Received: 7 January 2021 | Accepted: 5 April 2021
DOI: 10.1111/ppl.13428

SPECIAL ISSUE ARTICLE

Physiologia Plantarum

Photosynthetic hydrogen production: Novel protocols, promising engineering approaches and application of semi-synthetic hydrogenases

Sergey Kosourov[1] | Maximilian Böhm[2] | Moritz Senger[2] | Gustav Berggren[2] | Karin Stensjö[3] | Fikret Mamedov[2] | Peter Lindblad[3] | Yagut Allahverdiyeva[1]

[1]Molecular Plant Biology, Department of Life Technologies, University of Turku, Turku, Finland

[2]Molecular Biomimetics, Department of Chemistry-Ångström, Ångström Laboratory, Uppsala University, Uppsala, Sweden

[3]Microbial Chemistry, Department of Chemistry-Ångström, Ångström Laboratory, Uppsala University, Uppsala, Sweden

Correspondence
Sergey Kosourov and Yagut Allahverdiyeva, Molecular Plant Biology, Department of Life Technologies, University of Turku, FI-20014 Turku, Finland.
Email: serkos@utu.fi and allahve@utu.fi

Peter Lindblad, Microbial Chemistry, Department of Chemistry-Ångström, Ångström Laboratory, Uppsala University, SE-75120 Uppsala, Sweden.
Email: peter.lindblad@kemi.uu.se

Funding information
Carl Tryggers Foundation, Grant/Award Number: CTS 19:230; Energimyndigheten, Grant/Award Number: 48574-1; FP7: European Research Council, Grant/Award Number: 714102; H2020 Future and Emerging Technologies, Grant/Award Number: 899576; H2020 Marie Skłodowska-Curie Actions, Grant/Award Number: 897555; Koneen Säätiö, Grant/Award Number: 201608799; NordForsk, Grant/Award Number: 82845

Edited by: C. Funk

Abstract

Photosynthetic production of molecular hydrogen (H_2) by cyanobacteria and green algae is a potential source of renewable energy. These organisms are capable of water biophotolysis by taking advantage of photosynthetic apparatus that links water oxidation at Photosystem II and reduction of protons to H_2 downstream of Photosystem I. Although the process has a theoretical potential to displace fossil fuels, photosynthetic H_2 production in its current state is not yet efficient enough for industrial applications due to a number of physiological, biochemical, and engineering barriers. This article presents a short overview of the metabolic pathways and enzymes involved in H_2 photoproduction in cyanobacteria and green algae and our present understanding of the mechanisms of this process. We also summarize recent advances in engineering photosynthetic cell factories capable of overcoming the major barriers to efficient and sustainable H_2 production.

1 | INTRODUCTION

Molecular hydrogen (H_2) represents the highest energy density per mass unit among currently employed fuels and is a zero-carbon emission solution for the sustainable economy. Besides excellent properties as a fuel, H_2 is an important feedstock for many industrial processes, including methanation of CO and CO_2, methanol synthesis from CO_2 hydrogenation, and hydrogenation of nitrogen (N_2) for the production of ammonia. H_2 is also used as a reducing agent in a number of different applications. Compared to conventional methods of H_2 generation, photobiological H_2 production represents an attractive renewable alternative since it utilizes water and solar light, the world's most abundant resources.

Many species of cyanobacteria and eukaryotic green algae are capable of photosynthetic water splitting to H_2 and oxygen (O_2) under specific conditions using dedicated enzymes. Although a

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
© 2021 The Authors. *Physiologia Plantarum* published by John Wiley & Sons Ltd on behalf of Scandinavian Plant Physiology Society.

theoretical maximum of light energy to H_2 energy conversion efficiency (LHCE) is as high as 10–13% for green algae and 6% for N_2-fixing cyanobacteria, only a fraction of these values has been achieved (1.5–2% in algae and up to 4% in cyanobacteria) under controlled laboratory conditions typically employing low light illumination (Bolton & Hall, 1991; Kosourov et al., 2017; Sakurai et al., 2015). A recent study showed that green alga *Chlorella vulgaris* is capable of producing H_2 with the maximum LHCE up to 8% (3% on the average basis), though in the presence of glucose (Touloupakis et al., 2021). Importantly, volumetric H_2 yields in algal and cyanobacterial cultures are still quite low even in the controlled environment due to a number of physiological and biochemical barriers limiting H_2 production rates.

A deep understanding of the photosynthetic H_2 metabolism and its effect on cell bioenergetics opens new possibilities for the development of H_2-producing cell factories with improved performance and enhanced H_2 production yields. This mini-review will summarize recent advances in understanding the mechanisms of light-driven H_2 production in cyanobacteria and green algae and give a short overview of strategies and approaches for engineering efficient H_2-producing cell factories, including whole-cell applications of semi-synthetic hydrogenases.

2 | A SHORT OVERVIEW OF H_2 METABOLISM IN CYANOBACTERIA

Cyanobacteria are a large and diverse group of oxygenic photoautotrophic prokaryotes. Many of them have an innate capacity to produce H_2. Cyanobacteria have two sets of enzymes involved in H_2 metabolism: nitrogenases and hydrogenases (Figure 1A,C). Nitrogenases (encoded by *nifHDK*) are found in N_2-fixing unicellular or filamentous cyanobacteria, which produce H_2 as a byproduct of N_2 fixation and its reduction into ammonia. Cyanobacteria also possess two types of [NiFe]-hydrogenases: bidirectional hydrogenase (encoded by *hoxEFUYH*) and uptake hydrogenase (encoded by *hupSL*) enzymes (Barz et al., 2010; Lindblad, 2018; Puggioni et al., 2016). Unlike uptake hydrogenase, which recycles H_2 produced by nitrogenase, the bidirectional hydrogenase both oxidizes and produces H_2 and can be present in diazotrophic and non-diazotrophic strains. Since O_2 is a byproduct of photosynthesis and all enzymes involved in H_2 metabolism are O_2 sensitive, cyanobacteria have developed spatial and temporal strategies for their protection: (1) heterocystous filamentous cyanobacteria spatially separate oxygenic photosynthesis (occurring in vegetative cells) and N_2 fixation (occurring in heterocysts, specialized cells that provide a low-oxygen environment) and

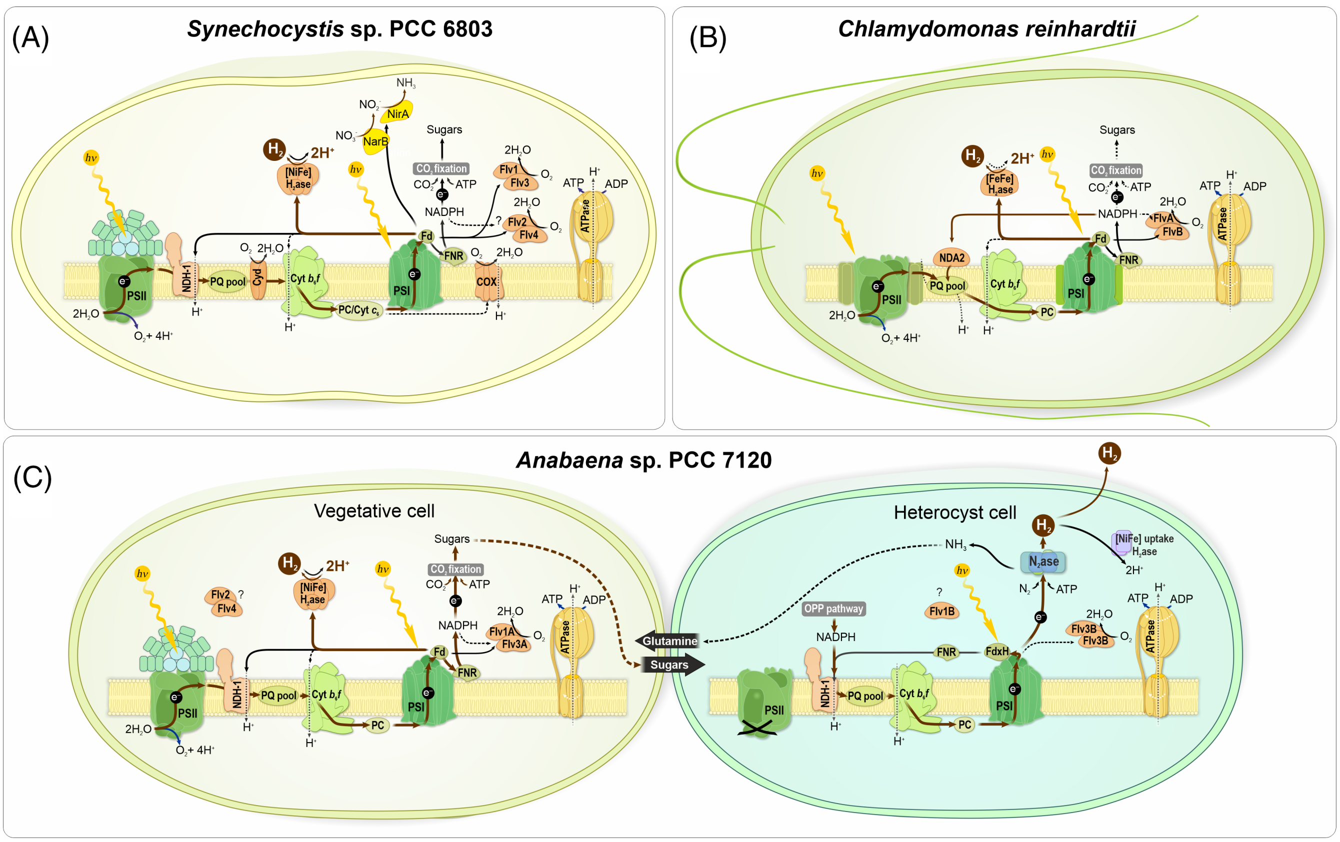

FIGURE 1 A schematic representation of the H_2 photoproduction (brown lines) and competing (black lines) metabolic pathways in (A) unicellular cyanobacterium *Synechocystis* sp. PCC 6803, (B) green alga *Chlamydomonas reinhardtii*, and (C) N_2-fixing heterocystous cyanobacterium *Anabaena* sp. PCC 7120

(2) non-heterocystous cyanobacteria (e.g., filamentous *Trichodesmium*, unicellular *Cyanothece*) perform N_2 fixation, and thus H_2 production, typically under anoxic conditions but also under oxic conditions (e.g., due to the control of their circadian rhythm or intracellular compartmentation). The processes that support H_2 production in these organisms under oxic conditions have yet to be fully resolved. In contrast, the bidirectional hydrogenase particularly operates under microoxic or anaerobic environment and could utilize reductants produced directly by the photosynthetic electron transport chain (PETC). Cyanobacteria bearing this enzyme typically photoproduce H_2 on exposure to light, but only for a very short period followed by H_2 uptake. The O_2 sensitivity of the bidirectional hydrogenase thus represents a major biotechnological hurdle for efficient H_2 photoproduction via this enzyme.

