Synthetic biology for improved hydrogen production in *Chlamydomonas reinhardtii*

Samuel J. King, Ante Jerkovic, Louise J. Brown, Kerstin Petroll and Robert D. Willows

Department of Molecular Sciences, Macquarie University, Sydney, NSW, Australia.

Summary

Hydrogen is a clean alternative to fossil fuels. It has applications for electricity generation and transportation and is used for the manufacturing of ammonia and steel. However, today, H₂ is almost exclusively produced from coal and natural gas. As such, methods to produce H₂ that do not use fossil fuels need to be developed and adopted. The biological manufacturing of H₂ may be one promising solution as this process is clean and renewable. Hydrogen is produced biologically via enzymes called hydrogenases. There are three classes of hydrogenases namely [FeFe], [NiFe] and [Fe] hydrogenases. The [FeFe] hydrogenase HydA1 from the model unicellular algae *Chlamydomonas reinhardtii* has been studied extensively and belongs to the A1 subclass of [FeFe] hydrogenases that have the highest turnover frequencies amongst hydrogenases (21,000 ± 12,000 H₂ s⁻¹ for CaHydA from *Clostridium acetobutylicum*). Yet to date, limitations in *C. reinhardtii* H₂ production pathways have hampered commercial scale implementation, in part due to O₂ sensitivity of hydrogenases and competing metabolic pathways, resulting in low H₂ production efficiency. Here, we describe key processes in the biogenesis of HydA1 and H₂ production pathways in *C. reinhardtii*. We also summarize recent advancements of algal H₂ production using synthetic biology and describe valuable tools such as high-throughput screening (HTS) assays to accelerate the process of engineering algae for commercial biological H₂ production.

Introduction

Hydrogen is the most abundant element in the universe, but on earth, it mostly exists as compounds with other elements. Thus, molecular H₂ is not readily available and needs to be manufactured. Hydrogen has a high gravimetric energy density (~120 MJ kg⁻¹), approximately triple that of gasoline (~46 MJ kg⁻¹) (Ley et al., 2014), and can be directly converted to electricity via a H₂ fuel cell, only emitting heat and water as local by-products (Wu et al., 2012). Furthermore, H₂ is used for manufacturing of chemicals including the production of ammonia for fertilizer, for steel manufacturing, and also the methanol industry (Ramachandran and Menon, 1998). To date, commercially available H₂ is sourced primarily from fossil fuels with approximately 99% manufactured from coal (76%) and natural gas (23%), typically via coal gasification and steam reforming (IEA, 2019). As such, renewable and sustainable methods for H₂ production are required to decarbonize the H₂ economy.

One means to supply clean H₂ is through water electrolysis. However, the worldwide large scale electrolyser capacity is only sufficient to provide less than 0.1% of the current H₂ demand (IEA, 2019). Commercial electrolyser today also require highly purified water and typically must operate continuously, which is generally not compatible with intermittent electricity supply from solar and wind. While there is ongoing work to improve the efficiency and scale of electrolysis to be more economically viable (Burton et al., 2021), other sustainable and low-carbon methods for producing renewable H₂ should also be examined to help accelerate the transition to achieving a renewable H₂ economy.

The field of microbial biotechnology has the potential to contribute to the production of carbon-neutral and renewable H₂. Organisms from all 3 domains of life can achieve a renewable H₂ economy.
Chlamydomonas hydrogenase

One of the best-characterized [FeFe] hydrogenases is the [FeFe] hydrogenase HydA1 from *C. reinhardtii*. *C. reinhardtii* is a unicellular phototrophic green algae and a model organism, which has been studied extensively since the mid-20th century for fundamental processes such as photosynthesis and H₂ production (Sasso et al., 2018). Phototrophic microalgae, like *C. reinhardtii*, are of great interest to the biotechnology industry since they can be grown to high densities in photobioreactors and harness solar energy for producing biomass and a wealth of valuable compounds, including H₂ (Fabris et al., 2020). In *C. reinhardtii*, there are two endogenous [FeFe] hydrogenases capable of producing H₂: HydA1 (accounting for ~80% of the total H₂ production) and HydA2 (accounting for ~20% of the total H₂ production) (Godman et al., 2010; Meuser et al., 2012). *C. reinhardtii* also contains a HydEF fusion protein that is analogous to the individual HydE and HydF proteins found in other organisms (Posewitz et al., 2004a). Both HydA1 and HydA2 belong to the [FeFe] A1 subclass that evolves H₂ via oxidation of ferredoxins (Søndergaard et al., 2016), which are Fe-S proteins that function as electron carriers in several important metabolic processes including H₂ production, nitrogen fixation and photosynthesis (Zanetti and Pandini, 2013). Specifically, HydA1 and HydA2 catalyse the reversible reduction in protons into H₂ either using energy from light (biophotolysis), or by oxidizing organic compounds such as starch (dark fermentation). Biophotolysis, the process of splitting water into H₂ and O₂, is unique to green algae and cyanobacteria and is suggested to function as a transient electron sink for excess reductive energy when *C. reinhardtii* is transitioned from the dark to light under anoxic conditions. This rapid restoration of redox balance is essential for the onset of photosynthesis and CO₂ assimilation (Ghysels et al., 2013).

To successfully adopt *C. reinhardtii* H₂ production into industrial applications, several technical limitations must be overcome. A major limitation is the low efficiency of conversion of light to H₂ due to competition of the electrons with CO₂ fixation through the Calvin–Benson–Bassham (CBB) cycle. One of the highest reported efficiencies of light to H₂ conversion is only about 10% of the theoretical maximum (Kanygin et al., 2020). Second, the rapid inactivation of [FeFe] hydrogenases by O₂ is a challenge limiting H₂ production, especially when considering that O₂ is also generated during the biophotolysis process. Hence, H₂ production in *C. reinhardtii* is generally a short-lived process lasting only a few minutes. The sustained production of H₂ can however be achieved over several days through the use of nutrient-deprived media supplemented with an organic carbon source (Sri-rangan et al., 2011), or through implementation of tightly
controlled dark-light cycles (Kosouroff et al., 2018). However, both these solutions are not ideal for commercial scale-up processes as they are considered as non-ideal growth conditions. To overcome these limitations, the design-build-test-learn framework of synthetic biology can be applied to improving H2 production by *C. reinhardtii* (Fig. 1).

The increased availability of sequenced genomes, as well as the development of new genetic tools in Synthetic Biology, is greatly facilitating the engineering of microorganisms for robust, biotechnological applications (Salomé and Merchant, 2019). Synthetic Biology is an emergent technology that simplifies and modularizes natural systems into standardized parts with distinct functions and defined design rules. By rationally combining novel or heterologous parts, altering existing systems and undertaking iterative design-build-test-learn cycles, the aim is to engineer new and better systems with specific outcomes and applications.

To date, many advances have been made in engineering microalgae such as *C. reinhardtii* for improved production of high-value compounds and biofuels including H2 (Gimpel et al., 2015; Jagadevan et al., 2018; Fabris et al., 2020; Ng et al., 2020; Wichmann et al., 2020). These advances include targeted recombinant protein expression in the chloroplast and nucleus using modularized toolkits (Lauersen et al., 2015); development of several inducible and constitutive native and synthetic promoters (Fischer and Rochaix, 2001; Fei and Deng, 2007; Scranton et al., 2016); editing tools such as precise gene editing through zinc-finger-nucleases technology and CRISPR/Cas9 technology (Shin et al., 2016; Greiner et al., 2017; Guzmán-Zapata et al., 2019); and miRNA gene silencing for metabolic engineering (Crozet et al., 2018). Riboswitches, specific components of an mRNA molecule that regulate gene expression have also been used for metabolic engineering in the cyanobacterium *Synechococcus elongatus* and could be applied to *C. reinhardtii* (Nakahira et al., 2013). In addition, new HTS tools are being developed to screen large algal strain libraries for superior traits, including for H2 production (Schrader et al., 2008; Stapleton and Swartz, 2010a, 2010b; Wecker and Ghirardi, 2014; Land et al., 2019). Typically, knowledge about the organism, its genome, gene regulations, metabolic and signalling pathways can be of great benefit for the targeted design of improved traits and phenotypes.

Therefore, this review aimed to identify Synthetic Biology targets for improving H2 production in *C. reinhardtii*, by summarizing current knowledge of HydA1-mediated H2 production in *C. reinhardtii* holistically, including transcription, translation, transport, maturation and mechanistic limitations for H2 production. We then highlight examples of how Synthetic Biology has been successfully used to alter and improve the native H2 production system in *C. reinhardtii* and describe several available HTS assays that may help accelerate future screening of engineered *C. reinhardtii* for improved H2 production.

**Biogenesis of HydA1**

Many processes are involved in the biogenesis of HydA1 in *C. reinhardtii* including transcription, translation, transport into the chloroplast and maturation of the active site. Understanding these processes is important to improve HydA1-mediated H2 production using Synthetic Biology tools and are described in the following sections. Most research studies to date have focused on HydA1 and less so on HydA2. Only where findings specifically involve HydA2, is it mentioned here.

**The transcription and translation of HydA1 occur under copper or O2 deficiency**

As introduced earlier, *C. reinhardtii* has two hydrogenases, HydA1 and HydA2. Both belong to the [FeFe] A1 hydrogenase subclass and are localized in the chloroplast. HydA1 and HydA2 are encoded in the nucleus by *HydA1* and *HydA2*, respectively, and are translated by cytosolic ribosomes (Happe and Naber, 1993). HydA2 shares 68% protein sequence identity to HydA1 (Fosterier et al., 2003). Transcript levels of *C. reinhardtii* HydA1, HydEF and HydG are increased during anaerobiosis or copper deficiency and are regulated by the transcription factor copper response regulator I (CRR1) (Casstria et al., 2011), in concert with potentially other unidentified genes.
transcription factors (Pape et al., 2012). HydA1 transcripts have also been shown to increase by 20% when inducing anaerobiosis by culturing C. reinhardtii cells in S depleted media (Gonzalez-Ballester et al., 2010). Generally, it was found that transcript levels of HydA1 reach a maximum 1.5–2 h after inducing anaerobiosis (Posewitz et al., 2004b; Mus et al., 2005), while functional HydA1 in chloroplasts was observed between 1 and 2 h post-anaerobiosis (Happe et al., 1994).

Phototropic and autotrophic growth conditions affect the transcription of HydA1 and HydA2. When culturing C. reinhardtii autotrophically, upon inducing anaerobiosis, the transcripts of HydA1 and HydA2 reached the maximum levels, threefold slower compared with cultures grown heterotrophically (i.e. in presence of acetate) (Forestier et al., 2003). HydA1/HydA2 transcripts also degrade more rapidly in presence of O2 when C. reinhardtii is grown autotrophically, that is without acetate supplementation (Forestier et al., 2003). These findings suggest that HydA1/HydA2 transcription is initiated more rapidly and that the transcripts remain intact longer when C. reinhardtii is cultured heterotrophically in presence of an additional carbon source such as acetate. Acetate addition has been suggested to facilitate higher starch accumulation concomitant with faster respiratory O2 consumption rates, which results in a more rapid onset of anoxia (Fouchard et al., 2005). In support of this suggestion are the properties of a mutant of C. reinhardtii (sta7-10 mutant) which is deficient in isoamylase activity, a starch hydrolysing enzyme. This mutant shows much lower HydA1/HydA2 transcript levels compared with wild-type cells (Posewitz et al., 2004b). The HydA1/HydA2 transcripts’ turnover rates in this sta7-10 mutant are also enhanced, resulting in shorter H2 photoproduction cycles (1.5 h) than observed for wild-type cells (> 10 h) under anoxic conditions (Posewitz et al., 2004b). The wild-type phenotype could be restored in the sta7-10 mutant strain through plasmid expression of the gene encoding the isoamylase enzyme. The results from this study support the hypothesis that supplementing the culture media with an additional carbon source is beneficial for extending the duration of H2 production. The additional carbon source facilitates starch accumulation concomitant with the rapid occurrence of anoxia, and accumulation of HydA1/HydA2 transcripts with slower degradation rates.

While HydA1/HydA2 enzymes are irreversibly damaged by O2, HydA1/HydA2 proteins are present in cells cultured aerobically under conditions where intracellular O2 levels are quickly consumed due to high respiratory activities (Liran et al., 2016; Kosourova et al., 2018). However, HydA1/HydA2 protein levels typically increase with decreasing O2 levels (Kosourova et al., 2018).

