New Phytologist

Alcohol dehydrogenase and hydrogenase transcript fluctuations during a day–night cycle in *Chlamydomonas reinhardtii*: the role of anoxia

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Received: *29 July 2010*
Accepted: *2 September 2010*

New Phytologist (2011) **190**: 488–498
doi: 10.1111/j.1469-8137.2010.03503.x

Key words: alcohol dehydrogenase, anoxia, *Chlamydomonas reinhardtii,* day–night cycle, hydrogenases.

Summary

• The unicellular green alga *Chlamydomonas reinhardtii* contains two iron (Fe)-hydrogenases which are responsible for hydrogen production under anoxia. In the present work the patterns of expression of alcohol dehydrogenase, a typical anaerobic gene in plants, of the hydrogenases genes (*HYD1*, *HYD2*) and of the genes responsible for their maturation (*HYDEF*, *HYDG*), were analysed.

• The expression patterns were analysed by real-time reverse-transcription polymerase chain reaction in *Chlamydomonas* cultures during the day–night cycle, as well as in response to oxygen availability.

• The results indicated that *ADH1*, *HYD1*, *HYD2*, *HYDEF* and *HYDG* were expressed following precise day–night fluctuations. *ADH1* and *HYD2* were modulated by the day–night cycle. Low oxygen plays an important role for the induction of *HYD1*, *HYDEF* and *HYDG*, while *ADH1* and *HYD2* expression was relatively insensitive to oxygen availability.

• The regulation of the anaerobic gene expression in *Chlamydomonas* is only partly explained by responses to anoxia. The cell cycle and light–dark cycles are equally important elements in the regulatory network modulating the anaerobic response in *Chlamydomonas*.

Introduction

The unicellular green alga *Chlamydomonas reinhardtii* (referred to here as *Chlamydomonas* throughout) has been used for years as a model organism to study a variety of cell processes, including photosynthesis, cell division and flagellar function (Rochaix, 1995; Harris, 2001a).

Hydrogen production in *Chlamydomonas* was reported nearly 70 yr ago, (Gaffron & Rubin, 1942) although it has only been the subject of significant attention by scientists for the past decade. (Melis *et al.*, 2000). Its ability to synthesize hydrogen, if a way were found to harness it, could potentially revolutionize the renewable energies market (Lee *et al.*, 2010). An advantage of hydrogen over other alternatives to fossil fuels is that the final product of its combustion is water rather than CO_2, and therefore it does not negatively contribute to climate change (Lee *et al.*, 2010).

Chlamydomonas is an aerobic organism, although it may experience hypoxia on a daily basis depending on the environment. *Chlamydomonas* contains two iron (Fe)-hydrogenase encoding genes, usually found with strict anaerobes, allowing hydrogen production under anoxic conditions (Happe & Kaminski, 2002; Forestier *et al.*, 2003; Mus *et al.*, 2007). Anoxia is required because the hydrogenase enzyme catalytic site is inactivated by oxygen and so does not function in an oxygenic environment (Ghirardi *et al.*, 2007; Stripp *et al.*, 2009).

Chlamydomonas must therefore be in anoxia as a precondition to activate hydrogen production (Happe *et al.*, 1994; Ghirardi *et al.*, 2007), but it is likely that it can survive anoxia only for limited periods. Cells may be grown in anoxia in sealed containers either in the dark, to avoid photosynthetic oxygen generation (dark anoxia), or in the light, provided that photosynthesis somehow be reduced to a level low enough so that all the oxygen it produces is consumed by cellular respiration (light anoxia) (Melis, 2007). This latter approach may be carried out by starving cells of sulfur (Melis *et al.*, 2000). In dark anoxia all electrons for H_2

synthesis come from the fermentation of organic substrates accumulated in the cell.

Under anoxia, cells potentially confront an energy crisis, as O_2, their favored final electron acceptor, is not available. In such conditions, NAD(P)H deriving from respiration cannot be reoxidized and the whole process is blocked (Greenway & Gibbs, 2003). It has been proposed that H_2 synthesis may act as a back-up mechanism to sequester electrons permitting NAD(P)H re-oxidation and the generation of ATP, which is essential for cell maintenance and repair functions, and ultimately for survival (Melis *et al.*, 2000; Happe & Kaminski, 2002).

In other systems, such as Arabidopsis, acclimatization to anoxia comprises whole array of metabolic adaptations, including the induction of alcohol dehydrogenase (ADH) (Ellis *et al.*, 1999) whose role is to reoxidize NADH produced in the glycolytic pathway (Perata & Alpi, 1993). Together, they determine the outcome: survival or death (Licausi & Perata, 2009).

The *Chlamydomonas* genome sequence (available online at http://genome.jgi-psf.org/Chlre4/Chlre4.home.html) contains a significant number of genes encoding proteins involved in anaerobic metabolism (Grossman *et al.*, 2007; Merchant *et al.*, 2007). Networks of pathways that ferment pyruvate derived from starch terminate in the production of a range of metabolites (heterofermentation) including acetate, ethanol, formate and small amounts of malate, CO_2 and H_2 (Kreuzberg, 1984; Mus *et al.*, 2007). The ratio of fermentation products may change with culture conditions and the interruption of one of these main pathways may activate additional alternative pathways (Gfeller & Gibbs, 1984; Kosourov *et al.*, 2003). A mutant (*hydEF-1*) defective in hydrogenase activity activates an otherwise non functioning pathway terminating in the production of succinate, seemingly to compensate for the loss of the ability to reduce protons to H_2 as final electron acceptor (Posewitz *et al.*, 2004; Dubini *et al.*, 2009). While these fermentative pathways have been identified, some questions remain regarding the individual role and contribution of each pathway as well as factors involved in their regulation.

