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High-CO_2 Response Mechanisms in Microalgae

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1. Introduction

The concentrations of atmospheric CO_2 and aquatic inorganic carbon have decreased over geologic time with minor fluctuations. In contrast, O_2 concentration has increased through the actions of photosynthetic organisms. Therefore, photosynthetic organisms must adapt to such dramatic environmental change. Aquatic photosynthetic microorganisms, namely eukaryotic microalgae, cyanobacteria, and non-oxygen-evolving photosynthetic bacteria, have developed the ability to utilize CO_2 efficiently for photosynthesis because CO_2 is a substrate for the primary CO_2-fixing enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and its related metabolic pathways such as the Calvin–Benson cycle (C_3 cycle). As the Rubisco carboxylase reaction is suppressed by elevated O_2 concentrations via competition with CO_2, photosynthetic organisms have developed special mechanisms for acclimating and adapting to changes in both CO_2 and O_2 concentrations. Examples of such mechanisms are the microalgal CO_2-concentrating mechanisms (CCM), the facilitation of "indirect CO_2 supply" with the aid of carbonic anhydrase and dissolved inorganic carbon (DIC)-transporters (see Section 3), and C_4-photoysnthesis (for review, see Giordano et al., 2005; Raven, 2010). Many reports on low-CO_2-acclimation/adaptation mechanisms have been published, particularly in relation to certain cyanobacteria and unicellular eukaryotes. However, knowledge of high-CO_2-acclimation/adaptation mechanisms is very limited. We recently identified an acceptable high-CO_2-inducible extracellular marker protein, H43/Fea1 (Hanawa et al., 2007) and a *cis*-element involved in high-CO_2-inducible gene expression in the unicellular green alga *Chlamydomonas reinhardtii* (Baba et al., 2011a). We also identified other high-CO_2-inducible proteins in the same alga using proteomic analysis (Baba et al. 2011b). In this chapter, we briefly introduce low-CO_2-inducible phenomena and mechanisms as background and then review recent progress in elucidating the molecular mechanisms of the high-CO_2 response in microalgae.

2. Aquatic inorganic carbon system

The CO_2 concentration dissolved in aqueous solution (dCO_2) is equilibrated with the partial pressure of atmospheric CO_2 (pCO_2) by Henry's law and depends on various environmental factors such as temperature, Ca^{2+} and Mg^{2+} levels, and salinity (e.g., Falkowski & Raven,

2007). The dCO_2 dissociates into bicarbonate (HCO_3^-), and carbonate (CO_3^{2-}) and these three species of DIC attain equilibrium at a certain ratio depending on pH, ion concentrations, and salinity (Fig. 1). HCO_3^- is the dominant species at physiological pH (around 8), which is similar to that in the chloroplast stroma where photosynthetic CO_2 fixation is actively driven (for review, see Bartlett et al., 2007). However, Rubisco [E.C. 4.1.1.39] reacts only with dCO_2, not bicarbonate or carbonate ions. At a pH of 8, the dCO_2/HCO_3^- ratio becomes extremely small (approximately 1/100) resulting in a high bicarbonate concentration and an increase in the total DIC pool size. The dCO_2 concentration equilibrates with atmospheric CO_2 at approximately 10 μM, whereas the bicarbonate concentration is approximately 2 mM at the surface of the ocean (Falkowski & Raven, 2007).

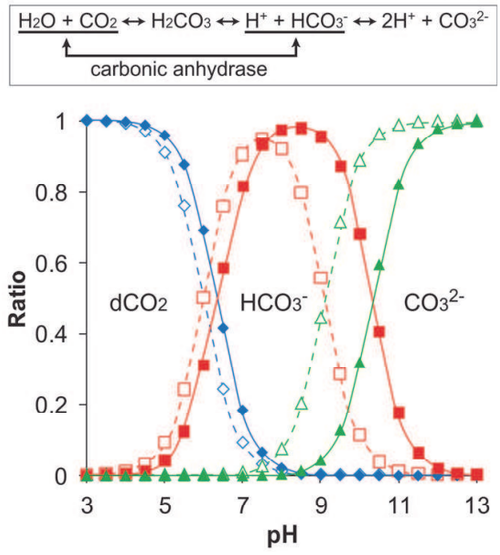

Fig. 1. Equilibration of dissolved inorganic carbon species in freshwater and seawater. Parameters used were as follows (at 25°C): For freshwater, pKa_1= 6.35, pKa_2 = 10.33; for seawater, pKa_1 = 6.00, pKa_2 = 9.10 (Table 5.2, Falkowski & Raven, 2007). Filled symbols and solid line, freshwater; clear symbols and dotted line, seawater; diamonds, dCO_2; squares, bicarbonate; triangles, carbonate.

CO_2 must be supplied rapidly when it is actively fixed by Rubisco in the chloroplast stroma during photosynthesis. CO_2 is supplied by both diffusion from outside of cells and the conversion of bicarbonate. However, these processes are very slow and become limiting for photosynthetic CO_2 fixation. In the former case, CO_2 must be continuously transported from outside of the cells via the cytoplasm through the plasmalemma and the chloroplast envelope. The diffusion rate of CO_2 in water is approximately 10,000-fold lower than that in the atmosphere (Jones, 1992). In the latter case, bicarbonate accumulated in the stroma can be a substrate when the dehydration rate to convert bicarbonate to CO_2 is comparable to Rubisco activity. However, the rate of chemical equilibration between CO_2 and the bicarbonate ion is very slow relative to photosynthetic consumption of CO_2 (Badger & Price, 1994; Raven, 2001); the first-order rate constants of hydration (CO_2 to bicarbonate) and dehydration (bicarbonate to CO_2) are 0.025–0.04 s^{-1} and 10–20 s^{-1}, respectively, at 25°C (Ishii et al., 2000). Such CO_2-limiting stress becomes a motive for photosynthetic organisms to develop unique CO_2-response mechanisms.