The import and maturation of HydA1 and HydA2

To be functionally active, HydA1 and HydA2 proteins first need to be imported into the chloroplast and matured. Following translation of HydA1 and HydA2 transcripts, the resulting pre-proteins are imported into the chloroplast stroma using the general import machinery that spans the chloroplast outer and inner membranes. This import machinery, known as the TOC-TIC import machinery (TOC for translocon at the outer envelope membrane of chloroplasts and TIC for translocon at the inner envelope membrane of chloroplasts), is a common feature in both plants and green algae and functions to import nuclear-encoded plastid proteins into the chloroplast (Bolte, 2018). An N-terminal chloroplast targeting sequence of 20–60 amino acids is required on the cytosolic protein to be imported by the TOC-TIC complex. The import targeting peptide becomes cleaved by a peptidyl processing protein protease after import into the chloroplast. Once imported, the HydA1/HydA2 proteins require maturation in the chloroplast to become active (Sawyer et al., 2017). HydA1/HydA2 proteins are only functional when matured to their catalytically active form and under anoxic conditions (Kosourova et al., 2018).

The maturation of HydA1 occurs through a stepwise biosynthesis and insertion of a H-cluster cofactor to form the active site of the enzyme (Britt et al., 2020). The H-cluster is a characteristic feature of [FeFe] hydrogenases and consists of two Fe–S clusters, a 4Fe-4S cubane subcluster [4Fe–4S]H and a 2Fe subcluster [2Fe]H (Fig. 2) (Mulder et al., 2010; Esselborn et al., 2016). A cysteine S atom links [2Fe]H and [4Fe–4S]H together in the protein, constituting the full H-cluster active site (Britt et al., 2020).

Fig. 2. Active site H-cluster of [FeFe] hydrogenases. The H-cluster constitutes two subclusters connected via a cysteine S: (i) [4Fe–4S]H (green), a classical 4Fe–4S cubane; and (ii) [2Fe]H (yellow), a unique di-iron Fe-S cluster coordinated with CO and CN ligands, and CO and azadithiolate bridges.
The [2Fe]₄₄ subcluster is a unique di-iron cluster found only in [FeFe] hydrogenases and is thought to be the active site of [FeFe] hydrogenases where H⁺ or H₂ substrates bind (Mulder et al., 2017; Pelmenschikov et al., 2017; Reijerse et al., 2017; Land et al., 2020). Both Fe atoms of the [2Fe]₄₄ subcluster have terminal CO and CN ligands, and the Fe atoms are further bridged by CO and azadithiolate ligands. The [2Fe]₄₄ subcluster is biosynthesized and inserted into HydA1 via the enzymes HydEF and HydG (Posewitz et al., 2004a).

The [4Fe-4S]₄₄ subcluster balances the redox state of the enzyme and is required for hydrogenase activity (Rodríguez-Macia et al., 2020). Four cysteine residues anchor the [4Fe-4S]₄₄ subcluster in [FeFe] hydrogenases (C115, C170, C362 and C366 in HydA1) (Kertess et al., 2017). The housekeeping SUF Fe-S cluster assembly machinery synthesizes and inserts [4Fe-4S]₄₄ into the apo form of HydA1 followed by insertion of the [2Fe]₄₄ subcluster (Godman and Balk, 2008; Bai et al., 2018). The order of insertion is supported by the requirement of [4Fe-4S]₄₄ to be present for [2Fe]₄₄ to produce a functional enzyme (Mulder et al., 2009).

During O₂ inactivation of [FeFe] hydrogenases, the distal Fe of [2Fe]₄₄, Fe₆, is the first site to be ‘attacked’ and degraded by O₂. In contrast, the [4Fe-4S]₄₄ cluster can remain stable in the presence of up to 17% O₂. If the [4Fe-4S]₄₄ subcluster remains intact, the HydEF and HydG proteins can once again mature the [2Fe]₄₄ subcluster and the hydrogenase into its active state upon returning to hypoxic conditions (Swanson et al., 2015).

**Biosynthesis of [4Fe-4S]₄₄**

Green algae and plants have three different systems for assembly of 4Fe-4S clusters – (i) SUF, (ii) cytosolic Fe-S cluster assembly (CIA) and (iii) Fe-S cluster assembly (ISC) systems (Gomez-Casati et al., 2021). Each of these 4Fe-4S cluster assembly systems operate in different parts of the cell. The SUF system in algae is localized in the chloroplast, the CIA system is in the cytosol, and the ISC system is in the mitochondria (Gomez-Casati et al., 2021). Interestingly, the CIA system requires a functional ISC to work suggesting co-dependence between these two systems (Godman and Balk, 2008; Xu and Möller, 2011).

The synthesis and insertion of the H-cluster into HydA1 occurs after import into the chloroplast stroma, since folded or partially folded proteins cannot be transported across the chloroplast membranes by the TOC-TIC translocon (Paila et al., 2015; Sawyer et al., 2017). As such, the SUF system, and not CIA or ISC, is responsible for the assembly and insertion of [4Fe-4S]₄₄ into HydA1 and functions under O₂ and iron limitation conditions (Takahashi and Tokumoto, 2002; Pérard and Choudens, 2018).

The SUF-mediated 4Fe-4S cluster biosynthesis is poorly characterized in *C. reinhardtii*; however, it is well characterized in *E. coli* (Fig. 3) (Blahut et al., 2020). *C. reinhardtii* produces 6 orthologues of the *E. coli* system: SufA, SufB, SufC, SufD, SufE and SufS (Godman and Balk, 2008). Sequence homology between the *C. reinhardtii* and *E. coli* SUF systems suggests they function similarly to produce 4Fe-4S clusters, although there is little direct experimental evidence to support this (Bai et al., 2018). In *E. coli*, assembly of the Fe-S complex and transfer to the target protein is initiated by the cysteine desulfurase SufS and the S shuttling protein SufE forming the SufSE complex (Fig. 3) (Bai et al., 2018; Pérard and Choudens, 2018; Blahut et al., 2020). This SufSE complex then extracts S from cysteine and transfers it to a SufBC₂D scaffold complex where the 4Fe-4S cluster is assembled (Fig. 3) (Bai et al., 2018; Pérard and Choudens, 2018; Blahut et al., 2020). Formation of the SufSE complex increases the cysteine desulfurase activity of SufS by 10- to 50-fold compared with free SufS. This increased activity has also been shown to be dependent on the sulfurization state of SufSE (Dai and Outten, 2012; Seibach et al., 2013). Furthermore, SufBC₂D in the presence of SufSE further increases the desulfurase activity of SufS 20- to 30-fold (Outten et al., 2003; Dai and Outten, 2012). Evidence suggests that S from SufSE, electrons from FADH₂ and Fe from an unknown source are used to build the 4Fe-4S cluster at the SufB-SufD interface of SufBC₂D (Pérard and Choudens, 2018; Blahut et al., 2020). The synthesized 4Fe-4S cluster is then transferred to the carrier protein SufA which finally shuttles the assembled cluster to the target protein (Fig. 3) (Blahut et al., 2020).

**Biosynthesis of [2Fe]₄₄**

In *C. reinhardtii*, HydA1 containing a [4Fe-4S]₄₄ cluster acts as the site to which the [2Fe]₄₄ subcluster can bind to form the full H-cluster (Fig. 2). The formation and assembly of this [2Fe]₄₄ subcluster in [FeFe] hydrogenases require the activity from three maturase enzymes: the two radical S-adenosyl-L-methionine (rSAM) enzymes HydE and HydG, and a guanosine triphosphatase (GTPase) enzyme, HydF. The details of the different stages of the biosynthesis of the [FeFe] hydrogenase 2Fe subcluster by these three enzymes, HydE, HydF and HydG, have been described in a recent review and are summarized in Fig. 4 (Britt et al., 2020).

The first step in the biosynthesis of the [2Fe]₄₄ subcluster is carried out by HydG. HydG is a free radical SAM enzyme that has two different Fe-S clusters sitting at each end of a conserved triosephosphate isomerase (TIM) barrel channel. These two Fe-S clusters carry out separate reactions to produce an Fe (II) intermediate.
species. Specifically, the Fe-S cluster at the N-terminus of the TIM barrel channel is a \([4\text{Fe}-4\text{S}]\) cluster and cleaves tyrosine to produce both the CO (Shepard et al., 2010) and CN ligands (Driesener et al., 2010). The Fe-S cluster at the C-terminal of the TIM barrel channel is a unique \([5\text{Fe}-5\text{S}]\) cluster, having a classical \([4\text{Fe}-4\text{S}]\) cubane structure linked to an auxiliary Fe via a bridging sulphide. Evidence points towards the auxiliary Fe of \([5\text{Fe}-5\text{S}]\) acting as a receiver for coordination of the CO and CN ligands derived from the cleaved tyrosine (Dinis et al., 2015). A second CO ligand, from a subsequent tyrosine cleavage, forms another bond with the auxiliary Fe, while the second CN ligand derived from the cleaved tyrosine (Dinis et al., 2015). A second CO ligand, from a subsequent tyrosine cleavage, forms another bond with the auxiliary Fe, while the second CN ligand derived from the cleaved tyrosine (Dinis et al., 2015). A second CO ligand, from a subsequent tyrosine cleavage, forms another bond with the auxiliary Fe, while the second CN ligand derived from the cleaved tyrosine (Dinis et al., 2015).

**Fig. 3.** Schematic Illustration of the SUF-mediated biosynthesis of \([4\text{Fe}-4\text{S}]\) clusters in *E. coli*.

A. SufS binds SufE to form the SufSE complex, increasing the desulfurase activity of SufS up to 50-fold.

B. Sulfur is acquired from L-cysteine by the SufSE complex. The SufSE complex then binds with the scaffold complex SufBC2D, further increasing desulfurase activity by another 20- to 30-fold. Sulfur acquired from cysteine and iron from an unknown source is used to build the \([4\text{Fe}-4\text{S}]\) cluster at the interface of SufB and SufD.

C. The complete \([4\text{Fe}-4\text{S}]\) cluster (shown as a cubane molecule) is transferred to SufA.

D. SufA transports \([4\text{Fe}-4\text{S}]\) to the target protein.

condenses into the \([2\text{Fe}]_{\text{H}}\) precursor within HydE, as shown Fig. 4B (top of figure), or if the intermediate is transferred to HydF prior to the formation of this precursor (Britt et al., 2020). Whether the formation of the CO and azadithiolate bridges are mediated by HydE or HydF remains unresolved. The addition of two serine molecules to form the azadithiolate bridge, Fig 3C (top), occurs by an unknown mechanism (Rao et al., 2020). It has been suggested that one of the terminal CO ligands of the \([2\text{Fe}]_{\text{H}}\) precursor is reconfigured into the CO bridge. Whether or not HydF is involved in these final stages of \([2\text{Fe}]_{\text{H}}\) cluster biosynthesis is unclear. Evidence points towards HydF being involved in these final stages of \([2\text{Fe}]_{\text{H}}\) cluster biosynthesis is unclear. Evidence points towards HydF being involved in these final stages of \([2\text{Fe}]_{\text{H}}\) cluster biosynthesis is unclear. Evidence points towards HydF being involved in these final stages of \([2\text{Fe}]_{\text{H}}\) cluster biosynthesis is unclear.

### Hydrogen production pathways in *C. reinhardtii*

There are three pathways through which HydA1 produces \(H_2\) in *C. reinhardtii*. These are photosystem (PS) II-dependent \(H_2\) photoproduction, PS II-independent \(H_2\) photoproduction and \(H_2\) production during dark
anaerobic fermentation (Fig. 5). In each of these three pathways, electrons are donated to HydA1 through a reduced ferredoxin (Fd) species. There are eight Fd isoforms that have been shown or predicted to be expressed by the *C. reinhardtii* chloroplast (PetF, FDX2-3 and FDX5-9) (Sawyer and Winkler, 2017). The particular isoform that donates electrons to HydA1 is still up for debate, although PetF is hypothesized to fill this role.

However, a recent transcriptome study has implicated FDX9 as the prime electron carrier as it is the only chloroplastic Fd isoform with an expression pattern that matches the expression of HydA1 and HydA2 over a 24-hr period during natural light/dark cycles (Strenkert et al., 2019). For clarity, we will refer to the electron donor in the H₂ production pathway herein to be PetF as it is currently accepted as the isoform that donates electrons to HydA1.

