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ORIGINAL ARTICLE

The hypothetical protein Ycf46 is involved in regulation of CO_2 utilization in the cyanobacterium *Synechocystis* sp. PCC 6803

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Abstract

Main conclusion **The Ycf46 mutant of *Synechocystis* showed growth inhibition under low dissolved CO_2 conditions, suggesting a role for the Ycf46 protein in the process of photosynthetic CO_2 uptake and utilization.**

Abstract Hypothetical chloroplast open reading frame Ycf46 proteins are highly conserved in all cyanobacterial lineages and most algal chloroplast genomes, but their exact function is still unknown. In the cyanobacterium *Synechocystis* sp. PCC 6803, the Ycf46 encoding gene *slr0374* is part of an operon (with *slr0373* and *slr0376*) and responds to many environmental stresses. Transcript levels of the *slr0373*, *slr0374* and *slr0376* genes were increased under a low concentration of dissolved inorganic carbon (C_i). Compared with the wild type, the mutant lacking *slr0374* showed growth arrest under C_i-deficient conditions but not under iron-deficient or low-light conditions. In addition, the mutant grew more slowly than the wild type under pH 6.0 conditions in which CO_2 was the dominant C_i source, indicating the mutant cells had weak CO_2 uptake and/or utilization ability. Supplying a high concentration of CO_2 (5 %, v/v) to the mutant restored its phenotype to the wild type level. The photosynthetic activity of the mutant was inhibited to a lesser extent by a carbonic anhydrase inhibitor than that of the wild type, which specifically blocked CO_2 uptake. Inactivation of *slr0374* decreased expression of the *ecaB* gene and reduced carbonic anhydrase activity. A subcellular localization assay indicated that the Ycf46 protein was soluble. By co-immunoprecipitation assay using Slr0374 as a bait-protein, potential interacting proteins in the size range of 30 kDa were identified. These results suggest that the Ycf46 protein plays a role in the regulation of photosynthesis in cyanobacteria, especially in CO_2 uptake and utilization.

Keywords Carbonic anhydrase · Cyanobacteria · Inorganic carbon utilization · Ycf46

Abbreviations

AAA	ATPases associated with diverse cellular activities
CA	Carbonic anhydrase
CCM	CO_2-concentrating mechanism
C_i	Dissolved inorganic carbon
Co-IP	Co-immunoprecipitation
EZ	Ethoxyzolamide
LC–MS/MS	Liquid chromatography with tandem mass spectrometry
ncRNA	Non-coding RNA
qRT-PCR	Quantitative reverse transcription PCR
Ycf	Hypothetical chloroplast open reading frame

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Introduction

Cyanobacteria, the only prokaryotic organism that perform oxygenic photosynthesis, have excellent capability to adapt to various environmental stresses. During their long-term

evolution, cyanobacteria have developed efficient CO_2-concentrating mechanisms (CCMs) by which cells can raise their CO_2 concentration up to 1,000-fold around the active site of Rubisco and perform efficient photosynthesis even under dissolved inorganic carbon (C_i or DIC) limiting conditions (Badger and Price 2003). In the past 30 years there has been a rapid increase in our understanding of these mechanisms, especially of the genes and protein components involved in cyanobacterial CCMs. To date, five DIC transport systems have been identified including three HCO_3^- transporters and two CO_2 transporters: (1) BCT1, an inducible high-affinity Na^+-independent HCO_3^- transporter encoded by the *cmpABCD* operon (Omata et al. 1999); (2) SbtA, an inducible high-affinity Na^+-dependent HCO_3^- transporter encoded by a single *sbtA* gene (Shibata et al. 2002); (3) BicA, a low-affinity high-flux Na^+-dependent HCO_3^- transporter encoded by *bicA* (Price et al. 2004); (4) a constitutive CO_2 uptake system based on the NDH-I_4 complex and potentially located on the plasma membrane, encoded by *ndhD4/ndhF4/cupB* (Shibata et al. 2001); and (5) an inducible CO_2 uptake system based on the NDH-I_3 complex and located on the thylakoid membrane, encoded by *ndhD3/ndhF3/cupA* (Shibata et al. 2001).

Despite a good understanding of CCM components, our knowledge of how cyanobacterial cells sense C_i-limitation signals and regulate inorganic carbon utilization is still limited. When exposed to C_i-limitation, cyanobacteria enhance the expression of inducible transporters and increase the number of carboxysomes (Price et al. 2008). It has been reported that one or two LysR-type transcription regulators, known as CcmR (NdhR) and CmpR, control the regulation process (Omata et al. 2001; Wang et al. 2004), although the precise signal transduction pathways are still unknown. To further clarify the CCM regulation mechanisms in cyanobacteria, DNA microarray analyses were carried out in the model species *Synechocystis* sp. PCC 6803 (Wang et al. 2004; Eisenhut et al. 2007). The microarray results indicated that when cells were transferred from high C_i (5 % CO_2) to low C_i (0.035 % CO_2) conditions, dozens of genes with unknown functions were strongly up-regulated, besides the genes encoding inducible inorganic carbon transporters such as NDH-1_3, BCT1, and SbtA (Eisenhut et al. 2007).

Using genetic transformation, we knocked out 22 of these unknown genes in *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803), to identify the functional genes involved in inorganic carbon utilization. Among them, a hypothetical chloroplast open reading frame 46 encoding gene *ycf46* (*slr0374*) was previously reported to be responsive to many environmental stresses, but its exact function is still unknown (Singh and Sherman 2002). In this study, *ycf46* (*slr0374*) was characterized as being involved in inorganic carbon utilization.

