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Thin cell layer cultures of *Chlamydomonas reinhardtii* L159I-N230Y, *pgrl1* and *pgr5* mutants perform enhanced hydrogen production at sunlight intensity

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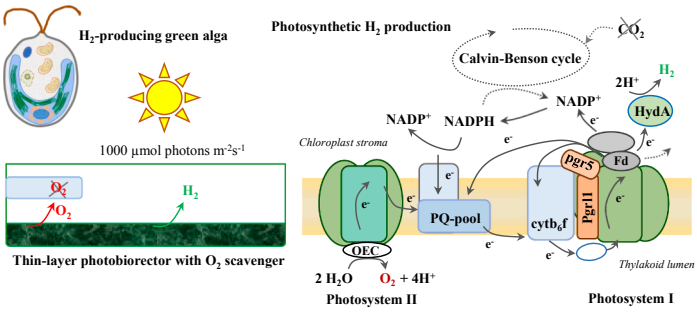
HIGHLIGHTS

- Anaerobiosis-induced photoautotrophic H_2 production lasts days in continuous light.
- Dense thin-layer cultures have improved H_2 production at sunlight intensity.
- H_2 productivities of the L159I-N230Y, *pgrl1*, and *pgr5* mutants are enhanced.

GRAPHICAL ABSTRACT



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ABSTRACT

Photobiological hydrogen (H_2) production is a promising renewable energy source. HydA hydrogenases of green algae are efficient but O_2-sensitive and compete for electrons with CO_2-fixation. Recently, we established a photoautotrophic H_2 production system based on anaerobic induction, where the Calvin-Benson cycle is inactive and O_2 scavenged by an absorbent. Here, we employed thin layer cultures, resulting in a three-fold increase in H_2 production relative to bulk CC-124 cultures (50 µg chlorophyll/ml, 350 µmol photons m^{-2} s^{-1}). Productivity was maintained when increasing the light intensity to 1000 µmol photons $m^{-2}s^{-1}$ and the cell density to 150 µg chlorophyll/ml. Remarkably, the L159I-N230Y photosystem II mutant and the *pgrl1* photosystem I cyclic electron transport mutant produced 50% more H_2 than CC-124, while the *pgr5* mutant generated 250% more (1.2 ml H_2/ml culture in six days). The photosynthetic apparatus of the *pgr5* mutant and its *in vitro* HydA activity remained remarkably stable.

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1. Introduction

Employing algal cells as whole-cell biocatalysts is a promising strategy to produce biofuels and high-value products, owing to their easy and rapid cultivation and remarkable CO_2 mitigation capacity. Biohydrogen production by green algae stands out in particular as it is directly linked to photosynthesis and has high theoretical energy conversion efficiency. While hydrogen (H_2) has a wide range of applications that are continuously expanding, it is presently produced mainly by steam reforming of fossil fuels, which contributes to the rise of atmospheric CO_2.

In the natural environment, the anoxic conditions established for instance during the night favor the expression of HydA hydrogenases in green algae. Upon illumination, photosynthetic electron transport is initiated, and electrons are transferred to HydA until the Calvin-Benson cycle becomes fully activated. HydA acts as a safety valve, protecting the photosynthetic electron transport chain from over-reduction (Godaux et al., 2015). Hydrogen production also promotes the increase of chloroplast stromal pH, required for activation of the Calvin-Benson cycle. Once activated, the Calvin-Benson cycle outcompetes HydA for electrons, thereby halting H_2 production (Milrad et al., 2018, Nagy et al., 2018a). Moreover, alternative electron transport processes around photosystem I (PSI) compete with HydA (Godaux et al., 2015, Burlacot et al., 2018), and the O_2 evolved by photosystem II (PSII) inactivates the catalytic site of HydA and downregulates *HYDA* expression (Eivazova and Markov, 2012).

While in nature H_2 production lasts only for a few minutes upon dark-light transitions, in a laboratory setting, it can be prolonged by e.g., sulfur deprivation, resulting in the downregulation of PSII activity (and thereby O_2 evolution) and increased expression and activity of HydA (Melis et al., 2000). However, this system is unsustainable because 1) the degradation of the photosynthetic apparatus limits H_2 production (Nagy et al., 2018b), and 2) sulfur-deprivation-induced H_2 production is dependent on organic carbon.

Long-term H_2 production can be attained while sustaining photosynthesis by directly driving the electrons derived from the water-splitting activity of PSII to HydA via photosystem I (PSI). In Nagy et al. (2018a) we demonstrated that algal cultures subjected to dark anaerobic incubation followed by continuous illumination in CO_2- and acetate-free conditions could produce H_2 for at least four days. H_2 production can be increased by applying O_2 absorbents, protecting HydA from inactivation (Nagy et al., 2018a, Khosravitabar and Hippler, 2019). The H_2 production protocol developed by Kosourov et al. (2018) is based on the application of short light pulses. These methods are similar because they both prevent the activation of the Calvin-Benson cycle upon illumination, thereby attenuating the competition for electrons in favor of H_2 production. Since HydA is a less efficient electron acceptor than the Calvin-Benson cycle, photosynthetic electron transport components, including the plastoquinone (PQ) pool, become reduced, slowing down linear electron transport. This so-called "photosynthetic control" entails decreased O_2 evolution, thereby sustaining HydA activity (Nagy et al., 2018a).

Hydrogen photoproduction upon sulfur deprivation performs relatively well at moderate light intensities (usually up to 200 μmol photons m^{-2} s^{-1}), whereas it strongly decreases near sunlight intensity (Scoma et al., 2012a, Geier et al., 2012). As the final goal of this biotechnology is to exploit full sunlight, it would be highly advantageous to develop robust H_2 production systems able to perform at the intensity of sunlight.

We have shown earlier that anaerobiosis-induced photoautotrophic H_2 production enables the utilization of somewhat higher light intensities than the traditional sulfur-deprivation method (350 μmol photons m^{-2} s^{-1}, Nagy et al., 2018a). Here, we achieved a three-fold increase in H_2 production by employing dense thin-layer cultures instead of traditional bulk cultures, with productivity maintained at 1000 μmol photons m^{-2} s^{-1} for six days. Furthermore, the PSI cyclic electron transport (PSI-CET) mutant *pgr5* produced 2.5-fold more H_2 than the wild-type CC-124 strain (1.2 ml H_2/ml culture) and its photosynthetic apparatus was largely preserved at sunlight intensity.