Illuminating *C. reinhardtii* cells can activate either PS II-dependent or PS II-independent H₂ production (Chochois et al., 2009). Hydrogen production by the PS II-dependent pathway occurs post-illumination when PS II is functioning normally. However, H₂ production via this pathway is a short-lived process, lasting only 6 min (Ben-Zvi et al., 2019). This time frame of H₂ production is rather impractical for commercial applications. The second pathway, the PS II-independent pathway, can be activated upon illumination by partly inhibiting PS II through nutrient deprivation, addition of the PS II specific inhibitor (DCMU), or genetically through modifying PS II gene expression. PS II-independent H₂ photoproduction has been shown to significantly prolong H₂ production for up to 200 h. However, inducing the PS II-independent route for H₂ production through nutrient deprivation limits cell growth (Chochois et al., 2009). The third H₂ production pathway, which is activated through dark anaerobic fermentation, can be sustained for over 6 hours (Kanygin et al., 2020). These three pathways are described in more detail in the following sections.

**PS II-dependent photoproduction of hydrogen**

During PS II-dependent photoproduction of H₂, PS II absorbs photons via the light-harvesting complex (LHC) II antenna and splits water into protons, electrons and O₂ (Fig. 5, orange arrows). In general, the electrons are shuffled in a linear electron flow (LEF) across the

---

**Fig. 4.** Biosynthesis of the [2Fe]₉ subcluster.

(A) HydG catalyses two reactions to form the Fe (II) intermediate species. Two tyrosine molecules are cleaved by [4Fe-4S] into CO and CN ligands. The auxiliary Fe of [5Fe-SS] is chelated by cysteine and coordinated by the CO and CN ligands to complete the intermediate.

(B) The Fe(II) centre of the intermediate is reduced to Fe(I) by HydE. Subsequent reactions form the [2Fe]₉ precursor through an unknown mechanism; however, it has been suggested that the transformation occurs through pairwise condensation of the Fe(I) intermediate.

(C) The [2Fe]₉ precursor is transformed into the final [2Fe]₉ subcluster by forming the azadithiolate and CO bridges. The mechanisms by which the bridges form are unclear; however, two serine molecules are involved in formation of the azadithiolate bridge with HydF possibly playing a role, and it has been suggested a terminal CO ligand on the [2Fe]₉ precursor is reconfigured into the CO bridge. The complete [2Fe]₉ subcluster is transferred to HydA1 by HydF.
photosynthetic electron transport chain (PETC) via three pigment-proteins PS II, the b6/f cytochrome complex (Cytb6f) and PS I. Electron transport between these three proteins is mediated by two electron carriers plastoquinone (PQ) and plastocyanin (PC). Both these proteins can freely diffuse through the lipid layers. Electrons leaving PS I are used to reduce PetF which then distributes the electrons to either NAD(P)H ferredoxin reductase (FNR) (the primary electron acceptor) or HydA1 (the secondary electron acceptor) (Hemschemeier and Happe, 2011; Sawyer and Winkler, 2017). FNR catalyses the reduction in NADP into NADPH for use in the CBB carbon fixation cycle (Shin, 2004).

Hydrogen production via the PS II-dependent pathway has been shown to occur upon illuminating anoxic C. reinhardtii cells after they have been placed in the dark for a minimum of 30 min (Happe et al., 1994). Hydrogen production typically begins at the onset of illumination and rates of H2 evolution increase for ~2 min before steadily declining and ceasing at ~6-min post-illumination (Ben-Zvi et al., 2019). These changes in rates of H2 production resemble changes from LEF to cyclic electron flow (CEF) with CEF activity reaching a maximum at ~2 min post-illumination, and the activation of the CBB cycle at ~2–4 min (Godaux et al., 2015). When CEF occurs, electrons are cycling through PS I by channelling electrons from PetF back towards the PQ pool instead of FNR or HydA1 (Fig. 5, yellow, dashed arrow). These findings suggest PS II-dependent H2 production is driven by PS I-CEF until the CBB cycle becomes active and the electron flux is entirely directed towards carbon assimilation.

By producing H2, HydA1 also removes protons from the stroma and thereby establishes a proton gradient for ATP synthesis by ATPase which is required for carbon assimilation (Fig. 5). Removing protons also results in the stroma becoming alkalized (pH 6.5 increases to pH 8.5), which is necessary for RuBisCO activity, the key enzyme of the CBB cycle. A pH change closely resembling the stromal pH change before and after illumination (pH 7 to pH 9) enhanced the HydA1-PetF rate constant for binding threefold in silico (Diakonova et al., 2016).

One of the major factors contributing to the cessation of H2 production 6-min post-illumination in the PS II-dependent pathway has been hypothesized to be the inactivation of HydA1 by O2 (Ghirardi, 2015). However, recent evidence supports an alternative hypothesis where HydA1 activity ceases due to electron competition with FNR. For example, it was observed that electron...
flow is channelled exclusively through FNR for carbon fixation before the irreversible inactivation of HydA1 by O₂ occurs (Milrad et al., 2018; Ben-Zvi et al., 2019). This alternative hypothesis is further supported by the observation that HydA1 can remain active in aerobically grown cultures as there are high rates of local respiratory O₂ consumption, creating microoxic niches at the stromal interface of the thylakoid membrane where HydA1 is typically located (Liran et al., 2016). In these aerobic cultures, HydA1 was shown to be a minor sink for electrons leaving the PETC, particularly for reductants produced upon the transition from low to high light.

As intermittent H₂ production is problematic for scaled production, there has been ongoing efforts to prolong H₂ production via the PS II-dependent route. By exposing cultures to a cycle of very short (1 s) pulses of light followed by dark intervals (9 s), cultures can maintain low O₂ levels and H₂ production can be prolonged for up to three days (Kosourov et al., 2018). Under such controlled light and dark cycling conditions, the maximum achieved reported rate of H₂ production was 25 𝜇mol H₂ g⁻¹ Chl h⁻¹. However, given that chlorophyll concentration varies with cell density and growth conditions, these units of H₂ production are not particularly useful for comparing with other biological H₂ production rates in the literature which are typically presented as mmol H₂ l⁻¹ h⁻¹ (where I represents the cell culture volume). In this review, we have therefore recalculated and presented all H₂ production units as H₂ l⁻¹ M⁻¹ h⁻¹ using reported cell densities (where available) or using a medium cell density of at 1 × 10⁷ cells per ml, and the reported chlorophyll amounts per cell of 2.4–3.5 × 10⁻¹⁵ mol (Polle et al., 2000). Thus, 25 𝜇mol H₂ g⁻¹ Chl h⁻¹ as reported above is equivalent to 0.37–0.54 mmol H₂ l⁻¹ h⁻¹.

**PS II-independent photoproduction of hydrogen**

Hydrogen can also be produced via a PS II-independent pathway through partial inhibition of PS II (Fig. 5, yellow arrows). In this pathway, reduction in PQ occurs from electrons derived through oxidation of starch reserves and residual PS II activity. The non-photochemical reduction in the PQ pool by NAD(P)H is suggested to be mediated either through ferredoxin:plastoquinone oxidoreductase (FQR) or a type-II NADH dehydrogenase NAD(P)H-PQ oxidoreductase (NDH-2) (Cournac et al., 2002; Mus et al., 2005; Takabayashi et al., 2005; Antal et al., 2009). As per the PS II-dependent pathway, the reduced PQ pool then supplies electrons directly through Cytb₅₆ to PS I. PS I then reduces PetF, which can feed electrons back to the PQ pool via CEF through FQR (Fig. 5, yellow, dashed arrows), or directly to HydA1 to generate H₂ (Fig. 5, yellow arrows) (Antal et al., 2009). Competition for electrons from PetF between HydA1 and CEF was shown to slow H₂ production more than two-fold (Antal et al., 2009).

PS II-independent H₂ photoproduction in *C. reinhardtii* can be achieved by starving cells of S (Melis et al., 2000) or phosphorus (P) (Batyrova et al., 2012). It can also be achieved via magnesium (Mg) depletion, a key ion of chlorophyll, which is a main component of the two light-harvesting complexes LHC II and LHC I (Volgusheva et al., 2015). All these nutrient depletion treatments result in impaired PS II assembly or repair and thus reduced PS II activity. The implications of a reduction in the PS II activity are both decreased O₂ evolution rates and carbon assimilation, which in turn leads to respiration and increased cellular stress levels.

There are four distinct cellular stages for cells that are nutrient-deprived and continuously illuminated. These are (i) O₂ increase and starch accumulation; (ii) decrease in O₂ levels; (iii) onset of anaerobiosis accompanied by starch degradation; and (iv) H₂ production (Nagy et al., 2018). While starch accumulation during stage 1 and subsequent starch catabolism during stage 3 both contribute to H₂ production via the PS II-independent pathway, more than 92% of the electrons for H₂ production under nutrient-deprived conditions derive from light-driven water splitting as a result of residual PS II activity (Kosourov et al., 2020).

Under nutrient limitation, H₂ production periods have been observed to last for up to 96 h for S-deprived cells (Nagy et al., 2018), 150 h for P-deprived cells (Batyrova et al., 2012) and 200 h for Mg-deprived cells (Volgusheva et al., 2015). The maximum rates of H₂ production for these different conditions are 0.14–0.20 M h⁻¹ under S-deprivation (Kosourov et al., 2003), 0.05 M⁻¹ h⁻¹ under P-deprivation (at ≈ 5.7 × 10⁻⁶ cells) (Batyrova et al., 2012) and 0.25–0.37 M h⁻¹ under Mg-deprivation (Volgusheva et al., 2015). While these rates are lower than the 0.37–0.54 mmol H₂ l⁻¹ h⁻¹ observed in the PS II-dependent pathway (Kosourov et al., 2018), the extended period of H₂ production increasing from a matter of minutes to over a week is an attractive quality for industrial biohydrogen production.

**Dark fermentation production of hydrogen**

A final pathway for H₂ production in *C. reinhardtii* is via dark fermentation (Fig. 5, purple arrows). Production of H₂ by dark fermentation is suggested to originate from oxidation of starch or other carbon sources and reduction in PetF by pyruvate ferredoxin oxidoreductase (PFR1) (Noth et al., 2013), or the accumulation of NADPH from other catabolic processes causing FNR to operate in reverse, reducing Fd (Petrova et al., 2020). Hydrogen production via dark fermentation is limited by the accumulation of fermentation products, notably acetic acid.
acid, that can impair H₂ production and cell growth, and compete with other fermentation pathways in the cell (Fakhimi et al., 2020). The rate of H₂ production by C. reinhardtii HydA1 during dark fermentation was reported to be nearly 50 times lower (~0.009–0.013 mmol H₂ l⁻¹ h⁻¹) (Kanygin et al., 2020) compared with PS II-independent H₂ photoproduction in Mg-deprived C. reinhardtii (~0.25–0.37 mmol H₂ l⁻¹ h⁻¹) (Volgusheva et al., 2015). Furthermore, H₂ production dark fermentation can be sustained for over 6 h (Kanygin et al., 2020), significantly longer than the 6-min H₂ production period of PS II-dependent photoproduction (Ben-Zvi et al., 2019), although much shorter than the 200 h H₂ production period of PS II-independent photoproduction (Volgusheva et al., 2015).

Strategies to improve H₂ production in C. reinhardtii using Synthetic Biology

A promising strategy for producing H₂ at industrial scale is by extending the C. reinhardtii H₂ production period through induction of the PS II-independent pathway. As such, efforts to induce the PS II-independent pathway using simpler methods than nutrient deprivation or expensive chemical inhibitors have been attempted. In addition to increasing the length of the H₂ production period, the efficiency of H₂ production needs to be improved in order to achieve the successful scaled production of H₂ in C. reinhardtii. Light-to-H₂ conversion efficiencies for C. reinhardtii are reported to be below 2% which is approximately 10% of the theoretical maximum (Kanygin et al., 2020). Major factors contributing to the low H₂ production efficiency in C. reinhardtii include synthesis and maturation of active HydA1, O₂ sensitivity of HydA1 as well as competition for electrons between HydA1, FNR and CEF. In this next section, we discuss these limitations and how Synthetic Biology has helped to overcome them.

