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Technical Communication

A truncated antenna mutant of *Chlamydomonas reinhardtii* can produce more hydrogen than the parental strain

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ABSTRACT

Photoproduction of H_2 gas was examined in the *Chlamydomonas reinhardtii tla1* strain, CC-4169, containing a truncated light-harvesting antenna, along with its parental CC-425 strain. Although enhanced photosynthetic performance of truncated antenna algae has been demonstrated previously (Polle et al. Planta 2003; 217:49–59), improved H_2 photoproduction has yet to be reported. Preliminary experiments showed that sulfur-deprived, suspension cultures of the *tla1* mutant could not establish anaerobiosis in a photobioreactor, and thus, could not photoproduce H_2 gas under conditions typical for the sulfur-deprived wild-type cells (Kosourov et al. Biotech Bioeng 2002; 78:731–40). However, they did produce H_2 gas when deprived of sulfur and phosphorus after immobilization within thin (~300 μm) alginate films. These films were monitored for long-term H_2 photoproduction activity under light intensities ranging from 19 to 350 μE m^{-2} s^{-1} PAR. Both the *tla1* mutant and the CC-425 parental strain produced H_2 gas for over 250 h under all light conditions tested. Relative to the parental strain, the CC-4169 mutant had lower maximum specific rates of H_2 production at low and medium light intensities (19 and 184 μE m^{-2} s^{-1}), but it exhibited a 4-times higher maximum specific rate at 285 μE m^{-2} s^{-1} and an 8.5-times higher rate at 350 μE m^{-2} s^{-1} when immobilized at approximately the same cell density as the parental strain. As a result, the CC-4169 strain accumulated almost 4-times more H_2 than CC-425 at 285 μE m^{-2} s^{-1} and over 6-times more at 350 μE m^{-2} s^{-1} during 250-h experiments. These results are the first demonstration that truncating light-harvesting antennae in algal cells can increase the efficiency of H_2 photoproduction in mass culture at high light intensity.

1. Introduction

The green alga, *Chlamydomonas reinhardtii*, is a common model organism used to study photobiological H_2 production. Algal H_2 production is catalyzed by a reversible [Fe-Fe]-hydrogenase enzyme that is maximally expressed after a period of 1–4 h of dark, anaerobic adaptation [1,2]. The algal [Fe-Fe]-hydrogenase accepts electrons from reduced ferredoxin, the terminal acceptor of the photosynthetic electron-transport chain, and reduces protons to molecular H_2 [3]. The rate of the reaction in healthy algal cells is very high [4], but *in vivo* H_2 photoproduction cannot be sustained due to rapid

Abbreviations: Chl, chlorophyll; PAR, photosynthetic active radiation; PSII, photosystem II; TAP, TRIS-acetate-phosphate.

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inactivation of the enzyme by O_2 co-evolved during photosynthesis [5]. Sustained H_2 photoproduction in algae is only possible when photosystem II (PSII)-driven, O_2-evolving activity is partly inactivated either by sulfur-deprivation [6,7] or by other methods that manipulate the expression level of the PSII D1 reaction center protein [8]. However, by reducing photosynthetic electron transport activity, the algal cultures evolve H_2 gas at rates that are only a fraction of the total potential capacity of the organism [9]. Recent technoeconomic analyses [10,11] suggested that such a process is not currently attractive commercially, although when optimized, it may yield H_2 gas a cost of $3.00 to $6.00/kg (1 kg $H_2 \approx$ the energy content of 1 gallon of gasoline) depending on the exact technology examined. Recent efforts have focused on a number of approaches to optimize the two-stage, sulfur-deprivation system for improved H_2-production rates [12–17].