2. Materials and methods

2.1. Algal growth conditions and H_2 production

Wild-type CC-124 and CC-409 strains were obtained from the Chlamydomonas Resource Center (University of Minnesota, USA) The *pgrl1* and *pgr5 C. reinhardtii* mutants were kindly provided by Prof. Michael Hippler (University of Münster, Germany). The L159I-N230Y mutant was generated and kindly provided by Prof. Udo Johanningmeier (Martin-Luther-Univeärsitt Halle-Wittenberg, Germany). All strains were grown initially at 22 °C in 500 ml Erlenmeyer flasks containing 300 ml Tris-acetate-phosphate (TAP) medium, shaken at 120 rpm in under continuous illumination of 80–90 μmol photons m^{-2} s^{-1} photosynthetically active radiation, provided by T8 cool white fluorescent light tubes (Sylvania luxline plus).

After three days of cultivation, cells were transferred to high-salt (HS) medium (http://www.chlamycollection.org/methods/media-recipes/) and the chlorophyll (Chl) content was set (based on the method by Porra et al., 1989) to 50 or 150 μg Chl (a + b)/ml.

For H_2 production in bulk cultures, 30 ml of culture processed as above was placed in 100-ml glass serum bottles (52 mm × 95 mm, total volume: 120 ml, light path: 22 mm, surface-to-volume ratio: 7.065 cm^2/120 cm^3 = 0.059 cm^{-1}) and sealed with rubber septa under sterile conditions. Thin layer cell cultures were established in modified 1-L Pyrex® Roux culture bottles (55 mm × 120 mm × 255 mm), which were placed horizontally with 100 ml cell culture, resulting in approx. 5 mm light path and a surface-to-volume ratio of approx. 240 cm^2/1000 cm^3 = 0.24 cm^{-1} (TCL-PBR).

An iron-salt-based, non-cytotoxic O_2 absorbent (O2Zero-50 cc loose; Global Reach Ltd, London, UK) was used to diminish the O_2 concentration below 0.05% in the headspace. 1.3 g of O_2 absorbent was put into a 2 ml-tube, which was introduced into the headspace of the serum bottles (Fig. 1A). In the case of the TCL-PBR, a holder was fabricated with slots on the side in which 20 g of O_2 absorbent was placed (Fig. 1A). Both reactor types had comparable amounts of absorbent in the gas phase (approx. 0.016 g/ml).

Dark anaerobic incubation was performed by flushing the headspace with N_2 gas for 20 min and keeping the cultures in the dark for 4 h. Afterward, algal cultures were placed under warm white LED panels, providing approx. 350 or 1000 μmol photons m^{-2} s^{-1} light. The cultures were illuminated continuously, kept at 25 °C for 96 or 144 h, and shaken at 120 rpm.

2.2. Determination of the amount of H_2 and O_2 by gas chromatography

The net amounts of H_2 and O_2 were determined by collecting a 100 μl aliquot from the gas phase of the cultures with a gas-tight Hamilton microsyringe. These samples were injected manually into a Hewlett Packard 5890 gas chromatograph (GC) equipped with an HP-PLOT Molesieve column (30 m × 0.53 mm × 0.25 μm) set at 40 °C and connected to a thermal conductivity detector set at 160 °C. The carrier gas was argon, linear velocity 115 cm/s. After gas sampling every 24 h, reactors were flushed with N_2 gas to prevent H_2 accumulation above 5% in the gas phase (Kosourov et al., 2012).

2.3. Chl a fluorescence measurements

Fluorescence measurements were carried out as described earlier (Nagy et al., 2018a; 2018b). Briefly, *C. reinhardtii* cultures were dark-adapted for about 15 min, and then 60 μl of cell suspension (150 μg Chl (a + b)/ml) was placed onto a Whatman glass microfibre filter (GF/B) that was placed in a Handy-PEA clip and measured with a HandyPEA

