Biochimica et Biophysica Acta 1860 (2016) 2576–2588

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Overexpression of AhpC enhances stress tolerance and N_2–fixation in *Anabaena* by upregulating stress responsive genes

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ARTICLE INFO

Article history:
Received 6 May 2016
Received in revised form 18 July 2016
Accepted 28 July 2016
Available online 31 July 2016

Keywords:
Anabaena
AhpC
Transformation
Gene cloning
Heterocyst
Transcript regulation

ABSTRACT

The study explores the significance of peroxides in regulating the CO_2- and N_2-fixation capacities in *Anabaena* sp. PCC7120. To this end *Anabaena* strains were generated carrying an extra copy of *ahpC* (An + ahpC) or by deleting from their endogenous functional *ahpC* (AnΔahpC). *AhpC* levels were 2.2- to 6.0-fold higher in An + ahpC than in wild type. An + ahpC revealed 1.4- to 2-fold upregulation of photosystems I and II, nitrogenase, superoxide dismutase and catalase activities while same activities were 1.3- to 2.5-fold downregulated in the insertional mutant (AnΔahpC) compared to the wild type. Peroxide, superoxide and malondialdehyde contents were low in An + ahpC and high in AnΔahpC. Growth was inhibited in AnΔahpC by approximately 40–60% compared to a 33–40% enhanced growth in An + ahpC under selected stresses. Most interestingly, heterocyst frequency was increased in An + ahpC. In order to address transcriptional and posttranscriptional effects, transcripts of genes including *groEL, fld, kat, gor, gst, dps, bfr, tf, sodA, dnaK, prx, uspA, pcs* and *apx* were quantified and found to be increased 1.33- to 7.70-fold in unstressed and 1.76- to 13.80-fold in stressed An + ahpC. In a converse manner, they were downregulated by 1.20- to 7.50-fold in unstressed and 1.23 to 10.20-fold in stressed AnΔahpC. It is concluded that the level of AhpC controls a major set of metabolic and developmental genes in normal and stress conditions and thus likely is in the core of the redox regulatory system of *Anabaena*.

1. Introduction

Cyanobacteria were the first organisms with oxygenic photosynthesis which colonized the earth >2 billion years ago. Cyanobacterial cells served as ancestors of chloroplasts in algae and plants [1]. Diazotrophic cyanobacteria combine two agriculturally important traits namely photosynthetic CO_2- and nitrogen fixation [2]. They occupy a wide range of environmental niches including extreme environments. They acclimatize to abiotic stresses including drought, salinity, heat, UV-B, heavy metals and xenobiotics [3–5]. This adaptability makes them an excellent model for studying photosynthesis [6,7], cellular differentiation [8], tolerance mechanisms to environmental stresses [9,10] and evolution [11,12]. Reactive oxygen species which are generated in normal metabolism and at enhanced rates upon abiotic stresses trigger upregulation of antioxidant enzymes such as superoxide dismutases, peroxiredoxins, catalases and accumulation of low molecular weight antioxidants like ascorbic acid and glutathione to maintain cellular redox homeostasis. Alkyl hydroperoxide reductase C (AhpC) is a major antioxidant enzyme which detoxifies both organic and inorganic peroxides [13–15] produced in regular metabolism and under abiotic stresses.

Cyanobacterial genomes code for a set of stress tolerance genes. Well studied examples are *smtA* from *Synechococcus* PCC7942 [16,17], glutaredoxin from *Synechocystis* sp. PCC 6803 [18], *pds* (phytoene desaturase) from *Synechococcus* PCC 7942, *fld* (isiB) (flavodoxin) from *Anabaena* sp., *desC* (D9-desaturase) from *Anacystis nidulans*, *pcs* (phytochelatin synthase), *dps* (DNA binding protein under starvation), *alr0882, all0195* and *alr1105* (hypothetical proteins) from *Anabaena* sp. PCC 7120 [19–25]. Surprisingly, the role of cyanobacterial *ahpC* in modulating abiotic stresses has remained largely unaddressed.

