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BIOENERGY AND BIOFUELS

Impacts of CO_2 concentration on growth, lipid accumulation, and carbon-concentrating-mechanism-related gene expression in oleaginous *Chlorella*

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Abstract Biodiesel production by microalgae with photosynthetic CO_2 biofixation is thought to be a feasible way in the field of bioenergy and carbon emission reduction. Knowledge of the carbon-concentrating mechanism plays an important role in improving microalgae carbon fixation efficiency. However, little information is available regarding the dramatic changes of cells suffered upon different environmental factors, such as CO_2 concentration. The aim of this study was to investigate the growth, lipid accumulation, carbon fixation rate, and carbon metabolism gene expression under different CO_2 concentrations in oleaginous *Chlorella*. It was found that *Chlorella pyrenoidosa* grew well under CO_2 concentrations ranging from 1 to 20 %. The highest biomass and lipid productivity were 4.3 g/L and 107 mg/L/day under 5 % CO_2 condition. Switch from high (5 %) to low (0.03 %, air) CO_2 concentration showed significant inhibitory effect on growth and CO_2 fixation rate. The amount of the saturated fatty acids was increased obviously along with the transition. Low CO_2 concentration (0.03 %) was suitable for the accumulation of saturated fatty acids. Reducing the CO_2 concentration could significantly decrease the polyunsaturated degree in fatty acids. Moreover, the carbon-concentrating mechanism-related gene expression revealed that most of them, especially *CAH2*, *LCIB*, and *HLA3*, had remarkable change after 1, 4, and 24 h of the transition, which suggests that *Chlorella* has similar carbon-concentrating mechanism with *Chlamydomonas reinhardtii*. The findings of the present study revealed that *C. pyrenoidosa* is an ideal candidate for mitigating CO_2 and biodiesel production and is appropriate as a model for mechanism research of carbon sequestration.

Keywords *Chlorella* · CO_2 · Growth · Lipid · Carbon-concentrating mechanism · Gene expression

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Introduction

Greenhouse effect has been recognized as a major factor of the global warming. Among which, carbon dioxide (CO_2) is the most important cause of greenhouse gases, thus making the CO_2 mitigation attracting much attention from both environmental and economical societies. In nature, aquatic photosynthetic microorganisms account for almost 50 % of the world's photosynthesis by acquiring CO_2 from the environment. Microalgae are expected to have strong CO_2 biofixation efficiency by dozens of times higher than terrestrial plants owing to their fast growth rate and short generation time (Tredici 2010). Therefore, using photoautotrophic microalgae for biological CO_2 mitigation has become a popular topic in this research area (Lam and Lee 2013; Zheng et al. 2014).

In fact, through photosynthesis, certain microalgae biofix CO_2 in the form of carbohydrate, protein, and lipids inside their cells, making the whole algal biomass a treasure, whereas only parts are valuable in the land plants. Microalgae can fix CO_2 from different sources, including CO_2 from the atmosphere and industrial exhaust gas and also in the form of soluble carbonates (Lam and Lee 2013). Nevertheless, there are several challenges existing in acquiring CO_2 by microalgae from the environment, especially the low solubility of CO_2

in water. Based on the fact that the diffusion of CO_2 in an aqueous solution is 10^4 times slower than the diffusion of CO_2 in air (Spalding 2008), the first challenge these organisms face is the ability to capture CO_2 as quickly as possible. The second challenge is rendered by the properties of ribulose bisphosphate carboxylase-oxygenase (Rubisco). However, Rubisco has a poor apparent affinity for CO_2 because the concentration of dissolved CO_2 is less than the Km (CO_2) of Rubisco and due to the relatively high concentration of O_2 which competes with CO_2. So, the relative rate of oxidation and carboxylation is the key factor to decide the efficiency of photosynthesis as well as CO_2 fixation rate. Third, dissolved inorganic carbon (DIC) has significant fluctuations in three forms: CO_2, HCO_3^-, and CO_3^{2-}. Inorganic carbon (Ci) in the form of CO_2 has an acidic pH value, while the overwhelming majority of Ci is in the form of HCO_3^- at an alkaline pH, which makes the microalgae suffer from dramatic changes in the supply of DIC (Kim 2014).

In order to overcome these challenges, a number of algae, including both the prokaryotic and eukaryotic algae, have developed a CO_2-concentrating mechanism (CCM) to maximize photosynthesis under limiting CO_2 conditions (Singh et al. 2014). The CCM increases the CO_2 concentration at the site of Rubisco which supplies sufficient Ci to augment photosynthetic productivity in algal cells. *Chlamydomonas reinhardtii*, a unicellular green alga, has served as a model system to study the CCM, including the energy-coupled Ci uptake and transport systems and various carbonic anhydrases (CAs) which catalyze interconversion of CO_2 and HCO_3^- (Jungnick et al. 2014; Spalding 2008). All of them play important roles in the CCM. Interestingly, the CCM can only be induced in low-CO_2 (LC, ~0.04 % CO_2) or very-low-CO_2 (VLC, <0.02 % CO_2) conditions. Maybe the high CO_2 (HC, >1 % CO_2) significantly suppresses the activity of CAs. Various CAs are induced when cells grown under HC are transferred to LC. To date, nine carbonic anhydrase genes (CAH1–CAH9) and putative transporters or other low CO_2-inducible genes have been discovered and have been hypothesized to relate to the CCM in *C. reinhardtii* (Duanmu et al. 2009b; Ohnishi et al. 2010). In addition, several transcription regulators have also been identified and characterized for induction of the CCM, e.g., Cia5 or Ccm1 (Fang et al. 2012). Although some progress has been achieved in this model organism, the detail of the CCM remains unclear, not to mention the understanding in other algae. Simple mechanisms for carbon concentration and carbon assimilation have been described in several algae; these mechanisms typically rely on protein function prediction with next-generation genome and transcriptome sequencing (Brueggeman et al. 2012; Fang et al. 2012; Radakovits et al. 2012). However, an actual mechanism for a CCM in single-cell alga has not been fully characterized, and further studies are needed to biochemically verify the proposed carbon-concentrating mechanisms in algae (Eckardt 2012; Jungnick et al. 2014).

