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

Seth Noone¹, Kathleen Ratcliff^{1†}, ReAnna Davis¹, Venkataramanan Subramanian¹, Jonathan Meuser^{3§}, Matthew C. Posewitz², Paul W. King^{1*} and Maria L. Ghirardi^{1*}

¹Biosciences Center, National Renewable Energy Laboratory, Golden CO U.S.A.

²Department of Chemistry, Colorado School of Mines, Golden CO U.S.A.

[†]current affiliation: U.S. Department of Energy, Washington DC

[§]current affiliation: Algenol, Fort Myers, FL

*corresponding authors email: paul.king@nrel.gov; maria.ghirardi@nrel.gov

Abstract

The high oxygen (O₂) sensitivity of green algal [FeFe]-hydrogenases is a significant limitation for the sustained production of hydrogen gas (H₂) from photosynthetic water splitting. To address this limitation we replaced the native [FeFe]-hydrogenases with a more O₂-tolerant clostridial [FeFe]-hydrogenase Cal in *Chlamydomonas reinhardtii* strain D66ΔHYD (*hydA1*⁻ *hydA2*⁻) that contains insertionally inactivated [FeFe]-hydrogenases genes. Expression and translocation of Cal in D66ΔHYD led to the recovery of H₂ 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 O₂ inactivation of Cal led to more sustained H₂ photoproduction when cultures were challenged with O₂ or kept under prolonged illumination at solar intensities. These results provide new insights into the requisites for attaining photobiological H₂ production from water splitting using a more O₂-tolerant hydrogenase.

Keywords:

Hydrogen photoproduction, hydrogenase, heterologous expression, O₂ tolerance.

1. Introduction

The photoproduction of H₂ 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-H₂ (STH) efficiency of algal photobiological H₂ production is 10-13% of incident solar energy¹⁻³, but actual measured efficiencies are around 0.9%⁴⁻⁶ due to several constraints. One limiting factor is the sensitivity of hydrogenase to O₂⁷⁻¹⁰ that results in a rapid and complete inactivation by O₂^{11,12} as well as loss of hydrogenase transcript and protein abundance¹³⁻¹⁵.

Chlamydomonas reinhardtii is one of the most well-developed model systems for investigating photosynthetic H₂ production^{2,16-18}. Under anaerobic induction, *C. reinhardtii* expresses two [FeFe]-hydrogenases, HYDA1 and HYDA2¹⁴, that couple to photosynthetic electron transport under illumination. To address O₂ inactivation there have been efforts to limit the levels of O₂ production (i.e., water oxidation) by down-regulation of Photosystem II (PSII) activity through either nutrient deprivation¹⁹, mutations of PSII reaction centers^{6,20} and other gene targets^{5,21,22} or by a combination of nutrient deprivation and physical means⁴. However, each of these strategies lowers the STH efficiency and underscores a need to develop strains and techniques that sustain H₂ production without compromising PSII activity levels.

The clostridial [FeFe]-hydrogenase I from *Clostridium acetobutylicum* (Cal) is known to have a 200-fold higher tolerance to O₂ compared to the [FeFe]-hydrogenases from *C. reinhardtii*²³, suggesting that the exchange of the native algal [FeFe]-hydrogenase for Cal in *C. reinhardtii* will contribute to more sustained H₂ photoproduction *in vivo*. To investigate this we developed a genetic construct for the nuclear transformation and targeted translocation of Cal 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 Cal in D66ΔHYD led to the catalytic production of H₂ from the coupling of Cal to photosynthetic electron transport. Importantly, when cells were cultured under solar intensities the rates of photosynthetic H₂ production were maintained for longer time periods, even at atmospheric levels of O₂.

