ428516).

Table 1 Arbitrary primers used in RAPD-RT PCR differential display

Primer	Sequence
DD1	5'-CTGACGCTGA-3'
DD2	5'-GAATGGCGAG-3'
DD3	5'-CGATGCGATG-3'
DD4	5'-GGTGATGAGG-3'
DD5	5'-TCGCTTCCCT-3'

Table 2 Gene specific primers used in semi-quantitative RT-PCR. *(F forward; R reverse)

Primer*	Sequence	Amplicon Size
<i>16S rRNA</i> -F	5'CAAGCGTTATCCGGAATGAT3'	479 bp
<i>16S rRNA</i> -R	5'ACTCTCGGCTTTCACCAAGA3'	
<i>Hstk</i> -F	5' GTGGGAATTGGGGAGGTT3'	283 bp
<i>Hstk</i> -R	5'GAGGCCCTGTGACGGAAC3'	

Semi-quantitative RT-PCR

The gene specific primers were synthesized for the differentially expressed genes (*Ocimum biosolutions*), for further expression profile analysis using semi-quantitative RT-PCR. We selected the expression of a housekeeping gene (*16S rRNA*) as internal control. The gene specific primers used and its amplicon sizes were listed in Table 2. Semi-quantitative RT-PCR was carried out using One step RT-PCR kit. The conditions were so chosen that RNAs analyzed were in the exponential phase of amplification. RT-PCR condition included initial activation at 50 °C for 30 min and denaturation at 94 °C for 15 min. Primer touchdown of 25–35 cycles included 94 °C for 60 s, ~46 °C for 60 s, and 72 °C for 60 s with a final extension of 7 min. The cDNA was quantified by agarose-gel electrophoresis using 1.5 % agarose with known concentrations of DNA Ladder Mix (MBI-Fermentas). The gel images were captured using the Gene-Genius gel documentation system with GeneSnap software (Syngene, Cambridge, UK) and densitometry values were measured by using Gene Tool software (Syngene). Statistical significance of the densitometry values were demonstrated using a Student's *t*-test ($p=0.05$).

Sequence analysis

The nucleotide sequence analysis of the gene was performed using the BLAST software at NCBI server (<http://www.ncbi.nlm.nih.gov/BLAST>). Sequence alignment was obtained using T-Coffee, a multiple sequence alignment program and the picture was generated using JALVIEW program. Domain architecture of protein was derived by Conserved Domain Database (NCBI) (<http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>).

Results

Steady-state room temperature fluorescence spectra

To study the effect of nitrogen starvation on intact cells of *Spirulina platensis* PCC 7345 and its thylakoid membrane, we have compared the emission intensity of both in NO_3^- depleted versus NO_3^- repleted filaments. We find that in comparison with the NO_3^- depleted culture, the emission intensity of NO_3^- repleted filaments showed an increase at 680 nm (Fig. 1). In the filaments as well as their thylakoids, F680 intensity increases in the presence of NO_3^- . The main emission bands around 680 nm showed a red-shift of 2 nm (Fig. 1).

RAPD/RT-PCR differential display

Amplified transcripts of RNA extracted from the differently treated filaments were clearly visualized after separation by PAGE. For every combination of arbitrary primers, numerous identical bands of amplified cDNA were displayed (Fig. 2a).

Fig. 1 Fluorescence emission spectra of **a**) cells and **b**) isolated thylakoid membrane. Cells grown in nitrate free medium were used as control against the cells grown in 10 mM KNO_3 for 2 hrs. The fluorescence spectra were recorded on equal Chl *a* basis ($5 \mu\text{g ml}^{-1}$) at excitation wavelength of 440 nm. These experiments were done in triplicates yielded identical spectra

Fig. 2 **a** RAPD RT-PCR with primer sets giving promising cDNA pattern visualized by EtBr staining; **b** Differential display gel showing cDNAs expressed only in NO_3^- repleted filaments: [NO_3^- depleted controls marked with (-) and NO_3^- repleted filaments marked with (+), superscript to the primer combinations used]. **c** Differentially expressed cDNA was eluted from the gel and reamplified using primers DD2 and DD5 for sequencing

