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Comparative proteomics of wild type, An + ahpC and An Δ ahpC strains of *Anabaena* sp. PCC7120 demonstrates AhpC mediated augmentation of photosynthesis, N_2-fixation and modulation of regulatory network of antioxidative proteins

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ABSTRACT

Alkylhydroperoxide reductase (AhpC), a 1-Cys peroxiredoxin is well known for maintaining the cellular homeostasis. Present study employs proteome approach to analyze and compare alterations in proteome of *Anabaena* PCC7120 in overexpressing (An + ahpC), deletion (An Δ ahpC) and its wild type. 2-DE based analysis revealed that the major portion of identified protein belongs to energy metabolism, protein folding, modification and stress related proteins and carbohydrate metabolism. The two major traits discernible from An + ahpC were (i) augmentation of photosynthesis and nitrogen fixation (ii) modulation of regulatory network of antioxidative proteins. Increased accumulation of proteins of light reaction, dark reaction, pentose phosphate pathway and electron transfer agent FDX for nitrogenase in An + ahpC and their simultaneous downregulation in AnΔahpC demonstrates its role in augmenting photosynthesis and nitrogen fixation. Proteomic data was nicely corroborated with physiological, biochemical parameters displaying upregulation of nitrogenase (1.6 fold) PSI (1.08) and PSII (2.137) in An + ahpC. Furthermore, in silico analysis not only attested association of AhpC with peroxiredoxins but also with other players of antioxidative defense system viz. thioredoxin and thioredoxin reductase. Above mentioned findings are in agreement with 33–40% and 40–60% better growth performance of An + ahpC over wild type and AnΔahpC respectively under abiotic stresses, suggesting its role in maintenance of metabolic machinery under stress.

Significance: Present work explores key role of AhpC in mitigating stress in *Anabaena* PCC7120 through combined proteomic, biochemical and in silico investigations. This study is the first attempt to analyze and compare alterations in proteome of *Anabaena* PCC7120 following addition (overexpressing strain An + ahpC) and deletion (mutant AnΔahpC) of AhpC against its wild type. The effort resulted in two major traits in An + ahpC as (i) augmentation of photosynthesis and nitrogen fixation (ii) modulation of regulatory network of antioxidative proteins.

1. Introduction

Cyanobacteria, the photosynthetic ancestors of chloroplasts in algae and plants occupy a wide range of environmental niches including the extremely hostile environments which make them an excellent model to study photosynthesis [1,2], cellular differentiation [3], tolerance to environmental stresses [4,5] and evolution [6,7]. *Anabaena* PCC7120 has been particularly interesting because it has a rare combination of two agriculturally important traits viz. photosynthesis and nitrogen fixation [8]. It is well known fact that abiotic stresses including drought, salinity, heat, UV-B, heavy metals and pesticide are exceedingly toxic to diazotrophic cyanobacteria [9,10,11,12], which negatively affect its nitrogen fixation potential hence nitrogen economy of soil [13].

AhpCs, members of peroxiredoxin family are found in organisms as diverse as cyanobacteria and humans where they are crucial for the detoxification of a variety of hydroperoxides, including hydrogen peroxide, alkyl hydroperoxides, lipid hydroperoxides as well as peroxynitrite [14,15]. Due to functional versatility and ubiquity, the AhpC proteins have been studied in some detail [16,17,18,19]. While peroxiredoxins (Prx1 and Prx6) are generally known to detoxify peroxide they also work as molecular chaperone under stress condition. For chaperone function the peroxiredoxin form decameric rings when exposed to oxidative stress. This type of structural rearrangements has been reported from yeast [20], *Helicobacter pylori* [21], *Schistosoma mansoni* [22], *Homo sapiens* [23] and *Arabidopsis* [24].

AhpC activity can be regulated by phosphorylation and possibly by change in oligomerization state [25,26,27]. Majority of AhpC belongs to 2-cys peroxiredoxin family. In contrast to this AhpC of *Mycobacterium tuberculosis* is an exception having 3-cys residue. Interestingly,

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however, the AhpC of *Anabaena* PCC7120 belongs to 1-cys peroxiredoxin family [19].