Materials and methods

Strains and culture conditions

The glucose-tolerant strain *Synechocystis* 6803 was used in this study (Williams 1988). Cyanobacterial mutants were generated by inserting a kanamycin-resistant cassette, C.K2, into the genome of wild-type cells as described previously (Jiang et al. 2010, 2012). The *slr0374-flag* strain was generated by fusing the FLAG coding sequence (GAC TAC AAG GAC GAC GAC GAC AAG) to the C-terminus of the *slr0374* gene. Complete segregation strains were verified by PCR (primers are listed in Supplemental Table S1). The wild-type and mutant strains were cultured in BG11 liquid medium buffered with 20 mM HEPES–KOH (pH 8.0) at 30 °C under continuous fluorescent cool white lights (40 μmol photons m^{-2} s^{-1}). To prepare strict C_i-limited cultures, Na_2CO_3 was removed from the BG11 medium. Different pH treatments were conducted using 20 mM 2-(4-morpholino) ethanesulfonic acid (MES) for pH 6.0, and 20 mM 3-(cyclohexylamino)-1-propanesuhinic acid (CAPS) for pH 10.0. The pH value of the growth medium was measured using a pH meter (Mettler Toledo, Shanghai, China). The growth of the cultures was monitored by measuring the optical density at 730 nm (OD_{730}) with a Cary-300 UV/visible light spectrophotometer. Specific growth rates were calculated as follows: $[(\log OD_{t2} - \log OD_{t1})/\log 2]/(t_2 - t_1)$, where OD_{t1} is the turbidity (optical density at 730 nm) after t_1 days and OD_{t2} is the turbidity (optical density at 730 nm) after t_2 days.

RNA isolation and transcriptional level analysis

Synechocystis 6803 cells of the wild-type and mutant strains were collected by centrifugation and total RNA was isolated using the TRIzol Reagent (Invitrogen). To remove DNA contamination, the RNA solutions were digested with 4 units of RNase-free DNase (Promega) according to the manufacturer's instructions. The digest was extracted with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1), and the RNA was pelleted by centrifugation after overnight precipitation with 200 mM LiCl and 75 % (v/v) ethanol at −80 °C. For quantitative RT-PCR analysis, first-strand cDNA was synthesized using the M-MLV Reverse Transcriptase (Promega) according to the manufacturer's instructions. The cDNA products and primers specific for the CCM-related genes *cmpA* (*slr0040*), *bicA* (*sll0834*), *sbtA* (*slr1512*), *ndhF3* (*sll1732*), *ndhF4* (*sll0026*), *ccaA* (*slr1347*), *ecaB* (*slr0051*) and *ccmN* (*sll1032*) were then used for PCR amplification. The RNase P subunit B gene (*rnpB*) was selected as the internal control for normalizing target transcript abundance. Amplifications were performed in 20-μL reaction mixtures using the SYBR Green

Realtime PCR Master Mix (TOYOBO); the primers used in these experiments are shown in Supplemental Table S1. PCR amplification was performed under the following conditions: DNA polymerase activation at 95 °C for 1 min, denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s, product extension at 72 °C for 45 s, and signal acquisition at 85 °C for 1 s. After the last cycle of amplification, the rotor temperature was ramped from 55 to 99 °C at 0.2 °C s^{-1} for melting curve analysis. Relative quantification of transcripts in this study was estimated using the $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen (2001): $\Delta\Delta C_T = (C_{T,Target} - C_{T,rnpB})_{slr1336\ \mathrm{mutant}} - (C_{T,Target} - C_{T,rnpB})_{WT}$.

Cellular location of the *slr0374* gene product

The *slr0374-flag* strain cells were collected by centrifugation, resuspended in 40 mM Tris–HCl (pH 8.0) with 1 mM phenylmethylsulfonyl fluoride (PMSF), and ruptured with a UP200S ultrasonic processor (Hielscher Ultrasound Technology, Germany) in an ice bath for 15 min at 40 % amplitude and a 30 % duty cycle. Cell debris and unbroken cells were removed by centrifugation at 11,000*g* at 4 °C for 10 min. Total membrane proteins and cytosolic soluble proteins were separated by centrifugation at 103,000*g* and 4 °C for 30 min. The total membranes and supernatant were then used for western blot detection. SDS-PAGE and western blotting were performed with standard methods. Briefly, equal amounts of the proteins were loaded, separated by 12 % SDS-PAGE, transferred to nitrocellulose filters (Millipore, Ireland), detected with anti-Flag or anti-CP47 antibody, respectively, and visualized with goat anti-mouse or anti-rabbit alkaline phosphatase antibody with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (Amresco) as the substrates. The anti-Flag antibody was purchased from the Sigma company and the anti-CP47 antibody was a kind gift from Professor Xudong Xu (Institute of Hydrobiology, Chinese Academy of Sciences) (Gao and Xu 2009).

CA activity assays and oxygen evolution measurements

A CA assay was conducted by measuring the rate of pH change after the injection of a standard amount of CO_2-saturated water as described previously in Jiang et al. (2013). Steady-state oxygen evolution (P) was measured with a Clark-type oxygen electrode (Hansatech) at 30 °C under saturating light. Before measurement, the cells were collected and resuspended in fresh BG11 medium containing 20 mM HEPES (pH 8.0) and 1 mM $KHCO_3$, at a chlorophyll concentration of 4 μg mL^{-1}. The measurements were performed by exposing a 2-mL cell suspension to 560 μmol photons m^{-2} s^{-1} white light, with 300 μM EZ added to inhibit CA activity. Relative O_2 evolution rates were calculated using the following equation: relative O_2 evolution rate $= (P_{-EZ} - P_{EZ})/P_{-EZ}$.

