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Expression of the [FeFe] hydrogenase in the chloroplast of *Chlamydomonas reinhardtii*

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ABSTRACT

Biological hydrogen generation from phototrophic organisms is a promising source of renewable fuel. The nuclear-expressed [FeFe] hydrogenase from *Chlamydomonas reinhardtii* has an extremely high turnover rate, and so has been a target of intense research. Here, we demonstrate that a codon-optimized native hydrogenase can be successfully expressed in the chloroplast. We also demonstrate a curiously strong negative selective pressure resulting from unregulated hydrogenase expression in this location, and discuss management of its expression with a vitamin-controlled gene repression system. To the best of our knowledge, this represents the first example of a nuclear-expressed, chloroplast-localized metalloprotein being synthesized *in situ*. Control of this process opens up several bioengineering possibilities for the production of biohydrogen.

1. Introduction

Interest in hydrogen as an alternative fuel derives from its clean-burning combustion, high efficiency in fuel cell applications and potential for domestic production. Unfortunately, the current industrial method of generating hydrogen is steam reformation of natural gas, requiring heavy use of fossil fuels for the input of both source material and energy [1]. Many microorganisms have the capability to produce molecular hydrogen [2]. When undertaken by oxygenic photosynthetic organisms, this involves the use of sunlight as energy source and water as electron source, both of which are abundant. In such phototrophs, electrons obtained either from the splitting of water at Photosystem II (PSII) or from fermentative catabolism are transferred through a series of electron transfer cofactors to Photosystem I (PSI), which passes the electrons on to ferredoxin (Fd). When growth conditions suppress CO_2 fixation, alternative electron sinks like hydrogen production are activated. Under these conditions, the organism can shunt the flow of electrons from Fd to the enzyme hydrogenase, which evolves H_2 via proton reduction. The hydrogenase enzyme is usually inactivated by O_2 produced by PSII, resulting in only a short-lived production of H_2, unless the conditions are managed to keep the steady-state levels of O_2 very low.

Hydrogenases catalyze the reversible cleavage of molecular hydrogen: $H_2 \leftrightarrows 2H^+ + 2e^-$. Two chloroplast hydrogenases (HydA1 and HydA2) have been identified in the green alga *Chlamydomonas reinhardtii* [3,4]. Both belong to the [FeFe] hydrogenase class. They readily catalyze hydrogen evolution, have been shown to be monomeric in structure, and

Abbreviations: Chl, chlorophyll; EDTA, ethylenediamine tetraacetic acid; IMAC, immobilized metal affinity chromatography; PAR, photosynthetically active radiation; TPP, thiamine pyrophosphate; UTR, untranslated region.

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contain a simple and unique active site consisting of a [4Fe-4S] cluster linked to a catalytically active 2-Fe sub-cluster using CO and CN ligands for redox tuning.

Despite an apparently simple structure, the *C. reinhardtii* hydrogenases have a high specific activity, up to 100-fold higher than that of other hydrogenases [5]. This makes the organism especially well suited for the photoproduction of biohydrogen. However, with the sulfur-deprivation methods currently in use [7], the reported *in vivo* hydrogen yields from this organism are far shy of the theoretical maximum. Temporal separation of photosynthetic oxygen evolution and carbon accumulation from metabolite catabolism and H_2 production addresses the vexing problem of oxygen sensitivity of the [FeFe] hydrogenase, but the adaptation is time-intensive, and the absence of sulfur is detrimental to production of cellular proteins [8] and eventually lethal for the cells. Several groups have had good success in linking hydrogenases *in vitro* to various photosynthetic components and increasing H_2 production by rerouting or controlling electron flow [9–11], but this requires intensive work in the isolation and attachment, as well as the addition of artificial electron donors. When considering a long-term production platform, an engineered *in vivo* system containing an electron-routing fusion may be a stronger contender.

In *Chlamydomonas*, the *HYDA1* gene is nuclear and the gene product appears to be imported into the chloroplast following synthesis in the cytosol [12]. It is assumed that the required maturation proteins are imported as well. HydA, as well as the maturation proteins HydEF and HydG, all possess predicted chloroplast transport peptides [13,14]. The homologous recombination that occurs in the chloroplast of *C. reinhardtii* allows precise genomic engineering that is simply unavailable in the nucleus. In this work we demonstrate successful chloroplast expression of an endogenous *C. reinhardtii* codon-optimized *HYDA1* (cp*hydA*). We also demonstrate the unexpected deleterious effect of unregulated chloroplast hydrogenase expression, and how it was overcome using a gene expression system incorporating a vitamin-repressible promoter and riboswitch.

2. Materials and methods

2.1. Cell culture

Unless otherwise noted, *C. reinhardtii* strains FUD50 (Institut de Biologie Physico-Chimique, Paris) [15], A31 (Silvia Ramundo, Université de Genève) [16], *hydA1-1 hydA2-1* (Matthew Posewitz, Colorado School of Mines) [4], and a 137c wild type (WT) were cultured in Tris-acetate phosphate (TAP) media [17] under ambient light with agitation. The minimal media Tris-bicarbonate phosphate (TBP) was prepared as for TAP but replaced acetic acid with 25 mM bicarbonate and was titrated to pH 7.0. Where required, the antibiotics ampicillin (Fisher) and spectinomycin (Gold Biotechnology) were used at 100 mg/L unless otherwise noted. Vitamins B_1 (Acros) and B_{12} (Sigma Aldrich) were used at 50 μM and 37 nM respectively.

