Proteomic Analysis of High-CO2-Induceable Extracellular Proteins in the Unicellular Green Alga, *Chlamydomonas reinhardtii*

Masato Baba, Iwane Suzuki and Yoshihiro Shiraiwa*

Graduate School of Life and Environmental Sciences, 1-1-1 Tennodai, University of Tsukuba, Tsukuba, 305-8572 Japan

*Corresponding author: E-mail, emilhux@biol.tsukuba.ac.jp; Fax, +81-29-853-6614

The unicellular green alga *Chlamydomonas reinhardtii* can acclimate to a wide range of CO2 concentrations through the regulation of a CO2-concentrating mechanism (CCM). By proteomic analysis, here we identified the proteins which were specifically accumulated under high-CO2 conditions in a cell wall-less strain of *C. reinhardtii* which releases extracellular matrices to the medium. When CO2 concentration was elevated from the air-level to 3% during culture, the algal growth rate increased 1.5-fold and the composition of extracellular proteins, but not intracellular-soluble and -insoluble proteins, clearly changed. Proteomic analysis data showed that the levels of 22 among 129 extracellular proteins increased for 1 and 3 days and such multiple high-CO2-inducible proteins include gametogenesis-related proteins and hydroxyproline-rich-glycoproteins. However, we could not prove the induction of gametogenesis under high-CO2 conditions, suggesting that the inductive signal might be incomplete, not strong enough, or only high-CO2 conditions might be not sufficient for proceeding cell stage to the formation of sexually active gamates. In any case, those gametogenesis-related proteins and/or hydroxyproline-rich-glycoproteins may take novel roles outside the cell under high-CO2 conditions.
**Keywords:** *Chlamydomonas reinhardtii* • extracellular proteins • gametogenesis • high-CO₂-inducible protein • high-CO₂-acclimation • proteomics

**Abbreviations:** CAH, carbonic anhydrase; CCM, CO₂-concentrating mechanism; DIC, dissolved inorganic carbon; emPAI, exponentially modified Protein Abundance Index; FAP, flagellar-associated protein; GAS, gamete-specific; GP, glycoprotein; H43/FEA1, high-CO₂-inducible 43 kDa protein/Fe-assimilation 1; HRGP, hydroxyproline-rich glycoprotein; ISG, inversion-specific glycoprotein; MMP, matrix metalloproteinase; MS, mass spectrometry; NSG, nitrogen-starved gametogenesis; PHC, pherophorin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
Introduction

Aquatic photosynthetic organisms such as microalgae and cyanobacteria have an ability to acclimate to a broad range of CO₂ concentrations. CO₂ is the substrate of photosynthetic carbon fixation and therefore the rate of CO₂ supply is a key factor for efficient photosynthetic reactions. The process of dissolving atmospheric CO₂ into water, the subsequent processes of equilibration of dissolved CO₂, bicarbonate, and carbonate, and the diffusion of those dissolved inorganic carbons (DIC) to cells and the CO₂ fixation site in chloroplasts are extremely slow physical and chemical processes, compared to other enzymatic reactions in photosynthesis. Furthermore, these processes are strongly affected by various environmental factors such as pH, temperature, and salinity. The atmospheric and oceanic CO₂ concentrations decreased markedly during certain geological periods and there have been several incidences of minor fluctuations in CO₂. This would suggest that photosynthetic organisms have developed special mechanisms for DIC utilization and for metabolic pathways to adapt and acclimate to changes in CO₂ concentration (e.g., Badger 1987; Falkowski and Raven 2007). However, some properties of a CO₂-fixing enzyme ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) are less developed; e.g., the relative specificity of Rubisco to CO₂/O₂ and an affinity of Rubisco to CO₂ (e.g., Falkowski and Raven 2007).

Microalgae induce a CO₂-concentrating mechanism (CCM) that facilitates the utilization of DIC through the de novo synthesis of inorganic carbon transporters and carbonic anhydrases (CAHs) when cells are exposed to air-level CO₂ conditions (i.e., ca. 10 μM CO₂ in the medium) (Badger et al. 1980; Aizawa and Miyachi 1986; Kaplan and Reinhold 1999; Miyachi et al. 2003; Badger et al. 2006; Raven et al. 2008; Spalding 2008; Moroney and Ynalvez 2007; Yamano and Fukuzawa 2009). The induction of CCM
is immediately suppressed and its activity decreases gradually under high-CO₂ conditions (for review, see Miyachi et al. 2003).

In contrast to the low-CO₂-inducible phenomena, high-CO₂-inducible and low-CO₂-suppressive phenomena have not been well-studied. Even though some microalgae and cyanobacteria are able to grow under extremely high-CO₂ (e.g., 40–100% CO₂), in general, they are susceptible to extremely high-CO₂ conditions (for review, see Miyachi et al. 2003). The effects of extremely high-CO₂ on cellular responses have been studied extensively in the high-CO₂-tolerant marine chlorophyte *Chlorococcum littorale*. When cells were transferred to extremely high-CO₂ conditions, photosynthetic activity was spontaneously decreased by chloroplastic and cytosolic acidifications. Then *C. littorale* recovers to acclimate via state transition for protecting photosystems from damage (Iwasaki et al. 1998; Sasaki et al. 1998; Satoh et al. 2001, 2002, 2004). However, the half-saturation concentration of CO₂ of high-CO₂-acclimated cells to be adequate for changing cellular characteristics has been reported to be 0.5% in a unicellular green alga *Chlorella kessleri* 211-11h (formerly *C. vulgaris* 11h; Shiraiwa and Miyachi 1985). Accordingly, the cellular acclimation to high-CO₂ conditions was suggested to be different from that to extremely high-CO₂.

A unicellular green alga, *Chlamydomonas reinhardtii* has been used widely as a model organism for photosynthesis research. It lives in aquatic environments and even in soil where CO₂ concentration change drastically between the atmospheric level and ≥10% (v/v) (for review, see Buyanovsky and Wagner 1983; Stolzy 1974). To survive in such habitats, this alga needs to acclimate and adapt to high-CO₂ conditions rather than low-CO₂. We previously demonstrated that a change in CO₂ concentration from air-level to 3% CO₂ in air induces a dramatic change in the composition of extracellular proteins in
C. reinhardtii (Kobayashi et al. 1997; Hanawa et al. 2004; Hanawa et al. 2007). We found that carbonic anhydrase 1 (CAH1), the most abundant extracellular protein in the low-CO2 cells, is replaced by high-CO2-inducible 43 kDa protein/Fe-assimilation 1 (H43/FEA1), a function-unknown protein, when cells were exposed to high-CO2 conditions (Allen et al. 2007; Baba et al. 2011; Hanawa et al. 2004, 2007; Kobayashi et al. 1997). Previous studies demonstrate that the expression of H43/FEA1 is separately regulated by CO2 and iron concentrations via independent cis-elements (Allen et al. 2007; Hanawa et al. 2007; Fei et al. 2009; Baba et al. 2011). It has been suggested that the homologous genes of H43/Fea1 can be found in the genomic sequences of the chlorophytes Scenedesmus obliquus, Volvox carteri, and C. littorale and the dinoflagellate Heterocapsa triquerta (Allen et al. 2007). A homolog of H43/Fea1 in C. littorale, Hcr1, had been identified previously as a high-CO2-responsive gene (Sasaki et al. 1998). These results suggest that the orthologs of H43/Fea1 may play a role in high-CO2 acclimation in these algae. In addition to H43/FEA1, carbonic anhydrase 2 (CAH2) (Fujiwara et al. 1996) and Rhesus1 (Soupene et al. 2004) have also been reported as high-CO2-inducible proteins in C. reinhardtii; however, their physiological functions have not yet been revealed.

These findings of high-CO2-inducible proteins indicate that C. reinhardtii cells can actively acclimate to high-CO2 conditions by not only reducing low-CO2-inducible CCM and CAH activities, but also through a high-CO2-inducible mechanism. To understand the details of such acclimation, we conducted an exhaustive search of proteins using genome-based liquid chromatography-mass spectrometry (LC-MS) methods to characterize the entire profile involved in the cellular response to high-CO2 conditions in C. reinhardtii.
Results

Effect of high-CO$_2$ on cell growth and protein content

We used the cell wall-less strain *C. reinhardtii* CC-400 cw-15 mt$^+$ in this study because
the strain largely releases extracellular matrices, including periplasmic proteins, into the
medium (Hanawa et al. 2007). We accurately called such proteins released to the medium
as extracellular proteins of which major components are periplasmic proteins.