3. The CO_2-concentrating mechanism and phenomena induced by CO_2 limitation

The atmospheric CO_2 level has gradually decreased over recent geological time with some fluctuations (Condie & Sloan, 1998; Falkowski & Raven, 2007; Giordano et al., 2005; Inoue, 2007), although it has been increasing rapidly due to CO_2 emissions from fossil fuels since the industrial revolution. Thus, photosynthetic organisms have adapted to utilize CO_2 efficiently for photosynthesis. Generally, eukaryotic microalgae and cyanobacteria have developed efficient CO_2-utilization mechanisms and exhibit high photosynthetic affinity for CO_2 when grown under CO_2-limiting conditions. Under elevated CO_2 conditions, they exhibit low affinity for CO_2, as enough CO_2 is available for photosynthesis. These properties can change over hours when photosynthetic microorganisms are grown under various CO_2 conditions (for review, see Miyachi et al., 2003) (Fig. 2).

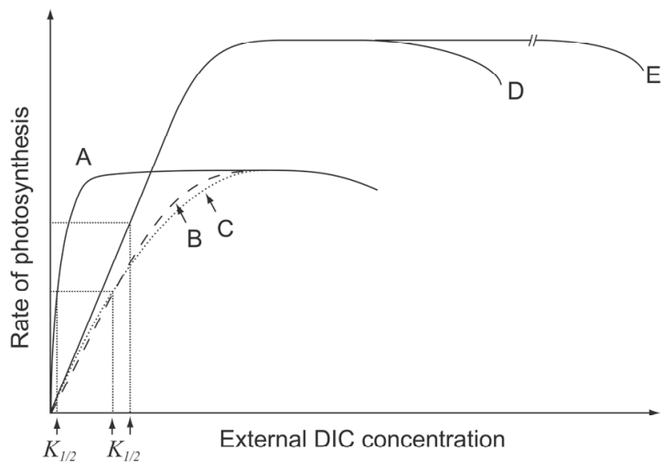

Fig. 2. Relationship between photosynthetic rate and external dissolved inorganic carbon (DIC) concentration in microalgae grown under low-, high-, and extremely high-CO_2 conditions. A, low-CO_2-acclimated cells (grown in air with 0.04% CO_2); B, low-CO_2-acclimated cells treated with a carbonic anhydrase inhibitor (e.g., *Chlorella*); C, high-CO_2-acclimated cells (grown in air containing 1–5% CO_2) (e.g., *Chlamydomonas*); D, high-CO_2-acclimated cells (e.g., *Chlorella*); D, extremely high-CO_2-acclimated cells grown under >40% CO_2 conditions. This figure is modified from Miyachi et al. (2003).

High photosynthetic activity of low-CO_2-grown cells under a CO_2-limiting concentration is due to the CCM, which is induced by cellular acclimation to limiting CO_2 (e.g., Aizawa and Miyachi, 1986). Two main factors are essential for CCM: inorganic carbon transporters that facilitate DIC membrane transport of CO_2 and/or bicarbonate through the plasmalemma and the chloroplast envelope, and carbonic anhydrases (CAs), which facilitate diffusion by stimulating the indirect supply of CO_2 from outside of cells to Rubisco. CA catalyzes the equilibration reaction of the hydration and dehydration of CO_2 and bicarbonate, respectively. The rapid equilibration catalyzed by CA stimulates the increase in bicarbonate concentration at physiological pH and augments the contribution of bicarbonate for diffusion. Finally, the processes driven by CA induce increases in the amount of bicarbonate

carried near Rubisco and then CO_2 produced from bicarbonate is immediately supplied to Rubisco when CA is located near Rubisco (see also Fig. 4). The relative specificity to CO_2/O_2 and affinity to CO_2 of Rubisco became more efficient over evolutionary time, indicating that Rubisco in eukaryotic microalgae is more efficient for CO_2 fixation than that in cyanobacteria (Falkowski & Raven, 2007). Such species-specific properties remain unchanged in present living organisms. However, even in eukaryotic algae, the affinity of Rubisco for CO_2 is insufficient to saturate activity at present atmospheric CO_2 concentrations. Therefore, the cells continuously activate mechanisms such as CCM to increase their affinity for CO_2. For further information on CCM, we recommend reading several previously published reviews (e.g., Aizawa & Miyachi, 1986; Badger et al., 2006; Giordano et al., 2005; Kaplan & Reinhold, 1999; Miyachi et al., 2003; Moroney & Ynalvez, 2007; Raven et al., 2008; Raven, 2010; Spalding, 2008; Yamano & Fukuzawa, 2009).

CCM is reversibly induced/suppressed by the decrease/increase in CO_2 concentration, respectively, in cyanobacteria and eukaryotic microalgae when the duration of acclimation is on an hour- or day-order length. However, in the unicellular green alga *Chlamydomonas reinhardtii* in which CCM is the most characterized among eukaryotic microalgae, cells grown for 1000 generations under high-CO_2 conditions are unable to re-acclimate to low-CO_2 conditions, exhibiting low photosynthetic affinity for CO_2 even when the cells are re-exposed to low CO_2 conditions (Collins & Bell, 2004; Collins et al., 2006). This suggests that CCM can be irreversibly lost when cells undergo prolonged acclimation/adaptation to high-CO_2 conditions. Such adaptation has been suggested to occur in natural populations (Collins & Bell, 2006). Although CCM-deficient mutants of cyanobacteria and the green alga *C. reinhardtii* are lethal, such lethality is prevented by elevated CO_2 concentration (e.g., Price & Badger, 1989; Spalding et al., 1983; Suzuki & Spalding, 1989). In *C. reinhardtii,* CCM is induced under either 1.2% CO_2 in air at 1000 μmol photons m^{-2} s^{-1} or 0.04% CO_2 in air at 120 μmol photons m^{-2} s^{-1}, suggesting that CCM induction can be regulated by not only external CO_2 concentration but also other signals derived from photorespiratory and/or excess photoenergy stresses, although the detailed mechanisms are not yet known (Yamano et al., 2008). CCM can be induced by an artificially produced strong limitation in CO_2 supply in large-scale photobioreactors where dCO_2 is consumed via photosynthesis (Yun & Park, 1997).