2. Materials and Methods

2.1 Cal expression construct

Vector pSL18 was digested with NdeI and XbaI and gel-purified with the Qiagen QIAquick Gel Extraction Kit. The sequence of the [FeFe]-hydrogenase I (Cal) gene from *Clostridium acetobutylicum* ATCC strain 824 was codon-optimized and synthesized for expression in *C. reinhardtii*. Gene Calv1

(synthesized by GeneArt) or Calv2 (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::*hydA2*, Zeo::*hydA1*)²⁴ was obtained from Dr. Matthew Posewitz at the Colorado School of Mines. The latter was used to create recombinant *C. reinhardtii* expressing the Cal hydrogenase. *C. reinhardtii* cultures were routinely maintained under 10 $\mu\text{E m}^{-2} \text{s}^{-1}$ PAR white fluorescent light on Tris-Acetate-Phosphate (TAP) plates. For H₂ 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 $\mu\text{g mL}^{-1}$. Enough culture was used to inoculate 500-750 mL of TAP media in Roux bottles at 0.4 $\mu\text{g mL}^{-1}$, and cultures were sparged under stirring with 2% CO₂ at 50-90 $\mu\text{E (m}^{-2} \text{s}^{-1})$ of cool white fluorescent light. Cells were harvested for H₂ photoproduction activity during mid-logarithmic growth phase at 10-20 $\mu\text{g Chl mL}^{-1}$.

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

Electrotransformation was performed as outlined in Shimogawara²⁵. 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 ($\sim 10 \mu\text{E m}^{-2} \text{s}^{-1}$ of cool white fluorescent) with constant shaking. After centrifugation, cells were resuspended in TAP medium and placed onto three TAP plates containing 15 $\mu\text{g mL}^{-1}$ hygromycin, 10 $\mu\text{g mL}^{-1}$ paromomycin and 7.5 $\mu\text{g mL}^{-1}$ zeocin (TAP-ZPH). Plates were incubated under benchtop light for 1-2 weeks until single colonies could be isolated and re-plated 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 1:10000, 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 protocol²⁶ and digested with the ScaI restriction enzyme. This DNA was used as template for transformant screening using the primers F (5'-TGCGGTGTGTGCATGGTCGAG-3') and R (5'-GCTGGGAGCCATGGCGACGAT-3'), which yielded a 600 bp product upon successful Cal 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 $\sim 10 \mu\text{E m}^{-2} \text{s}^{-1}$ 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 ($10\text{--}20\ \mu\text{g mL}^{-1}$ Chl) *C. reinhardtii* cultures as previously described¹⁹, 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 $1\ \text{cm}^2$ and a volume of 2 mL. Actinic light was provided by PAR LEDs at $2,000\ \mu\text{E m}^{-2}\ \text{s}^{-1}$ (equivalent to the PAR region at one sun intensity at noon). For O_2 evolution and respiration measurements, aerobic-grown cultures were 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 $400\ \mu\text{g mL}^{-1}$ chlorophyll in sealed serum vials in anaerobic induction buffer (AIB; $10\ \text{mM KH}_2\text{PO}_4$, $40\ \text{mM K}_2\text{HPO}_4$, $3\ \text{mM MgCl}_2$) and incubated overnight under illumination with $50\text{--}90\ \mu\text{E m}^{-2}\ \text{s}^{-1}$ cool white fluorescent light and argon sparging. Light induction was required due to regulation of Cal 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 period⁴. The uncoupler carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP, 2 μ M) was added to the H₂-production measurements to prevent inhibition of electron transport by the concomitant accumulation of the proton gradient.

Long-term H₂ measurements were conducted as above, except that the cultures were illuminated for a total period of 30 min. Rates of H₂ diffusion were measured in the same manner but in the absence of algal samples. Rates of H₂ uptake in the dark were measured immediately after the light was turned off.

2.7 Hydrogenase protein levels

Western Blotting performed as previously described²⁹. Briefly, equal amounts of total protein were separated on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. Membranes were hybridized with a Cal specific primary antibody raised in rabbit against the Cal peptide sequence 5'-HKLLHVKYTKDKNVSKHE-3' (YenZym, USA), and proteins were detected using the horseradish peroxidase conjugated secondary antibody (GE Healthcare). In parallel, proteins were also stained with Coomassie Blue to be used as a loading control.