The logarithmic growth phase of CC-400 was maintained only for about 24 h in a
batch culture, irrespective of CO$_2$ concentrations (Fig. 1A). The growth rate $\mu$ (d$^{-1}$) and
average doubling time (h; shown in parenthesis), were 1.8 (8.95), 2.2 (7.60), and 2.4
(6.81) for air-grown cells transferred to air (Air), air-grown cells transferred to 3% CO$_2$ in
air (Air to CO$_2$), and 3% CO$_2$-grown cells transferred to 3% CO$_2$ in air (CO$_2$), respectively
(Fig. 1B). When the growth reached the linear growth phase by increasing cell
concentration, the cell growth became especially slow under air (Fig. 1B).

To avoid such growth limitation, a semi-continuous culture method in which a cell
suspension was diluted once per day with fresh medium was introduced for preparing
samples for proteomic analysis (Fig. 2). The experiments were repeated three times and
data presented here are average values of them. Algal samples acclimated to low- and
high-CO$_2$ conditions were provided for protein analysis, as follows: cells grown under
ambient atmospheric air, namely CO$_2$-limiting conditions (Air), cells grown for 1 day
under high-CO$_2$ conditions (CO$_2$-1d), and cells grown for 3 days under high-CO$_2$
conditions (CO$_2$-3d) (Fig. 2A). The growth rates $\mu$ (d$^{-1}$) and average doubling times (h) in
parenthesis were 1.81 ± 0.06 (9.19 ± 0.32), 2.7 ± 0.23 (6.19 ± 0.55), and 2.78 ± 0.04
(5.98 ± 0.09) under Air, CO$_2$-1d, and CO$_2$-3d, respectively (Fig. 2B). The logarithmic
growth rate ($\mu$) of CO2-3d was 1.5-fold higher than that of Air. The amount of proteins released into the medium was slightly greater in CO2-3d cells than in Air cells (Fig. 2C). The fluorescent gel images of extracellular proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) clearly showed the induction of CAH1 and H43/FEA1, which are known to be low- and high-CO2-inducible markers, in air-acclimated cells and high-CO2-acclimated cells, respectively (Fig. 2D). Such different profiles of CAH1 and H43/FEA1 demonstrate that the cells were fully acclimated to low- and high-CO2 conditions, respectively.

Intracellular-soluble and -insoluble fractions were applied separately to 2D-gel analysis of low- and high-CO2-acclimated cells. The major proteins were Rubisco (Fig. S1A, B) and the photosystem-associated proteins disturbed clear separation of proteins in the intracellular-soluble and intracellular-insoluble fractions, respectively, but no clear difference was observed between the low- and high-CO2-acclimated cells (Fig. S1C). We only found significant changes in the profile of extracellular proteins and therefore we focused on these profiles in subsequent analyses.

One-dimensional SDS-PAGE was sufficient to separate the extracellular proteins for mass spectrometric analysis. Consequently, we identified 89, 69, and 98 proteins from culture media of Air, CO2-1d, and CO2-3d cells, corresponding to the samples presented in Fig. 2A (Table S1). The total number of proteins, identified at least once in triplicate experiments with a MASCOT score >50, was 129. The data are presented together with the exponentially modified Protein Abundance Index (emPAI) because the emPAI is useful for estimating the absolute amount of protein (Ishihama et al. 2005). According to the SignalP 3.0 server prediction, number (percent of total proteins) of proteins predicted to be secretory was 32 (36.0%), 33 (47.8%), and 40 (40.8%) in Air, CO2-1d cells, and
CO₂-3d cells, respectively, where total number was 43 (33.3%) (Fig. 3). On the other hand, the percentage of total putative secretory proteins calculated on the basis of protein amounts was 46.5%, 63.0%, and 65.9% in Air, CO₂-1d, and CO₂-3d cells, respectively, indicating that high-CO₂-acclimated cells secreted 1.4-fold more proteins than did low-CO₂-acclimated cells. These proteins were annotated based on the results of BlastX analyses and are listed separately up to 20 in order of their amounts in Air, CO₂-1d, and CO₂-3d cells in Tables 1–3 and Fig. 4. Proteins highly induced under high-CO₂ conditions were renamed as high-CO₂-inducible proteins (HCl) (Table S1). Other extracellular proteins that had no name were designated extracellular proteins (EXC).

Highly induced extracellular proteins in air-acclimated cells

Out of 89 proteins, 31 were identified in Air cells tested in triplicate (Table S1). Among them, the ratios of the amounts (mol%) of CAH1 and glyceraldehyde-3-phosphate dehydrogenase 3 (GAP3) in Air to CO₂-3d cells (Air/CO₂-3d) were 5.85 and 5.25 (p <0.05), respectively (Table 1, Fig. 4). CAH1 was the most abundant protein in Air cells, amounting to 10.11 ± 2.84% of the total extracellular proteins. CAH1 localizes in the periplasmic space (Kimpel et al., 1983, Coleman et al., 1984, Yang et al., 1985, Fukuzawa et al., 1990). Although CAH2, generally known as a high-CO₂-inducible protein, was identified as a low-CO₂-inducible protein by database, the identification contains uncertainty because CAH2 has a similar amino acid sequence to CAH1 and very low protein content (data not shown). Therefore, we hereby described it as CAH1/CAH2 (Table 1, Fig. 4). The location of GAP3 predicted by SignalP was in the cytoplasm, but this protein has also been reported in flagella proteome (Pazour et al., 2005), suggesting that it is a multi-protein. As such, the annotation of proteins contained
some less-reliable cases.

The levels of other proteins of low content were not significantly different between Air and CO$_2$-3d cells.

**Highly induced extracellular proteins in 1-day high-CO$_2$-acclimated cells**

Similarly, 44 of 69 proteins were identified in triplicate experiments in CO$_2$-1d cells (Table S1). Among them, the amounts of seven proteins (H43/FEA1, two nitrogen-starved gametogenesis [NSG] family proteins [HClI and HCl2], two glycoproteins [GP1 and FAP102], and two inversion-specific glycoproteins [ISG-C1 and ISG-C4]) were significantly higher ($p < 0.05$) in CO$_2$-1d cells than in Air cells (Table 2, Fig. 4). H43/FEA1 was the most abundant protein, accounting for $22.09 \pm 8.16$ (mol%) of the total extracellular proteins in CO$_2$-1d cells. The ratios of the amount (mol%) of proteins in CO$_2$-1d to Air cells (CO$_2$-1d/Air) were 3.66, 3.57, 2.26, and 2.07 for H43/FEA1, ISG-C1 (similar to *V. carteri* ISG and *C. reinhardtii* VSP-3), FAP102 (similar to GP3), and HClI (similar to NSG1), respectively (Fig. 4).

**Highly induced extracellular proteins in 3-day high-CO$_2$-acclimated cells**

Of 98 proteins, 41 were identified in triplicate experiments in all CO$_2$-3d cells (Table S1). Among them, the amounts (mol%) of eight proteins (H43/FEA1, three NSG family proteins [FAP212, HCl2, and HCl3], two GPs [FAP102 and HCl4], and two ISGs [ISG-C1 and ISG-C2]) were significantly higher in CO$_2$-3d cells than in Air cells ($p < 0.05$) (Table 3, Fig. 4). H43/FEA1 was the most abundant protein, amounting to $26.01 \pm 4.30$ (mol%) of total extracellular proteins in CO$_2$-3d cells (Table 3, Fig. 4). The ratios of the amount of proteins in CO$_2$-3d to Air cells (CO$_2$-3d/Air) were 4.36, 4.31, 3.03,
Mating efficiency of high-CO2 cells

According to the proteomic analysis data suggesting that gametogenesis might be induced under high-CO2 conditions, we examined the mating efficiency under the same culture conditions. For the purpose we used high-mating strains of *C. reinhardtii* strains CC-620 and CC-621 since the cell wall-less strain generally is known to show low mating efficiency. As a result, when those were grown under high-CO2, both strains did not show mating profile whereas gamete formation was triggered by nitrogen-depletion and the gamates showed normal mating profile (Fig. 5). A mating efficiency of gamates induced by nitrogen-depletion was approximately 75% (data not shown).

