Plant Cell Physiol. 49(11): 1672–1677 (2008)
doi:10.1093/pcp/pcn139, available online at www.pcp.oxfordjournals.org

Properties of Mutants of *Synechocystis* sp. Strain PCC 6803 Lacking Inorganic Carbon Sequestration Systems

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A mutant (Δ*5*) of *Synechocystis* sp. strain PCC 6803 constructed by inactivating five inorganic carbon sequestration systems did not take up CO_2 or HCO_3^- and was unable to grow in air with or without glucose. The Δ*4* mutant in which BicA is the only active inorganic carbon sequestration system showed low activity of HCO_3^- uptake and grew under these conditions but more slowly than the wild-type strain. The Δ*5* mutant required 1.7% CO_2 to attain half the maximal growth rate. Electron transport activity of the mutants was strongly inhibited under high light intensities, with the Δ*5* mutant more susceptible to high light than the Δ*4* mutant. The results implicated the significance of carbon sequestration in dissipating excess light energy.

Keywords: CO_2 and HCO_3^- uptake — CO_2-concentrating mechanism (CCM) — Cyanobacteria — Electron transport — Inorganic carbon sequestration.

Abbreviations: CCM, CO_2-concentrating mechanism; Ci, inorganic carbon; (r)ETR, (relative) electron transport rate; H, high CO_2-grown; L, low CO_2-induced; PAR, photosynthetically active radiation; WT, wild type.

Introduction

Cyanobacteria possess a CO_2-concentrating mechanism (CCM) that involves the uptake of CO_2 and HCO_3^- driven by light energy (Giordano et al. 2005, Kaplan et al. 2008, Price et al. 2008). Two CO_2 uptake systems and three HCO_3^- transporters have been identified in cyanobacterial strains. These are (i) the low CO_2-inducible high affinity CO_2 uptake system dependent on NdhD3/NdhF3/CupA/CupS (Shibata et al. 2001, Maeda et al. 2002, Zhang et al. 2004, Folea et al. 2008); (ii) the constitutively expressed low affinity CO_2 uptake system dependent on NdhD4/NdhF4/CupB (Shibata et al. 2001, Maeda et al. 2002, Xu et al. 2008); the low CO_2-inducible high affinity HCO_3^- transporters dependent on (iii) CmpA/CmpB/CmpC/CmpD (Omata et al. 1999, Koropatkin et al. 2007) and (iv) SbtA (Shibata et al. 2002); and (v) the low affinity HCO_3^- transporter dependent on BicA (Price et al. 2005). Both CO_2 and HCO_3^- are taken up by these systems and are accumulated within the cells as HCO_3^-, which enters into carboxysomes and is converted to CO_2 by the action of carbonic anhydrase to raise the CO_2 concentration in the vicinity of Rubisco (Kaplan et al. 2008).

The uptake of CO_2 and HCO_3^-, and leakage of these inorganic carbon (Ci) species occur during the operation of the CCM (Tchernov et al. 2003, Kaplan et al. 2008). This carbon cycling maintains an ambient CO_2 concentration substantially above or below that expected at chemical equilibrium with the prevailing HCO_3^- concentration in the medium (Tchernov et al. 2003). It has been suggested that the cycling of Ci species may play an important role in protecting the cells from high light stress by dissipating excess light energy (Kaplan et al. 2008).

In an attempt to see the significance of Ci sequestration for dissipation of excess energy in cyanobacterial cells, we constructed a mutant (Δ*5*) of *Synechocystis* sp. strain PCC 6803 (hereafter *Synechocystis* PCC 6803) by inactivating the *bicA* gene (*sll0834*) in the Δ*4* mutant (Δ*ndhD3*/*D4*/*sbtA*/*cmpA*; Shibata et al. 2002). The Δ*5* mutant did not have any Ci sequestration activity. Analyses of the growth and other physiological characteristics of the Δ*4* and Δ*5* mutants under various conditions revealed a role for Ci sequestration in dissipating excess light energy required for cyanobacterial cells to survive under diverse environmental conditions.