The *ADH* gene, encoding alcohol dehydrogenase, has been largely associated with the hypoxia/anoxia response in the plant kingdom, although it may also play a role in other environmental stress, including cold, dehydration and salinity (Dolferus *et al.*, 1994; Dennis *et al.*, 2000; Ismond *et al.*, 2003; Senthil-Kumar *et al.*, 2010). Most plant species upregulate *ADH* in response to low oxygen, although exceptions have been reported (Kennedy *et al.*, 1992). *ADH* is necessary to survive flooding in *Arabidopsis thaliana*, as highlighted by the decreased tolerance in an *ADH*-null mutant, although over-expressing *ADH* does not increase tolerance (Ismond *et al.*, 2003).

In *Chlamydomonas* ADH1 catalyses the conversion of acetyl-CoA into ethanol, and the presence of an ADH able to convert acetaldehyde into ethanol, thus resembling plant ADH, has been hypothesized (Mus *et al.*, 2007).

In *Chlamydomonas* the expression of the hydrogenase genes (*HYD*) has similarly been studied. Two hydrogenase genes were characterized in *Chlamydomonas*: *HYD1* and *HYD2* (Happe & Kaminski, 2002; Forestier *et al.*, 2003). They show 68% identity in their amino acid sequence (Forestier *et al.*, 2003). Both *HYD1* and *HYD2* are upregulated in response to dark anoxia (Mus *et al.*, 2007), and to light anoxia in sulfur starvation (Forestier *et al.*, 2003). In addition, *Chlamydomonas* possesses two hydrogenase maturation genes, *HYDEF* and *HYDG*, whose proteins are essential for constructing the metal core present in the active site of the hydrogenase themselves (Posewitz *et al.*, 2004).

Chlamydomonas, like most other organisms (Moore-Ede *et al.*, 1982; Johnson & Golden, 1999) shows temporal organization of its behavioral, physiological and biochemical processes to adapt them to the 24-h cycle of its environment (Takahashi, 1991; Dunlap, 1999). For example, its division has been observed to coincide with the night period, and the survival of *Chlamydomonas* cells after irradiation by UV light depended heavily on the time of day the treatment was carried out (Spudich & Sager, 1980; Nikaido & Johnson, 2000).

Evolution wise, an organism may posses a competitive advantage if its cellular physiology were organized such that oxygen sensitive reactions could be restricted to times when photosynthesis does not occur (e.g. the night). In this way energy would not be wasted in synthesizing proteins that cannot be active when oxygen or light is present. (Johnson & Golden, 1999; Nikaido & Johnson, 2000). In this context, it would be logical to expect that some phases of the daily cycle are more favorable to hydrogen production than others. During the day, *Chlamydomonas* photosynthesizes and produces its own oxygen but at night photosynthetic oxygen production ceases and whatever oxygen is dissolved in the water may quickly be consumed. This, together with the slow rate of diffusion of oxygen (from the atmosphere in this case) in water, makes it possible that *Chlamydomonas* experiences a few hours of hypoxia, or even anoxia on a daily basis. An experiment performed on a synchronous culture of wild-type strain CC124 following anoxic induction by sulfur starvation demonstrated that the amount of hydrogen produced varies according to the time of day the treatment starts (Tsygankov *et al.*, 2002).

Industrial-scale H_2 production, will likely involve growing *Chlamydomonas* outdoors and subject to day–night rhythms. In this context it is important to take cell cycle factors into consideration in scientific studies. In this article, we demonstrate that ability to survive dark anoxia depends on the time of day treatment starts. We further show that C*hlamydomonas ADH1*, *HYD1*, *HYD2*, *HYDEF* and *HYDG* expression displays a day–night fluctuation pattern, which can be only partly explained by oxygen availability in the medium.

Materials and Methods

Strains and growth conditions

The *C. reinhardtii* 11-32c wild-type strain was obtained from the algae collection of the Gottingen University, Germany. Cells were grown in Tris acetate-phosphate (TAP) medium (pH 7.2) as described by Harris (2001b). Cell suspensions were grown to a concentration of 1×10^6 cells ml^{-1} (7×10^5 cells ml^{-1} for the anoxia tolerance experiment). Synchronous *Chlamydomonas* cell cultures were obtained by alternating light (12 h) and dark (12 h) periods for 6 d and maintained by daily dilution of the cultures to a starting density of *c.* 10^6 cells ml^{-1}. Cell division was monitored microscopically and by counting cells with a Bürker chamber. The light intensity during the light period was 70 μmol m^{-2} s^{-1}. During 48-h experiments, samples were collected every 4 h and corresponding volume of fresh media added. For the continuous darkness or light experiments, the culture was divided and one part was transferred to continuous darkness, one part was transferred to continuous light at the intensity given earlier and one part was left in a 12 h : 12 h light : dark photoperiod. For experiments done on agar plates TAP agar 1.2% was used.

Anoxic treatments were carried out in the dark. An enclosed anaerobic workstation (Anaerobic System model 1025; Forma Scientific, Marietta, OH, USA) was used to provide an oxygen-free environment in which to incubate *Chlamydomonas* cultures. This chamber uses palladium catalyst wafers and desiccant wafers to maintain strict anaerobiosis to < 10 μg ml^{-1} O_2 (according to the manufacturer's specifications). High-purity N_2 was used to initially purge the chamber, and the working anaerobic gas mixture was N_2 : H_2 with a ratio of 90 : 10.

The experiments described in Fig. 9 were performed by continuously fluxing 1% oxygen or air (21% oxygen) in the flasks containing the cultures in a 12 h : 12 h light : dark photoperiod. Oxygen at 1% was used to induce hypoxic conditions to simulate the naturally low oxygen status of the culture medium during the night. No gas was fluxed in the control flask.

Oxygen readings

A synchronized culture prepared as described earlier was assayed every 4 h using a Jenway oxygen meter model 9071 (Jenway, Stone, UK) following the manufacturer instructions.