Discussion

**General features of high-CO2-acclimated cells**

The major component of the cellular response to limited CO2 is the activation of CCM, which is reversibly inactivated under high-CO2 conditions (for review, see Aizawa and...
Miyachi 1986, Badger 1987, Kaplan and Reinhold 1999, Miyachi et al. 2003). In this study, we analyzed high-CO$_2$-inducible proteins in *C. reinhardtii* by proteomic analysis. Although we did not find any significant changes in intracellular proteins after the transfer of cells from air to 3% CO$_2$ in air (Fig. S1), we observed remarkable changes in the amount and composition of extracellular proteins (Figs. 2D, 4 and Table S1). The algal growth rate and the amount of total proteins increased by only 1.5-fold, even when the CO$_2$ concentration increased ca. 75-fold from ca. 0.04 to 3% in a wall-less mutant of *C. reinhardtii* CC-400 (Fig. 1). These results indicate that air-acclimated cells could grow quickly, at a rate close to the maximum growth potential, and this may be due to the organism having established a mechanism for the efficient utilization of ambient CO$_2$ such as CCM. The big difference in growth rates between Air- and 3% CO$_2$-acclimated cells was obvious during the linear growth phase and this seems to be a reason why air-grown cultures take longer to attain a high algal density.

**Low- and high-CO$_2$-inducible extracellular proteins**

The induction of CAH1 and H43/FEA1, which are known as low- and high-CO$_2$-inducible proteins, respectively, demonstrated that our proteomic analysis was performed under adequate conditions (Fig. 4). Interestingly, GAP3 was dominantly induced under low-CO$_2$ (Table 1, Fig. 4). GAP3 has been implicated in flagellar activity (Pazour et al. 2005). GAP activity has been shown to correlate with cell motility in *Dunaliella salina* (Jia et al. 2009), implying that decreased CO$_2$ availability may stimulate cell motility.

We also found that two mastigoneme-like proteins, MST1 (a flagellar component; Pazour et al. 2005) and HCI5, were induced under high-CO$_2$ conditions (Table S1). We
also found some function-unknown flagellar associated proteins, or FAPs (Pazour et al. 2005), although the expression pattern of each FAP depended on the levels of CO₂ (e.g., FAP211 and FAP102). In our study, FAPs were found in the excreted protein fraction and therefore we cannot exclude the possibility that the annotation of FAPs contains some uncertainty. Consequently, our results suggest that a high-CO₂ signal may induce the expression of each flagellar component, but the detailed mechanism needs to be analyzed.

Some NSG family proteins were specifically induced under high-CO₂ (Table 3, Fig. 4). NSG family genes were previously identified in synchronized early G1 cells of C. reinhardtii grown in nitrogen-free medium (Abe et al. 2004).

We found that GP and ISG family proteins were significantly induced under high-CO₂ conditions (Table 3, Fig. 4). GP has been isolated from major outer layers of cell walls (W6 and W4) using sodium perchlorate or other chaotropes (Goodenough et al. 1986). Although GPs are thought to be ones of major components of cell wall, the expression of the proteins are rather enhanced in the cell wall-less mutant. Lack of cell wall might release a feedback control by products. ISG is an extracellular glycoprotein of V. carteri that may be synthesized for only a few minutes in inverting embryos and sperm cell packets and is thought to be involved in the early processes of extracellular matrix biogenesis (Ertl et al. 1992). Both GP and ISG were classified as hydroxyproline-rich glycoproteins (HRGPs) together with pherophorin (PHC), gamete-specific (GAS) protein, and sexual agglutinin with a shared origin (Adair 1985). PHC, a common protein in volvocales (Hallmann 2006), is abundant in the extracellular matrix and some of them have been reported to be strongly induced by sex inducers that trigger sexual development as well as by mechanical wounding (Hallmann 2006). GAS proteins are related to PHCs (Hallmann 2006). Transcripts for GAS28, GAS30, and GAS31
accumulate in the late phase of gametogenesis and in young zygotes (Hoffmann and Beck 2005). In our experiments, a GAS family protein (HCI6) and three PHC proteins (HCI7, HCI8, and PHC14) accumulated in cells grown under high-CO₂ conditions (Table S1). These findings suggest that high-CO₂ signals may induce HRGPs, which have been reported to be generally involved in sexual recognition of mating-type plus and minus gametes in the *Chlamydomonas* lineage (Lee et al. 2007).

Furthermore, we found that two matrix metalloproteinases (MMPs), MMP1 and HCI9, which are gamete-lytic enzymes, were induced under high-CO₂ conditions (Table S1). Gamete-lytic enzymes degrade cell walls during gametogenesis (Buchanan and Snell 1988; Kinoshita et al. 1992) and the MMP1 gene is induced during gametogenesis (Kubo et al. 2001). The expression of gamete-lytic enzymes is restricted under nitrogen-deficient conditions.

These proteomic results indicate that multiple extracellular HRGPs proteins, such as NSG, ISG, and GP proteins, together with PHC, GAS, and gamete-lytic enzymes (Table S1) are induced under high-CO₂ conditions. Among these proteins, NSG, GAS, and gamete-lytic enzymes are generally known to be induced during the gametogenetic process, which is triggered by nitrogen-depletion.

**Gametogenesis-related proteins expressed under high-CO₂ conditions**

Sears et al. (1980) previously reported that the vegetative cells of *C. reinhardtii* logarithmically grown in HS medium contained 6-10 μg N (10⁶ cells⁻¹). Daily increments of cells under Air, CO₂-1d, and CO₂-3d were 2.4×10⁶, 5.2×10⁶, 7.7×10⁶, respectively, where cell densities were maintained less than 10⁷ cells ml⁻¹ by daily dilution in the present experiments (Fig. 2B). Thus the nitrogen consumption by cells under Air, CO₂-1d,
and, CO$_2$-3d can be estimated to be 14-24, 31-52, and 46-77 mg l$^{-1}$ in a day. As HS medium firstly contains 500 mg l$^{-1}$ NH$_4$Cl (9.35 mM), the nitrogen contents can be estimated to remain between 7.91-9.09 mM in any culture. In previous studies, gametogenesis of *C. reinhardtii* was immediately and strongly inhibited by 7.5 mM NH$_4$Cl (Beck and Acker 1992). Accordingly, the significant induction of NSG, GAS, and gamete-lytic enzymes would be due to high-CO$_2$ conditions, and not to external nitrogen-depletion (Table 3, Fig. 4).

Nitrogen-depletion is an important inducing factor for gametogenesis (Sager and Granick 1954); however, Goodenough et al. (2007) reported that nitrogen-depletion is a necessary but not essential process for activating the gametogenic program in *C. reinhardtii*. Because the gene expressions for gametogenesis started with a certain length of lag phase after the depletion of nitrogen from the medium, the external nitrogen concentration seems to be a triggering factor, but not a regulatory signal. In terrestrial plants, carbon and nitrogen metabolism interact tightly with each other (for review, see Reichi et al. 2006), and carbon–nitrogen ratio signaling plays an important role in environmental responses (for review, see Zheng, 2009). Taking our results into consideration, a particular carbon–nitrogen ratio, generated under high-CO$_2$ conditions or nitrogen-depletion, is likely to act as a signal for gametogenesis.

Some interesting consistencies have been reported in proteins that facilitate DIC and nitrogen utilization, although their induction mechanisms are different. LCIA (also named NAR1.2), which is involved in chloroplast-located bicarbonate transport (Duanmu et al. 2009), was identified as a low-CO$_2$-inducible gene by EST analysis and was shown to be regulated by changes in CO$_2$ but not nitrogen availability (Miura et al. 2004). On the other hand, NAR1 genes are generally known to involve members of the
Formate/Nitrite Transporter (FNT) family (Rexach et al. 2000). In fact, LCIA-containing Xenopus oocytes display both low-affinity bicarbonate transport and high-affinity nitrite transport (Mariscal et al. 2006), suggesting that LCIA is involved not only in bicarbonate uptake but also nitrite uptake under low-CO2 conditions; in other words, the suppression of LCIA by high-CO2 may reduce nitrogen availability. In addition, the molecular structure of the high-affinity-bicarbonate transporter cmpABCD is very similar to the nitrate/nitrite transporter nrtABCD in Synechococcus sp. PCC7942, suggesting a close regulatory relationship between carbon and nitrogen assimilation (for review, see Badger and Price 2003). These data suggest the possibility that changes in CO2 availability may also affect nitrogen availability.