2.8 In vivo O₂ sensitivity of hydrogenases

Cells were evaluated for photosynthetic-hydrogen-production rates in the Clark-type electrode as described above, following exposure to increasing amounts of oxygenated buffer for a total of 30 s before the light was turned on. Initial rates were estimated based on the initial slope of each curve upon illumination, and the results were fitted to a single (for Cal recombinants) or double exponential decay curve (for native algal hydrogenases) from which half-lives were calculated.

3. Results and Discussion

Strain D66ΔHYD (*hydA1-1 hydA2-1*)²⁴ is a derivative of wild-type strain D66 that has insertions into each of the HYDA1 and HydA2 structural genes. As a result, D66ΔHYD expresses reduced levels of HYDA1 and HYDA2, but retains [FeFe]-hydrogenase maturation capability. Due to the lower expression of native [FeFe]-hydrogenases, D66ΔHYD has severely reduced levels of H₂ photoproduction activity of ~0.1–0.5% compared to D66²⁴. We therefore used D66ΔHYD as the host for the expression of [FeFe]-hydrogenase I from *Clostridium acetobutylicum* (Cal). A codon-optimized Cal gene was fused to the light-inducible *C. reinhardtii* PsaD promoter that regulates expression of the PsaD subunit of Photosystem I³⁰. The Cal coding regions included a ferredoxin (PETF) transit peptide in frame and upstream of the N-terminus of Cal for translocation of Cal into the chloroplast³¹ (Fig. S1). Initially, the circular plasmid was used for transformation. A total of 70 hygromycin-resistant transformants were selected and 10 were screened for the presence of the Cal gene through genomic PCR (Fig. S1). Five positive clones were cultured photoheterotrophically, induced anaerobically, and tested for H₂ photoproduction rates. Strain 55 Cal_{cir} showed the highest rates, up to 8% of D66 activity and was selected for further study (Table I).

Table I. Photosynthetic parameters for D66 and D66ΔHYD Cal expression strains.

Strain	O ₂ evolution rate ^a	O ₂ respiration rate ^a	Initial H ₂ photoproduction rate ^a		Inhibition by DCMU (%)	Fraction of reductant converted into H ₂ (%) ^c
			No Additives	DCMU		
D66	154 ± 10 (n=2)	33 ± 2.7 (n=4)	164 ± 34 n=6)	9 ± 5 (n=6)	94	50
D66ΔHYD Cal _{cir}	59 ± 16 (38%) ^b (n=4)	29 ± 7 (n=3)	13.3 ± 7.6 (8.0 ± 4.4 %) ^b n=15)	ND	ND	ND
D66ΔHYD Cal _{ex}	53 ± 7 (35%) ^b (n=4)	35 ± 6 (n=4)	35 ± 22 (21 ± 13 %) ^b (n=4)	11 ± 2 (n=6)	69	23

^a μmol H₂ or O₂ (mg Chl h)⁻¹. Values are averages of two replicates ± standard errors, or means ± standard deviation of more than three replicates (number of replicates shown as “n” in the table). ND=not determined.

^b % of wild-type D66.

^c Calculated based on $(\text{PSII-dependent rate of H}_2 \text{ photoproduction} \times 2 \text{ electrons per-H}_2) \div (\text{O}_2 \text{ evolution rate} \times 4 \text{ electrons per-O}_2) \times 100\%$.