Similar to *C. reinhardtii*, *Chlorella* spp. were used to be a model species for the study of photosynthesis for a long time. In recent years, they were found to have a fast growth rate both by heterotrophic and phototrophic cultivation (Liang et al. 2009). They are also considered to be one of the most promising commercial algae candidates due to the cells rich in nutrients. Moreover, *Chlorella* can accumulate large amounts of lipids from trophic transition or environmental stress factors, such as nutrient deficiency, so they were known as one of the potential strains for biodiesel production (Fan et al. 2014a). In the application for removal of CO_2, *Chlorella* have been studied for CO_2 reduction using industrial flue gas or pure CO_2 (Lam and Lee 2013). Despite these interesting studies to understanding the performance of *Chlorella*, large-scale production of biomass has still turned out to be problematic. One reason for that is most of the previous researches were mainly focused on the cultivation process level and little information is available regarding the dramatic changes of cells suffered upon different environmental factors, such as CO_2 concentration. Therefore, knowledge of physical and biochemical signs as well as metabolic changes and nutritional needs plays a central role in process development (Sydney et al. 2010).

Chlorella pyrenoidosa, a robust industrial strain that is already cultivated widely, mainly for food, supplement, and biofuel production, was selected as a research object in the present study. *C. pyrenoidosa* was firstly cultivated under different CO_2 concentrations ranging from 0.03 (air) to 20 % (*v*/*v*), and then, switch from 5 to 0.03 % was also conducted. The impacts of different CO_2 concentrations on the growth characteristics, lipid content, fatty acid profiles, and CO_2 biofixation rate, as well as CCM metabolism-related gene expression were fully investigated.

Materials and methods

Strain, medium, and culture conditions

C. pyrenoidosa (FACHB 9) was obtained from the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China), and purified aseptically for further study. Seed cells were grown in Endo medium and maintained at 30 °C for 3.5 days with a reciprocating shaker (150 rpm) under low-intensity light (50 μmol/m^2/s). Two percent of cells were inoculated into air-lift column photobioreactors (60 cm high and 5 cm in diameter) containing 800 mL Bold's basal medium (BBM) and cultured at 30 °C under continuous illumination using white fluorescent lamps (approximately 150 μmol/m^2/s) with one-side illumination. Photobioreactors were aerated with various concentrations of CO_2: filtered ambient air

(LC) and HC ranging from 1 to 20 % CO_2 (*v/v*) at a flow rate of 0.3 L/min for comparison. After that, four group experiments supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution (pH 7.2) were cultured with the same conditions but with bubbling of 5 % CO_2 for the first 5 days, and then, two groups were shifted from HC to LC conditions until the end of cultivation. Two biological replicates of each induction condition were processed.

Measurements of biomass density

The cell density was measured by spectrophotometer (optical density 680) and dry cell weight (DCW). Whatman GF/A glass microfiber filters (47-mm Φ circles, Whatman, England) were dried in an electric oven at 80 °C overnight and then cooled in a desiccator and weighed. Three to ten milliliters of the algal sample was filtered by vacuum filtration and was dried and cooled before weighing again. Biomass concentration was determined by the difference between the two weights.

Lipid extraction and content analysis

Total lipid extraction followed the method reported by Bligh and Dyer with some modifications. The microalgal cells were collected by centrifugation at 4000*g* for 10 min and washed with deionized water twice. Freeze-dried cells were obtained by lyophilization and weighed (*W*). Each sample (approximately 300 mg) was extracted with a mixture of methanol/chloroform (2:1, *v/v*) for 40 min by ultrasonication and then centrifuged at 6000*g* for 10 min after precipitation. After that, the extracts (supernatant) were transferred into a preweighed dry glass tube (G1) and evaporated by Termovap Sample Concentrator. The procedure was repeated until the organic solvent had no color, which meant the total lipids were entirely extracted. Finally, the residue and glass tube were dried in an electric oven at 80 °C for 12 h and cooled in a desiccator until the weight (*G*2) was constant. The total lipid content of the sample was calculated as follows:

$$\text{Total lipid content (\%)} = (G2 - G1)\Big/W \times 100\,\%$$

Chemical and physiological parameter analysis

pH in each sample was measured immediately after collection with an ISFET pH meter which was calibrated daily using standard solutions of pH 4 and 7. The method of total chlorophyll contents was referred to Dere et al. (1998) with some modifications. The cells were centrifuged, and the residue was dealt with 96 % methanol (*v/v*, fresh) in an ultrasonic cleaner (dark and cool environment) for 30 min to extract pigments. After that, cellular debris was pelleted by centrifugation at 6000 *g* for 10 min and chlorophyll a and b levels in the supernatant were estimated spectrophotometrically by recording optical absorbance at 653 and 666 nm.

Measurements of CO_2 fixation rate

The CO_2 biofixation rate R_{CO_z} was calculated by using the following equation:

$$R_{CO_z} = C_C P\left(\frac{\mathrm{M_{CO_z}}}{\mathrm{M_C}}\right)$$

where C_C is the carbon content of the microalgal cell (%, *w/w*), which was measured by an element analyzer (Elementar Vario ELIII, Germany); M_C is the molecular weight of carbon; M_{CO_z} is the molecular weight of CO_2; and P is the biomass productivity (g/L/day).

RNA preparation and isolation

During the whole experiment, the pH values of the four groups were maintained with 20 mM HEPES buffer solution in case of the pH fluctuation. On the fifth day, these samples were collected from the bioreactors at 0, 1, 4, 8, 24, and 48 h after shifting from HC to LC conditions and then centrifuged at 5000*g* for 5 min at 4 °C immediately. The supernatant was discarded, and the centrifuge tube was immersed in liquid nitrogen to freeze the cellular pellet. Samples were stored at −80 °C until RNA extraction.

Total RNA was isolated using the TRIzol Reagent (Invitrogen, USA). The previous sample was grinded in liquid nitrogen and transferred rapidly to a microcentrifuge tube with 1 mL TRIzol Reagent. The cellular pellet was resuspended by Mixplus and incubated for 10 min at room temperature and centrifuged at 12,000*g* for 10 min at 4 °C. The sample was added into 0.2 mL chloroform and shaken vigorously by hand for 15 s and incubated for 3 min at room temperature. Next, samples were centrifuged at 12,000*g* for 15 min at 4 °C. The aqueous phase was transferred to a fresh tube. Isopropanol was added to the aqueous phase, and the resulting solution was incubated for 10 min at room temperature and centrifuged at 12,000*g* for 10 min at 4 °C to precipitate RNA. Isopropanol was removed, and the RNA pellet was washed with 75 % ethanol. Following further centrifugation (7500*g* for 5 min at 4 °C), the supernatant was removed and the RNA pellets were dried briefly for 5 min. Finally, the air-dried RNA pellets were dissolved in 20–40 mL of RNase-free

water. The integrity of RNA was examined by agarose gel electrophoresis, and the concentration was analyzed by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) to verify RNA quantity and purity. The RNA solutions were stored at −80 °C for further experiments.