However, we could not find any effect of high-CO2 signal alone on mating (Fig. 5). This result suggest that high-CO2 signal induced gametogenesis-related proteins but the signal was not strong enough or still missing some factors required for triggering mating. Otherwise, it may also be possible that the gametogenesis-related protein families and/or hydroxyproline-rich-glycoproteins play another role under high-CO2 conditions.

The present results suggest that high-CO2 may be associated with sexual differentiation, by participating in gametogenesis and the sexual program. For further, detailed analysis of the relationship between high-CO2 and gametogenesis, whole-cell proteome analysis would be necessary. Targeted proteomics of whole C. reinhardtii established by Wienkoop et al. (2010) might be useful for such an analysis. Future works are needed to determine which factor is essential for triggering gametogenesis and mating, namely high-CO2, nitrogen-depletion or C/N ratio alone or in combination. Our findings also provide important clues for understanding the behavior of this organism in the natural environment.
Materials and Methods

Strains and culture conditions

A cell wall-less strain of a unicellular green microalga, *C. reinhardtii* CC-400cw-15mt⁺, was obtained from the Chlamydomonas Center at Duke University for use in proteomic analyses. A pair of high-mating strains of *C. reinhardtii*, CC-620 mt⁺ and CC-621 mt⁻, was obtained from Dr. Y. Hanawa, International Patent Organism Depositary (IPOD), National Institute of Advanced Industrial Science and Technology (AIST), Japan for use in mating analysis. Cells were grown at 25°C in Erlenmeyer flasks containing 500 ml of modified HS medium (Sueoka 1960) supplemented with 30 mM 3-(N-morpholino)propanesulfonic acid (MOPS)-NaOH (pH 6.8), and grown under continuous illumination at a photosynthetic photon flux density (PPFD) of 150 μmol m⁻² s⁻¹. Cells grown for 3 days were transferred to either atmospheric air or high (3%)-CO₂ conditions, as described previously (Hanawa et al., 2007).

For proteomic analysis, cells were grown in a semi-continuous culture by diluting each cell suspension with fresh media once per day to maintain a logarithmic growth phase. The algal cells were grown under the bubbling of air (0.04% [v/v] CO₂) for several days to fully acclimate to low-CO₂. After harvesting to transfer to fresh medium, cells were washed three times with fresh media to remove a tiny amount of extracellular proteins on the cell surface. Then, the washed cells were transferred to a new culture under continuous bubbling of air enriched with 3% (v/v) CO₂ (Fig. 2A).

For mating analysis, gamates triggered by nitrogen-depletion were prepared under either high-CO₂ or nitrogen-free conditions in modified HS medium supplemented with 30 mM MOPS-NaOH (pH 6.8) but no NH₄Cl.
Sample preparation

Aliquots (150 ml) of cultures were withdrawn and centrifuged at 2,300 \( \times \) g for 10 min at 4\(^\circ\)C to separate culture media and algal cells. Then, 0.12 mg ml\(^{-1}\) of complete protease inhibitor cocktail (Roche diagnostics, Basel, Switzerland) was added to the collected culture medium. Tiny floating particles in the culture media were removed by filtration through a cellulose acetate membrane (430624, 0.22 \( \mu \)m, Corning, Corning, NY) and the filtrate was lyophilized. The extracellular proteins were dissolved in 2 ml H\(_2\)O and then dialyzed against H\(_2\)O. The protein concentration was determined using a commercial assay kit (Bio-Rad Laboratories, Hercules, CA).

To obtain intracellular-soluble and -insoluble fractions, cells were washed twice with fresh modified HS medium at 4\(^\circ\)C and suspended in 1/50 volume of disruption buffer containing 50 mM piperazine-N,N’-bis(2-ethanesulfonic acid) (PIPES)-NaOH (pH 7.0), 5 mM ethylene diamine tetraacetic acid (EDTA), 5 mM ethylene glycol tetraacetic acid (EGTA), 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1.2 mg ml\(^{-1}\) complete protein inhibitor cocktail. Then the cells were disrupted by sonication on ice and centrifuged to remove cell debris. The resultant supernatants were ultracentrifuged twice: first at 50,000 \( \times \) g and then at 98,000 \( \times \) g for 30 min each. The final supernatants were collected as the soluble proteins. Both precipitates were combined and washed twice with disruption buffer and then used to prepare the insoluble proteins.

The intracellular-soluble and -insoluble proteins were precipitated with four volumes of cold acetone. The precipitated soluble proteins were suspended in 8.5 M urea, 0.2% (w/v) SDS, 2% (v/v) Triton X-100, 65 mM dithiothreitol (DTT), 2% (v/v) pharmalyte (pH 3-10) (GE healthcare Japan, Tokyo, Japan), and 1.2 mg ml\(^{-1}\) complete protease.
inhibitor cocktail. The precipitated insoluble proteins were suspended in 5 M urea, 2 M thiourea, 2% (w/v) 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonate (CHAPS), 0.2% (w/v) SDS, 65 mM DTT, 2% (v/v) pharmalyte (pH 3-10), and 1.2 mg ml⁻¹ complete protein inhibitor cocktail.

### SDS-PAGE

Protein samples (0.9 µg) from the culture medium were denatured in 1/6 volume of sample buffer containing 0.125 M Tris-HCl (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, and 0.004% bromphenol blue at 65°C for 15 min. The samples were resolved using 5–20% (w/v) gradient SDS-PAGE. The proteins in the gels were stained and visualized using Flamingo™ fluorescent gel stain (Bio-Rad Laboratories) or Quick CBB (Wako, Osaka, Japan), according to the manufacturers’ protocols.

### 2D-gel analysis

Each protein sample (50 µg) was applied to isoelectrofocusing (IEF) gel strips with an immobilized linear pH gradient (Immobiline™ DryStrip pH 3–10 NL, 18 cm, GE Healthcare, Japan). The strips were rehydrated at 20°C for 12 h at 100 V in solutions containing 6 M urea, 2 M thiourea, 2% (v/v) Triton X-100, 13 mM DTT, 1% (v/v) pharmalyte (pH 3-10), 2.5 mM acetate, and 0.025‰ (w/v) Orange G. The samples were applied to IEF at 20°C on a Cool phoreStar IPG-IEF Type-P system (Anatech, Poughkeepsie, NY) with a stepwise increase in voltage (500 V [2 h], 700 V [1 h], 1,000 V [1 h], 1,500 V [1 h], 2,000 V [1 h], 2,500 V [1 h], 3,000 V [1 h], and 3,500 V [10 h]). The gel strips were equilibrated in a denaturing solution containing 6 M urea, 13 mM DTT,
30% (w/v) glycerol, 2% (w/v) SDS, and 25 mM Tris-HCl (pH 6.8). Denatured gel strips were equilibrated in a reducing and alkylating solution containing 25 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 0.025‰ (w/v) bromophenol blue, 30% (w/v) glycerol, and 0.24 M iodoacetamide. Next, the gel strips were subjected to 12.5% SDS-PAGE. The protein spots on the gels were stained and visualized using Flamingo™ fluorescent gel stain, according to the manufacturer’s instructions.

Peptide preparation for LC-MS/MS analysis

We separated the proteins recovered from each medium using SDS-PAGE. Aliquots (0.9 μg) of each protein sample were loaded in duplicate and the two lanes for each sample were treated at the same time. The gel sections containing protein bands were sliced into four pieces per sample. Flamingo-stained gels were washed twice with 30% (v/v) HPLC-grade acetonitrile (Kanto Chemical, Tokyo, Japan), washed with 100% acetonitrile and dried under vacuum. The dried gel pieces were treated with 2 μl 0.5 μg μl⁻¹ trypsin (sequence grade; Promega, Madison, WI) in 50 mM ammonium bicarbonate (Shevchenko and Shevchenko 2001) and incubated at 37°C for 16 h. The digested peptides in the gel pieces were recovered twice with 20 μl 5% (v/v) formic acid/50% (v/v) acetonitrile. Finally, combined extracts were concentrated under vacuum.