3 | A SYSTEM BIOLOGY APPROACH TO UNDERSTANDING THE MECHANISM OF H_2 PRODUCTION IN CYANOBACTERIA

To achieve a greater understanding of how cell metabolism and bioenergetics navigate during H_2 photoproduction and to identify potential targets for further metabolic engineering, a system-level omics approach, which includes genomics, transcriptomics, proteomics, and metabolomics analysis, is necessary. Most of the omics approaches have been focused on understanding the metabolism of nitrogenase-based H_2 production (see, e.g., Aryal et al., 2013; Bernstein et al., 2015; Ekman et al., 2011; Kourpa et al., 2019; Sadler et al., 2016; Stensjö et al., 2007). The differential proteomes of N_2-fixing and non-N_2-fixing cultures of both *Nostoc* sp. and *Cyanothece* sp. have been resolved, and the heterocyst-specific Cys-proteome was detailed (Sandh et al., 2014). The reprograming of metabolism in the H_2-producing mutant (ΔHupL) of *Nostoc* (also known as *Anabaena*) sp. PCC 7120, which lacks the uptake hydrogenase in heterocysts (Figure 1C), involved >100 differentially expressed proteins (Ekman et al., 2011). Also, in the unicellular diazotrophic cyanobacterium *Cyanothece* sp., which produces H_2 at very high rates (Bandyopadhyay et al., 2010), proteomics was used to identify targets for improving H_2 production yields (Aryal et al., 2013). General important factors to drive nitrogenase-dependent H_2 production in both *Cyanothece* sp. and *Nostoc* sp. were (1) to provide sugars, that is, glycogen oxidation in the dark for *Cyanothece* and import of sucrose from the vegetative cell to heterocysts in *Nostoc*; (2) to provide enough reducing equivalents by an efficient oxidative pentose phosphate pathway; and (3) to provide ATP by cyclic electron flow (CEF) around Photosystem I (PSI).

For *Cyanothece*, this view was later challenged when it was shown that the defined key components of H_2 metabolism in *Cyanothece* differed substantially from that in *Nostoc*. For example, a positive quantitative correlation between the H_2 production profile and the abundance of proteins of Photosystem II (PSII) was demonstrated (Bernstein et al., 2015). This suggests that in contrast to heterocystous cyanobacteria, the PSII-driven linear electron flow in unicellular *Cyanothece* directly provides reducing equivalents and ATP for nitrogenase-dependent H_2 photoproduction. These findings bring new strategies for future metabolic engineering and optimization of growth conditions for efficient and sustainable photobiological H_2 production.

Although the usefulness of multi-omics and in silico strategies are well accepted, the attempts to use them for designing H_2-producing cyanobacteria are still infrequent. One of the delays in the effort to use systems biology as a fundament for bioengineering is the lack of genome-scale metabolic models. Recently, two such models have been constructed to predict pathways and protein targets for improvement of H_2 photoproduction in the diazotrophic heterocyst-forming model organisms, *Anabaena* sp. PCC 7120 (Malatinszky et al., 2017) and *Anabaena variabilis*sp. ATCC 29413 (Malek Shahkouhi & Motamedian, 2020). The in silico studies, for the most part, confirmed the targets of importance for improving heterocyst-based H_2 production emphasized in the quantitative proteomics investigations. Thus, the initial simulations and modeling attempts display the value for further development of these methods.

4 | ENGINEERING CYANOBACTERIA FOR SUSTAINABLE AND EFFICIENT H_2 PRODUCTION

Since cyanobacteria are capable of catalyzing H_2 photoproduction by the direct (e.g., in unicellular cyanobacteria via bidirectional hydrogenase) and indirect water biophotolysis (e.g., in N_2-fixing heterocystous cyanobacteria via nitrogenase localized in heterocysts) mechanisms, challenges and engineering strategies for improving H_2 photoproduction yields also differ to some extent. In general, the major metabolic bottlenecks of cyanobacterial H_2 production include (1) high O_2 sensitivity of the enzymes involved in H_2 metabolism (nitrogenases and/or hydrogenases), (2) low photosynthetic yield, (3) alternative electron transport pathways competing for photosynthetic electrons, and (4) H_2 uptake by the cells (via bidirectional or uptake hydrogenases). Therefore, the following challenges are to be addressed: (1) modulation of competing pathways for boosting the H_2 production yield without compromising cell fitness; (2) improvement of photosynthesis, including optimization of NADPH/ATP ratio, for efficient bioproduction; (3) screening available cyanobacterial culture collections; (4) mining and introducing novel, more efficient, and O_2-tolerant enzymes; and (5) development of novel cultivation protocols for efficient H_2 production. Throughout the years, there have been numerous attempts to overcome these barriers and challenges by employing a diversity of genetic engineering approaches. Here we focus on the most recent and novel advances in engineering cyanobacteria for sustainable H_2 photoproduction.

4.1 | Deletion of the capacity to take up hydrogen

There are many examples in the literature of either disrupting or deleting a structural gene, or a gene encoding a protein of the maturation machinery, of the uptake HupSL hydrogenase in cyanobacteria (see, e.g., Lindblad, 2018). The result will be H_2-producing cells, where H_2 evolution is catalyzed by a native nitrogenase. Using a ΔHupW strain of *Anabaena* sp. PCC 7120 (a strain lacking the last step in the maturation machinery) analyzed in a 5.0 L flat-panel photobioreactor system with a 3.0 L culture volume and illuminated with a mixture of red and white LED lights, Nyberg et al. (2015) showed a maximal LHCE of ca. 4.0% (in the PAR region) and a maximal hydrogen concentration of 6.89%. However, it is also important to note that inactivation of uptake hydrogenase reprograms cell metabolism (Ekman et al., 2011; Kourpa et al., 2019) and may significantly decrease the duration of the H_2 production process and cell fitness, particularly under a high C/N environment (Kosourov et al., 2014).

Another approach is to modify the flow of electrons from H_2 uptake toward H_2 evolution in the HupSL hydrogenase. The NiFe-containing active site is present in the large subunit, whereas the small subunit (HupS) harbors three FeS-clusters. HupS of the filamentous cyanobacterium *Nostoc punctiforme* ATCC 29133 was heterologously expressed as a fusion protein in *Escherichia coli* (f-HupS), purified, and characterized (Raleiras et al., 2013). Thereafter, the proximal Fe-S cluster of HupS was modified by changing the cysteine in position 12 to proline (C12P). C12P f-HupS investigated by EPR spectroscopy demonstrated a conversion from a 4Fe4S to a 3Fe4S proximal cluster. C12P HupSL was then introduced and expressed in *N. punctiforme*, which resulted in cells with increased H_2 production. The modified proximal Fe-S cluster in HupS changed the flow of electrons toward hydrogen production (Raleiras et al., 2016).

4.2 | Modulation of alternative electron transport routes

Regardless of hydrogenase, a steady flow of electrons to the hydrogenase enzyme, provided by primary electron donors such as reduced ferredoxin, is needed for efficient and sustainable photobiological H_2 production (Gutekunst et al., 2014). However, there is no "bag-of-electrons"; the available photosynthetic electrons are utilized in other assimilatory pathways, such as the respiratory electron transport system, nitrate assimilation, and carbon fixation via the Calvin–Benson–Bassham (CBB) cycle. From a biotechnological aspect, these are competing routes. Moreover, alternative electron transport pathways such as the flavodiiron proteins (FDPs)-driven water–water cycle and the cyclic electron transport around PSI can be considered as "waste" points for photosynthetic production. Therefore, a genetic engineering strategy should be applied for re-directing the electron flow away from the existing native assimilatory and auxiliary pathways toward H_2 production. This has been experimentally demonstrated in *Synechocystis* sp. PCC 6803 by eliminating the electron flow to (1) the respiratory terminal oxidases (Gutthann et al., 2007), (2) the nitrate-assimilation pathway (Baebprasert et al., 2011), and (3) the NDH-1 complex involved in CEF, respiration, and carbon concentrating mechanism (Cournac et al., 2004). These examples all resulted in increased H_2 production yields. Specifically, removing nitrate from the growth medium, or creating strains lacking a functional pathway to assimilate nitrate, significantly increases photobiological H_2 production.

Despite the fact that filamentous heterocystous N_2-fixing cyanobacteria are the most fascinating organisms for biotechnological applications, the electron-transport pathways inside heterocysts are largely unknown. A recent study demonstrated that the heterocyst-specific Flv3B enzyme actively eliminates O_2 thus enabling microoxic interior inside of the heterocyst, which supports diazotrophic growth and nitrogenase enzyme activity under illumination (Ermakova et al., 2014). Accordingly, overexpression of the Flv3B protein resulted in high H_2 photoproduction, even though this phenotype was not linked to enhanced nitrogenase activity (Roumezi et al., 2020). The latter requires further investigations.

4.3 | PSI–hydrogenase fusion *in vivo*

Semiartificial *in vitro* techniques can be created in which hydrogenases are attached to isolated photosystems for H_2 production. However, generally such systems are short lived. Recently, Appel et al. (2020) reported photosynthetic H_2 production using a PSI-hydrogenase fusion *in vivo*. HoxYH of the NiFe-hydrogenase of *Synechocystis* sp. PCC 6803 was fused to its PSI subunit PsaD. The recombinant *Synechocystis* strain showed long-lived H_2 production in a light-dependent manner with otherwise undisturbed metabolism.