Co-immunoprecipitation (Co-IP) and pull-down assay

To screen for proteins interacting with Slr0374, anti-Flag monoclonal mouse antibody (Sigma) and a Protein A/G Plus-Agarose Immunoprecipitation Reagent (SC2003, Santa Cruz Biotechnology) were used for Co-IP assay of the total proteins of the *Synechocystis slr0374-flag* strain. The *Synechocystis slr0374-flag* strain was grown to exponential phase in BG11 medium under the standard conditions described above. The cells were collected by centrifugation, resuspended in lysis buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1 % Triton-X-100, 0.1 % SDS] with part of a protease inhibitor cocktail (Roche), and ruptured with a UP200S ultrasonic processor (Hielscher Ultrasound Technology, Germany) in an ice bath for 20 min at 40 % amplitude and a 30 % duty cycle. Cell debris and unbroken cells were removed by centrifugation at 11,000*g* at 4 °C for 10 min. The supernatant was pre-incubated with about 1 μg normal mouse IgG antibody and 20 μL Protein A/G Plus-Agarose at 4 °C for 30 min, and then centrifuged at 1,000*g* and 4 °C for 5 min. About 1 mL of the supernatant (including 100–500 μg total proteins) was transferred to a new 1.5 mL Eppendorf tube. About 1 μg anti-Flag antibody (Sigma) was added to the tube and incubated at 4 °C for 1 h, and then 20 μL Protein A/G Plus-Agarose was added and incubated at 4 °C for overnight. The beads were then harvested by centrifugation at 1,000*g* and 4 °C for 5 min, and washed with lysis buffer three times. After the final wash, the beads were resuspended with 1× SDS loading buffer and analyzed by SDS-PAGE. The bands differing between the *Synechocystis slr0374-flag* strain and the wild type were cut out for identification by LC–MS/MS analysis. This analysis was performed over a 70-min run on an LTQ-Orbitrap mass spectrometer (Thermo-Fisher Scientific) connected to an Agilent 1200 quaternary HPLC Pump (Pandy and Mann 2000; Xu and Peng 2006). To confirm the identification by LC–MS/MS, the proteins were expressed in the *E. coli* BL21 (DE3) strain with a His-tag. The His-tag fused proteins from the recombinant *E. coli* strains together with the proteins from the *Synechocystis slr0374-flag* strain were pulled down by purification with an Ni-NTA His·Bind® resin (Novagen). The resulting proteins were detected by SDS-PAGE and western blotting with anti-Flag and anti-His-tag antiserum, respectively. SDS-PAGE and western blotting were performed using standard methods. Equal amounts of proteins were loaded, separated by 12 % SDS-PAGE, transferred to nitrocellulose filters (Millipore, Ireland), detected with anti-Flag monoclonal mouse antiserum

(Sigma) or anti-His-tag monoclonal mouse antiserum (Sigma), and visualized with goat anti-mouse alkaline phosphatase antibody with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (Amresco) as the substrates.

Results and discussion

There are two hypothetical chloroplast open reading frame 46 genes (*slr0374* and *slr0480*) in *Synechocystis* 6803, but only *slr0374* is required for acclimation to C_i-limiting conditions

Twenty-two genes or gene clusters in *Synechocystis* 6803 were knocked out (gene names are listed in Supplemental Table S2). These genes were chosen according to the DNA microarray results of Wang et al. (2004) and Eisenhut et al. (2007). All of the genes were highly up-regulated by a change from high C_i (5 % CO_2) to low C_i (0.035 % CO_2) conditions (Wang et al. 2004; Eisenhut et al. 2007). Through genetic transformation and mutant phenotype analyses, we found that all of the mutants had a similar growth phenotype to the wild type when they were cultured in standard BG11 medium. However, when the mutants were transferred to a more C_i-limiting medium, in which Na_2CO_3 was removed (without shaking and CO_2 aeration), the *slr0374* mutant showed a much lower growth rate compared with the wild type. Note that 'C_i-limiting conditions' in this study refers to culture in BG11 medium without carbonate or bicarbonate addition. Under these conditions, cells can only obtain carbon from the dissolution of atmospheric CO_2 into the medium.

The *slr0374* gene is annotated as a hypothetical chloroplast open reading frame 46 (*ycf46*). Another *ycf46* homologous gene, *slr048*0, was also found in the genome of *Synechocystis* 6803 with 61 % amino acid sequence similarity. Ycf46 homologues are highly conserved in different algae, but missing in green algae and higher plants (Table 1; Supplemental Fig. S1). By investigating the model of Martin et al. (1998) for gene transfer to the nucleus and the evolution of chloroplasts, we found that Slr0374 in *Synechocystis* 6803 had high similarities with homologues from *Porphyra yezoensis* (E-value, $1e^{-94}$), *Porphyra purpurea* (E-value, $1e^{-91}$), *Guillardia theta* (E-value, $2e^{-96}$), *Odontella sinensis* (E-value, $2e^{-95}$) and *Emiliania huxleyi* (E-value, $1e^{-90}$) (Table 1). Interestingly, no homologues were found in green algae or higher plants, suggesting the loss of the *ycf46* gene was a relatively late evolutionary event.

Generally, conserved chloroplast open reading frame proteins are involved in photosynthesis, but the exact functions of many *ycf* genes including *ycf46* in cyanobacteria remain unknown (Mäenpää et al. 2000). To clarify the exact roles of the two *ycf46* genes, we constructed two single mutants (*Δslr0374* and *Δslr0480*) and a double mutant (*Δslr0374/slr0480*). Complete segregation mutants were obtained after repeatedly growing cells in medium containing antibiotics and were confirmed by PCR on genomic DNA (Fig. 1a). In phenotype analysis, the *Δslr0374* mutant, but not the *Δslr0480* mutant, showed a significantly reduced growth rate compared with the wild type under C_i-limiting conditions (t test, $P < 0.05$) (Fig. 1b). The double mutant *Δslr0374/slr0480* showed a similar growth rate to the *Δslr0374* single mutant (Fig. 1b). These results demonstrated that *slr0374* played an important role in the acclimation to C_i-limitation, but the role of the *slr0480* gene in this process was negligible. Hence, we focused on only the *slr0374* gene in the following research.

The *ycf46* gene *slr0374* was induced by C_i-limitation

The *slr0374* gene, together with *slr0373* and *slr0376*, belongs to the *slr0373-slr0374-slr0376* operon (Singh and Sherman 2002). There is no gene named "*slr0375*" in the genome of *Synechocystis* 6803, but it has been noted that there is a non-coding RNA (ncRNA) between the *slr0374* and *slr0376* genes (Voß et al. 2009). The *slr0374* gene encodes a putative Ycf46 protein (501 aa) that contains an AAA (ATPases associated with diverse cellular activities) domain. Members of the AAA^+-ATPase superfamily are found in all kingdoms of life and are involved in very diverse cellular processes, including protein degradation, membrane fusion and cell division (Bussemer et al. 2009). The upstream gene encodes the protein Slr0373 (126 aa), which contains a helix-turn-helix motif and is a typical ATP-binding domain. The downstream gene *slr0376* encodes a putative Ycf35 protein (116 aa), whose function is also unknown.

