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Expression of a clostridial [FeFe]-hydrogenase in *Chlamydomonas reinhardtii* prolongs photo-production of hydrogen from water splitting

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ABSTRACT

The high oxygen (O_2) sensitivity of green algal [FeFe]-hydrogenases is a significant limitation for the sustained production of hydrogen gas (H_2) from photosynthetic water splitting. To address this limitation we replaced the native [FeFe]-hydrogenases with a more O_2-tolerant clostridial [FeFe]-hydrogenase CaI in *Chlamydomonas reinhardtii* strain D66ΔHYD (*hydA1*$^-$ *hydA2*$^-$) that contains insertionally inactivated [FeFe]-hydrogenases genes. Expression and translocation of CaI in D66ΔHYD led to the recovery of H_2 photoproduction at ~20% of the rates of the wild-type parent strain D66. We show for the first time that a bacterial [FeFe]-hydrogenase can be expressed, localized and matured to a catalytically active form that couples to photosynthetic electron transport in the green alga *C. reinhardtii*. The lower rates of O_2 inactivation of CaI led to more sustained H_2 photoproduction when cultures were challenged with O_2 or kept under prolonged illumination at solar intensities. These results provide new insights into the requisites for attaining photobiological H_2 production from water splitting using a more O_2-tolerant hydrogenase.

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1. Introduction

The photoproduction of H_2 gas by unicellular green algae was discovered 75 years ago and has long held promise as a renewable alternative to non-renewable production from natural gas reforming. The theoretical maximum solar-to-H_2 (STH) efficiency of algal photobiological H_2 production is 10–13% of incident solar energy [1–3], but actual measured efficiencies are around 0.9% [4–6] due to several constraints. One limiting factor is the sensitivity of hydrogenase to O_2 [7–10] that results in a rapid and complete inactivation by O_2 [11,12] as well as loss of hydrogenase transcript and protein abundance [13–15].

Chlamydomonas reinhardtii is one of the most well-developed model systems for investigating photosynthetic H_2 production [2,16–18]. Under anaerobic induction, *C. reinhardtii* expresses two [FeFe]-hydrogenases, HYDA1 and HYDA2 [14], that couple to photosynthetic electron transport under illumination. To address O_2 inactivation there have been efforts to limit the levels of O_2 production (i.e., water oxidation) by down-regulation of Photosystem II (PSII) activity through either nutrient deprivation [19], mutations of PSII reaction centers [6,20] and other gene targets [5,21,22] or by a combination of nutrient deprivation and physical means [4]. However, each of these strategies lowers the STH efficiency and underscores a need to develop strains and techniques that sustain H_2 production without compromising PSII activity levels.

The clostridial [FeFe]-hydrogenase I from *Clostridium acetobutylicum* (CaI) is known to have a 200-fold higher tolerance to O_2 compared to the [FeFe]-hydrogenases from *C. reinhardtii* [23], suggesting that the exchange of the native algal [FeFe]-hydrogenase for CaI in *C. reinhardtii* will contribute to more sustained H_2 photoproduction in vivo. To investigate this we developed a genetic construct for the nuclear transformation and targeted translocation of CaI to the chloroplast. A *C. reinhardtii* strain that is defective in the expression of native [FeFe]-hydrogenases, (D66ΔHYD) [24] was used as the host strain. We demonstrated that expression of CaI in D66ΔHYD led to the catalytic production of H_2 from

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the coupling of CaI to photosynthetic electron transport. Importantly, when cells were cultured under solar intensities the rates of photosynthetic H_2 production were maintained for longer time periods, even at atmospheric levels of O_2.

2. Materials and methods

2.1. CaI expression construct

Vector pSL18 was digested with *Nde*I and *Xba*I and gel-purified with the Qiagen QIAquick Gel Extraction Kit. The sequence of the [FeFe]-hydrogenase I (CaI) gene from *Clostridium acetobutylicum* ATCC strain 824 was codon-optimized and synthesized for expression in *C. reinhardtii*. Gene CaIv1 (synthesized by GeneArt) or CaIv2 (synthesized by GenScript) were ligated into pSL18 such that their expression was under the control of the *C. reinhardtii* PsaD promoter. Both a circular and an excised version (Fig. S1) were transformed into *E. coli* strain NovaBlue (Novagen). Transformants were screened by PCR, and plasmid sequences were confirmed by sequencing using the primers 5′-GGCCAGGGACGATTATGTATC-3′ and 5′-TCCCGTATCAATCAGCGAAAT-3′ to the PsaD promoter and terminator sequences.

2.2. Algal strains and culture conditions

C. reinhardtii strain D66 (*nit*$^-$, cw15, and *mt*$^+$) was obtained from the *Chlamydomonas* culture collection center; D66ΔHYD (Paro::*hydA*2, Zeo::*hydA*1) [24] was obtained from Dr. Matthew Posewitz at the Colorado School of Mines. The latter was used to create recombinant *C. reinhardtii* expressing the CaI hydrogenase. *C. reinhardtii* cultures were routinely maintained under 10 μE m^{-2} s^{-1} PAR white fluorescent light on Tris-Acetate-Phosphate (TAP) plates. For H_2 photoproduction experiments, fresh plate stocks (<2 weeks) were used to inoculate 50 mL of liquid TAP media in 250 mL Erlenmeyer flasks and grown under the same illumination to an OD of 15–30 μg mL^{-1}. Enough culture was used to inoculate 500–750 mL of TAP media in Roux bottles at 0.4 μg mL^{-1}, and cultures were sparged under stirring with 2% CO_2 at 50–90 μE (m^{-2} s^{-1}) of cool white fluorescent light. Cells were harvested for H_2 photoproduction activity during mid-logarithmic growth phase at 10–20 μg Chl mL^{-1}.