4.4 | Introduction of [FeFe]-hydrogenases in cyanobacteria

Cyanobacteria contain [NiFe]-hydrogenases. However, [FeFe]-hydrogenases, found in green algae and diverse bacteria, are metabolically much more active toward H_2 production and with higher turnover numbers. Already in 2011, Ducat and co-authors introduced and expressed a [FeFe]-hydrogenase (HydA) together with the HydEFG maturation machinery from *Clostridium acetobutylicum* in the non-N_2-fixing cyanobacterium *Synechococcus elongatus* PCC 7942. The heterologously expressed [FeFe]-hydrogenase showed the most significant *in vivo* hydrogenase activity connected to the light-dependent reactions of PETC under anoxic conditions (Ducat et al., 2011). Moreover, the introduced hydrogenase supported limited growth in the light using solely H_2 as a source of reducing equivalents. A challenge with introducing a very oxygen-sensitive [FeFe]-hydrogenase was addressed by Avilan et al. (2018) who expressed the clostridial [FeFe]-hydrogenase in the heterocysts of *Anabaena* sp. PCC 7120. Unlike wild type, the mutant strain showed negligible H_2 photoproduction under argon atmosphere, however substantially more H_2 photoproduction was observed in the presence of DCMU (3-[3,4-dichlorophenyl]-1,1-dimethylurea), an inhibitor of PSII. To further decrease the oxygen levels in the heterocysts, an oxygen scavenger, a GlbN cyanoglobin from *Nostoc commune*, was co-expressed with HydA from *Clostridium acetobutylicum*. The obtained mutant strain showed significantly higher H_2 production under an argon atmosphere, but weak H_2 photoproduction was also recorded in the presence of air (Avilan et al., 2018).

As further discussed below, another strategy is now available that circumvents the biological maturation of [FeFe]-hydrogenase by an artificial synthetic activation of the heterologously expressed HydA protein in living cells. Whereas the structural HydA apoprotein (from e.g. the green alga *Chlamydomonas reinhardtii*) is expressed using a designed gene fragment, a functional HydA is created by the addition of a synthetic analogue of the $[2Fe]_H$ subsite outside the cells (Wegelius et al., 2018). The experiments showed that the non-native, semi-synthetic enzyme retains its H_2 production capacity for several days after synthetic activation with the regulation of activity based on the availability of electrons.

4.5 | Whole-cell immobilization approach

The immobilization of enzymes and microbes within polymer matrices is an attractive approach to extending the lifetime of catalysts and enabling an easy and continuous production platform. Improved cell fitness and H_2 photoproduction activity have been demonstrated in cyanobacteria and green algae immobilized in thin-films (Jämsä et al., 2018; Kosourov et al., 2017; Wutthithien et al., 2019). Recently, a proof-of-concept for the sustained nitrogenase-based H_2 photoproduction was demonstrated by engineered thin-layer alginate films with entrapped heterocysts cells (Volgusheva et al., 2019). Immobilized heterocysts were able to produce H_2 in the presence of externally added sucrose for about 20 days, whereas heterocysts in suspensions showed similar activity for only 24 h.

5 | RECENT ADVANCES IN UNDERSTANDING H_2 METABOLISM IN GREEN ALGAE

Hydrogen metabolism in eukaryotic green algae is mediated by [FeFe]-hydrogenase enzymes that catalyze the reversible reduction of protons to molecular hydrogen using a biologically unique diiron cofactor (the $[2Fe]_H$ subsite). Depending on the redox state of the cells and the partial pressure of H_2 in the environment, algae could either produce or oxidize H_2. Concerning H_2 metabolism, *C. reinhardtii* is the most studied alga and thereafter it would be a primary focus of this review.

Similar to many other green algae, *C. reinhardtii* possesses two distinct hydrogenase genes in the nuclear genome that encode for two proteins, *Cr*HYDA1 and *Cr*HYDA2 (Forestier et al., 2003; Happe & Kaminski, 2002). Some algal species carry three hydrogenases (Skjånes et al., 2010). Until recently, the physiological basis for the presence of multiple hydrogenases in cells has not been yet determined. The electrochemical study performed by Engelbrecht et al. (2021) showed the higher catalytic preference of *Cr*HYDA2 to H_2 oxidation, thus revealing its potential role in H_2 uptake in *C. reinhardtii* cells. The biosynthesis and assembly of the complete active site of [FeFe]-hydrogenases is a comparatively simple process that involves three specific maturase enzymes: HydE, HydF, and HydG (Posewitz et al., 2004). In *C. reinhardtii*, genes encoding HydE and HydF proteins are fused, but in the majority of other organisms *hydE*, *hydF*, and *hydG* exist as separately transcribed genes.

It has been widely accepted that *C. reinhardtii* induces [FeFe]-hydrogenases and activates H_2 metabolic pathways only under strictly anoxic conditions (see, e.g., Dubini & Ghirardi, 2015). The extreme sensitivity of isolated [FeFe]-hydrogenases to atmospheric O_2 with oxidation and irreversible inactivation of their catalytic center supported the anaerobic nature of these enzymes (Stripp et al., 2009). The first evidence on the presence of hydrogenase transcripts in aerobic samples was reported by Forestier et al. (2003), who observed a prominent level of *hydA2* transcripts in aerobic samples. Later, the presence of *hydA1*, *hydA2* transcripts at the beginning of sulfur deprivation, when cultures are still aerobic, was shown by microarray analysis (Nguyen et al., 2008) and RNA-Seq (González-Ballester et al., 2010). The authors suggested that O_2 may not be the only trigger for the induction of *hydA* transcription. The presence of a very small amount of HYDA1 in aerobic samples was later confirmed at the protein level (Nikolova et al., 2018).

The availability of transcripts or even proteins in cells does not always mean the presence of active enzymes. Employing a high-sensitive membrane-inlet mass spectrometry (MIMS) approach, Liran et al. (2016) showed H_2 photoproduction in *C. reinhardtii* cultures under aerobic conditions, and in particular, on a shift from low to high light conditions. The authors proposed the existence of microoxic niches in the thylakoid stroma, where the enzyme resides and remains active (Liran et al., 2016). In these microoxic niches, O_2 is eliminated either by chlororespiration catalyzed by the plastid terminal oxidase (PTOX) or O_2 photoreduction driven by FDPs. Though, the role of FDPs in the creation of microoxic niches was put under question soon after Liran's et al. publication (Burlacot et al., 2018). Similarly, the presence of the [FeFe]-hydrogenase in aerobic samples was proposed by Kosourov et al. (2018), but in contrast to Liran's et al.'s (2016) study, authors could not detect the production of H_2 in aerobic cultures. Instead, they observed the appearance of the active [FeFe]-hydrogenase in cells within less than 3 min of transition from fully aerobic (21% of O_2) to microoxic (0.01–0.04% of O_2) environment (Kosourov et al., 2018, 2020). Since [FeFe]-hydrogenases are nuclear-encoded and their transportation to the chloroplast with following maturation requires time, the authors suggested the expression of hydrogenases during aerobic cultivation and activation of these enzymes under a microoxic environment. It should be noted, however, that Kosourov and co-authors worked with synchronized cultures, which were sampled during the period of active photosynthesis. Therefore, it is possible that Liran et al. study (employing unsynchronized cultures) monitored H_2 production from a fraction of algae in a different metabolic state, either cells with reduced photosynthetic activity or cells with enhanced respiration. Further experiments are necessary to prove this suggestion. Anyway, the presence of the [FeFe]-hydrogenase in aerobically-grown *C. reinhardtii* algae is now proved by two independent physiological studies. The most obvious role of hydrogenases in aerobically grown algal cells is the protection of the photosynthetic machinery of overreduction during (or immediately after) a shift to the microoxic environment. In such a scenario, algae should constitutively express [FeFe]-hydrogenases and activate the enzymes as soon as microoxic conditions are established. On the contrary, the transition from microoxic to aerobic conditions results in degradation of the major pool of the [FeFe]-hydrogenase enzyme as demonstrated in the pulse-illuminated algae on the switch to continuous light (Kosourov et al., 2018).

Interestingly, another alga *Chlorella vulgaris* that possesses the [FeFe]-hydrogenase has been shown to sustain H_2 photoproduction under air atmosphere, though at comparatively low production rates (Hwang et al., 2014). The mechanism of such O_2 tolerance in photoautotrophically grown algae has not been revealed yet. This work demonstrates that the inhibitory threshold of [FeFe]-hydrogenases to extracellular levels of O_2 could be different in different strains and species and may vary depending on photosynthetic and respiratory activities of algae. Thus, the adverse effect of O_2 on algal [FeFe]-hydrogenase *in vivo* has been dramatically overestimated in early studies. This conclusion, however, does not necessarily contradict to the well-known fact of extreme O_2-sensitivity of isolated [FeFe]-hydrogenase (Stripp et al., 2009). Indeed, the enzyme inside the algal chloroplast could be less susceptible to O_2 inhibition due to a number of different factors such as a change in the catalytic state or in the local environment surrounding [FeFe]-hydrogenase/its active site (Winkler et al., 2021), enhanced respiration rate, reduced photosynthetic activity, or yet unknown protection mechanisms.

The most efficient H_2 photoproduction in *C. reinhardtii* cultures occurs on their exposure to light after the period of the dark anaerobic adaptation. According to Boichenko et al. (2004), some active algal producers placed under optimal conditions could reach production rates up to 300 μmol H_2 h^{-1} (mg Chl)$^{-1}$, which is close to the maximal steady-state rate of CO_2-dependent O_2 evolution (Boichenko et al., 2004). Thus, H_2 photoproduction in green algae has a great potential for industrial applications, but the process proceeds for only a short time (from a few seconds to a few minutes).