It is worth stating that increased expression of AhpC has been reported under variety of stresses such as iron deficiency, heat shock, oxidative stress, nalidixic acid, ethanol and hydrogen peroxide [28,29,30,31,32]. *Arabidopsis* with downregulated AhpC activity demonstrated impaired photosynthesis, degradation of D1 protein, and LHCPII and enhanced ATP synthase activity [33]. Heterologous expression of *Arabidopsis thaliana* gene *At-1-CysPrx* in *E. coli* cells made it sensitive to cumene hydroperoxide [34].

Proteomics provides a snapshot of events occurring in an organism under normal and stress conditions which are largely corroborated with physiology of the organism [35,36]. Apart from this proteomics has also been applied to decipher changes at mutant level. For example Koksharova et al. [37] compared the proteomes of wild-type and cell division mutants *Ftn2* and *Ftn6* of *Synechococcus* sp. PCC7942 and observed a nice correlation between proteome and physiological attributes [37]. Likewise, proteomic characterization of wild type and ΔcrhR, ΔHik33 strains of *Synechocystis* sp. PCC 6803 has been done under cold and salt acclimation [38].

While analyzing the proteome of *Anabaena* subjected to heat stress [36], Mishra et al. (2009b) observed accumulation of AhpC/TSA protein which on heterologous expression in *E. coli* conferred tolerance to several abiotic stresses [18]. However, its regulatory role in different metabolic pathways has remained unexplored. Present study is an effort to understand how AhpC modulates the proteome and associated metabolic pathways. To address the above question, the proteome of AhpC overexpressing (An + ahpC), mutant AhpC (An Δ ahpC) and the wild type *Anabaena* have been compared using 2DE followed by MALDI-TOF MS/MS analysis.

2. Materials and methods

2.1. Culture and growth conditions

All three strains of *Anabaena* sp. PCC7120 (here after *Anabaena*) wild type, AhpC null mutant (AnΔahpC) and AhpC overexpressing strain (An + ahpC) were grown in combined nitrogen-free BG-11 [39] liquid medium [BG-11(N^-)] at 27 °C, pH 7.5 under continuous illumination (30 μE/m2/s) and aeration. Cells were inoculated at a chlorophyll *a* density of 1 μg ml^{-1} for all the experiments. *Escherichia coli* strains were grown in Luria Bertani (LB) medium at 37 °C in an incubator at 120 rpm. Cells were inoculated with 0.05/ml OD_{600} for all the experiments and growth was measured in terms of turbidity (OD_{600}). Ampicillin and kanamycin were used at 100 μg ml^{-1} and 50 μg ml^{-1} concentrations respectively for *E. coli* [40] and for *Anabaena* transformants 25 μg ml^{-1} neomycin was used in BG-11 agar media or 12.5 μg ml^{-1} in BG-11 liquid medium. Source of strains used were mentioned in Supplementary Table 1.

2.2. Characterization of ahpC overexpressing and mutant strains of Anabaena sp. PCC7120

Genomic DNA from *Anabaena* sp. PCC 7120 was isolated as per the method of Srivastava et al. [41] and *ahpC* gene was amplified through PCR by using gene specific primer (Supplementary Table 2). The amplified PCR product was purified and cloned into an integrative expression vector pFPN digested with the same restriction enzymes (Fig. 2A–C). The resulting construct was designated as pFPN-*ahpC*. Further for disruption of *ahpC* gene PCR amplification of 1939 nt long sequence (650 nt long up and down stream sequence from *ahpC* gene) including 639 nt long *ahpC* was done using gene specific primers AhpCF* and AhpCR* (Supplementary Table 2). The amplified PCR product was purified and cloned into a suicidal vector pBS (Fig. 3A–D). The resultant construct was designated as pBS*ahpC* in which *ahpC* was disrupted by incorporation of neomycin cassette. After neomycin insertion within ahpC sequence of pBS*ahpC* construct the resultant construct was designated as pBSahpC* (disrupted *ahpC gene*) and selected on neomycin containing agar plate (Fig. 3E–F). Transformation of recombinant plasmid pFPN-ahpC and pBSahpC* into *E. coli* was done as per the method in Sambrook and Russell [40] which was then grown in LB medium [40]. Thereafter, the recombinant plasmid was isolated, double digested and the DNA sequence of cloned fragment was confirmed by sequencing (Macrogen, Korea).