Mass spectrometric analysis and database search

LC-MS/MS analyses were performed using an LTQ-Orbitrap XL-HTC-PAL-Paradigm MS4 system (Thermo Fisher Scientific, Bremen, Germany). Trypsin-digested peptides were loaded on the column (100 μm i.d. × 15 cm; L-Column, CERI, Auburn, CA) using a Paradigm MS4 HPLC pump (Michrom BioResources) and HTC-PAL autosampler (CTC
analytics, Zwingen, Switzerland). The digests were applied to a column equilibrated with 6.4% acetonitrile and 0.1% acetic acid. The proteins were eluted under a linear gradient from 6.4 to 41.6% acetonitrile solution containing 0.1% acetic acid over 25 min. The eluted peptides were applied directly to the LTQ-Orbitrap mass spectrometer at a flow rate of 300 nl min\(^{-1}\) and a spray voltage of 2.0 kV. The range of MS scan was \(m/z\) 200–2,000 and the top three peaks were subjected to MS/MS analysis. The obtained spectra were compared against a genome database of \textit{Chlamydomonas reinhardtii} v3.0 from the Joint Genome Institute (http://genome.jgi-psf.org/Chlre3/Chlre3.home.html) using the MASCOT server (version 2.1 Matrix Science, London, UK). The MASCOT search parameters were as follows: threshold at 0.05 in the ion score cut-off, peptide tolerance at 10 ppm, MS/MS tolerance at ± 0.8 Da, peptide charge of 2 + or 3 +, trypsin as the enzyme allowing up to one missed cleavage, carbamidomethylation on cysteine as a fixed modification, and oxidation on methionine as a variable modification. To predict the subcellular localization of identified proteins, we used SignalP, ChloroP, and TargetP from the CBS prediction servers (http://www.cbs.dtu.dk/services/).

Observation of mating of gamates

\textit{C. reinhardtii} strains mt\(^+\) and mt\(^-\) were mixed and then microscope image was taken 10 minutes later. The mating efficiency was determined as described by Chiang et al. (1970).

Acknowledgments

This work was supported by a Grant in-Aid for Scientific Research for a Plant Graduate Student from the Nara Institute of Science and Technology (NAIST), Japan (M.B.). We are grateful to Drs. Y. Fukao and M. Fujiwara of NAIST for their assistance with
proteome analysis, to Dr. T. Sasaki of Okayama University, Japan, for his consultation on
the preparation of extracellular and cellular proteins, and to Dr. Y. Hanawa of the National
Institute of Advanced Industrial Science and Technology, International Patent Organism
Depositary of Japan for his helpful suggestions.

References

transcriptional program of synchronous gametogenesis in *Chlamydomonas reinhardtii*. 


*FRE1*, encoding two homologous secreted proteins and a candidate ferrireductase, are
expressed coordinately with *FOXI* and *FTR1* in iron-deficient *Chlamydomonas

Baba, M., Hanawa, Y., Suzuki, I. and Shiraiwa, Y. (2011) Regulation of the expression of


Badger, M.R., Kaplan, A. and Berry, J.A. (1980) Internal inorganic carbon pool of
*Chlamydomonas reinhardtii*. Evidence for a CO\(_2\) concentrating mechanism. *Plant


polypeptide in an unicellular green alga *Chlamydomonas reinhardtii* (abstract no. 493).  

*Plant Physiol.* 114: S112.


Figure legends

Fig. 1. Growth parameters of the wall-less strain *Chlamydomonas reinhardtii* CC-400 under various CO₂ conditions in a batch culture. A, Growth curves. Air (●), cells pre-grown in ordinary air for 3 days were transferred to fresh medium under the same conditions; Air to CO₂ (■), cells pre-grown in ordinary air for 3 days were transferred to fresh medium under high-CO₂ conditions (3% CO₂ in air); CO₂ (▲), cells pre-grown in air containing 3% CO₂ for 3 days were transferred to fresh medium under the same conditions. B, Specific growth rate (y-axis) and the doubling time (numbers on the columns) during the logarithmic growth phase under various CO₂ conditions. Values were calculated from those in Fig. 1A.

Fig. 2. Semi-continuous culture of the wall-less strain *Chlamydomonas reinhardtii* CC-400 for the preparation of samples for proteomic analysis. A, Experimental plan of semi-continuous culture with dilution of culture once per day to maintain logarithmic growth. Algal cells were grown in ordinary air for 3 days and then transferred to 3% CO₂-enriched air. Cells were harvested 0 (1), 1 (2), and 3 (3) days after the transfer of cells from air to high-CO₂. Three independent replicates were used. B, Specific growth rates and the doubling time of cells in cultures (1), (2), and (3) were 9.19 ± 0.32, 6.19 ± 0.55, and 5.98 ± 0.09 h, respectively. C, Concentrations of total proteins released into the medium in cultures (1)–(3) shown in Fig. 2A. D, SDS-PAGE image stained with Flamingo™ gel stain. CAH1 and H43/FEA1 are markers of air- and high-CO₂-inducible proteins in *C. reinhardtii*, respectively. Lanes 1–3 show triplicate samples.
Fig. 3. A Venn diagram of extracellular proteins identified in air-, 1-day-high-CO₂-, and 3-day-high-CO₂-acclimated cells. Numbers in parenthesis indicate numbers of secretory proteins which were identified in air-, 1-day-high-CO₂-, and/or 3-day-high-CO₂-acclimated cells, respectively. Percentages indicate contents of secretory protein in total.

Fig. 4. Lists of top 10 extracellular proteins aligned by its protein content and by its ratio of protein content in air- to 1-day-high-CO₂- or 3-day-high-CO₂-acclimated cells.

Fig. 5. Microscopic images of mating. A, the mixture of *C. reinhardtii* CC-620 and CC-621 which had been grown under high-CO₂ conditions. B, higher magnification image of A. C, the mixture of *C. reinhardtii* CC-620 and CC-621 which had been grown under nitrogen-free conditions. D, higher magnification image of C.

(Additional information)

The English in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, please see: http://www.textcheck.com/certificate/ZtGAp9
A diagram illustrates the dilution process with time points labeled [1], [2], [3]. The legend explains that white lines represent air bubbling (a day) and black lines represent 3% CO₂ bubbling (a day).

Bar graph B shows the growth rate (d⁻¹) with time points 1d and 3d for air and CO₂ conditions. The values are 9.19 for air, 6.19 for 1d CO₂, and 5.98 for 3d CO₂.

Bar graph C shows the protein content in media (mg L⁻¹) with time points 1d and 3d for air and CO₂ conditions. The values are 1.50 for air, 2.00 for 1d CO₂, and 3.00 for 3d CO₂.