In some microalgae, the supply of CO_2, not bicarbonate, is strongly limited at alkaline pHs in closed culture systems and such a limitation may be a factor or signal for inducing CCM (Colman & Balkos, 2005; Diaz & Maberly, 2009; Verma et al., 2009). The euglenophyte *Euglena mutabilis* and an acid-tolerant strain of *Chlamydomonas* do not induce CCM under any conditions (Balkos & Colman, 2007; Colman & Balkos, 2005), suggesting that photosynthetic carbon fixation is not limited by CO_2 supply even under ambient atmospheric conditions. These results indicate that there is species-specific variation in the induction mechanism of CCM depending on physiological and ecological conditions (for review, see Giordano et al., 2005; Raven, 2010).

4. High-CO_2 response phenomena

The atmospheric CO_2 level is presumed to have been very high during the ancient geological era (Condie & Sloan, 1998; Falkowski & Raven, 2007; Giordano et al., 2005; Inoue, 2007), so microalgae are believed to have been high-CO_2-adapted/acclimated cells. Microalgae preserve their ancient physiological properties at present, and the relative

specificity of Rubisco is a typical example. Even in the present environment, high-CO_2 conditions occur in soil where CO_2 concentration changes drastically between the atmospheric level and ≥10% (v/v) (for review, see Buyanovsky & Wagner, 1983; Stolzy, 1974). Accordingly, phenomena that are induced under high-CO_2 conditions, such as high-CO_2 acclimation, remain important for microalgae to survive in various environments.

Among the various phenomena induced by high CO_2 concentrations, keenly interesting topics are how to maximize inorganic CO_2 fixation and organic production by microalgae for CO_2 mitigation and mass cultivation. The most frequently used species for studies on fast growth and tolerance to high CO_2 levels is *Chlorella sp.*, followed by *Scenedesmus sp.*, *Nannochloropsis sp.*, and *Chlorococcum sp*. The CO_2 concentration used for such studies varies from atmospheric levels to 100% (Kurano et al., 1995; Maeda et al., 1995; Olaizola, 2003; Seckbach et al., 1970). Appropriate CO_2 supply for saturation of microalgal growth is approximately 5% in the unicellular green alga *Chlorella* (Nielsen 1955). The growth of microalgae and cyanobacteria is generally inhibited under very high concentrations of CO_2. Some species isolated from extreme environments can grow rapidly with tolerance to very high and extremely high CO_2 conditions such as >40% (for review, see Miyachi et al., 2003). Even in a high-CO_2-tolerant microalga, growth is suppressed at > 60% CO_2 in air (Satoh et al., 2004). The rate of maximum photosynthesis per packed cell volume increases in some species, such as *Chlorella,* but not in other species, such as *Chlamydomonas*, even when cells are acclimated to high-CO_2 conditions (Miyachi et al., 2003) (Fig. 2). However, the detailed mechanism on such high CO_2 tolerance needs to be clarified.

Many reports have focused on lipid biosynthesis for biofuel production, and response surface methodology (Box & Wilson, 1951) has been used very effectively to evaluate multiple factors associated with total biomass production. Excellent review articles on large-scale cultivation for biofuel production by microalgae and cyanobacteria have focused on how to obtain the best productivity under high-CO_2 conditions (Ho et al., 2011; Kumar et al., 2010; Lee J.S. & Lee J.P., 2003), but not on the underlying mechanisms of how cells provide high productivity under fine regulation.

One of the best examples of sequential analysis was performed systematically in the high-CO_2-tolerant unicellular green alga *Chlorococcum littorale* (for review, see Miyachi et al., 2003). *C. littorale* is a unicellular marine chlorophyte that was isolated from a saline pond in Kamaishi City, Japan; it grows rapidly under extremely high CO_2 conditions (e.g., 40%, and even at 60% CO_2; Chihara et al., 1994; Kodama et al., 1993; Satoh et al., 2004). Several experiments have revealed that cellular responses, namely the regulation of photosystem (PS) I and PS II, the production of ATP, and pH homeostasis are well maintained particularly in *C. littorale*, but not in high-CO_2-sensitive species such as the green soil alga *Stichococcus bacillaris*, during a lag period when cells are transferred from low to extremely high levels of CO_2 (Demidov et al., 2000; Iwasaki et al., 1996, 1998; Pescheva et al., 1994; Pronina et al., 1993; Sasaki et al., 1999; Satoh et al., 2001, 2002). However, many of the processes that make it possible for cells to grow under such extremely high-CO_2 conditions remain to be understood.

Photosynthesis in acidic environment, the influence by ocean acidification, and the effect of O_2 on photorespiration are also deeply associated with high-CO_2-induced phenomena. Some microalgal species have been isolated mainly from acidic environments where only CO_2 is predominant and supplied to algal cells as a substrate for photosynthesis (Balkos & Colman, 2007; Colman & Balkos, 2005; Diaz & Maberly, 2009; Verma et al., 2009; for review see Raven, 2010). Three synurophyte algae, *Synura petersenii*, *Synura uvella,* and *Tessellaria*

volvocina, have been studied in detail for the DIC uptake mechanism and show unique photosynthetic properties (Bhatti & Coleman, 2008). These species have no external carbonic anhydrase on the cell surface, no bicarbonate uptake ability, and exhibit a low affinity for DIC during photosynthesis, indicating a lack of CCM as in high-CO_2-grown/acclimated cells. However, their Rubisco shows a relatively high affinity for CO_2, and cells such as *S. petersenii* accumulate large amounts of internal DIC via diffusive uptake of CO_2 facilitated by a pH gradient across the cell membranes, as reported previously in spinach chloroplasts (Heldt et al., 1973). These data suggest that the affinity of Rubisco for CO_2 and the homeostasis of the pH gradient play key roles in the whole-cell affinity for CO_2 and the pH-tolerance of microalgae. Under high-CO_2 conditions, Rubisco can get enough CO_2 supply although CCM is usually lost in high-CO_2 cells. The physiological status of synurophyte algae living at acidic pH may be similar to cells that are exposed to high-CO_2 conditions even under low-CO_2 conditions.