Although the *slr0373-slr0374-slr0376* operon has been reported to be responsive to various environmental stresses, including low Fe (Singh and Sherman 2000), low S, low N, and high NaCl (Singh and Sherman 2002), the exact functions of the encoded proteins are still unknown. More importantly, as conserved chloroplast open reading frames, the functions of Ycf46 (Slr0374) and Ycf35 (Slr0376) in photosynthesis have never been studied. Microarray results showed that transcript levels of the *slr0373*, *slr0374*, and *slr0376* genes in *Synechocystis* 6803 were significantly up-regulated when cells were cultured under low C_i (0.03 % CO_2) versus high C_i (5 % CO_2) conditions (Eisenhut et al. 2007). In the present study, to confirm the linkage of these *ycf* genes with photosynthesis and CO_2 utilization, we compared their transcription levels by RT-PCR from cells grown in BG11 medium with and without carbon source (Na_2CO_3) addition. The results showed that all three genes

Table 1 Ycf46 homologues in cyanobacteria, algae and phototrophic bacteria

Species	Phylum	Gene name	Function annotation	*E*-value
Chlorobium tepidum[a]	Chlorobi	TLSCT0297	Cell division protein FtsH	$4e^{-23}$
Rhodopseudomonas palustris[a]	Proteobacteria	CGA009RPA4084	AAA ATPase	$5e^{-21}$
Acaryochloris marina MBIC11017	Cyanophyta	AM1_6050	Conserved hypothetical protein	0.0
Arthrospira platensis NIES-39	Cyanophyta	NIES39_L03880	Hypothetical protein	0.0
Cyanothece sp. PCC 7424	Cyanophyta	PCC7424_4640	AAA ATPase central domain protein	0.0
Gloeobacter violaceus PCC 7421	Cyanophyta	glr4289	Hypothetical protein	0.0
Microcystis aeruginosa NIES-843	Cyanophyta	MAE26800	AAA famiry ATPase centra	0.0
Nostoc punctiforme ATCC 29133	Cyanophyta	Npun_F0518	ATPase domain-containing protein	0.0
Nostoc sp. PCC 7120	Cyanophyta	all1872	Hypothetical protein	0.0
Prochlorococcus marinus MIT9515	Cyanophyta	P9515_13591	AAA ATPase, central region	e^{-103}
Synechococcus elongates PCC 7942	Cyanophyta	Synpcc7942_0417	ATPase of the AAA+ family	0.0
Synechococcus sp. WH7803	Cyanophyta	SynWH7803_1903	ATPase of the AAA+ family	$2e^{-94}$
Thermosynechococcus elongates BP-1	Cyanophyta	tlr1876	ATPase	0.0
Trichodesmiumery thraeum IMS101	Cyanophyta	Tery_1047	Hypothetical protein	0.0
Porphyra yezoensis	Rhodophyta	Poyecp003	Ycf46	$1e^{-94}$
Porphyra purpurea	Rhodophyta	Popucp004	Ycf46 AAA domain-containing protein	$1e^{-91}$
Gracilaria tenuistipitat	Rhodophyta	Grc000001	Ycf46	$9e^{-88}$
Cyanidium caldarium	Rhodophyta	Cycacp077	Ftsh cell division protein	$2e^{-21}$
Guillardia theta	Cryptophyta	Guthcp031	Ycf46	$2e^{-96}$
Odontella sinensis	Heterokontophyta	Odsicp048	Ycf46 AAA domain-containing protein	$2e^{-95}$
Emiliania huxleyi	Heterokontophyta	Emhucp015	Ycf46 hypothetical protein	$1e^{-90}$

Homology analysis showed that Ycf46 (Slr0374) is highly conserved in cyanobacteria and some eukaryotic algae, but has been lost in green algae and higher plants

[a] These two species are phototrophic bacteria

were clearly up-regulated when the only inorganic carbon source (Na_2CO_3) was removed from the culture medium after 24 h (Fig. 2). A decreasing trend in gene transcriptional levels was detected from upstream to downstream genes in the gene cluster (in the order *slr0373* > *slr0374* > *slr0376*), which was consistent with the suggestion by Singh and Sherman (2002) that the three genes could be co-transcribed as an operon. These results, including the increased gene transcription in C_i-limiting cells and the growth arrest of the *Δslr0374* mutant in C_i-limitation conditions, suggested that the product of the putative *ycf46* gene played a positive role in C_i utilization.

The *ycf46* gene (*slr0374*) was independent from the regulation of the known CCM transcription regulators CcmR and CmpR

Considering its potential roles in C_i utilization or CCM, we were interested in whether *slr0374* was regulated by the LysR-type transcription regulators CcmR and CmpR, which are the main transcription regulators functioning in CCM (Omata et al. 2001; Wang et al. 2004). CcmR is a transcription repressor and can repress the transcription of SbtA and Ndh-I_3 when cells are cultured in high C_i conditions, while this repression is removed when cells are transferred back to C_i-limitation environments (Omata et al. 2001). CmpR functions as a transcription activator and activates the transcription of the *Cmp* operon, which encodes a HCO_3^- transporter, BCT1 (Wang et al. 2004). We constructed mutants of the two transcription regulators CcmR (Sll1594) and CmpR (Sll0030) and cultured them in C_i-limitation conditions with the wild type as a control. The transcript level of the *ycf46* gene (*slr0374*) was monitored by RT-PCR in the wild type and the two mutants (Supplemental Fig. S2). The results from two independent experiments showed that there was no significant change in the expression level of *slr0374* in the wild type and the two mutants, suggesting that the gene was not regulated by these two transcription regulators (Supplemental Fig. S2). Considering that the *ycf46* gene (*slr0374*) was up-regulated under various environmental stresses, we speculated that other transcription regulators responsive to environmental stresses might regulate its transcription.