The majority of these bands were present in nearly the same expression level, even after NO_3^- repletion. Only one of these cDNA (from primer set DD2 and DD5) showed a differential expression pattern (Fig. 2b). This differentially expressed gene band was recovered from gel and used as the template for the re-amplification, which was performed with the corresponding arbitrary primer used in the mRNA differential display (DD2 and DD5 primers) (Fig. 2c). The reamplification was specific and reproducibly

gave a high yield of cDNA, and allowed direct sequencing of the reamplified product without purification or cloning. Sequence was deposited at the GenBank (Genebank accession no: GQ428516).

Semiquantitative RT-PCR

Semiquantitative RT-PCR was then conducted using the EST specific primers (Fig. 3) with the total RNA of *S. platensis* isolated from both NO_3^- depleted and NO_3^- repleted filaments. cDNA showed expected amplicons with RT-PCR. The intensities of these bands increased in proportion to the increasing amounts of total RNA from 0.5 to 2 $\mu\text{g}/\text{reaction}$. Following nitrate starvation, expression level of gene in NO_3^- repleted filaments was significantly ($p < 0.05$) higher than the NO_3^- depleted filaments (Fig. 3) (no significant change in expression levels of 16S *rRNA*). This data was consistent with the differential-display results, clearly showing that NO_3^- repletion induces the expression of this gene.

Fig. 3 Semi-quantitative RT-PCR analysis of *hstK* (283 bp) and 16S *rRNA* (479 bp). [Lane 1: marker; lane 2: no RNA control; lane 3: 16S *rRNA* from nitrate treated cells; lane 4: 16S *rRNA* from non-treated; lane 5: *hstK* from nitrate treated cells; lane 6: *hstK* from non-treated]. The bar graph shows the amount of mRNA, expressed as the ratio of densitometric measurement. Data are shown as mean $\pm$ SEM for 3 independent experiments, * $p < 0.05$ vs lane 6 (NO_3^- Depleted)

Sequence analysis

The nucleotide sequence analysis of this gene revealed that it was homologous to multi sensor histidine kinase (*hstK*) from *Spirulina platensis* NIES-39 and *Spirulina maxima* CS-328 genes. The sequence prediction was carried out using the GenScan software. An open reading frame encoding 98 amino acids was found in the 297 bp cDNA sequence. The predicted protein sequence of *hstK* acquired from *S. platensis* PCC 7345 was 94 % identical to *S. platensis* NEIS-39 (protein id: BAI88177.1) and 81 % identical to *S. maxima* CS-328 (protein id: ZP_03275569.1) (Fig. 4a). Figure 4b shows sensory box domain belonging to the PAS domain family identified within the predicted protein sequence.

Discussion

In non-nitrogen fixing cyanobacteria the controlled rate of nitrate (NO_3^-) assimilation becomes necessary to maintain C:N homeostasis. Thus, when the cells suffer nitrogen starvation, they tend to cease active photosynthesis. This is reported due to the inactivation of PSII (involving change in the structural organization of phycobilisomes) accompanied with decrease in the amount of carbohydrates (Peter et al. 2010a, b). Cyanobacteria grown in nitrate-free medium for at least 48 h are ingenious as an experimental system to study the effect of nitrogen starvation (Gombos and Vigh 1986; Sauer et al. 2001).

After 48 h of NO_3^- depletion *S. platensis* have shown an inhibition in PSII catalyzed electron transport (Peter et al. 2010b). The reason for this inhibition was the decline in Chl *a* present in the pigment apparatus in PSII reaction centre and disorganization of the thylakoid membranes induced by starvation (Billi and Grilli Caiola 1996; Berges et al. 1996).