Increasing pCO_2 induces a decrease in oceanic pH and causes gradual equilibrium shifts from bicarbonate ions to CO_2 in seawater. Therefore, ocean acidification is said to be another high-CO_2 problem (for review, see Doney et al., 2008). Coccolithophorids, marine phytoplankton that form cells covered with $CaCO_3$, are very sensitive to calcium carbonate saturation and pH shifts in seawater. The effects of ocean acidification on algal physiology have been studied in several coccolithophorid species such as *Emiliania huxleyi* and *Pleurochrysis carterae,* although some conflicting results have been reported (Fukuda et al., 2011; Igresiaz-Rhodorigez et al., 2008; Riebesell et al., 2000). Hurd et al. (2009) indicated the importance of maitaining pH in experiments and demonstrated that doing so via high-CO_2 bubbling creates conditions that are much closer to actual ocean acidification than acidification by adding HCl. The effects of high-CO_2 conditions on calcification and photosynthesis would be closed up in later analyses. Fukuda et al. (2011) reported that the coccolithophorid *E. huxleyi* possesses alkalization activity, which helps compensate for acidification when photosynthesis is actively driven. Furthermore, when oceanic acidification is caused by the bubbling of air with elevated CO_2, coccolithophorid cells increase both photosynthetic activity and growth and are not damaged because of the stimulation of photosynthesis (unpublished data by S. Fukuda, Y. Suzuki & Y. Shiraiwa). These results suggest that ocean acidification will not immediately harm coccolithophorids. However, long-term experimental evidence is strongly required on this topic.

Badger et al. (2000) described how low-CO_2-grown microalgae tend to have low photorespiratory activity, as determined by photosynthetic O_2 uptake in C_4 plants because of the function of CCM. O_2 uptake under illumination is relatively insensitive to changes in CO_2 concentration, because the activity depends predominantly on the activity of non-photorespiratory reactions probably such as the Mehler reaction and oxidizing reaction in the mitochondria (Badger et al., 2000). CO_2 insensitivity is also observed in *C. reinhardtii* (Sültemeyer et al., 1987) although photosynthetic O_2 uptake increases considerably with increasing light intensity (Sültemeyer et al., 1986). Accordingly, the photosynthetic productivity of microalgae may not be significantly enhanced by suppressing photorespiration. The rate of maximum photosynthesis, calculated on a cell volume, increases clearly in *Chlorella* but not so in *Chlamydomonas* when cells are acclimated to high-CO_2 conditions (Miyachi et al., 2003) (Fig. 2). In *C. reinhardtii*, growth rate is only slightly higher (1.3–1.8-fold) in cells grown under high-CO_2 than in those grown under ordinary air (Baba et al., 2011b; Hanawa, 2007). These results suggest that low-CO_2-acclimated/grown cells have a very highly efficient carbon-fixation mechanism for maintaining high growth

rates even under atmospheric CO_2 levels, so we need to carefully optimize growth conditions when we want to obtain high algal growth and production using CO_2 enrichment (see also section 5).

5. Molecular mechanisms for high-CO_2 responses

Microalgae can acclimate to high-CO_2 conditions by changing their photosynthetic properties such as CCM. The half-saturation concentration of CO_2 for changing cellular photosynthetic characteristics, i.e., CO_2 affinity, is 0.5% in the unicellular green alga *Chlorella kessleri* 211-11h (formerly *C. vulgaris* 11h; Shiraiwa & Miyachi, 1985). CCM-related proteins are also degraded simultaneously when cells are transferred from low- to high-CO_2 conditions (see references in section 3). Yang et al. (1985) found that, during acclimation to high-CO_2 conditions, CA, an essential component of CCM, was passively degraded and thus the process took almost 1 week.

C. reinhardtii cells in freshwater and in soil are exposed to drastically fluctuating concentrations of CO_2 between atmospheric level and ≥10% (v/v) (for review, see Stolzy, 1974; Buyanovsky & Wagner, 1983). To grow in such habitats and maintain optimum growth, the alga needs to rapidly change its physiology. Such rapid acclimation was in fact observed in *C. reinhardtii* cells that were successfully acclimated to 20% CO_2 within a few days (Hanawa, 2007). The specific growth rate (μ) of *C. reinhardtii* was 0.176 in ordinary air containing 0.04% CO_2 where dCO_2 and total DIC were 1.62 and 6.19 μM, respectively, at pH 6.8 (Hanawa, 2007) (Fig. 3). Although dCO_2 and total DIC concentrations in the culture media, which were equilibrated with 0.3, 1.0, and 3.0% CO_2 (v/v) in air, were 28-, 121-, and 489-fold higher than that in ordinary air, respectively, alga-specific growth rates under the respective conditions were only 1.3-, 1.8-, and 1.7-fold higher than that in air (Hanawa, 2007) (Fig. 3). In a wall-less mutant of *C. reinhardtii* CC-400 (same as *CW-15*), the growth rate and the amount of total proteins increased only 1.5-fold even when the CO_2 concentration was increased from atmospheric level to 3% (Baba et al., 2011b). These results clearly indicate that, in *C. reinhardtii*, CO_2 enrichment is not advantageous to increase in growth rate, as the fully low-CO_2-acclimated cells acquire CCM and grow quickly with a near-maximum growth rate even under atmospheric levels of CO_2. These results are true when cells are growing logarithmically at low cell density to prevent self-shading. However, when cell density is quite high, the ratio of growth at high to low CO_2 is usually quite high. This is probably due to the decrease in growth under air conditions. Under such conditions, CO_2 supply is strongly limited resulting in very low growth rates under air-level CO_2. Nevertheless, the growth rate does not exceed the specific growth rate obtained at the logarithmic growth stage.

High-CO_2-grown *C. reinhardtii* declines CCM physiologically by losing CA and active DIC transport systems in order to avoid secondary inhibitory effects caused by excess DIC accumulation (for review, see Miyachi et al., 2003; Spalding, 2008; Yamano & Fukuzawa, 2009) but no other significant responses have been reported until recently. Recently, we found drastic changes in extracellular protein composition (Baba et al., 2011b) including induction of the H43/Fea1 protein (Hanawa et al., 2004, 2007; Kobayashi et al., 1997).