2.2. Construction of cp*hydA* strains

Plasmid pKTR1 (Fig. 1A) was constructed by cutting the codon-optimized *HYDA1* gene out of its pET-Duet vector (modified from the original [18] and sent to us by Matthew Posewitz, Colorado School of Mines) with complete BglII and partial NcoI digestions. This gene codes for the sequence of the mature hydrogenase protein, with an N-terminal hexahistidine (His_6) tag replacing the chloroplast import peptide. The chloroplast expression vector cg13 containing the *atpB* locus and flanking areas (provided by Jörg Nickelsen, Ludwig-Maximilians-Universität München) was cut with BamHI and NcoI. Ligation of the linearized cg13 and pETDuet-*HYDA1* insert was performed with the USB Ligate-IT kit then transformed into chemically competent cells (New England Biolabs). Colonies selected on ampicillin were test digested and confirmed by sequencing.

For the creation of plasmid pKTR3 (Fig. 1B), plasmid pKTR1 was linearized with EcoRV, which cuts between 5′-*psbD* and *atpB* flanking region. An internal aminoglycoside-resistance cassette (*aadA*) with flanking 5′-*psbC* repeats was excised from plasmid pKR102 [19] with ClaI and SphI. The *aadA* cassette flanked by direct repeats of the *psbC*-promoter/5′-UTR (untranslated region) was blunted using the 'Quick Blunting Kit' (NEB) followed by blunt-end ligation with EcoRV-linearized pKTR1 at an insert to vector molar ratio of 3:1 and transformation as above. Colonies selected on ampicillin and spectinomycin were confirmed by sequencing.

Bioballistic chloroplast transformation of *C. reinhardtii* was performed by the method of Boynton [20], delivering 200 ng of DNA in suspension with 1-μm tungsten particles (50 mg/mL),

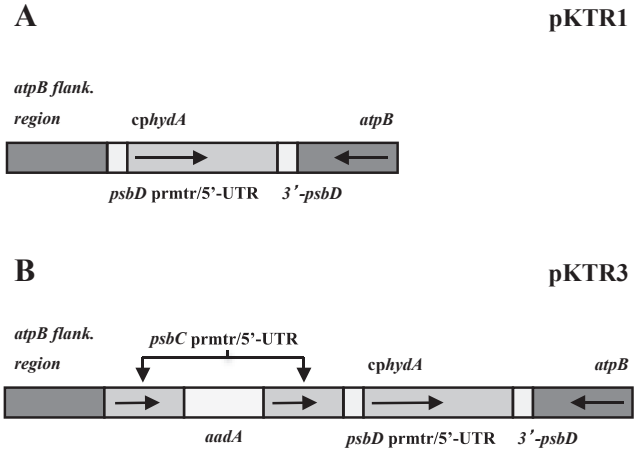

Fig. 1 – Schematic representation of plasmids pKTR1 and pKTR3. In both plasmids, the hydrogenase gene is controlled by the *psbD* promoter and 5′-UTR (*psbD* prmtr/5′-UTR) and is bordered by the 3′-end of *atpB* and a portion of the *atpB* flanking region to direct homologous recombination in the chloroplast. Plasmid pKTR3 contains an *aadA* (aminoglycoside resistance) cassette upstream of the 5′-*psbD* UTR and it is controlled by the *psbC* promoter and 5′-UTR. The *psbC* repeat allows excision of the *aadA* gene by homologous recombination and subsequent loss after selection for antibiotic-resistance is released.

$CaCl_2$ (1 M), and spermidine (20 mM) to each plate spread with ~10^7 cells. Selection for photosynthetic complementation of the *atpB*Δ strain FUD50 was performed on TBP under 175 μmol photons m^{-2} s^{-1} photosynthetically active radiation (PAR). Selection for the 137c WT co-transformed with pKTR1 and the antibiotic resistance cassette pORF472:*aadA* [19] was on TAP containing ampicillin and spectinomycin in either the ambient light or darkness as noted. Selection for transformed A31 was performed on TAP media containing ampicillin, spectinomycin, and both B_1 and B_{12}.