AhpC homologues belong to the peroxiredoxin (prx), usually to the subclass of typical 2-CysPrx which are widely distributed among prokaryotes and eukaryotes [25–28]. An exception is AhpC of *Anabaena* sp. PCC 7120 which belongs to the 1-Cys Prx group and thus lacks the regular resolving Cys residue. AhpCs from *Helicobacter pylori* and *Mycobacterium tuberculosis* exist as high molecular weight oligomers, function as chaperone [29,30], regulate signal transduction under peroxide stress [31,32] and exhibit mucin binding ability for cell adhesion and

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http://dx.doi.org/10.1016/j.bbagen.2016.07.031
0304-4165/

colonization [33,34]. AhpC expression increases in response to various stresses such as iron deficiency, heat shock, oxidative stress, or exposure to nalidixic acid, ethanol and hydrogen peroxide [35–37]. *Arabidopsis* with downregulated 2-CysPrx activity, the homologue of AhpC, demonstrated impaired photosynthesis, degradation of D1 protein and LHCPII as well as enhanced ATP synthase protein levels [38]. The doubling time of mutant *Synechocystis* increased from 8 to 14 h while the quantum yield of PS II was severely decreased in light and photoinhibition occurred. Hosoya-Matsuda et al. [39] demonstrated that insertional inactivation of slr1198 having 79% homology with *ahpC* of *Anabaena* sp. PCC7120 partially inhibits the growth of *Synechocystis* PCC6803 under control as well as stressed conditions.

In an attempt to analyse the proteome of *Anabaena* subjected to 53 °C, Mishra et al. [40] observed accumulation of the AhpC thiol-specific antioxidant protein under heat stress. The heterologous expression of this *ahpC* in *E. coli* conferred tolerance to abiotic stresses [41]. In view of lack of data on the role of *ahpC* (*alr4404*) in regulating abiotic stresses in the cyanobacterium *Anabaena* sp. PCC7120, it was decided to develop *ahpC* mutant (AnΔahpC) and *ahpC* overexpressing (An + ahpC) strains of *Anabaena* in order to address its role in abiotic stress management in cyanobacteria. Subsequently the study was developed to cover the cell response to control and stress conditions. Counterintuitive responses of some parameters such as SOD activity under control conditions are suggestive of a central function of AhpC in cell signalling both under control and stress conditions. This will be discussed.

2. Materials and methods

2.1. Culture and growth conditions

Anabaena sp. PCC7120 (here after *Anabaena*) cells were grown in combined nitrogen-free BG-11 liquid medium [BG-11(N^-)] at 27 °C, pH 7.5 under continuous illumination (30 μmol quanta/m^2 s) and with aeration. Cells were inoculated at a chlorophyll *a* density of 1 μg ml^{-1} for all experiments. Cell growth was measured as turbidity (OD_{665}). *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 OD_{600} ml^{-1} for all experiments and growth was assessed either as turbidity (OD_{600}) or colony forming unit (cfu) ml^{-1} on solid media after overnight growth at 37 °C. Ampicillin and kanamycin were used at 100 μg ml^{-1} and 50 μg ml^{-1} concentrations respectively for *E. coli* [42] 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. Bacterial strains and plasmid used in the present study are compiled in Table 1.

Table 1
Bacterial strains and plasmids used in the present study.

Bacterial strains	Relevant characteristics	Source and/or reference
E. coli DH5α	F− recA41 endA1 gyrA96 thi-1 hsd R17(rk − mk −) supE44 relAλ ΔlacU1	Novagen
Anabaena sp. strain PCC7120	Fresh water filamentous heterocystous diazotroph	Novagen
An + ahpC	*Anabaena* 7120 transformed with pFPN	This study
AnΔahpC	*Anabaena* 7120 transformed with pBSahpC*	This study
Plasmids		
pBluescript II SK (+)	A phagemid cloning vector	Chaurasia A.K. et al. (2008)
pFPN	An integrative expression vector for *Anabaena* 7120	Chaurasia A.K. et al. (2008)
pFPN-ahpC	AhpC gene cloned in pFPN at *Nde*I and *Bam*HI	This study
pBSahpC*	Disrupted ahpC gene cloned in pBS plasmid	This study