Hydrogen photoproduction in algae (Figure 1B) depends on two metabolic pathways: (1) direct water biophotolysis, which involves water oxidation at PSII, and (2) indirect process, which supplies reductants originating from the oxidation of organic substrates to the PQ-pool via the Type II NADPH dehydrogenase (NDA2) (see, e.g., Dubini & Ghirardi, 2015). The contribution of each pathway varies to a different degree depending on the metabolic state of the cell and stage of hydrogen production. While the availability of the indirect pathway in algae was shown by multiple studies with photosynthetic inhibitors, the role of the PSII-dependent process in H_2 photoproduction has been mostly probed indirectly. The reason for that was the absence of O_2 evolution during the period of efficient H_2 photoproduction and inhibition of the latter as soon as O_2 starts to accumulate in the culture. Even if the absence of net O_2 production could be explained by the removal of O_2 in respiration (especially for the sulfur-deprived algae with their residual PSII activity; Melis et al., 2000; Tsygankov et al., 2006), the other research data indicate the domination of PSII-independent pathway in algal cells. Many short-term experiments performed with dark-adapted algae showed the quantum yield of H_2 evolution significantly exceeding 0.25 (Boichenko et al., 2004), the maximum theoretical value for the process depending on two photosystems. The latter means that H_2 photoproduction in green algae occurs at least partially through the mechanism independent of water oxidation. Moreover, PSII-deficient strains do not lose the capacity to photoproduce H_2, at least in the short-term process (Kosourov et al., 2020).

The direct photosynthetic pathway involving both photosystems is the most promising for the development of sustainable H_2 production. It utilizes water oxidation by PSII as an electron source for the hydrogenase-driven H^+ reduction but also co-evolves O_2, a by-product of water oxidation. Since O_2 inhibits the [FeFe]-hydrogenase enzyme, it is clear that the PSII activity must be contained to a level where respiration overtakes O_2 evolution thus creating an anoxic state in the cells (or at least in the pockets where the [FeFe]-hydrogenase operates) and allowing prolonged H_2 evolution. Early strategies for sustaining H_2 production in algal cultures pursued this goal. The nutrient limitation approach and, in particular, the most-studied sulfur (S)-deprivation protocol is one of them. This approach allows effective termination of the de novo PSII synthesis leading to a gradual inhibition of the PSII-dependent water oxidation activity about 20% of the original level and enabling spontaneous anoxia in algal cultures with following induction of hydrogenase enzymes in cells (Melis et al., 2000; Volgusheva et al., 2013). Another important consequence of S-deprivation is the reduced state of the PQ pool, which in some cases enables H_2 photoproduction for more than a week (Volgusheva et al., 2013). Since S-deprivation gradually degrades PSII centers in algal cells, their contribution to H_2 photoproduction declines over time and is replaced by the degradation of organic substrates through the PSII-independent pathway, primarily by the degradation of starch. The involvement of the residual PSII activity in the H_2 photoproduction yield in S-deprived algae has been proven by spectroscopic studies, fluorescence data, and inhibitory analysis (Melis et al., 2000; Volgusheva et al., 2013, 2016).

Alternatively, the PSII activity could be restricted by specific mutations leading to similar results. However, we have to emphasize that strategies aiming at the restriction of PSII activity in cells are a temporary solution for sustaining H_2 production in algal cultures. They significantly decrease the efficiency of the process and should be replaced by the methods aiming at the natural regulation of photosynthetic electron flow from PSII to the [FeFe]-hydrogenase.

6 | A SYSTEM BIOLOGY APPROACH FOR UNDERSTANDING AND IMPROVING H_2 PRODUCTION IN GREEN ALGAE

Multi-omics approaches, which include data from genomics, transcriptomics, proteomics, and metabolomic analyses, have been used for identification of genes and proteins involved in H_2 production in *C. reinhardtii* and some other algal species (see, e.g., Xu et al., 2019). These genes and proteins are potential new targets for metabolic engineering and improving H_2 production yields. Although genetic analysis has been plentifully employed, the analytical proteome studies of H_2-producing algae are quite rare. As a result, the experimental data at the protein level are still limited, especially in combination with other omics approaches. Comparative proteomics has been applied to sulfur- and nitrogen-deprived algae at different stages of nutrient starvation (Chen et al., 2010; Li et al., 2021) and to anaerobically adapted *C. reinhardtii* cultures under continuous illumination (Terashima et al., 2010). In dark-adapted anaerobic *C. reinhardtii*, the proteome analysis has been combined with transcriptomics and metabolomics data (Subramanian et al., 2014). The authors of these studies identified new candidates for further investigations and targeted engineering, which could clarify peculiarities of H_2 metabolism in algae. However, no complex metabolic engineering strategies, which are based on findings from multi-omics data, have been implemented yet for improving the algal H_2 production yield.

7 | NOVEL APPROACHES FOR SUSTAINING H_2 PHOTOPRODUCTION IN GREEN ALGAE

7.1 | Balancing PSII to PSI ratio

One of the important conclusions derived from S-deprivation studies of *C. reinhardtii* is that the complete reduction of the PQ-pool is the initial condition for induction of H_2 photoproduction in algal cells (Volgusheva et al., 2015, 2016). The reduction of the PQ-pool reflects the overall reduced state in the chloroplast and also results in the decreased PSII activity. Both conditions (the reduced PQ-pool and the decrease PSII activity) facilitate H_2 evolution as a way to relieve the electron pressure in the cell.

Although the redox state of the PQ-pool in the thylakoid membrane of *C. reinhardtii* depends on many factors, a proper condition for the induction of H_2 photoproduction can be achieved by simply altering the balance between two photosystems, PSII and PSI. A decreased amount of PSI can potentially modulate the electron transfer from the PSII by generating the reduced PQ-pool, thus also the decreased PSII activity. Indeed, the C3 mutant strain of *C. reinhardtii* with an abnormally low PSI content exhibits a reduced state of the PQ-pool already at standard growth conditions (Krishna et al., 2019). Incubation of C3 mutant in closed photobioreactor resulted in the establishment of anaerobiosis within 17 h (allowing respiration to overtake O_2 evolution) and stimulated H_2 photoproduction for an unprecedented period of at least 6 weeks under the standard continuous light and growth conditions (Krishna et al., 2019). Although a low amount of PSI was pre-request for quick initiation H_2 photoproduction, after 6 days it has significantly exceeded the amount of PSII, demonstrating the importance of PSI at later stages. It is possible that by optimizing PSII/PSI ratio in the cells even longer H_2 photoproduction can be achieved.

7.2 | Avoiding competition with CO_2 fixation and other metabolic pathways

As mentioned above, H_2 photoproduction in the nutrient-replete green algae is a transient phenomenon, which is typically observed in dark-adapted anaerobic cultures after their exposure to light. Under saturating illumination, algae evolve H_2 for only a few seconds and start accumulating O_2. Therefore, the inactivation of H_2 photoproduction in cells was primarily attributed to the irreversible inhibition of hydrogenase enzymes by O_2 accumulated in algal cultures. For a while, the O_2-sensitivity hypothesis became dominating despite (1) the proposed role of hydrogenases in the activation of the CBB cycle (see, e.g., Godaux et al., 2015) and (2) the available data on the direct competition of H_2 photoproduction for reducing equivalents with CO_2 fixation. The O_2-sensitivity concept has been reconsidered after three independent studies (Kosourov et al., 2018; Milrad et al., 2018; Nagy et al., 2018), which were published almost simultaneously.

By employing a high-sensitive MIMS approach, Milrad et al. (2018) showed that inactivation of H_2 photoproduction in *C. reinhardtii* during the shift from dark to light conditions is caused by an increasing flow of electrons toward photosynthetic CO_2 fixation. The competition for photosynthetic reductants between these two pathways occurs long before the irreversible inactivation of [FeFe]-hydrogenases by O_2 accumulated in the culture. Thus, the hydrogenase remains active during the period of induction of oxygenic photosynthesis and could even participate in H_2 uptake later on. Not only CO_2 fixation but also O_2 photoreduction driven by FDPs could compete with H_2 evolution for photosynthetic reductants. Similar to hydrogenases, FDPs are crucial for the rapid activation of the CBB cycle but negatively affect H_2 production yields in *C. reinhardtii* cultures under increasing O_2 levels (Burlacot et al., 2018). Elimination of FDPs as a competing electron sink resulted in the substantial increase in the long-term H_2 photoproduction yield (Jokel et al., 2019). These studies indicate that the adverse effect of O_2 on H_2 photoproduction also occurs long before the direct inhibition of [FeFe]-hydrogenases by constantly increasing levels of O_2 co-produced in photosynthesis during the shift from dark anaerobic to light aerobic conditions.

Since activation of the CBB cycle in dark-adapted algae requires time, Kosourov et al. (2018) proposed that a sequence of short light pulses applied to cultures instead of continuous light will prevent induction of CO_2 fixation, eliminate the competition, and thus, make a substantial number of photosynthetic electrons available to hydrogenase. The performed research corroborated the prediction. A train of 1-s light pulses interrupted by 9-s dark phases indeed induced efficient H_2 photoproduction in *C. reinhardtii* cultures and sustained the process for up to 3 days. The maximum rates were observed in the first 8 h. In contrast to the classical dark adaptation protocol, only a very short period (3–5 min) of purging with argon was needed for activation of the hydrogenase enzyme in cells. The cell-wall deficient strain (CC-4533) of *C. reinhardtii* showed H_2 evolution already on the first light pulse (Kosourov et al., 2020), indicating that hydrogenase proteins were already available in aerobic cultures. As expected, the pulse-illuminated algae could not fix CO_2 and produce biomass (Jokel et al., 2019; Kosourov et al., 2018). Instead, the PSII-dependent CO_2 release was detected by MIMS in pulse-illuminated algal cultures (Kosourov et al., 2020). The CO_2 consumption and biomass accumulation were detected when longer light pulses (5–8 s) activated the CBB cycle (Jokel et al., 2019). The latter was accompanied by the boost in the production of O_2 in the cultures and pronounced H_2 uptake.

The application of $^{18}O_2$-labeled water, DCMU, and PSII- and NDA2-deficient mutants showed that up to 92% of H_2 in the pulse-illuminated algae originate from water oxidation activity, while the remaining H_2 is evolved by fermentation during the prolonged dark periods (Kosourov et al., 2020).

