Biochimica et Biophysica Acta 1817 (2012) 1248–1255

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Identification and functional role of the carbonic anhydrase Cah3 in thylakoid membranes of pyrenoid of *Chlamydomonas reinhardtii*☆,☆☆

Maria A. Sinetova, Elena V. Kupriyanova, Alexandra G. Markelova, Suleyman I. Allakhverdiev, Natalia A. Pronina *

Timiryazev Institute of Plant Physiology, Russian Academy of Sciences, Botanicheskaya Street 35, Moscow, 127276 Russia

ARTICLE INFO

Article history:
Received 25 October 2011
Received in revised form 9 February 2012
Accepted 10 February 2012
Available online 21 February 2012

Keywords:
Chlamydomonas reinhardtii
Carbonic anhydrase Cah3
CCM-mutant *cia3*
Fatty acids
Immunocytochemical localization
Pyrenoid

ABSTRACT

The distribution of the luminal carbonic anhydrase Cah3 associated with thylakoid membranes in the chloroplast and pyrenoid was studied in wild-type cells of *Chlamydomonas reinhardtii* and in its *cia3* mutant deficient in the activity of the Cah3 protein. In addition, the effect of CO_2 concentration on fatty acid composition of photosynthetic membranes was examined in wild-type cells and in the *cia3* mutant. In the *cia3* mutant, the rate of growth was lower as compared to wild-type, especially in the cells grown at 0.03% CO_2. This might indicate a participation of thylakoid Cah3 in the CO_2-concentrating mechanism (CCM) of chloroplast and reflect the dysfunction of the CCM in the *cia3* mutant. In both strains, a decrease in the CO_2 concentration from 2% to 0.03% caused an increase in the content of polyunsaturated fatty acids in membrane lipids. At the same time, in the *cia3* mutant, the increase in the majority of polyunsaturated fatty acids was less pronounced as compared to wild-type cells, whereas the amount of 16:4ω3 did not increase at all. Immunoelectron microscopy demonstrated that luminal Cah3 is mostly located in the thylakoid membranes that pass through the pyrenoid. In the cells of CCM-mutant, *cia3*, the Cah3 protein was much less abundant, and it was evenly distributed throughout the pyrenoid matrix. The results support our hypothesis that CO_2 might be generated from HCO_3^- by Cah3 in the thylakoid lumen with the following CO_2 diffusion into the pyrenoid, where the CO_2 fixing Rubisco is located. This ensures the maintenance of active photosynthesis under CO_2-limiting conditions, and, as a result, the active growth of cells. The relationships between the induction of CCM and restructuring of the photosynthetic membranes, as well as the involvement of the Cah3 of the pyrenoid in these events, are discussed. This article is part of a Special Issue entitled: Photosynthesis Research for Sustainability: from Natural to Artificial.

1. Introduction

At present, three mechanisms of CO_2 concentrating in the photosynthetic cells are known. Two mechanisms operate in higher plants with C_4- and CAM-type of carbon metabolism. The third type of CO_2-concentrating mechanism functions in aquatic photosynthesizing organisms, namely, in microalgae and cyanobacteria that fix CO_2 via C_3 biochemistry. The latter mechanism, called CCM (CO_2-concentrating *m*echanism), was discovered in the 1980s [1]. It permits the maintenance of the photosynthetic activity at low concentrations of CO_2 in the environment. Unlike the mechanisms of CO_2 concentrating that is characteristic for the C_4- and CAM-type plants, the third CCM operates before the incorporation of inorganic carbon into organic compounds, and it is associated with the active transport and with the intracellular conversion of inorganic carbon (C_i).

In cyanobacteria, active transport of C_i into the cells has been well studied. It involves three HCO_3^- transporters and two systems of facilitated diffusion of CO_2 [2]. In eukaryotic algae, several genes for the putative C_i transporters had been discovered during the last few years [3].

The second important element of the CCM consists of a system of CO_2/HCO_3^- conversion, which is better understood in microalgae [4]. The CO_2/HCO_3^- conversion is performed by the enzyme carbonic anhydrase (CA; carbonate hydrolyase; EC 4.2.1.1), which catalyzes the reversible hydration of carbon dioxide: $CO_2 + H_2O \leftrightarrows HCO_3^- + H^+$. According to modern nomenclature, all CAs are divided into five main classes (α, β, γ, δ, and ζ) that do not have significant homology in amino acid sequences and, presumably, have evolved independently [5,6].

Abbreviations: CA, carbonic anhydrase; CCM, CO_2-concentrating mechanism; C_i, inorganic carbon compounds (CO_2 and HCO_3^-); H-CO_2 cells, cells grown at 2% CO_2; L-CO_2 cells, cells grown at 0.03% CO_2; FA, fatty acid; PUFA, polyunsaturated fatty acid

☆ This article is dedicated to the memory of Geda L. Klyachko-Gurvich, a remarkable and talented person and scientist, whose data has also contributed to this work.

☆☆ This article is part of a Special Issue entitled: Photosynthesis Research for Sustainability: from Natural to Artificial.

* Corresponding author at: Timiryazev Institute of Plant, Physiology, Russian Academy of Science, Botanicheskaya Street 35, Moscow, 127276 Russia. Tel.: +7 499 231 8352; fax: +7 499 977 8018.

E-mail address: pronina@ippras.ru (N.A. Pronina).

0005-2728/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2012.02.014

The unicellular green alga *Chlamydomonas reinhardtii* is a good model to study the organization of the CCM [4,7,8]. The cells of *C. reinhardtii* accumulate high concentrations of C_i in chloroplasts due to the activity of the influx transporters, which operate in the cytoplasmic membrane (Hla3) and in the envelope membranes of the chloroplast (LciA), in response to limitation in CO_2 [7]. Twelve genes that correspond to CA isozymes have been found in *C. reinhardtii*, which possesses three alpha, six beta, and three gamma CAs [9]. These enzymes are located in all cellular compartments: in the periplasm (Cah1, Cah2, and, perhaps, Cah8), in the cytoplasm (Cah9), mitochondria (Cah4, Cah5, Cag1, Cag2, and Cag3), in the stroma of the chloroplast (Cah6), and in the thylakoid membranes (Cah3). The exact localization of Cah7 has not yet been determined.

So far, the detailed mechanisms of C_i accumulation and CO_2 concentration remain unclear. Nevertheless, the data on the role of the molecular and structural organization of the CCM is steadily accumulating. Algal cells grown under low CO_2 concentration are characterized by well-developed pyrenoid surrounded by starch sheath [10–12]. These cells also exhibit alterations in the structure of their chloroplast envelopes, as well as in their thylakoid membranes [13,14].

In electron micrographs, the pyrenoid appears as an electron-dense inclusion usually surrounded by a starch sheath in the chloroplast. The chloroplast thylakoids pass through or extend into the pyrenoid protein matrix, where they may form a random or a regular array. The pyrenoids, similarly to cyanobacterial carboxysomes, contain almost all cellular Rubisco [15,16], which is involved in the formation of pyrenoid [17,18].

The presence of Cah3 inside the pyrenoid has been previously demonstrated [19,20]. Various types of evidence have been accumulated on the importance of the algal pyrenoid in functioning of the CCM. It was suggested that the enrichment in CO_2 is attributed and limited to the area of pyrenoid, but not to the whole cell volume [4,21–26]. It is generally accepted that the CO_2 formation in the pyrenoid is catalyzed by lumenal Cah3 located in close proximity to the active Rubisco centers. At the same time, Cah3 is supposedly associated with the PSII, and it is involved in the maintenance of PSII activity [27].

In the present study, we investigated the localization and distribution of Cah3 protein in the thylakoid membranes of the chloroplast stroma and pyrenoid of *C. reinhardtii*. We also discuss the interactions between the induction of the CCM and the changes in the structure of the photosynthetic membranes at low concentrations of CO_2 in the environment.