A Western blot image D is presented with lanes labeled Air, CO₂-1d, and CO₂-3d. The bands indicate proteins H43/FEA1 and CAH1.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Amount</th>
<th>Protein</th>
<th>Amount</th>
<th>Protein</th>
<th>Amount</th>
<th>Protein</th>
<th>Amount</th>
<th>Protein</th>
<th>Amount</th>
<th>Protein</th>
<th>Amount</th>
<th>Protein</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAH1</td>
<td>10.11</td>
<td>CAH1</td>
<td>5.85</td>
<td>H43/FEA1</td>
<td>22.09</td>
<td>H43/FEA1</td>
<td>3.66</td>
<td>EXC2</td>
<td>4.92</td>
<td>ISG-C1</td>
<td>4.36</td>
<td>ISG-C1</td>
<td>3.76</td>
</tr>
<tr>
<td>H43/FEA1</td>
<td>6.04</td>
<td>GAP3</td>
<td>5.25</td>
<td>PHC21</td>
<td>4.82</td>
<td>PHC21</td>
<td>3.45</td>
<td>EXC1</td>
<td>4.77</td>
<td>FAP102</td>
<td>3.48</td>
<td>FAP102</td>
<td>3.48</td>
</tr>
<tr>
<td>EXC1</td>
<td>5.37</td>
<td>CAH1/CAH2</td>
<td>2.29</td>
<td>HCl1</td>
<td>3.43</td>
<td>HCl1</td>
<td>2.07</td>
<td>HCl1</td>
<td>3.36</td>
<td>HCl2</td>
<td>2.46</td>
<td>HCl2</td>
<td>2.46</td>
</tr>
<tr>
<td>GAP3</td>
<td>4.95</td>
<td>PHC4</td>
<td>1.90</td>
<td>HCl2</td>
<td>3.36</td>
<td>HCl2</td>
<td>1.93</td>
<td>HCl2</td>
<td>3.36</td>
<td>HCl2</td>
<td>2.46</td>
<td>GAS31</td>
<td>1.80</td>
</tr>
<tr>
<td>PHC21</td>
<td>3.96</td>
<td>PHOT</td>
<td>1.67</td>
<td>FAP102</td>
<td>3.16</td>
<td>FAP102</td>
<td>1.74</td>
<td>GP1</td>
<td>3.06</td>
<td>GP2</td>
<td>2.36</td>
<td>GP2</td>
<td>2.36</td>
</tr>
<tr>
<td>EXC2</td>
<td>3.49</td>
<td>GP1</td>
<td>1.49</td>
<td>FAP102</td>
<td>2.87</td>
<td>FAP102</td>
<td>1.46</td>
<td>CAH1</td>
<td>1.73</td>
<td>GP2</td>
<td>2.36</td>
<td>CAH1</td>
<td>1.73</td>
</tr>
<tr>
<td>GP1</td>
<td>2.09</td>
<td>EXC1</td>
<td>1.43</td>
<td>GP1</td>
<td>2.32</td>
<td>EXC2</td>
<td>1.41</td>
<td>ISG-C1</td>
<td>2.27</td>
<td>HCl2</td>
<td>1.41</td>
<td>SRR16</td>
<td>1.12</td>
</tr>
<tr>
<td>FAP211</td>
<td>1.90</td>
<td>PCY1</td>
<td>1.41</td>
<td>GP2</td>
<td>2.87</td>
<td>GP1</td>
<td>1.46</td>
<td>GP2</td>
<td>2.87</td>
<td>GP2</td>
<td>1.46</td>
<td>GP2</td>
<td>1.32</td>
</tr>
<tr>
<td>GP2</td>
<td>1.79</td>
<td>FAP211</td>
<td>1.26</td>
<td>GP2</td>
<td>2.32</td>
<td>FAP211</td>
<td>1.41</td>
<td>ISG-C2</td>
<td>2.27</td>
<td>HCl2</td>
<td>1.41</td>
<td>SRR16</td>
<td>1.12</td>
</tr>
<tr>
<td>HCl2</td>
<td>1.75</td>
<td>RPS14</td>
<td>1.14</td>
<td>FAP211</td>
<td>2.32</td>
<td>FAP211</td>
<td>1.41</td>
<td>GAS31</td>
<td>1.93</td>
<td>GP2</td>
<td>1.46</td>
<td>GP2</td>
<td>1.32</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. S1. 2D-GE analysis of proteins in the intracellular-soluble and -insoluble fractions from a wall-less strain of *Chlamydomonas reinhardtii* CC-400 grown under various CO₂ conditions. A, SDS-PAGE of the cellular-soluble and -insoluble proteins stained with Quick-CBB. M indicates a molecular weight marker. The arrowhead indicates a band corresponding to the large subunit of Rubisco. B and C, 2D-GE profiles of the cellular-soluble and -insoluble proteins stained with Flamingo™ gel stain. Air, CO₂-1d and CO₂-3d represent samples (1), (2), and (3) from Fig. 2A, respectively.
Table 1 List of top 20 extracellular proteins aligned by its amount in air-acclimated cells.

<table>
<thead>
<tr>
<th>Ranking</th>
<th>Assigned name</th>
<th>JGI protein ID</th>
<th>Protein content in Air (mol%)</th>
<th>Air/CO₂-3d</th>
<th>SignalP</th>
<th>Function and/or similarities to known proteins</th>
<th>Grouping</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CAH1</td>
<td>24120 *</td>
<td>10.11 ± 2.84</td>
<td>5.85</td>
<td>S</td>
<td>Carbonic anhydrase1(CAH1), low-CO₂ inducible gene regulated by LCR1 [PMID: 15155888] and CCM1 [PMID: 11287669] high-CO₂ inducible, iron-deficiency inducible, periplasmic protein [PMID: 17660359]; Also known as H43 [PMID: 17202179]</td>
<td>CAH</td>
</tr>
<tr>
<td>2</td>
<td>H43/FEA1</td>
<td>129929</td>
<td>6.04 ± 5.50</td>
<td>0.23</td>
<td>S</td>
<td>high-CO₂ inducible, iron deficiency inducible, periplasmic protein [PMID: 17660359]; Also known as H43 [PMID: 17202179]</td>
<td>other</td>
</tr>
<tr>
<td>3</td>
<td>EXC1</td>
<td>191447</td>
<td>5.37 ± 2.59</td>
<td>1.43</td>
<td>S</td>
<td>No domain</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>GAP3</td>
<td>129019 *</td>
<td>4.95 ± 3.22</td>
<td>5.25</td>
<td>C</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase A</td>
<td>other</td>
</tr>
<tr>
<td>5</td>
<td>PHC21</td>
<td>93464</td>
<td>3.96 ± 2.25</td>
<td>-</td>
<td>C</td>
<td>pherophorin-C21 (PHC21) [PMID: 16367971]; similar to pherophorin-C21</td>
<td>PHC</td>
</tr>
<tr>
<td>6</td>
<td>EXC2</td>
<td>152521</td>
<td>3.49 ± 2.04</td>
<td>-</td>
<td>C</td>
<td>No domain</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>GP1</td>
<td>34358</td>
<td>2.09 ± 0.29</td>
<td>1.49</td>
<td>S</td>
<td>GP1 [CAL91937], hydroxyproline-rich glycoprotein [PMID: 15998802], similar to NSG1 [PMID: 15459796]</td>
<td>GP</td>
</tr>
<tr>
<td>8</td>
<td>FAP211</td>
<td>186474</td>
<td>1.90 ± 0.95</td>
<td>1.26</td>
<td>S</td>
<td>FAP211 [PMID: 15998802], similar to NSG1 [PMID: 15459796]</td>
<td>NSG</td>
</tr>
<tr>
<td>9</td>
<td>GP2</td>
<td>195768</td>
<td>1.79 ± 0.63</td>
<td>0.76</td>
<td>S</td>
<td>GP2 [CAL91937], hydroxyproline-rich glycoprotein [PMID: 15998802], similar to flagella-associated protein; NSG1 [PMID: 15459796]</td>
<td>NSG</td>
</tr>
<tr>
<td>10</td>
<td>HCI2</td>
<td>190800</td>
<td>1.75 ± 0.76</td>
<td>0.71</td>
<td>-</td>
<td>similar to flagella-associated protein; NSG1 [PMID: 15459796]</td>
<td>NSG</td>
</tr>
<tr>
<td>11</td>
<td>PCY1</td>
<td>185915</td>
<td>1.68 ± 1.23</td>
<td>1.41</td>
<td>M</td>
<td>pre-apoplasticin, PETE [PMID: 2165059; PMID: 8940133]</td>
<td>other</td>
</tr>
<tr>
<td>12</td>
<td>FAP102</td>
<td>191022</td>
<td>1.40 ± 1.14</td>
<td>0.40</td>
<td>S</td>
<td>FAP102 [PMID: 15998802], similar to GP3 [CAJ98661]</td>
<td>GP</td>
</tr>
<tr>
<td>13</td>
<td>LCI5</td>
<td>196466</td>
<td>1.37 ± 0.91</td>
<td>-</td>
<td>C</td>
<td>low-CO₂ inducible protein, regulated by CCM1 [PMID: 15235119]</td>
<td>other</td>
</tr>
<tr>
<td>14</td>
<td>CAH1/CAH2</td>
<td>24120; 128726 *</td>
<td>1.37 ± 0.18</td>
<td>2.29</td>
<td>S</td>
<td>Carbonic anhydrase1 (CAH1); Carbonic anhydrase 2 (CAH2), high-CO₂ inducible [PMID: 2124702]</td>
<td>CAH</td>
</tr>
<tr>
<td>15</td>
<td>FSD1</td>
<td>182933</td>
<td>1.32 ± 0.30</td>
<td>-</td>
<td>C</td>
<td>superoxide dismutase [Fe]</td>
<td>other</td>
</tr>
<tr>
<td>16</td>
<td>SEBP1</td>
<td>185196</td>
<td>1.15 ± 0.76</td>
<td>-</td>
<td>C</td>
<td>Sedoheptulose-1,7-bisphosphatase</td>
<td>other</td>
</tr>
<tr>
<td>17</td>
<td>GAS31</td>
<td>193780</td>
<td>1.07 ± 0.92</td>
<td>0.56</td>
<td>S</td>
<td>GAS31 [PMID: 16183845], belongs to the large pherophorin-family</td>
<td>GAS</td>
</tr>
<tr>
<td>18</td>
<td>HCI3</td>
<td>186476</td>
<td>1.00 ± 0.37</td>
<td>0.33</td>
<td>-</td>
<td>similar to flagella-associated protein; NSG1 [PMID: 15459796]</td>
<td>NSG</td>
</tr>
<tr>
<td>19</td>
<td>HCI1</td>
<td>115272</td>
<td>0.96 ± 0.09</td>
<td>0.71</td>
<td>S</td>
<td>similar to NSG1 [nitrogen-starved gametogenesis] protein [PMID: 15459796]</td>
<td>NSG</td>
</tr>
<tr>
<td>20</td>
<td>FAP212</td>
<td>186478</td>
<td>0.90 ± 0.02</td>
<td>0.54</td>
<td>S</td>
<td>FAP212 [PMID: 15998802], similar to NSG1 [PMID: 15459796]</td>
<td>NSG</td>
</tr>
</tbody>
</table>

*: Protein content was significantly (p<0.05) higher than that of CO₂-3d
Table 2 List of top 20 extracellular proteins aligned by its amount in 1-day-high-CO$_2$-acclimated cells.