Another method for preventing competition with the CBB cycle was devised by Nagy et al. (2018). The method relies on the substrate limitation of the CBB cycle in very dense CO_2- and acetate-limited algal cultures. In this approach, anaerobic *C. reinhardtii* suspensions are dark-adapted for several hours with periodic re-flushing by N_2 (or Ar) for the CO_2 removal. The dark adaptation step is followed by exposure of algae to continuous illumination, which enables H_2 photoproduction. Periodic purging of H_2-producing algae with N_2 prevents the accumulation of CO_2 in cultures and sustains H_2 photoproduction for several days. It should be noted, however, that N_2 re-flushing removes not only CO_2 but also O_2 and H_2 from the cultures. The latter prevents inhibition of [FeFe]-hydrogenases and, in principle, should limit H_2 oxidation through the reversible process. The authors suggested that H_2 photoproduction in CO_2-limited algae is driven by direct water biophotolysis since DCMU completely blocks the process.

Besides competition with CO_2 assimilation and O_2 photoreduction, H_2 photoproduction in *C. reinhardtii* cells is negatively affected by the induction of CEF around PSI. Elimination of state transition and disruption of PGR5 and PGRL1 proteins, essential components of CEF, resulted in significantly enhanced H_2 photoproduction yields in S-deprived algae (Kruse et al., 2005; Steinbeck et al., 2015; Tolleter et al., 2011). The performance of these mutants may be further improved by eliminating the competition of H_2 photoproduction with the CBB cycle, thus redirecting most of photosynthetic electrons to [FeFe]-hydrogenase.

7.3 | Engineering photosynthesis for improved electron flow toward hydrogenase

A few recent studies have shown that the efficiency of photosynthetic electron flow toward hydrogenase could be significantly improved by applying protein fusion technology. For avoiding competition with ferredoxin-NADP$^+$ oxidoreductase (FNR), ferredoxin could be fused to hydrogenase (Yacoby et al., 2011). By fusing [FeFe]-hydrogenase to ferredoxin, Eilenberg et al. (2016) showed that the new synthetic enzyme supports ∼4.5-fold greater photosynthetic H_2 production rate *in vivo* and improved resistance to O_2 than the native [FeFe]-hydrogenase. Alternatively, [FeFe]-hydrogenase could be directly fused to PSI. Following this approach, Kanygin et al. (2020) designed and created the PSI-hydrogenase chimera where the HydA sequence was inserted into the PsaC subunit. The engineered strain placed under anoxic conditions demonstrated sustained H_2 photoproduction for 5 days at an average rate of 14 μmol H_2 h^{-1} (mg Chl)$^{-1}$. This work further supported the hypothesis that sustained H_2 photoproduction in nutrient-replete algae could be achieved by avoiding competition with the CBB cycle.

7.4 | Eliminating the H_2 uptake component

Under an increased H_2 partial pressure, algal hydrogenases could also function in H_2 uptake. In 2012, Kosourov and co-authors evaluated the effect of the H_2 partial pressure in S-deprived cultures and found an exponential decay in the H_2 photoproduction yield with the increased H_2 level (Kosourov et al., 2012). The authors also observed a transient H_2 uptake after injecting extra H_2 in the photobioreactor headspace. The presence of active H_2 uptake in sulfur-deprived algae was later confirmed by Scoma and Hemschemeier (2017). Yet, the mechanism and metabolic relevance of this reaction have not been revealed. The possibility of CO_2 photoreduction in sulfur-deprived algae is questionable because of the noticeable degradation of the RuBisCo enzyme by the time H_2 photoproduction begins. Nevertheless, as Scoma and Hemschemeier (2017) suggested, H_2 oxidation could be linked to fermentative CO_2 reduction via PFR1, which also interacts with [FeFe]-hydrogenases. Meanwhile, the oxyhydrogen reaction should be limited by the low levels of O_2 in algae due to the degradation of PSII centers, the only source of O_2 in the sealed sulfur-deprived cultures. Recently, the presence of strong H_2 uptake was demonstrated in the pulse-illuminated algae on the shift to darkness (Kosourov et al., 2018). The reaction occurred even in the DCMU-treated cells (thus under strong anaerobiosis) and does not show a strong correlation with CO_2 consumption (Kosourov et al., 2020). These data thus indicate another unknown terminal acceptor for H_2 oxidation. Recently, Milrad et al. (2021) presented evidence that H_2 uptake in the darkness is accompanied by restoration of the NADPH pool. The latter suggests that H_2 may serve as an electron donor to NADP$^+$ via [FeFe]-hydrogenase. Anyway, the elimination of H_2 uptake component should significantly improve the H_2 production yield in algal cultures. This could be achieved by physical removal of H_2 from H_2-producing algae or by disruption of H_2-uptake pathway(s) in cells. The latter, though, requires understanding the molecular mechanisms of H_2 oxidation and regulation of this process.

The findings presented in this section bring some optimism in the development of H_2-producing algal-based photosynthetic cell factories. Altogether they show the way how to (1) control the photosynthetic electron flow between photosystems for keeping the reduced state of the PQ-pool, (2) balance the PSII activity for preventing inactivation of hydrogenases by O_2 co-produced during water oxidation, and (3) eliminate H_2 uptake and competition of H_2 photoproduction with other metabolic pathways, primarily with CO_2 fixation (biomass production) and FDPs.

8 | SEMI-SYNTHETIC HYDROGENASES

Hydrogenases possess a remarkable capacity for the interconversion of H$^+$ to H_2 on par with noble metal artificial catalysts; however, the enzymes achieve this activity with first row transition metals and can thus be readily produced in abundant quantities (Kleinhaus et al., 2021; Land, Senger, et al., 2020). Therefore, they have become increasingly employed in H_2 production applications, especially

[FeFe]-hydrogenases. They possess the highest catalytic activity among different hydrogenases and a comparatively simple maturation, which involves three specific enzymes denoted as HydE, –G, and –F (Posewitz et al., 2004). Through the combined activities of two radical S-adenosyl-L-methionine enzymes (HydG, HydE), a pre-catalyst of the $[2Fe]_H$ subsite is assembled on HydF. The pre-catalyst is subsequently transferred to the apo [FeFe]-hydrogenase, containing a pre-assembled $[4Fe4S]_H$ cluster, to yield the active enzyme (Britt et al., 2020). Despite its relative simplicity, the requirement for co-expression of the $[2Fe]_H$ maturation enzymes to obtain active [FeFe]-hydrogenases initially complicated both large-scale screening as well as detailed studies of these biotechnologically relevant enzymes, as isolation was dependent on anaerobic isolation of the enzyme following homologous expression or the use of specifically engineered *E. coli* strains.

In 2013, synthetic chemists reported that the complex biological H-cluster maturation machinery of [FeFe]-hydrogenases could be avoided by insertion of chemically synthesized diiron sites into the apo protein (Berggren et al., 2013; Esselborn et al., 2013). More specifically, the synthetic $[2Fe]_H$ subsite analogue $[(\mu\text{-adt})Fe_2(CN)_2(CO)_4]^{2-}$ (adt = azadithioloate) generates a fully active enzyme, but modified cofactors can also be introduced to generate "organometallic mutants" with new properties. Utilizing this "artificial maturation" technique, target [FeFe]-hydrogenases can now be expressed in a wide range of host organisms and activated without the need for co-expression of the HydEFG machinery. Critically, this can be achieved both in vitro as well as in whole cells, as will be further outlined below.

In short, key applications of this technique in the context of biotechnology include (1) large-scale isolation of the active enzyme utilizing standard expression hosts, enabling detailed mechanistic investigations, (2) manipulation of the enzyme through synthetic chemistry, (3) screening of new [FeFe]-hydrogenases, and (4) screening of host organisms.

The large degree of freedom in creating new semi-synthetic enzymes obtained by artificial maturation was demonstrated by Siebel et al. (2015). They created enzymes with novel properties by introducing a range of synthetic analogues of the diiron site into apo [FeFe]-hydrogenase from *C. reinhardtii* (*Cr*HydA1). These non-native diiron sites with alternated dithiolate ligands and/or variations of the diatomic (CO/cyanide) ligands led to [FeFe]-hydrogenase enzymes with drastically different characteristics regarding hydrogen oxidation and proton reduction. For example, ~50% H_2 evolution activity was observed when the native diiron site was replaced by its mono cyanide variant. The importance of the azadithiolate amine moiety was demonstrated by the nearly complete loss of activity upon replacement of nitrogen by other elements. In a more mechanistic study, Duan et al. investigated the reactivity of *Cr*HydA1 diiron site variants with H_2 and CO by in situ and transient IR spectroscopy (Duan et al., 2019). The substrate affinity was monitored by tracing the redox state composition and the kinetics of the redox state (de)population. The differences observed suggest diverse interactions of ligands (e.g., CO or a terminal hydride) with the dithiolate head group, amino acid residues, and water molecules close to the distal Fe atom. This indicates a function beyond only proton transfer for the dithiolate head group.

9 | WHOLE-CELL APPLICATIONS OF THE SEMI-SYNTHETIC SYSTEMS

Inspired by the in vitro applications, the concept of artificial maturation of [FeFe]-hydrogenases was extended to *in vivo* conditions. In 2017, Khanna et al. reported the successful incorporation of the diiron site into heterologously expressed apo-hydrogenase inside living *E. coli* cells for the first time (Khanna et al., 2017). Importantly, the maturation occurs spontaneously via the simple addition of the synthetic cofactor to the growth medium. Perhaps, the most immediate advantage in the context of biotechnology is the more rapid screening for new [FeFe]-hydrogenases enabled by omitting the time-consuming process of enzyme isolation. Moreover, it has proven to be a highly useful tool for screening also non-native host organisms for [FeFe]-hydrogenases with higher throughput and lower experimental effort. Thus, both the catalyst ([FeFe]-hydrogenase) and the host organism can be screened for the optimal design of H_2 producing cells.

From an enzyme re-design perspective, it should be noted that artificial maturation *in vivo* also preserved the possibility to optimize [FeFe]-hydrogenases itself by creating organometallic variants by modified diiron sites. In addition, it opened the door for detailed mechanistic investigations of [FeFe]-hydrogenases in a more native cellular environment compared to studies in vitro.