RNA extraction and real-time reverse-transcription polymerase chain reaction (qPCR)

Cultures of *Chlamydomonas* 1×10^6 were pelleted at 4000 ***g*** for 1 min. The pellet was resuspended in the following buffer diluted 1 : 1 with water: 2% SDS, 400 mM EDTA, 100 mM Tris-HCl (pH 8.0). The resulting solution was extracted once with phenol-chloroform 1 : 1 (v/v) supplemented with sodium-acetate 0.3M (pH 5.0). The samples were vortexed briefly and centrifuged at 12 000 ***g*** for 10 min. The supernatant was extracted twice with phenol–chloroform (without sodium acetate), then extracted a final time in chloroform. Samples were precipitated using LiCl 8M added 1 : 1 (v : v) for 4 h at 4°C, centrifuged at 13 000 ***g*** and the pellet washed in 70% ethanol and finally resuspended in DEPC water. RNA quality was checked on 1% agarose gel, and quantified with spectrophotometric readings. RNA was subjected to a DNase treatment using a TURBO DNA free kit (Ambion, Inc., Austin, TX, USA). One microgram of each sample was reverse transcribed into cDNA with an iScript cDNA Synthesis kit (Bio-Rad). Real-time reverse-transcription polymerase chain reaction amplification was carried out with an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA), using the ribosomal protein L13 (*RPL13*) RNA as an endogenous control. The primers used are listed in the Supporting Information, Table S1. A cDNA pool of all samples was analysed for a standard dilution series to monitor the qPCR efficiency for each primer pair. The PCR reactions were carried out using 40 ng of cDNA and iQ SYBR Green SuperMix (Bio-Rad Laboratories Inc.) following the manufacturer's protocol. Relative quantification of each mRNA was performed using the comparative CT method, as described by the manufacturer (ABI PRISM 7700 Sequence Detection System User Bulletin #2; Applied Biosystems).

Results

Anoxia tolerance is influenced by the time of day

We suspected that *Chlamydomonas* cells would display a different response to anoxia at different times of the day, thus suggesting a time-dependent change in the ability to adjust the metabolism to anaerobic conditions. *Chlamydomonas* resistance to UV treatment indeed varied according to time of day (Nikaido & Johnson, 2000).

We used a culture of synchronized *Chlamydomonas* cells which, at four time-points, were exposed to dark anoxia for 3, 6 and 9 d. It is evident that the cells that were exposed to anoxia starting at 04:00 h survived the best, and the time of day the experiment started seems to be of importance for survival from anoxia (Fig. 1).

The concentration of O_2 dissolved in the media varies rhythmically over 24 h

We considered that the differential response to anoxia according to the time of the day could arise from a transient

acclimatization to low oxygen linked to a daily fluctuation of the oxygen content in the culture medium. The O_2 concentration in the medium was measured every 4 h for 48 h. As expected, the O_2 concentration during the night was much lower than during the day (Fig. 2a). The O_2 concentration began to rise at the beginning of the day, as soon as light became available and photosynthesis begins. The maximum O_2 concentration was measured at 12:00 h, and then decreased slowly until 20:00 h. As soon as the lights switched off, O_2 concentration dropped significantly and remained constantly low between 00:00 h and 08:00 h. This pattern was repeated over 48 h (Fig. 2a).

ADH1 expression in *Chlamydomonas* depends on time of day, not on O_2 concentration

Higher expression of *ADH* is often linked with survival in low oxygen conditions in plants (Perata & Alpi, 1993). We therefore checked whether *ADH* expression correlated with the anoxia survival of *Chlamydomonas* cells (Fig. 1). *ADH1* expression was determined every 4 h for 48 h (Fig. 2b, top panel). Surprisingly, *ADH1* was most highly expressed at 16:00 (black arrows) when oxygen levels in the media were high (Fig. 2a), and was least expressed at night when oxygen levels were low. This result contrasts with the common wisdom that *ADH* genes, including *Chlamydomonas ADH1*, are induced by low oxygen (Mus *et al.*, 2007).

To further investigate our idea that *ADH1* may be responding to light (or dark) signals rather than to oxygen availability, we carried out an experiment to see how *ADH1* responds to continuous darkness or light conditions. In continuous darkness, *ADH1* continued to show an afternoon peak (Fig. 2b, middle panel, black arrows), although at a much lower level. Generally, therefore, in continuous darkness, *ADH1* continued to fluctuate in a circadian manner, although darkness attenuated the afternoon peak of expression. In continuous light, during the first 12 h *ADH1* expression followed its regular pattern with a peak at 16:00 h (Fig. 2b, bottom panel, black arrow), as expected because light conditions during the day are normal. As soon as the cells were exposed to unexpected light (from 20:00 h onwards), they showed a change in the way they expressed *ADH1*, which no longer follows a regular pattern. These results suggest that *ADH1* in *Chlamydomonas* may be under circadian regulation, although continuous light disrupts the cycle of *ADH1* expression. Unlike with higher plants, the expression level of *ADH* did not correlate with anoxia tolerance (Fig. 1).

Hydrogenase genes in *Chlamydomonas* display a circadian expression pattern

The activity of hydrogenases may allow *Chlamydomonas* to reoxidize the excess NAD(P)H under anoxia thus enhancing

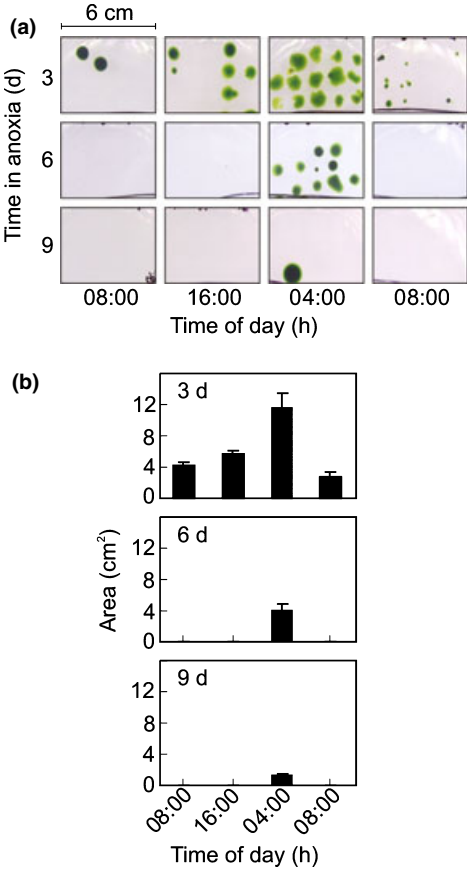

Fig. 1 Effect of anoxia on the survival of *Chlamydomonas* cells. (a) At four chosen time points (08:00 h, 16:00 h, and 04:00 h and 08:00 h the following day), 15 spots, each equal to 2 μl of culture were spotted on to Tris acetate-phosphate (TAP) agar plates. The plates were then transferred to the anoxic chamber and kept in the dark for 3, 6 and 9 d. At the end of the treatment, the plates were transferred back to normoxia to recover. The cells spotted onto the agar grew to form colonies. They were grown for 25 d, although no additional growth was observed after 20 d. (b) Total surface area of the 15 colonies at each time-point was measured (data are mean ± SD, n = 3 individual plates, each containing 15 colonies).