Genetic Engineering to alter H₂ production pathways

Synthetic Biology has been used to improve PS II-independent H₂ photoproduction in C. reinhardtii. In a synthetic C. reinhardtii strain, cyc6Nac2.49, expression of PS II was made inducible by fusing the Nac2 gene to the cyc6 promoter, which is induced under anaerobic conditions or copper deficiency (Surzycki et al., 2007). Nac2 is key for PS II expression as it binds to and stabilizes psbD mRNA, which translates to the PS II D2 protein. Addition of copper to cyc6Nac2.49 cultures was shown to reduce expression of Nac2 and diminished PS II D2 protein levels 8 h post-addition. Undetectable levels of PS II D2 coincided with reduction in PS II activity and reduced O₂ evolution rates, and resulted in H₂ production rates of up to 0.18–0.27 mmol H₂ l⁻¹ h⁻¹. However, H₂ production was sustained for only a short period of time (1.5 h) as PS II protein levels eventually recovered due to the onset of anaerobiosis, which restores expression of PS II D2 protein. The duration of H₂ production achieved here is 64-fold shorter than the 96 h reported for S-deprived cultures (Nagy et al., 2018). By fusing other genes to the cyc6 promoter and controlling O₂ or copper levels in the media, this inducible chloroplast gene expression system might be applied for other gene targets C. reinhardtii (Surzycki et al., 2007).

In a follow-up study, this same strain (cy6Nac2.49) was then tested for H₂ production during multiple dark-low-light cycles (Batyrova and Hallenbeck, 2017). The authors demonstrated that during illumination, the typical steep rise of O₂ in the culture was diminished due to O₂-induced downregulation of the engineered Nac2-PsbD system and diminished PS II expression. This resulted in a 10-fold lower O₂ accumulation overall than in the parental WT strain. As a consequence, increased H₂ production rates were observed, particularly under low light (10 W m⁻²). Through cycling of dark–light conditions, H₂ production could be sustained for over 30 h. While the rates were 4.5 higher than for the parental WT strain under these conditions, the resulting rates of the mutant strain (0.038–0.056 mmol H₂ l⁻¹ h⁻¹) (Batyrova and Hallenbeck, 2017) are much lower compared with wild-type C. reinhardtii grown in S-deprived media (0.14–0.20 M⁻¹ h⁻¹) (Kosourov et al., 2003) and Mg-deprived media (0.25–0.37 M⁻¹ h⁻¹) (Volgusheva et al., 2015). In addition, the mutant strain (cy6Nac2.49) showed poor cell growth during photoheterotrophic conditions as starch did not accumulate in this anaerobically inducible PS II strain. Nevertheless, because this is a powerful example of sustaining H₂ production through an O₂ and copper-mediated genetic circuit, this strain might be an interesting target for further strain engineering (Batyrova and Hallenbeck, 2017).

In addition to degrading or inhibiting the function of PS II, the PS II-independent pathway can also be further improved to sustain H₂ production for longer periods of time by other methods. Approaches that do not degrade PS II are beneficial as long-term loss of PS II function inhibits growth of cells. As mentioned previously, H₂ production via the PS II-independent pathway competes for electrons with PS I-mediated CEF (Cournac et al., 2002; Antal et al., 2009; Ghysels et al., 2013). Therefore, an effective strategy to increase PS II-independent photoproduction is by targeting the disruption of CEF. For example, treating cells with inhibitors of ferredoxin-plastoquinone reductase (FQR), a key mediator of CEF, was shown to increase PS II-independent H₂ photoproduction in S-starved cells, twofold, compared with untreated cells (Antal et al., 2009).
Alternatively, increased H₂ production rates were also achieved by disrupting CEF using Synthetic Biology techniques. For instance, a randomly generated mutant library of C. reinhardtii was first screened for mutants in which the LHC is locked in state 1 to favour LFE over CEF using fluorescence screening and spectroscopy (Kruse et al., 2005). Shortlisted mutants in which CEF was disrupted were then screened for enhanced H₂ production. This study has resulted in identification of a new mutant strain Strm6 with H₂ production rates 5–13 times higher than that of the wild-type strain over a range of conditions including light intensity and culturing time (Kruse et al., 2005).

Disruption of the CEF pathway and improved H₂ production has also been achieved by generating a C. reinhardtii knockout (KO) mutant (pgr1) incapable of producing the Proton Gradient Regulation-Like 1 (PGRL1) (Tolleter et al., 2011). PGRL1 is required for CEF in C. reinhardtii and was suggested to act as an alternative FQR, a key protein for CEF (Fig 4) (Hertle et al., 2013), and to interact with the proton gradient regulation 5 (PGR5) (Tolleter et al., 2011). In this pgr1 KO mutant, an approximately fourfold increase in the short-term photoproduction rate and yield of H₂ during high-light exposure, compared with the wild-type strain, was reported (Tolleter et al., 2011). A similar improvement in H₂ photoproduction by this mutant of nearly fourfold compared with the wild type was observed under S-deprivation over 6 days.

The duration and thus yield of H₂ production via the PS II-independent pathway are strongly controlled by the electrochemical gradient across the thylakoid membranes which restricts electron flow towards HydA1 (Cournac et al., 2002). This has been shown by the addition of an oxidative phosphorylation uncoupler (carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP)) (Cournac et al., 2002) or deletion of pgr5 (Johnson et al., 2014) and/or pgr1 (Steinbeck et al., 2015) which were observed to diminish the thylakoid proton gradient and uncoupling of ATP synthesis from the membrane proton transport system. For example, when the chemical inhibitor FCCP is used, H₂ production yields were shown to increase 10-fold in a PS II deficient C. reinhardtii mutant (Cournac et al., 2002), and by 37% in S-starved C. reinhardtii (Antal et al., 2009) due to prolonged H₂ production. Similarly, the pgr5 and pgr1 single mutant and a pgr5/pgr1 double C. reinhardtii mutants were reported to generate one of the highest H₂ production yields (610–850 ml H₂/L culture) (Steinbeck et al., 2015). The sustained H₂ production in these mutants was suggested to be a result of greater residual PS II activity than in WT cells, while the oxygen-sensitive hydrogenase was protected from O₂ for extended periods through increased rates of respiration (O₂ consuming) (Steinbeck et al., 2015). Engineering cells to uncouple ATP synthesis from the membrane proton transport system therefore seems to be a promising strategy to mimic the effects of FCCP and increase H₂ production in C. reinhardtii.

Disruption of the CEF pathway to increase H₂ production has also been done by generating a PS I-HydA2 fusion protein in a C. reinhardtii ΔHydA1, ΔHydA2 double knockout mutant strain (Kanygin et al., 2020). The authors intended to direct electrons towards HydA2 and H₂ production by eliminating competition of electrons with FNR. This was done by genetically fusing HydA2 to the PS I Fe–S centre C (PsaC), the terminal electron acceptor subunit of PS I. As a result, HydA2 intercepts electrons directly from PS I for H₂ production, which lasted up to 5 days. This strategy was extremely successful in efficiently mediating the electron flow directly between PS I and HydA2, and as such, bypassing both the CEF and FNR pathways. It was also successful in decreasing the O₂ evolution rates from PS II below the respirational O₂ consumption rates. The PsaC-HydA2 chimera showed a sevenfold increase in the rate of H₂ production at high-light intensity (2000 µmol photons m⁻² s⁻¹) compared with the WT strain which saturated at 100 µmol photons m⁻² s⁻¹. Such high-light intensities are usually detrimental to H₂ photoproduction as the O₂ evolution rates by PS II also increase under these conditions (Batyrova and Hallenbeck, 2017). In addition to the disruption of CEF, the CBB cycle was significantly downregulated. However, the ability of PetF to intercept electrons away from HydA2 remained. Overall, the efficiency of the conversion of light to H₂ was reported as 1.75%. This efficiency is only about 10% of the theoretical maximum (Kanygin et al., 2020) and over twofold short of the 5% efficiency target at which algal H₂ production is stated to be economically viable (Kruse et al., 2005). Further engineering the PetF binding site of HydA2 and O₂ sensitivity was suggested to be an important next step to further improve H₂ production.

**Optimizing the stable expression of a functional HydA1**

Controlling the synthesis and maturation of HydA1 to optimize its protein yields and stability in C. reinhardtii is another important target for improving H₂ production. A strategy to regulate the synthesis and maturation of HydA1 is to express HydA1 from the chloroplast genome under controllable conditions. The presence of homologous recombination in C. reinhardtii chloroplasts makes this strategy more feasible than genetic engineering of the nucleus, where non-homologous DNA end -joining dominates. Additionally, expression of HydA1 in the chloroplast overcomes the need for protein import and the regulatory elements involved.

© 2022 The Authors. *Microbial Biotechnology* published by Society for Applied Microbiology and John Wiley & Sons Ltd., *Microbial Biotechnology*, 15, 1946–1965
Using the native chloroplast homologous recombination system, HydA1 was integrated into the chloroplast genome of *C. reinhardtii* (Reifschneider-Wegner et al., 2014). Integration of the synthetic gene design into the chloroplast genome required extremely tight regulation of HydA1 expression using a double repressor system induced by vitamin B12 and thiamine pyrophosphate (TPP) due to a strong selective pressure against the synthetic HydA1. As a consequence, stable integration into the chloroplast genome was only achieved when culturing the cells in the dark making H2 photoproduction unfeasible.

Nevertheless, engineered strains were able to produce HydA1 transcripts and apoprotein under both aerobic and anaerobic conditions and increased H2 production yields during dark fermentation twofold when compared to its parent strain (Reifschneider-Wegner et al., 2014). Therefore, this strain might be a good starting point for further strain engineering, to allow for inducible H2 production through the more efficient PS II pathway and PS II-independent pathway.

**Heterologous and engineered hydrogenases with improved O2 tolerance**

Native *C. reinhardtii* HydEF and HydG maturases in conjunction with native [4Fe-4S] cluster machinery was demonstrated to be capable of maturing heterologous [FeFe] hydrogenases such as Cpi from *C. pasteurianum* (Sawyer et al., 2017). The capacity of HydEF and HydG to mature heterologous [FeFe] hydrogenases in this study was suggested to be due to the homology of the H-cluster between [FeFe] hydrogenases.

As mentioned earlier, a major limitation for photoproduction of H2 in *C. reinhardtii* is the O2 sensitivity of the [FeFe] hydrogenases (Ghirardi, 2015). To improve the tolerance of *C. reinhardtii* to O2, expression of heterologous hydrogenases in *C. reinhardtii* has been explored to find more O2 tolerant enzyme variants. For example, Cpi expressed heterologously from the *C. reinhardtii* chloroplast demonstrated higher O2 tolerance than HydA1/HydA2 (Noone et al., 2017). Additionally, H2 production in *C. reinhardtii* using a double point mutated variant of Cpi (Cpi<sup>T356V/S357T</sup>) (Koo and Swartz, 2018) showed improved O2 tolerance, retaining 85% of initial activity after exposure to O2 compared to 65% for the wild type (Elman et al., 2020).

More recently, another clostridial [FeFe] hydrogenase, CbA5H from *C. beijerinckii*, was shown to be O2 tolerant (Winkler et al., 2021). CbA5H is the only [FeFe] hydrogenase shown to protect the H-cluster from destruction by long-term O2 exposure. When CbA5H is exposed to air, the H-cluster changes from an active state to an inactive O2 protected state (H<sub>inact</sub>). The formation of H<sub>inact</sub> is dependent on the conserved cysteine residue C367, which is thought to act as a safety cap for the H-cluster preventing O2 attack. In the H<sub>inact</sub> state, residue C367 is in close proximity (3.1 Å) to the distal Fe atom, Fe<sub>d</sub>, of the 2Fe cluster, which is the site of degradation of the H-cluster by O2 in other [FeFe] hydrogenases (Swanson et al., 2015). The C367-Fe<sub>d</sub> distance is only slightly longer than the average distance for covalent Fe-S bonds (2.4 Å) and is small enough to stop small molecules including H2 and O2 from inserting between Fe<sub>d</sub> and C367. By comparison, the distance between Fe<sub>d</sub> and the corresponding cysteine residue (C299) of Cpi from *C. pasteurianum* is significantly longer (5.1 Å) accounting for the inability of Cpi to enter the H<sub>inact</sub> state. Residues L364, A561 and P386, which are in close proximity to the H-cluster in CbA5H, also contribute to increased O2 resistance of CbA5H. The mechanisms of O2 tolerance in CbA5H are similar to [NiFe] hydrogenases, where two conserved cysteine motifs in close proximity to the proximal Fe-S cluster confer O2 tolerance to the hydrogenase (Lukey et al., 2011).