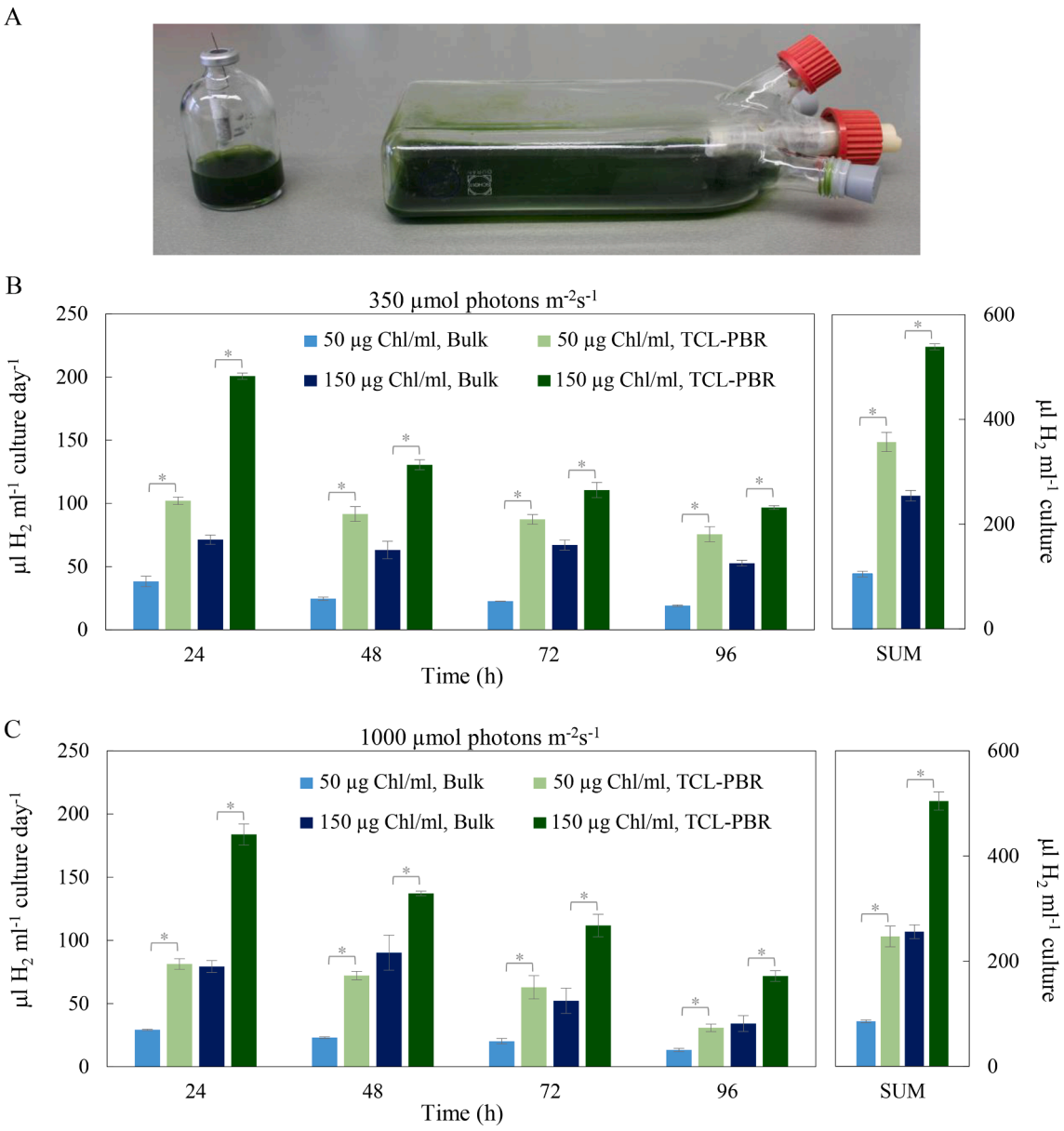

Fig. 1. Photoautotrophic, anaerobiosis-induced H_2 production by *Chlamydomonas reinhardtii* CC-124 cultures in serum bottles (bulk) and thin cell layer photobioreactors (TCL-PBR). A) Photos of bulk cultures and TCL-PBR. B) H_2 production by bulk cultures and in TCL-PBR with 50 and 150 µg Chl (a + b)/ml initial Chl contents at 350 µmol photons m^{-2} s^{-1} continuous white light. C) H_2 production by bulk cultures and in TCL-PBR with 50 and 150 µg Chl (a + b)/ml initial Chl contents at 1000 µmol photons m^{-2} s^{-1} continuous white light. H_2 production was measured once every 24 h after which the gas phase was flushed with N_2 to remove the produced gases. SUM is the total amount of H_2 produced in 96 h. The data represent means ± SE of three independent biological replicates. The significance of differences between bulk and TCL-PBR cultures at the same Chl concentration at the same time points was determined by Student's *t*-test. Asterisks indicate significantly different means ($p < 0.05$).

instrument (Hansatech Instruments Ltd, UK).

2.4. Immunoblot analysis

At each sampling point, 2 ml of culture were collected, spun-down for removal of the supernatant, and frozen in liquid nitrogen. The samples were then solubilized as described in Nagy et al. (2016). An amount of Chl(a + b) equivalent to 1 million cells was then mixed with 6x Laemmli buffer (375 mM Tris/HCl [pH 6.8], 60% [v/v] glycerin, 12.6% [w/v] sodium dodecyl sulfate, 600 mM dithiothreitol, 0.09% [w/v] bromophenol blue) and incubated at 75 °C for 10 min before loading. Protein separation and western blot were carried out as described in Podmaniczki et al. (2021). Specific polyclonal antibodies (produced in rabbit) against PsbA (N-terminal), PSBO, PSBP, CP47, PsaA, HydA were purchased from Agrisera AB.

2.5. In vitro hydrogenase activity assay

In vitro hydrogenase activity (in µl H_2/million cells/h) was measured after the 4-h dark anaerobic incubation and after 144 h of H_2 production in the light, similarly to Hemschemeier et al. (2009). The assay was carried out at 37 °C, in darkness in 13.5-ml serum bottles. The reaction mixture consisted of 1.9 ml of 100 mM potassium phosphate buffer, pH 6.8, 760 µl of deionized water, 100 µl of 10% Triton X-100, 40 µl of 1 M methyl viologen, 400 µl of anaerobic 1 M sodium dithionite and 200 µl of algal culture. The H_2 concentration in the headspace was measured by GC every 20 min and fitted with linear regression.

2.6. Analysis of HydA1 gene expression

One ml culture, containing approximately 150 μg chl (a + b) was collected. RNA isolation was carried out by the DirectZol RNA kit and the isolated RNA was treated with DNaseI (Zymo Research). Reverse transcription of 1 μg of RNA was primed with oligo dT using FIREScript reverse transcriptase (Solis BioDyne). Real-time qPCR analysis was performed using a Bio-Rad CFX384 Touch Real-Time PCR Detection System using HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (Solis BioDyne). To ensure correct normalization of the *HYDA1* (Cre03.g199800; primers: GGCGAGTGGGACAATCCAAT and TGCCCGTGAACAGCTCATAG) transcript level, four reference genes showing stable expression during H_2 production were used, namely *bTUB2* (Cre12.g549550; primers: ACTGGCTGTGATTGTGCTTCAGG and TGTCTGCTGCTGCACCTTTACG), *UBQ2* (Cre09.g396400; primers: GCGATTTCTCGTTGGGCAGT and TGGCCCATCCACTTGTCCTT), *CBLP* (Cre06.g278222; primers: ATCAAGATCTGGGACCTGGAGAGC and CTTGCTGGTGATGTTGAACTCGGG) and *RBCS2* (Cre02.g120150; primers: AACGGCGGTGGATGGAAGATAC and AAGACTGATCAGCACGAAACGG). The mRNA transcript abundance of *HYDA1* was normalized to the average of the reference genes and expressed relative to the samples collected after 4 h of anaerobiosis treatment. Three technical replicates and three or four biological replicates were used for the analysis and standard errors were calculated.

2.7. Statistical analysis

The presented data are based on at least three independent experiments. The exact number of the biological repetitions are indicated in the figure captions. When applicable, averages and standard errors (±SE) were calculated. The significance of the mean differences between the mutants and the CC-124 wild type strain under each growth condition were analyzed by Student's *t*-test or by two-way mixed ANOVA with Tukey post-hoc test at $p < 0.05$ level using OriginPro 9.5 software.

3. Results and discussion

3.1. Dense thin-layer cultures have improved H_2 production

HydA expression and activity are inhibited by O_2 that is produced by PSII (Swanson et al., 2015, Happe and Kaminski 2002, Eivazova and Markov, 2012), whereas high H_2 partial pressures lead to H_2 uptake by HydA (Kosourov et al., 2012). Diminution of both O_2 and H_2 concentration in the PBR is thus essential to improve H_2 production. Increasing the surface-to-volume ratio of liquid algal cultures promotes the diffusion of the gases produced by the cells to the gas phase, preventing or delaying possible inhibitory effects on H_2 production.

Thin cell layer photobioreactors (TCL-PBR) are cultivation systems characterized by low culture thickness, thereby short light paths (<10 mm) and high surface-to-volume ratios. These features allow TCL-PBR to be operated at very high cell densities (up to 1000 μg Chl(a + b)/ml culture, Masojídek et al., 2011, Masojídek et al., 2015) and improving photosynthetic productivity per unit of irradiated area. When coupled to turbulent mixing, high cell densities in TCL-PBR also enable short light/dark cycles (e.g. 470 ms^{-1} in TCL-PBR operated outdoors, Doucha and Lívanský, 1995, Masojídek et al., 2011). Short light/dark cycles improve light utilization efficiency by preventing over-reduction of the photosynthetic electron transport (Matthijs et al., 1996, Nedbal et al., 1996), and the activation of the Calvin-Benson cycle, which competes with H_2 production (Kosourov et al., 2018).

