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Inactivation of the conserved open reading frame *ycf34* of *Synechocystis* sp. PCC 6803 interferes with the photosynthetic electron transport chain

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ABSTRACT

Ycf34 is a hypothetical chloroplast open reading frame that is present in the chloroplast genomes of several non-green algae. Ycf34 homologues are also encoded in all sequenced genomes of cyanobacteria. To evaluate the role of Ycf34 we have constructed and analysed a cyanobacterial mutant strain. Inactivation of *ycf34* in *Synechocystis* sp. PCC 6803 showed no obvious phenotype under normal light intensity growth conditions. However, when the cells were grown under low light intensity they contained less and smaller phycobilisome antennae and showed a strongly retarded growth, suggesting an essential role of the Ycf34 polypeptide under light limiting conditions. Northern blot analysis revealed a very weak expression of the phycocyanin operon in the *ycf34* mutant under light limiting growth in contrast to the wild type and to normal light conditions. Oxygen evolution and P_{700} measurements showed impaired electron flow between photosystem II and photosystem I under these conditions which suggest that the impaired antenna size is most likely due to a highly reduced plastoquinone pool which triggers regulation on a transcriptional level. Using a FLAG-tagged Ycf34 we found that this protein is tightly bound to the thylakoid membranes. UV–vis and Mössbauer spectroscopy of the recombinant Ycf34 protein demonstrate the presence of an iron–sulphur cluster. Since Ycf34 lacks homology to known iron–sulphur cluster containing proteins, it might constitute a new type of iron–sulphur protein implicated in redox signalling or in optimising the photosynthetic electron transport chain.

1. Introduction

Cyanobacteria are the ancestors of chloroplasts due to an endosymbiotic event that occurred two billion years ago [1]. For nearly all proteins that are encoded by chloroplast genome orthologues exist in cyanobacteria. Amongst them are several highly conserved putative genes with still an unknown function. Although they are often annotated as *ycf* (hypothetical chloroplast open reading frame) most *ycf* gene products that have been analysed are functional and their cellular role is in many cases related to photosynthetic processes and pigment synthesis. An overview of the known functions of *ycfs* is provided in [2]. On the other hand many plant genes of cyanobacterial origin have been transferred to the nucleus during evolution. Based on a bioinformatic analysis more than 3600 open reading frames from *Arabidopsis thaliana* appear to originate from the ancestral cyanobacterial-like endosymbiont [3]. In another study nuclear-encoded chloroplast proteins of endosymbiotic origin (CPRENDOs) were identified by a combination of bioinformatics and experimental analyses [4]. Some of these genes are retained in the chloroplast genome of at least a few algae. Interestingly, inactivation of 40 of these CPRENDOs genes using the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803) yielded 22 mutants exhibiting a phenotype related to photosynthesis, thus, indicating important functions of these hypothetical open reading frames in photosynthetic organisms [4].

Besides *ycf* genes that are present virtually in all chloroplasts there are examples of *ycf* genes that have been retained only in the chloroplast genomes of red algae but are present in nearly all cyanobacteria like *ycf18* (*nblA*), that is involved in phycobilisome degradation [5,6]. Further, more conserved *ycfs* constitute subunits of the cytochrome b_6f complex (*ycf6*/*petN*) [7] or of photosystem (PS) II (*ycf8* and *ycf12* [8,9]). Others

Abbreviations: WT, wild type; *ycf*, hypothetical chloroplast open reading frame; CPRENDO, chloroplast proteins of endosymbiotic origin; ORF, open reading frame; PpBQ, phenyl-*p*-benzoquinone; β-DM, *n*-dodecyl-β-maltoside; PS I, photosystem I; PS II, photosystem II; PQ, plastoquinone; EPR, electron paramagnetic resonance

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encode proteins with a function in pigment synthesis (*ycf59* and *ycf53* [10,11]) or with a role in the biogenesis of *c*-type cytochromes (*ycf5* and *ycf44* [12–14]) that are essential elements of the electron transport chain.