2. Materials and methods

2.1. Strains and growth conditions

Cells of *C. reinhardtii* 137mt + (WT) and its CCM-mutant *cia3* [28] were used in this study. *C. reinhardtii* cells were grown photoautotrophically at 28 °C in high-salt medium [29], with continuous bubbling with air that contained 2% CO_2 (v/v). Continuous illumination was provided from cool white fluorescent lamps at 50 μmol photons m^{-2} s^{-1}. The growth rate, as assessed by the cell concentration in liquid cultures, was estimated from the optical density at 750 nm (OD_{750}) as determined with a Genesis 10UV spectrophotometer (Thermo Fisher Scientific, MA, USA). Low CO_2 concentration was provided to the cells by a decrease of the content of CO_2 in the air from 2 to 0.03%. All experiments were carried out with cells in the mid-exponential phase of growth.

2.2. Isolation of C. reinhardtii cell fractions

Intact *C. reinhardtii* cells were collected by centrifugation and washed once with a 30 mM HEPES-KOH buffer, pH 8.2 that contained 0.25 M NaCl. Cells were disrupted in a chilled buffer (30 mM HEPES-KOH, pH 8.2) with the French Press (8000 psi; SLM, American Instruments Co., Urbana, IL, USA). Unbroken cells were sedimented by centrifugation at 700 ×*g* for 10 min at 4 °C. The fractions enriched in soluble and membrane proteins (supernatant and pellet, respectively) were obtained by centrifugation of the total cell extract at 18,000 ×*g* for 40 min at 4 °C. To remove soluble protein contaminations, the pellet was washed once at the same conditions. The protease inhibitor mixture (P2714, Sigma, USA) was added to a buffer at all steps of the separation experiments to avoid protein degradation. Protein content was estimated with Bio-Rad kit with standard solutions (DC Protein Assay Kit, Bio-Rad Laboratories, Hercules, CA, USA).

2.3. Electrophoresis and immunodetection

Proteins were separated in 12% SDS-PAGE according to Laemmli [30]. Proteins were loaded at 45 μg per lane. A mixture of protein standards (Precision Plus Protein Standards, Bio-Rad) was used as molecular mass markers. The samples were solubilized by heating at 95 °C for 5 min in the sample buffer.

Western blotting was performed as described in the protocol provided by Bio-Rad Laboratories. The primary polyclonal antibodies were raised against: (1) recombinant Cah3 protein (α-CA) of *C. reinhardtii* (Agrisera, Vännäs, Sweden) or (2) recombinant Cah3-MBP fusion protein. The latter antibodies were kindly provided by Prof G. Samuelsson (Umeå Plant Science Centre, Sweden). Donkey anti-rabbit IgG conjugated with the horseradish peroxidase (GE Healthcare) were used as secondary antibodies. To detect an antibody–antigen conjugates, the membranes were incubated in 50 mM Tris–HCl (pH 7.5) buffer supplemented with 30 μl of 30% H_2O_2, 25 mg of 3,3′-diaminobenzidine, and 75 mg of 4-chloro-1-naphthol as the substrates for peroxidase (per 100 ml of the final buffer solution). Both substrates were pre-dissolved in 5 ml of methanol. The exposure time in a substrate-containing solution varied from 0.5 to 2 min.

2.4. Immunoelectron microscopy

For immunogold labeling experiments, *C. reinhardtii* cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for several days at 4 °C. Samples were rinsed three times in the same buffer, dehydrated with 70% ethanol, and embedded in the LR-White resin (62661, Sigma). Sections were prepared with an LKB microtome (Sweden) with a glass knife and placed on Formvar (F6146, Sigma) and carbon-coated nickel (G5651, Sigma) grids. To determine localization of Cah3 in WT cells of *C. reinhardtii,* we used affinity-purified antibodies to the recombinant Cah3 protein, that were kindly provided by Prof. G. Samuelsson. Nonspecific interaction of antibodies was blocked by pretreatment of nickel grids for 40 min in a drop of 1% BSA dissolved in 50 mM phosphate buffer (pH 7.4) with 0.85% NaCl (BSA-PBS). Then the grids were transferred into 100-μl drops of primary antibody solution in BSA-PBS (1:20) or in BSA-PBS without antibodies (control) for 4 h at room temperature and then for 12 h at 4 °C. After several washes in BSA-PBS, the grids were transferred into 100-μl drops of Protein-A-Gold solution in BSA-PBS (1 : 15) for 1.5 h at room temperature. Then the grids were rinsed successively in BSA-PBS, PBS, and mQ water. Gold colloids (size 20 nm) were obtained from the Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences (Saratov). After contrasting the sections were analyzed with a JEM JEOL X-100 electron microscope (Japan).

In experiments with WT and *cia3* mutant cells of *C. reinhardtii,* the antibodies raised against recombinant Cah3 protein (Agrisera; 1:16 dilution) or similarly diluted rabbit serum (R4505, Sigma) (negative control) were used as primary antibodies. Nonspecific interaction of antibodies was blocked by pretreatment of nickel grids for 1 h in a

drops of 20% goat serum (G9023, Sigma) in Tris-buffered saline (TBS), (2.4 g Tris–HCl, 8.76 g NaCl per liter, pH 7.4) supplemented with 1% BSA (BSA-TBS). The grids were transferred to 100-μl drops of the primary antibodies in BSA-TBS and incubated at 4 °C overnight. After several washes in TBS, grids were transferred to 100-μl drops of diluted (1:15) anti-rabbit IgG conjugated with 10 nm colloidal gold particles (G7402, Sigma) in BSA-TBS and exposed for 1 h at room temperature. Then the grids were washed sequentially in TBS and deionized water. Contrasting of thin sections was performed with uranyl acetate and lead citrate according to Reynolds [31]. Thin sections of samples were analyzed with the Libra-120 transmission electron microscope (Carl Zeiss, Germany).

The distribution of gold particles within sections was estimated as average value of series of independent experiments. Values represent the number of gold particles in the pyrenoid (means ± standard deviation). Statistical significance of difference in mean values was confirmed by unpaired *t*-test with *p* values less than 0.05.

2.5. Determination of fatty acids composition of lipids

Lipids were extracted by a mixture of methanol and chloroform (1:1, v/v). FAs were converted into their methyl esters by direct methanolysis of lipids after addition of 5% acetyl chloride. FAs methyl esters were analyzed using a Chrom-5 chromatograph equipped with a CI-100 integrator (Laboratorni pristroje, Czech Republic). Arachidic acid (20:0) was used as an internal standard. FAs analysis was performed on a 2.4 m column packed with 5% PEGA (polyethyleneglycol adipate) on Celite 545 at 185 °C. Nitrogen was used as a carrier gas. The rate of individual FA formation was calculated according to the equation: $V_m = \Delta m/(\Delta t \times p_{av})$, where Δm is the change in the FA content in unit volume for the Δt period, and p_{av} is the average content of proteins in the some volume of suspension. In agreement with the hypothesis of the sequential incorporation of double bonds during polyunsaturated FA (PUFA) biosynthesis, the rate of stearic acid formation can be approximately equal to $V_{(18:0)} = V_{m\ (18:0)} + V_{m\ (18:1)} + V_{m\ (18:2)} + V_{m\ (18:3)} + V_{m\ (18:4)}$, the rate of oleic acid formation can be $V_{(18:1)} = V_{m\ (18:1)} + V_{m\ (18:2)} + V_{m\ (18:3)} + V_{m\ (18:4)}$, etc. It is more difficult to perform a similar analysis for the acids of C_{16} series, because the pool of palmitic acid is consumed by the desaturases, and, at the same time, the palmitic acid is a substrate for elongase, which performs the conversion of 16:0 into 18:0 [32].