**Diverting electron flow to HydA1**

During illumination and photosynthesis by PS II, FNR is the primary electron acceptor of the PETC transferring electrons from reduced PetF to NADP<sup>+</sup> generating NADPH for carbon fixation (Shin, 2004). While photosynthesis is concomitant with O2 evolution, which itself inhibits HydA1 activity, it was suggested that HydA1 is outcompeted for electrons from PetF by FNR prior to the inactivation of HydA1 by O2 (Milrad et al., 2018; Ben-Zvi et al., 2019). As such, competition for electrons between HydA1 and FNR is hypothesized to be a major limitation during PS II-dependent H2 photoproduction. FNR protein levels are approximately 70-fold higher than that of HydA1 in *C. reinhardtii* (Nikolova et al., 2018) and the affinity of FNR to PetF is 4- to 13-fold higher than that of HydA1 (Km (FNR) = 0.8–2.6 mM, Km (HydA1) = 3.4–35 mM) (Kanygin et al., 2020). Thus, a strategy for improving H2 photoproduction by *C. reinhardtii* has been to engineer proteins to increase the interaction of PetF with HydA1 rather than FNR (Sawyer and Winkler, 2017). A PetF-HydA1 fusion protein was shown to successfully divert over 60% of the photosynthetic electron pool towards H2 production *in vitro* compared with less than 10% observed for HydA1 (Yacoby et al., 2011). This fusion protein also improved H2 production 4.5-fold *in vivo* compared with the wild type when expressed in *C. reinhardtii* (Eilenberg et al., 2016). The increase in H2 production was attributed to a tethering of PetF-HydA1 to PS I allowing for effective interception of photosynthetic electrons. The fusion protein also showed improved O2 resistance *in vitro* and *in vivo*, retaining
towards HydA1 and H2 production. More recently, site-
photosynthetic electrons were more likely to be directed
construct showed a reduced af
in vivo
(Rumpel et al., 2014). The PetF double mutant construct showed a reduced affinity for FNR, and so photosynthetic electrons were more likely to be directed towards HydA1 and H2 production. More recently, site-
directed mutagenesis of FNR and Fd from Synechocystis
PCC 6803 was undertaken to assess the protein to
protein interaction with recombinant HydA1 from C. rein-
hardtii (Wiegand et al., 2018). Single amino acid
exchange of aspartate to alanine near the C terminus
(Fd-D22A) and the N-terminus (Fd-D61A) of Fd increased Fd-HydA1 interaction and H2 photoproduction by 15 and 23% respectively (Wiegand et al., 2018). Exchange of a lysine residue to aspartate near the N-
terminus of the cyanobacterial FNR (FNR-K78-D),
together with the Fd-D61A mutant, significantly
decreased Fd/FNR interaction and conversely increased
Fd/HydA1 interaction and H2 photoproduction in vitro by
18-fold (Wiegand et al., 2018). Modifications to HydA1 to
improve binding affinity for PetF have not yet been
explored but could further improve H2 production. This is
a challenging task, suggested to likely require directed
evolution rather than rational site-directed mutagenesis
(Sawyer and Winkler, 2017).

A HydA1 and superoxide dismutase (SOD) fusion pro-
tein, originally designed to increase the O2 tolerance of
HydA1, greatly enhanced H2 production (Ben-Zvi and
Yacoby, 2016). While the HydA1-SOD fusion protein did
not protect HydA1 from O2, the fusion protein showed
H2 production up to 10–15 mmol H2 l−1 h−1 in C. rein-
hardtii, the highest photosynthetic rate of H2 production
reported to date. The HydA1-SOD fusion protein was
also shown to produce H2 continuously for up to
14 days. This was hypothesized to be due to the ability
of the HydA1-SOD fusion protein to compete with the
CBB cycle for photosynthetic electrons, and reduced O2
evolution (Ben-Zvi et al., 2019). This extended H2 pro-
duction period was also achieved with non-limiting media
rather than using nutrient-deprived media that is typically
used to activate PS II-independent H2 photoproduction.
The majority of electrons for H2 production using HydA1-
SOD were shown to be supplied by PS II LEF, with
starch catabolism playing a secondary role. The com-
petition with CBB does have some drawbacks, as C. rein-
hardtii HydA1-SOD mutants had slower growth rates,
likely due to the reduced flux through the CBB cycle and
impaired photosynthetic activity. The molecular mecha-
nism accounting for this improved H2 production by
HydA1-SOD is unclear, although two hypotheses were
put forward. First was that HydA1-SOD may be bound to
or in close proximity to PS I, hindering the access of
FNR to reduced PetF. The alternate hypothesis was that
HydA1-SOD outcompetes soluble FNR for electrons in
the stroma (Ben-Zvi et al., 2019).

High-throughput screening tools of hydrogenase
activity

Screening of large libraries of synthetic, engineered or
native hydrogenases and other proteins involved in bio-
ological H2 production for improved H2 production in C.
reinhardtii ideally requires accurate, efficient and reliable
HTS platforms. These HTS platforms can be automated
and integrated in automated strain engineering pipelines.
While several instruments and methods are available to
sensitively assay biological H2 production, such as the
Clark-type electrode (normally used for measuring dis-
solved O2) in reverse polarity configuration (Mislov et al.,
2015) or gas chromatography (WeiJun, 2015), these
methods can be slow and cumbersome and are typically
low-throughput. In order to process large sample numbers,
HTS methods (Ghirardi, 2015) have been developed
which include chemochromic films (Posewitz et al.,
2004b); biochemical assays using sulfonated Wilkinson’s
catalyst with a tetrazolium indicator (Schrader et al.,
2008); methyl-violagen assays in combination with cell-
free synthesis using single-molecule PCR expression
(SIMPLEX) (Stapleton and Swartz, 2010a); in vitro com-
partmentalization using C12-resorufin fluorescence (Sta-
pleton and Swartz, 2010b); or a Rhodobacter capsulatus
H2 biosensor (Wecker and Ghirardi, 2014). Another high-
throughput method that can be considered is the use of
chemically synthesized [2Fe]4 cluster mimics which can be
used to artificially mature the [FeFe] hydrogenases
(Land et al., 2019). These HTS assays and their applica-
tions for characterizing hydrogenases and measuring H2
production are briefly described in the following sections.
With the exception of chemochromic films and the
Rhodobacter biosensor, the HTS assays for measuring
H2 typically rely on redox reactions of the hydrogenase
with a non-natural substrate, and hence the measured
rates in vitro might not always reflect H2 production
in vivo.

Pd/WO3 chemochromic films

Chemochromic films are composed of Pd/WO3 layers
that turn blue when reduced by H2. These films are very
sensitive to H2 and can detect amounts of H2 as low as

© 2022 The Authors. Microbial Biotechnology published by Society for Applied Microbiology and John Wiley & Sons Ltd., Microbial
Biotechnology, 15, 1946–1965
was used to measure H₂ production in a strain of *C. reinhardtii* containing a mutated and non-functional isoamylase gene, STA7 (Posewitz *et al.*, 2004b). As STA7 is involved in the accumulation of starch, the decrease in H₂ production detected by the films provided a clear link of starch degradation to H₂ production by dark fermentation (Posewitz *et al.*, 2004b). In another study, Pd/WO₃ chemochromic films were used in a high-throughput H₂ production assay device (H₂-PAD) to screen over 10,000 *C. pasteurianum* Cpl hydrogenase mutants and 400 *Oryza sativa* FNR mutants, identifying a hydrogenase with threefold higher H₂ turnover rate than wild-type Cpl, and an FNR mutant that increased NADPH-driven H₂ production by 60% (Koo *et al.*, 2017). The H₂-PAD device consisted of a 96-well plate at the bottom where biological H₂ production took place, a filter plate containing a desiccant (CaSO₄) and at the top the Pd/WO₃ sensor plate.

**Sulfonated Wilkinson’s catalyst with tetrazolium indicator**

Biological H₂ production is also effectively measured using a semi-quantitative colorimetric assay containing a water-soluble tetrazolium indicator (WST-3) and soluble Wilkinson’s catalyst (Schrader *et al.*, 2008). This method was used to measure H₂ production by *Synechocystis* sp. PCC 6803 encapsulated in silica sol-gel (Dickson *et al.*, 2009). Wilkinson’s catalyst is used to catalyse the hydrogenation of primary and secondary alkenes. In this assay, the Wilkinson’s catalyst hydrogenates the tetrazole ring of WST-3, forming a formazan molecule which can be detected via a colour change at 433 nm. While not as sensitive as Pd/WO₃ chemochromic film based assays, the colorimetric assay using Wilkinson’s catalyst has been shown to detect H₂ from whole-cell systems in microtitre plates for amounts of H₂ as low as 20 nmol (Schrader *et al.*, 2008).

**SIMPLEX-based methyl-viologen HTS assay**

Single-molecule PCR-linked expression (SIMPLEX) in conjunction with cell-free protein synthesis (CFPS), methyl-viologen reduction and gas chromatography (GC) has been used to screen the effects of hydrogenase mutations on H₂ production *in vitro*. After screening ~30,000 hydA1 mutants with the SIMPLEX-based assay, a novel HydA1 mutant was identified which showed fourfold improved H₂ production compared with the wild type (Stapleton and Swartz, 2010a). SIMPLEX can amplify single molecules of DNA from a mutant library after serial dilution into microtitre plates. Subsequent expression of the mutant DNA using CFPS results in the plate wells containing on average just 2 mutant proteins which can then be characterized using high-throughput assays. After amplification with SIMPLEX and expression with CFPS, H₂ consumption rates of hydrogenases are measured using methyl viologen as substrate. Methyl viologen can be an electron donor and acceptor for [FeFe] hydrogenases similar to PetF. Hydrogenases that oxidise H₂ in solution reduce methyl viologen which is indicated by a colour change from clear to blue, detectable at 578 nm. Following identification of high H₂ consumers, a shortlist of promising hydrogenases can then be analysed for their H₂ production rates using low-throughput techniques such as GC. A particular advantage of the SIMPLEX-based assay is the accelerated protein expression offered by CFPS compared with the typically slow-growing *C. reinhardtii*.

**In vitro compartmentalization and fluorescence-activated cell sorting**

A HTS for O₂ tolerance has been developed using a combination of *in vitro* compartmentalization (IVC), microbead display and fluorescence-activated cell sorting (FACS) (Stapleton and Swartz, 2010b). The throughput of IVC-FACS screening is significantly greater than 96-well plate assays and is able to sort >1000 beads per second. This HTS tool is based on beads which bind to the DNA templates and are emulsified into droplets to create individual CFPS reactors. To demonstrate the suitability of this technique, a mock library of *C. pasteurianum* hydrogenase Cpl was successfully screened for O₂ tolerance. For this, streptavidin-coated beads are generated that each displayed a single biotinylated DNA template and biotinylated anti-hemagglutinin (HA) antibodies. The beads are then emulsified to form oil phase emulsion droplets to express hydrogenase proteins using emulsion CFPS (eCFPS). *E. coli* extracts containing the [FeFe] hydrogenase maturases HydE, HydF and HydG from *Shewanella oneidensis* are included in the emulsions to mature the hydrogenases. The hydrogenases contain three HA tags which once expressed bind to the anti-HA antibodies on the streptavidin-coated beads. After protein synthesis, the beads are removed from the emulsion droplets, washed and exposed to O₂. New emulsion droplets containing the beads and the reporter molecule C₁₂-resazurin are created after O₂ exposure. C₁₂-resazurin forms a fluorescent molecule C₁₂-resorufin when reduced by active hydrogenases in a H₂ consumption assay. Subsequently, the beads are again removed from the emulsion and FACS is used to detect and enrich fluorescent beads which contain hydrogenases that are tolerant to O₂ exposure. Since the beads still bind the respective DNA template, a direct genotype–phenotype link can be established to provide the sequence to the O₂ tolerant hydrogenase. While this

© 2022 The Authors. *Microbial Biotechnology* published by Society for Applied Microbiology and John Wiley & Sons Ltd., *Microbial Biotechnology*, 15, 1946–1965
particular assay was tested to screen for O2 tolerance of hydrogenases, it is useful for screening other important hydrogenase characteristics including thermostability and tolerance to other harsh conditions. The IVC-FACS assay was also confirmed to be compatible with HydA1 from C. reinhardtii, indicating that this screening platform may be used to screen [FeFe] hydrogenases from diverse organisms for a variety of characteristics (Stapleton and Swartz, 2010b).