Although, this inhibition in considered advantageous for the cells as in such stressful environment the formation of excited chlorophyll molecules generate toxic species of O_2 (Grossman et al. 1995). We observe that the addition of NO_3^- increases the intensity of a peak around 680 nm (Fig. 1). These peaks were originated from Chl *a* of PSII reaction centre and are universal in cyanobacterial emission spectra (Sudhir et al. 2005; Allen et al. 1990). Similar results were observed in *Chlorella vulgaris*, *Dunaliella tertiolecta*, *Synechococcus* sp. PCC 7002 and *Thalassiosira weissflogi* (El-Anwar et al. 1999; Berges et al. 1996).

The fluorescence yield increases when the PSII centers are closed (P680^+) (Campbell et al. 1998). Thus, an increase in steady state fluorescence intensity indicates increase in conversion of P680 to P680^+ . We thus hypothesize that addition of NO_3^- to a NO_3^- depleted *S. platensis* leads to the rearrangement of these chlorophyll molecules in the chlorophyll-protein complex, leading to increased PSII reaction centre efficiency. The filaments tend to regain their active photosynthesis setup, which they have lost due to NO_3^- starvation (Peter et al. 2010b; Allen et al. 1990).

With RAPD/RT PCR differential display and sequence analysis we confirm the role of *hstK* in nitrate assimilatory pathway of *Spirulina platensis* PCC 7345. In *Escherichia coli*, a similar two-component regulatory system controls the expression of genes in response to NO_3^- availability (Cavicchioli et al. 1996). In *E. coli*, NarX and NarQ sensor-transmitter proteins independently detect the presence of NO_3^- in the cell environment and transmit the signal to the response regulator, NarL (Cavicchioli et al. 1995). Upon activation, NarL binds DNA and modulates the expression of genes involved in NO_3^- assimilation (Cavicchioli et al. 1995, 1996).

Nevertheless, in *S. platensis* NIES-39, *hstK* gene cassette included a histidine kinases protein (of PAS and PAC superfamily) and two response regulator protein (of REC

Fig. 4 **a** Sequence alignment between HstK of *S. maxima* CS-328, *S. platensis* PCC 7345 and *S. platensis* NIES-39. **b** The putative domain of the protein encoded by HstK

superfamily) (protein id: BAI88175.1, BAI88176.1) (Fujisawa et al. 2010). Upon stimulation by a given signal, autophosphorylation of histidine kinase occurs on a conserved His residue. Subsequently, phosphate group is transferred to a conserved Asp residue of a response regulator protein (REC) (Stock et al. 2000). REC domain consisted of C-terminal GGDEF domain, with diguanylate cyclase activity (Fujisawa et al. 2010; Gao and Stock 2010; Ryjenkov et al. 2005). Upon receiving the signal, REC undergoes conformational changes to form dimers and relay the signals via C-terminal GGDEF domain (Gao and Stock 2010). GGDEF forms bis cyclic diguanosine monophosphate (c-di-GMP), a widely distributed second messenger signaling for light- or redox-response (Fujisawa et al. 2010; Galperin 2006). Perhaps, c-di-GMP would carry over the signaling pathway by controlling the expression of related genes/operons.

In cyanobacteria, the signal transduction protein P_{II} is required for any molecular event under nitrogen deprivation (Aldehni et al. 2003; Paz-Yepes et al. 2003). The activity of P_{II} is controlled by its phosphorylation state in a seryl (Ser49) residue (Vázquez-Bermudez et al. 2002; Lee et al. 1999). With our results, we propose that in *S. platensis* change in phosphorylation state of P_{II} protein could be one of the probable outcomes of the above-mentioned series of activating events involving HstK. Along with phosphorylation, activation of P_{II} requires the binding of ATP and 2-oxoglutarate (Flores et al. 2005; Merrick and Edwards 1995). Activated P_{II} binds to the global nitrogen regulator NtcA, which belongs to a catabolite activator protein (CAP) family. NtcA activates transcription of nitrogen assimilation genes in response to nitrogen step-down. It binds to the enhancer region of genes responsible for nitrate assimilation, photosynthesis and activates their transcription (Fujisawa et al. 2010; Su et al. 2005). However, the exact mechanism of this cascade involving HstK, c-di-GMP, PII, NtcA and its link photosynthesis is still to be explained.

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