Ycf34 is a hypothetical open reading frame conserved in all cyanobacterial lineages. Interestingly, the *ycf34* gene is specifically distributed in the chloroplast genomes of *Cyanophora paradoxa*, red algae and several brown algae harbouring red algal derived plastids. No orthologues of this gene are found in the nuclear genomes of higher plants or green algae. In the present study we analyse the function of Ycf34 in cyanobacteria using a *Synechocystis* 6803 *ycf34* (*ssr1425*, [15]) knockout strain and the recombinantly expressed gene product.

2. Material and methods

2.1. Bacterial strains and growth conditions

The motile strain of *Synechocystis* 6803 used in this study was originally obtained from S. Shestakov (Moscow State University, Russia) and propagated on BG11 agar plates (0.75% (w/v)) [16]. Liquid cultures of *Synechocystis* 6803 wild type (WT) and mutant strains were grown at 30 °C under continuous illumination with white light (Philips TLD Super 80/840) of 3.5 or 50 μmol photons m^{-2} s^{-1} in BG11 medium containing 10 mM TES buffer (pH 8.0). For the mutants described below, the media were supplemented with antibiotics at the following concentrations: chloramphenicol, 7 μg ml^{-1}; kanamycin, 80 μg ml^{-1}.

Escherichia coli strains DH5α and GM2163 were used for cloning procedures. *E. coli* cultures were grown in LB media, supplemented with antibiotics at standard concentrations.

2.2. Mutagenesis

For insertion mutagenesis, the *ycf34* ORF (locus *ssr1425*, [15]) was amplified as a 3950 bp fragment including the adjacent genes *pcnB/sll0825* (polyA polymerase) and *ndhF1/slr0844* (NADH dehydrogenase subunit 5) using the primers P1 and P2 (Table S1) and ligated into the pGEM-T vector (Promega, Germany). The kanamycin resistance cassette excised with PvuII from the pUC4K vector was inserted into a BsaBI restriction site within the *ycf34* gene sequence. The resulting construct was used for the transformation of *Synechocystis* 6803 WT cells as described by Ermakova et al. [17]. Transformants were restreaked eight times with increasing concentrations of kanamycin and analysed by Southern blot hybridization and PCR using primers P9 and P10 to validate the level of segregation of mutant genome copies.

The complementation of the *ycf34* gene disruption mutant was initially approached by amplification of the entire *ycf34* ORF using primers P3 and P4 and ligation into the pJET1.2 vector (Fermentas, Germany). The resulting 266 bp fragment was excised with NdeI and BglII and subsequently ligated into the pSK9 vector (S. Zinchenko, Moscow State University, Russia), digested with NdeI and BglII, respectively. Oligonucleotides encoding the 3x-FLAG epitope with artificial 5′-TA overhangs (P11 and P12) were hybridized and ligated into the vector pSK9-Ycf34, linearised with NdeI. Oligonucleotides containing the sequence of the *oop* transcription terminator from phage lambda and additional 5′-GATC overhangs (primer pair P7 and P8) were ligated into the vector pSK9-FLAG-Ycf34, digested with BglII. The resulting construct was used for the transformation of *ycf34::aph* mutant cells. Transformants were restreaked four times with increasing concentrations of chloramphenicol. Transformants were analysed by PCR (P13 and P14) to validate the level of segregation of the FLAG-*ycf34* gene copy. The probe used in the Southern blot analysis was amplified by PCR using primers P9 and P10. The resulting 251 bp fragment containing the sequence of *ycf34* was labelled with digoxigenin-dUTP using a DIG high prime DNA labelling kit (Roche Applied Science, Germany).