E. coli cells containing heterologously expressed [FeFe]-hydrogenases have been successfully matured with a monocyanide derivative of the diiron site, as well as synthetic cofactors in which the bridgehead amine of the adt-ligand has been replaced with a non-protonatable methylene group (pdt) (Khanna et al., 2017; Mészáros et al., 2018). Utilizing EPR spectroscopy, the latter study provided the first spectroscopic verification of the successful assembly of a semi-synthetic enzyme in the cytoplasm of a living cell. Both synthetic cofactor variants resulted in lower whole-cell H_2 producing activities as compared to cells containing the H-cluster generated by the addition of $[(\mu\text{-adt})Fe_2(CN)_2(CO)_4]^{2-}$ to the growth medium. However, both activity measurements and spectroscopy studies indicate that activity observed *in vitro* translates into whole-cell activity and that alternative diiron sites can be utilized *in vivo* as well.

To gain more molecular level insights into the hydrogenase mechanism inside living cells, Meszaros et al. expanded the analytic toolbox by FTIR spectroscopy, electrochemistry, and scattering scanning near field optical microscopy on whole cells (Mészáros et al., 2020). In combination with gas treatments, pH titrations and isotope editing a number of proposed catalytic intermediates of [FeFe]-hydrogenase *Cr*HydA1 were observed in living *E. coli* cells. In addition to the first observation of a reactive metal hydride species in living cells, only partially *in vitro* characterized hydride species could be characterized in greater detail under whole-cell conditions. This finding highlights how investigations *in vivo* can contribute to the understanding of H_2 catalysis in general. We have just started to gain insight into the enzyme chemistry on a molecular level in cellulo and *in vivo*, and these findings can now be used to optimize enzymes via rational design and directed evolution.

A proof-of-concept study by Land et al. showed how artificial maturation of heterologously expressed apo-hydrogenase in living cells can be applied for new screening procedures in the search for novel [FeFe]-hydrogenases (Land et al., 2019). A bioinformatic approach motivated the selection of the putative [FeFe]-hydrogenases encoding genes. Representative examples of uncharacterized [FeFe]-hydrogenases were then expressed in different strains of *E. coli*, artificially maturated, and screened *in vivo* and in vitro for H_2 evolution. As a result, two newly found active [FeFe]-hydrogenases were subsequently subjected to whole-cell (*in vivo*) and *in vitro* biophysical and electrochemical characterization. In the wider context of understanding H_2 metabolism, it is noteworthy that the screening study subsequently enabled the first detailed characterization of a putative sensory group D [FeFe]-hydrogenase, phylogenetically distinct from previously studied groups A and C [FeFe]-hydrogenases (Land, Sekretareva, et al., 2020).

In parallel, Wegelius et al. pushed artificial maturation beyond the *E. coli* system and introduced apo [FeFe]-hydrogenase into the model cyanobacterium *Synechocystis* sp. PCC 6803 (Wegelius et al., 2018), and showed how that could be activated via simple cofactor addition to the growth medium. The resulting semi-synthetic *in vivo* functional [FeFe]-hydrogenase links to the native metabolism of the cyanobacterial cells and is active both in light conditions and in darkness. Impressively, the heterologously introduced [FeFe]-hydrogenase outperformed the native [NiFe]-hydrogenase and deletion of [NiFe]-hydrogenase amplified the H_2 evolution activity of these cells even more. Importantly, once activated the enzyme remained stable on the time-scale of days in this non-native host, underscoring the high stability of these artificially matured [FeFe]-hydrogenases.

The increased H_2 evolution activity of [FeFe]-hydrogenase introduced into cyanobacteria serves as an example of how an artificial metalloenzyme can tune the performance of host cell systems and outperform the native catalysis pathways. Additionally, the possibility to re-design the H-cluster with synthesized metal cofactors opens the perspective of creating enzymes that catalyzes abiotic reactions not present in natural systems. In this respect, the concept of artificial maturation/semi-synthetic hydrogenases also provides a promising perspective for the design of non-natural metabolic pathways for the production of high-value chemicals.

10 | CONCLUSIONS

The release of H_2 by algae and cyanobacteria in the environment is bioenergetically expensive and results in a significant loss of metabolic energy. Therefore, photosynthetic microbes typically photoproduce H_2 only "on-demand" for a very short period or recycle produced H_2 via dedicated metabolic pathways. H_2 recycling has already been known for years in N_2-fixing cyanobacteria, where H_2 is imminently co-produced during N_2 fixation and then oxidized by the uptake hydrogenase. Elimination of HupSL and then its modification for H_2 evolution instead of H_2 oxidation, which resulted in the boost production of H_2 in N_2-fixing heterocystous cyanobacteria, were the first and the second most noticeable achievements in this field. Recently, the presence of the active H_2 uptake component has also been confirmed in green algae, but molecular mechanisms of this process are still not clear. We expect that elimination of this pathway, if achievable, may enhance H_2 photoproduction yields in green algae as well.

Besides H_2 recycling, photosynthetic H_2 production competes for the reducing equivalents with a number of assimilatory and auxiliary pathways, from which CO_2 fixation in the CBB cycle and O_2 photoreduction by FDPs represent the strongest sinks both in cyanobacteria and green algae. For years, the decline in H_2 photoproduction via the direct water biophotolysis pathway in green algae (but not in cyanobacteria where H_2 photoproduction via the bidirectional hydrogenase is followed by noticeable H_2 uptake) has been primarily attributed to O_2 inactivation of [FeFe]-hydrogenase. Recent studies mentioned in this mini-review have demonstrated that the competition for photosynthetic electrons occurs before inactivation of [FeFe]-hydrogenase by O_2 co-produced in photosynthesis. Suggested novel protocols (pulse-illumination and CO_2 limitation) specifically designed for prevention of the CBB cycle activation resulted in sustainable H_2 photoproduction in algal cultures without nutrient deprivation. Additional elimination of FDPs in algal cells enhanced H_2 photoproduction yields in these protocols. Similar strategies for preventing competition for photosynthetic reductants have been shown to improve H_2 photoproduction in cyanobacteria.

Without a doubt, the O_2 sensitivity of H_2 producing enzymes and pathways still remains one of the major issues of photosynthetic H_2 production. Although some progress with the heterologous expression of more O_2-tolerant hydrogenases has been achieved, direct modification of the enzymes for enhanced O_2 tolerance has not been successful yet. In this context, the development of strategies and approaches for the assembly of semi-synthetic hydrogenases *in vivo* with novel catalytic properties brings some optimism in this field.

ACKNOWLEDGMENTS

This work was funded by the NordForsk Nordic Center of Excellence "NordAqua" (no. 82845 to Y.A.); the Kone Foundation (project no. 201608799 to S.K.); the Swedish Energy Agency (STEM, grant agreement no. 48574-1 to G.B.); the European Research Council under the European Union's Seventh Framework Program (grant agreement no. 714102 to G.B.), the Carl Tryggers Foundation (grant no. CTS 19:230 to F.M.) and by Horizon 2020 research and innovation program (Marie Sklodowska-Curie grant agreement no. 897555 to M.S.). Y.A. and S.K. acknowledge EU FET Open project FuturoLEAF under grant agreement no. 899576. Authors also would like to thank Dr. Dmitry Shevela (ShevelaDesign AB, Sweden) for preparation of Figure 1.

AUTHOR CONTRIBUTIONS

All authors act as a team and contributed to the planning, writing and revision of the manuscript. Coordination of the work was performed by corresponding authors.

DATA AVAILABILITY STATEMENT

"n/a"

ORCID

Sergey Kosourov https://orcid.org/0000-0003-4025-8041
Moritz Senger https://orcid.org/0000-0001-9225-4910
Gustav Berggren https://orcid.org/0000-0002-6717-6612
Karin Stensjö https://orcid.org/0000-0001-6993-8476
Fikret Mamedov https://orcid.org/0000-0002-6218-3039
Peter Lindblad https://orcid.org/0000-0001-7256-0275
Yagut Allahverdiyeva https://orcid.org/0000-0002-9262-1757

REFERENCES

Appel, J., Hueren, V., Boehm, M. & Gutekunst, K. (2020) Cyanobacterial *in vivo* solar hydrogen production using a photosystem I–hydrogenase (PsaD-HoxYH) fusion complex. *Nature Energy*, 5, 458–467.

Aryal, U.K., Callister, S.J., Mishra, S., Zhang, X., Shutthanandan, J.I., Angel, T.E. et al. (2013) Proteome analyses of strains ATCC 51142 and PCC 7822 of the diazotrophic cyanobacterium *Cyanothece* sp. under culture conditions resulting in enhanced H_2 production. *Applied and Environmental Microbiology*, 79, 1070–1077.

Avilan, L., Roumezi, B., Risoul, V., Bernard, C.S., Kpebe, A., Belhadjhassine, M. et al. (2018) Phototrophic hydrogen production from a clostridial [FeFe]-hydrogenase expressed in the heterocysts of the cyanobacterium *Nostoc* PCC 7120. *Applied Microbiology and Biotechnology*, 102, 5775–5783.

Baebprasert, W., Jantaro, S., Khetkorn, W., Lindblad, P. & Incharoensakdi, A. (2011) Increased H_2 production in the cyanobacterium *Synechocystis* sp. strain PCC 6803 by redirecting the electron supply via genetic engineering of the nitrate assimilation pathway. *Metabolic Engineering*, 13, 610–616.

Bandyopadhyay, A., Stöckel, J., Min, H., Sherman, L.A. & Pakrasi, H.B. (2010) High rates of photobiological H_2 production by a cyanobacterium under aerobic conditions. *Nature Communications*, 1, 139.

Barz, M., Beimgraben, C., Staller, T., Germer, F., Opitz, F., Marquardt, C. et al. (2010) Distribution analysis of hydrogenases in surface waters of marine and freshwater environments. *PLoS One*, 5, e13846.

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.

Bernstein, H.C., Charania, M.A., McClure, R.S., Sadler, N.C., Melnicki, M.R., Hill, E.A. et al. (2015) Multi-Omic dynamics associate oxygenic photosynthesis with nitrogenase-mediated H_2 production in *Cyanothece* sp. ATCC 51142. *Sci Rep*, 5, 16004.