2.3. Detection of the cp*hydA* gene by PCR

Presence of the cp*hydA* gene was detected in genomic DNA by PCR (25 cycles: 94 °C denaturation for 30 s, 50 °C annealing for 30 s, 72 °C elongation for 30 s). Primers were designed to anneal within the chloroplast codon-optimized *HYDA1* gene (*HYDA1*-as: CAGCTGGTAAACATCGGCA), and upstream in the 5′-*psbD* region (*psbD*-5′-UTR-s: ATAATAAATTTAACGTAACGATGAG). This combination does not amplify the native hydrogenase gene and results in a 439-bp product from the cp*hydA* gene. Homoplasmicity was quantified using a primer set in the *atpB* region (*atpB*-3′-s: TACTTAGTAGGTAACATTACAGAAGC), and in the *atpB* flanking region (*atpB*-3′-UTR2: ATTATTAAATACACGTTTAA) using the same PCR cycle, but using an annealing temperature of 48 °C. In the absence of the cp*hydA* gene, a 219-bp product results. With the cp*hydA* insertion, the product is 1.6 kbp, and detection of this longer product was precluded by the PCR conditions used. The smaller product was visualized on a 1% TAE gel with ethidium bromide staining, and the relative amounts were quantified using the ImageJ program (NIH).

2.4. Reverse-transcriptase PCR

A selective method of RNA amplification using dUMP-containing primers and Uracil-DNA Glycosylase (UDG) was employed [21]. Cells were lysed using either bead beating (ZR BashingBead™, Zymo Research) or sonication. RNA was prepared using the RNeasy Prep Kit (QIAGEN) following manufacturer instructions. A dUMP adapter primer (cp*hydA*-adapt: GUCUCCAUCUCUGCAGUCAUAAUAAAUUUAACGUAACGAUGAG) was hybridized to RNA, followed by first strand synthesis with Reverse Transcriptase (Tetro, Bioline). RNA was degraded with RNAse H (New England Biolabs) and the gene-specific primer (cp*hydA*-GSP: CAGCTGGTAAACATCGGCA) hybridized to the now-exposed first strand. Second strand synthesis proceeded and the adapter primer was degraded with UDG (New England Biolabs). The amplification primer (cp*hydA*-amp: GTCTCCATCTCTGCAGTC) annealed to the region cleared by degradation of the adapter primer by UDG, and standard PCR amplification resulted in a 455-bp product.

2.5. Analysis of crude lysate from induced C. reinhardtii

Anaerobic induction was achieved by either four hours of nitrogen flushing or a 30-min argon purge at room temperature followed by four hours of sealed shaking in the dark. All procedures after induction were performed inside an anaerobic chamber (Coy). Cells were pelleted and then lysed by sonication on ice (5, 5 s pulses at maximum power with 10 s rests between) in 50 mM phosphate buffer (pH 8.0) containing 10 μL/mL water-soluble serine, cysteine and metalloprotease inhibitors (P2714 cocktail, Sigma). The supernatant was clarified by centrifugation (2,500 × g for 2 min), concentrated by acetone precipitation and solubilized by heating in lithium dodecyl sulfate (LDS) sample buffer (Invitrogen) at 75 °C for 10 min. Total protein was quantified by bicinchoninic acid (BCA) assay (Pierce). Samples were reduced with 5% β-mercaptoethanol at 75 °C for 10 min, then separated by SDS-PAGE on a 4%/10% Bis-Tris gel with MOPS buffer (50 mM MOPS, 50 mM Tris Base, 0.1% SDS, 1 mM ethylenediamine tetraacetic acid (EDTA), pH 7.7). Samples destined for immunoblotting were run with chemiluminescent molecular weight standards (Precision Plus Protein™ WesternC™ Standards, Bio-Rad). For immunoblotting, SDS-PAGE separation was followed by protein transfer to 0.45-μm Immobilon-P polyvinylidene fluoride (PVDF) membranes (Millipore) with Bis-Tris/Bicine transfer buffer (25 mM Bicine, 25 mM Bis-Tris, 1 mM EDTA, pH 7.2). Blots were blocked in 5% nonfat dry milk in TBST (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.05% Tween-20) overnight, and then probed with a primary antibody, either anti-HydA at 1:1000 (Agrisera) or anti-His_6 at 1:1000 (Genscript). The secondary goat anti-rabbit HRP antibody conjugate was used at 1:10,000 (Bio-Rad), as was the StrepTactin-HRP conjugate for the chemiluminescent molecular weight standard (Bio-Rad). Blots were visualized with ECL (SuperSignal West Femto Chemiluminescent Substrate, Thermo) and imaged using an UltraLum Omega12iC imager.

2.6. Affinity purification of cpHydA

After induction as in **2.5** above, cells were lysed as before but in binding buffer (50 mM phosphate buffer (pH 8.0), 300 mM NaCl and 10 μL/mL protease inhibitors). Clarified supernatant was loaded on a Ni-ITA (iminodiacetic acid) column (800 μg binding capacity, Affymetrix). The column was washed with 620 μL binding buffer, and protein eluted with 720 μL of the same buffer containing 250 mM imidazole. Fractions were desalted through a 5-kDa molecular weight cut off spin filter (Corning). All steps were carried out in an anaerobic chamber. SDS-PAGE separation and immunoblotting were carried out as in **2.5** above.

2.7. Strain characterization

Chlorophyll (Chl) concentration was determined by the method of Porra [22]. Starch granules were qualitatively imaged by staining with Lugol's iodine (Sigma), and soluble starch quantitated by colorimetric reaction with the same, using a standard curve of soluble starch and a glucose negative control. The reactive oxygen species (ROS)-scavenging enzymes catalase (CAT) and ascorbate peroxidase (APX) were assayed as described [23,24].