In this study, we aimed at improving H_2 production by employing closed TCL-PBRs (Fig. 1A). First, we compared the amount of H_2 produced by either bulk cultures in serum bottles or by thin cell layer algal cultures in horizontally-placed 1-L Roux bottles (culture thickness: approx. 22 *vs*. 5 mm, respectively). The surface-to-volume ratio is four-fold higher in the TCL-PBR than in the serum bottle (0.059 cm^{-1} *vs*. 0.24 cm^{-1}), presumably improving degassing. In the TCL-PBR, the gas-to-liquid ratio is increased by a factor three (3 *vs*. 9), contributing to the maintenance of low partial O_2 and H_2 pressures. We note that regular flat-panel PBRs (e.g., Gilbert et al., 2011, Skjånes et al., 2016) and laboratory-scale PBRs have minimal gas phases, i.e., the gas-to-liquid ratio is usually below 1.5 (e.g., Fedorov et al., 2005, Tsygankov et al., 2006).

Experiments were conducted at 50 or 150 μg Chl(a + b)/ml, and at 350 or 1000 μmol photons $m^{-2}s^{-1}$, using the widely tested CC-124 strain. At 50 Chl(a + b)/ml and 350 μmol photons $m^{-2}s^{-1}$ light intensity, the amount of H_2 produced in TCL-PBR increased 3.6 times compared to bulk cultures in serum bottles (in 96 h, approx. 360 *vs*. 100 μl H_2/ml; Fig. 1B). When Chl concentration was tripled (from 50 to 150 μg Chl(a + b)/ml), the amount of H_2 produced by bulk cultures increased 2.5-fold, whereas in thin-layer cultures the increase was less substantial (approx. 1.5 times, Fig. 1B). Thus, TCL-PBRs could still perform two times better than bulk cultures at 150 Chl(a + b)/ml (539 *vs*. 254 μl H_2/ml; Fig. 1B).

Next, H_2 productivity was tested in the range of sunlight intensity, i.e. 1000 μmol photons $m^{-2}s^{-1}$. For cultures at 50 Chl(a + b)/ml, this increase in light intensity diminished the amount of produced H_2 by approx. 20 and 30% in bulk cultures and TCL-PBRs, respectively (86 and 247 μl H_2/ml; Fig. 1C), probably due to photoinhibition. When employing high cell-density cultures (150 Chl(a+b)/ml), the total amount of H_2 produced remained essentially the same as obtained at 350 μmol photons $m^{-2}s^{-1}$ (255 and 504 μl H_2/ml by bulk cultures and TCL-PBR, respectively, compare Fig. 1B and 1C).

Calculating the amount of produced H_2 on a Chl(a + b) basis suggests that the highest light usage efficiency could be achieved by medium cell density in TCL-PBR at 350 μmol photons $m^{-2}s^{-1}$; the difference between bulk and thin-layer cultures was more than three-fold (approx. 2090 and 7100 μl H_2/mg Chl(a + b) was produced in 72 h, respectively).

Application of a highly performant O_2 scavenger (see Materials and Methods) resulted in O_2 concentrations in the gas phase below the detection limit of our GC system (approx. 0.05%), both in bulk cultures and TCL-PBRs, with either light intensity and Chl content.

The highest H_2 production was achieved in TCL-PBR at 150 Chl(a + b)/ml. While no improvement in H_2 accumulation occurred between moderate and very high light intensities (350 *vs*. 1000 μmol photons $m^{-2}s^{-1}$), no diminution occurred either. We point out that the photosynthetic apparatus of sulfur-deprived cultures is degraded within two days when subjected to intense light outdoors, with H_2 productivity decreased to 10–20% of cultures kept at moderate light (Scoma et al., 2012a, Geier et al., 2012). Further implementation of the present set up appears as the most promising alternative to scale up to outdoor, large-scale systems. The fact that increasing the light intensity about three times did not result in increased H_2 outputs suggests that the major limitation is not the excitation energy but the photosynthetic electron transport or HydA activity. In the following experiments, we tested three photosynthetic mutants affected in PSII and PSI-CET activities to validate this hypothesis.

3.2. Improved hydrogen production by the L159I-N230Y mutant in TCL-PBR at sunlight intensity

The L159I-N230Y mutant of *Chlamydomonas reinhardtii* carries a double amino acid substitution in its PsbA protein: the leucine residue L159 was replaced by isoleucine, and the N230 asparagine by tyrosine. It was reported that this strain has reduced amount of Chl per dry weight, approx. 20% higher photosynthetic capacity and 40% higher dark respiration rate on a Chl basis than its wild-type CC-409 strain (Torzillo et al., 2009). Along with this, the L159I-N230Y mutant produces about twice as much H_2 as the CC-124 strain upon sulfur deprivation (Scoma et al., 2012b), and it was also successfully applied in a long-term anaerobiosis-induced H_2 production experiment (Scoma et al., 2014).

We compared the H_2 production of the L159I-N230Y mutant, its CC-409 background strain, and the CC-124 control strain in TCL-PBR at

sunlight intensity (1000 μmol photons $m^{-2}s^{-1}$) and high Chl content (150 μg Chl(a + b)) in a 6-day experiment. The H_2 production of all three strains was comparable in the first 24 h, approx. 200 μl/ml (Fig. 2A). After the first day, there was a steady decrease of H_2 production by the CC-124 and the CC-409 strains, whereas it remained fairly stable in the L159I-N230Y mutant, which declined more consistently only by day 6 (144 h). In total, the L159I-N230Y strain produced approx. 980 μl H_2/ml culture in 6 days, whereas CC-124 and CC-409 produced approx. 640 and 730 μl/ml H_2, respectively (Fig. 2A). The amount of O_2 in each culture's headspace was below the detection limit of our GC system that is 0.05%.

The stability of the photosynthetic apparatus during six days of H_2 production was investigated in the three strains. The Chl content remained approx. at the initial level in the L159I-N230Y strain, whereas it decreased by approx. 30% and 50% in the CC-409 and CC-124 strains, respectively (Fig. 2B). The cell density increased slightly in the first 24 h in all strains, then remained relatively stable throughout the experiment (Fig. 2C). These data show no substantial cell lysis occurred, but Chl became partially degraded in the CC-409 and CC-124 strains.

Regarding the composition of the photosynthetic apparatus, an important observation is that various PSII subunits, including PsbA, CP47, PSBO, and PSBP degraded in the CC-124 strain, while they were more stable in the CC-409 and L159I-N230Y strains (Fig. 3). A similar trend occurred for the PsaA subunit of PSI (Fig. 3). The amount of HydA strongly decreased (by about 70%) in the CC-124 strain already after 24 h of H_2 production, whereas it was more stable in the other two strains (Fig. 3).

In agreement with these data, the F_V/F_M parameter, an indicator of photosynthetic efficiency, remained significantly higher in the CC-409 and L159I-N230Y strains than in CC-124 (Fig. 2D), further suggesting that they are more resistant to high-light exposure during anaerobiosis-induced H_2 production than the CC-124 strain.