2.3. Nuclear electrotransformation of C. reinhardtii and algal cell line generation

Electrotransformation was performed as outlined in Shimogawara [25]. Briefly, cells were grown to logarithmic phase in TAP media and resuspended to a cell density of 4×10^8 cells mL^{-1} in TAP + 40 mM sucrose medium. A 0.4 cm BIORAD electrotransformation cuvette was filled with 250 μL of resuspended cells and 150–750 ng of purified vector DNA was added; the cuvette was then incubated on ice for 10 min. A 0.8 kV potential with a 25 μF capacitance was applied to the cuvette using a BioRad Gene Pulser II electroporation device. Cells were then resuspended in 10 mL of TAP + 40 mM sucrose and allowed to recover overnight at room temperature in benchtop light (~10 μE m^{-2} s^{-1} of cool white fluorescent) with constant shaking. After centrifugation, cells were resuspended in TAP medium and placed onto three TAP plates containing 15 μg mL^{-1} hygromycin, 10 μg mL^{-1} paromomycin and 7.5 μg mL^{-1} zeocin (TAP-ZPH). Plates were incubated under benchtop light for 1–2 weeks until single colonies could be isolated and replated onto TAP-ZPH plates. After a week of growth, cells were assayed for the presence of the plasmid using genomic DNA PCR (see 2.4), or directly for H_2 production by the bacterial overlay system (see 2.5). High H_2-producing colonies were used to start liquid cultures and grown to logarithmic phase. Cultures were then diluted 1:10,000, and spread on TAP-ZPH plates with glass beads. After 1–2 weeks, colonies were assayed again by the bacterial overlay system. High H_2-producing colonies were maintained as stocks for further experiments.

2.4. PCR

Genomic DNA was isolated using a phenol-chloroform extraction protocol [26] and digested with the Sca1 restriction enzyme. This DNA was used as template for transformant screening using the primers F (5′-TGCGGTGTGTGCATGGTCGAG-3′) and R (5′-GCTGGGAGCCATGGCG ACGAT-3′), which yielded a 600 bp product upon successful CaI insertion.

2.5. Bacterial overlay biosensor system

Hydrogen photoproduction rates were initially assessed using the biosensor system described by Wecker [27,28]. Briefly, recombinant *C. reinhardtii* strains were plated onto TAP agar. After two days of growth at RT in ~10 μE m^{-2} s^{-1} of cool white fluorescent light, plates were overlayed with a *Rhodobacter* strain expressing the Green Fluorescent Protein (GFP) under the regulation of the native H_2-sensing, [NiFe]-hydrogenase promoter. *Rhodobacter* cells (OD_{660} of 0.5) were first mixed in a M1 media-agar solution before overlaying onto *C. reinhardtii* plates. The TAP plates were incubated for 24 h on the bench-top to allow the agar overlay to induce anaerobiosis in the *C. reinhardtii* colonies. Plates were then illuminated and imaged on a Cell Biosciences FluorChem Q system to evaluate GFP (as a proxy for hydrogen production) and chlorophyll fluorescence. Larger GFP fluorescence zones around the spotted *C. reinhardtii* strains indicate higher H_2 production.

2.6. Photosynthetic O_2 and H_2 production and dark respiration

Photosynthetic O_2 evolution and respiration rates were measured in actively growing, mid-logarithmic (10–20 μg mL^{-1} Chl) *C. reinhardtii* cultures as previously described [19], using a Clark-type Pt-Ag/AgCl polarographic electrode system (ALGI, Golden, CO, USA) equipped with a commercially available YSI 5331 electrode (Yellow Springs Instruments, Yellow Springs, OH, USA). The electrode chamber had an illuminated surface area of 1 cm^2 and a volume of 2 mL. Actinic light was provided by PAR LEDs at 2000 μE m^{-2} s^{-1} (equivalent to the PAR region at one sun intensity at noon). For O_2 evolution and respiration measurements, aerobic-grown cultures where placed in the Clark electrode chamber, briefly sparged with argon to get rid of oxygen and then subjected to 30 s in the dark before being illuminated for 2 min followed by 30 s in the dark. The second dark period was used to calculate dark respiration, which was then added to the measured O_2 evolution estimates to evaluate the actual O_2-evolution capacity of the cultures.

For light-induced H_2 measurements, cells were resuspended at 200 or 400 μg mL^{-1} chlorophyll in sealed serum vials in anaerobic induction buffer (AIB; 10 mM KH_2PO_4, 40 mM K_2HPO_4, 3 mM $MgCl_2$) and incubated overnight under illumination with 50–90 μE m^{-2} s^{-1} cool white fluorescent light and argon sparging. Light induction was required due to regulation of CaI by the light-inducible PsaD promoter. In vivo H_2 photoproduction rates were determined as previously described, following illumination for 30 s, after a 30 s dark incubation period [4]. The uncoupler carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP, 2 μM) was added to the H_2-production measurements to prevent inhibition of electron transport by the concomitant accumulation of the proton gradient.

Long-term H_2 measurements were conducted as above, except that the cultures were illuminated for a total period of 30 min. Rates of H_2 diffusion were measured in the same manner but in the absence of algal samples. Rates of H_2 uptake in the dark were measured immediately after the light was turned off.

2.7. Hydrogenase protein levels

Western Blotting performed as previously described [29]. Briefly, equal amounts of total protein were separated on a 10% SDS-PAGE gel

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