2.8. Hydrogen evolution measurements

Anaerobically-adapted cell cultures were assayed for hydrogen production by a method adapted from Happe [25]. Induced cell culture at 200 μg/mL Chl (40 μg total) was added to

a solution of methyl viologen (10 mM) in 50 mM potassium phosphate buffer (pH 6.9) containing 0.2% Triton-X (1.2 mL total). To this mixture, 200 μL of sodium dithionite (100 mM) in sodium hydroxide (30 mM) was added and the sample incubated at 37 °C for 15 min. Hydrogen production was assayed from an injection of 200 μL of headspace gas into an SRI model 310C Gas Chromatograph with a helium mobile phase. All transfers of cells, solutions, and gases were performed using argon-flushed, gas-tight syringes (Hamilton Company).

3. Results and discussion

3.1. *Transformation with cphydA and selective pressure against its expression*

Schematics of plasmids pKTR1 and pKTR3 are shown in Fig. 1. Both plasmids contain a synthetic *HYDA1* gene designed for expression in the chloroplast by removal of introns and having codons matching the bias of the chloroplast genome, reflecting the relatively AT-rich composition of the chloroplast genome compared to the GC-rich nuclear genome in *C. reinhardtii*. The sequence encoding the first 56 residues, which compose a putative chloroplast import peptide, was removed and replaced with a sequence encoding a hexahistidine (His_6) tag. As a result, the gene product should resemble that of the imported and processed HydA1. This synthetic gene will be referred to as cp*hydA*. In both plasmids, cp*hydA* was placed behind the *psbD* promoter and 5′-UTR. Targeted gene insertion is possible in the chloroplast if regions of chloroplast homology flank the gene of interest. The chloroplast transformation vectors pKTR1 and pKTR3 were designed to integrate downstream of the *atpB* gene and restore phototrophic (PS^+) growth to the FUD50 strain, which bears a deletion of the 3′-end of *atpB*.

The *C. reinhardtii* chloroplast contains ~80 copies of the genome [26]. Homoplasmicity refers to a state in which all copies of the genome contain the insertion. Immediately after transformation, very few copies in the chloroplast genome will contain the introduced gene. During subsequent cell divisions, the number of copies of the modified genome will increase or decrease in daughter cells due to unequal inheritance. Gene copy number was quantified by a combination of PCRs designed to detect different regions of the native and modified chloroplast genome. The PCR to detect cp*hydA* amplifies only the introduced chloroplast *hydA* gene, not the native nuclear *HYDA1* gene. PCR reactions normalized to total genomic DNA can thus be used to track the enrichment or disappearance of the cp*hydA* gene over generations. The homoplasmicity detection PCR uses primers in the *atpB-3'* and *atpB* flanking regions. In the absence of the cp*hydA* insertion, a ~200 bp product is formed. After insertion of the cp*hydA* gene, the span between the primers becomes too large to amplify under the conditions used (~1600 bp), and a product is no longer observed for that fraction of the population. Therefore, the cp*hydA* and homoplasmicity detection PCR results will be inversely related and may be used to track the level of the cp*hydA* gene (and the unmodified locus) after transformation. Until the insertion becomes homoplasmic (i.e. all copies of the genome are replaced), reversion to the untransformed state (i.e. loss of all copies of the modified genome) can occur, and will occur quickly in the presence of a negative selective pressure that penalizes daughter cells with higher copy numbers of the modified genome.

FUD50 transformed with cg13 successfully restored PS^+ growth, but no colonies were obtained with plasmid pKTR1, in which the *aadA* gene has been replaced by cp*hydA*. Cotransformation of a WT strain (137c genetic background) with pKTR1 and an aminoglycoside resistance cassette conferring resistance to spectinomycin and streptomycin resulted in colonies when selected on spectinomycin in the dark, but the cp*hydA* gene copy number was not subsequently maintained. While spectinomycin-resistant transformants were obtained, and the cp*hydA* gene was initially detected by PCR in many of them, the copy number of the cp*hydA* gene consistently dropped and was undetectable within a few subcloning steps.

We hypothesized that unregulated expression of cp*hydA* was in some way deleterious to growth, thereby generating negative selective pressure against retention of the gene. In order to counter this with positive selective pressure for the modified genome, the transformation construct was redesigned to make the plasmid pKTR3, which places the aminoglycoside adenylyltransferase gene (*aadA*) next to the cp*hydA* gene. This eliminates the need to perform cotransformations, and should allow the selective pressure for antibiotic resistance to drive increase in copy number of both *aadA* and the flanking cp*hydA* gene. To further reduce the total hydrogenase activity, and to make the cp*hydA* gene the only source of hydrogenase, this plasmid was transformed into a double hydrogenase knockout background (*hydA1-1 hydA2-1*). Although many spectinomycin-resistant colonies were obtained, none of them maintained the cp*hydA* gene for more than a few weeks. Thus, it would seem that the selective pressure against the cp*hydA* gene is very strong.