The wall-less mutant of *C. reinhardtii, CW-15*, releases a large amount of extracellular matrix, including periplasm-locating proteins, named as extracellular proteins, into the medium (Hanawa et al., 2007; Baba et al., 2011b). Our previous studies clearly showed that the extracellular protein composition changes drastically when *C. reinhardtii* cells are transferred

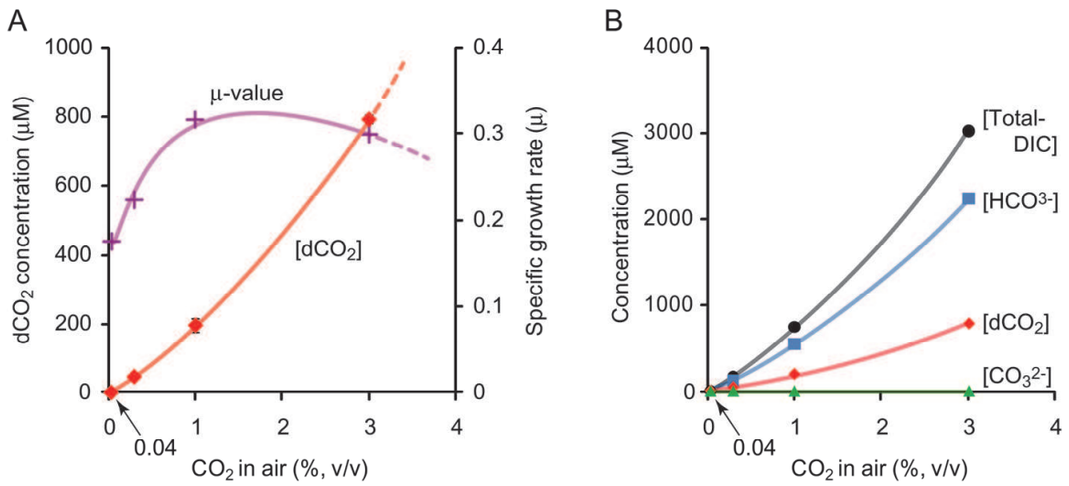

Fig. 3. Relationship between the specific growth rate and dCO_2 concentration in an air-bubbled culture of *Chlamydomonas reinhardtii* (A), and the concentrations of three dissolve inorganic carbon (DIC) species in the culture (B). The concentration of dCO_2 was experimentally determined. Each DIC species was calculated by Henley's law and the Henderson–Hasselbalch equation, respectively. The parameters were as follows (for freshwater at 25°C): $pKa_1 = 6.35$, $pka_2 = 10.33$. The culture medium used was a high salt medium supplemented with 30 mM MOPS (pH 6.8). Crosses, specific growth rate; diamonds, dCO_2; circles, total DIC; squares, bicarbonate; triangles, carbonate. Fig. 3A is modified from Hanawa, 2007.

from atmospheric air to 3% CO_2 in air (Hanawa et al., 2004, 2007; Kobayashi et al., 1997), whereas an SDS-PAGE profile of intracellular-soluble and -insoluble proteins showed no clear difference (Baba et al., 2011b). Recently, we analyzed 129 proteins by proteomic analysis and identified 22 high-CO_2-inducible proteins from *C. reinhardtii* cells transferred from low- to high-CO_2 conditions (Baba et al., 2011b). These high-CO_2-inducible proteins are multiple extracellular hydroxyproline-rich glycoproteins (HRGPs), such as nitrogen-starved gametogenesis (NSG) protein (Abe et al., 2004), inversion-specific glycoprotein (ISG) (Ertl et al., 1992), and cell wall glycoprotein (GP) (Goodenough et al., 1986), together with sexual pherophorin (PHC) (Hallmann, 2006), gamete-specific (GAS) protein (Hoffmann & Beck, 2005), and gamete-lytic enzymes (Buchanan & Snell, 1988; Kinoshita et al., 1992; Kubo et al., 2001). Both GP and ISG are classified as HRGPs together with PHC, GAS, and sexual agglutinin with a shared origin (Adair, 1985). HRGPs are generally involved in sexual recognition of mating-type, plus or minus gametes, in the *Chlamydomonas* lineage (Lee et al., 2007). Among these proteins, NSG, GAS, and gamete-lytic enzymes are generally known to be induced during the gametogenetic process. The sexual program, including gametogenesis in *Chlamydomonas*, is strictly regulated by nitrogen availability (for review, see Goodenough et al., 2007). Drastic changes in the expression of gametogenesis-related extracellular proteins were clearly observed in *C. reinhardtii* cells in response to high-CO_2 but not to environmental nitrogen concentrations, because the experiment was performed under nitrogen-sufficient conditions (Baba et al., 2011b). No visible effect of high-CO_2 signal alone was observed on mating (Baba et al., 2011b). From these results, we concluded that the high-CO_2 signal induced gametogenesis-related proteins but that the signal was not strong

enough or was still missing some necessary factors to trigger mating. Otherwise, these gametogenesis-related protein families and/or HRGPs may have another function under high-CO_2 conditions.

The biological meaning of the expression of gametogenesis-related proteins at the stage of vegetative growth is quite mysterious. CCM may be differentially regulated by changes in nitrogen availability, depending on the species (for review, see Giordano et al., 2005). In *C. reinhardtii*, mildly limited nitrogen availability suppresses CCM and mitochondrial β-CA expression (Giordano et al., 2003) and the increase in NH_4^+ concentration promotes the efficiency of photosynthetic CO_2 utilization (Beardall & Giordano, 2002). From these results, Giordano et al. (2005) suggested that the induction of CCM and related phenomena induced by CO_2 limitation is regulated to satisfy an adequate C/N ratio. Basically, cells growing under high-CO_2 conditions may require more nitrogen, at least no less than low-CO_2-acclimated cells, and tend to attain nitrogen-limitation status easily. In contrast, Giordano et al. (2005) suggested that activating CCM may reduce the loss of nitrogen through the photorespiratory nitrogen cycle. Namely, NH_4^+ produced by converting Gly to Ser through the C_2 cycle in mitochondria is transported to and re-fixed in the chloroplasts by the GS2/GOGAT cycle where chloroplastic GS2 is induced in response to CO_2 concentration in *C. reinhardtii* (Ramazanov & Cárdenas, 1994). In previous works, the NH_4^+ excretion rate from algal cells was lower in high-CO_2 cells than in low-CO_2 cells when monitored in the presence of 1 mM 1-methionine sulfoximine, a specific inhibitor of GS activity, to prevent re-fixation of NH_4^+ in *C. reinhardtii CW-15* (Ramazanov & Cárdenas, 1994) and similarly in *C. vulgaris* 211-11h (Shiraiwa & Schmid, 1986). A decrease in the intracellular NH_4^+ level was first reported to induce gametogenesis-related genes in *C. reinhardtii* (Matsuda et al., 1992). Thus, it is reasonable to hypothesize that gametogenesis is triggered by a decrease in intracellular NH_4^+ levels under high-CO_2 conditions when photorespiration is suppressed. However, further study is required, as photorespiratory activity in *C. reinhardtii* is very low (Badger et al., 2000).