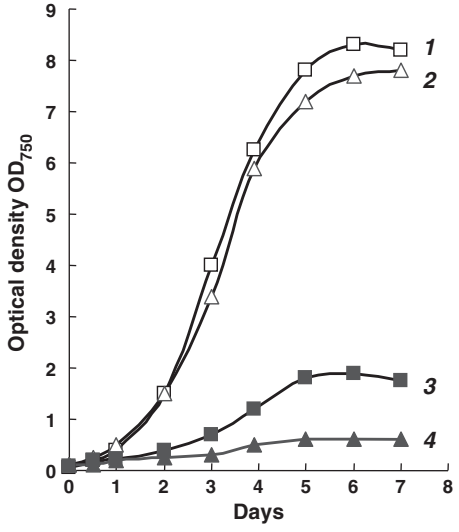

Fig. 1. The growth of WT (*1, 3*) and *cia3* mutant (*2, 4*) of *C. reinhardtii* grown at 2% (*1, 2*) and 0.03% (*3, 4*) CO_2.

Chlorophyll was extracted from microalgal cells with methanol. The absorption spectra were registered with an M-400 spectrophotometer (Carl Zeiss, Germany).

All experiments were carried out in 2–3 replicates. Biochemical characteristics were based on 4–6 analytical assays.

3. Results

3.1. Growth of C. reinhardtii

The rate of growth was measured in WT cells of *C. reinhardtii* and in its *cia3* mutant that lacks the active form of Cah3. The algae were grown under aeration with 2% (H-CO_2 cells) or 0.03% (L-CO_2 cells) of CO_2. The growth rate of WT depended on the CO_2 concentration, being higher at 2% CO_2 (Fig. 1). At this concentration of CO_2, the difference in the growth rate between the WT and *cia3* was insignificant, whereas at 0.03% CO_2 the growth rate of *cia3* mutant was lower than that of WT. At the high CO_2 concentration, a linear phase of growth of both WT and mutant cells was characterized by a doubling time of 17 h. At the low CO_2 concentration, the doubling time of WT cells was 26 h and that of *cia3* cells—45 h. It should be noted that in our experiments the doubling time was lower than that obtained for *C. reinhardtii* grown in less concentrated nutrient medium [28].

3.2. Fatty acids composition

H-CO_2 cells of the *cia3* mutant differed from WT by lower amounts of 16:0, E-16:1ω13, 16:3ω3, and 18:1ω9 acids and by higher amounts of 16:1, 18:0, 18:3ω3, and 18:4 acids, as well as minor components, 16:2ω6 and 16:3ω6 (Table 1). Total FAs content, as estimated on a chlorophyll basis (Fig. 2), increased during adaptation of both types of cells to low concentrations of CO_2 in the growth medium, mainly, due to an increase in the content of C_{18} acids. Under these conditions, the C_{18}/C_{16} ratio increased from 0.74 to 1.66 in WT cells, and from 0.79 to 1.46 in the *cia3* mutant cells. Upon a decrease in the concentration of CO_2, the content of saturated FAs has changed in a different way. The amount of palmitic acid (calculated either as mol%, or on a chlorophyll basis) decreased, whereas the amount of stearic acid increased (Table 1, Fig. 2).

The amount of E-16:1ω13 decreased in both strains (Table 1, Fig. 2). The relative amount of 16:1 remained almost unchanged in WT cells, whereas it sharply dropped in the *cia3* mutant cells. The relative amount of 18:1ω9 substantially decreased. The proportion of PUFAs increased in both strains, especially evident for 16:4ω3, 18:3ω3, and 18:4 acids in WT.

Table 1
The relative content of FA (mol%) in the cells of WT *C. reinhardtii* and *cia3* grown at 2% CO_2 (H-CO_2 cells) and adapted to 0.03% CO_2 (L-CO_2 cells).

	WT		*cia3*	
Fatty acid	H-CO_2 cells	L-CO_2 cells	H-CO_2 cells	L-CO_2 cells
16:0	45.7	23.2	39.5	29.7
16:1[a]	2.1	2.2	4.4	0.9
E-16:1ω13	5.7	2.5	5.0	3.3
16:2ω6	1.3	1.8	2.4	3.0
16:3ω6	0.7	2.1	1.2	2.1
16:3ω3	2.4	1.8	1.3	1.4
16:4ω3	4.2	5.8	4.3	4.6
18:0	2.1	11.1	4.6	9.5
18:1ω9	14.3	9.1	13.0	9.3
18:2ω6	9.7	7.9	9.5	11.0
18:3ω3	3.9	8.3	5.0	6.0
18:4[a]	7.7	24.2	9.9	19.3

Notes: FA: 16:0—palmitic; 16:1—hexadecenoic; E-16:1ω13—*trans*-3-hexadecenoic; 16:2ω6—hexadecadienoic; 16:3ω6—hexadecatrienoic; 16:4ω3—hexadecatetraenoic; 18:0—stearic; 18:1ω9—oleic; 18:2ω6—linoleic; 18:3ω3—linolenic; 18:4—octadecatetraenoic.

[a] The positions of double bonds are not determined.

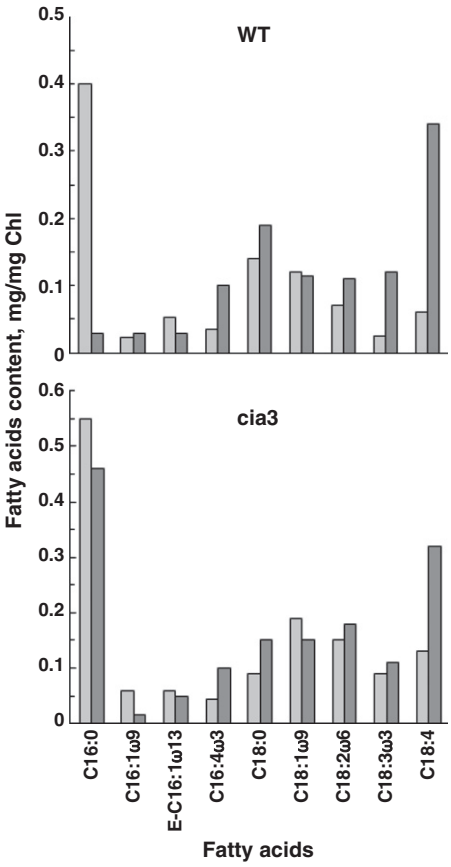

Fig. 2. FA composition of lipids in the cells of WT and *cia3* mutant of *C. reinhardtii* grown at 2% (light columns) and after 48-h exposure to 0.03% CO_2 (dark columns). See Table 1 for definitions of the FAs.

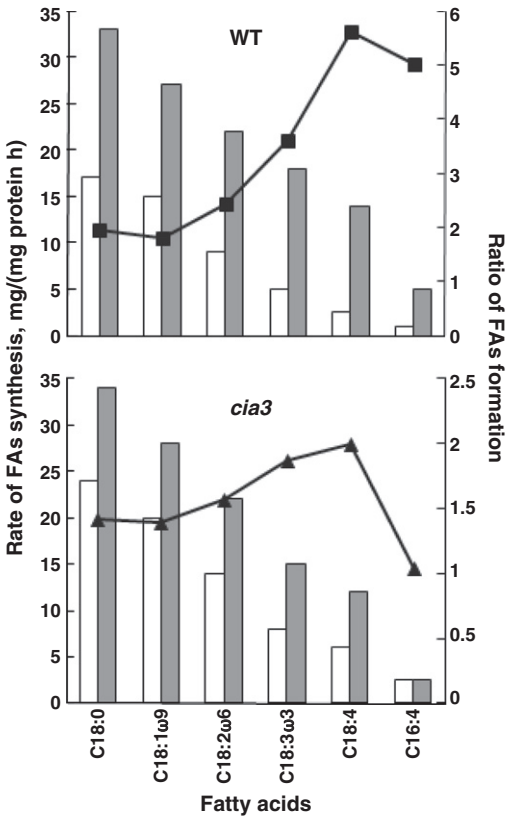

Fig. 3. The rate of major FAs formation in cells of the WT and the *cia3* mutant of *C. reinhardtii* grown at 2% (light columns) and exposed to 0.03% CO_2 for 48 h (dark columns). The ratio of FA formation rates at 0.03% CO_2 and at 2% CO_2 is presented as the curve referring to the right ordinates. See Table 1 for definitions of the FAs.