In order to ensure colony homogeneity we used a GFP bioassay (see section 2.5, Materials and Methods) to assess H₂ photoproduction activity by Strain 55 Cal_{cir} after serial propagation and screening under selection. After prolonged growth on selective media, colonies of Strain 55 Cal_{cir} showed loss of H₂ photoproduction (Fig. 1A) and clonal heterogeneity (Fig. 1B). Thus, single colonies originating from the parent transformant colony consisted of mixed populations of hygromycin-resistant cells. Therefore, multiple rounds of propagation and screening were conducted, which resulted in a more homogenous cell line of the Cal expressing Strain 55, designated as Cal_{cir} (Fig. 1C). As previously observed, the measured initial rate of H₂ photoproduction by Cal_{cir} was 8% of that of the parent strain (Table I). We addressed the low H₂ photoproduction rates by Cal_{cir} by excising the non-essential bacterial vector regions of Cal³² to generate Cal_{ex} (Fig. S1). Five strains were isolated and analyzed for H₂ photoproduction and two were observed to express elevated H₂ photoproduction rates that were ~21% of rates of D66 (Table 1).

Figure 1. Purification of a stable Cal-expressing *Chlamydomonas* strain from a heterogeneous population of Cal_{cir} transformants. (A) Loss of H₂ photoproduction by the Cal_{cir} Strain 55 over a period of four months. D66 and the D66ΔHYD strain (*hyd*⁻) are shown as positive and negative controls, respectively (B) Heterogeneity of Cal_{cir} Strain 55 isolates observed using the *Rhodobacter* overlay H₂ sensor system. (C) Stable Cal-expressing Strain 55 (designated as Cal_{cir} from now on) colonies after successive rounds of H₂-production screening. (D) Western Blot of proteins extracts probed with a α-Cal antibody followed by HRP-conjugated secondary antibody and detection with a chemiluminescent substrate. Lanes 1, 3, 5, proteins from oxic cultures of D66, Cal_{cir} and Cal_{ex} transformant, respectively; lanes 2, 4, 6, proteins from anaerobically induced cultures of D66, Cal_{cir}, and Cal_{ex} transformant; lane 7, 1 ng of purified Cal; lane 8, blank; lane 9, 2 ng of purified Cal.

Western blotting analysis compared expression levels of the Cal protein in the Cal_{ex} strain versus expression in Cal_{cir}. Fig. 1D shows that Cal_{cir} displayed low levels of Cal expression under both anoxic and oxic conditions (Fig. 1D, lanes 3 and 4, respectively), while the Cal protein from the Cal_{ex} strain was detected at significantly higher levels under anoxic conditions vs. oxic conditions (Fig. 1D, lane 6 compared to lane 5). At high culture densities, the O₂ tension declines, leading to low levels of Cal expression that are detected in cells prior to anaerobic incubation. As shown in Fig. 1D, lanes 5 and 6, the Cal protein is induced several-fold in the Cal_{ex} strain and, based on Western Blotting, its level in Cal_{ex} is 6-fold higher (>6 ng) than in Cal_{cir} (Fig. 1D, lane 4), even when corrected for the total protein level present in each lane (Fig. S3).

We assayed the ability of Cal_{cir} to photoproduce H₂ following exposure to increasing amounts of added O₂. As shown in Fig. 2A, 1 min exposure of cultures to increasing amounts of O₂ led to a decay in H₂ photoproduction rates for both D66 and Cal_{cir}. However, whereas the H₂ photoproduction activity was essentially abolished in D66 after exposure to 50 μM O₂, Cal_{cir} retained a significant fraction of activity after exposure to near atmospheric levels of 208 μM. The exponential fits of the decays (Fig. 2B) revealed that D66 inactivates at rates that are 14-285 fold faster than Cal_{cir}, confirming previous in vitro results²³. The range in relative rates is due to the biphasic decays of H₂ photoproduction activity in D66 compared to a predominantly monophasic decay in rate for Cal_{cir} and takes into consideration the results obtained with a total of four samples. It has been shown that the inactivation of [FeFe]-hydrogenase by O₂ is dependent on the redox state of the enzyme^{13,33}. Due to differences in the

reduction state populations of the two enzymes under turnover, D66 and Cal_{cir} show differences in the O₂ inactivation kinetics.