L159I-N230Y produced about 30% more than CC-409, although their photosynthetic apparatus and performance were comparable after 144 h. In an earlier study, it was demonstrated that the L159I-N230Y mutant is more sensitive to high light than the two wild-type strains (Scoma et al., 2012a). Therefore, it is likely that the improved H_2 production of the L159I-N230Y mutant relative to CC-409 is not related to increased high light tolerance but to its higher respiration rate (reported earlier by Torzillo et al., 2009) that may feed the electron transport towards HydA and at the same time minimize the level of intracellular O_2.

3.3. *H_2 production by the pgr5 and pgrl1 mutants in TCL-PBR at sunlight intensity*

Photosystem I cyclic electron transport is a mechanism enabling the adjustment of ATP to NADPH ratio in the chloroplast, and it plays a vital role in photoprotection, particularly upon the induction of photosynthesis (Nawrocki et al., 2019a). In *C. reinhardtii*, the primary route for PSI-CET is the ferredoxin (Fd)-dependent PSI cyclic electron flow in anoxia. PSI-CET recycles electrons, and in doing so, it generates a proton motive force that controls the rate of photosynthesis. The PROTON GRADIENT REGULATION LIKE 1 (PGRL1) and the PROTON GRADIENT REGULATION 5 (PGR5) proteins are involved in the regulation of this process (Tolleter et al., 2011, Johnson et al., 2014), although they are probably not directly transferring electrons from Fd to the plastoquinone (PQ) pool during PSI-CET (Nawrocki et al., 2019a; 2019b). PGRL1 is a transmembrane protein found in thylakoids, and PGR5 is a small, stroma-soluble protein, binding no cofactors, and it is tethered to the thylakoids by PGRL1 (Shikanai 2007, DalCorso et al., 2008). They are

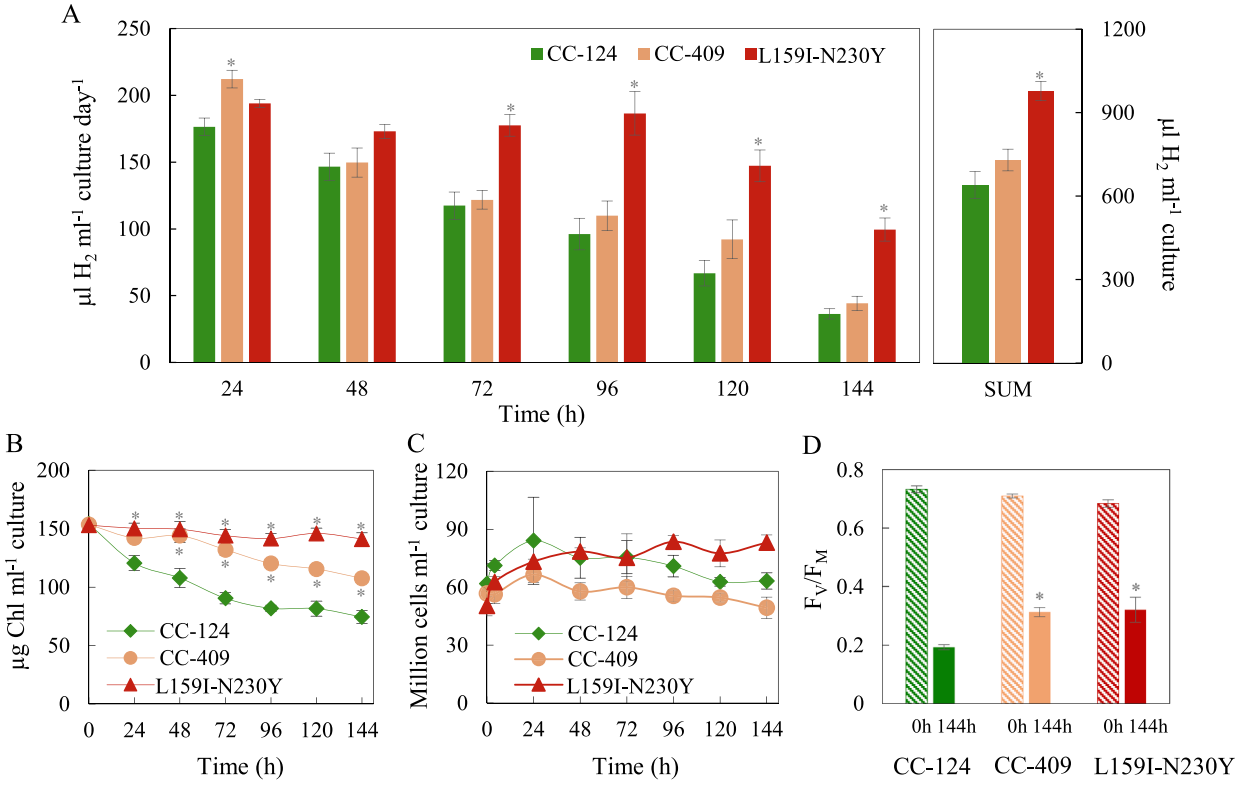

Fig. 2. Photoautotrophic, anaerobiosis-induced H_2 production by the PsbA mutant L159I-N230Y, its background CC-409 and by the CC-124 strain in thin cell layer photobioreactors (TCL-PBR) with 150 μg Chl (a + b)/ml initial Chl contents at 1000 μmol photons m^{-2} s^{-1} continuous white light A) Daily H_2 production and the total amount of H_2 produced in 144 h (SUM). B) Changes in Chl(a + b) content during H_2 production C) Changes in cell number D) The photosystem II parameter F_V/F_M of aerobic control cultures and after 144 h of H_2 production. The data represent means ± SE of seven to eight independent biological replicates. The significance of differences between means was determined by two-way mixed ANOVA with Tukey post-hoc test. Asterisks indicate significantly different means in comparison with CC-124 at each time point ($p < 0.05$).