FA desaturation is known to be carried out by several desaturases, which specifically incorporate double bonds into definite positions of FAs molecules, thus increasing the degree of their unsaturation. These desaturases differ in their response to changes in the external conditions [33]. To characterize the activity of these processes we estimated the rate of FAs biosynthesis [32]. This calculation takes into account not only the rate of FAs formation, but also the extent of their utilization as substrates for the next steps of desaturation.

Fig. 3 represents the results of such calculation for FAs of the C_{18} series. The figure demonstrates that the formation of stearic acid in both WT and *cia3* cells was accelerated upon a decrease in the CO_2 concentration. This reflects the increase in the ratio of C_{18} to C_{16} acids noted above, as well as in the activity of FA-synthetase II, which is responsible for the elongation of palmitic acid, C_{16}, into stearic acid, C_{18}.

The acceleration of rate of 18:1ω9 formation under decline of CO_2 concentration is not as much as that of 18:0 and of more unsaturated FAs, such as 18:2ω6, and, especially, 18:3ω3 or 18:4 (Fig. 3, curve). This was also confirmed by the fact that the amount of 18:1ω9 did not increase and even somewhat decreased in both WT and *cia3* mutant cells after their transfer to low CO_2 concentration (Fig. 2, Table 1). The acceleration of 18:3ω3 and 18:4 formation in L-CO_2 cells (Fig. 3, curve) resulted in a sharp increase in their contents, especially in WT cells (Table 1, Fig. 2). It was also noticeable that the low CO_2 caused the enhancement in the rate of 16:4ω3 biosynthesis in the WT, but not in the *cia3* cells (Fig. 3).

Thus, the deficit in CO_2 induces selective changes in the biosynthesis of FAs. The following features should be noted: (1) acceleration of 16:0→18:0 elongation; (2) relatively moderate increase in the synthesis of 18:1ω9 and the inhibition of E-16:1ω13 formation; and (3) a higher rate of PUFAs biosynthesis in WT cells as compares to that in *cia3*; this was especially expressed in the case of 16:4ω3 acid.

3.3. Ultrastructure of the Chlamydomonas chloroplast and detection of the Cah3

The *C. reinhardtii* cell has a cap-shaped chloroplast with a basal part that contains the pyrenoid surrounded by a starch sheath (Fig. 4A). The stacks of chloroplast thylakoids are oriented in parallel to the chloroplast envelope. Some thylakoids pass through the starch grains and may extend into the pyrenoid (Fig. 5A). Thylakoids that cross the pyrenoid were characterized by the reduced stacks and displayed a tubular shape (Fig. 4C; Fig. 5A, C). They formed a dense chaotic network inside pyrenoid, which retained their connections to the chloroplastic thylakoids (Fig. 5A).

Starch granules were separated from the lamellas by thin layers of the chloroplast matrix, which surrounded the granules and contacted the body of the pyrenoid (Fig. 4A).

For immunocytochemical localization of the Cah3 inside the cells, thin sections of WT cells were probed with the antibodies against Cah3 and secondary gold-labeled antibodies followed by the electron microscopy. In WT cells, the electron-dense markers (gold particles) were found in the chloroplast: specifically, in the intrapyrenoid thylakoids and, to much less extent, in the chloroplast thylakoids (Fig. 4A, C). The gold particles have not been detected in the stroma of chloroplast, or in other cellular compartments. The insignificant number of gold particles detected there may reflect nonspecific binding of antibodies. In control cells,

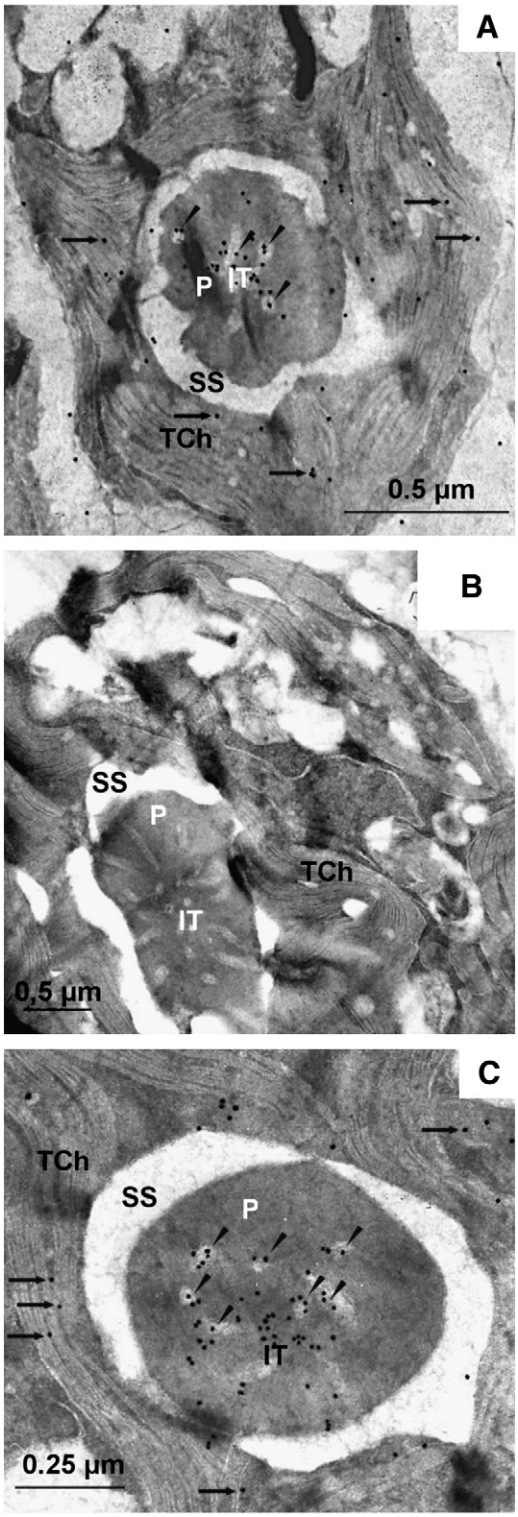

Fig. 4. Immunocytochemical localization of Cah3 in thin sections of the WT of *C. reinhardtii* cells. (A, B) experimental and control samples respectively; (C) pyrenoid section. For the immunocytochemical reaction, antibodies against *C. reinhardtii* Cah3 protein and Protein-A-Gold were used. For the control, the treatment of samples with primary antibodies was omitted. Abbreviations: IT—intrapyrenoid thylakoids; P—pyrenoid; SS—starch sheath; TCh—chloroplast thylakoids. Gold particles attached to chloroplast thylakoids are marked with arrows; gold particles in intrapyrenoid thylakoids are marked with arrowheads.

only a negligible number of gold particles were observed, and they were not specifically attributed to any cell compartment (Fig. 4B).

The CCM-mutant *cia3* has two point mutations in the thylakoid transit peptide of Cah3 [28]. This mutant was also subjected to immunodetection of Cah3 with the anti-Cah3 antibodies. Cells of the *cia3* mutant had the unusually large pyrenoid as compared with the WT cells. This pyrenoid, however, was surrounded by a very thin starch sheath in comparison with WT, or, alternatively, this sheath was absent at all. Intrapyrenoid thylakoids of the *cia3* mutant were quite rare and often formed a "tangle" in the center of the pyrenoid (Fig. 5B, D).