Quantitative real-time RT-PCR analysis

The first-strand complementary DNA (cDNA) synthesis and real-time quantitative PCR were performed using the ReverTra Ace qPCR RT Master Mix with gDNA Remover and SYBR Green Real-Time PCR Master Mix (Toyobo, Japan), respectively, according to the manufacturer's instruction. Using gene sequences retrieved from NCBI genome sequencing databases, primers of CCM-related key genes (Table 1) were designed and used for expression-level quantitative detection. The actin gene from *C. pyrenoidosa* was used as internal control for normalization of qPCR data. Each reaction has three repeats for fidelity assay and was employed in a final volume of 20 μL containing 1 μL of cDNA template, 1 μL of each primer (10 mM), 10 μL of SYBR Mix, and 7 μL distilled water. All PCR reactions were performed on a C1000 Thermal Cycler Real-Time PCR Detection System (Bio-Rad, USA) with a program of initial denaturation at 95 °C for 30 s, 39 cycles of 95 °C denaturation for 10 s, 65 °C annealing for 15 s, and 72 °C extension for 25 s. The $2^{-\Delta\Delta CT}$ method was used to analyze gene expression relative to control based on the average fold change.

Table 1 Sequences of primer pairs used for real-time quantitative RT-PCR

Gene name	Primer pairs (5′–3′)	Product size (bp)
Actin	F: GCTCAACTCCTCCACGCT R: GTCCTTGCGGATGTCCAC	187
CAH2	F: GACTCCAACATTGCGAAGAT R: GGAAGAGGTCGGTCAGGT	109
CAH4	F: GCCTGGTCAACCTGTGGAT R: GAACACCTGCCGCATCAC	128
HLA3	F: TGATGTGCTTCCTCACCCT R: TCCAAAGTGTCCTGGTCCT	189
LCIA	F: CTGCCTTCCTCCTTGACAAC R: CACTGCTTCCACACCTTCTT	124
LCIB	F: TGCCTTGTCTGTTGACGACT R: ATCTTGTCCTCCAGCACCAT	132
RBCL	F: CTTTCCAAGGTCCTCCTCAC R: TCTCTCCAACGCATAAATGG	208
RBCS	F: GGACTACATCGTGAACAACG R: TCCAGTAGCGGTTGTCGTAG	131
CIA5	F: GATGTAATCGCTCCTTTGCC R: CAGGTCCTTTCCACACTTTG	134
LCR1	F:CAGAGCAACTGGAGCCTGATAG R: AGTGGTTCTTGACAGCGTTCTC	220

Results

Growth characteristics and lipid accumulation of *C. pyrenoidosa* under different CO_2 concentrations

To investigate the effect of different CO_2 concentrations on the growth of *C. pyrenoidosa* in the process of photoautotrophy, algal cells were firstly cultured 3.5 days until the depletion of glucose and then inoculated into 1-L photobioreactors using BBM. In Fig. 1a, the initial pH was 6.5 and changed immediately when different CO_2 were aerated into the medium. Meanwhile, the curve of cell density showed no lag phase, and the growth was kept strong except for pure CO_2 and air, which suggested that in the process of growth, the algal cells adapted to the environment and adjusted the external environment to be suitable for growing at the same time (Richmond 2008). The difference of concentration of CO_2 has a significant bearing on pH value which decreased from approximately 9 to 5 with the increase of CO_2 concentrations from 0.03 (air) to 100 %. However, four treatment groups (1, 5, 10, and 20 % CO_2) showed less change in pH (between 6.5 and 7.5, approaching to neutral) over the whole cultivation period, whereas in the control group, the pH value of the broth aerated with air increased gradually during the first 4 days (from 6.5 to 9). In contrast, pH fell to 4.8 at once after 1 day and was too acidic in the presence of 100 % CO_2, and the growth of the cells was completely inhibited.

The difference between the biomass concentrations of the four HC conditions (1–20 % CO_2) was little especially during the first 7 days. From the eighth day, the biomass of cells under the four concentrations began to appear different, of which the biomass concentrations of 1 and 5 % CO_2 continued to increase and attained to over 4.3 g/L finally, while for 10 and 20 % CO_2, they had a tendency of declining and leveling off, and the biomass concentration was about 3.5 g/L.

As shown in Fig. 1c, the chlorophyll content of heterotrophic cells as seed was very low (about 15 mg/g DCW) for the lack of light. However, chlorophyll in cells had a sudden increase after 24-h photoautotrophy; several groups of CO_2 concentrations (except for 100 % CO_2) increased from 15 to about 40 mg/g DCW during 24 h. During the following 4 days, the chlorophyll contents of the four groups' CO_2 concentration (1–20 % CO_2) were in a linearly decreasing trend and down to less than 15 mg/g DCW in the fifth day which basically closed to normal heterotrophic levels. This is due to the high concentration of CO_2 which promoted the growth of algal cells; most of the carbon sources were used for the increase of cell biomass, which resulted in the decrease of chlorophyll content. After that, chlorophyll content had been maintaining at a low level (nearly 10 mg/g DCW) by the end of cultivation.

The initial total lipid content of the algal cells was about 12 % and showed sustained increase along the whole period of cultivation expected for pure CO_2, and finally reached to 25–

