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A second rhodopsin-like protein in *Cyanophora paradoxa*: Gene sequence and protein expression in a cell-free system

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ABSTRACT

Here we report the identification and expression of a second rhodopsin-like protein in the alga *Cyanophora paradoxa* (Glaucophyta), named Cyanophopsin_2. This new protein was identified due to a serendipity event, since the RACE reaction performed to complete the sequence of Cyanophopsin_1, (the first rhodopsin-like protein of *C. paradoxa* identified in 2009 by our group), amplified a 619 bp sequence corresponding to a portion of a new gene of the same protein family. The full sequence consists of 1175 bp consisting of 849 bp coding DNA sequence and 4 introns of 326 bp. The protein is characterized by an N-terminal region of 47 amino acids, followed by a region with 7 α-helices of 213 amino acids and a C-terminal region of 22 amino acids. This protein showed high identity with Cyanophopsin_1 and other rhodopsin-like proteins of Archea, Bacteria, Fungi and Algae. Cyanophosin_2 (CpR2) was expressed in a cell-free expression system, and characterized by means of absorption spectroscopy.

1. Introduction

Over the past 10 years, genome sequencing and sequence comparison tools have revealed that genes encoding rhodopsin-like photoreceptive proteins are shared among distant taxa, in all three domains of life: Archaea, Eubacteria, and Eukarya [1]. Reports from different research groups worldwide have provided examples of the broad distribution of type I rhodopsin-based photoreceptors among the different algal division and allowed the assumption that these proteins are present in all the algae of the supergroups of Plantae (Glaucophyta, Rhodophyta, and Chlorophyta), i.e. those originating from a primary symbiotic event, Chromoalveolata (Haptophyta, Cryptophyta, Ochrophyta, i.e. those originating from a secondary symbiotic event, and Chromoalveolata (Dinophyceae), i.e. those originating from a tertiary symbiotic event [2–4]. In fact, genes encoding functional rhodopsins probably related to photoreception or ionic transport have been detected in Cyanophyceae (prokaryotic algae) [5], and Cryptophyceae [6,7], Glaucophyceae [8], Chlorophyceae [9–12], Dasycladophyceae [13,14], Mesostygmatophyceae [15], Mamiellophyceae [16], prasinophytes [10], Trebouxiophyceae [10], Coccolithophyceae [17], and Dinophyceae [17–19], all eukaryotic algae. Moreover, spectroscopic and biochemical evidence of rhodopsin-based photoreceptors is available for algae belonging also to Euglenophyceae [20], Chrysophyceae [21] and Phaeophyceae [22].

Glaucophyta, one of the three phyla of the supergroup of Plantae and with the lowest number of species, became important in the past few years as model systems to study plastid evolution, since their plastids (called muroplasts) retain the remnants of a gram-negative bacterial peptidoglycan wall that would have been between the two membranes of the cyanobacterial ancestor symbiont, [23]. Recently, genome and transcriptome data from *Cyanophora paradoxa* Korschikov, one of the best known and most well-studied glaucophyte species, have provided evidence for a single origin of the primary plastid in the eukaryote supergroup Plantae, and confirmed the chimeric nature of this alga, which contains a unique combination of ancestral, novel and borrowed (via horizontal gene transfer) genes [24].

In 2009 we identified a first rhodopsin in *C. paradoxa*, and immunolocalized it on the surface of the plastid [8]. Recently, we have identified a second rhodopsin gene in this glaucophyte; we here report the full sequence of the gene, together with the analysis of the structure and *in vivo* and *in vitro* spectroscopic characterization of the protein. The expression of this protein was performed in both *Escherichia coli* and a cell-free expression system. Though the expression succeeded in both systems, the amount obtained by the *E. coli* expression was too low to allow any further manipulation of the protein. On the contrary, the amount obtained by the cell-free expression system allowed us to record the absorption spectrum of the purified protein and to immunodetect it. This is the first report of the expression of a eukaryotic rhodopsin in a ready-to-go cell-free system.

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2. Materials and methods

2.1. Algal cultures

C. paradoxa Korshikov strain 29.80 M was obtained from the Culture Collection of Algae of the University of Gottingen (SAG, Sammlung von Algenkulturen der Universität Göttingen, Germany). Cells were grown in medium CY-II [25] at 23 °C under continuous light (15 μmol photons m^{-2} s^{-1}).

2.2. Preparation of genomic DNA and cDNA

Cyanophora cells were harvested by centrifugation at 4000 xg for 5 min at 4 °C; genomic DNA and total RNA were extracted from 100 mg wet weight of cells using TriPure Isolation Reagent (Roche, USA). cDNA was synthesized from 2 μg of total RNA with oligo $(dT)_{12-18}$ primers (Invitrogen, USA) in reverse transcription PCR, using SuperScript™ II RNase H-RT (Invitrogen, USA) according to the manufacturer's suggested protocol.