The *ycf46* mutant is impaired in CO_2 utilization

To clarify the functions of the putative Ycf46 and Ycf35 proteins, detailed physiological experiments were

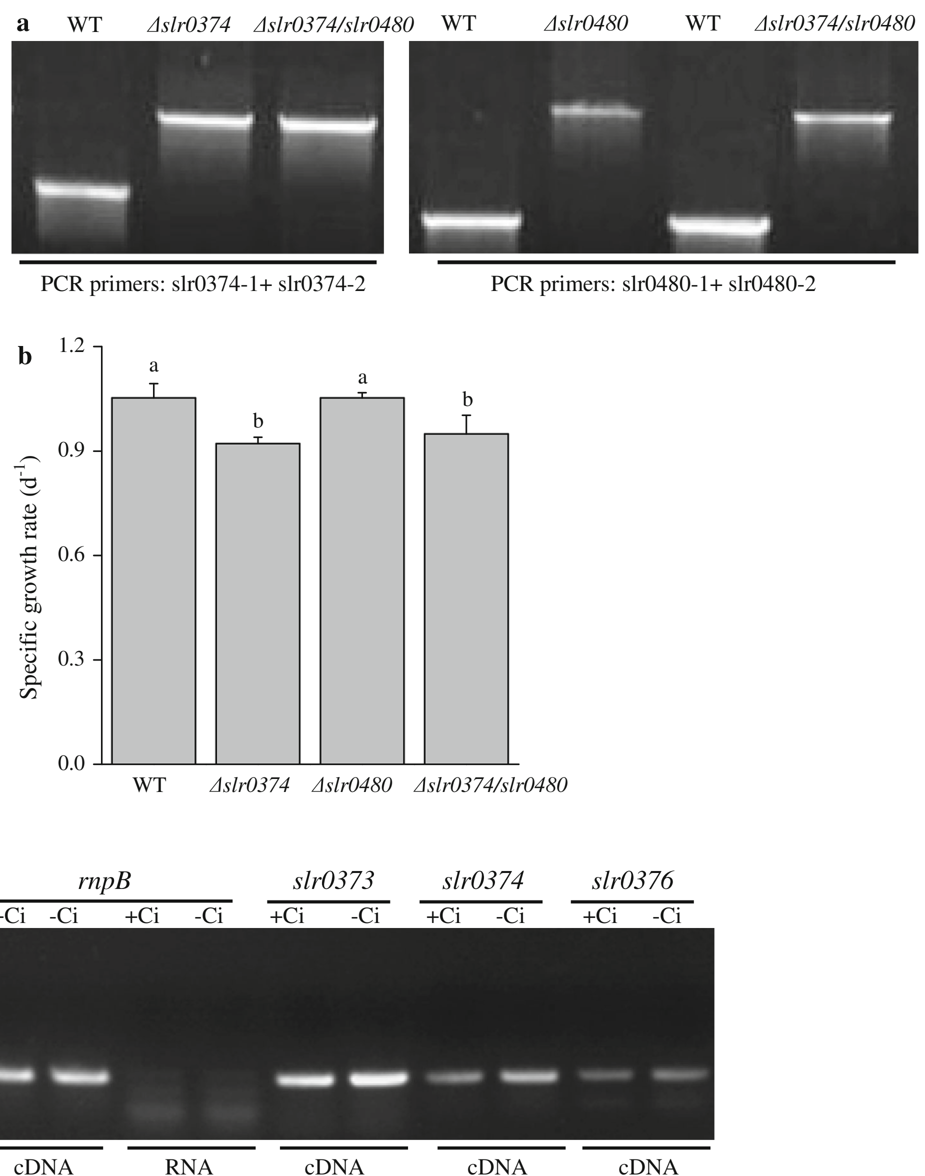

Fig. 1 PCR analysis (**a**) and specific growth rate (**b**) of the *Synechocystis* 6803 wild type and *ycf46* mutants cultured under C_i-limiting conditions. The wild-type and mutant strains were cultured in BG11 medium lacking Na_2CO_3 and buffered with 20 mM HEPES–KOH (pH 8.0). *ycf46* single mutants: *Δslr0374* and *Δslr0480*; double mutant: *Δslr0374/slr0480*. **a** Complete segregation of the mutants confirmed by PCR with corresponding primers; **b** inactivation of one *ycf46* gene (*slr0374*) resulted in growth arrest in C_i-limiting conditions, but inactivation of another *ycf46* gene (*slr0480*) did not change the growth rate. *Different letters* indicate significant difference (*t* test, $P < 0.05$). Mean ± SD ($n = 3$–4)

Fig. 2 RT-PCR analysis of the *slr0373*, *slr0374* and *slr0376* gene transcript levels in *Synechocystis* 6803 in BG11 media with Na_2CO_3 (+C_i) and without Na_2CO_3 (–C_i). Two independent experiments showed identical results. The housekeeping gene *rnpB* was used as a reference gene. PCR using RNA as a template confirmed the RNA solution was not contaminated by genomic DNA

performed with the *Δslr0374* mutant, *Δslr0374/slr0376* mutant (*slr0374*, ncRNA, and *slr0376* deletion) and wild type (Fig. 3a, c). The PCR results confirmed that the mutants were fully segregating (Fig. 3b, d). Although the transcriptional levels of *slr0374* or *slr0376* changed under various stresses such as iron starvation and oxidative stress (Singh and Sherman 2002), the *ycf46* mutant did not show obvious growth differences compared with the wild type when the cells were grown under a low iron concentration and low-light conditions (Supplemental Fig. S3). It also seemed to be even more tolerant to methyl viologen than the wild type (Supplemental Table S3). Since the pH value can change the ratio of C_i sources (CO_2, HCO_3^- and CO_3^{2-}) in liquid medium, we cultured the mutant and the wild type with standard BG11 medium under different pH conditions (pH 6.0, 8.0 and 10.0). In pH 6.0, 8.0 and 10.0 conditions the dominant C_i sources are CO_2, HCO_3^-, and CO_3^{2-}, respectively (Golterman et al. 1978). When the cells were cultured in the pH 8.0 medium (standard conditions), the specific growth rates of the two mutant strains (1.29 ± 0.05 for *Δslr0374* and 1.30 ± 0.01 for *Δslr0374/slr0376*) were similar to that of the wild type (1.31 ± 0.03) (*t* test, $P > 0.05$) (Fig. 3f). However, under pH 6.0 conditions, both the *Δslr0374* and