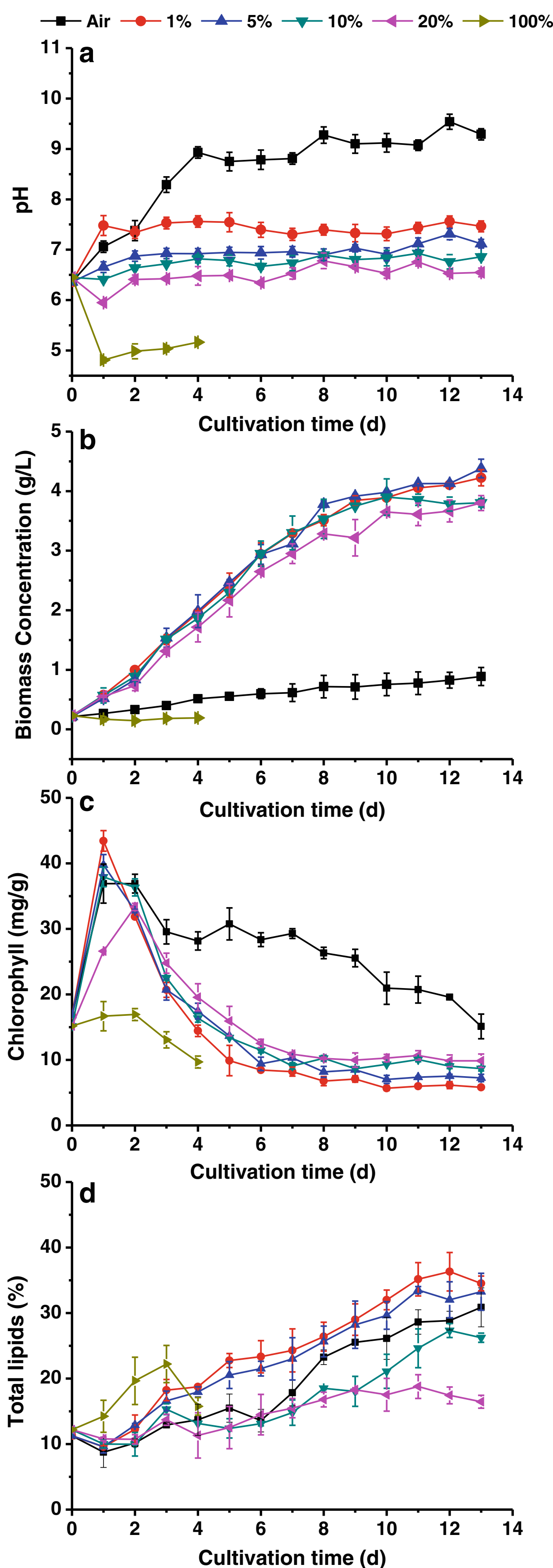

Fig. 1 Growth and intracellular component changes under different CO_2 concentrations in *Chlorella pyrenoidosa*: **a** pH value, **b** biomass concentration, **c** changes of chlorophyll, and **d** changes of total lipid content

35 % of the dry cell weight (Fig. 1d), which increased more than the double amount. In this study, the total lipid content exhibited the most marked increase under the 1 % condition, whereas the worst result was achieved by 20 % CO_2, even below that of air condition. The results suggested that the 1 and 5 % CO_2 strategy could result in the most favorable lipid productivity (about 107 mg/L/day), while in the LC way, the lipid productivity was only 16 mg/L/day, which suggested an efficient microalga lipid production mode by *C. pyrenoidosa* using CO_2 as the carbon source.

Effects of high CO_2 to low CO_2 transition under pH control on *C. pyrenoidosa* growth

As shown in Fig. 2a, after shifting to the air cultivation, the cell density of the experimental group increased slowly, which was lesser than that of the control group with continuous CO_2 due to the sudden reduction of carbon source. Finally, the dry weight of the control group reached 4.18 g/L, while that of experimental group was only 3.15 g/L. The chlorophyll content (Fig. 2e) showed an increasing trend at first and descending later as a whole; this trend was also consistent with the change of chlorophyll under different CO_2 concentrations. Comparing the two groups, there was little difference in the chlorophyll content after the switch.

In order to further explore the intracellular growth state, the carbon and nitrogen contents of the dry algal powder were measured by an elemental analyzer. As shown in Fig. 2c, the intracellular nitrogen content fell with the increase of culture time, especially in the first 5 days, with a sharp drop from 8.5 to 4.5 %. The nitrogen content decreased much slower from the fifth day which basically maintained at a constant level. By contrast, the change of intracellular carbon content was not obvious; it was kept at about 45 % over the whole culture period. Even though the cells were shifted to the air condition, the carbon content changed a little. It may be because the early high-CO_2 concentration made the carbon content saturated, and the fixed carbon content would not have a huge variation in a short time even after the switch to the air.

In Fig. 2d, the carbon fixation rate showed an early upregulation and then a downward trend overall. The carbon fixation rate of the switched group was lower than that of continuous 5 % CO_2 after the shift because there was no extraneous inorganic carbon source to be fixed which resulted in the carbon fixation rate being reduced correspondingly.

