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Novel heterocyst-specific flavodiiron proteins in *Anabaena* sp. PCC 7120

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ABSTRACT

Flavodiiron proteins present in many prokaryotic and some eukaryotic organisms have a capacity to protect cells against nitrosative or oxidative stress. In *Anabaena* sp. PCC 7120, Flv1 and Flv3 proteins are encoded by families of two genes. We demonstrate here that *flv1A* and *flv3A* genes are up-regulated in vegetative cells in low CO_2 and high light conditions. In contrast, *flv1B* and *flv3B* genes are expressed in N_2-fixing conditions and corresponding proteins are located exclusively in heterocysts. It is suggested that Flv1B and Flv3B protect enzymes of N_2-fixation in heterocysts of *Anabaena* 7120 by reducing molecular oxygen directly to water.

1. Introduction

Flavodiiron proteins (FDPs), earlier called A-type flavoproteins, participate in detoxification of O_2 and NO. FDPs were first characterized in anaerobic bacteria, but later they have been found in many other bacteria and archea species as well as in pathogenic protozoa (reviewed in [1]). Intriguingly, cyanobacteria have evolved a specific type of FDPs that conserve the typical module structure and are likely to be capable of performing electron transfer from NAD(P)H to O_2/NO. Among eukaryotic photosynthetic organisms, cyanobacteria-type FDP genes have been found in green algae, mosses and lycophytes, but not in higher plants [2,3].

FDPs of *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*), a non-N_2-fixing, unicellular model organism of cyanobacteria, have been investigated most thoroughly [2,4–8,15]. *Synechocystis* has four genes encoding FDPs; *sll1521, sll0219, sll0550* and *sll0217*, designated Flv1 to Flv4, respectively. Flv1 and Flv3 perform the cyanobacteria-specific Mehler-reaction by reducing O_2 directly to water with NADPH produced on the acceptor side of PS I [4,6,7]. Flv2 and Flv4 form a heterodimer that is involved in alternative electron flow, removing excess electrons from the reducing side of Photosystem (PS) II [2,8].

Anabaena sp. PCC 7120 (hereafter *Anabaena*) is a model filamentous, N_2-fixing cyanobacterium that forms heterocysts, which are special cells where alternative nitrogen metabolism is maintained. When combined nitrogen is present in the growth medium, *Anabaena* does not require the N_2-fixing apparatus located in heterocysts, and forms only vegetative cells for growth, while nitrogen limitation leads to formation of heterocysts. Heterocysts provide an appropriate micro-oxic environment for the key enzymes for N_2-fixation, nitrogenase and uptake hydrogenase, which are extremely sensitive to O_2 (reviewed in [9]).

Unlike *Synechocystis*, the *Anabaena* genome contains six genes encoding FDPs, designated in the present work as *flv1A*, *flv1B*, *flv2*, *flv3A*, *flv3B*, *flv4*. While *flv2* and *flv4* in *Anabaena* are highly orthologous with genes from *Synechocystis* [2], the phylogenetic relationships of the remaining four *flv* genes are more difficult to assess. We demonstrate here that all six *flv* genes are expressed in *Anabaena*, with four expressed only in vegetative cells in response to CO_2 deprivation and/or high light, similar to their orthologues in *Synechocystis*. The two remaining *flv* genes are abundantly expressed under N_2-fixing conditions, and the respective proteins are localized exclusively in heterocysts. We also discuss factors that may explain the duplication of *flv1* and *flv3* genes in the *Anabaena* genome.