To test the idea that expression of cp*hydA* was the root cause of the negative selective pressure, we made use of a vitamin-mediated gene repression system [16]. This system makes use of the native function of the Nac2 protein, which stabilizes the chloroplast *psbD* mRNA encoding the PSII polypeptide D2 [27] by interacting with the 5′-UTR. In the A31 strain, a mutated *NAC2* gene was supplanted with a nuclear *NAC2* gene under control of a vitamin B_{12}-responsive promoter and a TPP (thiamine pyrophosphate) riboswitch. The native *psbD* gene is placed under control of the *psaA* promoter and 5′-UTR, so its expression is no longer dependent upon Nac2. In the presence of B_{12}, transcription of the engineered *NAC2* gene is decreased; and in the presence of TPP, a riboswitch is stabilized that induces aberrant splicing of the *NAC2* mRNA, further tightening the repression. The presence of both vitamins will thus result in decreased expression of the gene placed behind the *psbD* promoter/5′UTR, due to loss of Nac2 and consequent instability of the mRNA containing the *psbD* 5′-UTR. A test transformation with an *aadA* cassette behind the *psbD* 5′UTR and promoter showed vitamin-mediated repression of the gene, although a small amount of growth on antibiotics in the presence of vitamins indicated that the *aadA* gene expression was not completely shut down (Fig. 2).

When A31 was transformed with cp*hydA*, several experimental conditions were established that greatly increased the

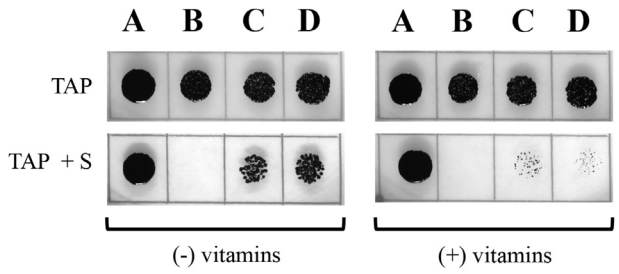

Fig. 2 – Growth assay of test transformants demonstrating vitamin-mediated repression of the *aadA* gene. The following strains were grown in liquid TAP medium and spotted on agar plates without (TAP) and with (TAP + S) 500 mg/L spectinomycin. A: spectinomycin-resistant control (*psbD*-driven *aadA* in a 137c strain); B: A31 parental strain; C and D: Independent A31[*aadA*] transformants.

number of transformants that possessed the cp*hydA* as judged by PCR. First, a light-dependent loss of copy number was observed (Figure S1 of Supplemental Information). A31 did not appear to be light sensitive, nor did the transformants, yet PCRs demonstrated a swift loss of cp*hydA* copy number when heteroplasmic strains were cultured in ambient light. Transformants were hence cultured in the dark. In addition to darkness, vitamins were judged essential for copy number maintenance. First, the number of transformants and their subsequent stability was significantly increased if the parental strain A31 was grown with both vitamins for several days prior to transformation (data not shown). If the transformations were then plated on antibiotic- and vitamin-containing media, colonies that maintained or increased the abundance of cp*hydA* were obtained.

To demonstrate the importance of vitamins in maintaining the cp*hydA* copy number, a single subclone that had been maintained on vitamins and in the dark was cultured in the dark and in the absence or presence of vitamins ("G_1" of Fig. 3A and B, respectively). In the continued presence of vitamins, the cp*hydA* abundance increased (Fig. 3B, "G_2") only to decrease when vitamin control was removed (Fig. 3B, "G_3"). The strong initial growth on vitamins was in stark contrast to the level of cp*hydA* after subculturing in the absence of vitamins once (Fig. 3A, "G_1"). When this subclone was returned to vitamin-containing media, the cp*hydA* copy number was maintained (Fig. 3A, "G_2 (S + V)"). In contrast, a second generation in the absence of vitamins resulted in the drop of cp*hydA* below detection limits (Fig. 3A, "G_2 (S)"). This is a striking demonstration of the strong selective pressure against expression of the cp*hydA* gene. After this discovery, strains were maintained only in the dark and on vitamin-containing media.

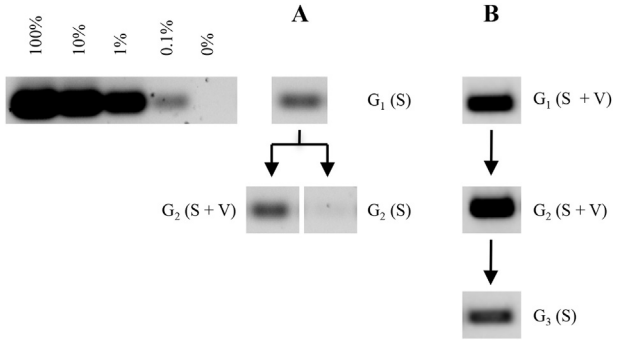

Fig. 3 – Loss of cp*hydA* gene upon cessation of vitamin-mediated repression. Each band shows the abundance of the cp*hydA* gene visualized by cp*hydA*-specific detection PCR after growth conditions as follows. All strains were grown in the dark. From the same initial colony of A31 [cp*hydA*] grown on media containing spectinomycin (S) at 500 mg/L and vitamins B_1 and B_{12} (V, 50 μM and 37 nM respectively), Panel A G_1 (S) shows the result of culturing on spectinomycin only, in the absence of vitamins. That cultured strain was differentially grown again on spectinomycin only G_2 (S), or returned to a vitamin-containing media in G_2 (S + V). In Panel B, the strain was maintained on vitamin-containing media for G_1 (S + V), and again for G_2 (S + V). Removal of vitamin conditions is shown in G_3 (S).