Results

CO_2 and HCO_3^- uptake

The activities of CO_2 and HCO_3^- uptake were low in high CO_2-grown (H) cells of the wild type (WT) and were increased 3–4 times in low CO_2-induced (L) cells. We did not detect any activities of CO_2 and HCO_3^- uptake in either H-cells or L-cells of the Δ*5* strain. This indicates that there are no more than five Ci sequestration systems in *Synechocystis* PCC 6803 functional under the experimental

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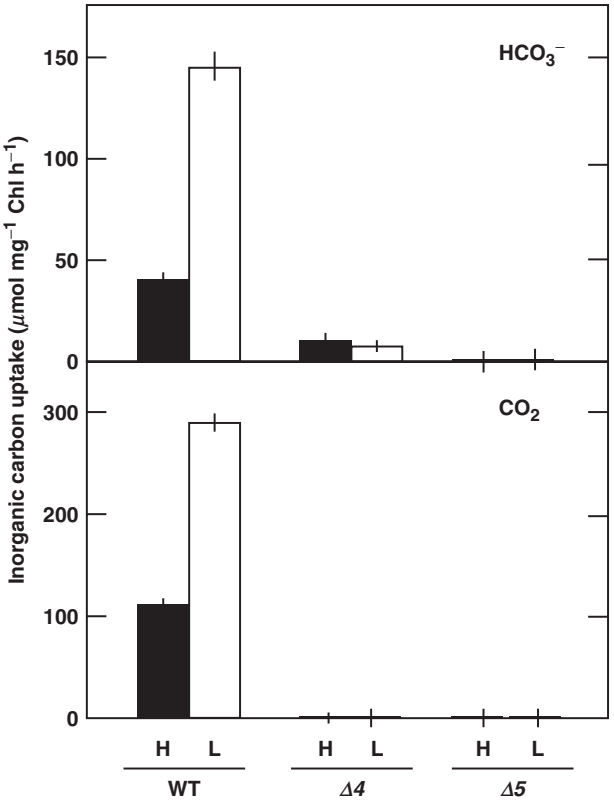

Fig. 1 The uptake of HCO_3^- (upper panel) and CO_2 (lower panel) by WT, *Δ4* and *Δ5* strains of *Synechocystis* PCC 6803. Cells grown at 3% CO_2 in air (v/v) (H-cells, filled bars) were aerated with air overnight (L-cells, open bars). Cells were suspended in BG11 medium buffered at pH 8.0 or 20 mM HEPES-KOH, pH 7.0, supplemented with 15 mM NaCl for the measurement of uptake of HCO_3^- and CO_2, respectively; for details see Materials and Methods. Vertical bars indicate SD ($n=4$).

conditions used here. The BicA-dependent HCO_3^- transporter is the only active Ci sequestration system in the *Δ4* strain. It has been reported that this HCO_3^- transporter is induced under low CO_2 and is a major contributor to HCO_3^- uptake in a marine cyanobacterium, *Synechococcus* sp strain PCC 7002 (Price et al. 2005). However, the activity of HCO_3^- uptake of the *Δ4* mutant was 25% that of the WT in H-cells and was only 6% in L-cells; the activity was not increased by bubbling H-cells of the *Δ4* mutant with air overnight (Fig. 1). As expected, the *Δ4* mutant did not have CO_2 uptake activity.

Effect of CO_2 concentration on growth rate

The *Δ5* mutant of *Synechocystis* PCC 6803 did not grow under air levels of CO_2. The *Δ4* mutant grew under these conditions, indicating that carbon supply by the BicA HCO_3^- transporter supported the growth of this mutant. However, the growth rate of the *Δ4* mutant was less than half that of the WT. The *Δ4* and *Δ5* mutants required about