cell survival and, indeed, the expression of *HYD* genes is known to be favored under oxygen limitation (Mus *et al.*, 2007). Expression of *HYD1* showed a significant rise in expression during the early part of the night (Fig. 3a, black arrows), when oxygen level decreased, while during the day, *HYD1* was expressed at a much lower level.

Under continuous darkness, *HYD1* expression level fluctuated (Fig. 3b). Upon the sensing of unexpected dark, the expression level increased to create a peak during the virtual 'day' (Fig. 3b, white arrows). This is especially noticeable between 08:00 h and 12:00 h on the first day of unexpected dark.

Under continuous light, *HYD1* showed its usual low expression for the first 12 h (Fig 3c). At 20:00 h, *HYD1* expression began to rise as usual, but the sensing of unexpected light

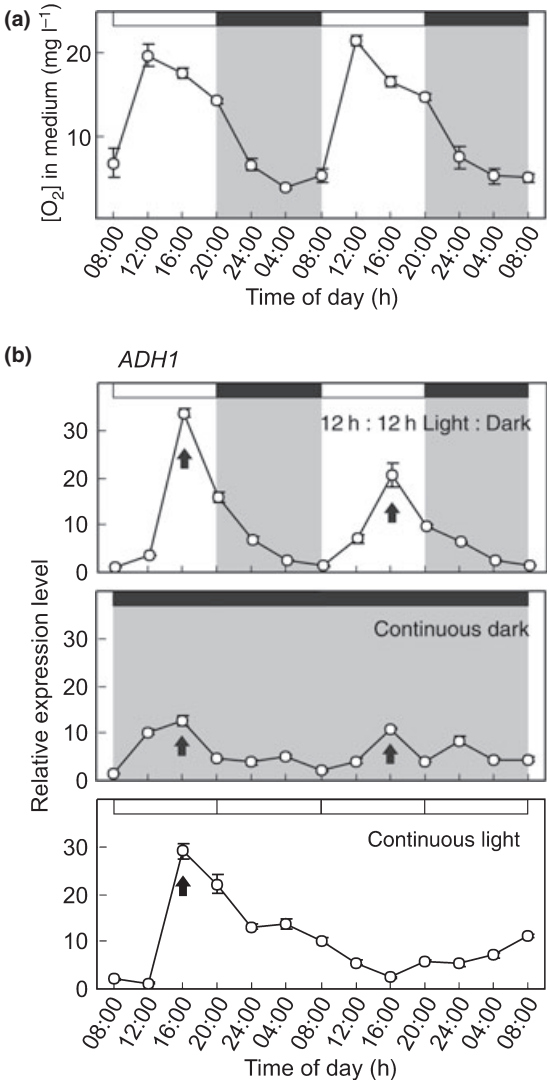

Fig. 2 Effect of day–night cycle on oxygen level and patterns of expression of *ADH1* in *Chlamydomonas* cells. (a) Oxygen in the media was measured every 4 h for 48 h in a culture of synchronized cells maintained in photoperiodic conditions. (b) mRNA levels for *ADH1* were measured in synchronized cells during photoperiod (top panel), continuous darkness (middle panel) and continuous light (bottom panel). Relative expression levels were measured by real-time reverse-transcription polymerase chain reaction (qPCR) (1 = the lowest value of expression measured in the three conditions). Data are mean ± SD, n = 3 technical replicates. When not shown, the error bars here smaller than the symbols. The experiment was replicated three times. A representative experiment depicting two consecutive days is shown.

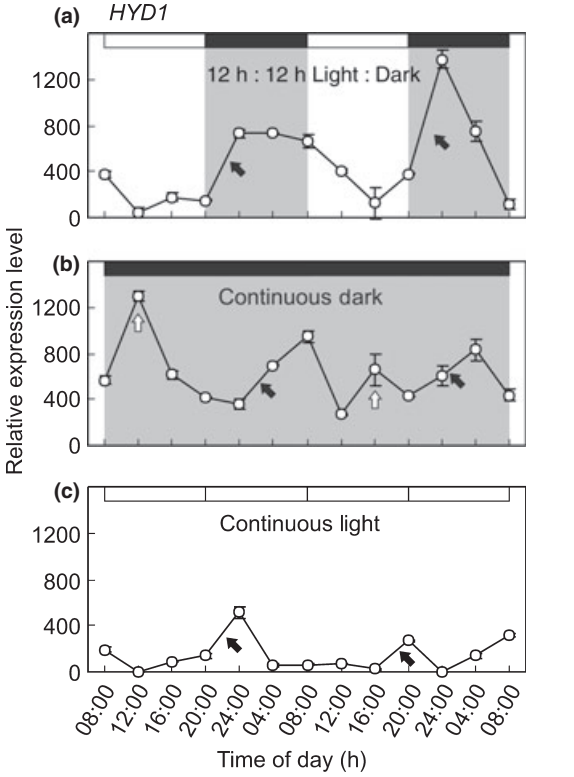

Fig. 3 Patterns of expression of *HYD1* in *Chlamydomonas* cells. (a) mRNA levels for *HYD1* were measured in synchronized cells during photoperiod (a), continuous darkness (b) and continuous light (c). Relative expression levels were measured by real-time reverse-transcription polymerase chain reaction (qPCR) (1 = the lowest value of expression measured in the three conditions). Data are mean ± SD, n = 3 technical replicates. When not shown, the error bars were smaller than the symbols. The experiment was replicated three times. A representative experiment depicting two consecutive days is shown.

quickly prevents this rise. Another small peak was also visible at the onset of the virtual night on the second day. The period between expression peaks was maintained close to 24 h.