**Rhodobacter biosensor**

A *Rhodobacter capsulatus* H2 biosensor has been engineered to fluoresce upon exposure to H2 (Wecker et al., 2011). This organism has a H2 sensory system composed of a H2 sensor protein (HupUV), a histidine kinase (HupT), a transcription regulator (HupR) and an uptake hydrogenase (HupSL). The fluorescent reporter emGFP was inserted downstream of HupSL on a plasmid in *E. coli* S17-1 and transferred by conjugation into the recipient strain *R. capsulatus* JP91. After exposing the *R. capsulatus* biosensor to 0.1% H2 for 4 h, the activation of emGFP by 1% O2 exposure for 2 h was found to be sufficient to generate a fluorescent signal that was approximately eightfold higher than cells not exposed to H2. The lower limit of detection using the biosensor was 200 pM H2 in solution. A 1:1 ratio of *C. reinhardtii* and the *R. capsulatus* biosensor was co-cultured in microtitre plates incubated under dark anaerobic conditions, followed by activation of emGFP by O2 exposure. Fluorescent measurements of the co-culture correlated with H2 concentrations in the headspace gas measured by GC.

The *R. capsulatus* biosensor has since been adapted for use on solid media to rapidly test for H2 production by overlaying the biosensor, grown on agar, over single colonies of H2 producing strains (Wecker and Ghirardi, 2014). The biosensor was recently used to assay the O2 tolerance of *Clostridium pasteurianum* CpI mutants expressed heterologously in *C. reinhardtii*, identifying the O2 tolerant CpI<sup>T<sup>356V/S357V</sup></sup> mutant (Elman et al., 2020). The advantage of this assay is that it measures actual H2 production of the cells and not reduction in alternative, non-native substrates.

**Artificial maturation using [2Fe]<sub>H</sub> mimics**

Recently, a HTS assay for recombinant [FeFe] hydrogenases in *E. coli* was described that circumvents the expression of HydEF and HydG (Land et al., 2019). When heterologously expressed in *E. coli*, [FeFe] hydrogenases lacking the [2Fe<sub>H</sub>] subcluster were artificially matured with a [2Fe<sub>H</sub>] subcluster mimic, [Fe<sub>2</sub>(ad) (CO)<sub>4</sub>(CN)<sub>2</sub>(3.6 µmol H2 min<sup>−1</sup> l<sup>−1</sup> OD<sub>600</sub>)<sup>−1</sup>], which led to the discovery that this enzyme has predominantly a H2 sensory function rather than a catalytic function. Further use of these combined techniques will be useful in future efforts to discover more efficient hydrogenases.

**Conclusion and future perspectives**

Many of the fundamental processes that drive H2 production in *C. reinhardtii* have been described. Knowledge of these processes in conjunction with the genetic engineering tools of *C. reinhardtii* and target proteins have been used to improve H2 production in *C. reinhardtii*. The major strategies explored were redirecting electron flow towards H2 production, improving hydrogenase O2 tolerance and using heterologous hydrogenases which may prove to be valuable for industrial biohydrogen production scales. Methods for assaying biological H2 production are also now moving towards HTS which allows mutant libraries or more hydrogenase variants to be more rapidly screened, accelerating the synthetic biology ‘design-build-test’ cycle. Future efforts to improve H2 production in *C. reinhardtii* should try to combine the proposed engineering targets mentioned above and select an appropriate HTS assay to efficiently screen the new constructs. In addition, new tools such as genetic circuits could be developed to couple H2 production to growth or positive selection to support enrichment of mutants with superior H2 producing traits. This use of selective pressure would allow for more randomized, evolutionary engineering approaches such as directed evolution and adaptive laboratory evolution which can rapidly increase the genetic diversity and broaden the
scale of the engineering. Altogether, knowledge of the fundamental processes underlying H₂ production in C. reinhardtii, discovery of O₂ tolerant hydrogenases, ever-evolving synthetic biology tools and development of HTS platforms has the potential to improve biological H₂ production to the point where it is economically viable to produce at an industrial scale.

Acknowledgements

SK is supported by a Macquarie University RTP Scholarship. TJ and KP are supported by the Australian Renewable Energy Agency (ARENA) grant RW0003 awarded to RDW and LB.

Conflict of interest

The authors declare no conflict of interest.

References

Albertini, M., Galazzo, L., Maso, L., Valles, F., Berto, P., De Rosa, E., et al. (2015) Characterization of the [FeFe]-hydrogenase maturation protein HydF by EPR techniques: insights into the catalytic mechanism. *Top Catal* **58**: 708–718.

Antal, T.K., Volgusheva, A.A., Kukarskii, G.P., Krendeleva, T.E., and Rubin, A.B. (2009) Relationships between H₂ photoproduction and different electron transport pathways in sulfur-deprived *Chlamydomonas reinhardtii*. *Int J Hydrog Energy* **34**: 9087–9094.

Bai, Y., Chen, T., Happe, T., Lu, Y., and Sawyer, A. (2018) Iron–sulphur cluster biogenesis via the SUF pathway. *Metalomics* **10**: 1038–1052.

Batyrova, K., and Hallenbeck, P.C. (2017) Hydrogen production by a *Chlamydomonas reinhardtii* strain with inducible expression of photosystem II. *Int J Mol Sci* **18**: 647.

Batyrova, K.A., Tsygankov, A.A., and Kosourov, S.N. (2012) Sustained hydrogen photoproduction in sulfur-deprived *Chlamydomonas reinhardtii*. *Int J Hydrog Energy* **37**: 8834–8839.

Ben-Zvi, O., Dafni, E., Feldman, Y., and Yacoby, I. (2019) Re-routing photosynthetic energy for continuous hydrogen production in vivo. *Biotechnol Biofuels* **12(1)**: 266.

Ben-Zvi, O., and Yacoby, I. (2016) The in-vitro enhancement of FeFe hydrogenase activity by superoxide dismutase. *Int J Hydrog Energy* **41**: 17274–17282.

Berggren, G., Adamska, A., Lambertz, C., Simmons, T.R., Esselborn, J., Atta, M., et al. (2013) Biomimetic assembly and activation of [FeFe]-hydrogenases. *Nature* **499**: 66–69.

Birrell, J.A., Wrede, K., Pawlak, K., Rodríguez-Mací, P., Rüdiger, O., Reijerse, E.J., and Lubitz, W. (2016) Artificial maturation of the highly active heterodimeric [FeFe] hydrogenase from *Desulfovibrio desulfuricans* ATCC 7757. *Isr J Chem* **56**: 852–863.

Blaht, M., Sanchez, E., Fisher, C.E., and Outten, F.W. (2020) Fe-S cluster biogenesis by the bacterial Suf pathway. *Biochim Biophys Acta Mol Cell Res* **1867**: 118829.

Böltcher, B. (2018) En route into chloroplasts: preproteins’ way home. *Photosynth Res* **136**: 263–275.

Britt, R.D., Rai, G., and Tao, L. (2020) Biosynthesis of the catalytic H-cluster of [FeFe] hydrogenase: the roles of the Fe-S maturase proteins HydE, HydF, and HydG. *Chem Sci* **11**: 10313–10323.

Burton, N.A., Padilla, R.V., Rose, A., and Habibullah, H. (2021) Increasing the efficiency of hydrogen production from solar powered water electrolysis. *Renew Sustain Energy Rev* **135**: 110255.

Caserta, G., Adamska-Venkatesh, A., Pecqueur, L., Atta, M., Artero, V., Roy, S., et al. (2016) Chemical assembly of multiple metal cofactors: the heterologously expressed multidomain [FeFe]-hydrogenase from *Megasphaera elsdenii*. *Biochim Biophys Acta Bioenerg* **1856**: 1734–1740.

Castruita, M., Casero, D., Karpowicz, S.J., Kropat, J., Vieler, A., Hsieh, S.I., et al. (2011) Systems biology approach in *Chlamydomonas* reveals connections between copper nutrition and multiple metabolic steps. *Plant Cell* **23**: 1273–1292.

Chochois, V., Dauvville, D., Bely, A., Tolleret, D., Cuine, S., Timpano, H., et al. (2009) Hydrogen production in *Chlamydomonas*: photosystem II-dependent and -independent pathways differ in their requirement for starch metabolism. *Plant Physiol* **151**: 631–640.

Chongdar, N., Birrell, J.A., Pawlak, K., Sommer, C., Reijerse, E.J., Rüdiger, O., et al. (2018) Unique spectroscopic properties of the h-cluster in a putative sensory [FeFe] hydrogenase. *J Am Chem Soc* **140**: 1057–1068.

Coumac, L., Mus, F., Bernard, L., Guedeney, G., Vignais, P., and Peltier, G. (2002) Limiting steps of hydrogen production in *Chlamydomonas reinhardtii* and *Synechocystis* PCC 6803 as analysed by light-induced gas exchange transients. *Int J Hydrog Energy* **27**: 1229–1237.

Crocknell, J.A., Wait, A.F., Lenz, O., Friedrich, B., and Armstrong, F.A. (2009) A kinetic and thermodynamic understanding of O₂ tolerance in [NiFe]-hydrogenases. *Proc Natl Acad Sci USA* **106**: 20681–20686.

Dai, Y., and Outten, F.W. (2012) The E. coli SulS - SulF sulfur transfer system is more resistant to oxidative stress than IscS - Iscu. *FEMS Lett* **16**: 4016–4022.

Diakonova, A.N., Khruishchev, S.S., Kovalenko, I.B., Rizinenko, G.Y., and Rubin, A.B. (2016) Influence of pH and ionic strength on electrostatic properties of ferredoxin, FNR, and hydrogenase and the rate constants of their interaction. *Phys Biol* **13**: 056004.

Dickson, D.J., Page, C.J., and Ely, R.L. (2009) Photobiological hydrogen production from *Synechocystis* sp. PCC 6803 encapsulated in silica sol-gel. *Int J Hydrog Energy* **34**: 204–215.

Dinis, P., Suess, D.L.M., Fox, S.J., Harner, J.E., Driesener, R.C., De La Paz, L., et al. (2015) X-ray crystallographic and EPR spectroscopic analysis of HydG, a maturase in [FeFe]-hydrogenase H-cluster assembly. *Proc Natl Acad Sci USA* **112**: 1362–1367.

Driesener, R.C., Challand, M.R., McGlynn, S.E., Shepard, E.M., Boyd, E.S., Broderick, J.B., et al. (2010) [FeFe]-
hydrogenase cyanide ligands derived from S-adenosylmethionine-dependent cleavage of tyrosine. *Angew Chem Int Ed* **49**: 1687–1690.

Eilenberg, H., Weiner, I., Ben-Zvi, O., Pundak, C., Marmari, A., Liran, O., et al. (2016) The dual effect of a ferredoxin-hydrogenase fusion protein in vivo: successful divergence of the photosynthetic electron flux towards hydrogen production and elevated oxygen tolerance. *Biotechnol Biofuels* **9**: 1–10.

Elman, T., Schweitzer, S., Shahar, N., Swartz, J., and Yacoby, I. (2020) Engineered cloridial [FeFe]-hydrogenase shows improved O2 tolerance in *Chlamydomonas reinhardtii*. *Int J Hydrog Energy* **45**: 30201–30210.

Esselborn, J., Lambertz, C., Adamska-Venkatesh, A., Simmons, T., Berggren, G., Noth, J., et al. (2013) Spontaneous activation of [FeFe]-hydrogenases by an inorganic [2Fe] active site mimic. *Nat Chem Biol* **9**: 607–609.

Esselborn, J., Muraki, N., Klein, K., Engelbrecht, V., Metzler-Nolte, N., Apfel, U.-P., et al. (2016) A structural view of synthetic cofactor integration into [FeFe]-hydrogenases. *Chem Sci* **7**: 959–968.