<table>
<thead>
<tr>
<th>Ranking</th>
<th>Assigned name</th>
<th>JGI protein ID</th>
<th>Protein content in CO$_2$-1d (mol%)</th>
<th>CO$_2$-1d/Air</th>
<th>SignalP</th>
<th>Function and/or similarities to known proteins</th>
<th>Grouping</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H43/FEA1</td>
<td>129929 *</td>
<td>22.09 ± 8.16</td>
<td>3.66 S</td>
<td>S</td>
<td>high-CO$_2$ inducible, iron-deficiency inducible, periplasmic protein [PMID: 17660359]; Also known as H43 [PMID: 17202179]</td>
<td>other</td>
</tr>
<tr>
<td>2</td>
<td>EXC2</td>
<td>152521</td>
<td>4.92 ± 0.62</td>
<td>1.41 C</td>
<td>C</td>
<td>No domain</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>PHC21</td>
<td>93464</td>
<td>4.82 ± 0.97</td>
<td>1.22 C</td>
<td>C</td>
<td>pherophorin-C21 (PHC21) [PMID: 16367971]; similar to PHC</td>
<td>PHC</td>
</tr>
<tr>
<td>4</td>
<td>EXC1</td>
<td>191447</td>
<td>4.77 ± 1.39</td>
<td>0.89 S</td>
<td>S</td>
<td>No domain</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>HCI3</td>
<td>186476</td>
<td>3.43 ± 3.09</td>
<td>3.45 _</td>
<td>_</td>
<td>similar to flagella associated protein; NSG1 protein [PMID: 15459796]</td>
<td>NSG</td>
</tr>
<tr>
<td>6</td>
<td>HCI2</td>
<td>190800 *</td>
<td>3.36 ± 0.79</td>
<td>1.93 _</td>
<td>_</td>
<td>similar to flagella associated protein; NSG1 protein [PMID: 15459796]</td>
<td>NSG</td>
</tr>
<tr>
<td>7</td>
<td>FAP102</td>
<td>191022 *</td>
<td>3.16 ± 0.40</td>
<td>2.26 S</td>
<td>S</td>
<td>FAP102 [PMID: 15998802], similar to GP3 [CAJ98661]</td>
<td>GP</td>
</tr>
<tr>
<td>8</td>
<td>GP1</td>
<td>34358 *</td>
<td>3.06 ± 0.30</td>
<td>1.46 S</td>
<td>S</td>
<td>GP1 [CAL91937], hydroxyproline-rich glycoprotein [PMID: 15998802], similar to NSG1 [PMID: 15459796]</td>
<td>GP</td>
</tr>
<tr>
<td>9</td>
<td>GP2</td>
<td>195768</td>
<td>2.87 ± 0.79</td>
<td>1.60 S</td>
<td>S</td>
<td>GP2 [CAL91937], hydroxyproline-rich glycoprotein [PMID: 15998802], similar to NSG1 [PMID: 15459796]</td>
<td>NSG</td>
</tr>
<tr>
<td>10</td>
<td>FAP211</td>
<td>186474</td>
<td>2.32 ± 1.35</td>
<td>1.22 S</td>
<td>S</td>
<td>FAP211 [PMID: 15998802], similar to NSG1 [PMID: 15459796]</td>
<td>NSG</td>
</tr>
<tr>
<td>11</td>
<td>ISG-C1</td>
<td>178049 *</td>
<td>2.02 ± 0.44</td>
<td>3.57 S</td>
<td>S</td>
<td>similar to Volvox ISG [PMID: 1600938] and Chlamydomonas VSP-3 [PMID: 8000007]; Also known as FAP40 [PMID: 15459796]</td>
<td>ISG</td>
</tr>
<tr>
<td>12</td>
<td>HCI1</td>
<td>115272 *</td>
<td>1.99 ± 0.68</td>
<td>2.07 S</td>
<td>S</td>
<td>similar to NSG1 (nitrogen-starved gametogenesis) protein [PMID: 15459796]</td>
<td>NSG</td>
</tr>
<tr>
<td>13</td>
<td>FAP212</td>
<td>186478</td>
<td>1.57 ± 0.66</td>
<td>1.74 S</td>
<td>S</td>
<td>FAP212 [PMID: 15998802], similar to NSG1 [PMID: 15459796]</td>
<td>NSG</td>
</tr>
<tr>
<td>14</td>
<td>ISG-C4</td>
<td>185383 *</td>
<td>1.51 ± 0.62</td>
<td>- S</td>
<td>- S</td>
<td>similar to Volvox ISG [PMID: 1600938] and Chlamydomonas VSP-3 [PMID: 8000007]; Also known as FAP137 [PMID: 15459796]</td>
<td>ISG</td>
</tr>
<tr>
<td>15</td>
<td>GAS31</td>
<td>193780</td>
<td>1.41 ± 0.79</td>
<td>1.32 S</td>
<td>S</td>
<td>similar to CoMSIA (CAH1), low-CO$_2$ inducible gene regulated by LCR1 [PMID: 15155888] and CCM1 [PMID: 11287669]</td>
<td>GAS</td>
</tr>
<tr>
<td>16</td>
<td>CAH1</td>
<td>24120</td>
<td>1.41 ± 0.17</td>
<td>0.14 S</td>
<td>S</td>
<td>Carbonic anhydrase 1 (CAH1), belongs to the large pherophorin-family</td>
<td>CAH</td>
</tr>
<tr>
<td>17</td>
<td>GAP3</td>
<td>129019</td>
<td>0.99 ± 1.08</td>
<td>0.20 C</td>
<td>C</td>
<td>Glyceroldehyde 3-phosphate dehydrogenase A</td>
<td>other</td>
</tr>
<tr>
<td>18</td>
<td>PHC1</td>
<td>196399</td>
<td>0.95 ± 0.36</td>
<td>- S</td>
<td>- S</td>
<td>pherophorin-C1 (PHC1) [PMID: 16367971]; belongs to the large pherophorin-family</td>
<td>PHC</td>
</tr>
<tr>
<td>19</td>
<td>EXC3</td>
<td>166267</td>
<td>0.82 ± 0.13</td>
<td>1.13 S</td>
<td>S</td>
<td>Hypothetical protein containing a DUF3707; pherophorin domain</td>
<td>PHC</td>
</tr>
<tr>
<td>20</td>
<td>PCY1</td>
<td>185915</td>
<td>0.72 ± 0.35</td>
<td>0.43 M</td>
<td>M</td>
<td>pre-apoplastocyanin, PETE [PMID: 2165059; PMID: 8940133]</td>
<td>other</td>
</tr>
</tbody>
</table>

