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Overcoming the expression barrier of the ferredoxin-hydrogenase chimera in *Chlamydomonas reinhardtii* supports a linear increment in photosynthetic hydrogen output

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ABSTRACT

While the prospect of producing hydrogen from photosynthetic microalgae has long been described as one of the promising directions towards achieving a renewable fuel source, current endeavors towards this goal face serious limitations including the inefficient electron supply to hydrogenase and the enzyme's sensitivity to molecular oxygen. In this work we express our ferredoxin-hydrogenase (fd-hyd) fusion enzyme in a hydrogenase knockout *Chlamydomonas reinhardtii* mutant and compare its hydrogen production traits to those of wild-type strains. We found that the active enzyme abundance in both systems is in linear correlation with photosynthetic hydrogen production, thus establishing that protein abundance is an additional important bottleneck in the process of hydrogen photo-production. We report here the isolation of two clones with high expression of fd-hyd; OP68 and R2D2, created by either nuclear or chloroplast transformation, respectively. The study of these clones shows that fd-hyd's roughly 4.5-fold higher H_2 production efficiency, compared to the native hydrogenase, is maintained in high expression regimes. By comparing a strain's active enzyme pool to its total protein amount, we observed that the engineered clones harbor a large non-mature enzyme pool, indicating that natural maturation of the fd-hyd saturates at a lower level than the native hydrogenase. Subsequently, we measured mRNA levels by quantitative PCR and observed that while the two clones express roughly the same amount of protein, R2D2 has considerably more mRNA. Thus improving transcription rates in nuclear transformants or translation efficiency in chloroplast transformants are both potential routes towards further increasing protein abundance. Finally, we show that these clones are able to continuously produce H_2 in sealed bioreactors for five days, significantly outcompeting their parental wild-type strain.

1. Introduction

Utilizing the microalgal machinery for gaseous H_2 production is one of the most promising directions towards sustainable large-scale production of this commodity. The potential of such algal H_2 production systems lays in the ability of algae to rapidly self-replicate while consuming sunlight, CO_2 and basic salts alone. Thus, an algal-based H_2 production farm would be relatively economical to set up and maintain, including in developing countries [1–6].

In *Chlamydomonas reinhardtii* - a model green alga - a portion of the photosynthetic electron flux is used to feed the hydrogenase enzyme which in turn catalyzes proton reduction into H_2. Hydrogenase is a special metalloenzyme which is naturally coded in the nucleus and imported into the chloroplast, where it undergoes maturation [7, 8]. Hydrogenase maturation requires the activity of three maturases: hydE, hydF and hydG (in *C. reinhardtii* hydE and hydF are fused and coded in a single gene hydEF), which are responsible for incorporating the catalytic H cluster in the hydrogenase protein scaffold [9–11].

Two well described limitations hinder the scaling of microalgal hydrogen production: (i) the enzyme's sensitivity to O_2 [12–15] and (ii) its poor ability to compete against alternative downstream pathways [16, 17]. Several notable endeavors tackling the enzyme's O_2 sensitivity have been reported; Li et al. have co-cultured algae with bacteria which consume oxygen from the medium while leaving the algal growth rate

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intact and thus supporting more efficient H_2 production [18]. From a different angle, Melis et al. have developed a novel approach for obtaining prolonged H_2 production; in this system algal O_2 evolution is inactivated by sulfur deprivation, resulting in continuous H_2 production [19].

In attempt to tilt the competition scale towards hydrogenase, several important works aimed at lowering the competition abilities of hydrogenase's adversaries were reported. For example, Happe et al. engineered a synthetic ferredoxin with reduced affinity towards Ferredoxin-$NADP^+$ reductase (FNR) while keeping its affinity to hydrogenase intact [20]. A different approach for maiming the same target was reported by Sun et al.; in this work FNR was knocked down using RNA interference [17]. Pursuing a different approach, Tolleter et al., Steinbeck et al., and Chen et al. focused on reducing cyclic electron flow, an additional competitor. This was achieved by isolating mutants lacking PGRL1 (proton gradient regulation like) or/and PGR5 which are principle components of this process [21–23]. All of these approaches have supported significant increments in H_2 production.

We have previously shown that fusing hydrogenase to ferredoxin generates a new synthetic enzyme (fd-hyd) with increased oxygen tolerance as well as better competition abilities [16, 24]. We have found that our fd-hyd supports a roughly 4.5-fold increment in photosynthetic enzyme activity compared to the native hydrogenase. However, these results were based on relatively low expression regimes of fd-hyd. Here, using sequence optimization and high throughput screening, we report the isolation of 26 new fd-hyd *Chlamydomonas reinhardtii* clones, out of which two had remarkably high abundance of the synthetic enzyme. Notably, all isolated fd-hyd clones maintain a similar ratio between their photosynthetic enzyme efficiency and that of the wild-type. These results highlight the role of enzyme abundance as an important rate limiting factor for *in vivo* H_2 production in microalgae.

2. Methods

See additional methods in the *supplementary methods*.