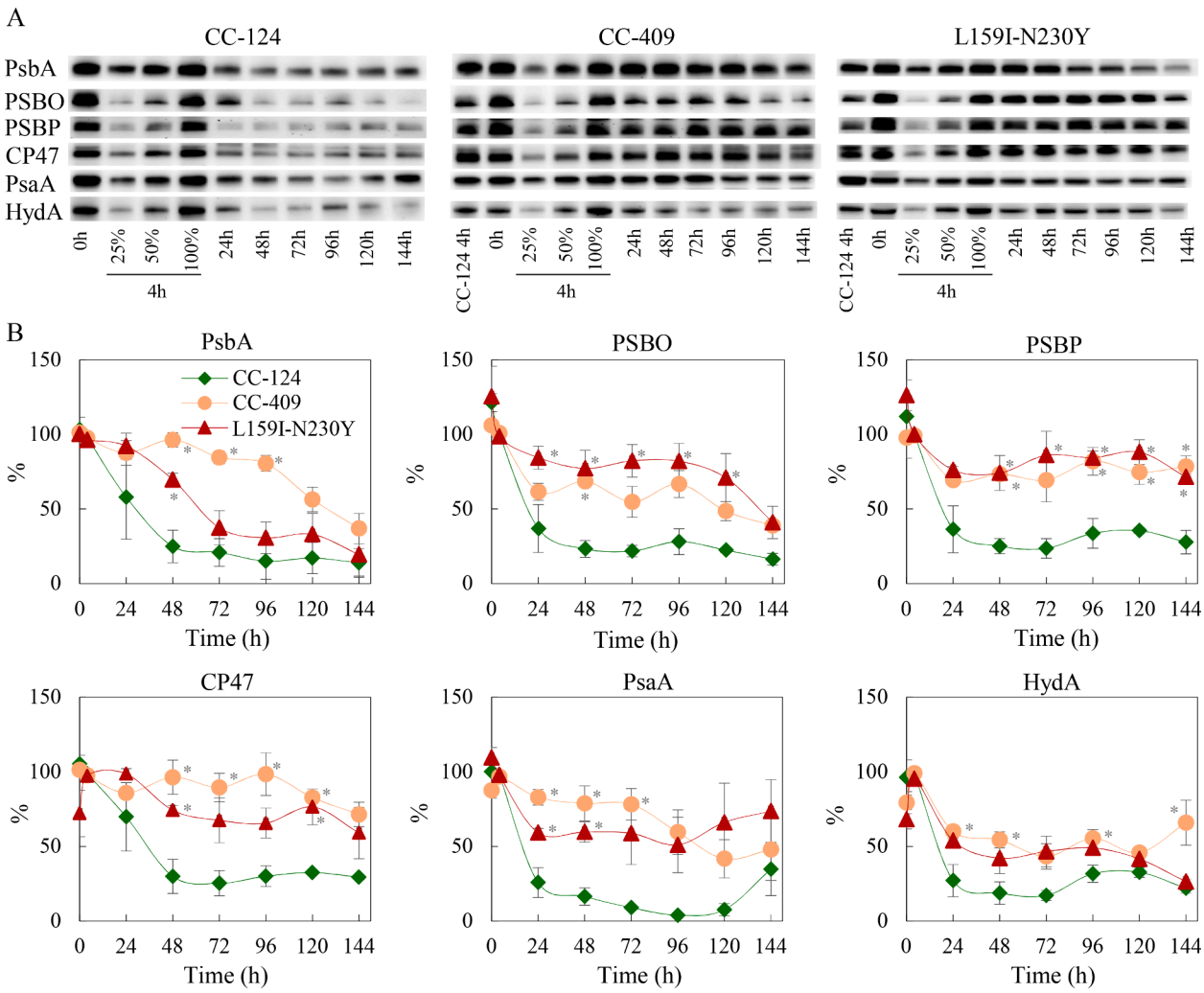

Fig. 3. Immunoblot analysis for the semi-quantitative determination of HydA and certain photosynthetic subunits of the L159I-N230Y mutant, its background CC-409, and the CC-124 strain during 144 h of H_2 production in thin cell layer photobioreactors (TCL-PBR) at 1000 μmol photons m^{-2} s^{-1} continuous white light. A) Representative immunoblots. An amount of Chl(a + b) equivalent to 1 million cells was loaded in each well. The 0 h samples represent the aerobic control, the 25%, 50%, and 100% samples were taken after 4 h of anaerobic induction (4 h-sample) and are for the approximate quantitation of the proteins by densitometry. For comparison, the 4 h-sample of the CC-124 strain was loaded on the blots of the CC-409 and the L159I-N230Y mutant as well. B) Densitometry analysis of the immunoblots, based on 3 to 4 independent experiments. The 4 h-sample of each genotype was used as the 100% reference for the densitometry analysis. The significance of differences between means was determined by two-way mixed ANOVA with Tukey post-hoc test. Asterisks indicate significantly different means in comparison with CC-124 at each time point ($p < 0.05$).

both components of a super-complex, necessary to promote PSI-CET in *C. reinhardtii*, particularly in anoxic conditions (Godaux et al. 2015, Petroutsos et al., 2009, Terashima et al., 2012, Steinbeck et al., 2018). PSI-CET is most required under conditions when Fd may become over-reduced, and PSI is subjected to photoinhibition, such as high light, limiting Calvin-Benson cycle activity and anoxia (Johnson et al., 2014).

Both PGR5 and PGRL1 deficiency in *C. reinhardtii* leads to a diminished proton gradient across the thylakoid membrane, accompanied by less effective PSI-CET capacity and increased light-induced respiration (Petroutsos et al., 2009, Dang et al., 2014, Tolleter et al., 2011, Steinbeck et al., 2015). Under photoautotrophic conditions, the absence of PGR5 also leads to enhanced light sensitivity (Johnson et al., 2014).

The proton gradient across the thylakoid membrane restricts electron flow toward HydA, both under sulfur deprivation (Antal et al., 2009, Tolleter et al., 2011) and anaerobiosis-induced H_2 production (Nagy et al., 2018a), therefore diminishing PSI-CET is expected to result in the improvement of H_2 production. Besides, both mutants have an increased mitochondrial respiration capacity, leading to decreased intracellular O_2 levels, which may allow more sustained HydA activity (Johnson et al., 2014, Godaux et al., 2015).

The *pgr5* and *pgrl1* mutants produce several times more H_2 than the CC-124 strain under sulfur deprivation-induced H_2 production in an acetate-containing medium at 60 μmol photons m^{-2} s^{-1} (Steinbeck et al., 2015). At 200 μmol photons m^{-2} s^{-1}, the *pgrl1* mutant produced H_2 for several days, whereas the total amount of H_2 produced by the *pgr5* mutant was diminished due to photoinhibition and PSI degradation, and the CC-124 strain produced practically no H_2 at all (Steinbeck et al., 2015).

In our photoautotrophic anaerobiosis-induced H_2 production system, the *pgr5* mutant performed very well at 1000 μmol photons m^{-2} s^{-1} and 150 μg Chl(a + b)/ml: on the first day, it produced already remarkably more H_2 than the CC-124 strain (by about 65%), and the difference became much larger towards the end of the experiment (Fig. 4A). On days 4 and 5, the *pgr5* mutant produced approx. five-fold more H_2 than CC-124; the total amount of H_2 was 2.5 times as much as that of the CC-124 strain. The *pgrl1* mutant showed a similar trend, but the improvement was only about 50% as compared to the CC-124 strain (Fig. 4A).