As shown above, the lumenal Cah3 was mostly located in the intrapyrenoid thylakoids of the WT cells of *C. reindhardtii* (Fig. 5A) [19,20]. In *cia3* mutant cells, the Cah3 was significantly less abundant, and it was found evenly distributed throughout the pyrenoid matrix (Fig. 5B). The statistics of distribution of gold particles in pyrenoids of the *cia3* mutant confirms that the detection of the Cah3 protein in the pyrenoid matrix is not just accidental: (a) sections probed by Cah3 antibodies provided as many as 6.1 ± 3.9 gold particles per pyrenoid (statistics from 17 sections); (b) sections probed by the pre-immune serum provided only 3.11 ± 1.9 gold particles per pyrenoid (statistics from 9 sections).

The presence of Cah3 protein in *cia3* mutant was also confirmed by western blot analysis with the same antibodies against Cah3 of *C. reinhardtii* (Fig. 6). In the cell fractions of *cia3*, antibodies cross-reacted with only one specific protein of about 30 kDa (Fig. 6). This signal was weaker as compared to the WT, and it was detected only at extended time of exposition in substrate-containing solution (Fig. 6B, C). The results of immunocytochemical detection, as well as western blotting, indicate that Cah3 is mislocated in *cia3* mutant cells, i.e., it was found in the matrix but not in thylakoids of the pyrenoid.

The analysis of the large number of electron micrographs of WT cells showed that the Cah3 immune markers were mostly bound to intrapyrenoid thylakoids rather than to stromal thylakoids. Moreover, in stromal thylakoids Cah3 was rather located in the thylakoids that adjoin the pyrenoid. Taking into account that the bulk of Rubisco protein is also localized in the algal pyrenoid [15,16,20,34], we suppose a co-localization of Rubisco and Cah3 in the pyrenoid of *C. reinhardtii.*

4. Discussion

Operation of the CCM requires the activities of CAs and the active transport of C_i. The mutants defective in CCM, such as *cia3* mutant of *C. reindhardtii,* exhibit poor growth under low CO_2 conditions (Fig. 1). It belongs to a group of so-called high-CO_2-requiring mutants [24,28,35]. It has been shown previously that the rate of photosynthetic oxygen evolution in this mutant is significantly lower than in the WT cells when CO_2 is limited [20,28,35]. It is known that the *cia3* mutant has two point mutations in the transit peptide of the Cah3 that is chloroplast lumen-located α-type CA [24]. As a result, the Cah3 protein is inactive, and the *cia3* mutant, being capable to over-accumulate C_i, cannot assimilate it [28].

The physiological function of the luminal Cah3 is still not completely clear. It was suggested that this enzyme might be responsible for the increase in the concentration of CO_2 in the chloroplast by conversion of HCO_3^- to CO_2 in the acidic compartment of the thylakoid lumen [9,21–23]. It might be also involved in the regulation of electron transport through the control of PSII activity [27].

Therefore, it is important to investigate the appearance of the Cah3 in two sites: (1) within stromal thylakoids, where the primary light reactions of photosynthesis occur, and (2) within thylakoids of the pyrenoid, where the bulk of Rubisco, the key CO_2-fixing enzyme, is present. Previously, the attempts have been made to localize the Cah3 in the intrapyrenoid thylakoids of *C. reinhardtii* by the immunocytochemical approach [19,20]. Here we confirm and specify these results by more precise demonstration of preferential localization of Cah3 in thylakoids of the pyrenoid (Figs. 4, 5). Our results also

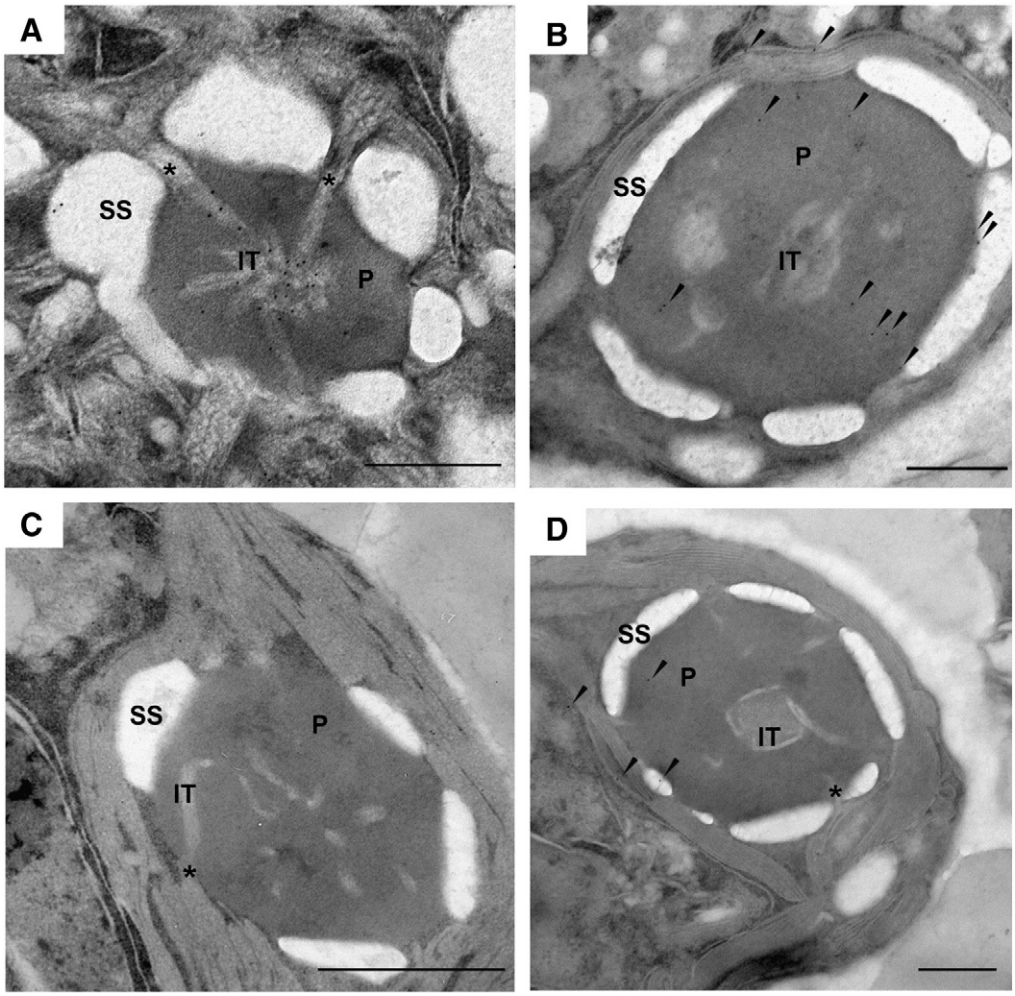

Fig. 5. Immunocytochemical localization of Cah3 in the cells of WT and *cia3* mutant of *C. reinhardtii.* Experimental samples, WT (A) and *cia3* mutant (B) respectively; control samples, WT (C) and *cia3* mutant (D) respectively. For the immunocytochemical reaction, antibodies against *C. reinhardtii* Cah3 protein and Protein-A-Gold were used. For the control, rabbit serum (Sigma) was used as primary antibodies. For abbreviations see Fig. 4. Gold particles in *cia3* mutant cells are marked with arrowheads. Asterisks show the entrance of thylakoid stacks into pyrenoid. Bars are 0.5 μm.

provide the evidence for the participation of Cah3 in the CCM of chloroplasts.