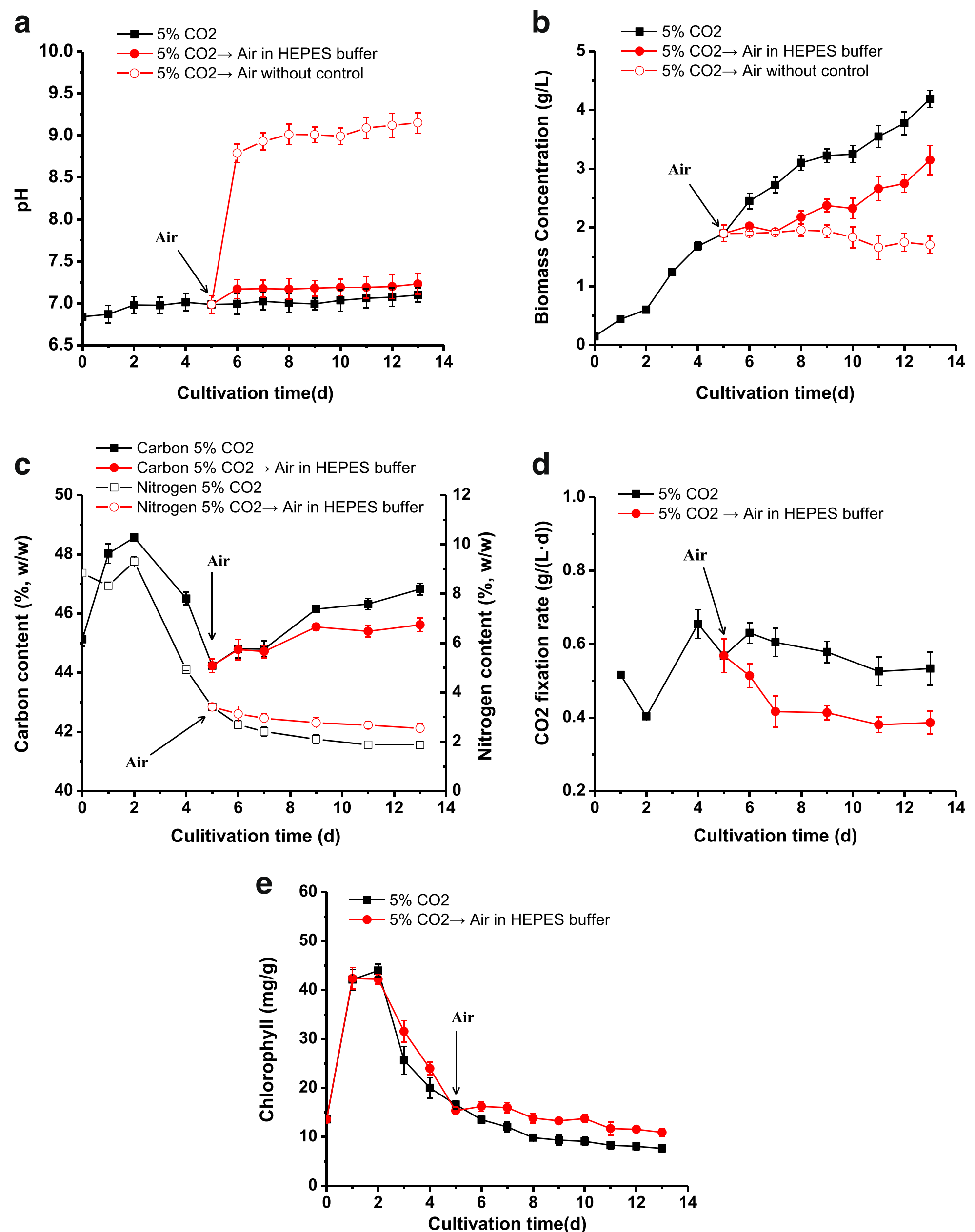

Fig. 2 The effect of high CO_2 (5 %) to low CO_2 (0.03 %) transition on growth and CO_2 fixation in *Chlorella pyrenoidosa*: **a** pH value, **b** biomass concentration, **c** changes of cellular carbon and nitrogen content, **d** changes of CO_2 fixation rate, and **e** changes of cellular chlorophyll content

Fatty acid composition of *C. pyrenoidosa* grown under high CO_2 (5 %) to low CO_2 (0.03 %) transition

The fatty acid composition of *C. pyrenoidosa* grown under high-CO_2 (5 %) to low CO_2 (0.03 %) transition was analyzed using gas chromatography (Table 2). The results showed that the main components of this alga were fatty acids with C16–C18 by photoautotrophy, accounting for about 72–92 % of the total fatty acid content in the whole cultivation process. The fatty acid profiles were favorable for biodiesel production. The amount of the saturated fatty acids was increased obviously along the time course cultivation, especially after the switch from 5 % CO_2 to air. Low CO_2 concentration (0.03 %) was suitable for the accumulation of saturated fatty acids, such as palmitic acid (C16:0) and short-chain fatty acid (C12:0).

Effects of CO_2 concentration changes on CCM-related gene expression

As shown in Fig. 3, the expression abundance of *CAH2* revealed a normal distribution. It began to be induced after 1 h and then increased gradually over time, showing a trend toward a higher peak at 8 h. Its expression quantity at 8 h was ten times greater than that of the control group. On the whole, *CAH2* was a moderately upregulated gene with severe

Table 2 Fatty acid composition of *Chlorella pyrenoidosa* grown under high CO_2 (5 %) to low CO_2 (0.03 %) transition

Fatty acids	0 day	4 days	6 days (1 day after switch)		9 days (4 days after switch)		12 days (7 days after switch)	
	5 % CO_2	5 % CO_2	5 % CO_2	0.03 % CO_2	5 % CO_2	0.03 % CO_2	5 % CO_2	0.03 % CO_2
Saturated fatty acids (%)								
C12:0	6.01	8.25	9.92	16.85	6.99	9.79	4.12	9.11
C14:0	2.94	3.73	4.73	7.46	3.31	4.63	2.24	4.48
C15:0	0.19	0.22	0.26	0.40	0.23	0.34	0.19	0.35
C16:0	22.00	34.50	39.36	40.03	36.71	44.40	39.40	52.34
C17:0	0.52	0.37	0.54	0.72	0.41	0.45	0.48	0.47
C18:0	6.77	3.17	3.96	4.83	4.04	4.08	3.16	4.93
C20:0	0.72	0.70	0.97	1.31	0.58	0.82	0.46	0.87
Sum	39.14	50.95	59.75	71.60	52.28	64.51	50.05	72.55
Monounsaturated fatty acids (%)								
C16:1	1.23	1.10	1.24	1.68	0.97	1.27	1.50	1.60
C18:1	0.73	1.04	0.87	0.63	1.09	1.17	1.83	1.10
C22:1	1.63	1.52	2.75	1.67	1.18	0.89	0.75	1.09
Sum	3.59	3.67	4.85	3.98	3.24	3.34	4.08	3.78
Polyunsaturated fatty acids (%)								
C16:2	2.51	4.63	2.62	2.08	2.88	2.48	2.80	1.67
C16:3	12.09	8.68	6.54	3.86	8.03	5.00	7.91	3.44
C18:2	14.25	14.55	10.57	7.90	15.52	12.20	16.56	9.02
C18:3	28.42	17.52	15.68	10.58	18.05	12.47	18.59	9.53
Sum	57.27	45.39	35.40	24.42	44.49	32.15	45.87	23.67

changeable points at 4 and 8 h in *C. pyrenoidosa*. The expression amount of *CAH4* was remarkably raised at 1 h which was also the peak value, and the expression quantity was about four times as much as that of the control group and then fell to a basic stable state which was similar to that of the control group. It is obvious that *HLA3* is a strong-expression gene in *C. pyrenoidosa*, reaching the peak value (about 110 times of that of the control group) at 1 h after the switch, and then, the expression abundance fell, but it still maintained at a high level, about 40 times at 4 h and 34 times at 8 h, which were significantly higher than those of the *CAH2* and *CAH4* genes.

In this study, CO_2 concentration decreased dramatically after sudden deprivation of CO_2, and this may be because *LCIA* encodes bicarbonate transporters, in order to make up for the limiting CO_2; the *LCIA* gene raised quickly within 1 h which promoted the accumulation of bicarbonate. This result is consistent with the report that its expression is the most intense within 1 h in *C. reinhardtii*. The strange thing is that the expression abundance of *LCIA* was not very high, and the highest point was just increased by about 3.4 times; after that, it even decreased a little compared to the control. It is likely to serve as an auxiliary synergy in the CCM regulation. *LCIB* is an upregulated gene in *C. pyrenoidosa*, and the highest expression abundance was at 4 h. Interestingly, it maintained at a high continuous expression level after 4 h when the expression abundance was about 210 times, 130 times, 160 times, and 120 times greater than the control group, respectively.