Remarkably, the Chl(a + b) content of the *pgr5* mutant remained

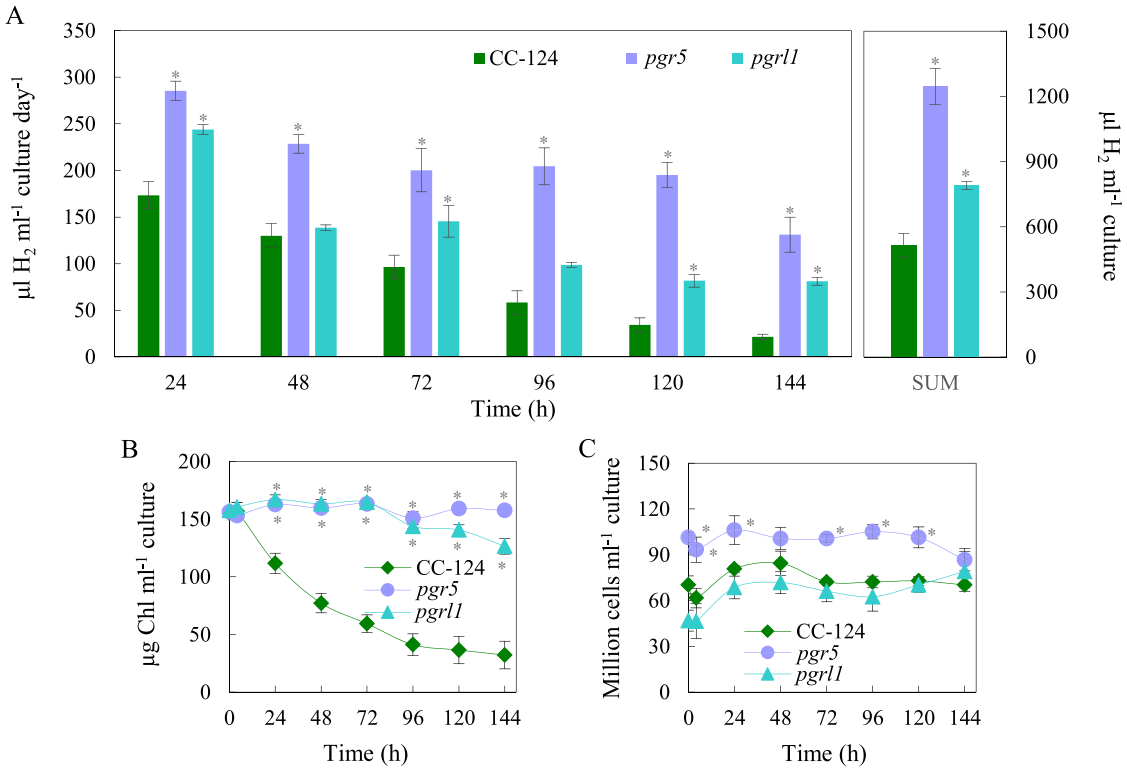

Fig. 4. Photoautotrophic, anaerobiosis-induced H_2 production by the photosystem I cyclic electron transport mutants *pgr5* and *pgrl1* and by their background strain CC-124 in thin cell layer photobioreactors (TCL-PBR) with 150 μg Chl(a + b)/ml initial Chl contents at 1000 μmol photons m^{-2} s^{-1} continuous white light A) Daily H_2 production and the total amount of H_2 produced in 144 h (SUM). B) Changes in Chl(a + b) content during H_2 production C) Changes in cell number. The data represent means ± SE of three to four independent biological replicates. The significance of differences between means was determined by two-way mixed ANOVA with Tukey post-hoc test. Asterisks indicate significantly different means in comparison with CC-124 at each time point ($p < 0.05$).

unchanged during six days of H_2 production at a very intense light, whereas that of the *pgrl1* mutant decreased slightly, and in the case of the CC-124 strain, a substantial decrease was observed (Fig. 4B); the cell densities remained relatively stable for all three strains apart from a moderate increase in the first 24 h (Fig. 4C).

The photosynthetic apparatus was preserved in the PSI-CET mutants, especially in the *pgr5* mutant: in this strain, about 50% of PsbA was detected at the end of the experiment, and practically all CP47, PSBO, PSBP subunits were retained. Regarding HydA, a large proportion (about 70%) of HydA was degraded within 24 h in CC-124 and in the *pgrl1* mutant. In stark contrast, HydA was retained at almost 100% in the *pgr5* mutant throughout the 6-day H_2 production experiment (Fig. 5).

The *in vitro* H_2 production activity of the *pgrl1* mutant, calculated on a cell number basis, was almost 3-fold higher than that of the CC-124 strain at the beginning of the experiment (after the 4-h dark incubation period, Table 1), which is in agreement with the higher amount of HydA relative to CC-124 (Fig. 5A). The *pgr5* mutant had approx. 60% higher *in vitro* hydrogenase activity than the CC-124 upon a 4-h dark incubation (Table 1). After six days of H_2 production, the *in vitro* hydrogenase activity remained at a considerably high level in all strains. In the CC-124 strain, about 34% of the initial *in vitro* activity was retained, whereas in the *pgrl1* mutant 11% remained. Remarkably, 44% of the initial *in vitro* HydA activity of the *pgr5* mutant remained by the end of the experiment. The relative transcript abundance of *HydA1* was preserved to a similar extent: in CC-124 about 30%, in the *pgr5* mutant about 23%, and in the *pgrl1* mutant about 10% of the initial transcript abundance could be detected after 144 h of H_2 production (Table 1).

In our previous study, only about 7% of the dark-induced HydA activity was retained after 24 h (Nagy et al., 2018a; 2018b), and earlier studies show that HydA gets completely inactivated within minutes in the presence of a few % of O_2 (Ghirardi et al., 1997).

The higher initial *in vitro* hydrogenase activity in the PSI-CET mutants was unexpected, though the available data suggest that when PSI-CET is impaired, other photoprotective mechanisms come into play, including H_2 production. It was also reported that these mutants have increased respiration rates (Petroutsos et al., 2009, Steinbeck et al., 2015), contributing to the maintenance of low intracellular O_2 levels and HydA activity. However, the reasons for the large differences between the *pgr5* and *pgrl1* mutants in terms of H_2 productivity merits further investigation.

4. Conclusions

Anaerobiosis-induced photoautotrophic H_2 production in TCL-PBR is increased approx. three-fold as compared to traditional bulk cultures. TCL-PBR also enables continuous H_2 production at sunlight intensity. The sustained H_2 production at sunlight intensity is in stark contrast with earlier achievements, making the present protocol a significant step toward upscaling algal H_2 production. H_2 productivity was enhanced in a PsbA mutant and in mutants deficient in PSI-CET. The *pgr5* mutant performed surprisingly well: Its photosynthetic apparatus and HydA were retained for six days, and it produced as high as 1.2 ml H_2/ml culture, which is the highest H_2 photoproduction achieved so far.