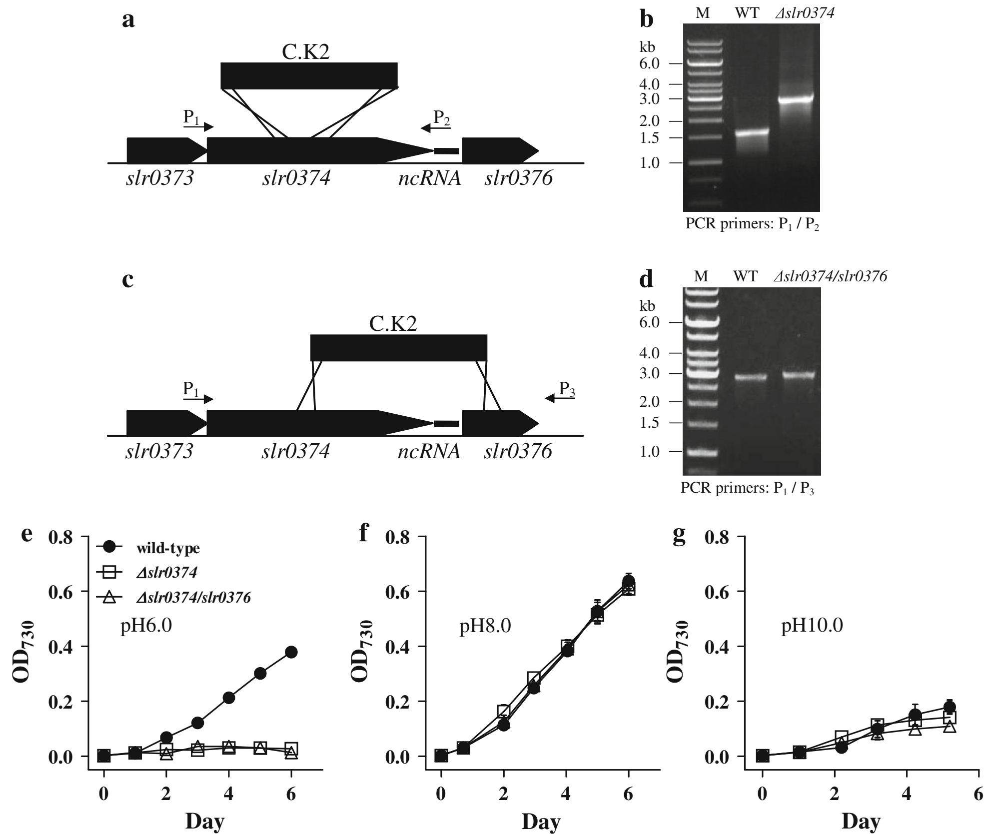

Fig. 3 Construction (**a**, **c**), PCR analysis (**b**, **d**), and growth characteristics under different pH conditions (**e**–**g**) of the *Synechocystis* 6803 *Δslr0374* and *Δslr0374/slr0376* mutants. A kanamycin-resistant cassette, C.K2, was inserted at different loci of the gene cluster to obtain *Δslr0374* and *Δslr0374/slr0376* mutants (**a**, **c**). PCR results confirmed that the mutants were completely segregating (**b**, **d**). PCR was carried out with the P_1/P_2 or P_1/P_3 primer pairs. P_1, P_2 and P_3 represent the primers Slr0374-1, Slr0374-2 and Slr0374-3 in Table S1, respectively. The growth curves for the *Synechocystis* strains were obtained in standard BG11 medium buffered at pH 6.0, 8.0 or 10.0, respectively (**e**–**g**)

Δslr0374/slr0376 mutants showed significantly lower growth rates (0.63 ± 0.02 and 0.68 ± 0.04, respectively) than the wild type (1.17 ± 0.01) (*t* test, $P < 0.05$) (Fig. 3e). pH 10.0 conditions are a strict stress on cyanobacterial cells, but the mutants showed similar growth rates (0.98 ± 0.01 for *Δslr0374*, 0.91 ± 0.02 for *Δslr0374/slr0376*) to the wild type (0.99 ± 0.06) (*t* test, $P > 0.05$) (Fig. 3g). These results suggested that the inactivation of Ycf46 and/or Ycf35 probably decreased the cells' capability to utilize CO_2, rather than HCO_3^- or CO_3^{2-}. As there were no differences in growth rates between the Ycf46 single mutant and the Ycf46/Ycf35 double mutant, these two genes were probably co-transcribed in the same operon as suggested by Singh and Sherman (2002).

Supplying a high concentration of CO_2 (5 %, v/v) to the *ycf46* mutant restored its phenotype under pH 6.0 conditions to the wild type level (Supplemental Fig. S4), which further confirmed that the *ycf46* mutant was impaired in CO_2 utilization. A complementation strain of the *ycf46* mutant, in which the *ycf46* gene was expressed with a highly efficient *psbA2* promoter (Jiang et al. 2012), grew even better than the wild type, suggesting that the increased copies of *ycf46* were helpful to cells grown in low pH conditions (Supplemental Fig. S4).

The *ycf46* mutant showed decreased expression of the *ecaB* gene and reduced carbonic anhydrase (CA) activity

Quantitative reverse transcription PCR (qRT-PCR) was used to evaluate the transcript levels of several CCM component genes in the wild-type and *Δslr0374/slr0376* mutant strains. The cells used for qRT-PCR analysis were cultured in standard growth conditions (pH 8.0). Most CCM component genes showed no significant difference in their transcriptional levels between the wild-type and mutant cells, while *ecaB*, which encodes a possible non-carboxysome carbonic anhydrase (EcaB) (So et al. 1998; Anthony et al. 2002), showed a decreased transcriptional level in the mutant (Table 2). The total carbonic anhydrase activity of the *Δslr0374/slr0376* mutant was 43.6 ± 27.0 WAU (mg Chl a)$^{-1}$, which was significantly lower than that of the wild type, 176.5 ± 8.3 WAU (mg Chl a)$^{-1}$ (*t* test, $P < 0.05$; Fig. 4a). This result was consistent with the lower transcriptional level of *ecaB* in the mutant cells. EcaB is thought to be an external carbonic anhydrase outside of the carboxysome (So et al. 1998). CA is absent from the cytosol in cyanobacteria, which means that the enzyme is located either in the periplasm, or on the outer surface of the plasma membrane or S-layer (Kupriyanova

Table 2 Transcript levels of CCM components in wild-type *Synechocystis* 6803 and the *Δslr0374/slr0376* mutant cultured in standard growth medium