Fabris, M., Abbriano, R.M., Pernice, M., Sutherland, D.L., Commault, A.S., Hall, C.C., et al. (2020) Emerging technologies in algal biotechnology: toward the establishment of a sustainable, algae-based bioeconomy. *Frontiers in Plant Science* **11**: 279.

Fakhimi, N., Gonzalez-Ballester, D., Fernández, E., Galván, A., and Dubini, A. (2020) Algae-bacteria consortia as a strategy to enhance H2 production. *Cells* **9**: 1353.

Fei, X., and Deng, X. (2007) A novel Fe deficiency-responsive element (FeRE) regulates the expression of atx1 in *Chlamydomonas reinhardtii*. *Plant Cell Physiol* **48**: 1496–1503.

Fischer, N., and Rochaix, J.-D. (2001) The flanking regions of PsaD drive efficient gene expression in the nucleus of the green alga *Chlamydomonas reinhardtii*. *Mol Genet Genom* **265**: 888–894.

Forestier, M., King, P., Zhang, L., Posewitz, M., Schwarzer, S., Happe, T., et al. (2003) Expression of two [Fe]-hydrogenases in *Chlamydomonas reinhardtii* under anaerobic conditions. *Eur J Biochem* **270**: 2750–2758.

Fouchard, S., Hemschemeier, A., Caruana, A., Pruvojost, J., Legrand, J., Happe, T., et al. (2005) Autotrophic and mixotrophic hydrogen photoproduction in sulfur-deprived *Chlamydomonas cells*. *Appl Environ Microbiol* **71**: 6199–6205.

Ghirardi, M.L. (2015) Implementation of photobiological H2 production: the O2 sensitivity of hydrogenases. *Photosynth Res* **152**: 383–393.

Ghysels, B., Godaux, D., Matagne, R.F., Cardol, P., and Franck, F. (2013) Function of the chloroplast hydrogenase in the microalgae chlamydomonas: the role of hydrogenase and state transitions during photosynthetic activation in anaerobiosis. *PLoS One* **8**: e64161.

Gimpel, J.A., Hyun, J.S., Schoepp, N.G., and Mayfield, S.P. (2015) Production of recombinant proteins in microalgae at pilot greenhouse scale. *Biotechnol Bioeng* **112**: 339–345.

Godaux, D., Bailleul, B., Beme, N., and Cardol, P. (2015) Induction of photosynthetic carbon fixation in anoxia relies on hydrogenase activity and proton-gradient regulation. Like1-mediated cyclic electron flow in *Chlamydomonas reinhardtii*. *Plant Physiol* **168**: 648–658.

Godman, J., and Balk, J. (2008) Genome analysis of *Chlamydomonas reinhardtii* reveals the existence of multiple, compartmentalized iron-sulfur protein assembly machineries of different evolutionary origins. *Genetics* **179**(1): 59–68.

Godman, J.E., Molnár, A., Baulcomb, D.C., and Balk, J. (2010) RNA silencing of hydrogenase-(like) genes and investigation of their physiological roles in the green alga *Chlamydomonas reinhardtii*. *Biochem J* **431**: 345–351.

Gomez-Casati, D.F., Busi, M.V., Barchiesi, J., Pagani, M.A., Marchetti-Acosta, N.S., and Terenzi, A. (2021) Fe-S protein synthesis in green algae mitochondria. *Plants* **10**: 200.

Gonzalez-Ballester, D., Casero, D., Cokus, S., Pellegrini, M., Merchant, S.S., and Grossman, A.R. (2010) RNA-Seq analysis of sulfur-deprived *Chlamydomonas* cells reveals aspects of acclimation critical for cell survival. *Plant Cell* **22**: 2058–2084.

Greiner, A., Kelterborn, S., Evers, H., Kreimer, G., Sizova, I., and Hegemann, P. (2017) Targeting of photonceptor genes in *Chlamydomonas reinhardtii* via zinc-finger nucleases and CRISPR/Cas9. *Plant Cell* **29**: 2498–2518.

Gutekunst, K., Hoffmann, D., Westerbro, U., Schulz, R., Garbe-Schönberg, D., and Appel, J. (2018) In-vivo turnover frequency of the cyanobacterial NiFe-hydrogenase during photohydrogen production outperforms in-vitro systems. *Sci Rep* **8**(1): 6083.

Guzmán-Zapata, D., Sandoval-Vargas, J., Macedo-Osorio, K., Salgado-Manjarrez, E., Castrojón-Flores, J., Oliver-Salvador, M., et al. (2019) Efficient editing of the nuclear APT reporter gene in chlamydomonas reinhardtii via expression of a CRISPR-Cas9 module. *Int J Mol Sci* **20**: 1247.

Happe, T., Mosler, B., and Naber, J.D. (1994) Induction, localization and metal content of hydrogenase in the green alga *Chlamydomonas reinhardtii*. *Eur J Biochem* **222**: 769–774.

Happe, T., and Naber, J.D. (1993) Isolation, characterization and N-terminal amino acid sequence of hydrogenase from the green alga *Chlamydomonas reinhardtii*. *Eur J Biochem* **214**: 475–481.

Hemschemeier, A., and Happe, T. (2011) Alternative photosynthetic electron transport pathways during anaerobiosis in the green alga *Chlamydomonas reinhardtii*. *Biochim Biophys Acta Bioenerg* **1807**: 919–926.

Hertle, A.P., Blunder, T., Wunder, T., Pesaresi, P., Pribil, M., Armbruster, U., and Leister, D. (2013) PGRL1 is the elusive ferredoxin-plastoquinone reductase in photosynthetic cyclic electron flow. *Mol Cell** **49**: 511–523.

IEA (2019) *The Future of Hydrogen*. Paris: IEA. URL: https://www.iea.org/Reports/the-future-of-hydrogen.

Jagadevan, S., Banerjee, A., Banerjee, C., Guria, C., Tiwari, R., Baweja, M., and Shukla, P. (2018) Recent developments in synthetic biology and metabolic engineering in microalgae towards biofuel production. *Biotechnol Biofuels* **11**: 185.

Johnson, X., Steinbeck, J., Dent, R.M., Takahashi, H., Richaud, P., Ozawa, S.-I., et al. (2014) Proton gradient
Kanygin, A., Milrad, Y., Thummala, C., Reifschneider, K., Baker, P., Marco, P., et al. (2020) Rewiring photosynthesis: a photosystem I-hydrogenase chimera that makes H₂ in vivo. *Energy Environ Sci* **13**(9): 2903–2914.

Kertess, L., Adamska-Venkatesh, A., Rodriguez-Macía, P., Rudiger, O., Lubitz, W., and Happe, T. (2017) Influence of the [4Fe-4S] cluster coordinating cysteines on active site maturation and catalytic properties of *C. reinhardtii* [FeFe]-hydrogenase. *Chem Sci* **8**: 8127–8137.

Koo, J., Schnabel, T., Liong, S., Evitt, N.H., and Swartz, J.R. (2018) System analysis and improved [FeFe] hydrogenase O₂ tolerance suggest feasibility for photosynthetic H₂ production. *Metab Eng* **49**: 21–27.

Kosourova, S., Jokel, M., Aro, E.-M., and Allahverdiyeva, Y. (2018) A new approach for sustained and efficient H₂ photoproduction by *Chlamydomonas reinhardtii*. *Energy Environ Sci* **11**: 1431–1436.

Kosourov, S., Nagy, V., Shevela, D., Jokel, M., Messinger, J., and Allahverdiyev, Y. (2020) Water oxidation by photosystem II is the primary source of electrons for sustained H₂ photoproduction in nutrient-replete green algae. *Proc Natl Acad Sci USA* **117**: 29629–29636.

Kosourov, S., Seibert, M., and Ghirardi, M.L. (2003) Effects of extracellular pH on the metabolic pathways in sulfur-deprived, H₂-producing *Chlamydomonas reinhardtii* cultures. *Plant Cell Physiol* **44**: 146–155.

Kruse, O., Rupprech, J., Bader, K.-P., Thomas-Hall, S., Schen, P.M., Finazzi, G., and Harkamer, B. (2005) Improved photosynthetic H₂ production in engineered green algal cells. *J Biol Chem* **280**: 34170–34177.

Land, H., Ceccaldi, P., Meszaros, L.S., Lorenzi, M., Redman, H.J., Senger, M., et al. (2019) Discovery of novel [FeFe]-hydrogenases for biocatalytic H₂-production. *Chem Sci* **10**: 9941–9948.

Land, H., Senger, M., Berggren, G., and Stripp, S.T. (2020) Current state of [FeFe]-hydrogenase research: biodiversity and spectroscopic investigations. *ACS Catal* **10**: 7069–7086.

Lauersen, K.J., Kruse, O., and Mussgnug, J.H. (2015) Targeted expression of nuclear transgenes in *Chlamydomonas reinhardtii* with a versatile, modular vector toolkit. *Appl Microbiol Biotechnol* **99**: 3491–3503.

Ley, M.B., Jepsen, L.H., Lee, Y.-S., Cho, Y.W., Bellosta von Colbe, J.M., Domhein, M., et al. (2014) Complex hydrides for hydrogen storage – new perspectives. *Mater Today* **17**: 122–128.

Liran, O., Semyatic, R., Milrad, Y., Eilenberg, H., Weiner, I., and Yacoby, I. (2016) Microoxic niches within the thylakoid stroma of air-grown *Chlamydomonas reinhardtii* protect [FeFe]-hydrogenase and support hydrogen production under fully aerobic environment. *Plant Physiol* **172**: 264–271.

Lukey, M.J., Roessler, M.M., Parkin, A., Evans, R.M., Davies, R.A., Lenz, O., et al. (2011) Oxygen-tolerant [NiFe]-hydrogenases: the individual and collective importance of supernumerary cysteines at the proximal Fe-S cluster. *J Am Chem Soc* **133**: 16881–16892.

Madden, C., Vaughn, M.D., Diez-Pérez, I., Brown, K.A., King, P.W., Gust, D., et al. (2012) Catalytic turnover of [FeFe]-hydrogenase based on single-molecule imaging. *J Am Chem Soc* **134**: 1577–1582.

Melis, A., Zhang, L., Forestier, M., Ghirardi, M.L., and Seibert, M. (2000) Sustained photobiological hydrogen gas production upon reversible inactivation of oxygen evolution in the green alga *Chlamydomonas reinhardtii*. *Plant Physiol* **122**: 127–135.

Meuser, J.E., D’Adamo, S., Jinkerson, R.E., Mus, F., Yang, W., Ghirardi, M.L., et al. (2012) Genetic disruption of both *Chlamydomonas reinhardtii* [FeFe]-hydrogenases: Insight into the role of HYDA2 in H₂ production. *Biochem Biophys Res Commun* **417**: 704–709.

Milrad, Y., Schweitzer, S., Feldman, Y., and Yacoby, I. (2018) Green algal hydrogenase activity is outcompeted by carbon fixation before inactivation by oxygen takes place. *Plant Physiol* **177**: 918–926.

Mislov, D., Cifrek, M., Krois, I., and Dzapo, H. (2015) Measurement of dissolved hydrogen concentration with Clark electrode, 2015 IEEE Sensors Applications Symposium (SAS): 1-5.

Mulder, D.W., Boyd, E.S., Samra, R., Lange, R.K., Endrizzi, J.A., Broderick, J.B., and Peters, J.W. (2010) Stepwise [FeFe]-hydrogenase H-cluster assembly revealed in the structure of HydA<sub>lEF<sub>G</sub></sub>. *Nature* **465**: 248–252.

Mulder, D.W., Guo, Y., Ratzlaff, M.W., and King, P.W. (2017) Identification of a catalytic iron-hydride at the H-Cluster of [FeFe]-hydrogenase. *J Am Chem Soc* **139**: 83–86.

Mulder, D.W., Ortiz, D.O., Gardenghi, D.J., Naumov, A.V., Ruebush, S.S., Szilagyi, R.K., et al. (2009) Activation of HydA<sub>lEF<sub>G</sub></sub> requires a preformed [4Fe-4S] cluster. *Biochemistry* **48**: 6240–6248.

Mus, F., Cournac, L., Cardetti, V., Caruana, A., and Pelletier, G. (2005) Inhibitor studies on non-photochemical plastoquinone reduction and H₂ photoproduction in *Chlamydomonas reinhardtii*. *Biochim Biophys Acta Bioenerg* **1708**: 322–332.