The expression pattern of *HYD2* under photoperiodic conditions, showed a peak of expression at 16:00 h (Fig. 4a, black arrows), a point in time where oxygen levels are high, proving that induction of this gene is not strictly correlated to oxygen status, as would have been expected because of the extreme oxygen sensitivity of its protein (Stripp *et al.*, 2009). *HYD2* expression, however, decreased only slightly until 20:00 h, and then remained constant throughout the night (Fig. 4a).

Under continuous darkness, an accentuation of the 16:00 h *HYD2* peak was observed, which was stronger on the first day (Fig. 4b, black arrows).

Under continuous light, only the expected peak of expression during the first day was retained (Fig. 4c, black arrow). The loss of fluctuation in *HYD2* expression when under continuous light conditions (Fig. 4c) suggests that, in addition to the circadian clock, other factors might also affect the expression of this gene.

The hydrogenase proteins require HYDEF and HYDG maturation proteins to be activated (Posewitz *et al.*, 2004). Both *HYDEF* and *HYDG* showed higher expression during the night along the day–night cycle (Fig. 5, top panels in a

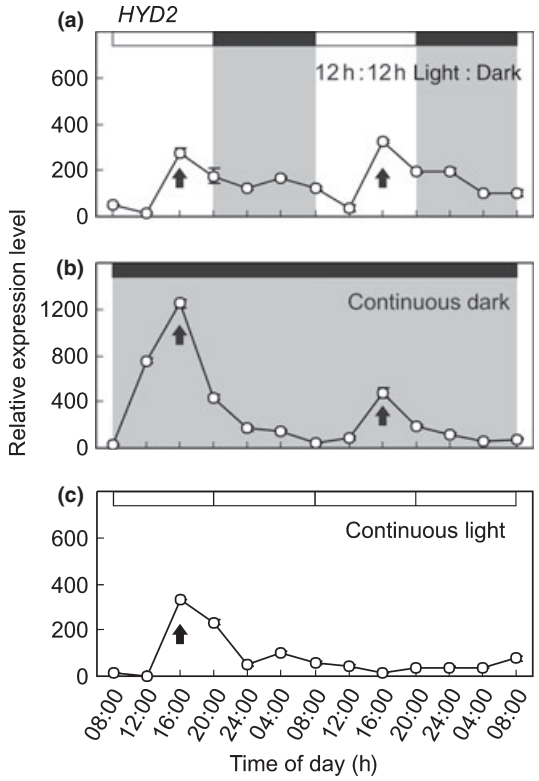

Fig. 4 Patterns of expression of *HYD2* in *Chlamydomonas* cells. (a) mRNA levels for *HYD2* were measured in synchronized cells during photoperiod (a), continuous darkness (b) and continuous light (c). Relative expression levels were measured by real-time reverse-transcription polymerase chain reaction (qPCR) (1 = the lowest value of expression measured in the three conditions). Data are mean ± SD, n = 3 technical replicates. When not shown, the error bars were smaller than the symbols. The experiment was replicated three times. A representative experiment depicting two consecutive days is shown.

and b) and the unexpected darkness at the beginning of the dark treatment strongly enhanced their expression (Fig. 5, middle panels in a and b). The darkness-related peaks of expression of *HYDEF* and *HYDG* were lost when *Chlamydomonas* was grown under continuous light (Fig. 5, bottom panels in a and b).

Effects of continuous light on circadian and cell-cycle related genes

The genes studied in this work displayed a day–night fluctuation in their expression. Most of them retained an apparently circadian pattern of expression under continuous darkness but only *HYD1* behaved in this way under continuous light (Fig. 3c). This suggested that either the circadian clock or the cell cycle were disrupted by the continuous light conditions used.

We found that the expression pattern of *tufA*, a circadian gene (Fig. 6a; Hwang *et al.*, 1996), retained its circadian