2.2. Cloning of ahpC gene from Anabaena sp. PCC 7120 in pFPN vector

Genomic DNA from *Anabaena* was isolated as per the method of Srivastava et al. [43]. Gene sequence of *ahpC* was taken from cyanobase (www.genome.kazusa) and gene specific primers were designed using PRIMER 3 software. An open reading frame alr4404, encoding AhpC from the family of thiol-specific antioxidants was amplified by polymerase chain reaction (PCR) using genomic DNA as the template with a pair of forward (AhpCF) and reverse (AhpCR) primers respectively. The underlined bases (Table 2) are restriction sites for *Nde*I and *Bam*HI endonucleases respectively. The PCR was developed in a reaction mixture of 25 μl for 30 cycles at 94 °C for 1.0 min, 63 °C for 1.5 min, and 72 °C for 2 min using standard PCR conditions in an Icycler (Bio-Rad, USA). The amplified PCR product was purified and digested with *Bam*HI and *Nde*I (NEB), and the resultant DNA fragment was cloned into an integrative expression vector pFPN (courtesy Prof. S.K. Apte, BARC, Mumbai) digested with the same restriction enzymes. Transformation of *E. coli* with recombinant plasmid pFPN-ahpC was achieved using the method described by Sambrook and Russell [42]. The recombinant plasmid pFPN-ahpC was introduced into *E. coli* DH5α which was then grown in LB medium. Thereafter the recombinant plasmid was isolated, double-digested and the correctness of the DNA sequence of *alr4404* confirmed by sequencing (Macrogen, Korea).

2.3. Construction of ahpC null mutant cassette

A 1939 nt long sequence including the 650 nt up- and downstream sequence of the *ahpC* gene and the 639 nt long *ahpC* sequence was derived from the cyanobase database. Suitable primers were designed using PRIMER 3 software (Table 2). PCR amplification of the 1939 nt long gene sequence from *Anabaena* sp. PCC 7120 was achieved by using gene specific primers ΔAhpCF and ΔAhpCR (Table 2) where the underlined bases are restriction sites for *Bam*HI and *Not*I endonucleases respectively. The amplified PCR product was purified and digested with *Bam*HI and *Not*I (NEB), and the resultant DNA fragment cloned into a suicidal vector pBS which was digested with the same restriction enzymes. The recombinant plasmid DNA was transformed into *E. coli* [42], and the transformants were selected on ampicillin plates. The plasmid was then isolated, double digested and the correctness of the insert confirmed by sequencing. Plasmid DNA was isolated from *E. coli* and the genomic *ahpC* sequence disrupted by integration of the neomycin cassette. The resultant pBSahpC construct was designated as pBSahpC* (disrupted *ahpC* gene) and selected on neomycin-containing agar plate.

2.4. 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, which allows integration at a defined risk-free site in the *Anabaena* genome and expression from a strong cyanobacterial *psbA1* promoter [44]. The resulting construct designated as pFPN-*ahpC* was electroporated into *Anabaena* 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

Table 2
Primers designed for PCR amplification and DNA sequencing.

Primer name	Primer sequence
AhpCF	5′ GAATTC**CATATG**GAATTCATGGCTCTCCGTCTTGGT 3′
AhpCR	5′CG**GGATCC**CGTTACTTGTTAGGTTGAGGAGT 3′
AnΔAhpCF	5′ CG**GGATCC**CGGTGCCGTTCTTTAACTGATT 3′
AnΔAhpCR	5′ ATTT**GCGGCCGC**TTTAGACATTCTTCTGATTGCA3′
PpsbA1	5′ GAGCTGCAGGGATTCCCAAAGATAGGG 3′