Boichenko, V.A., Greenbaum, E. & Seibert, M. (2004) Hydrogen production by photosynthetic microorganisms. In: Barber, J. & Archer, M.D. (Eds.) *Molecular to global photosynthesis: Photoconversion of solar energy*. London: Imperial College Press, pp. 397–451.

Bolton, J.R. & Hall, D.O. (1991) The maximum efficiency of photosynthesis. *Photochemistry and Photobiology*, 53, 545–548.

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

Burlacot, A., Sawyer, A., Cuiné, S., Auroy-Tarrago, P., Blangy, S., Happe, T. et al. (2018) Flavodiiron-mediated O_2 photoreduction links H2 production with CO_2 fixation during the anaerobic induction of photosynthesis. *Plant Physiology*, 177, 1639–1649.

Chen, M., Zhao, L., Sun, Y.-L., Cui, S.-X., Zhang, L.-F., Yang, B. et al. (2010) Proteomic analysis of hydrogen photoproduction in sulfur-deprived Chlamydomonas cells. *Journal of Proteome Research*, 9, 3854–3866.

Cournac, L., Guedeney, G., Peltier, G. & Vignais, P.M. (2004) Sustained photoevolution of molecular hydrogen in a mutant of *Synechocystis* sp. strain PCC 6803 deficient in the type I NADPH-dehydrogenase complex. *Journal of Bacteriology*, 186, 1737–1746.

Duan, J., Mebs, S., Laun, K., Wittkamp, F., Heberle, J., Happe, T. et al. (2019) Geometry of the catalytic active site in [FeFe]-hydrogenase is determined by hydrogen bonding and proton transfer. *ACS Catalysis*, 9, 9140–9149.

Dubini, A. & Ghirardi, M.L. (2015) Engineering photosynthetic organisms for the production of biohydrogen. *Photosynthesis Research*, 123, 241–253.

Ducat, D.C., Sachdeva, G. & Silver, P.A. (2011) Rewiring hydrogenase-dependent redox circuits in cyanobacteria. *Proceedings of the National Academy of Sciences*, 108, 3941–3946.

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. *Biotechnology for Biofuels*, 9, 182.

Ekman, M., Ow, S.Y., Holmqvist, M., Zhang, X., van Wagenen, J., Wright, P. C. et al. (2011) Metabolic adaptations in a H2 producing heterocyst-forming cyanobacterium: potentials and implications for biological engineering. *Journal of Proteome Research*, 10, 1772–1784.

Engelbrecht, V., Liedtke, K., Rutz, A., Yadav, S., Günzel, A. & Happe, T. (2021) One isoform for one task? The second hydrogenase of *Chlamydomonas reinhardtii* prefers hydrogen uptake. *International Journal of Hydrogen Energy*, 46, 7165–7175.

Ermakova, M., Battchikova, N., Richaud, P., Leino, H., Kosourov, S., Isojarvi, J. et al. (2014) Heterocyst-specific flavodiiron protein Flv3B enables oxic diazotrophic growth of the filamentous cyanobacterium *Anabaena* sp. PCC 7120. *Proceedings of the National Academy of Sciences*, 111, 11205–11210.

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. *Nature Chemical Biology*, 9, 607–609.

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. *European Journal of Biochemistry*, 270, 2750–2758.

Godaux, D., Bailleul, B., Berne, N. & 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 Physiology*, 168, 648–658.

González-Ballester, D., Casero, D., Cokus, S., Pellegrini, M., Merchant, S. S. & 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.

Gutekunst, K., Chen, X., Schreiber, K., Kaspar, U., Makam, S. & Appel, J. (2014) The bidirectional NiFe-hydrogenase in *Synechocystis* sp. PCC 6803 is reduced by flavodoxin and ferredoxin and is essential under mixotrophic, nitrate-limiting conditions. *The Journal of Biological Chemistry*, 289, 1930–1937.

Gutthann, F., Egert, M., Marques, A. & Appel, J. (2007) Inhibition of respiration and nitrate assimilation enhances photohydrogen evolution under low oxygen concentrations in *Synechocystis* sp. PCC 6803. *Biochimica et Biophysica Acta–Bioenergetics*, 1767, 161–169.

Happe, T. & Kaminski, A. (2002) Differential regulation of the Fe-hydrogenase during anaerobic adaptation in the green alga

Chlamydomonas reinhardtii. *European Journal of Biochemistry*, 269, 1022–1032.

Hwang, J.-H., Kim, H.-C., Choi, J.-A., Abou-Shanab, R.A.I., Dempsey, B.A., Regan, J.M. et al. (2014) Photoautotrophic hydrogen production by eukaryotic microalgae under aerobic conditions. *Nature Communications*, 5, 3234.

Jämsä, M., Kosourov, S., Rissanen, V., Hakalahti, M., Pere, J., Ketoja, J.A. et al. (2018) Versatile templates from cellulose nanofibrils for photosynthetic microbial biofuel production. *Journal of Materials Chemistry A*, 6, 5825–5835.

Jokel, M., Nagy, V., Tóth, S.Z., Kosourov, S. & Allahverdiyeva, Y. (2019) Elimination of the flavodiiron electron sink facilitates long-term H_2 photoproduction in green algae. *Biotechnology for Biofuels*, 12, 280.

Kanygin, A., Milrad, Y., Thummala, C., Reifschneider, K.T., Baker, P., Marcu, P. et al. (2020) Rewiring photosynthesis: a photosystem I-hydrogenase chimera that makes H_2 *in vivo*. *Energy and Environmental Science*, 13, 2903–2914.

Khanna, N., Esmieu, C., Mészáros, L.S., Lindblad, P. & Berggren, G. (2017) In vivo activation of an [FeFe]-hydrogenase using synthetic cofactors. *Energy and Environmental Science*, 10, 1563–1567.

Kleinhaus, J.T., Wittkamp, F., Yadav, S., Siegmund, D. & Apfel, U.-P. (2021) [FeFe]-Hydrogenases: maturation and reactivity of enzymatic systems and overview of biomimetic models. *Chemical Society Reviews*, 50, 1668–1784.

Kosourov, S., Jokel, M., Aro, E.-M. & Allahverdiyeva, Y. (2018) A new approach for sustained and efficient H_2 photoproduction by *Chlamydomonas reinhardtii*. *Energy and Environmental Science*, 11, 1431–1436.

Kosourov, S., Leino, H., Murukesan, G., Lynch, F., Sivonen, K., Tsygankov, A.A. et al. (2014) Hydrogen photoproduction by immobilized N_2 -fixing cyanobacteria: understanding the role of the uptake hydrogenase in the long-term process. *Applied and Environmental Microbiology*, 80, 5807–5817.

Kosourov, S., Murukesan, G., Seibert, M. & Allahverdiyeva, Y. (2017) Evaluation of light energy to H_2 energy conversion efficiency in thin films of cyanobacteria and green alga under photoautotrophic conditions. *Algal Research*, 28, 253–263.

Kosourov, S., Nagy, V., Shevela, D., Jokel, M., Messinger, J. & Allahverdiyeva, Y. (2020) Water oxidation by photosystem II is the primary source of electrons for sustained H_2 photoproduction in nutrient-replete green algae. *Proceedings of the National Academy of Sciences*, 117, 29629–29636.

Kosourov, S.N., Batyrova, K.A., Petushkova, E.P., Tsygankov, A.A., Ghirardi, M.L. & Seibert, M. (2012) Maximizing the hydrogen photoproduction yields in *Chlamydomonas reinhardtii* cultures: the effect of the H_2 partial pressure. *International Journal of Hydrogen Energy*, 37, 8850–8858.

Kourpa, K., Manarolaki, E., Lyratzakis, A., Strataki, V., Rupprecht, F., Langer, J.D. et al. (2019) Proteome analysis of enriched heterocysts from two hydrogenase mutants from *Anabaena* sp. PCC 7120. *Proteomics*, 19, 1800332.

Krishna, P.S., Styring, S. & Mamedov, F. (2019) Photosystem ratio imbalance promotes direct sustainable H_2 production in: *Chlamydomonas reinhardtii*. *Green Chemistry*, 21, 4683–4690.

Kruse, O., Rupprecht, J., Bader, K.-P., Thomas-Hall, S., Schenk, P.M., Finazzi, G. et al. (2005) Improved photobiological H_2 production in engineered green algal cells. *The Journal of Biological Chemistry*, 280, 34170–34177.

Land, H., Ceccaldi, P., Mészáros, L.S., Lorenzi, M., Redman, H.J., Senger, M. et al. (2019) Discovery of novel [FeFe]-hydrogenases for biocatalytic H_2-production. *Chemical Science*, 10, 9941–9948.

Land, H., Sekretareva, A., Huang, P., Redman, H.J., Németh, B., Polidori, N. et al. (2020) Characterization of a putative sensory [FeFe]-hydrogenase provides new insight into the role of the active site architecture. *Chemical Science*, 11, 12789–12801.

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

Li, L., Zhang, L. & Liu, J. (2021) Proteomic analysis of hydrogen production in *Chlorella pyrenoidosa* under nitrogen deprivation. *Algal Research*, 53, 102143.

Lindblad, P. (2018) Chapter 13. Hydrogen production using novel photosynthetic cell factories. Cyanobacterial hydrogen production: design of efficient organisms. In: Seibert, M. & Torzillo, G. (Eds.) *Microalgal hydrogen production: achievements and perspectives*. London, UK: The Royal Society of Chemistry, pp. 323–334.

Liran, O., Semyatich, R., Milrad, Y., Eilenberg, H., Weiner, I. & 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 Physiology*, 172, 264–271.

Malatinszky, D., Steuer, R. & Jones, P.R. (2017) A comprehensively curated genome-scale two-cell model for the heterocystous cyanobacterium *Anabaena* sp. PCC 7120. *Plant Physiology*, 173, 509–523.

Malek Shahkouhi, A. & Motamedian, E. (2020) Reconstruction of a regulated two-cell metabolic model to study biohydrogen production in a diazotrophic cyanobacterium *Anabaena variabilis* ATCC 29413. *PLoS One*, 15, e0227977.