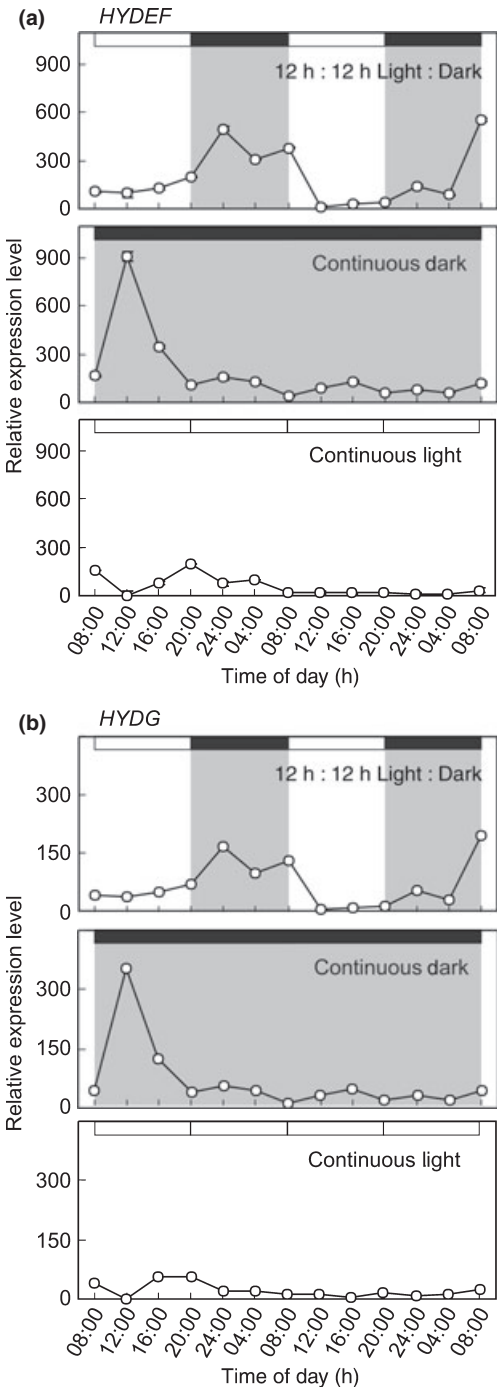

Fig. 5 Patterns of expression of *HYDEF* and *HYDG* in *Chlamydomonas* cells. mRNA levels for (a) *HYDEF* and (b) *HYDG* were measured in synchronized cells during photoperiod (top panel), continuous darkness (middle panel) and continuous light (bottom panel). Relative expression levels were measured by real-time reverse-transcription polymerase chain reaction (qPCR) (1 = the lowest value of expression measured in the three conditions). Data are mean ± SD, n = 3 technical replicates. When not shown, the error bars were smaller than the symbols. The experiment was replicated three times. A representative experiment depicting two consecutive days is shown.

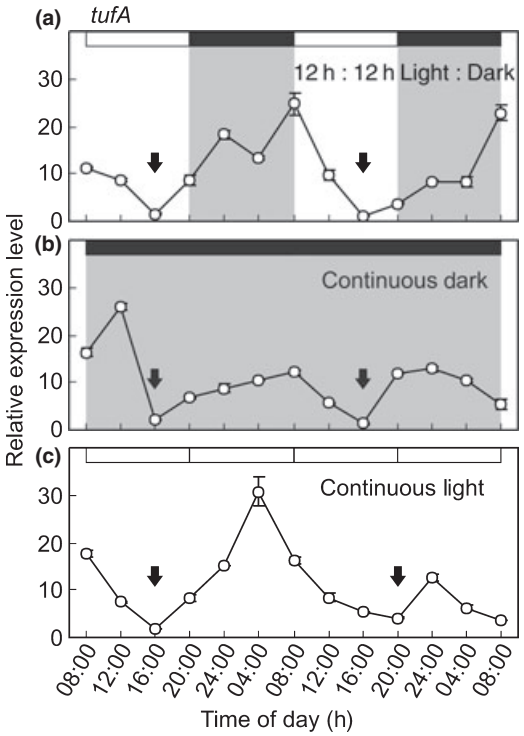

Fig. 6 Patterns of expression of *tufA* in *Chlamydomonas* cells. mRNA levels for *tufA* were measured in synchronized cells during photoperiod (a), continuous darkness (b) and continuous light (c). Relative expression levels were measured by real-time reverse-transcription polymerase chain reaction (qPCR) (1 = the lowest value of expression measured in the three conditions). Data are mean ± SD, n = 3 technical replicates. When not shown, the error bars were smaller than the symbols. The experiment was replicated three times. A representative experiment depicting two consecutive days is shown.

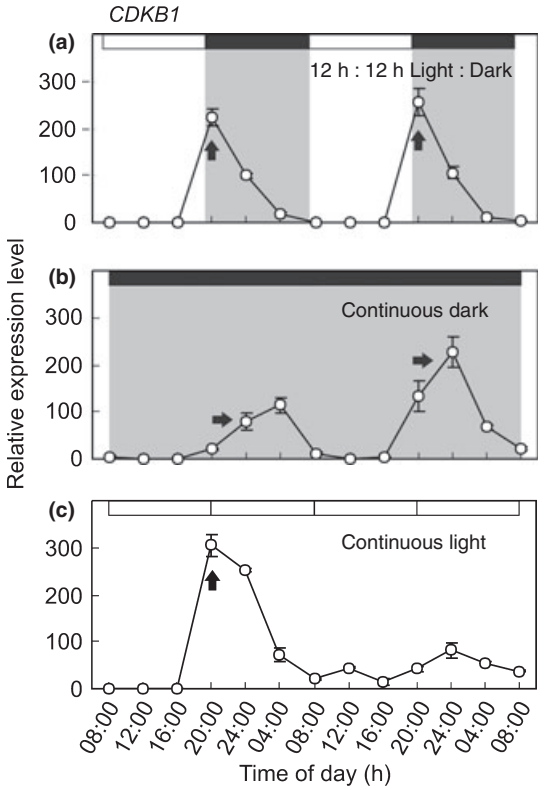

Fig. 7 Patterns of expression of *CDKB1* in *Chlamydomonas* cells. mRNA levels for *CDKB1* were measured in synchronized cells during photoperiod (a), continuous darkness (b) and continuous light (c). Relative expression levels were measured by real-time reverse-transcription polymerase chain reaction (qPCR) (1 = the lowest value of expression measured in the three conditions). Data are mean ± SD, n = 3 technical replicates. When not shown, the error bars were smaller than the symbols. The experiment was replicated three times. A representative experiment depicting two consecutive days is shown.

pattern even under continuous light, similarly to the expression pattern of *HYD1* (cf. Figs 3, 6c). While the 24-h period is maintained under continuous darkness, the sensing of unexpected dark triggered a temporary enhancement in the expression of *tufA* (Fig. 6b), as was observed for *HYD1* (Fig. 3b). These results suggest that the circadian clock was not altered by the continuous light conditions.

Continuous light may alter the cell cycle, indirectly influencing expression of genes whose regulation is possibly linked to cell division and growth. To test this hypothesis, *CDKB1*, a gene expressed only during the S/M phase of the cell cycle (Bisova *et al.*, 2005) was examined over 48 h (Fig. 7).

In normal conditions of alternating dark and light (Fig. 7a), *CDKB1* showed a peak in expression at 20:00 h, at the onset of the dark phase (black arrows). Its expression was reduced at 00:00 h, and was virtually zero at all other time-points. In continuous darkness, the *CDKB1* pattern was not only maintained, but its peaks in expression became higher during the second cycle (Fig. 7b). Under continuous darkness the *CDKB1* expression peak was delayed, occurring 8 h later during the first cycle, and 4 h later during the next cycle, as indicated by the black arrows (Fig. 7b).