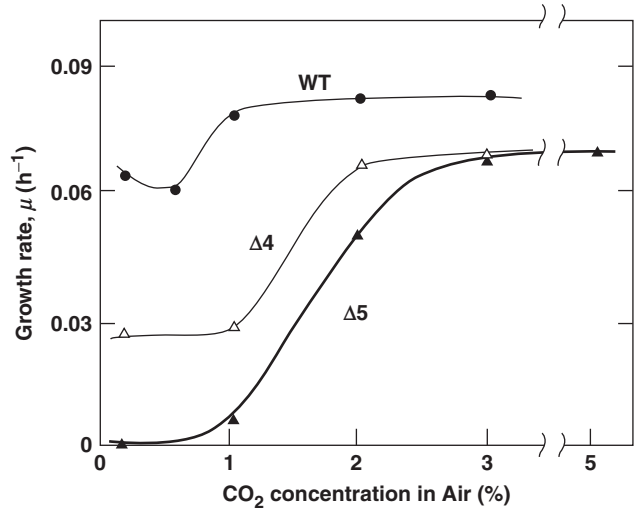

Fig. 2 Growth rates (μ) of the WT (filled circles), *Δ4* (open triangles) and *Δ5* (filled triangles) strains as a function of CO_2 concentration in air, obtained at the light intensity of 50 μE m^{-2} s^{-1}. $\mu = \ln(N/N_0)/t$, where N_0 and N represent the OD_{730} values at $t=0$ and t h after the start of the growth, respectively. The doubling time (h) = ln2/μ.

1.3 and 1.7% CO_2, respectively, to attain half the maximal rate (Fig. 2). The maximal growth rate of these mutants was attained at 2 and 3% CO_2, respectively, where the growth is supported by passive diffusion of Ci, predominantly in the *Δ4* mutant and totally in the *Δ5* mutant. The WT cells grew more slowly under air than under high CO_2 concentrations. The growth rate started to increase at around 0.6% CO_2 to reach the maximal level at 1% CO_2, where the growth rate of the *Δ5* mutant was nearly zero.

Growth under photomixotrophic conditions

Synechocyctis PCC 6803 used to construct the mutants is the strain that is able to utilize glucose and to grow under photomixotrophic conditions as well as under photoautotrophic conditions (Fig. 3A, curves b and a, respectively). It was assumed that the *Δ5* mutant was also able to grow in air using glucose as a carbon source. However, the mutant which was unable to grow under bubbling with air died after a certain period of slow growth in the presence of glucose (Fig. 3C, curves e and f, respectively). The mutant grew under high CO_2 (curve g), and glucose accelerated the growth under these conditions (curve h). The *Δ4* strain grew slowly under air, and glucose accelerated the growth (curves c and d), but to a level lower than that of the WT (curve b).

Effect of light intensity on relative electron transport rate (rETR)

Fig. 4 shows the ETR (relative) values obtained for the WT and mutant cells under various light intensities.

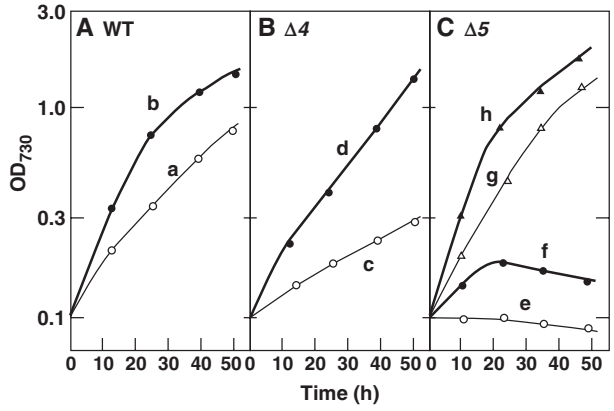

Fig. 3 Growth curves of the WT (A), Δ*4* (B) and Δ*5* (C) strains under air (curves a–f) or 3% CO_2 (g and h) in the absence (a, c, e, g) and presence (b, d, f, h) of 5 mM glucose obtained at the light intensity of 50 μE m^{-2} s^{-1}

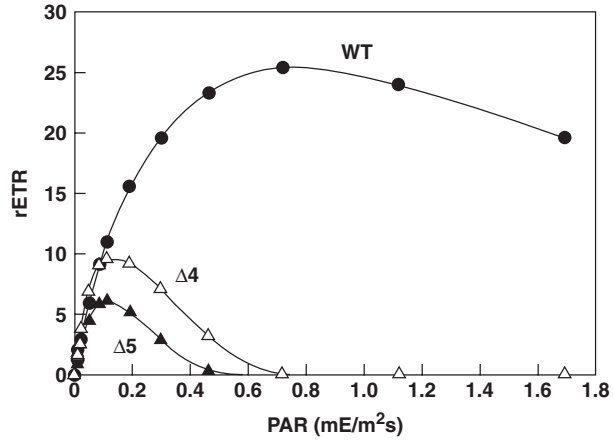

Fig. 4 Effect of light intensity on the (relative) electron transport rate (rETR) of the WT (filled circles), Δ*4* (open triangles) and Δ*5* (filled triangles) strains. H-cells collected by centrifugation were suspended in BG11 medium containing about 1 mM Ci and used for the experiments.