Earlier, based on the results of immunoblots and immunochemical localization, Mitra et al. [19], have concluded that *cia3* mutant completely lacks the Cah3 protein. Our results, obtained with the cells of CCM-mutant *cia3*, show that Cah3 protein is present in the pyrenoid matrix of the mutant. This fact was confirmed both by the immune-gold localization of the Cah3 (Fig. 5B) and by western blot analysis (Fig. 6 A, C). The latter was performed with two different types of antibodies: 1) raised against Cah3 protein of *C. reinhardtii*, and 2) raised against recombinant Cah3-MBP fusion protein (for details, see Section 2.3). Cross-reaction with antibodies raised against the fusion protein, was clearly detected in the *cia3* cell fractions (Fig. 6A). Cross-reaction with antibodies raised against the Cah3 alone was also detected if the exposure time to visualize the antibodies was extended (Fig. 6C), No signal was detected with antibodies raised against Cah3 alone under short exposition (Fig. 6B). Similar results have been obtained by Mitra et al. [19], who concluded that the antibodies raised against the fusion protein might recognize some other protein that has similar molecular weight as Cah3. In our experiments, however, the specific signal of Cah3 was amplified in the membrane fraction.

Thus, our immunoblotting data (Fig. 6C), as well as the results of immunoelectron microscopy (Fig. 5B), support the suggestion that the cells of the *cia3* mutant still contain the Cah3 protein. One may assume that *cia3* has lost the ability to concentrate CO_2 because the Cah3 is not embedded in the lumen due to two mutations in the leader sequence that targets the protein into the lumen [24]. The appearance of Cah3 in *cia3* cells at rather low amounts might be the result of incomplete degradation or decreased synthesis of the protein.

The requirement of the luminal CA for operation of the CCM was proposed 30 years ago [21,22]. The recent developments in the understanding of this mechanism are presented in Fig. 7. The C_i absorbed by the cell is accumulated in the form of HCO_3^- in the chloroplast stroma, where pH is slightly alkaline (about 8.0). This leads to the accumulation of the bicarbonate pool. Since HCO_3^- is required for intracellular accumulation and CO_2—for the photosynthetic fixation of C_i, it is necessary to convert a massive pool of HCO_3^- into the CO_2 molecules that are fixed by Rubisco for further formation of the organic compounds. This reaction can be accomplished only in the "acidic" cell compartments and requires close cooperation of the CA and the Rubisco enzymes. The pyrenoid is a suitable compartment: it contains Rubisco, and its thylakoids are filled with the Cah3 (Figs. 4A, 5A). The main compartment where CO_2 is produced from the HCO_3^- pool is the acid lumen of intrapyrenoid thylakoids rather than the whole pyrenoid. According to the proposed scheme (Fig. 7), the pyrenoid acts as an autonomous metabolic microcompartment, where Cah3 plays the key role in the production and concentrating CO_2 for Rubisco, thus increasing the rate of photosynthesis.

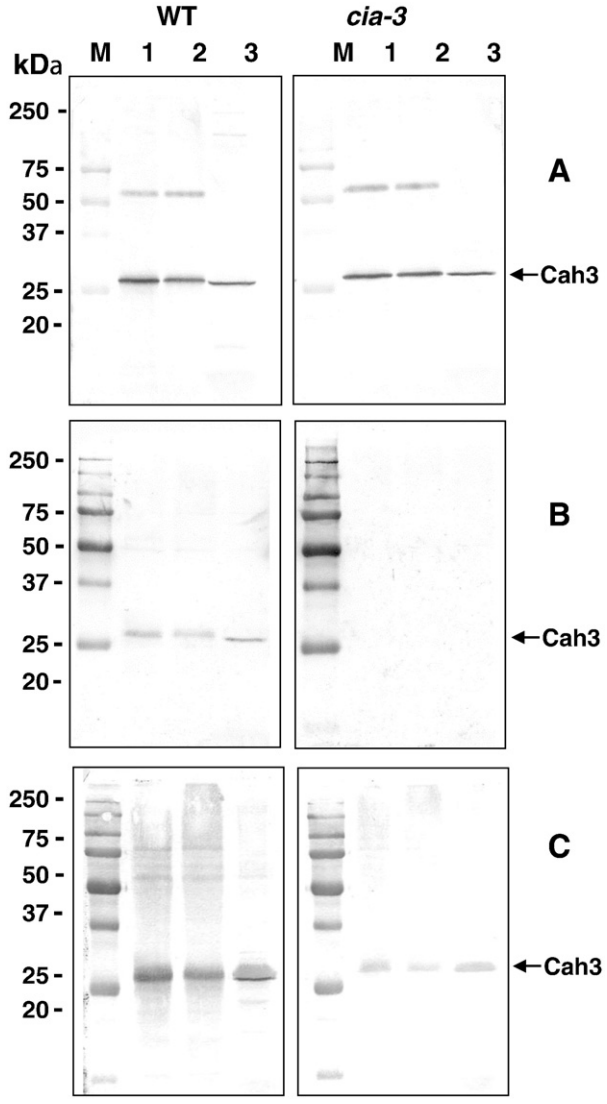

Fig. 6. Western blotting detection of Cah3 protein in the cell fractions of WT and *cia3* mutant of *C. reinhardtii*. (A) The blotted membrane was probed with antibodies raised against recombinant Cah3-MBP fusion protein and exposed in a substrate solution for 0.5 min. (B) The blot was probed with Cah3 antibodies and exposed in a substrate solution for 0.5 min. (C) The blot was probed by Cah3 antibodies and exposed in a substrate solution for 2 min. Panels A, B, C: (1) total cell homogenate; (2) soluble proteins; (3) membrane proteins; M—molecular weight markers. Each lane contained 45 μg of protein. 12% SDS-PAGE was used to resolve the polypeptides for western blotting analysis.

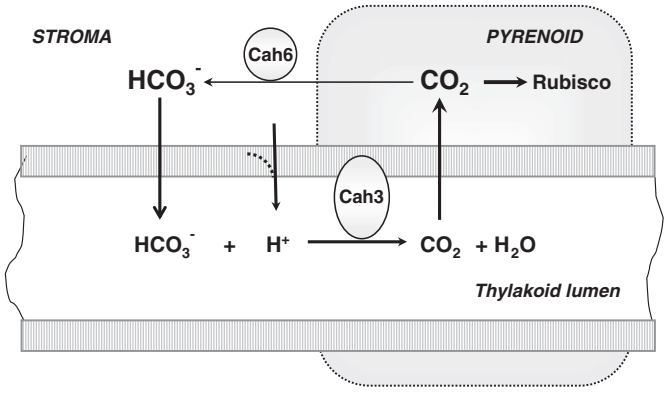

Fig. 7. The scheme for the proposed mechanism of Cah3-mediated CO_2 generation in the pyrenoid. Dashed line shows proton generation during the dehydrogenation of FAs.

Strong evidence that the pyrenoid plays an important role in the CCM was provided during investigation of the new CCM-mutant, *cia6* [36]. The stromal CA Cia6 is located outside the pyrenoid. Surprisingly, when the corresponding *cia6* gene is knocked out in *C. reinhardtii*, cells have disrupted pyrenoids and dysfunction of the CCM. These results indicate that Cia6 is also important for fixation of CO_2. Probably, the stromal Cia6 recaptures CO_2 that diffuses out of the pyrenoid, and converts CO_2 to HCO_3^-, thereby reducing the leakage of CO_2 out of the chloroplast.

The proposed model of CCM in the chloroplast is related to ion (H^+ и HCO_3^-) transport across the thylakoid membrane, and it inevitably depends on the physical properties (fluidity) of the membranes. Fluidity, in turn, is determined by the membrane composition and, first of all, by the level of the PUFAs of membrane lipids [37,38].