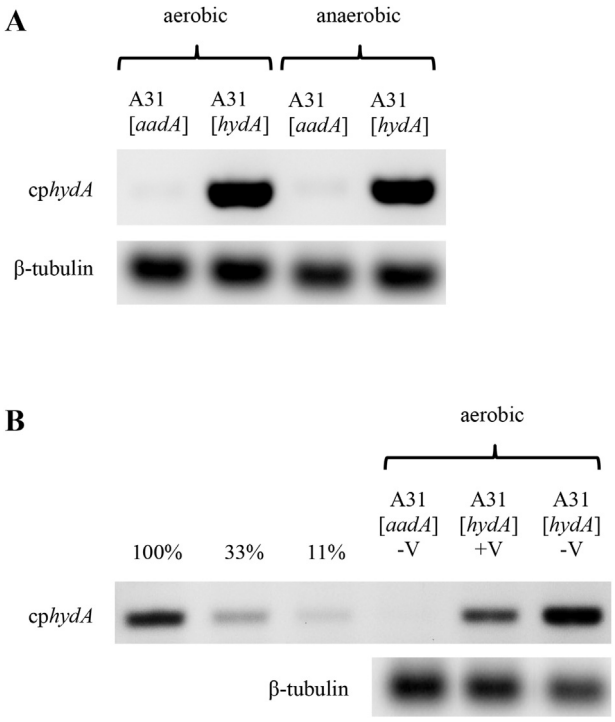

Fig. 4 – Measurement of cp*hydA* mRNA levels by reverse-transcriptase PCR. The mRNA levels of cp*hydA* and beta-tubulin (as control) were assessed by an RT-PCR protocol (see Materials and Methods for details). Panel A: RNA was isolated from the A31[*aadA*] and A31[cp*hydA*] transformants that had been grown aerobically or anaerobically. Panel B: The same strains were grown aerobically or anaerobically in absence or presence of vitamins B_1 and B_{12} (V) and RNA was isolated. The first 3 lanes are RT-PCR results using RNA isolated from anaerobically-grown A31[cp*hydA*] cells and then diluted into RNA isolated from the A31[*aadA*] strain (at the same concentration) at 1/3 or 1/9 to allow quantification of the cp*hydA* mRNA.

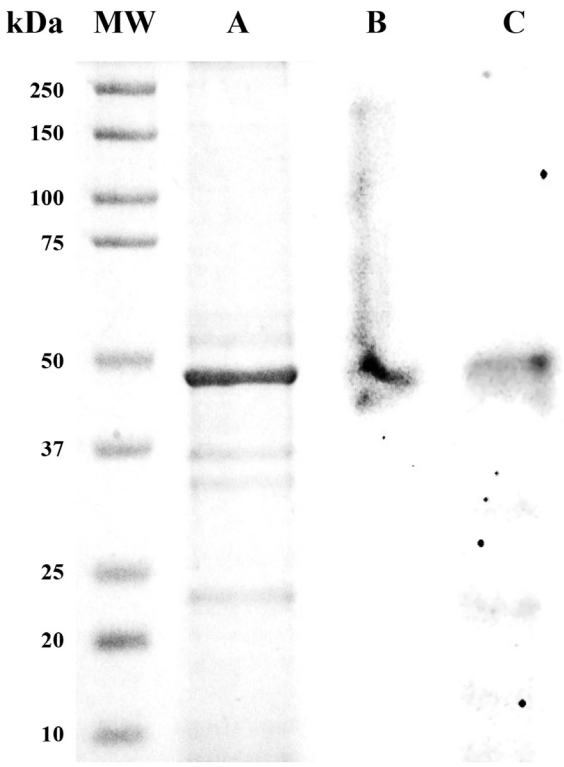

Fig. 5 – Partial purification of the cpHydA apoprotein by IMAC. The cpHydA protein was isolated by IMAC as described in the text. The preparation was subjected to SDS-PAGE (A) and immunoblots with anti-HydA (B) and anti-His_6 (C) antibodies.

Despite all this, the strains remained persistently heteroplasmic for the cp*hydA* gene. Starvation for nitrogen causes a drastic decrease in the chloroplast genome copy number [17]. Thus, nitrogen depletion followed by nitrogen repletion should cause the chloroplast chromosomes of the transformants to pass through a genetic bottleneck, as the chloroplast genome population after addition of nitrogen will have descended from a small number of surviving chromosomes. Some of the descendants of A31[cp*hydA*] transformants treated in this way became greater than 99% homoplasmic, which is the detection limit of our PCR method (Figure S2 of Supplemental Infomation).