At light intensities below 100 μE m^{-2} s^{-1} photosynthetically active radiation (PAR), there was no significant difference between the WT and mutant strains in their ETR. The ETR values increased in the WT by increasing the light intensity to attain the maximal value at around 750 μE m^{-2} s^{-1} and then decreased at higher intensities. In contrast, ETR values in the Δ*4* and Δ*5* mutants decreased at light intensities above 100 μE m^{-2} s^{-1} to reach zero value at 460 and 720 μE m^{-2} s^{-1}, respectively. The Δ*5* mutant was more susceptible to high light than the Δ*4* mutant at the intensities above 50 μE m^{-2} s^{-1}.

ΔpH build-up

Fig. 5 shows the time courses of build-up of the proton gradient across the thylakoid membranes of the WT and

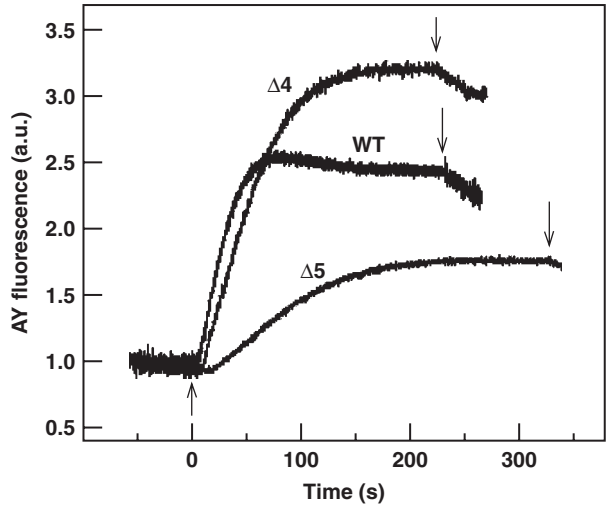

Fig. 5 Build-up of the proton gradient, followed by acridine yellow fluorescence in the WT, Δ*4* and Δ*5* strains. Up- and downward-facing arrows represent actinic light switched on and off, respectively. Sample preparation was as in Fig. 4.

Table 1 Kinetic parameters of the ΔpH build-up in WT and mutant *Synechocystis* PCC 6803[a]

	$t_{1/2}$ (s)	$\Delta F/F$
WT	20.5 ± 1.6	1.45 ± 0.09
Δ*4*	57 ± 8	2.13 ± 0.09
Δ*5*	73 ± 13	1.09 ± 0.21

[a]Expressed as mean ± SD; individual data ($n = 4$ independent cultures) were obtained from acridine yellow experiments as shown in Fig. 5.

mutants, as measured by acridine yellow fluorescence. Kinetic parameters of the proton gradient build-up, calculated from the curves as shown in Fig. 5, are summarized in Table 1. These results indicated the build-up of the pH gradient in the mutants as well as in the WT, although this was significantly slower in the mutants, decreasing from Δ*4* to Δ*5* mutants. However, the equilibrium pH gradient (shown by the $\Delta F/F$ ratio at the steady-state level) was higher in the Δ*4* mutant and lower in the Δ*5* mutant, respectively, as compared with that in the WT (Table 1).