Melis, A., Zhang, L., Forestier, M., Ghirardi, M.L. & Seibert, M. (2000) Sustained photobiological hydrogen gas production upon reversible inactivation of oxygen evolution in the green alga *Chlamydomonas reinhardtii*. *Plant Physiology*, 122, 127–136.

Mészáros, L.S., Ceccaldi, P., Lorenzi, M., Redman, H.J., Pfitzner, E., Heberle, J. et al. (2020) Spectroscopic investigations under whole-cell conditions provide new insight into the metal hydride chemistry of [FeFe]-hydrogenase. *Chemical Science*, 11, 4608–4617.

Mészáros, L.S., Németh, B., Esmieu, C., Ceccaldi, P. & Berggren, G. (2018) In vivo EPR characterization of semi-synthetic [FeFe]-hydrogenases. *Angewandte Chemie International Edition*, 57, 2596–2599.

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

Milrad, Y., Schweitzer, S., Feldman, Y. & Yacoby, I. (2021) Bi-directional electron transfer between H_2 and NADPH mitigates light fluctuation responses in green algae. *Plant Physiol*, kiab051. doi.org/10.1093/plphys/kiab051.

Nagy, V., Podmaniczki, A., Vidal-Meireles, A., Tengölics, R., Kovács, L., Rákhely, G. et al. (2018) Water-splitting-based, sustainable and efficient H_2 production in green algae as achieved by substrate limitation of the Calvin–Benson–Bassham cycle. *Biotechnology for Biofuels*, 11, 69.

Nguyen, A.V., Thomas-Hall, S.R., Malnoë, A., Timmins, M., Mussgnug, J.H., Rupprecht, J. et al. (2008) Transcriptome for photobiological hydrogen production induced by sulfur deprivation in the green alga *Chlamydomonas reinhardtii*. *Eukaryotic Cell*, 7, 1965–1979.

Nikolova, D., Heilmann, C., Hawat, S., Gäbelein, P. & Hippler, M. (2018) Absolute quantification of selected photosynthetic electron transfer proteins in *Chlamydomonas reinhardtii* in the presence and absence of oxygen. *Photosynthesis Research*, 137, 281–293.

Nyberg, M., Heidorn, T. & Lindblad, P. (2015) Hydrogen production by the engineered cyanobacterial strain *Nostoc* PCC 7120 ΔhupW examined in a flat panel photobioreactor system. *Journal of Biotechnology*, 215, 35–43.

Posewitz, M.C., King, P.W., Smolinski, S.L., Zhang, L., Seibert, M. & Ghirardi, M. L. (2004) Discovery of two novel radical S-adenosylmethionine proteins required for the assembly of an active [Fe] hydrogenase. *The Journal of Biological Chemistry*, 279, 25711–25720.

Puggioni, V., Tempel, S. & Latifi, A. (2016) Distribution of hydrogenases in cyanobacteria: a phylum-wide genomic survey. *Frontiers in Genetics*, 7, 223.

Raleiras, P., Kellers, P., Lindblad, P., Styring, S. & Magnuson, A. (2013) Isolation and characterization of the small subunit of the uptake hydrogenase from the cyanobacterium *Nostoc punctiforme*. *The Journal of Biological Chemistry*, 288, 18345–18352.

Raleiras, P., Khanna, N., Miranda, H., Mészáros, L.S., Krassen, H., Ho, F. et al. (2016) Turning around the electron flow in an uptake hydrogenase. EPR spectroscopy and *in vivo* activity of a designed mutant in HupSL from *Nostoc punctiforme*. *Energy and Environmental Science*, 9, 581–594.

Roumezi, B., Avilan, L., Risoul, V., Brugna, M., Rabouille, S. & Latifi, A. (2020) Overproduction of the Flv3B flavodiiron, enhances the photobiological hydrogen production by the nitrogen-fixing cyanobacterium *Nostoc* PCC 7120. *Microbial Cell Factories*, 19, 65.

Sadler, N.C., Bernstein, H.C., Melnicki, M.R., Charania, M.A., Hill, E.A., Anderson, L.N. et al. (2016) Dinitrogenase-driven photobiological hydrogen production combats oxidative stress in *Cyanothece* sp. strain ATCC 51142. *Applied and Environmental Microbiology*, 82, 7227–7235.

Sakurai, H., Masukawa, H., Kitashima, M. & Inoue, K. (2015) How close we are to achieving commercially viable large-scale photobiological hydrogen production by cyanobacteria: a review of the biological aspects. *Life*, 5, 997–1018.

Sandh, G., Ramström, M. & Stensjö, K. (2014) Analysis of the early heterocyst Cys-proteome in the multicellular cyanobacterium *Nostoc punctiforme* reveals novel insights into the division of labor within diazotrophic filaments. *BMC Genomics*, 15, 1064.

Scoma, A. & Hemschemeier, A. (2017) The hydrogen metabolism of sulfur deprived *Chlamydomonas reinhardtii* cells involves hydrogen uptake activities. *Algal Research*, 26, 341–347.

Siebel, J.F., Adamska-Venkatesh, A., Weber, K., Rumpel, S., Reijerse, E. & Lubitz, W. (2015) Hybrid [FeFe]-hydrogenases with modified active sites show remarkable residual enzymatic activity. *Biochemistry*, 54, 1474–1483.

Skjånes, K., Pinto, F.L. & Lindblad, P. (2010) Evidence for transcription of three genes with characteristics of hydrogenases in the green alga *Chlamydomonas noctigama*. *International Journal of Hydrogen Energy*, 35, 1074–1088.

Steinbeck, J., Nikolova, D., Weingarten, R., Johnson, X., Richaud, P., Peltier, G. et al. (2015) Deletion of proton gradient regulation 5 (PGR5) and PGR5-like 1 (PGRL1) proteins promote sustainable light-driven hydrogen production in *Chlamydomonas reinhardtii* due to increased PSII activity under sulfur deprivation. *Frontiers in Plant Science*, 6, 1–11.

Stensjö, K., Ow, S.Y., Barrios-Llerena, M.E., Lindblad, P. & Wright, P.C. (2007) An iTRAQ-based quantitative analysis to elaborate the proteomic response of *Nostoc* sp. PCC 7120 under N_2-fixing conditions. *Journal of Proteome Research*, 6, 621–635.

Stripp, S.T., Goldet, G., Brandmayr, C., Sanganas, O., Vincent, K.A., Haumann, M. et al. (2009) How oxygen attacks [FeFe]-hydrogenases from photosynthetic organisms. *Proceedings of the National Academy of Sciences of the United States of America*, 106, 17331–17336.

Subramanian, V., Dubini, A., Astling, D.P., Laurens, L.M.L., Old, W.M., Grossman, A.R. et al. (2014) Profiling chlamydomonas metabolism under dark, anoxic H_2-producing conditions using a combined proteomic, transcriptomic, and metabolomic approach. *Journal of Proteome Research*, 13, 5431–5451.

Terashima, M., Specht, M., Naumann, B. & Hippler, M. (2010) Characterizing the anaerobic response of *Chlamydomonas reinhardtii* by quantitative proteomics. *Molecular and Cellular Proteomics*, 9, 1514–1532.

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

Touloupakis, E., Faraloni, C., Silva Benavides, A.M., Masojídek, J. & Torzillo, G. (2021) Sustained photobiological hydrogen production by *Chlorella vulgaris* without nutrient starvation. *International Journal of Hydrogen Energy*, 46, 3684–3694.

Tsygankov, A.A., Kosourov, S.N., Tolstygina, I.V., Ghirardi, M.L. & Seibert, M. (2006) Hydrogen production by sulfur-deprived *Chlamydomonas reinhardtii* under photoautotrophic conditions. *International Journal of Hydrogen Energy*, 31, 1574–1584.

Volgusheva, A., Kosourov, S., Lynch, F. & Allahverdiyeva, Y. (2019) Immobilized heterocysts as microbial factories for sustainable nitrogen fixation. *Journal of Biotechnology: X*, 4, 100016.

Volgusheva, A., Kruse, O., Styring, S. & Mamedov, F. (2016) Changes in the photosystem II complex associated with hydrogen formation in sulfur deprived *Chlamydomonas reinhardtii*. *Algal Research*, 18, 296–304.

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

Volgusheva, A., Styring, S. & Mamedov, F. (2013) Increased photosystem II stability promotes H_2 production in sulfur-deprived *Chlamydomonas reinhardtii*. *Proceedings of the National Academy of Sciences*, 110, 7223–7228.

Wegelius, A., Khanna, N., Esmieu, C., Barone, G.D., Pinto, F., Tamagnini, P. et al. (2018) Generation of a functional, semi-synthetic [FeFe]-hydrogenase in a photosynthetic microorganism. *Energy and Environmental Science*, 11, 3163–3167.

Winkler, M., Duan, J., Rutz, A., Felbek, C., Scholtysek, L., Lampret, O. et al. (2021) A safety cap protects hydrogenase from oxygen attack. *Nature Communications*, 12, 1–10.

Wutthithien, P., Lindblad, P. & Incharoensakdi, A. (2019) Improvement of photobiological hydrogen production by suspended and immobilized cells of the N_2-fixing cyanobacterium *Fischerella muscicola* TISTR 8215. *Journal of Applied Phycology*, 30, 1–10.

Xu, L., Fan, J. & Wang, Q. (2019) Omics application of bio-hydrogen production through green alga *Chlamydomonas reinhardtii*. *Frontiers in Bioengineering and Biotechnology*, 7, 201.

Yacoby, I., Pochekailov, S., Toporik, H., Ghirardi, M.L., King, P.W. & Zhang, S. (2011) Photosynthetic electron partitioning between [FeFe]-hydrogenase and ferredoxin:NADP+-oxidoreductase (FNR) enzymes in vitro. *Proceedings of the National Academy of Sciences of the United States of America*, 108, 9396–93401.

How to cite this article: Kosourov S, Böhm M, Senger M, et al. Photosynthetic hydrogen production: Novel protocols, promising engineering approaches and application of semi-synthetic hydrogenases. *Physiologia Plantarum*. 2021;1–13. https://doi.org/10.1111/ppl.13428