Genes	*NdhF3*	*NdhF4*	*CmpA*	*sbtA*	*bicA*	*ccaA*	*ecaB*	*ccmN*
Ratios (mutant/wild type)	1.52 ± 0.61	1.64 ± 0.57	1.37 ± 0.73	0.79 ± 0.47	0.56 ± 0.41	1.13 ± 0.42	0.46 ± 0.09[a]	1.59 ± 0.68

[a] Transcript levels of the gene in the wild type and the *Δslr0374/slr0376* mutant are significantly different ($P < 0.05$, *t* test). Data are mean ± SD ($n = 4$)

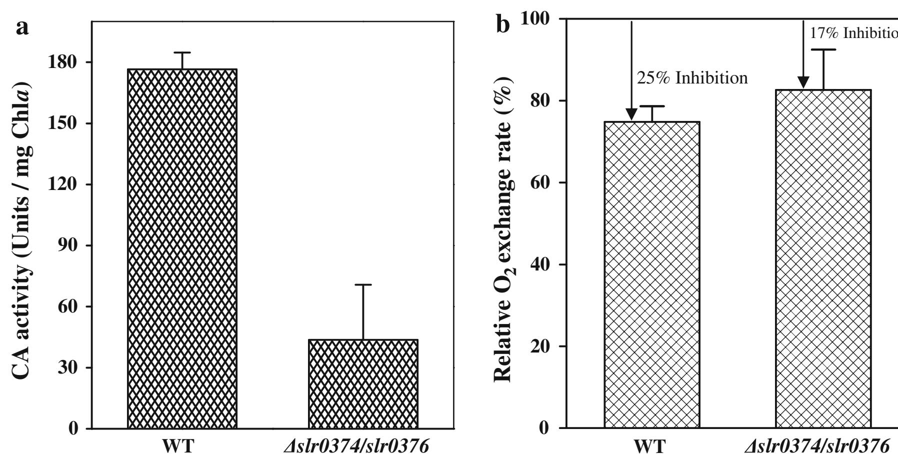

Fig. 4 CA activity (**a**) and inhibition of a specific CA activity inhibitor (EZ) on O_2 exchange rates (**b**) of the *Synechocystis* 6803 wild type (WT) and the *Δslr0374/slr0376* mutant. The relative rate shows the O_2 exchange rates with EZ versus without EZ. Exponential cells were measured with a Clark-type oxygen electrode (Chlorolab 2, Hansatech)

et al. 2007, 2011). Although limitations in methodology restricted our identification of the subcellular location of the Slr0374 protein in the periplasmic space, we hypothesize that the Ycf46 protein in *Synechocystis* is involved in the regulation of CCM either directly or indirectly, which might be mediated by a non-carboxysome carbonic anhydrase.

The photosynthetic activity of the *ycf46* mutant was less sensitive to a carbonic anhydrase inhibitor compared with the wild type

The O_2 evolution rates of the wild-type and mutant strains were measured in the presence of 300 μM EZ (ethoxyzolamide), which is a membrane-permeable inhibitor of CA and specifically inhibits active CO_2 uptake (Price and Badger 1989). The two strains showed similar O_2 evolution rate (wild type, 4.33 ± 0.71 μmol O_2 mg^{-1} Chl *a* min^{-1}; *ycf46* mutant, 3.54 ± 1.21 μmol O_2 mg^{-1} Chl *a* min^{-1}) under normal growth conditions without EZ treatment. The EZ treatment results showed that 25 % of the relative O_2 evolution rate was supported by the CO_2 uptake system in wild-type cells. While in the *Δslr0374/slr0376* mutant cells, the O_2 evolution rate was less inhibited by EZ (about 17 %) (Fig. 4b), indicating a relatively lower CO_2 uptake capability in the mutant ($P < 0.05$, *t* test). This result was consistent with the growth arrest in pH 6.0 medium (Fig. 3e), and further indicated that inactivation of the *slr0374-slr0376* genes decreased the CO_2 uptake capability and carbonic anhydrase activity.

The Ycf46 protein Slr0374 is a soluble protein

Because of the difficulty in obtaining a heterologous recombinant protein, we introduced a FLAG tag into the end (upstream of the stop codon) of the Slr0374 protein in *Synechocystis* 6803 and obtained a transgenic *slr0374-flag* strain. After isolating the total membrane and soluble fractions of the *slr0374-flag* strain, we used SDS-PAGE and western blotting to reveal the location of the Slr0374-

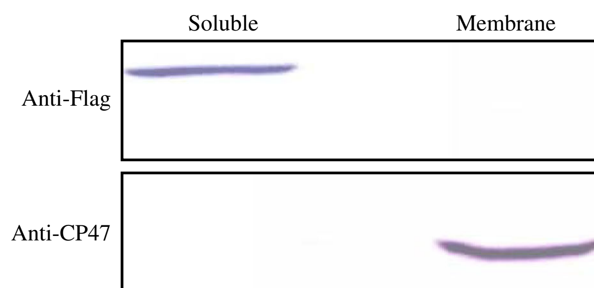

Fig. 5 Subcellular localization of the Slr0374 protein in *Synechocystis* 6803. Total protein was extracted from *Synechocystis* 6803 *slr0374-flag* cells, and then centrifuged at 103,000*g* and 4 °C for 30 min to isolate soluble and membrane protein solutions. The solutions were detected with an SDS-polyacrylamide gel and western blotting with anti-flag antibody. The anti-CP47 antibody was used as a membrane protein marker