Discussion

The present study clearly showed that *Synechocystis* PCC 6803 does not possess more than five Ci sequestration systems functional under the experimental conditions used here, since inactivation of all of these systems in the Δ*5* mutant completely abolished the activity of CO_2 and

HCO_3^- uptake (Fig. 1). The mutant was unable to grow under air levels of CO_2 whereas the Δ*4* mutant, where the BicA HCO_3^- transporter is the only active Ci sequestration system, grew under air but much more slowly than the WT (Fig. 2). Thus, under air levels of CO_2, the growth is limited by the supply of carbon in the Δ*4* mutant. The growth rate of the WT in air was lower than that under high CO_2 concentrations; the maximal growth rate was attained at 1% CO_2 where the growth of the Δ*5* mutant was nearly zero (Fig. 2). Under air levels of CO_2, all of the five Ci sequestration systems are expressed in the WT and, therefore, its growth may be not limited by the availability of carbon. This is supported by the observation of leakage of CO_2 and HCO_3^- during uptake of these species under air levels of CO_2 by the Ci sequestration systems (Tchernov et al. 2001, Tchernov et al. 2003). On the other hand, a considerable level of glycolate is produced under these conditions but not under high CO_2, suggesting that Rubisco is not saturated with CO_2 under air levels of CO_2 (Colman 1989, Eisenhut et al. 2006). It is possible that, although the WT strain takes up Ci in excess under these conditions, the rates of transfer of HCO_3^- accumulated within the cytoplasm and conversion to CO_2 in the carboxysomes are not high enough to saturate Rubisco with CO_2. The lower growth rate of the WT at CO_2 concentrations below 1% might be attributed to the following reasons: (i) Rubisco is not saturated with CO_2 under low CO_2 as discussed above; (ii) changes in cell physiology related to the down-regulation of expression of many genes under low CO_2 such as those encoding ribosomal proteins (Wang et al. 2004); and/or (iii) a significant portion of the available energy is consumed for Ci uptake under low CO_2 at a relatively low light intensity of 50 μE m^{-1} s^{-1} and limitation in energy retards the growth. The Δ*5* mutant, in which CO_2 is supplied from the external medium by diffusion, possibly through a water channel (Tchernov et al. 2001), required about 1.7% CO_2 to attain half the maximal growth rate (Fig. 2). The concentration of CO_2 in water at 30°C in equilibrium with 1.7% CO_2 in air is calculated to be 455 μM, which is much higher than the $K_m(CO_2)$ value of Rubisco of *Synechocystis* PCC 6803 (= 162–202 μM; Marcus et al. 2003). In this mutant, CO_2 enters the cells only by diffusion. The requirement for a CO_2 concentration for growth much higher than that expected from the $K_m(CO_2)$ value of Rubisco indicates the presence of high resistance to CO_2 diffusion at the site of entry to the cells and/or to the carboxysomes.

One of the most significant findings of this study is that both Δ*4* and Δ*5* mutants are susceptible to high light intensities (Fig. 4). Previous data of Helman and co-workers (2005) show that electron flow to O_2 can reach up to 40% of the photosynthetic electron flow originating from PSII in cyanobacteria. Also, genes encoding A-type flavoproteins involved in photoreduction of O_2 have been identified (Helman et al. 2003). In the Δ*5* and Δ*4* mutants, most of the electrons leaving PSII may be used to reduce O_2 due to the absence or low activity of carbon uptake and fixation (Fig. 1). This might cause damage to the cells at high light intensities when electron supply by PSII is increased.

Excess electrons from PSII inhibit their electron transport activity by fully reducing the electron transport chain and causing photodamage, especially in the mutants where there is no or a highly limited amount of carbon supply (Fig. 1). The Δ*5* mutant was unable to grow under air even in the presence of glucose, and the Δ*4* mutant grew slowly under air and glucose accelerated the growth, but to a level lower than that observed with the WT (Fig. 3). The result supports the above view that excess electrons (from PSII and glucose) inhibit the electron transport activity. The growth curves were obtained at 50 μE m^{-2} s^{-1} where the inhibition of ETR was not observed (Fig. 4). However, the growth is highly dependent on the availability of Ci species, which are much lower in the mutants than in the WT (Fig. 1).