2.3. Electroporation of recombinant plasmid pFPNahpC and pBSahpC* (disrupted ahpC) into Anabaena sp. PCC 7120

The *ahpC* gene was cloned into the *Nde*I-*Bam*HI restriction sites of plasmid vector pFPN (Fig. 1) which allows integration at a defined risk-free site in the *Anabaena* PCC7120 genome and expression from a strong cyanobacterial *psbA1* promoter [42]. The resulting construct designated as pFPN-*ahpC* was electroporated into *Anabaena* PCC7120 using an electroporator (Eppendorf, Germany). The electroporation was done at 1.2 KV cm^{-1} for 5 ms employing an external autoclavable electrode with a 2-mm gap. The electrotransformants were selected on BG11 + 25 μg ml^{-1} neomycin plates by repeated subculturing for at least 24 weeks to achieve complete segregation and designated as An + ahpC (Fig. 2D). PBSahpC*(disrupted *ahpC* gene) construct was also electroporated into *Anabaena* PCC7120 using the same method and the resulting strain of *Anabaena* PCC7120 was designated as null mutant of *ahpC* (AnΔahpC, Fig. 3F). The electroporation buffer contained high concentrations of salt (10 mM HEPES, 100 mM LiCl, 50 mM $CaCl_2$), as has been recommended for plant cells and other cell types [43,44].

2.4. Measurement of photosynthesis, respiration and nitrogenase activity

A thylakoid membrane was prepared by sonication following the method of Lien [45].and activity of the photosystem I (PS I) and photosystem II (PS II) was determined as per Srivastava et al. [35]. The rate of respiration was determined by measuring the total O_2 consumed in the dark for a given time period minus nonspecific O_2 uptake [46].The nitrogenase activity was determined by acetylene reduction assay [47]. For this the cultures were transferred to 13 ml glass vials and sealed with Suba seals. The sealed vials were evacuated and flushed with argon twice and then sparged with argon for 10–15 min. For nitrogenase assay, acetylene was injected at 10% concentration by volume and incubated for two hours under continuous irradiance of 72 μmol photon m^{-2} s^{-1} PAR. The reaction was terminated by injecting 0.8 ml of 15% TCA. The ethylene formed was measured in a Varian CP-3800 gas chromatograph equipped with a Porapak R column and a flame ionization detector. Activity was expressed in terms of nmol C_2H_2 formed mg^{-1} protein h^{-1}.

2.5. Measurement of survival and growth of Anabaena to selected stresses

To decide the dose for treatment of cyanobacteria, cells were treated with different concentrations of Cd^{2+} (0.0–50.0 μM), Cu^{2+} (0.0–50.0 μM), temperature (40–60 °C), UV-B (0–50 min), NaCl (0.0–250 mM) and carbofuran (60–180 ppm). The LC_{50} and lethal doses for Cd^{2+}, Cu^{2+}, temperature, UV-B, NaCl and carbofuran against control *Anabaena* PCC7120 (here after wild type) was determined by the plate colony count method of Rai and Raizada [48]. In this method, the cyanobacterial suspension was homogenized to generate filaments of almost equal length (10–15 cells per filament). Thirty such filaments were counted and the average number of cells per filament was calculated [49]. Aliquots of this culture suspension (0.03 ml) containing 4.39×10^4 cells ml^{-1} were plated onto agar plates supplemented with various doses of the above-mentioned stresses. Colonies were counted after 3 weeks and the survival percentage scored. Growth was estimated by measuring optical density of the cyanobacterial cultures at