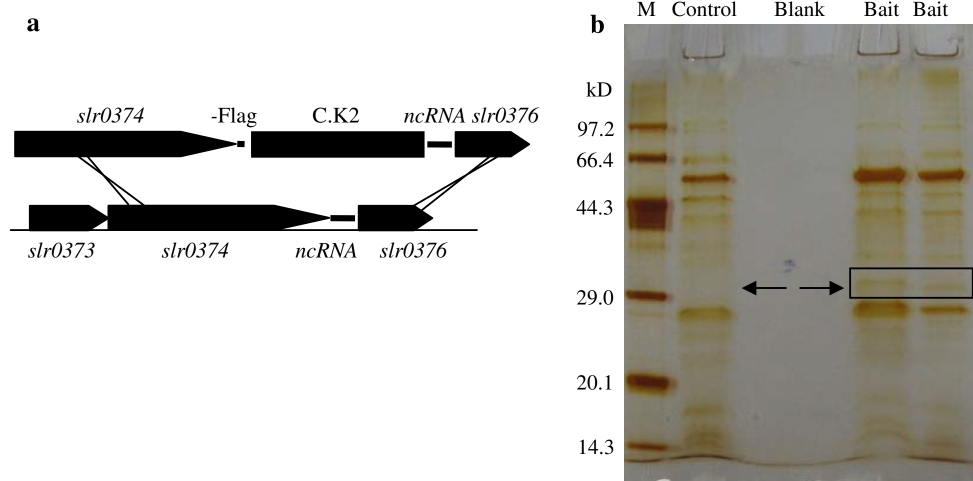

Fig. 6 Co-IP analysis with Slr0374 as a bait-protein. **a** Construction of the *Synechocystis slr0374*-flag strain. A flag tag was fused at the end of *slr0374*, a C.K2 fragment was inserted between the flag tag and ncRNA for resistance selection, and thus the expression of *slr0374* and *slr0376* were not influenced. **b** Co-IP with Slr0374 as a bait-protein. Total protein solutions of the *Synechocystis slr0374*-flag strain (bait) and wild type (control) were incubated with anti-flag antibody that was coupled with protein A/G. The eluted solutions were electrophoresed on an SDS-PAG, and then dyed with $AgNO_3$

Table 3 Proteins that may interact with Slr0374 identified by LC–MS/MS analysis

Protein denotation	Gene name	Protein size	Sequence coverage (%)	Peptide count
Septum site-determining protein MinD NP_442592.1	*sll0289*	29.047 KD	57.9	13
iron-stress chlorophyll-binding protein NP_441268.1	*sll0247*	37.221 KD	45.6	14
Enoyl-(acyl carrier protein) reductase NP_440356.1	*slr1051*	27.626 KD	33.8	5
Diaminopimelate epimerase NP_442973.1	*slr1665*	30.096 KD	30.8	5
3-Hydroxyisobutyrate dehydrogenase NP_442163.1	*slr0229*	31.9 KD	29.7	5
Photosystem II D1 protein NP_441550.1	*slr1311*	39.6 KD	23.9	6
Photosystem II manganese-stabilizing polypeptide NP_441796.1	*sll0427*	29.912 KD	23.4	3
ABC transporter NP_442473.1	*slr0075*	28.419 KD	17.2	3

Flag protein with an anti-Flag antibody. The results showed that Slr0374 was located in the soluble fraction, but not in the membrane fraction (Fig. 5). An antibody to the thylakoid membrane protein CP47 was used as a control marker. These results confirmed that the Ycf46 (Slr0374) protein was a soluble protein, not a thylakoid membrane protein as previously expected (Singh and Sherman 2002).

Co-immunoprecipitation (Co-IP) results with Slr0374 as a bait-protein

Since a Flag tag was introduced into the genome in the *Synechocystis slr0374-flag* strain (Fig. 6a), Co-IP was a good way to find proteins that have protein–protein interactions with Slr0374 in vivo. As shown in Fig. 6b, a protein band of about 30 kDa was found in the Co-IP protein solution of the *slr0374-flag* strain (marked with arrow in Fig. 6b), but not in the wild-type solution. By LC–MS/MS, nine candidate proteins were identified (Table 3). We tried to express these nine proteins fused with a His-tag in *Escherichia coli* and confirm the protein–protein interactions in vitro by pull-down assay combined with a western blot. Unfortunately, only three proteins were successfully expressed in soluble fractions. The other six proteins were expressed in inclusion bodies, and were not suitable for further protein–protein interaction study. These three recombinant proteins (Slr1051-histag, Slr1665-histag, and Slr0075-histag) were pulled down with an Ni-NTA His·Bind® resin (Novagen) together with the soluble proteins of the *Synechocystis slr0374-flag* strain. As shown in Supplemental Fig. S5, all three proteins were detected by western blotting with an anti-His-tag antibody, as expected (upper panel). When testing with an anti-Flag antibody, weak Slr0374 protein bands (about 56 kDa) were found in the Slr0075-histag and Slr1665-histag recombinant protein solutions, but not for the Slr1051-histag recombinant protein. These results further suggested that Slr0075 and Slr1665 exhibited protein–protein interaction with Slr0374.

Although no CA-encoding protein was recovered by the Co-IP assay, our results provide valuable information about Slr0374 and its regulation mechanism in the cyanobacterial CCM. It is probable that Slr0374 regulates CA activity in an indirect manner. We noticed that Slr0075 is also a conserved chloroplast open reading frame, Ycf16, which is predicted to encode a soluble SufC protein involved in Fe/S cluster assembly. Similar to *slr0374*, the *slr0075* gene also responds to many environmental stresses, such as low iron and oxygenic stress (Singh et al. 2003; Kobayashi et al. 2004). Our unpublished data showed that the transcriptional level of the *slr0075* gene was significantly lower in the *Δslr0374* mutant, consistent with the suggestion that they have protein–protein interaction with each other. The lower efficiency of Fe–S assembly in the *Δslr0374* mutant likely influences the CA activity, and the mechanisms are being investigated by our group and should be elucidated in the future.

The putative Ycf46 (Slr0374) from the unicellular cyanobacterium *Synechocystis* 6803 is highly conserved in all lineages of cyanobacteria and a limited number of algal plastid genomes, but has been lost in the green algae and higher plants. Structural analysis of potential functional motif indicated that Ycf46 (Slr0374) has an AAA-type ATPase domain, a putative leucine zipper, and a phosphorylation site. AAA modules are widespread among bacteria and archaea, but their common cellular functions are still not well characterized, besides ATP binding and/or hydrolysis. Based on recent researches, it has been proposed that the AAA domain-containing proteins have chaperone-like function for the maturation of specific protein complexes and regulate a number of cellular processes (Snider and Houry 2006; El Bakkouri et al. 2010). Ycf46 (Slr0374) as well as Ycf35 (Slr0376), may be involved in the regulation of CO_2 utilization in cells by some CCM components, particularly carbonic anhydrase. The evidence for the direct interaction between carbonic anhydrase and the Ycf46 protein needs further investigation.

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