In many algae, 18:3ω3 and 16:4ω3 are predominantly localized in the monogalactosyl diacylglycerols, the main thylakoid lipids, which are suggested to be structurally and functionally related to the core complex of PSI [39]. The concentration of these PUFAs is tightly correlated with the activity of the photosynthetic apparatus [32,39,40]. It is generally accepted that 16:1ω13 stabilizes the oligomeric structure of the light-harvesting chlorophyll *a*/*b*-protein complex (LHC) of PSII [41]. A considerable decrease in 16:1ω13 and a simultaneous increase in PUFAs content at low CO_2 (Table 1, Fig. 2) suggests that LHC may not be sufficiently developed under such conditions, whereas PSI may operate actively. Both activation of PSI and ATP synthesis due to the cyclic electron transport are necessary for providing energy for the functioning of CCM [42]. This, in turn, results in the enhancement of PUFAs biosynthesis.

Thus, the present results (namely, the observed correlation between the increase in the content of PUFAs (Fig. 2) and the acceleration of the activity of photosynthesis) demonstrate the importance of the thylakoid structure for cell adaptation to low CO_2 environments. The enhanced increase in the relative content (% of total FAs) (Table 1) and in the rate of accumulation of the ω3-PUFAs (Fig. 3) in the WT cells compared to the *cia3* mutant cells, implies the cross-talk between the metabolic pathways that link the activity of CAs, CCM, photosynthesis, and the synthesis of PUFAs.

It has been shown earlier that FA desaturation is primarily related to PSI activity [39]. The reduced parameters for growth (Fig. 1) and photosynthesis [20] at low CO_2 concentration in *cia3* cells if compared to WT, may reflect a decrease in PUFAs biosynthesis in the mutant. FA desaturation, which causes the release of the excess of H^+, implies the presence of an acceptor of these protons. The role of such an acceptor is regularly attributed to the photosynthetic oxygen produced by the water-splitting complex. However, the mutant of *C. reinhardtii* that lacks the water-splitting complex can successfully perform FA desaturation [43]. In this mutant, the cyclic electron transport around PSI provides all the components necessary for FA desaturation, except oxygen. These observations allowed us to conclude that, in the mutant cells, the acceptor of H^+ may be not limited to O_2.

Here, we suggest that the local excess of H^+ may be neutralized by the CA (Cah3) through the reaction with HCO_3^- in the lumen [22,39] with the ultimate formation of CO_2 and H_2O.

In *cia3* mutant cells, Cah3 protein was detected in the pyrenoid matrix (Fig. 5B), which has slightly alkaline pH, similar to stroma of a chloroplast. Thus, in *cia3* cells, non-functional Cah3 cannot perform the reaction $HCO_3^- + H^+ \rightarrow CO_2 + H_2O$, under acidic conditions in the lumen. This reaction, however, is obligatory for CO_2 generation and for the removal of protons during FA dehydrogenation. Putative disturbance in the system of O_2 evolution and HCO_3^- to CO_2 conversion in the thylakoids of the *cia3* mutant cells makes it impossible to carry out FAs desaturation with the same efficiency as in WT cells.

The multiplicity of CA functions had been repeatedly discussed [2,4,20,23]. CAs may facilitate the transport of CO_2 and HCO_3^- by supplying these carbon forms to the C_i transporters. CAs may also catalyze reactions, whose substrates or products require the supply of

CO_2 or HCO_3^-. This is achieved by providing of the appropriate concentrations of CO_2 or HCO_3^- near the enzyme active center (including FA-synthase) or, alternatively, by removal of CO_2/HCO_3^- products. CAs may also participate in the removal of protons, which are generated during the reaction of water oxidation or FA desaturation (dehydrogenation) in thylakoid membranes, by protonation of bicarbonate in the lumen.

Acknowledgements

We are very grateful to Prof. J. Moroney (Louisiana State University, USA) for generously providing the strain of *C. reinhardtii* 137mt$^+$ and CCM-mutant *cia3*, which were presented to Culture Collection of the Institute of Plant Physiology (IPPAS); Prof. G. Samuelsson (Umea University, Sweden) who kindly supplied us with primary antibodies against recombinant Cah3 and Cah3-MBP fusion protein. We thank Prof. D.A. Los and Dr. N. L. Klyachko (Institute of Plant Physiology, Russia) for helpful discussions and advice at the stage of manuscript preparation. This work was supported by a grant from the Russian Foundation for Basic Research (no. 10-04-01463) to E.V.K., by a grant from the Presidium of Russian Academy of Sciences (program "Molecular and Cell Biology"), and by a grant from the Russian Ministry of Education and Science (no. 16.740.11.0176).

