

II.D.2 Biological Systems for Hydrogen Photoproduction

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Project Start Date: October 1, 2000

Project End Date: Project continuation and direction determined annually by DOE

Overall Objective

Develop photobiological systems for large-scale, low-cost, and efficient H₂ production from water to meet DOE's targets (see Table 1).

Fiscal Year (FY) 2013 Objectives

- Develop and characterize deoxyribonucleic acid (DNA) constructs for stable expression of the more O₂-tolerant *Clostridium acetobutylicum* [FeFe]-hydrogenase Ca1 in *Chlamydomonas reinhardtii*
- Investigate the efficiency of H₂ photoproduction in recombinant organisms

Technical Barriers

This project addresses the following technical barriers from the Hydrogen Production section of the Fuel Cell Technologies Office Multi-Year Research, Development, and Demonstration Plan.

(AO) Rate of Hydrogen Production

(AP) Oxygen Accumulation

Technical Targets

The technical targets for this project are listed in Table 1.

TABLE 1. Technical Targets for Photobiological Hydrogen Production (<http://www1.eere.energy.gov/hydrogenandfuelcells/mypp/pdfs/production.pdf>)

	2013 Status	2015 Target	2020 Target	Ultimate Target
Duration of continuous H ₂ production under full sunlight intensity	2 min	30 min	4 h	8 h
Solar-to-Hydrogen Energy Conversion Ratio	NA	2%	5%	17%

FY 2013 Accomplishments

- Increased the stability and rates of the recombinant Ca1 hydrogenase under the regulation of the light-dependent PsaD promoter in *C. reinhardtii*.
- Demonstrated that the *in vivo* O₂-tolerance of the recombinant Ca1 is 10-20-fold higher than that of the native hydrogenases.
- Observed expression of Ca1 under the regulation of the anaerobiosis-dependent *C. reinhardtii* [FeFe]-hydrogenase (HYDA)1 promoter.
- Generated DNA expression constructs incorporating introns from other algal genes to further improve rates of H₂ photoproduction; currently testing the activity of the positive transformants.
- Achieved a 2.5-fold increase in H₂ photoproduction rates by optimizing induction of the PsaD-Ca1 gene.



INTRODUCTION

The green alga *C. reinhardtii* has the biochemical machinery necessary to photoproduce H₂ from water efficiently by using hydrogenase enzymes as endogenous biological catalysts. Currently, under conditions where O₂ is a by-product of photosynthesis, sustained H₂ photoproduction cannot be maintained for more than a few minutes. This limitation is due to a number of factors, including the sensitivity of the hydrogenase enzyme to O₂ co-evolved from photosynthetic water oxidation. Other contributing factors include the down-regulation of photosynthesis by the proton gradient that is established during electron transport at high light intensity, the competition for photosynthetic reductant between H₂ photoproduction and CO₂ fixation, and the low-light saturation of the organism.

Our current project addresses the O₂ sensitivity and concomitant low rate of H₂ photoproduction by expressing

a gene encoding a more O₂-tolerant hydrogenase (from *Clostridium acetobutylicum*) in the green alga *C. reinhardtii*.

APPROACH

Previous efforts at expressing the clostridial hydrogenase in *Chlamydomonas* were hampered by the presence of two native algal hydrogenases, HYDA1 and HYDA2, which confounded the analysis of our results. Since then, we switched our host organism to an algal strain, developed by Prof. Posewitz under Ghirardi's Office of Science/Basic Energy Sciences-funded project, that lacks native hydrogenase activity. To achieve expression of the Ca1 hydrogenase in *Chlamydomonas*, we generated DNA constructs comprised of a promoter (to turn the recombinant gene ON or OFF), a signal peptide (to direct the translated protein to the chloroplast), a codon-optimized Ca1 gene (to match the high guanine/cytosine content of the algal genome), and an algal terminator (to ensure proper conclusion of mRNA translation). Two major promoters were tested this year; the PsaD promoter is responsive to light, and the HYDA1 promoter responds to anaerobiosis. Finally, we are also incorporating intron sequences within the prokaryotic Ca1 gene, an approach that has been shown by others to promote expression of heterologous genes in *Chlamydomonas*.