Figure 2. *In vivo* measurement of hydrogenase O₂ tolerance (representative curve of four replicates). (A) Plots of the decay in the H₂ photoproduction rates of D66 (—) and Cal_{cir} (—) after O₂ exposure. Dotted lines are exponential fits. Cultures were anaerobically induced in the presence of 2 μM FCCP, and challenged with fixed amounts of O₂ saturated buffer. Rates for Cal_{cir} were normalized to D66. (B) Table of O₂ inactivation constants derived from exponential fits of (A). The inactivation of native [FeFe]-hydrogenase activity in D66 fits a double exponential decay ($\chi^2 = 1.3 \times 10^{-7}$), whereas the inactivation of Cal activity fit to a monoexponential decay ($\chi^2 = 6.2 \times 10^{-7}$).

We measured the rates of photosynthetic electron transport and respiration in wild-type and recombinant strains. Table I shows reduced photosynthetic O₂-evolution rates under aerobic conditions by the Cal strains, whereas the respiration rates are similar in all strains. Both water-splitting at

Photosystem II (PSII) and starch degradation can contribute to the reductant pools required for photosynthetic H₂ production. The amount from water splitting in all strains was determined by measuring rates both in the presence and absence of the PSII inhibitor DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea). As shown in Table I, the addition of DCMU inhibited PSII-dependent H₂ photoproduction^{34,35} on D66 by 94%, whereas Cal_{ex} was inhibited by 69%. The residual rates of H₂-photoproduction with DCMU were ~9-11 μmol H₂ (mg Chl h)⁻¹ in both strains, indicating the PSII-independent rates arising from starch degradation are similar for algal and Cal hydrogenases. PSII-dependent, H₂-production rates in Cal_{ex} were 16% of D66 (48.4 versus 308.8 μmol electrons (mg Chl h)⁻¹, respectively, Table S1) and, given the variation in the measurements, this difference could be attributed to the difference in photosynthetic electron transport rates between Cal_{ex} and D66 measured under aerobic conditions (35%). We also estimated a decreased efficiency of photosynthetic reductant conversion into H₂ in the recombinant strain (23 vs. 50% in D66). Thus, H₂ photoproduction in Cal strains is derived from (a) water-splitting at PSII at reduced levels than D66; (b) coupling of Cal to photosynthetic electron transport chain; and (c) reductant from photofermentative starch degradation.

To evaluate whether Cal expression led to more prolonged H₂ photoproduction during photosynthetic water splitting and O₂ production, strains Cal_{cir}, Cal_{ex} and D66 were induced overnight and H₂ photoproduction rates were compared over a period of 30 min (Fig. 3). The initial rate of H₂

Figure 3. Long-term measurement of H₂ levels upon exposure of D66 (blue), Cal_{cir} (red) and Cal_{ex} (green) to solar-intensity illumination for a total of 30 min. Insert: decline in H₂ levels immediately right before and after the lights are turned off. Notice difference in behavior between D66 and Cal expressing strains. The figure shows representative curves from a total of 7 WT, 10 Cal_{cir} and 4 Cal_{ex} replicates with different Chl concentrations (notice that only those around 20-40 µg /ml Chl were used for Table I)

photoproduction by D66 cultures was high during the first few seconds and then declined, due to H₂ uptake and eventual loss of hydrogenase activity from O₂ inactivation. Maximum H₂ accumulation occurred at ~2-3 min. In contrast, Cal_{cir} and Cal_{ex} cultures both showed lower initial rates of H₂ photoproduction but with a more prolonged decline. In this case, maximum H₂ accumulation was observed at ~8 min. In all cultures, both H₂ diffusion out of the chamber and enzymatic H₂ uptake by hydrogenase lead to the decline in H₂ levels¹³. The measured rate of H₂ diffusion was 3.2 µmol H₂ h⁻¹ (Fig. S4), a value that is too low to completely account for the observed decline in H₂ rates in all three strains. This demonstrates that catalytic H₂ uptake is a major contributor to the loss of H₂ levels in the reaction cell. Dark rates of H₂ uptake were also evaluated (Fig. 3). We observed that Cal remains active throughout illumination and that the decline in H₂ levels in the dark was due to a combination of H₂ uptake and O₂ inactivation of the enzyme (Fig.3, inset; see immediate decrease in H₂ levels when the