Another important factor precluding the practical application of algae for H_2 production is their low light utilization efficiency in mass culture [18–21]. Algae possess large light-harvesting Chl antennae that allow them to absorb photons efficiently at low light intensity. However, under high (up to solar) levels of light, the rate of light absorption exceeds that of photosynthetic reductant utilization, and the antennae Chls dissipate up to 80% or more of the incident energy as fluorescence and heat [19]. As a consequence, the cells near the surface of a pond or a photobioreactor waste light energy and in addition shade the cells located deeper in the suspension. The shading can be significant in dense cultures, and as a result, overall light utilization efficiency by the culture is low. The problem can be addressed in part by cultivating or immobilizing algae and other phototrophs in thin layers [17,22,23]. Another approach, though, is to find or generate algal mutants with small chlorophyll antenna sizes (i.e. low numbers of total Chls per reaction center). The rate of photosynthesis in algal cultures with truncated antennae is expected to saturate at much higher light intensity than in organisms containing a WT antenna size, with up to 460 Chls (a+b) per PSII reaction center [18]. This type of mutant should decrease wasteful dissipation of excitation energy in mass culture. Recently, a few algal mutants of *C. reinhardtii* with truncated Chl antennae were generated and characterized [20,24]. They indeed showed promise in increasing light utilization efficiency and overall productivity in mass cultures [19,20]; however, improved H_2 production was never demonstrated.

In the present work, we have investigated the effect of the truncation of about 50% of the photosynthetic light-harvesting antennae on long-term H_2 photoproduction in *C. reinhardtii*. The results reported in this study prove conclusively the hypothesis that truncating light-harvesting antennae in algal cells not only improves the photosynthetic productivity in mass cultures, but also increases the efficiency of H_2 photoproduction per unit of illuminated culture surface area under high light intensity for long periods of time.

2. Materials and methods

The *tla1* mutant, CC-4169 (*tla1 cw15 sr-u-2-60 mt*$^+$) with truncated antennae and the arginine-dependent CC-425 (*arg2 cw15 sr-u-2-60 mt*$^+$) parental strain were obtained from the Chlamydomonas Center. The CC-4169 strain was isolated from a DNA-insertional mutagenesis library [20]. The antenna mutant and the parental CC-425 strain were pre-grown for three days photomixotrophically under continuous illumination from one side (~110 μE m^{-2} s^{-1} PAR) on TAP medium [25] supplemented with 500 μg ml^{-1} arginine. Mature algal cultures were then harvested by centrifugation, washed with TAP-minus-sulfur or TAP-minus-sulfur-minus-phosphorus media to remove sulfate, and re-suspended in the same medium, as required. All nutrient-deprived media were also supplemented with 500 μg ml^{-1} arginine. Sulfur-deprived-only cells were used in suspension cultures experiments, as described before [12]. In these experiments, the initial Chl concentrations varied from 9 to 12 μg ml^{-1} in the mutant cultures and from 18 to 22 μg ml^{-1} in CC-425 cultures. Sulfur/phosphorus-deprived cells were immobilized in Ca^{2+}-alginate-film strips according to the protocol developed by Kosourov and Seibert [17]. In this case, all algae/alginate formulations were made based on the same fresh cell weight (1 g per 1 ml of 4% alginate), which resulted in approximately the same volumetric cell density but different amounts of Chl in each strip, as demonstrated in Table 1. After fabrication, the alginate film strips (6-cm^2) were transferred into 75-ml vials containing 10 ml of TAP-minus-sulfur-minus-phosphorus medium supplemented with arginine. The vials were then sparged with argon for 20 min and placed under different light intensities, ranging from 19 to 350 μE m^{-2} s^{-1}. The latter was the highest intensity that we were able to obtain from compact fluorescent lamps (25 W, Feit Electric, Inc., Pico Rivera, CA), placed at a distance that did not cause excessive heating of the cultures. Two fans were also used to cool the lamps and cultures on a continuous basis. The H_2 concentration in the vials was monitored by gas chromatography (7890A GC system, Agilent Technologies, Inc., Santa Clara, CA) once a day. All experiments were repeated six to nine times.

Table 1 – Conversion effeciencies of incident light energy into energy in the H_2 produced by algae entrapped in alginate film strips.