Another report suggested the close participation of CO_2 in inorganic nitrogen assimilation (for review, see Fernández et al., 2009). LCIA, or NAR1.2, is involved in the bicarbonate transport system in chloroplasts (Duanmu et al., 2009) but is not regulated by nitrogen availability, and has been identified as a low-CO_2-inducible gene by expressed sequence tag (EST) analysis (Miura et al., 2004). However, NAR1 genes generally involve members of the formate/nitrite transporter family (Rexach et al., 2000). In fact, LCIA-expressing *Xenopus* oocytes display both low-affinity bicarbonate transport and high-affinity nitrite transport activities (Mariscal et al., 2006), suggesting that LCIA is involved in both bicarbonate uptake and nitrite uptake induced under low-CO_2 conditions. In other words, the suppression of LCIA by high-CO_2 conditions may reduce nitrogen availability in the chloroplast. Additionally, the molecular structure of the high-affinity-bicarbonate transporter *cmpABCD* is very similar to that of the nitrate/nitrite transporter *nrtABCD* in *Synechococcus* sp. PCC7942 (for review, see Badger & Price, 2003). The expression of high-affinity nitrate and nitrite transporter (HANT/HANiT) system IV is triggered by a sensing signal of low CO_2 but not NH_4^+ (Galván et al., 1996; Rexach et al., 1999). These data suggest that changes in CO_2 concentration may also affect intracellular nitrogen availability. Further study should be conducted to identify the cooperative effect of CO_2 and nitrogen availability on the expression of CO_2, nitrogen, and gametogenesis-responsive proteins.

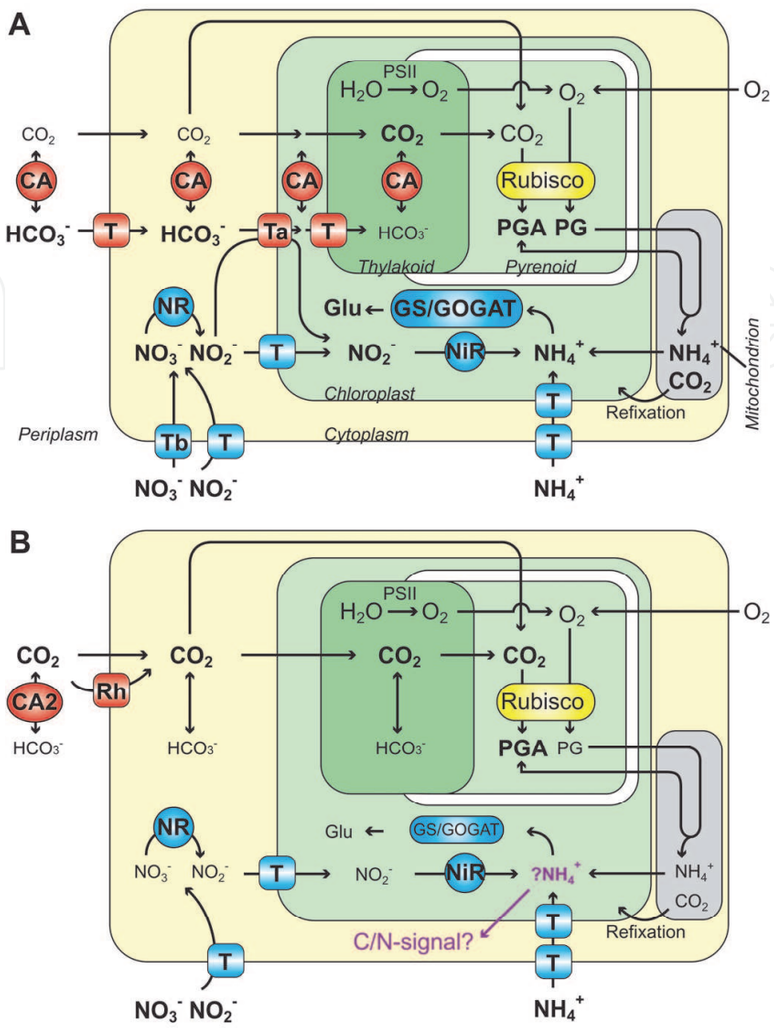

Fig. 4. Schematic illustration of a C/N-status model in low- (A) and high-CO_2-acclimated cells (B) under respective CO_2 conditions produced during acclimation in *C. reinhardtii*. Dissolved inorganic carbon and nitrogen species drawn in bold dominate. CA, carbonic anhydrases; CA2, CAH2 (Fujiwara et al., 1990; Rawat & Moroney, 1991; Tachiki et al., 1992); NiR, nitrite reductase; NR, nitrate reductase; PG, 2-phosphoglycolate; PGA, 3-phosphoglycerate; PSII, photosystem II; Rh, Rh1 (Soupene et al., 2002; Yoshihara et al., 2008); T, (putative) transporters; Ta, LCIA (Duanmu et al., 2009; Mariscal et al., 2006); Tb, HANT/HANiT system IV (Galván et al., 1996; Rexach et al., 1999). CCM models of WT/LC cells, inorganic nitrogen assimilation, and photorespiratory carbon oxidation in *C. reinhardtii* are modified from Yamano et al. (2010), Fernández et al. (2009), and Spalding (2009), respectively.