The transcript level of the Rubisco small subunit under a limiting CO_2 condition was relatively constant, and the variation between the treatment and the control was minor, indicating that the *rbcS* might be a constitutive expression in *C. pyrenoidosa*. In contrast, the expression abundance of *rbcL* showed a downward trend overall during the whole period, but the variability of the population was modest except when it was at 8 h. The expression abundance at 8 h in the continuous-CO_2 condition was about 20 times higher than that in the CO_2-limiting condition.

In Fig. 3, the variation of the expression level of *CIA5* was not obvious, suggesting that the constitutive expression of the gene led to the stable control of the putative Ci transporters and induced CAs. With regard to *LCR1*, the overall tendency of its expression level was upregulated and showed the highest change for 24 h, which was about 5.7 times higher than that of the control group. Compared to *CIA5*, both of them revealed the most expression quantity in a 24-h time point after the switch.

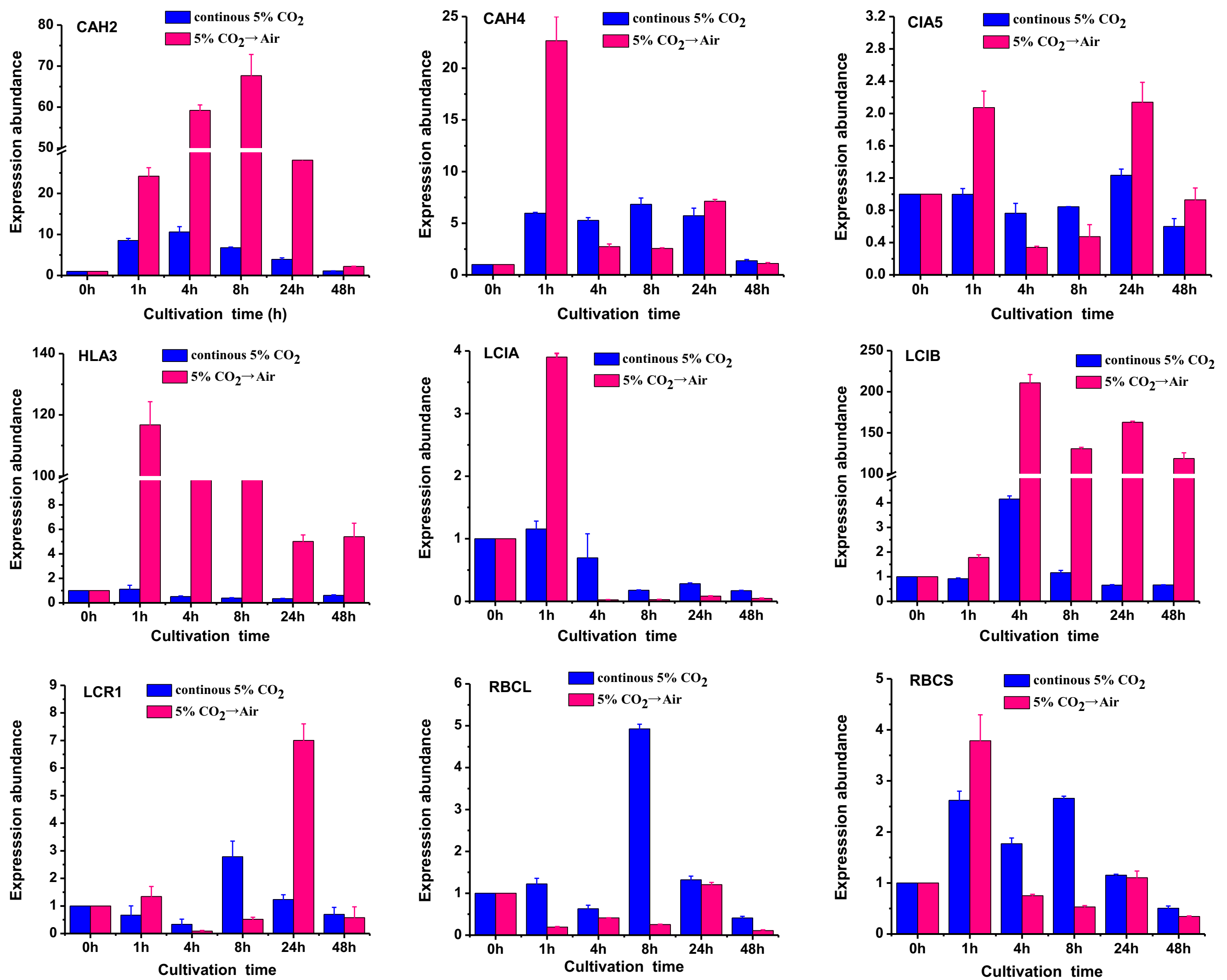

Fig. 3 Expression of photosynthesis and CCM-related genes in *Chlorella pyrenoidosa* subject to high CO_2 (5 %) to low CO_2 (0.03 %) transition. Transcript abundance corresponding to specific genes involved in photosynthesis and CCM-related metabolism and pathways was analyzed by real-time quantitative RT-PCR

Discussion

Biodiesel production by microalgae with photosynthetic CO_2 biofixation is thought to be a feasible way in the field of bioenergy and carbon emission reduction. The results obtained in the present study demonstrated that the cultures of *C. pyrenoidosa* using CO_2-enriched air as the sole carbon source exhibited high biomass concentrations (4.3 g/L in 5 % CO_2). CO_2 concentration influenced pH, growth, and lipid content. The pH value of the broth aerated with air increased gradually in the first few days; however, the pH did not increase without limit, and cells would gradually die when the nutrition is not enough to reproduce again; then, the dead cells would release reserve substances which made the medium acidic, slowed down the increase of pH, and prevented algal cells from absorbing and utilizing CO_2 (Cheng et al. 2004). So, that was why pH showed less change and maintained a certain range of 9 basically. On the whole, in either HC or LC conditions, pH did not change all the time in the period of cultivation.

Since CO_2 was used as the sole carbon source in the medium, the higher the CO_2 concentration is, the higher the DIC concentration becomes; in theory, that meant the more carbon source would be used for the increase of biomass concentration. Quite the opposite has been the case; cells in the presence of 10 and 20 % CO_2 did not obtain a better cell density than those with 1 and 5 % CO_2 (Fig. 1b) because higher CO_2 concentration could result in lower pH as shown in Fig. 1a. This was especially true for 100 % CO_2 level; the *Chlorella* cells ceased growing basically and approached to death after 4 days. Another control of air showed a low biomass due to the deficient in carbon source compared to the HC conditions.

Maybe early cells were hungrier and could consume the increasing DIC. As time went on, intracellular carbon was gradually saturated and the rate of growth tended to be relatively stable.