Strain	Total Chl concentration, μg per film	The efficiencies[a] of light energy conversion to H_2 energy (%) at different light intensities (μE m^{-2} s^{-1})			
		19	184	285	350
CC-425	421 ± 36	0.40 ± 0.15	0.07 ± 0.02	0.02 ± 0.02	0.007 ± 0.009
CC-4169	232 ± 64	0.07 ± 0.01	0.03 ± 0.02	0.08 ± 0.04	0.06 ± 0.03

a The effeciencies were calculated for the total amount of H_2 produced over 240 h of nutrient deprivation under different light intensities.

Maximum specific rates (Fig. 2) were calculated based on initial Chl concentrations.

The efficiency of light energy conversion to H_2 energy was calculated using the following equation:

$$\eta(\%) = (100\ (\Delta G^{o} - R\ T\ \ln\ (P^{o}/P))\ R_H) / E_S\ A,$$

where ΔG^{o} is the standard Gibbs free energy of H_2 (237,200 J mol^{-1} at 25 °C), R is the universal gas constant in J K^{-1} mol^{-1}, T is the absolute temperature in °K, P^{o} and P are the standard and observed H_2 pressures (atm), R_H is the rate of H_2 photoproduction (mol s^{-1}), E_S is the energy of the incident light radiation in J (averaged over 400–700 nm; 1 μmol = 0.214 J), and A is the illuminated surface area in m^2.

3. Results and discussion

Sulfur deprivation causes the reversible inhibition of PSII-dependent, O_2-evolving activity in *C. reinhardtii* cells without significantly affecting cellular respiration [6,7]. As a consequence, algal cultures become anaerobic in the light within 20–30 h when cultivated in a sealed photobioreactor. The transition to anaerobiosis depends significantly on the cell density inside the reactor, and we have shown that denser cell suspensions become anaerobic more quickly than dilute cultures due to active respiration of cells inside the shaded area of the photobioreactor [12]. However, under typical sulfur-deprived conditions [12] at the Chl concentrations described in the Materials and Methods, we have observed that sulfur-deprived suspension cultures of the *tla1* antenna mutant do not become anaerobic (data not shown) in a photobioreactor. Hence, while the mutant strain did not produce any H_2 gas, the parental CC-425 strain evolved up to 30 ml H_2 gas per liter of the culture (data not shown). The absence of a transition to anaerobiosis in the CC-4169 *tla1* strain is most probably the result of the low Chl concentration in the mutant suspension culture (see Table 1), which resulted in less shading and higher effective O_2 production even under sulfur-deprived conditions. It is possible of course that a *tla1* mutant can establish anaerobiosis and produce H_2 gas at twice the cell (Chl) concentrations in a photobioreactor. However, the increase in Chl concentration would double the volumetric cell density of the mutant culture compared to the parental strain and consequently would not permit us to examine the underlying hypothesis posed in this investigation.

To demonstrate H_2 production by the antenna mutant, we immobilized sulfur/phosphorus-deprived algal cultures within thin alginate matrices. Phosphorus deprivation in addition to sulfur deprivation was necessary to stabilize the Ca^{2+}-alginate films, as discussed previously [17,26]. Alginate immobilization provided the means to further increase cell density above what was possible in suspension cultures. Indeed, immobilization significantly increases the volumetric cell density of the algae (up to 2000 μg Chl per ml of matrix as observed previously in alginate films with entrapped wild-type CC-124 cells [17]). Moreover, we demonstrated recently that alginate-entrapped, nutrient-deprived *C. reinhardtii* cultures photoproduce H_2 gas even when O_2 was present in the headspace of a photobioreactor [17]. Therefore, our expectation was to see H_2 production in the sulfur/phosphorus-deprived antenna mutant, even if the cultures could not establish full anaerobiosis in the photobioreactor.