The DNA constructs are introduced into a hygromycin-resistance-carrying vector that has been used extensively by the *Chlamydomonas* community. The success of each transformation is determined by the survival of cells in the presence of different levels of hygromycin. Positive transformants are screened on plates for hydrogenase activity using a novel, *in vivo* bioassay developed by Dr. Wecker under Ghirardi's Office of Science/Biological and Environmental Research Program funding. The bioassay detects H₂ as fluorescence emission by a green fluorescent protein (GFP), which is expressed from a H₂-sensing promoter in a modified bacterium *Rhodobacter capsulatus* [1]. Hydrogenase-expressing *C. reinhardtii* transformants are detected by the presence of a green, GFP halo around the colony. Each positive transformant is then assessed for H₂-photoproduction capability, stability of the phenotype, and tolerance to O₂. Modifications to the construct are then made, as needed, to achieve higher rates, stability and O₂-tolerance.

RESULTS

At the last progress report, we described the successful transformation of a double-hydrogenase knock-out strain of *Chlamydomonas* with the Ca1 gene and showed light-induced H₂ photoproduction by this transformant. Unfortunately, its H₂-photoproduction capability decreased over time. This year, we used the GFP assay to assess the homogeneity of our transformants. Figure 1A shows a plate of individual

cells originated from a single transformant colony; the red dots indicate individual clones, while the green halo corresponds to H₂-producing colonies. Clearly, this sample shows a high level of heterogeneity; the best H₂-producing clone was re-plated, re-grown in liquid medium, and again assessed for H₂ production. Figure 1B indicates that the clone is now stable and homogeneous. We are investigating its H₂-production capability over time in order to determine whether this re-purification step solved the decrease in H₂-production rate observed earlier. So far, we have not observed any negative effect.

We have since generated constructs under the regulation of the HYDA promoter and tested hundreds of transformants for H₂ photoproduction. As in the case of the PsaD constructs, we successfully detected H₂ production by the GFP assay and by the Clark electrode (results not shown), although the observed rates were not as high as those obtained with the PsaD constructs.

With stable Ca1-expressing transformants in hand, we are then able to measure their tolerance to added O₂. Figure 2 shows a representative series of curves showing light-induced H₂ production by wild-type (A) and Ca1-expressing mutants (B) upon exposure to different levels of O₂. Rates of H₂ photoproduction were determined from the initial slope of each curve and fitted to a two-exponential decay equation. The results are shown in Table 2. At this point it is not clear what the origins of the slow and fast components are. However, the transformant's rate constants are significantly higher than the ones derived for the wild-type strain. These results confirm that Ca1, when expressed in *Chlamydomonas*, does display a higher tolerance to O₂. It is important to point out, though, that these measurements were done in the presence of the uncoupler carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone, which dissipates the proton gradient. As such, the observed H₂ photoproduction activity is not limited by the proton gradient during the experiment.

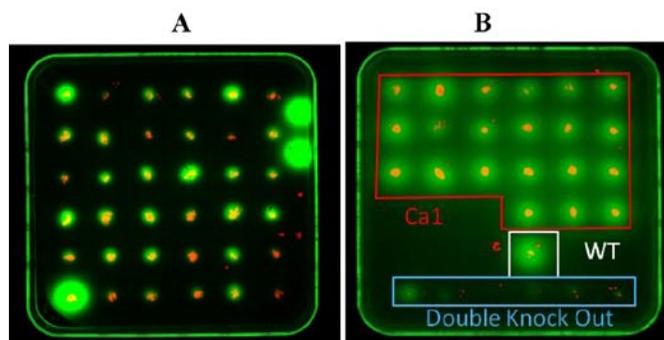


FIGURE 1. Results from the GFP screening of the PsaD-Ca1-expressing transformant (A) and from clones derived from a high H₂-producing strain from A, re-grown in liquid medium and re-plated (B). Controls: Wild-type (expressing algal hydrogenases), double knock-out (unable to express algal hydrogenases and host for Ca1 hydrogenase gene).