References

[1] K. Aizawa, S. Miyachi, Carbonic anhydrase and CO_2-concentrating mechanism in microalgae and cyanobacteria, FEMS Microbiol. Rev. 39 (1986) 215–233.
[2] G.D. Price, M.R. Badger, F.J. Woodger, B.M. Long, Advances in understanding the cyanobacterial CO_2-concentrating mechanism (CCM): functional components, C_i transporters, diversity, genetic regulation and prospects for engineering into plants, J. Exp. Bot. 59 (2008) 1441–1461.
[3] N. Yamano, H. Fukuzawa, Carbon-concentrating mechanism in green alga, *Chlamydomonas reinhardtii*, revealed by transcriptome analyses, J. Basic Microbiol. 49 (2009) 42–51.
[4] J.V. Moroney, R.A. Ynalvez, Proposed carbon dioxide concentrating mechanism in *Chlamydomonas reinhardtii*, Eukaryot. Cell 6 (2007) 1251–1259.
[5] C.T. Supuran, Carbonic anhydrases—an overview, Curr. Pharm. Des. 14 (2008) 603–614.
[6] Y. Xu, L. Feng, P.D. Jeffrey, Y. Shi, F.M. Morel, Structure and metal exchange in the cadmium carbonic anhydrase of marine diatoms, Nature 452 (2008) 56–61.
[7] M.H. Spalding, Microalgal carbon-dioxide-concentrating mechanisms: *Chlamydomonas* inorganic carbon transporters, J. Exp. Bot. 59 (2008) 1463–1473.
[8] T. Yamano, T. Tsujikawa, K. Hatano, S. Ozawa, Y. Takanashi, H. Fukuzawa, Light and low CO_2-dependent LCID–LCIC complex localization in the chloroplast supports the carbon-concentrating mechanism in *Chlamydomonas reinhardtii*, Plant Cell Physiol. 51 (2010) 1453–1468.
[9] J.V. Moroney, Y. Ma, W.D. Frey, K.A. Fusilier, T.T. Pham, T.A. Simms, R.J. DiMario, J. Yang, B. Mukherjee, The carbonic anhydrase isoforms of *Chlamydomonas reinhardtii*: intracellular location, expression, and physiological roles, Photosynth. Res. 109 (2011) 133–149.
[10] M.R. Badger, G.D. Price, The CO_2 concentrating mechanism in cyanobacteria and microalgae, Physiol. Plant. 84 (1992) 606–615.
[11] R.M.L. McKay, S.P. Gibbs, Composition and function of pyrenoids: cytochemical and immunocytochemical approaches, Can. J. Bot. 69 (1991) 1040–1052.
[12] Z. Ramazanov, M. Rawat, M.C. Henk, C.B. Mason, S.W. Matthews, J.V. Moroney, The induction of the CO_2-concentrating mechanism is correlated with the formation of the starch sheath around the pyrenoid of *Chlamydomonas reinhardtii*, Planta 195 (1994) 210–216.
[13] M. Tsuzuki, E. Ohnuma, N. Sato, T. Takaku, A. Kawaguchi, Effects of CO_2 concentration during growth on fatty acid composition in microalgae, Plant Physiol. 93 (1990) 851–856.
[14] N.A. Pronina, N.B. Rogova, S. Furnadjieva, G.L. Klyachko-Gurvich, Effect of CO_2 concentration on the fatty acid composition of lipids in *Chlamydomonas reinhardtii cia-3*, a mutant deficient in CO_2-concentrating mechanism, Russ. J. Plant Physiol. 45 (1998) 447–455.
[15] A.G. Markelova, M.G. Vladimirova, V.E. Semenenko, Ultrastructural localization of Rubisco in algal cells, Sov. Plant Physiol. 37 (1990) 907–911.
[16] K. Kuchitsu, M. Tsuzuki, S. Miyachi, Polypeptide composition and enzyme activities of the pyrenoid and its regulation by CO_2 concentration in unicellular green algae, Can. J. Bot. 69 (1991) 1062–1069.
[17] M. Rawat, M.C. Henk, L.L. Lavigne, J.V. Moroney, *Chlamydomonas reinhardtii* mutants without ribulose-1,5-bisphosphate carboxylase-oxygenase lack a detectable pyrenoid, Planta 198 (1996) 263–270.
[18] T. Genkov, M. Meyer, H. Griffiths, R.J. Spreitzer, Functional hybrid Rubisco enzymes with plant small subunits and algal large subunits: engineered rbcS cDNA for expression in *Chlamydomonas*, J. Biol. Chem. 285 (2010) 19833–19841.
[19] M. Mitra, C.B. Masson, Y. Xiao, R.A. Ynalvez, S.M. Lato, J.V. Moroney, The carbonic anhydrase gene families of *Chlamydomonas reinhardtii*, Can. J. Bot. 83 (2005) 780–795.
[20] A.G. Markelova, M.P. Sinetova, E.V. Kupriyanova, N.A. Pronina, Distribution and functional role of carbonic anhydrase Cah3 associated with thylakoid in chloroplast and pyrenoid of *Chlamydomonas reinhardtii*, Russ. J. Plant Physiol. 56 (2009) 761–768.
[21] N.A. Pronina, S. Avramova, D. Georgiev, V.E. Semenenko, Dynamics of carbonic anhydrase activity in *Chlorella* and *Scenedesmus* during adaptation of cells to light of high intensity and low CO_2 concentration, Sov. Plant Physiol. 28 (1981) 32–40.
[22] N.A. Pronina, V.V. Borodin, CO_2-stress and CO_2-concentration mechanism: investigation by means of photosystem-deficient and carbonic anhydrase-deficient mutants of *Chlamydomonas reinhardtii*, Photosynthetica 28 (1993) 515–522.
[23] J.A. Raven, CO_2 concentrating mechanisms: a role for thylakoid lumen acidification? Plant Cell Environ. 20 (1997) 147–154.
[24] J. Karlsson, A.K. Clarke, Z.Y. Chen, S.Y. Hugghins, Y.I. Park, H.D. Husic, J.V. Moroney, G. Samuelsson, A novel alpha-type carbonic anhydrase associated with the thylakoid membrane in *Chlamydomonas reinhardtii* is required at ambient CO_2, EMBO J. 17 (1998) 1208–1216.
[25] D.T. Hanson, L.A. Franklin, G. Samuelsson, M.R. Badger, The *Chlamydomonas reinhardtii cia3* mutant lacking a thylakoid lumen-localized carbonic anhydrase is limited by supply to Rubisco and not photosystem II function *in vivo*, Plant Physiol. 132 (2003) 2267–2275.
[26] D. Duanmu, Y. Wang, M.H. Spalding, Thylakoid lumen carbonic anhydrase (CAH3) mutation suppresses air-Dier phenotype of LCIB mutant in *Chlamydomonas reinhardtii*, Plant Physiol. 149 (2009) 929–937.
[27] T. Shutova, H. Kenneweg, J. Buchta, J. Nikitina, V. Terentyev, S. Chernyshov, S.I. Allakhverdiev, V.V. Klimov, H. Dau, G. Samuelsson, The Photosystem II-associated Cah3 in *Chlamydomonas* enhance the O_2 evolution rate by proton removal, EMBO J. 27 (2008) 782–791.
[28] J.V. Moroney, H.D. Husic, N.E. Tolbert, M. Kitayama, L.J. Manuel, R.K. Togasaki, Isolation and characterization of a mutant of *Chlamydomonas reinhardtii* deficient in the CO_2 concentrating mechanism, Plant Physiol. 89 (1989) 897–903.
[29] M.G. Vladimirova, A.G. Markelova, Autotrophic growth of the cell-wall-free mutant of *Chlamydomonas reinhardtii* CW-15 under conditions of intensive culture, Sov. Plant Physiol. 27 (1980) 878–889.
[30] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of the bacteriophage T4, Nature 227 (1970) 680–685.
[31] E.S. Reynolds, The use of lead citrate at high pH as an electron-opaque stain in electron microscopy, J. Cell Biol. 17 (1963) 208–212.
[32] G.L. Klyachko-Gurvich, A.N. Semenova, V.E. Semenenko, Lipid metabolism of the chloroplasts during adaptation of *Chlorella* cells to lowering of illumination, Sov. Plant Physiol. 27 (1980) 290–298.
[33] D.A. Los, N. Murata, Structure and expression of fatty acid desaturases, Biochim. Biophys. Acta 1394 (1998) 3–15.
[34] N.A. Pronina, V.E. Semenenko, Pyrenoid role in CO_2 concentrating and fixation in microalga chloroplast, Russ. J. Plant Physiol. 39 (1992) 723–732.
[35] M.H. Spalding, R.J. Spreitzer, W.L. Ogren, Carbonic anhydrase-deficient mutant of *Chlamydomonas reinhardtii* requires elevated carbon-dioxide concentration for photoautotrophic growth, Plant Physiol. 73 (1983) 268–272.
[36] Y. Ma, S.V. Pollock, Y. Xiao, K. Cunnusamy, J. Moroney, Identification of a novel gene, Cia6, required for normal pyrenoid formation in *Chlamydomonas reinhardtii*, Plant Physiol. 156 (2011) 884–896.
[37] S.I. Allakhverdiev, D.A. Los, N. Murata, Regulatory roles of unsaturated fatty acids in membrane lipids in photosynthesis, in: H. Wada, N. Murata (Eds.), Lipids in Photosynthesis. Essential and Regulatory Functions, Springer Science + Business Media B.V., 2009, pp. 373–388.
[38] L. Renberg, A.I. Johansson, T. Shutova, H. Stenlund, A. Aksmann, J.A. Raven, P. Gardeström, T. Moritz, G. Samuelsson, A metabolomic approach to study major metabolite changes during acclimation to limiting CO_2 in *Chlamydomonas reinhardtii*, Plant Physiol. 154 (2010) 187–196.
[39] G.L. Klyachko-Gurvich, L.N. Tsoglin, J. Doucha, J. Kopetskii, I.B. Shebalina (Ryabykh), V.E. Semenenko, Desaturation of fatty acids as an adaptive response to shifts in light intensity, Physiol. Plant. 107 (1999) 240–249.
[40] P. Dörman, G. Höltz, The role of glycolipids in photosynthesis, in: H. Wada, N. Murata (Eds.), Lipids in Photosynthesis. Essential and Regulatory Functions, Springer Science + Business Media B.V., 2009, pp. 265–282.
[41] J. Maroc, A. Tremolieres, J. Garnier, D. Guyon, Oligomeric form of the light-harvesting chlorophyll *a* + *b* protein complex CPII, phosphatidylglycerol, 3-*trans*-hexadecenoic acid and energy transfer in *Chlamydomonas reinhardtii* wild type and mutants, Biochim. Biophys. Acta 893 (1987) 91–99.
[42] E.D. Demidov, Activation of the cyclic photophosphorylation during the induction of CO_2-concentrating mechanism in *Chlamydomonas reinhardtii* cells, Russ. J. Plant Physiol. 41 (1994) 166–171.
[43] G.L. Klyachko-Gurvich, N.A. Pronina, V.G. Ladygin, Tsoglin, V.E. Semenenko, Uncoupled functioning of separate photosystems: 1. Characteristics of fatty acid desaturation and its role, Russ. J. Plant Physiol. 47 (2000) 603–612.