2. Materials and methods

2.1. Cyanobacteria growth conditions

Wild type *Anabaena* sp. PCC 7120 was routinely grown in BG11 medium contained 10 mM TES–KOH, pH 8.2, and $NaNO_3$ as a

Abbreviations: FDP, flavodiiron protein; PS, photosystem; ROS, reactive oxygen species; RT-qPCR, real-time quantative RT-PCR

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source of nitrogen at a temperature of 30 °C and under continuous illumination of 50 μmol photons m^{-2} s^{-1}. The cells were supplied with 3% of CO_2 and cultivated under gentle agitation. Exponentially growing cultures at OD_{750} = 1.0 were then shifted to different conditions: (1) from 3% (high carbon, HC) to ambient (low carbon, LC) CO_2 level for 48 h; (2) from BG11 to BG11o growth medium (without source of combined nitrogen) for 24 h; (3) from the growth light (GL) intensity of 50 μmol photons m^{-2} $s^{-1}$to either high light (HL, 220 μmol photons m^{-2} s^{-1}), dim light (DL, 10 μmol photons m^{-2} s^{-1}), or darkness for 1 h.

The mutants Flv1A-YFP, Flv1B-YFP, Flv3A-YFP and Flv3B-YFP were grown constantly on 1,5% agar plates with BG11o supplemented with 25 μg/ml spectinomycin under ambient CO_2.

2.2. Construction of mutant strains with Flv-YFP fusion protein

In order to generate *Anabaena* mutants containing the Flv protein of interest fused to a C-terminal yellow fluorescent protein (YFP) the ORFs of *all0177*, *all3891*, *all0178* and *all3895,* including their upstream regions, were amplified by PCR using all0177-YFP-L-Sal-Fw and all0177-YFP-L-Bam-Rev primers (respective primers for other genes, Table 1) followed by the restriction with SalI and BamHI. The regions downstream to *all0177* and *all3891* were amplified using all0177-YFP-R-RI-Fw and all0177-YFP-R-Spe-Rev primers (respective primers for *all3891*), followed by restriction with EcoRI and SpeI.

all0177 and *all3891* constructs included the SalI–SpeI vector, which carries the *sacB* gene for the positive selection of double recombinants that was isolated from plasmid pRL278, as well as the YFP ORF and the spectinomycin/streptomycin resistance cassette, which were both included in the BamHI–EcoRI fragment obtained from pEYFP-His6-SpR plasmid described in [10]. These four fragments were ligated together, and the resulting plasmid was transferred to *Anabaena* by triparental mating [11]. Clones exhibiting resistance to spectinomycin and sucrose were selected and named Flv1B-YFP and Flv1A-YFP, respectively.

In the case of *all0178* and *all3895*, the SalI–BamI fragment, described below, was fused with vector fragment SalI–XbaI and with BamHI–XbaI containing YFP and resistance cassette. The resulting plasmids were similarly transferred to *Anabaena*, and mutants Flv3B-YFP and Flv3A-YFP were selected with spectinomycin.

2.3. Isolation of total RNA

Whole filaments from 10 ml of culture were harvested by centrifugation at 7000 rpm for 5 min at 4 °C, washed with 20 mM Tris–HCl, pH 7.4, and immediately frozen at −80 °C. Total RNA was extracted by TRIsure reagent (Bioline) treatment at 95 °C for 5 min and purified with 1 unit of DNase (Ambion Turbo DNase kit, USA) to remove genomic DNA. RNA concentration and purity were measured using a NanoDrop spectrophotometer (Thermo Scientific). PCR was performed with 1 μg of total RNA, primers for *rnpB,* and DyNAzyme II DNA-polymerase (Finnzyme), in order to detect any traces of DNA. Integrity of RNA was verified by agarose gel electrophoresis.

2.4. Reverse transcription and RT-qPCR Analysis

One microgram of purified RNA was used for cDNA synthesis. Reverse transcription was performed with random hexamer primers (Promega) and SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer protocol. The generated cDNA was diluted five-fold and used as a template for RT-PCR.

The primer pairs used in this study are summarized in Table 2. The primers were designed to generate amplicons of similar lengths (180–250 bp) so as to obtain uniform amplification with high efficiency ($r^2 > 0.98$; 97% <*E*> 102%). The primer pairs were compared using BLAST against the *Anabaena* PCC 7120 genome (http://www.kazusa.or.jp/cyano) in order to ensure their gene-specificity. Amplification of the expected amplicon fragments was confirmed using agarose gel electrophoresis. The amount of transcripts of the constitutively expressed gene *rnpB*, which encodes the RNA subunit of RNaseP, served as a control [12]. *rpoA* was used as an additional reference gene for samples shifted from BG11 to BG11o [13].