The trans-thylakoid proton gradient build-up is highly dependent on the ETR and CCM (Teuber et al. 2001, Berry et al. 2005). The observation that the proton gradient is formed in the mutants indicates the presence of electron transport activity even at the high light intensity (1,700 μE $m^{-2}s^{-1}$) where the apparent rETR value was nearly zero (Figs. 4, 5). It is possible that the formation of ΔpH in the mutants depends mostly on cyclic electron transport and/or there is a certain linear electron transport activity even when plastoquinone is highly reduced (giving a very low apparent rETR value). While linear and cyclic electron transport are directly coupled to proton transduction (build up of ΔpH), ATP synthesis essential for CO_2 fixation is driven by a pH gradient. The steady-state ΔpH level is determined by the balance of these opposing processes. The larger $\Delta F/F$ ratio of the Δ*4* mutant in spite of the slower ΔpH formation, as compared with that in the WT (Fig, 5 and Table 1), suggests that the utilization of the proton gradient is more limited in the Δ*4* mutant than the ΔpH formation by electron transport processes. The low $\Delta F/F$ ratio in the Δ*5* mutant indicates that in this strain, the build-up of ΔpH is very low, most probably because of the low electron transport activity.

The way by which the Ci sequestration systems are energized is not known, except for the ABC-type HCO_3^- transporter (Omata et al. 1999). It has been reported that CO_2 uptake systems are energized by PSI-dependent cyclic electron flow whereas HCO_3^- transport is dependent on linear electron transport (Li and Canvin 1998). The uptake and leakage of CO_2 and HCO_3^- occur simultaneously during the operation of the CCM (Tchernov et al. 2003, Kaplan et al. 2008), producing a cycling of Ci species. It has been suggested that this carbon cycling may play an

important role in protecting the cells from high light stress by dissipating excess light energy (Tchernov et al. 2003, Kaplan et al. 2008). Our present work provided the experimental evidence to support this hypothesis.

Materials and Methods

Growth conditions

WT and mutant cells of *Synechocystis* PCC 6803 were grown at 30°C in BG11 medium (Stanier et al. 1971) buffered with 20 mM TES-KOH (pH 8.0) and bubbled with ambient (with about 380 p.p.m. CO_2, L-cells) or CO_2-enriched [with 3% (v/v) CO_2, H-cells] air under continuous illumination of 50 μE m^{-2} s^{-1} PAR provided by white fluorescent lamps.

Construction of mutants

Construction of the Δ*ndhD3*/*ndhD4*/*cmpA*/*sbtA* (designated Δ*4*) mutant has been described previously (Shibata et al. 2002) and deposited in the web site CyanoMutants (http//www.kazusa.or.jp/cyano/mutants/). The cassettes used to inactivate *ndhD3*, *ndhD4*, *cmpA* and *sbtA* are those which give resistance to spectinomycin, kanamycin, hygromycin and chloramphenicol, respectively. A gentamycin-resistant cassette was used to inactivate *bicA (sll0834)* in the Δ*4* mutant to create the Δ*5* mutant. The DNA region containing *bicA* was amplified by PCR using the following primers: 68BicEcoF, GGGGAATTCGGTGGTTGGTTAGA; and 68BicR, TCATTCAAGCCATCAGC.

The PCR product was digested with *Eco*RI and *Hin*dIII, and cloned into *p*UC18. The gentamycin cassette was then inserted to the *Pst*I site inside *bicA* and the plasmid thus constructed was used to transform the Δ*4* mutant to generate the Δ*5* mutant in which all of the five Ci uptake systems were inactivated. PCR analysis of the mutant confirmed complete segregation of the inactivated *bicA*.

Determination of growth rates

H-cells from WT and mutant strains were collected by centrifugation at 3,000 *g* for 5 min and resuspended in fresh BG11 medium to an OD_{730} of 0.1 Glucose (5 mM) was added for photomixotrophic growth. Tubes containing 50 ml of the cell suspension were placed at 30°C and aerated with CO_2-enriched (3% v/v) or ambient air under continuous illumination with fluorescent lamps at 50 μE m^{-2} s^{-1} PAR. The OD_{730} was measured using a spectrophotometer, model UV755B (MC Scientific Instruments, Shanghai, PR China).

Measurement of CO_2 uptake

WT and mutant H- and L-cells (the latter bubbled with air for 18 h) were harvested by centrifugation and suspended in 25 ml of 20 mM HEPES-KOH pH 7.0 supplemented with 15 mM NaCl at a chlorophyll level of 5.0 μg ml^{-1}, and placed in a reaction vessel (Ogawa et al. 1985). CO_2 exchange of the cell suspension was measured at 30°C using an open gas analysis system, which measures the rate of CO_2 exchange as a function of time. Ambient air containing 377–380 p.p.m. CO_2 was passed into the reaction vessel at a flow rate of 1.0 liter min^{-1}, the exchanged gas was dried, and then the CO_2 concentration was analyzed using an infrared gas analyzer, model WMA-4 (PP Systems, Amesbury, MA, USA).