3.2. *Constitutive expression of cphydA transcript and cpHydA apopolypeptide*

Transcription of the native hydrogenase is tightly regulated by oxygen [28,29]. We used the method of Buchman et al. [21] to develop a reverse-transcriptase PCR (RT-PCR) method that would be specific for the mRNA from the cp*hydA* gene (see Materials and Methods for details). Data from this method demonstrated that the cp*hydA* transcript was present under both aerobic and anaerobic conditions (Fig. 4A), as expected. Repression by vitamins was operational but incomplete, as we saw only a ~2-fold reduction of transcript in the presence of vitamins under aerobic conditions (Fig. 4B). This was unexpected, as selected essential chloroplast genes controlled with A31 have demonstrated much higher repression after 72 h with vitamins, when assayed by immunoblotting [16]. At this time, we do not understand why we see a weaker effect on the cp*hydA* gene.

Antibodies raised against either HydA1 or the His_6 tag were used to detect the cp*hydA* gene product. We first expressed cpHydA in *E. coli* and purified the apopolypeptide from a bacterial lysate by immobilized metal affinity chromatography (IMAC). The recombinant polypeptide was used to optimize the immunoblotting conditions with both antibodies. The cpHydA protein was then isolated from a clarified cell lysate of anaerobically-induced A31[cp*hydA*] cells by IMAC. Fig. 5 shows the predominant band migrating at 48.5 ± 1.0 kDa, which is very close to the predicted molecular weight of 49.3 kDa for cpHydA, calculated assuming no processing of the polypeptide. A band of the same size reacted with both anti-HydA and anti-His_6 antibodies, further confirming its identity as the cpHydA apoprotein.

Immunoblots of cleared cell lysates with the anti-His_6 antibody revealed the cpHydA polypeptide in the A31[cp*hydA*] strain under both anaerobic and aerobic conditions (Fig. 6). This was expected, given the RT-PCR results (Fig. 4A). No such polypeptide was detected in lysates from A31[*aadA*] cells or hydrogenase mutants that had been treated similarly (Fig. 6). As was the case with the purified cpHydA protein (Fig. 5), the cell extract also contained a polypeptide of the same molecular weight that reacted with anti-HydA antibodies and more anti-HydA reactive polypeptide under anaerobic conditions, consistent with an additional contribution of the native HydA polypeptide (data not shown).

From these results, we can conclude that removal of the native promoter and UTR regions in our construct, as well as changing its location from the nucleus to the chloroplast, has replaced the native regulatory control with an engineered one responsive to an orthogonal exogenous signal – the presence of B_{12} and TPP. The cp*hydA* gene is transcribed in the chloroplast and the transcript is translated there as well. The

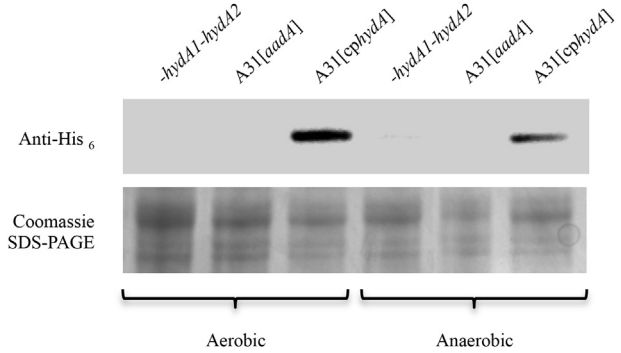

Fig. 6 – Expression of cpHydA apoprotein under aerobic and anaerobic conditions. Clarified cell extracts from the hydrogenase double mutant (-*hydA1*-*hydA2*), A31[*aadA*], and A31[cp*hydA*] strains grown aerobically or anaerobically were separated by SDS-PAGE and immunoblotted with the anti-His_6 antibodies as in Fig. 5. (A separate gel run the same way and Coomassie-stained is shown below to assess protein loading.)

cpHydA polypeptide is thus present under both aerobic and anaerobic conditions and it carries the introduced His_6-tag.

3.3. *Morphological and biochemical characterization of A31[cphydA]*

During routine microscopic examination, it was observed that A31[cp*hydA*] cells fixed with Lugol's iodine appeared to contain significantly more starch granules than the control transformant. This was tested in a quantitative starch assay. The A31[cp*hydA*] strain produced 4 to 10 times more starch than the control A31[*aadA*] transformant (Table S1, Supplemental Information). Starch accumulation is often observed under stress conditions [30]. We measured the activities of the ROS-scavenging enzymes catalase and ascorbate peroxidase to see if they were elevated in the A31[cp*hydA*] cells, and they were marginally higher than in the control strain (Table S2, Supplemental Information).