light is turned off). In D66, the native algal [FeFe]-hydrogenase is inactivated prior to the end of the illumination period so that no further loss of H₂ is observed when the light is turned off (Fig. 3, inset; see no change in H₂ levels after lights are off). These results are in agreement with the differences in K_i for O₂ measured for algal versus the Cal hydrogenase¹³.

We determined whether the observed differences between D66 and the Cal strains could be due to the lower H₂ photoproduction levels by the Cal strains by testing long-term changes in H₂ photoproduction at increasing Chl concentrations, which should yield increasing levels of photosynthetically-evolved H₂ and O₂. Both the time required to achieve peak H₂ levels and the increased dark H₂ uptake rates (observed as a decline in H₂ levels after lights are turned off, Fig. S5) follow similar patterns in each Cal strain independent of the Chl concentration. The differences in native versus Cal-expressing strains are attributed to differences in the biochemical properties of algal and clostridial hydrogenases (i.e., higher O₂ tolerance and kinetic differences in coupling to ferredoxin-catalyzed H₂ production and uptake).

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 higher O₂ tolerance of Cal directly resulted in longer photohydrogen evolution from water splitting. Reductant for photohydrogen production was derived from both photosynthetic water oxidation and photofermentative starch degradation, with each having a differential contribution to the observed rates in the D66 and Cal strains. Rates of O₂ evolution (i.e., photosynthetic water oxidation, Table I) by Cal_{ex} strains were ~35% that of D66, and are consistent with the lower photohydrogen production rates from water oxidation (~16% of PSII-dependent rates in D66) observed under anaerobic conditions. The effect of the absence of native hydrogenases and H₂ metabolism on photosynthetic electron transport rates (i.e., rates of PSII O₂ evolution) under aerobic conditions was not recovered by expression of Cal. This reduced rates of PSII activity partially accounts for the lower photohydrogen

production rates in Cal-expressing strains (Table 1). Finally, the improved Cal-expression levels in Cal_{ex} versus Cal_{cir} led to more efficient conversion of reductant into H₂ (21% versus 8%, respectively, Table 1).

Because our measurements were performed in a 2 ml-volume Clark electrode chamber that has a minimal headspace volume, we could ascertain the effects of O₂ and H₂ on equilibrium rates and better compare the effect of Cal on H₂ metabolism. Preferential O₂ binding to HYDA1 under H₂ oxidation conditions, and similar O₂ inactivation of H₂ production and oxidation by HYDA1¹³ provide the basis for our conclusion that, when expressed in *C. reinhardtii*, Cal maintains its higher tolerance to H₂ inhibition and O₂ inactivation, which is evidenced by a prolonged H₂ photoproduction rate in the light and lower rates of H₂-oxidation (Fig. 3).

Our results unequivocally demonstrate that the successful co-integration of hydrogenases from bacterial microorganisms with microalgae can be achieved for the ultimate purpose of producing renewable H₂ fuel from photosynthetic water oxidation to provide a potential alternative to water electrolysis or fermentation. This work shows the introduction of alternative enzymes can be used to address limitations of native biochemical reactions and metabolic pathways. Engineering of enzymes, and enzyme expression, that catalyze conversion of reductant pools into reduced products effectively alters how metabolic pathways integrate with photosynthetic electron transport, and provides a means to resolve regulatory phenomena that control metabolism in photosynthetic systems.

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