Measurement of HCO_3^- uptake

The rate of HCO_3^- uptake was measured using $H^{14}CO_3^-$ in BG11 medium (pH 8.0) which had been bubbled with ambient air (~380 p.p.m. CO_2) for several hours. Cells were suspended in the aerated BG11 and 1 ml of cell suspension was mixed with an equal volume of the aerated BG11 containing $NaH^{14}CO_3$ and then 0.9 ml of the mixture was taken up by an Eppendorf pipet. Ci uptake was initiated by illuminating the cells in the pipet by white light (800 μE m^{-2} s^{-1}) and terminated after 30 s by rapid filtration of the cells onto a glass filter (GF/B, Whatman) by suction, followed by immediate washing of the filter with 5 ml of BG11 medium. The filter was washed once more and then subjected to the measurement of radioactivity.

Measurement of rETR

Changes in chlorophyll fluorescence yield in the WT and mutant cells were measured using a Dual-PAM-100 measuring system (Walz, Effeltrich, Germany). The actinic light intensity was increased stepwise from 0 to 1,700 μE m^{-2} s^{-1}; steady-state (F) and maximal (F_m') fluorescence levels were determined after 30 s adaptation periods at each light intensity. F_m' was obtained by applying 300 ms saturating pulses with an intensity of 10,000 μE m^{-2} s^{-1}.

The rETR values were calculated using the following equations as reported by Genty et al. (1989).

$$\mathrm{ETR} = \Phi_{(\mathrm{PSII})}\mathrm{PAR}\ a^* n_{(\mathrm{PS2})} \quad (1)$$

where $\Phi_{(\mathrm{PSII})} = (F_m' - F)/F_m'$ is the effective PSII quantum yield, a^* is the PSII (optical) cross-section and $n_{(\mathrm{PSII})}$ is the number of PSII centers.

As a^* and $n_{(\mathrm{PSII})}$ can be considered equal in the WT, Δ*4* and Δ*5* mutants, the rETR can be calculated as

$$\mathrm{rETR} = \mathrm{PAR}\,(F_{m\prime} - F)F_{m\prime} \quad (2)$$

ΔpH measurements

Relative pH gradient changes across the thylakoid membranes were determined by using the pH indicator acridine yellow as described by Teuber et al. (2001). Excitation and detection of the fluorescence was done by the Dual-PAM-100 modules DUAL-EAY and DUAL-DAY, respectively, in a 90° arrangement; saturated red light (PAR = 1,700 μE m^{-2} s^{-1}) was provided by the LED (light-emitting diode) array of DUAL-DAY.

Other methods

Unless otherwise stated, standard techniques were used for DNA manipulation. Pigments in the cells were extracted in methanol, and the concentration of chlorophyll in the extract was determined (Ogawa and Shibata 1965).

Funding

The National Natural Science Foundation of China (No. 306701620 to H.M.); the State Key Basic Research and Development Plan (No. 20007CB108804 to H.M.); the Key foundation of CAS (No. KSCX2-YW-N-059 to H.M.); the Federal Ministry of Education and Research (BMBF; to G.B.); the EU/NEST project 'Solar-H' (to M.R.); the German Research Foundation (DFG, project C1 in SFB

480, to M.R.), National Science Foundation-FIBR program (Grant EF0425749 to A.S.); the Membrane Biology EMSL Scientific Grand Challenge Project at the W.R. Wiley Environmental Molecular Sciences Laboratory, a national scientific user facility sponsored by the US Department of Energy Office of Biological and Environmental Research program located at Pacific Northwest National Laboratory (to T.O. and H.P.). Pacific Northwest National Laboratory is operated for the Department of Energy by Battelle.

Acknowledgments

The technical assistance of Claudia König and Erdmut Thomas is greatly acknowledged.

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(Received June 25, 2008; Accepted September 8, 2008)