The contents of chlorophyll in the whole cultivation showed a downward trend overall. It just had a surge response process in the early stage because of the switch of heterotrophic cells for light environment and reboot their photosynthesis ability. Because, on the one hand, the light can activate light energy utilization-related enzyme systems which were closed in the stage of heterotrophy, especially for the restart of photosynthetic carbon sequestration reaction, making algal cells from the heterotrophic nutrition by using organic carbon source and releasing CO_2 into the photosynthetic nutrition by intake CO_2. On the other hand, the optimal light intensity for algal cells under the normal growth condition is about 100–200 μmol/m^2/s; in general, high light has a strong chemical and thermal damage effect on algal cells. Chlorophyll, as the main biochemical component converted by absorbing sun energy, has a direct protection on the algal cells which are exposed to the strong high lights. So, this sharp increase maybe associated with the self-protection mechanism of algal cells (Fan et al. 2012a), and the sudden light was a strong factor for algal cells in the long time of darkness or even an intimidation factor, so the algal cells need to synthesize relevant protective substances, such as pigment for protecting cells. Compared to the two ways of air and pure CO_2, it was found that algal cells bubbled with air grew slowly and had low cell density because of the low CO_2 concentration; so, a similar light intensity of the stimulus to algal cells under the above situation was obviously higher than that of high density under higher CO_2 concentrations. Thus, the high light had much more intense entrainment to the synthesis of chlorophyll under this condition, and algal cells suffered from the physiological pressure and the chlorophyll content was significantly higher. The condition of pure CO_2 (may be due to low pH value) harmed the growth of algal cells at all and the further synthesis of chlorophyll.

Lipid productivity is of particular importance in large-scale microalgal lipid production processes since it takes into account both lipid content and biomass production rate. The trophic switch from heterotrophy to photoautotrophy might be a critical reason that contributes to lipid overproduction. It was also a protective mechanism by the synthesis of total lipid when algal cells were under light pressure. Previous studies revealed that the heterotrophic *Chlorella* seeds exhibited a superiority on both biomass growth and lipid accumulation in the subsequent photoautotrophic cultivation (Han et al. 2012). Consistent with this, it was found that the transition from heterotrophy to photoinduction culture can stimulate a dramatic increase of lipid in three *Chlorella* species (Fan et al. 2014b; 2012b).

Effects of switch from high CO_2 to low CO_2 in buffered BBM on *C. pyrenoidosa* growth and CO_2 biofixation were studied in this section. From the above experimental results, it was concluded that the growth of cells under 1 and 5 % CO_2 concentrations was better than that under 10 and 20 % CO_2 concentrations. Furthermore, considering that the objective of the experiment was to investigate the CCM-related response when cells were switched from high CO_2 concentration to low CO_2 concentration, so finally, we chose the higher concentration (5 % CO_2) as the research model of culture process and carbon sequestration mechanism.

In this new experiment, the experimental groups were firstly aerated with 5 % CO_2 (HC), and then, the cells were shifted from HC to LC (air), as for the control group was aerated with continuous 5 % CO_2, both of them were conducted in buffered BBM by HEPES buffer. A preliminary experiment about the effect of HEPES buffer on algae cell growth was done to determine the concentration and the potential toxicity before this formal experiment (data not shown). In the previous results (Fig. 1a), the pH rose sharply under the air condition without adding HEPES buffer. However, as shown in Fig. 2a, HEPES buffer had a very good control of the pH value of the culture after the switch from 5 % CO_2 to the air; the pH was maintained at around 7.2 till the end of the switch. There was little difference of pH value between continuous 5 % CO_2 and 5 % CO_2 to air transition under HEPES buffer. This approximate neutral pH was conducive to the growth of algal cells. In addition, the control of pH eliminated the effect of pH change on CCM-related gene expression.

In the early stage of cultivation, the carbon fixation rate had a small amplitude increase, because the early algal cells were physiologically vigorous, and at this time, they were in a state of hunger because the algal cells were transferred from a heterotrophic organic carbon source to a autotrophic inorganic carbon source, so they began to fix a large number of inorganic carbon. Subsequently, the physiological and biological activities of the cells went down which affected the ability of carbon sequestration and the intracellular carbon content approached to saturated value in the late state of growth, so the carbon fixation rate declined gradually.

It is worth noting that reducing the CO_2 concentration could significantly decrease the polyunsaturated degree in fatty acids, dropped by more than 22 % (absolute value) compared to the control group. It seems that little influence was found upon monounsaturated fatty acids, as shown in Table 2; the contents of this part were only around 3–4 % along the process. It was reported that at low CO_2 concentrations, the desaturation rate was slower than the elongation rate, making the production of less polyunsaturated fatty acids (Ota et al. 2009). The regular patterns of fatty acid profiles in this study were consistent

with those in the study of Ota et al. (2009) and Tang et al. (2011).

To the best of our knowledge, nine different α- and β-CA genes have been identified in *C. reinhardtii* by far, but it is not entirely clear what roles are played by these CAs; some of these proteins are implicated to have possible functions in the CCM (Jungnick et al. 2014). In this study, we just focused on the *CAH2* and *CAH4* genes in *C. pyrenoidosa*. CAH2 is a periplasmic soluble α-CA in *C. reinhardtii*. Tachiki et al. isolated a carbonic anhydrase from high CO_2 (5 % CO_2) grown *C. reinhardtii* which was identified as the isozyme from CAH2. They found that *CAH1* and *CAH2* shared high sequence similarity, and both of them were present in the wild-type cells of *C. reinhardtii* (Tachiki et al. 1992). Surprisingly, they are differentially regulated by the CO_2 concentration. *CAH1* is induced by limiting CO_2 and light, while *CAH2* is poorly expressed and downregulated under limiting CO_2 conditions (Moroney and Ynalvez 2007). However, the results were opposite to those in the present study.

CAH4 is a mitochondrial β-CA (mtCA) which is induced under limiting CO_2 conditions, but it is not very clear how it plays a role in CCM, and multiple hypotheses have been advanced regarding the potential function of the protein. One assumed that mtCAs buffer the mitochondrial matrix due to the complete induction of the CCM, and photorespiratory glycine decarboxylation produces equivalent amounts of NH_3 and CO_2. The mtCA might serve to catalyze the hydration of CO_2, producing H^+, which would prevent alkalinization in the mitochondrial matrix as a result of the generation of NH_3 by glycine decarboxylation (Moroney and Ynalvez 2007). Alternatively, the mtCAs might play a role in converting the CO_2 generated by respiration and photorespiration to HCO_3^- (Raven 2001). The change of *CAH4* in *C. pyrenoidosa* was consistent with the reports in the literature in *C. reinhardtii*.