*: Protein content was significantly (p<0.05) higher than that of Air
<table>
<thead>
<tr>
<th>Ranking</th>
<th>Assigned name</th>
<th>JGI protein ID</th>
<th>Protein content in CO2-3d (mol%)</th>
<th>CO2-3d/Air</th>
<th>SignalP</th>
<th>Function and/or similarities to known proteins</th>
<th>Grouping</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H43/FEA1</td>
<td>129929 *</td>
<td>26.01 ± 4.30</td>
<td>4.31</td>
<td>S</td>
<td>high-CO2 inducible, iron-deficiency inducible, periplasmic protein [PMID: 17660359]; Also known as H43 [PMID: 17202179]</td>
<td>other</td>
</tr>
<tr>
<td>2</td>
<td>EXC1</td>
<td>191447</td>
<td>3.76 ± 2.61</td>
<td>0.70</td>
<td>S</td>
<td>No domain</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>FAP102</td>
<td>191022 *</td>
<td>3.48 ± 0.63</td>
<td>2.48</td>
<td>S</td>
<td>FAP102 [PMID: 15998802], similar to GP3 [CAJ98661]</td>
<td>GP</td>
</tr>
<tr>
<td>4</td>
<td>HCI3</td>
<td>186476 *</td>
<td>3.02 ± 1.52</td>
<td>3.03</td>
<td>-</td>
<td>similar to flagella associated protein; NSG1protein [PMID: 15459796] similar to Volvox ISG [PMID: 1600938] and Chlamydomonas VSP-3 [PMID: 8000007]; Also known as FAP40 [PMID: 15459796] similar to flagella associated protein; NSG1protein [PMID: 15459796]</td>
<td>NSG</td>
</tr>
<tr>
<td>5</td>
<td>ISG-C1</td>
<td>178049 *</td>
<td>2.46 ± 0.70</td>
<td>4.36</td>
<td>S</td>
<td>similar to Volvox ISG [PMID: 15459796] and Chlamydomonas VSP-3 [PMID: 8000007]; Also known as FAP40 [PMID: 15459796] similar to flagella associated protein; NSG1protein [PMID: 15459796]</td>
<td>ISG</td>
</tr>
<tr>
<td>6</td>
<td>HCI2</td>
<td>190800 *</td>
<td>2.46 ± 0.27</td>
<td>1.41</td>
<td>-</td>
<td>GP2[CAL91937], hydroxyproline-rich glycoprotein [PMID: 15459796]; similar to Volvox ISG [PMID: 1600938] and Chlamydomonas VSP-3 [PMID: 8000007]</td>
<td>GP</td>
</tr>
<tr>
<td>7</td>
<td>GP2</td>
<td>195768</td>
<td>2.36 ± 0.49</td>
<td>1.32</td>
<td>S</td>
<td>similar to Volvox ISG [PMID: 1600938] and Chlamydomonas VSP-3 [PMID: 8000007]</td>
<td>other</td>
</tr>
<tr>
<td>8</td>
<td>ISG-C2</td>
<td>193727 *</td>
<td>2.27 ± 0.75</td>
<td>-</td>
<td>S</td>
<td>similar to Volvox ISG [PMID: 1600938] and Chlamydomonas VSP-3 [PMID: 8000007]</td>
<td>ISG</td>
</tr>
<tr>
<td>9</td>
<td>GAS31</td>
<td>193780</td>
<td>1.93 ± 0.52</td>
<td>1.80</td>
<td>S</td>
<td>GAS31[PMID: 16183845], belongs to the large pherophorin-family</td>
<td>GAS</td>
</tr>
<tr>
<td>10</td>
<td>CAH1</td>
<td>24120</td>
<td>1.73 ± 0.24</td>
<td>0.17</td>
<td>S</td>
<td>Carbonic anhydrase1(CAH1), low-CO2 inducible gene regulated by LCR1 [PMID: 15155888] and CCM1 [PMID: 11287669]</td>
<td>CAH</td>
</tr>
<tr>
<td>11</td>
<td>FAP103</td>
<td>58944</td>
<td>1.69 ± 1.10</td>
<td>-</td>
<td>_</td>
<td>Flagellar Associated Protein similar to ncleoside diphosphate kinase, found in the flagellar proteome [PMID: 15998802]</td>
<td>other</td>
</tr>
<tr>
<td>12</td>
<td>FAP212</td>
<td>186478 *</td>
<td>1.66 ± 0.34</td>
<td>1.85</td>
<td>S</td>
<td>FAP212 [PMID: 15998802], similar to NSG1[PMID: 15459796]</td>
<td>NSG</td>
</tr>
<tr>
<td>13</td>
<td>PHC15</td>
<td>148333</td>
<td>1.54 ± 0.87</td>
<td>-</td>
<td>S</td>
<td>pherophorin-C15(CAH15) [PMID: 16367971]; similar to FAP211 [PMID: 15998802], similar to NSG1[PMID: 15459796]</td>
<td>PHC</td>
</tr>
<tr>
<td>14</td>
<td>FAP211</td>
<td>186474</td>
<td>1.51 ± 0.23</td>
<td>0.79</td>
<td>S</td>
<td>similar to Volvox ISG [PMID: 1600938] and Chlamydomonas VSP-3 [PMID: 8000007]; Also known as FAP137 [PMID: 15459796]</td>
<td>NSG</td>
</tr>
<tr>
<td>15</td>
<td>ISG-C4</td>
<td>185383</td>
<td>1.47 ± 0.24</td>
<td>-</td>
<td>S</td>
<td>GP1[CAL91937], hydroxyproline-rich glycoprotein [PMID: 15459796]; similar to NSG1(nitrogen-starved gametogenesis) protein [PMID: 15459796]</td>
<td>ISG</td>
</tr>
<tr>
<td>16</td>
<td>GP1</td>
<td>34358</td>
<td>1.40 ± 0.43</td>
<td>0.67</td>
<td>S</td>
<td>similar to GP3 [CAJ98661]</td>
<td>GP</td>
</tr>
<tr>
<td>17</td>
<td>HCI1</td>
<td>115272</td>
<td>1.36 ± 0.73</td>
<td>1.41</td>
<td>S</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase A</td>
<td>other</td>
</tr>
<tr>
<td>18</td>
<td>PCY1</td>
<td>185915</td>
<td>1.19 ± 0.50</td>
<td>0.71</td>
<td>M</td>
<td>pre-apoplastocyanin, PETE [PMID: 2165059;PMID: 8940133]</td>
<td>other</td>
</tr>
<tr>
<td>19</td>
<td>HCI4</td>
<td>157979 *</td>
<td>0.98 ± 0.59</td>
<td>0.71</td>
<td>C</td>
<td>similar to GP3 [CAJ98661]</td>
<td>GP</td>
</tr>
<tr>
<td>20</td>
<td>GAP3</td>
<td>129019</td>
<td>0.94 ± 0.16</td>
<td>0.19</td>
<td>C</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase A</td>
<td>other</td>
</tr>
</tbody>
</table>

*: Protein content was significantly(p<0.05) higher than that of Air
<table>
<thead>
<tr>
<th>Table S1. List of extracellular proteins in Air-, CO2-1d, and CO2-3d acclimated cells.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protein content (mol%)</strong></td>
</tr>
<tr>
<td>----------------------------</td>
</tr>
<tr>
<td>1.938 ± 0.030</td>
</tr>
<tr>
<td>0.417 ± 0.027</td>
</tr>
<tr>
<td>-</td>
</tr>
<tr>
<td>0.500 ± 0.030</td>
</tr>
<tr>
<td>0.361 ± 0.023</td>
</tr>
<tr>
<td>-</td>
</tr>
<tr>
<td>-</td>
</tr>
<tr>
<td>-</td>
</tr>
<tr>
<td>0.549 ± 0.030</td>
</tr>
<tr>
<td>1.747 ± 0.756</td>
</tr>
<tr>
<td>0.179 ± 0.145</td>
</tr>
<tr>
<td>0.265 ± 0.355</td>
</tr>
<tr>
<td>-</td>
</tr>
<tr>
<td>-</td>
</tr>
<tr>
<td>-</td>
</tr>
<tr>
<td>-</td>
</tr>
<tr>
<td>-</td>
</tr>
<tr>
<td>1.100 ± 0.118</td>
</tr>
<tr>
<td>0.397 ± 0.576</td>
</tr>
<tr>
<td>0.099 ± 0.027</td>
</tr>
<tr>
<td>-</td>
</tr>
<tr>
<td>0.397 ± 0.576</td>
</tr>
<tr>
<td>6.042 ± 5.501</td>
</tr>
<tr>
<td>1.366 ± 0.179</td>
</tr>
<tr>
<td>2.682 ± 1.150</td>
</tr>
<tr>
<td>3.963 ± 2.249</td>
</tr>
<tr>
<td>0.241 ± 0.155</td>
</tr>
<tr>
<td>4.712 ± 1.100</td>
</tr>
<tr>
<td>-</td>
</tr>
<tr>
<td>10.109 ± 2.841</td>
</tr>
<tr>
<td>0.066 ± 0.027</td>
</tr>
<tr>
<td>0.763 ± 0.418</td>
</tr>
</tbody>
</table>