CAH2 was first reported as an active α-type carbonic anhydrase induced under high-CO_2 conditions and light (Fujiwara et al., 1990; Rawat & Moroney, 1991; Tachiki et al., 1992), but it is poorly expressed and located in the periplasmic space (Rawat & Moroney, 1991). However, the physiological roles and expressional regulation of high-CO_2-inducible CAH2 are not well understood. Another high-CO_2-inducible protein, Rh1, has been identified as a

human Rhesus protein in a homology search and is a paralog of the ammonium and/or CO_2 channels (Soupene et al., 2002). The lack of Rh1 impairs cell growth in *C. reinhardtii* under high-CO_2 conditions (Soupene et al., 2004). Fong et al. (2007) proposed that Rh proteins served as H_2CO_3 transporters in *Escherichia coli* under high-CO_2 conditions. Rh1 was originally expected to be located on the chloroplast envelope *in silico* but the Rh1-GFP fusion protein is located in the plasma membrane in transgenic *C. reinhardtii* cells (Yoshihara et al., 2008).

Some mechanisms of CCM, the photorespiratory nitrogen cycle, and the nitrate/nitrite transport system, and the interactions among them, are summarized in relation to high- and low-CO_2-acclimated cells in Figure 4.

6. High-CO_2 signaling

How can microalgal cells sense the CO_2 signal and respond to changes in CO_2 concentration? The most abundant extracellular carbonic anhydrase, CAH1, in low-CO_2 cells is replaced by high-CO_2-inducible extracellular 43 kDa protein/Fe-assimilation 1 (H43/FEA1) when low-CO_2-cells are transferred to high-CO_2 conditions (Allen et al., 2007; Baba et al., 2011a; Hanawa et al., 2004, 2007; Kobayashi et al., 1997). We found that H43/FEA1 was the most abundant extracellular soluble protein, which occupied about 26% of the total extracellular proteins of high (3%)-CO_2-grown cells for 3 days (Baba et al., 2011b). *H43/FEA1* homologous genes are found in the genomic sequences of the chlorophytes *Scenedesmus obliquus*, *Chlorococcum littorale*, and *Volvox carteri*, and the dinoflagellate *Heterocapsa triquerta* (Allen et al., 2007). This suggests that the *H43/FEA1* orthologs may be widely distributed among at least chlorophyte algae.

The function of H43/FEA1 is not completely understood but one possible role may be in iron assimilation (Allen et al., 2007; Rubinelli et al., 2002). Allen et al. (2007) identified *FEA1*, *FEA2*, and a candidate ferrireductase (*FRE1*) are expressed coordinately with iron assimilation components, and it was hypothesized that the proteins may facilitate iron uptake with high affinity by concentrating iron in the vicinity of the cells (Allen et al., 2007). *FEA1* and *FRE1* homologs were previously identified as the high-CO_2-responsive genes *HCR1* and *HCR2* in the marine chlorophyte *C. littorale*, suggesting that the components of the iron-assimilation pathway are responsive to changes in CO_2 concentration (Sasaki et al., 1998). A homology search of DNA sequences showed that *H43, FEA1,* and *HCR1* are identical (Allen et al., 2007; Hanawa et al., 2007), indicating that *H43/FEA1* expression was also induced by iron deficiency with transcriptional regulation. Therefore, we proposed that the gene is expressed as *H43/FEA1* (Baba et al., 2011a, 2011b).

In *C. reinhardtii*, 0.3% (v/v) CO_2 in air is sufficient to trigger the expression of the high-CO_2-inducible *H43/FEA1* and expression is correlated linearly between 0.04% and 0.3% (Hanawa et al., 2007). *H43/FEA1* can also be induced under heterotrophic conditions in the presence of acetate as an organic carbon source even under low-CO_2 conditions (Hanawa et al., 2007). In a previous study, the dCO_2 concentration in a cell suspension increased about 28 times from 1 to approximately 28 μM, which was identical to that equilibrated under the bubbling of 0.22% CO_2 in light, when cells were incubated in the presence of acetate and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) (Hanawa et al., 2007). From these data, the authors concluded that the induction of *H43/FEA1* is triggered by the CO_2 signal, even CO_2 generated from respiration, but not acetate itself or the change in carbon metabolite

abundance. Thus, *H43/FEA1* expression can be regulated by a high-CO_2 signal at the transcriptional level, irrespective of high-CO_2 conditions. H43/FEA1 is highly reliable as a high-CO_2 response marker. The signal for *H43/FEA1* expression might be sensed by putative proteins localized on the cell membrane, which are influenced by protein modifiers and send the signal for *H43/FEA1* expression (Hanawa et al., 2007).

H43/FEA1 expression is induced under excessive levels of Cd (>25 μM) or iron-deficient conditions (<1 μM) (Allen et al., 2007; Rubinelli et al., 2002). Fei et al. (2009) reported two transcriptional *cis*-elements that are responsive to the Fe-deficient signal (FeREs) for *H43/FEA1* expression, namely FeRE1 and FeRE2, which are located at -273/-259 and -106/-85 upstream from the *H43/FEA1* transcriptional initiation site. The conserved sequence motif was identified from some iron-deficiency-inducible genes (Fei et al., 2009). However, according to our recent study, the two *cis*-elements are not necessary for the high-CO_2-induced expression of the *H43/FEA1* gene (Baba et al., 2011a). The high-CO_2-responsive *cis*-element (HCRE) was located at a -537/-370 upstream region from the *H43/FEA1* transcriptional initiation site, although the precise location has not yet been determined (Baba et al., 2011a). These results show that *H43/FEA1* expression is regulated by the high-CO_2 signal alone via the HCRE, which is located distantly from the iron-deficient-responsive element. This observation indicates that *H43/FEA1* is a multi-signal-regulated gene (Fig. 5). We have not yet determined whether all of these signals may affect the expression of other high-CO_2-inducible proteins (Baba et al., 2011b). Allen et al. (2007) reported some proteins that are iron-deficient-responsive but not CO_2-responsive, so those proteins are considered components of the iron-assimilation system. In addition, an iron-assimilation component was not found among high-CO_2-inducible extracellular proteins analyzed experimentally (Baba et al., 2011b). The expression by either high-CO_2 or iron-deficient signals is a unique feature of *H43/FEA1*.