2.3. RNA isolation and Northern blot hybridization

Exponentially growing *Synechocystis* 6803 liquid cultures ($OD_{750\ nm}$ = 0.4–0.6, UV-2401 PC, Shimadzu, Japan) were collected by quenching on ice and immediate centrifugation at 4 °C. Cell pellets were resuspended in 1 ml PGTX reagent [18] per 20 ml aliquot and total RNA was isolated according to the author's instructions with following modifications: After the first extraction the aqueous phase was additionally mixed with one volume of 1-bromo-3-chloropropane and the two phases were separated again. The RNA in the retrieved aqueous phase was precipitated overnight at −20 °C. The purified RNA was quantified using a NanoDrop ND-1000 spectrophotometer (PEQLAB Biotechnology, Germany), separated by electrophoresis on 1.3% agarose/formaldehyde gels and blotted onto Roti-Nylon plus (Carl Roth, Germany) membranes [19]. Hybridization probes were generated either by random prime labelling (Rediprime II labelling kit, GE Healthcare, Germany) of PCR products (*ndhF3*: P24 and P25; 16S rRNA: P26 and 27, Table S1) with [α-^{32}P]-dCTP (Hartmann Analytic, Germany) or by in vitro transcription of PCR fragments from the T7 promoter (*cpcBAC2C1D*: P20 and P21; *glnA*: P22 and P23, Table S1) in the presence of [α-^{32}P]-UTP (Hartmann Analytic) using the T7 polymerase Maxiscript kit (Ambion, USA). Following prehybridization at 55 °C (for DNA probes) or 68 °C (using riboprobes) for at least 30 min in 50% deionised formamide, 7% SDS, 250 mM NaCl and 120 mM sodium phosphate (pH 7.2), the labelled probes were added and hybridized for 3–16 h. The membranes were rinsed in 2× SSC/0.5% SDS and washed twice for 15 min at 68 °C in 2× SSC/0.5% SDS and 0.1× SSC/0.1% SDS, respectively. Signals were detected and analysed on a Personal Molecular Imager FX system using the Quantity One software (Bio-Rad, USA). All DNA oligonucleotides are listed in Table S1.

2.4. Mapping of the transcription initiation start site

The 5′-RACE was carried out as described previously [20,21], with following modifications: 5′-triphosphates were converted to 5′-monophosphates by treatment of 2 μg total RNA (obtained from WT cells grown to an OD_{750} of 0.6) with 10 U of tobacco acid pyrophosphatase (TAP, Epicentre Technologies, USA) at 37 °C for 60 min. Control RNA was incubated in the absence of TAP. Reactions were stopped by phenol/chloroform extraction, followed by ethanol/sodium acetate precipitation. Pellets were dissolved in water, mixed with 40 pmol of 5′-RNA adapter (P15), and ligated at 37 °C for 1 h with 4 U T4-RNA ligase (Epicentre Technologies). Phenol/chloroform-extracted, ethanol-precipitated RNA was then reverse-transcribed using 2 pmol gene specific primer (P16) and the SuperScriptIII RT system (Invitrogen, Germany) in a total volume of 20 μl. Reverse transcription was performed at 55 °C for 1 h. The RT enzyme was inactivated at 70 °C for 10 min, followed by RNase H (Fermentas, 5 U)-treatment at 37 °C for 20 min. 2 μl of this cDNA served as template in a standard PCR using Phusion polymerase (Finnzymes, Finland), and 10 pmol each of a gene specific primer (P16) and a linker specific primer (P17). Products were separated on 2% agarose gels, bands of interest excised, gel eluted (QiaQuick gel extraction, Qiagen, Germany) and amplified in a second PCR using nested primers (P18 and P19) to increase the DNA yield for subsequent cloning.

2.5. Determination of the pigment and protein content

Chlorophyll contents of thylakoid membranes were measured in 80% acetone according to MacKinney [22]. Phycocyanin and allophycocyanin contents were determined in the soluble protein fraction of cell extracts [23]. The total protein concentration from whole cell extracts was assayed by a modified Lowry method [24] and used for normalization of the pigment content.