In fact, both the *tla1* mutant and its parental CC-425 strain started to photoproduce H_2 gas soon after immobilized sulfur/phosphorus-deprived cells were purged with argon (Fig. 1). All cultures were able to sustain H_2 production in the light for about 250 h, and the behavior of the mutant under nutrient-deprived conditions mimicked the behavior of the parental (Fig. 1) as well as that of the wild-type CC-124 strain [17]. As

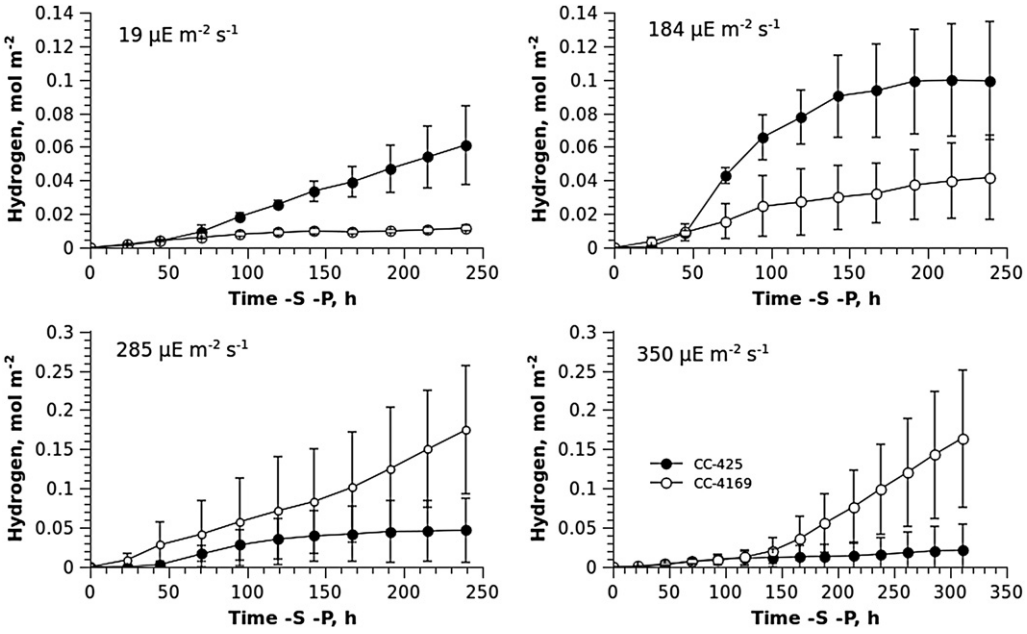

Fig. 1 – The effect of light intensity on H_2 photoproduction by the immobilized, sulfur/phosphorus-deprived *tla1* antenna mutant (CC-4169) and the parental strain (CC-425). Each curve represents an average of four to eight independent experiments.

shown in Fig. 1, the *tla1* mutant exhibited lower overall H_2-photoproduction activity than the parental strain under low illumination. At 19 μE m^{-2} s^{-1}, H_2 yields in the mutant cultures were less than half those observed in the parental strain. Since the light-harvesting antennae in this mutant are about 50% as large as those of the parental strain, and hence, capture photon energy at approximately half the rate of the parental strain on a per cell basis, the mutant algae indeed should exhibit decreased photosynthetic activity under light-limiting conditions when cultivated at lower Chl concentration (Figs. 1 and 2). An increase in the light intensity from 19 to 184 μE m^{-2} s^{-1} slightly improved the rate of H_2 production in the CC-4169 mutant, but the H_2-production yield was still significantly lower than in the CC-425 strain (Fig. 1). A further increase in the light intensity to 285 μE m^{-2} s^{-1} significantly improved both the yield and rate of H_2 photoproduction in the CC-4169 strain (Figs. 1 and 2). At this intensity, which is known to be saturating for the WT strain [19], the maximum specific rate by the CC-4169 culture was about 4-times higher than in the parental CC-425 strain (Fig. 2), which is what would be expected for a mutant with a smaller antenna size that had not yet reached light saturation. Moreover, the CC-425 strain experienced significant photoinhibition at 350 μE m^{-2} s^{-1}, as demonstrated by the lower yield and specific rate of H_2 production compared to those observed at lower light intensities. A decrease in the H_2-production activity under high light was also observed for the wild-type CC-124 strain both in suspension [27] and immobilized (data not shown) cultures. This is to be expected, since nutrient-deprived algae cannot efficiently synthesize proteins and, hence, cannot effectively repair their photosynthetic apparatus, especially under high light [28,29]. The CC-4169 strain was also subject to photoinhibition, but the effect was less pronounced (Fig. 2).