2.3. cDNA amplification, cloning and sequencing

Cyanophopsin_2 (CpR2) was identified due a serendipity event, since the RACE reaction performed to complete the sequence of Cyanophopsin_1, (GQ402542) amplified a 619 bp sequence corresponding to a portion of a new gene of the same protein family. In particular, cDNA was subjected to PCR amplification, using the primer Rev_1 (5′-GGAGATGCCGAACCAGCC-3′) in combination with the anchor primer at the 5′-terminus in a 5′RACE-reaction (5′RACE System for Rapid Amplification of cDNA Ends, version 2.0, Invitrogen, USA). PCR conditions were: 15 min at 95 °C; 35 cycles of 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C; 7 min at 72 °C. The amplified product was purified using NucleoSpin extract kit (Macherey–Nagel, Germany) and cloned in the pGem®-T Vector System I (Promega, Madison, WI). 5′RACE products were sequenced by EUROFINS MWG|OPERON (Germany). A gene-specific primer Fw_1 (5′-TGGGATCGCTATGCGGAGTGG-3′) was designed on the 619 bp sequence and used in combination with the anchor primer at the 3′-terminus in a 3′RACE-reaction. PCR conditions were: 15 min at 95 °C; 35 cycles of 1 min at 94 °C, 1 min at 59 °C, 1 min at 72 °C; 7 min at 72 °C. 3′RACE products were cloned and sequenced as described above, resulting in a 663 bp sequence. The 5′ and 3′RACE sequences showed an overlapping region of 186 bp, resulting in a full length cDNA sequence of 1096 bp. PCR amplification using the gene-specific primers, Fw_2 (5′-CATATGGCGTCCGTCCTGATGGCATTC-3′) introducing a NdeI recognition site, and Rev_2 (5′-GAATTCTTACAGCTTCGACGTCTTCAC-3′) introducing a EcoRI recognition site, produced a 849 bp Coding DNA Sequence (CDS) of the new protein CpR2. This PCR product was cloned into the pGem®-T vector sistem I (Promega, USA) and sequenced by EUROFINS MWG|OPERON (Germany).

2.4. Amplification of genomic DNA

The two gene-specific primers (Fw_2 and Rev_2) were used for the amplification of the rhodopsin-coding gene from genomic DNA. A 1175 bp band was produced, consisting of 849 bp exons and 326 bp introns. This band was cloned into the pGem®-T vector sistem I (Promega, USA) and sequenced from both sides using T7 and Sp6 primers (EUROFINS MWG|OPERON, Germany).

2.5. Plasmid construction

The cDNA was PCR-amplified with the Fw_2 primer and the new primer Rev_3 (5′-GAATTCAGCTTCGACGTCTTCACGAG-3′) introducing a EcoRI recognition site, and in frame with the 6*x*His-tag at the C-terminal of the expression vector pET20b(+) (Novagen, CA, USA). The amplified product was cloned in pGem®-T vector sistem I (Promega, Madison, WI), and the selected colony used for the plasmid extraction using EuroGOLD Plasmid Miniprep Kit I (EuroClone, Italy). The isolated plasmid-DNA was digested with the restriction endonucleases NdeI and EcoRI (New England BioLabs). Finally, the digestion product was cloned into the expression vector pET20b(+) and the plasmid purified using PureLink™ HiPure Plasmid Midiprep kit (Invitrogen, USA), yielding the plasmid named pET20b_CpR2.

2.6. Expression and purification of Cyanophopsin_2 in E. coli

E. coli BL21(DE3) harboring pET20b_CpR2 was grown at 37 °C in a shaking incubator set at 200 rpm, in LB medium supplemented with ampicillin (100 μg ml^{-1} final concentration) and 10 μM all-trans retinal. When the absorbance at 600 nm of the cultures reached 0.7, isopropyl-β-D-thiogalactopyranoside (IPTG) (Promega, USA) was added to a final concentration of 0.4 mM to induce the gene expression, together with 10 μM all-trans retinal. After an additional incubation of 120 min, cells were harvested, sonicated on ice and centrifuged at 12,000 xg for 20 min at 4 °C. The pellet was collected and washed three times with Tris–HCl 50 mM, pH 7.4. Protein purification according to the protocol of Kelemen et al. [26], achieved no results.

2.7. Cell-free protein expression and purification of Cyanophopsin_2

A 1 ml cell-free protein synthesis reaction was performed using Membrane Max Protein Expression Kit (Invitrogen, USA) combined with our T7-based expression vector pET20b_CpR2 and 10 μl 10 mM all-trans retinal. The standard reaction mixture was optimized in the amount of the reagent used, and incubated 150 min at 29 °C in a shaking incubator set at 300 rpm. The native recombinant protein, polyhistidine-tagged, was incubated with His-Tag affinity resin in TALONspin™ column (Clontech, USA) that had been equilibrated with 1 ml wash buffer containing 0.05 M sodium phosphate (pH 7.0) and 300 mM NaCl. The resin was washed three times with 1 ml wash buffer containing 0.05 M sodium phosphate (pH 7.0) and 300 mM NaCl. CpR2 were eluted from the resin two times with 0.4 ml elution buffer containing 0.05 M sodium phosphate (pH 7.0) and 300 mM NaCl and 150 mM imidazole. The eluted CpR2 samples were pooled together and acetone precipitated. The pellet obtained was resuspended in 550 μl of DNAase and RNAase free water.

2.8. Spectroscopy

About 500 μl of the purified Cyanophopsin_2 solution was kept overnight in the dark at room temperature, and the absorption spectrum of the dark adapted sample was measured from 300 nm to 700 nm, using a a Jasco V-550 spectrophotometer (Jasco Inc., Easton, MD, USA).

2.9. Western blot

About 50 μl of the purified Cyanophopsin_2 solution was added to 200 μl acetone and well mixed. The solution was centrifuged at 1000 xg for 5 min at room temperature. After removal the supernatant the pellet was resuspended in 20 μl of denaturing sample buffer, subjected to electrophoresis in denaturing 12% polyacrilamyde gel and transferred to nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk, (w/v) in PBS (pH 7.3) and incubated overnight with the antibody Anti-His (C-term) alkaline phosphatase (AP) – conjugated (Novex® by Life