High light-induced gene 3 (*HLA3*) was identified when *C. reinhardtii* cells were exposed to high light, by encoding a putative ATP-binding cassette-type transporter of the multi-drug resistance-related protein subfamily (Im and Grossman 2001). Unlike other low CO_2-induced genes, its expression is activated by both high light and low CO_2, for which it was shown to be under the control of zinc-finger transcription factor Ci accumulation 5 (CIA5), a master regulator for the response of *C. reinhardtii* to limiting CO_2 conditions (Miura et al. 2004). High expression of this gene may be because it is related to its function of activating the ATP, and it can be induced under low CO_2.

Low CO_2-induced gene A (*LCIA*) is an attractive candidate transporter which encodes a chloroplast membrane protein reported to transport bicarbonate in *C. reinhardtii* (Moroney and Ynalvez 2007). According to some findings which have been reported, it is no surprise that the expression of *LCIA* is induced under limiting CO_2 conditions; the point is that it is also partially under the control of CIA5, a transcription factor required for induction of most other CCM genes. Moreover, simultaneous knockdown of *LCIA* and *HLA3* in wild-type *Chlamydomonas* cells showed high pH-sensitive phenotype and low photosynthetic Ci affinity, which had more severe phenotypic effect than *HLA3* knockdown alone, indicating that LCIA and HLA3 are key synergistic or complementary components of the active Ci transport pathway in limiting Ci-acclimated cells (Duanmu et al. 2009a).

Low CO_2-induced gene B (*LCIB*) encodes a soluble protein which localized in the vicinity of pyrenoid, a prominent structure in the chloroplast, and it is responsible for the normal Ci uptake or accumulation in low-CO_2 conditions. This gene belongs to a novel gene family which has three homologous genes in *Chlamydomonas*, *LCIC*, *LCID*, and *LCIE*. All of them are responsive to limiting CO_2, but LCIB and LCIC are the most abundant transcripts upon the limiting CO_2 induction (Wang et al. 2011). Recent report found that LCIB interacts with LCIC and forms a hexamer complex of approximately 350 kDa which gathered around the pyrenoid from the matrix under light illumination and LC conditions during active operation of the CCM. In contrast, in the dark or under high-CO_2 conditions when the CCM was inactive, the related proteins immediately diffused away from the pyrenoid to the matrix (Yamano et al. 2010); it seems that the pyrenoid related to location is very important for the function of LCIB. Combining the HLA3 knockdown with both the off-target LCIA knockdown and an LCIB mutation confirmed the overlapping functions of HLA3, LCIA, and LCIB (Duanmu et al. 2009a). LCIB is suggested to have two functional possibilities (Yamano et al. 2010): one is that it mainly traps CO_2 leaking from the pyrenoid matrix and transfers the captured CO_2 to stromal carbonic anhydrase CAH6; the other is that LCIB combined with LCIC to form a complex as a structural barrier to prevent the leakage of CO_2 and maintain CO_2 concentration in the pyrenoid matrix. So, in either case, the continuous and abundant expression at a later stage was likely to maintain cell growth and photosynthesis under LC. In *Chlamydomonas*, overexpression of *LCIA* and *LCIB* could enhance the growth rate and biomass density by about 40–80 % comparing with the wild type under normal CO_2 conditions (Spalding 2012).

Rubisco is short for ribulose 1,5-bisphosphate carboxylase/oxygenase, a hexadecamer consisting of eight 55-kDa large subunits and eight 12-kDa small subunits. It catalyzes the first major step of carbon fixation in the Calvin cycle, a process by which the carbon dioxide in the atmosphere is converted to energy-rich molecules. So, it is a key enzyme deciding the rate of carbon assimilation in photosynthesis. It is also involved in the photorespiratory pathway when it reacts with CO_2 or O_2. The Rubisco large subunit (RBCL) is encoded by the single-copy *rbcL* gene of the chloroplast genome and is translated by chloroplast ribosomes, while the Rubisco small subunit (RBCS) is encoded by a multigene family of *rbcS* on the

nuclear genome and is posttranslationally transported to the chloroplast. The different conclusions were obtained by Winder et al. (1992) that the expression level of *rbcL* in *C. reinhardtii* did not vary during the time course of adaptation to limiting CO_2, while the *rbcS2* messenger RNA (mRNA) which contributed most of the mRNA to the *rbcS* mRNA pool remained essentially unchanged in abundance, but a more distinct shift in abundance was observed for *rbcS1* mRNA in cell wall-deficient mutant cells (but not in the wild-type strain). The obvious difference between these two studies maybe the light; the algae cells were cultured under illumination in our study, but they were not in Winder's study. It should not be surprising that the illumination has a similar effect on the biosynthesis of Rubisco as well as CO_2. In view of the evidence that synthesis of the large subunit of Rubisco dropped remarkably within 15 min and only gradually recovered at about 6 h when *C. reinhardtii* cells were transferred from low light to higher light intensities, the Rubisco small subunit was not dramatically affected, and the further research revealed that downregulation of large subunit synthesis was not related with a decline in the steady-state level of the *rbcL* transcript. So, we conclude that maybe the relatively stable expression abundance of *rbcL* and *rbcS* in the present study is a function of the presence of light, if at all; the results of the transcripts of *rbcL* and *rbcS* were insufficient to overlook the importance of Rubisco in the control over CCM. Besides, the low catalytic activity of Rubisco raises the possibility of the results in this study.

CIA5 and low CO_2-induced gene1 (LCR1) have been confirmed as the most two important transcription regulators in *C. reinhardtii* CCM regulatory mechanisms (Fang et al. 2012). CIA5 is a zinc-finger-type transcription regulator. It was found that the expression of most putative Ci transporters and induced CAs relies on CIA5. However, the transcription of *CIA5* itself does not depend on the CO_2 level, whereas posttranslational activation in low CO_2 apparently is required for regulation. As to LCR1, it has a Myb domain and appears to regulate the expression of several low CO_2-induced genes. In addition, *LCR1* itself is also regulated by limiting CO_2, and this induction requires CIA5. The results also supported the conclusion that the expression of the *CIA5* gene is not influenced by CO_2 concentration (Miura et al. 2004). The transition from high CO_2 concentration to a low-CO_2 condition showed significant impacts on the CCM-related gene expression. In short, *C. pyrenoidosa* could be successfully exploited to simultaneously mitigate CO_2 and to produce biofuels as well as to study the carbon sequestration mechanism.

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