Table 1 shows the conversion efficiencies of incident light energy into H_2 energy by the algal cultures over the 240-h period of nutrient deprivation. Both strains showed decreased efficiency as a function of light intensity. However, the decay in efficiency with light intensity was less pronounced in the *tla1* mutant, as expected. The CC-4169 strain also exhibited an 8.5-fold higher efficiency than the parental CC-425 strain under the highest light condition tested, which is still well below solar irradiation levels. Furthermore, the experiments performed in our laboratory showed that the parental CC-425 strain had low H_2 photoproduction yields when compared to the wild-type CC-124 strain. The latter has been the strain of choice in many previous sulfur-deprivation experiments. Under approximately the same light intensity (17 vs. 19 μE m^{-2} s^{-1}), the maximum specific rate of H_2 production in the CC-124 culture was almost 4-times higher than in CC-425 (2.97 vs. 0.75 μmol mg^{-1} Chl h^{-1}, respectively). The reasons for the low H_2 production activity in the CC-425 strain are not clear. However, the low activity in the parental strain can significantly diminish the effect of the *tla1* mutation on H_2 production in the daughter strain. Indeed, one of the most important functions of the [Fe-Fe]-hydrogenase in algal cells is the restoration of linear electron flow under anaerobic conditions, when most components of the photosynthetic electron-transport chain are over-reduced [30]. Since low hydrogenase activity in anaerobic cells may increase 'back-pressure' on the photosynthetic apparatus under high light conditions, especially in nutrient-deprived cells, photoinhibition, the condition that we indeed observed in our experiments, is an expected outcome. Therefore, for better understanding of the role of the light-harvesting antenna size in H_2 photoproduction, we suggest transferring the *tla1* phenotype into strains more stable to photoinhibition and that demonstrate high H_2-production activities.

In summary, this work is the first report of improved H_2 photoproduction in an algal, truncated antenna mutant. As originally hypothesized, the strain affected in the *TLA1* gene demonstrates increasing yields and efficiencies of H_2 photoproduction with increasing the light intensity in caparison to the parental strain. As such, improved *tla1*-type mutants may show promise for future biotechnology applications, if the accompanying photoinhibition response can be addressed.

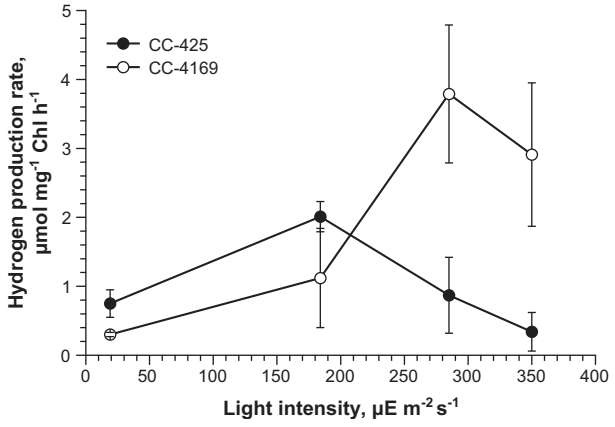

Fig. 2 – Changes in the maximum specific rate of H_2 production in the immobilized *tla1* antenna mutant (CC-4169) and the immobilized parental strain (CC-425) under different light intensities. Values are means ± SD of four to eight independent experiments. Since all individual kinetic curves have slightly different shapes, the maximum specific rates were calculated for each curve independently.

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