The regulation of CCM-related gene expression, which is positively induced by a low-CO_2 signal and negatively induced by a high-CO_2 signal, has been well characterized in *C. reinhardtii*. A zinc-finger protein named CCM1/CIA5 has been identified as a candidate of the CCM master regulator (Fukuzawa et al., 2001; Miura et al., 2004; Xiang et al., 2001). CCM1/CIA5 is a protein complex with a molecular mass of approximately 290–580 kDa that is induced independently by DIC availability; Zn is necessary for its enzymatic function (Kohinata et al., 2008). One of the CCM1/CIA5-mediated signaling systems functions in the expression of *CAH1,* which encodes a low-CO_2-inducible periplasmic carbonic anhydrase (Fukuzawa et al., 1990) and the signaling is mediated by a Myb-type transcriptional regulator named LCR1 (Yoshioka et al., 2004). CCM1/CIA5 may possibly function as an amplifier for the CO_2 signaling cascade (Yamano et al., 2008). A direct signaling factor for CCM induction has not been identified, although some candidates have been reported (Giordano et al., 2005; Kaplan & Reinhold, 1999; Yamano et al., 2008). The *CCM1/CIA5* mutant lacks suppression of *H43/FEA1* expression under both low-CO_2 and iron-sufficient conditions (Allen et al., 2007), suggesting that *H43/FEA1* expression is regulated by the CCM1/CIA5-dependent signaling cascade. However, the regulatory mechanism seems to be complex. The responses of CAH1 and H43/FEA1 expression are not an all-or-none type to the signals for a change in environmental CO_2 concentration, acetate concentration, and light intensity (Hanawa et al., 2007). Signaling for *H43/FEA1* expression may be partially associated with CCM1/CIA5 signaling, although additional signals may also exist (Fig. 5). CAH2 is continuously expressed in a *CCM1/CIA5* mutant independent of CO_2 concentration

(Rawat & Moroney, 1991), suggesting that CAH2 expression is regulated by CCM1/CIA5 in the wild type. However, another high-CO_2-inducible protein, Rh1, is not likely regulated by CCM1/CIA5 (Wang et al., 2005).

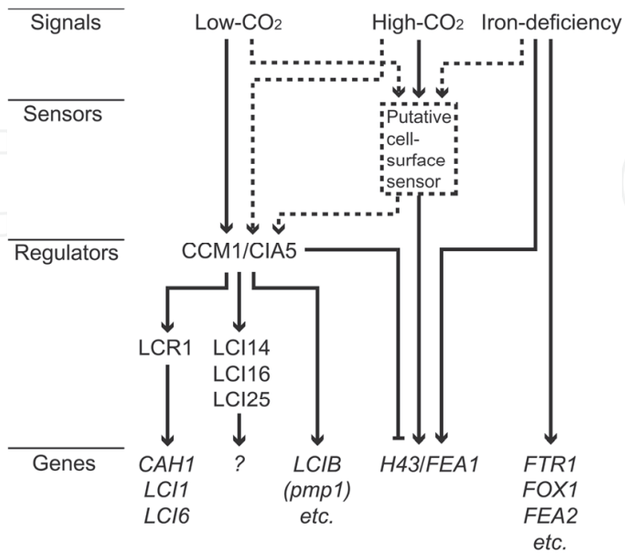

Fig. 5. Schematic model of high-CO_2 signaling for *H43/FEA1* induction. Solid and broken lines are expective and putative signaling flows, respectively. Low-CO_2 signaling is modified from Miura et al. (2004) and Yamano and Fukuzawa (2009). Iron-deficient-inducible genes are according to Allen et al. (2007). Cd signaling on *H43/FEA1* induction, proposed by Rubinelli et al. (2002), is not drawn because little about it is known.

7. Conclusion

Compared to low-CO_2-inducible mechanisms that are well understood, analyses of high-CO_2-responsive mechanisms in microalgae at the molecular level have just started using the unicellular green alga *C. reinhardtii*. An accurate characterization of the acclimation mechanisms to high-CO_2 conditions will be important for both a detailed understanding of sensing and responding to environmental CO_2 changes and maximizing algal biomass productivity in mass cultivation. H43/FEA1, the most abundant extracellular protein in high-CO_2-acclimated cells, is expressed in response to multiple signals, including high-CO_2, iron-deficiency, or Cd-stress conditions. This suggests that, in addition to the high-CO_2 signal itself, abnormally stressful conditions such as strong nutrient depletion caused by rapid growth under high-CO_2 conditions may trigger expression of the gene. Targeted proteomics of whole *C. reinhardtii* established by Wienkoop et al. (2010) and a cDNA array (Yamano et al., 2008) or transcriptomics (Yamano & Fukuzawa, 2009), which has been applied to an expression analysis of CCM-associated genes,, would be useful for further detailed analysis of high-CO_2 response phenomena. Our recent data indicate that the expression of gametogenesis-related proteins, which are strictly regulated by nitrogen availability, is triggered by high-CO_2 signals with a drastic change in extracellular proteins. These gametogenesis-related proteins in the periplasmic space of *C. reinhardtii* cells may play novel and crucial roles when *C. reinhardtii* is grown under high-CO_2 conditions.

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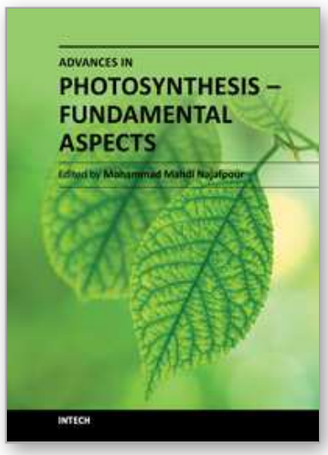

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Photosynthesis is one of the most important reactions on Earth. It is a scientific field that is the topic of many research groups. This book is aimed at providing the fundamental aspects of photosynthesis, and the results collected from different research groups. There are three sections in this book: light and photosynthesis, the path of carbon in photosynthesis, and special topics in photosynthesis. In each section important topics in the subject are discussed and (or) reviewed by experts in each book chapter.

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