The regular expression pattern of *CDKB1* was disrupted by continuous light, and only the first peak of expression is retained during the first day (Fig. 7c, black arrow).

HYD1, *HYD2*, *HYDEF*, *HYDG*, but not *ADH1* are induced by anoxia

The day–night fluctuation in the expression of genes can result from the oxygen concentration fluctuations (Fig. 2a), and indeed *HYD1*, *HYDEF*, and *HYDG* behaved as expected in relation to the oxygen content in the medium (Figs 3–5).

We verified the responsiveness of *ADH1* to 4 h of dark anoxia at different times of day (Fig. 8). Interestingly, no *ADH1* induction was visible that differentiated anoxia-treated cells (open bars in Fig. 8) from those treated in air in the dark (closed bars in Fig. 8). Shorter anoxic treatments

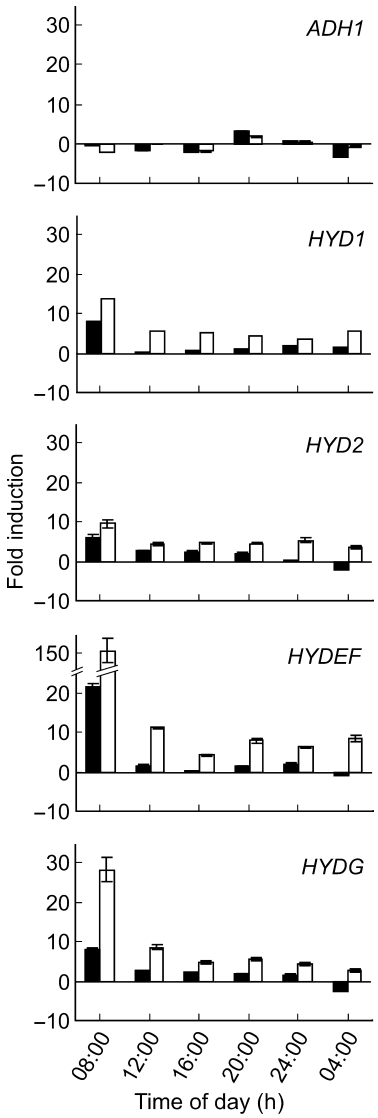

Fig. 8 Effect of anoxia on the expression level of *ADH1*, *HYD1*, *HYD2, HYDEF*, *HYDG* in *Chlamydomonas* cells. mRNA levels for *ADH1*, *HYD1*, *HYD2*, *HYDEF*, *HYDG* were measured in synchronized cells treated for 4 h in dark under aerobic conditions (closed bars) or in dark under anoxia (open bars) at different time of day. Relative expression levels were measured by real-time reverse-transcription polymerase chain reaction (qPCR) (1 = expression value measured after 4 h in photoperiod in air). Data are mean ± SD, n = 3 technical replicates. When not shown, the error bars where smaller than the symbols. The experiment was replicated three times. A representative experiment is shown.

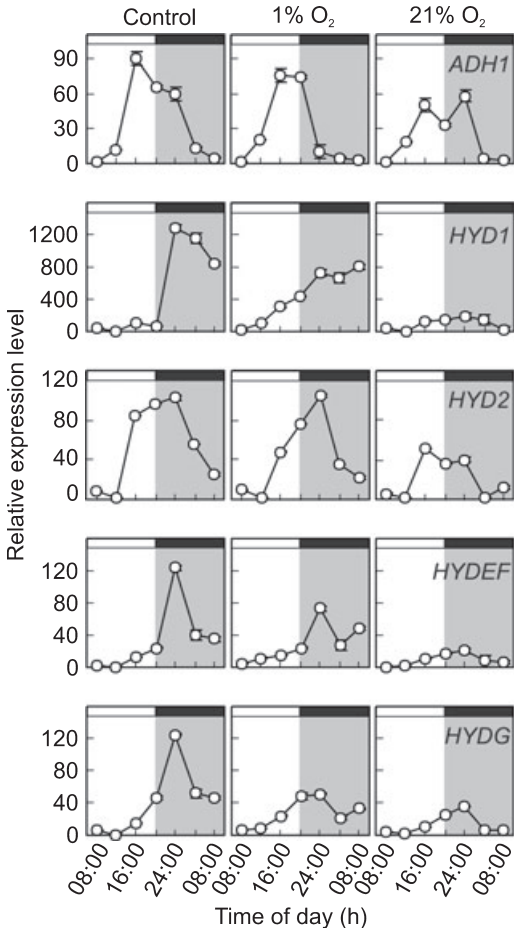

Fig. 9 Effect of oxygen on the expression level of *ADH1*, *HYD1*, *HYD2*, *HYDEF*, *HYDG* in *Chlamydomonas* cells. mRNA levels for *ADH1*, *HYD1*, *HYD2*, *HYDEF*, *HYDG* were measured in synchronized cells. Treatments were as follows: 'Control', untreated; 1%O_2, culture treated by bubbling 1%O_2; 21%O_2, culture treated by bubbling air. Relative expression levels were measured by real-time reverse-transcription polymerase chain reaction (qPCR) (1 = the lowest value of expression measured in the three conditions). Data are mean ± SD, n = 3 technical replicates. When not shown, the error bars where smaller than the symbols. The experiment was replicated three times. A representative experiment is shown.

(0.5–4 h) were also unable to significantly induce *ADH1* (data not shown).

HYD1, however, was induced by anoxia at all times of day (Fig. 8). Dark treatment in normoxia did increase *HYD1*, but the effect of anoxia was stronger (Fig. 8). *HYD2* induction by anoxia was limited to a twofold induction, when compared with the effect of dark normoxia. Only at the 24:00 h and 4:00 time points, was it significantly more induced (Fig. 8). *HYDEF* and *HYDG* were induced by 4 h of anoxia, and were induced to a lesser extent by normoxia in the dark (Fig. 8). *HYDEF* and *HYDG* showed a tendency to be induced to a higher level when the beginning of the light phase was expected (time: 08:00 h), likely a response to the unexpected darkness (see Fig. 5, middle panels in a and b).