The negative selective pressure associated with expressing a hydrogenase in the chloroplast, which we observed by the drop in gene copy number (Fig. 3), thus appears to be manifested in stress-specific physiological responses. At this time, we do not understand the root cause of the stress. We tested the hypothesis that the cpHydA apopolypeptide was aggregating and forming inclusion bodies by performing immunoblots of particulate and soluble fractions, but found very little of the cpHydA polypeptide in the insoluble fraction (data not shown). The elevation in ROS-scavenging enzymes indicates that the cpHydA protein may be responsible for generation of ROS. Given the low-to-nonexistent activity of the hydrogenase maturation factors in aerobic conditions, we think it is unlikely that an active hydrogenase is being made from cpHydA in those conditions. However, it is possible that assembly and/or subsequent degradation of the [4Fe-4S] cluster in HydA1, which does not require these factors [31,32], could generate superoxide or other ROS [33]. Further work will be required to test this hypothesis. It is also possible that the cp*hydA* transformants have adapted to expression of the gene after the initial transformation event, given that vitamin-mediated repression of this gene has become incomplete by the time the modified chloroplast genome has become homoplasmic (Fig. 4).

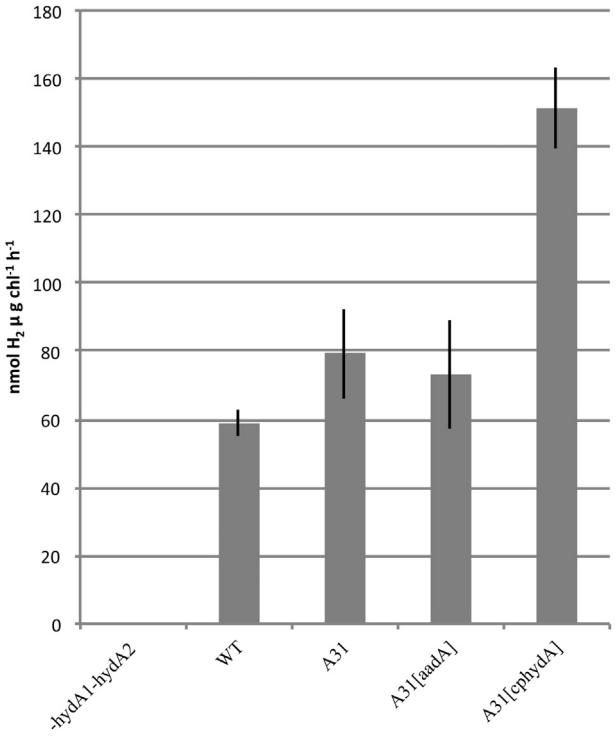

Fig. 7 – MV-mediated H_2 production in permeabilized cells. Hydrogen production was measured in anaerobically-grown cells that were permeabilized by detergent and provided reduced methyl viologen as electron donor to hydrogenase (see Materials and Methods for details). The following strains were assayed: the hydrogenase double mutant (-*hydA1-hydA2*), a 137c wild-type strain (WT), the A31 parental strain, and the A31[*aadA*] and A31[cp*hydA*] transformants.

3.4. *Hydrogen production is elevated in cphydA strain*

In order to test the hypothesis that the cp*hydA* gene was expressing an active hydrogenase enzyme, we measured hydrogen production in the cp*hydA* transformant and control strains. Total dark anaerobic hydrogen production *in vivo* using electrons from fermentation in A31[cp*hydA*] was twice that of the controls (data not shown). However, given the higher starch content in the A31[cp*hydA*] strain (Table S1), and the role of starch fermentation in dark production of hydrogen [34], we did not know if expression of cpHydA was the primary cause of the higher dark production *in vivo*.

In order to measure the maximum H_2 evolution activity, we used reduced methyl viologen (MV) as an electron donor in detergent-permeabilized cells. This removes variations due to *in vivo* changes of electron transfer sources and sinks, and the measurement of evolved H_2 should be limited only by the amount of hydrogenase activity. MV-mediated H_2 generation in the A31[cp*hydA*] strain was slightly more than twice that of the A31 parent strain, which was indistinguishable from the A31[*aadA*] control transformants (Fig. 7). Hydrogen production in the A31 background was also about the same as in a non-engineered wild-type strain (137c background), indicating that the increase in production seen in the cp*hydA* transformant was not due to reversion of a low-H_2 production phenotype in the parental A31 strain arising during the numerous genetic modifications it underwent.

4. Conclusions

We have re-engineered the chloroplast genome of *C. reinhardtii* to express a version of the endogenous algal [FeFe] hydrogenase that is no longer regulated by the native control system. The relocated cp*hydA* gene is transcribed and translated in the chloroplast under both aerobic and anaerobic conditions; in the latter, the polypeptide appears to be assembled into an active hydrogenase enzyme. We observed significant deleterious effects from unregulated expression of the hydrogenase apoprotein, resulting in a strong negative selective pressure against expressing the hydrogenase in the chloroplast. This was relatively well managed by the vitamin-controlled gene

repression system, but did result in a persistently stressed state in the cp*hydA* transformant, as manifested by elevated levels of starch and ROS-detoxifying enzymes. As work proceeds in the development of an *in vivo* fusion system for the production of hydrogen, the lessons here will be applied. Unchecked chloroplast hydrogenase expression is not well tolerated by *Chlamydomonas*, and further study will tease out the direct mechanism of impact and work to design the appropriate control systems for more complete repression of the introduced gene.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijhydene.2013.12.157

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