CRediT authorship contribution statement

Valéria Nagy: Investigation, Data curation, Methodology, Visualization. **Anna Podmaniczki:** Investigation, Data curation, Methodology. **André Vidal-Meireles:** Investigation, Data curation. **Soujanya Kuntam:** Investigation. **Éva Herman:** Investigation. **László Kovács:** Data curation, Formal analysis. **Dávid Tóth:** Investigation. **Alberto Scoma:** Conceptualization, Writing - review & editing. **Szilvia Z. Tóth:**

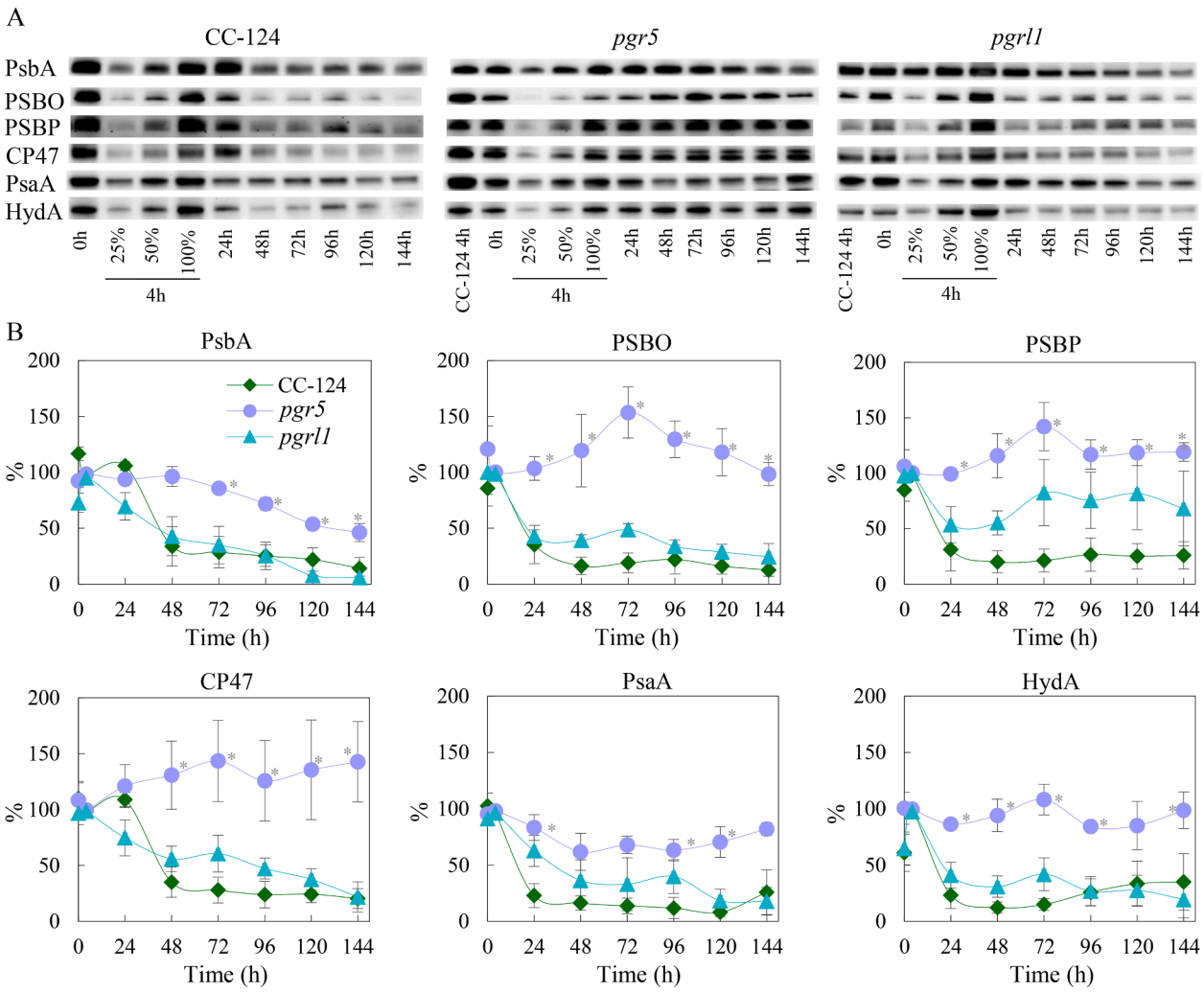

Fig. 5. Immunoblot analysis for the semi-quantitative determination of HydA and certain photosynthetic subunits of the photosystem I cyclic electron transport mutants *pgr5* and *pgrl1*, and their background strain CC-124 during 144 h of H_2 production in thin cell layer photobioreactors (TCL-PBR) at 1000 μmol photons m^{-2} s^{-1} continuous light. A) Representative immunoblots. An amount of Chl(a + b) equivalent to 1 million cells was loaded in each well. The 0 h samples represent the aerobic control, the 25%, 50%, and 100% samples were taken after 4 h of anaerobic induction (4 h-sample) and are for the approximate quantitation of the proteins. For comparison, the 4 h-sample of the CC-124 strain was loaded on the blots of the *pgr5* and the *pgrl1* mutants as well. B) Densitometry analysis of the immunoblots, based on 3 to 4 independent experiments. The 4 h-sample of each genotype was used as the 100% reference for the densitometry analysis. The significance of differences between means was determined by two-way mixed ANOVA with Tukey post-hoc test. Asterisks indicate significantly different means in comparison with CC-124 at each time point ($p < 0.05$).

Table 1
In vitro H_2 production determined on a cell number basis and relative transcript abundance of *HydA1* in the CC-124 strain, the *pgr5* and *pgrl1* mutants, after 4 h of anaerobic induction and at the end of H_2 production experiment (144 h) carried out at 1000 μmol photons m^{-2} s^{-1}, 150 μg Chl(a + b)/ml. The data represent means ± SE (in brackets) of three or four independent biological replicates. The remaining activities after 144 h of H_2 production are also indicated (in %).

	In vitro H_2 production (μl H_2 /million cells/h)			Rel. transcript abundance of *HydA1* at 144 h (rel. to 4 h)
	4 h	144 h	%	
CC-124	1.13 (0.15)	0.38 (0.14)	33.6%	30.3% (0.07)
pgr5	1.62 (0.17)	0.70 (0.22)	43.5%	23.0% (0.04)
pgrl1	3.10 (0.70)	0.34 (0.05)	11.0%	10.0% (0.02)

Conceptualization, Funding acquisition, Project administration, Supervision, Writing - original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data Availability

Data sharing does not apply to this article as all newly created data is already contained within this article and in its supplementary material.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2021.125217.

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