The expression of the genes that were analysed in this study may be divided into two different categories. The first includes *HYD1*, *HYDEF* and *HYDG*, which were predominantly expressed during the night (Figs 3–5) and are inducible by anoxia (Fig. 8). The second includes *ADH1* and

HYD2, whose expression increased during the day (Figs 2, 4), suggesting that the oxygen level in the medium played a minor role in their regulation, although *HYD2* was slightly induced by anoxia during the night (Fig. 8). To verify whether oxygen might modulate the expression of *HYD1*, *HYDEF* and *HYDG* but not that of *ADH1* and *HYD2*, we attempted to change the expression of these genes by artificially altering the oxygen status of the culture medium. Two cultures, one fluxed with 1% oxygen and one fluxed with air (21% oxygen) were compared with a control culture in which no gas was fluxed. The expression pattern of *ADH1* and *HYD2* were unchanged by 1% O_2, while fluxing 21% O_2 slightly reduced their expression (Fig. 9).

The 21% oxygen treatment had a dramatic effect on the expression of *HYD1*, *HYDEF* and *HYDG*, whose expression was strongly reduced by the treatment (Fig. 9).

When cultures were fluxed with 1% O_2, the expression of *HYD1* was not enhanced as usual when the cultures were exposed to darkness (cf. 'Control' and '1% O_2' in Fig. 9), suggesting that exposure to both darkness and a drop in oxygen concentration (Fig. 2a) rather than exposure to hypoxia alone was responsible for the up-regulation of these genes during the night.

Discussion

Chlamydomonas possesses a variety of fermentative pathways, which are activated when oxygen is not available (Mus *et al.*, 2007). The hydrogenase genes themselves, encoding the proteins responsible for hydrogen production, are part of anaerobic pathways (Mus *et al.*, 2007).

In this work, we demonstrated that the expression of anaerobic genes fluctuates during the day–night cycles, in line with the different tolerance to low oxygen displayed by cultures exposed to anoxia at different times of the day. The most obvious explanation for a day–night fluctuation of genes reported to be induced by low oxygen (Mus *et al.*, 2007) is related to oxygen availability. Photosynthetic O_2 generation varies over the 24-h period and photosynthesis itself is reported to be under circadian regulation in higher plants (Dodd *et al.*, 2005; Fukushima *et al.*, 2009).

The O_2 levels in the medium of synchronous cultures of *Chlamydomonas* were, as expected, high during the day and low at night (Fig. 2a). However, counter-intuitively, *ADH1* expression was high during the day when oxygen is high (Fig. 2b, top panel), while its expression was actually lower in continuous darkness (Fig. 2b, middle panel), when no photosynthesis occurred and dissolved O_2 levels were quite low (Fig. 2a).

One explanation could be that *ADH1* is not hypoxia responsive in *Chlamydomonas*. Indeed, we did not observe *ADH1* induction in response to dark anoxia (Fig. 8), and increasing oxygen availability did not inhibit *ADH1* expression (Fig. 9). Upregulation of *ADH1* by anoxia was observed in *Chlamydomonas* by Mus *et al.* (2007), but the different experimental set-up may explain the different conclusions reached in our work.

The function of *ADH1* in *Chlamydomonas*, when uncoupled from its otherwise obvious role in the hypoxic metabolism, remains obscure. Ethanol production through the action of ADH recycles NADH and allows ATP production through glycolysis to continue in absence of oxygen. For this reason ADH is important for survival under anoxia (Perata & Alpi, 1993; Gibbs & Greenway, 2003). In *Chlamydomonas* the picture might be different, as ethanol and H_2 production pathways may compete for reductants during anoxia. Following anoxia induction by sulfur starvation in the light, ethanol production is inhibited when H_2 production is maximized, possibly because reductants from starch may be preferentially used by NAD(P)H-PQ oxidoreductase to fuel H_2 production (Kosourov *et al.*, 2003). The expression of *ADH1* might therefore be downregulated during the night (Fig. 2b) to avoid the competition for reducing agents required for the action of hydrogenases.

Overall, the hydrogenases (*HYD1* and *HYD2*) and hydrogenase maturation genes (*HYDEF*, and *HYDG*) (Fig. 8) displayed a pattern of expression that is consistent with hydrogen production during dark-induced anaerobiosis as reported by Mus *et al.* (2007).

ADH1 and *HYD2* deregulation in continuous light showed that the dark phase is essential to maintain their daily pattern (Fig. 2b, bottom panel).

Deregulation of *ADH1* and *HYD2* in continuous light could be a direct response to light conditions, or an indirect response to a light-dependent disruption of the cell cycle. Under continuous light the circadian rhythm seemed to be maintained, as demonstrated by the *tufA* gene (Fig. 4), leaving the cell cycle hypothesis viable.

The green alga *Ostreococcus* was reported to modify its cell division rhythms in response to light conditions (Moulager *et al.*, 2007). Interrupted cell synchrony in continuous light was also observed in *Chlamydomonas* (Hwang *et al.*, 1996). Interestingly, *CDKB1*, a cell cycle marker gene (Bisova *et al.*, 2005), was deregulated in continuous light, whereas it maintained a regular oscillation in continuous darkness (Fig. 6b,c). These results suggested that the regulation of *ADH1* and of *HYD2*, could be linked, at least in part, to the cell cycle. This hypothesis certainly deserves further investigation.

In conclusion, our results demonstrated that fermentative genes are expressed following precise day–night fluctuations. The regulation of the anaerobic metabolism of *Chlamydomonas* can only be partly explained by responses to anoxia, but the cell cycle and light–dark cycles are equally important elements in the regulatory network modulating the anaerobic response in *Chlamydomonas*. Intriguingly, the regulation and metabolic role of *ADH1* in *Chlamydomonas* is apparently not explained by our knowledge of the

fermentative metabolism in higher plants and further work is warranted on this topic.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 List of primers for real-time PCR analysis

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