Gene expression was measured in triplicate for each sample by Quantitative Real Time PCR using a BioRad iQ5 Detection System. iQ SYBR Green Supermix (BioRad) supplied with SyBR Green was used as a fluorescent dye to detect accumulation of double-stranded amplicons. Reactions with 5 μl of cDNA and 10 μmol of each primer in a total volume of 25 μl were prepared according to manufacturer's protocol. To confirm the presence and specificity of the expected amplicon product a melting curve analysis was performed after 40 cycles of PCR with amplification at 58 °C. Quantification cycle (C_q) for each reaction was determined using iQ5 Optical System software 2.0 and the relative changes in gene expression were calculated as described earlier in [2]. Samples lacking reverse transcriptase and template were used as negative controls.

2.5. Confocal microscopy

Anabaena cells were visualized using a laser scanning microscope with multi-spectral analyzer Zeiss LSM510 META with Plan apochromat 63x (100x) / 1,4 Oil DIC objective lens attached. YFP was excited using 488-nm irradiation from an argon ion laser. Fluorescent emission was monitored by collection across windows of 510–550 nm (YFP imaging) and 680–720 nm (cyanobacterial autofluorescence).

Table 1
Oligonucleotide sequences used for obtaining mutants with Flv-YFP fusion proteins.

all0177-YFP-L-Sal-Fw	gcaaggtcgacgaaaggggtataggggtgta
all0177-YFP-L-Bam-Rev	cagtcggatccccaccactgccacctccataatgattgccagttttccg
all3891-YFP-L-Sal-Fw	gcatcgtcgacctactaaaactataatcgctc
all3891-YFP-L-Bam-Rev	cagtcggatccccaccactgccacctccataatggttacctgatttgcg
all0178-YFP-L-Sal-Fw	gctccgtcgaccttatgaagtgaggaaatcta
all0178-YFP-L-Bam-Rev	cagtcggatccccaccactgccacctccgtaatagttgcctactttgc
all3895-YFP-L-Sal-Fw	gcaacgtcgacacaaatattccgtgtcacaat
all3895-YFP-L-Bam-Rev	cagtcggatccccaccactgccacctccataatgattacctaccttgcg
all0177-YFP-R-RI-Fw	gctccgaattccagtgtaagggtgtagggg
all0177-YFP-R-Spe-Rev	gtcctactagtctttgaatgtagcaattacatcc
all3891-YFP-R-RI-Fw	gcctcgaattcagactgttttaattacttttttg
all3891-YFP-R-Spe-Rev	gacctactagtctaaacccataagagttagg

Table 2
Oligonucleotide sequences used to perform RT-qPCR.

Gene name	Forward primer (5′ →3 ′)	Reverse primer (3′ →5 ′)
flv1B (*all0177*)	attgtcctcaaggttggtgat	tagcagcttgttggggtaca
flv1A (*all3891*)	tgcagaaacgactagatgtca	aaatccgggttttctagcac
flv3B (*all0178*)	atttggatgtgttgactgagtg	cttgaatttctgctgtgctga
flv3A (*all3895*)	gaggaactcaccggacgtt	gctgcacccaaatctacaac
flv2 (*all4444*)	cgacttttgcccaaacttta	gatcgccatcataattcctg
flv4 (*all4446*)	ctgctattcgctgtttggat	ttcactaagccgctatggtc
rnpB	ggactaggggttggggact	acgagggcgattatctatctg
rpoA	caactctctgtacgggccta	gcttcttttcttggggtaagg
nifH (*all1455*)	cctacgacgtattgggtgac	taaacgtacaccaccggagt