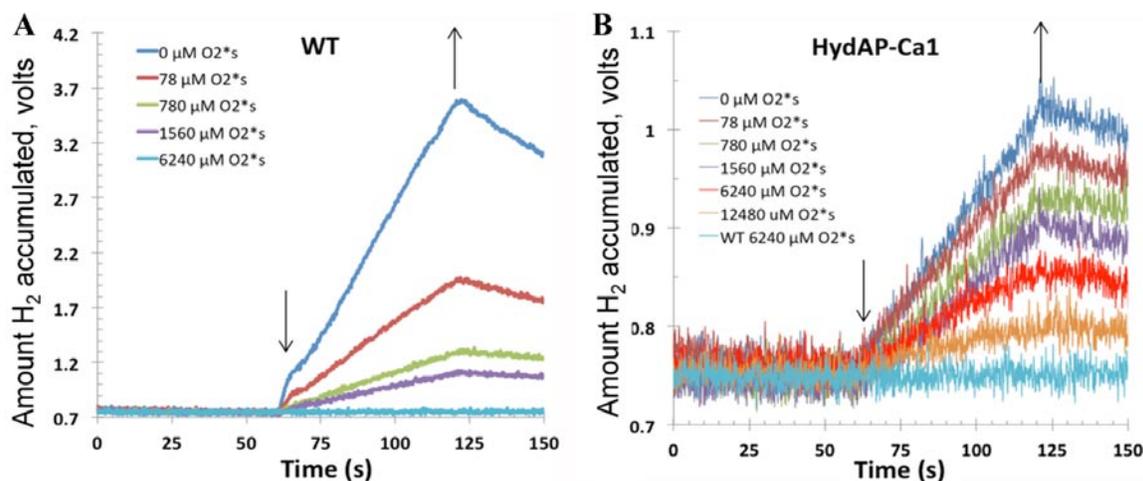


FIGURE 2. Kinetic analysis of the effect of added O_2 on the rates of H_2 photoproduction as measured by the Clark electrode by wild-type (A) and a HYDA-Ca1-expressing transformant (B). Initial rates were estimated from the slope of each curve and plotted as a function of added O_2 concentration. The results are shown in Table 2.

TABLE 2. Kinetic components derived from Figures 2A and 2B and data collected from the HYDA-Ca1 transformant. The data were better fitted to a two-exponential decay equation.

Enzyme/ Strain	O_2 Inactivation Rate Constants (τ_1 and τ_2) ($\mu M O_2^{-1} \cdot s^{-1}$)		O_2 Tolerance (Ratio to WT)	
	Fast	Slow	Fast	Slow
WT	$2.7 \times 10^2 \pm 2 \times 10^3$	$1.7 \times 10^3 \pm 5.2 \times 10^4$	NA	NA
PsaDP-Ca1	1.4×10^3	$1 \times 10^4 \pm 2 \times 10^5$	19	17
HydAP-Ca1	3×10^3	3.5×10^4	9	5

Finally, we designed alternative constructs containing introns from the Rubisco small subunit algal gene and transformed them into *Chlamydomonas*. Unfortunately, our initial selection in hygromycin suggested that the host double knock-out strain had lost some of its sensitivity to the antibiotic for unknown reasons. We are in the process of repeating the transformation with another knock-out clone.

Overall, we were able to improve the stability of the PsaD-based construct, as described above, and, by manipulating the induction conditions, have shown a 2.5-fold increase in the initial rates of H_2 photoproduction, from 5 to 12 $\mu moles H_2 \times mg Chl^{-1} \times h^{-1}$.

CONCLUSION AND FUTURE DIRECTIONS

- The data have confirmed the hypothesis that the introduction into *Chlamydomonas* of a clostridial hydrogenase that is more O_2 -tolerant *in vitro* than algal hydrogenases results in a recombinant organism that has increased O_2 -tolerant H_2 photoproduction *in vivo*.

- A 2.5-fold increase in the rates of H_2 photoproduction was achieved by manipulating induction conditions.
- The investigation of the effect of introns in Ca1 expression will continue in FY 2014.
- A genetic effort will be initiated to combine multiple useful traits into a single algal strain, including Ca1 expression, truncated antenna, and a mutation that eliminates the formation of a proton gradient.

SPECIAL RECOGNITIONS AND AWARDS/ PATENTS ISSUED

- Maria Ghirardi was selected as one of the two newest NREL Fellows.

FY 2013 PUBLICATIONS

- Ghirardi, M.L. 2013. "Photobiological H_2 production: theoretical maximum light conversion efficiency and strategies to achieve it". ECS Trans. 50:47-50.

FY 2013 PRESENTATIONS

- Ghirardi, M.L. "Photobiological H_2 production: theoretical maximum light conversion efficiency and strategies to achieve it". ECS meeting, October 2012.
- Ghirardi, M.L. "Algal Systems for H_2 Photoproduction". EERE/FCTO Annual Merit Program Review Meeting, May 2013.

REFERENCES

- Wecker et al. 2011. Int. J. Hydrogen Energy 36:11229