Nagy, V., Vidal-Meireles, A., Podmaniczki, A., Szentmihalyi, K., Rakhey, G., Zsigmond, L., et al. (2018) The mechanism of photosystem-II inactivation during sulphur deprivation-induced H₂ production in *Chlamydomonas reinhardtii*. *Plant J* **94**: 548–561.

Nakahira, Y., Ogawa, A., Asano, H., Oyama, T., and Tazawa, Y. (2013) How [Fe]-hydrogenase metabolizes dihydrides for hydrogen storage. *Nat Catal* **2**: 481–482.

Nicolò, G. and Miele, G. (2009) How [Fe]-hydrogenase metabolizes dihydrogen. *Nat Catal* **2**: 481–482.

Nicolò, G., Heilmann, C., Hewat, S., Gäbelein, P., and Hippler, M. (2018) Absolute quantification of selected photosynthetic electron transfer proteins in *Chlamydomonas*
reinhardtii in the presence and absence of oxygen. Photosynth Res 137: 281–293.
Noone, S., Ratcliff, K., Davis, R.A., Subramanian, V., Meuser, J., Posewitz, M.C., et al. (2017) Expression of a cislabyrinthine [FeFe]-hydrogenase in Chlamydomonas reinhardtii prolongs photo-production of hydrogen from water splitting. Algal Res 22: 116–121.
Noth, J., Krawietz, D., Hemscheier, A., and Happe, T. (2013) Pyruvate ferredoxin oxidoreductase is coupled to light-independent hydrogen production in Chlamydomonas reinhardtii. J Biol Chem 288: 4368–4377.
Outten, F.W., Wood, M.J., Munoz, F.M., and Storz, G. (2003) The SuF protein and the SuFBCD complex enhance sfs cysteine desulfurase activity as part of a sulfur transfer pathway for Fe-S cluster assembly in Escherichia coli. J Biol Chem 278: 45713–45719.
Paila, Y.D., Richardson, L.G.L., and Schnell, D.J. (2015) New insights into the mechanism of chloroplast protein import and its integration with protein quality control, organelle biogenesis and development. J Mol Biol 13: 1038–1060.
Pape, M., Lambertz, C., Happe, T., and Hemscheier, A. (2012) Differential expression of the Chlamydomonas [FeFe]-hydrogenase-encoding HYDA1 gene is regulated by the copper response regulator1. Plant Physiol 159: 1700–1712.
Pelmenschikov, V., Birrell, J.A., Pham, C.C., Mishra, N., Wang, H., Sommer, C., et al. (2017) Reaction coordinate leading to H2 production in [FeFe]-hydrogenase identified by nuclear resonance vibrational spectroscopy and density functional theory. J Am Chem Soc 139: 16894–16902.
Pérard, J., and Ollagnier de Choudens, S. (2018) Iron–sulfur clusters biogenesis by the SUF machinery: close to the molecular mechanism understanding. J Biol Inorg Chem 23: 581–596.
Peters, J.W., Schut, G.J., Boyd, E.S., Mulder, D.W., Shepard, E.M., Broderick, J.B., et al. (2015) [FeFe]- and [NiFe]-hydrogenase diversity, mechanism, and maturation. Biochim Biophys Acta Mol Cell Res 1853: 1350–1369.
Petrova, E.V., Kukarskich, G.P., Krendeleva, T.E., and Antal, K. (2020) The mechanisms and role of photosynthetic hydrogen production by green microalgae. Microbiology 89: 251–265.
Pollie, J.E.W., Benemann, J.R., Tanaka, A., and Melis, A. (2000) Photosynthetic apparatus organization and function in the wild type and a chlorophyll b-less mutant of Chlamydomonas reinhardtii. dependence on carbon source. Planta 211: 335–344.
Posewitz, M.C., King, P.W., Smolinski, S.L., Zhang, L., Seibert, M., and Ghiardi, M.L. (2004a) Discovery of two novel radical S-adenosylmethionine proteins required for the assembly of an active [Fe] hydrogenase. J Biol Chem 24: 25711–25720.
Posewitz, M.C., Smolinski, S.L., Kanakagiri, S., Melis, A., Seibert, M., and Ghiardi, M.L. (2004b) Hydrogen photoproduction is attenuated by disruption of an isoamylase gene in Chlamydomonas reinhardtii. Plant Cell 16: 2151–2163.
Rangachandran, R., and Menon, R.K. (1998) An overview of industrial uses of hydrogen. Int J Hydrog Energy 23: 593–598.
Rao, G., Tao, L., and Brit, R.D. (2020) Serine is the molecular source of the NH(CH2)2 bridgehead moiety of the in vitro assembled [FeFe] hydrogenase H-cluster. Chem Sci 11: 1241–1247.
Reifschneider-Wegner, K., Kanygin, A., and Redding, K.E. (2014) Expression of the [FeFe] hydrogenase in the chloroplast of Chlamydomonas reinhardtii. Int J Hydrog Energy 39: 3657–3665.
Reijerse, E.J., Pham, C.C., Pelmenschikov, V., Gilbert-Wilson, R., Adamska-Venkatesh, A., Siebel, J.F., et al. (2017) Direct observation of an iron-bound terminal hydride in [FeFe]-hydrogenase by nuclear resonance vibrational spectroscopy. J Am Chem Soc 139: 4306–4309.
Rodríguez-Macia, P., Breuer, N., DeBeer, S., and Birrell, J.A. (2020) Insight into the redox behavior of the [4Fe–4S] subcluster in [FeFe] hydrogenases. ACS Catal 10: 13084–13095.
Rumpel, S., Siebel, J.F., Fairé, C., Duan, J., Reijerse, E., Happe, T., et al. (2014) Enhancing hydrogen production of microalgae by redirecting electrons from photosystem I to hydrogenase. Energy Environ Sci 7: 3296–3301.
Salomé, P.A., and Merchant, S.S. (2019) A series of fortunate events: introducing chlamydomonas as a reference organism. Plant Cell 31: 1682–1707.
Sasso, S., Stibor, H., Mittag, M., and Grossman, A.R. (2018) From molecular manipulation of domesticated Chlamydomonas reinhardtii to survival in nature. eLife 7: e39233. doi: 10.7554/eLife.39233.001
Sawyer, A., Bai, Y., Lu, Y., Hemscheier, A., and Happe, T. (2017) Compartmentalisation of [FeFe]-hydrogenase maturation in Chlamydomonas reinhardtii. Plant J 90: 1134–1143.
Sawyer, A., and Winkler, M. (2017) Evolution of Chlamydomonas reinhardtii ferredoxins and their interactions with [FeFe]-hydrogenases. Photosynth Res 134: 307–316.
Schrader, P.S., Burrows, E.H., and Ely, R.L. (2008) High-throughput screening assay for biological hydrogen production. Anal Chem 80: 4014–4019.
Scranton, M.A., Ostrand, J.T., Georgianna, D.R., Lofgren, S.M., Li, D., Ellis, R.C., et al. (2016) Synthetic promoters capable of driving robust nuclear gene expression in the green alga Chlamydomonas reinhardtii. Algal Res 15: 135–142.
Selbach, B.P., Pradhan, P.K., and Santos, P.C.D. (2013) Protected sulfur transfer reactions by the Escherichia coli sul system. Biochemistry 52: 4089–4096.
Shepard, E.M., Duffus, B.R., George, S.J., McGlynn, S.E., Challand, M.R., Swanson, K.D., et al. (2010) [FeFe]-hydrogenase maturation: HydG-catalyzed synthesis of carbon monoxide. J Am Chem Soc 132: 9247–9249.
Shin, M. (2004) How is ferredoxin-NADP reductase involved in the NADP photoreduction of chloroplasts? Photosynth Res 80: 307–313.
Shin, S.-E., Lim, J.-M., Koh, H., Kim, E.K., Kang, N.K., Jeon, S., et al. (2016) CRISPR/Cas9-induced knockout and knock-in mutations in Chlamydomonas reinhardtii. Sci Rep 6: 27810.
Siebert, M., Benson, D.K., and Flynn, T.M. (2001) Method and Apparatus for Rapid Biohydrogen Phenotypic Screening of Microorganisms Using a Chemochromatic Sensor.
Takahashi, Y., and Tokumoto, U. (2002) A third bacterial system for the assembly of iron-sulfur clusters with homologs in archaea and plastids. J Biol Chem 277: 28380–28383.

Swanson, K.D., Ratzloff, M.W., Mulder, D.W., Artz, J.H., Ghose, S., Hoffman, A., et al. (2015) [FeFe]-hydrogenase oxygen inactivation is initiated at the H cluster 2Fe subcluster. J Am Chem Soc 137: 1809–1816.

Takabayashi, A., Kishine, M., Asada, K., Endo, T., and Sato, F. (2005) Differential use of two cyclic electron flows around photosystem I for driving CO2-concentration mechanism in C4 photosynthesis. Proc Natl Acad Sci USA 102: 16896–16903.

Takahashi, Y., and Tokumoto, U. (2002) A third bacterial system for the assembly of iron-sulfur clusters with homologs in archaea and plastids. J Biol Chem 277: 28380–28383.

Tao, L., Pattenaude, S.A., Joshi, S., Begley, T.P., Rauchfuss, T.B., and Britt, R.D. (2020) Radical SAM enzyme hyde generates adenosylated Fe(I) intermediates En route to the [FeFe]-hydrogenase catalytic H-Cluster. J Am Chem Soc 142: 10841–10848.

Tolleter, D., Ghysels, B., Alric, J., Petroutsos, D., Tolstyagina, I., Krawietz, D., et al. (2011) Control of hydrogen production by the proton gradient generated by cyclic electron flow in Chlamydomonas reinhardtii. Plant Cell 23: 2619–2630.

Volgusheva, A., Kukarskikh, G., Krendeleva, T., Rubin, A., and Mamedov, F. (2015) Hydrogen photoproduction in green algae Chlamydomonas reinhardtii under magnesium deprivation. RSC Adv 5: 5633–5637.

Wecker, M.S.A., and Ghirardi, M.L. (2014) High-throughput biosensor discriminates between different algal H2-photoproducing strains. Biotechnol Bioeng 111: 1332–1340.

Wecker, M.S.A., Meuser, J.E., Posewitz, M.C., and Ghirardi, M.L. (2011) Design of a new biosensor for algal H2 production based on the H2-sensing system of Rhodobacter capsulatus. Int J Hydrog Energy 36: 11229–11237.

Weijun, Y. (2015) Analytical accuracy of hydrogen measurement using gas chromatography with thermal conductivity detection. J Sep. Science 38: 2640–2646.

Wichmann, J., Lauer sen, K.J., and Kruse, O. (2020) Green algal hydrocarbon metabolism is an exceptional source of sustainable chemicals. Curr Opin Biotechnol 61: 28–37.

Wiegand, K., Winkler, M., Rumpel, S., Kannchen, D., Rexroth, S., Hase, T., et al. (2018) Rational redesign of the ferredoxin-NADP+-oxidoreductase/ferredoxin-interaction for photosynthesis-dependent H2-production. Biochim Biophys Acta Bioenerg 1859: 253–262.

Winkler, M., Duan, J., Rutz, A., Felbik, C., Scholtysek, L., Lampret, O., et al. (2021) A safety cap protects hydrogenase from oxygen attack. Nat Commun 12(1): 756.

Wu, B., Matian, M., and Offer, G.J. (2012) Hydrogen PEMFC system for automotive applications. Int J Low-Carbon Technol 7: 28–37.

Xu, X.M., and Möller, S.G. (2011) Iron-sulfur clusters: Biogenesis, molecular mechanisms, and their functional significance. Antioxid Redox Signal 15: 271–307.

Yacoby, I., Poche Balkov, S., Topork, H., Ghirardi, M.L., King, P.W., and Zhang, S. (2011) Photosynthetic electron partitioning between [FeFe]-hydrogenase and ferredoxin: NADP+-oxidoreductase (FNR) enzymes in vitro. Proc Natl Acad Sci USA 108: 9396–9401.

Zanetti, G., and Pandini, V. (2013) Ferredoxin. In Encyclopedia of Biological Chemistry. Lennarz, W.J., and Lane, M.D. (eds). Cambridge, MA: Academic Press, pp. 286–298.