2.1. Methyl-Viologen protein quantification

Total amount of hydrogenase was quantified as described in [24, 25]. Following 2 h of dark anaerobiosis, cells were transferred into a buffer containing reduced Methyl-Viologen, NaCl, and Triton X-100 for lysing the cells. A 500 μL sample was drawn from the headspace and H_2 concentration was determined by gas chromatography (GC). The amount of enzyme was calculated based on the fd-hyd specific activity constant. In this work we further optimized this protocol by adding salt (NaCl) to the MV buffer, which slightly increased the MV reads in comparison to [24].

2.2. Immunoblot analysis

Soluble proteins were isolated from 50 mL mid-log phase *C. reinhardtii* (3×10^6 cells mL^{-1}) cultures. A volume that corresponded to 140 μg chlorophyll was concentrated (2800 × *g* for 7 min) and taken for induction (see *supplementary methods*). Immediately thereafter cells were precipitated (3200 × *g*, 5 min) and re-suspended in buffer A (50 mM Tris–HCl pH 8.5, 20 mM Na-dithionite, 60 mM NaCl and 1 mM protease inhibitor cocktail). The cell suspension was lysed in a Minilys tissue lyser (Bertin technologies) by three 5000 rpm cycles of 45 s each in the presence of Sigma glass beads (425–600 μm). The soluble proteins were isolated by centrifugation (10 min, 4 °C, 14,000 × *g*) and the total soluble protein concentration was determined by commercial Bradford solution (BioRad). The protein concentrations were all in the range of 1.84 ± 0.26 μg $μL^{-1}$. After adding sample buffer (Bolt™ LDS Sample Buffer) and reducing agent, samples were boiled at 95 °C for 4 min. Either 15 or 30 μg of soluble proteins were loaded on 4–12% Bis–Tris Plus PAGE gels (Novex by Life technologies) and analyzed by immunoblotting using iBind™ blotter and its specific blocking reagents (Life technologies). As a primary antibody we used a rabbit polyclonal HydA1/2 antibody (http://www.agrisera.com/en/artiklar/hyda-iron-hydrogenase-hyda1_hyda2.html). Membrane images were taken using a DNR-MicroChemi station whereas the size markers were overlaid using the RGB camera included in the DNR. Band intensities were analyzed by standard pixel intensity quantification using MATLAB.

2.3. Quantitative PCR

Quantitative PCR was performed as described in [25]. Following induction (see *supplementary methods*), a cell pellet (100 mg) was taken for total RNA extraction using RNeasy plant Mini Kit (QIAGEN 74903). Purified RNA (2 μg) from each sample was used for complementary DNA (cDNA) synthesis. Serial template dilutions provided a standard curve to which the samples were fitted. The qPCR reaction was performed with Applied Biosystems StepOnePlus™ real time PCR system. A sample from each cDNA pool was put through a subsequent qPCR analysis, quantifying the reference gene: receptor of activated protein kinase C (*cblp*). All target transcript levels were normalized by their corresponding *cblp* transcript levels.

2.4. Long term hydrogen production assay

1 L of algal culture was grown in normal conditions (see *supplementary methods*) to high density (10^7 cells mL^{-1}). The cells were concentrated, re-suspended in 1 L of fresh TAP with 5 mM $MgSO_4$ (this addition was found somewhat beneficial for H_2 production in all strains) and transferred to 1.2 L BlueSens bioreactors [22]. The bioreactors were kept at room temperature and with constant stirring. Following the transfer to the BlueSens system, the cultures were flushed with N_2 for 2 min and subsequently kept in the dark for 2 h to achieve anaerobic conditions. At this point light (LED, 150 μE) was turned on and both O_2 and H_2 were continuously measured (1 min intervals) for 5 days.

3. Results

3.1. Protein abundance is a rate limiting factor for photosynthetic H_2 production

To retrieve clones with high expression of fd-hyd, we took two separate approaches; nuclear and plastid expression. For both targets we carried out coding sequence optimization based upon inference of *C. reinhardtii* genomic data [25, 26]. For nuclear expression we placed the gene under control of the psaD promoter in the psL18 vector [27] (Nfd-hyd), whereas for chloroplast expression we placed it under the control of the psaA promoter in the pLM21 vector [28] (Cfd-hyd). The *C. reinhardtii* hydrogenase *hydA1 hydA2* double knockout mutant [29] was transformed using each of the vectors and initially screened on selective agar. A subsequent phenotypic screen was carried out using the *R. capsulatus* high throughput assay which detects H_2 producing clones [30]. Interestingly, while nuclear transformations yielded the regular 15–20% positive clones in secondary screening, the chloroplast transformation yielded nearly perfect efficiency (Fig. S1).

To obtain a gradient of Nfd-hyd expression levels, we took advantage of the random integration which leads to varying mRNA levels [31] and selected 24 different positive clones. Since chloroplast transformation occurs *via* homologous recombination, we only selected a single clone and brought it to homoplasmicity by PCR selection rounds.

To check the relation between protein abundance and H_2 photoproduction activity, we grew each of these 25 clones to mid-log phase and incubated the cultures in 14 mL septum sealed Wheaton vials in dark anaerobiosis for 2 h. At this point the samples were divided in two; one aliquot was drawn from the bottle and its active enzyme abundance was determined using the MV-based protein quantification assay [24,