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# Photosystem ratio imbalance promotes direct sustainable H<sub>2</sub> production in *Chlamydomonas reinhardtii*†

Pilla Sankara Krishna,‡ Stenbjörn Styring and Fikret Mamedov  \*

The green alga *Chlamydomonas reinhardtii* can photoproduce H<sub>2</sub> gas for only a few minutes under anaerobic conditions due to the inhibition of hydrogenase by O<sub>2</sub> produced by Photosystem II (PSII). A few days of sustained H<sub>2</sub> production can only be achieved when O<sub>2</sub> and H<sub>2</sub> production are temporally separated under two-stage processes such as sulfur deprivation. Under sulfur deprivation, H<sub>2</sub> production is initiated after the over-reduction of the plastoquinone pool and decreased PSII activity in the thylakoid membrane. As a result, activated hydrogenase consumes the excess of electrons produced by PSII [Volgusheva *et al.*, *Proc. Natl. Acad. Sci. U. S. A.*, 2013, **110**, 7223]. Here, we report that similar conditions can be achieved by simply altering the ratio between photosystem I (PSI) and PSII. In the C3 mutant of *C. reinhardtii*, we found a lower PSI/PSII ratio than in the wild type, 0.33 vs. 0.85, respectively. This imbalance of photosystems resulted in the over-reduced state of the plastoquinone pool and activation of hydrogenase in the C3 mutant that allowed the photoproduction of H<sub>2</sub> continuously for 42 days. This is an unprecedented duration of H<sub>2</sub> production in green algae under standard growth conditions without any nutrient limitation. Photosynthetic electron flow from PSII to hydrogenase was closely regulated during this long-term H<sub>2</sub> production. The amount of PSII was decreased and the amount of PSI was increased reaching a PSI/PSII ratio of more than 5 as shown by EPR and fluorescence spectroscopy. This fine-tuning of photosystems allows to sustain the long-term production of H<sub>2</sub> in *C. reinhardtii* by a direct photosynthetic pathway.

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## 1. Introduction

Solar energy is a renewable energy source that is sufficient to meet future energy demands, provided it can be efficiently harnessed and stored.<sup>1</sup> Conserving solar energy in the form of fuel is the most effective way of storage for multiple uses. Therefore, so-called solar fuels are recognized as likely to become an important component of the future energy landscape<sup>2</sup> on both the local and global scales.

Molecular H<sub>2</sub> is a unique energy source which becomes indispensable if can be produced as a solar fuel. It has the highest energy density (*ca.* 3 times higher than diesel in the liquid form) and, if produced by the direct photolysis of water, is not involved in any CO<sub>2</sub> release or CO<sub>2</sub> regeneration cycles.<sup>3,4</sup> This makes it an environmentally friendly potential

energy source and there already exists an appreciable industry making and using H<sub>2</sub>. Thus, many technical aspects concerning safety, storage and transportation have been established already. Conventional methods of H<sub>2</sub> production are either based on fossil fuels (natural gas) or dependent on the electrolysis of water.<sup>5</sup> There are, as yet, few technological processes available making H<sub>2</sub> from solar energy.

Photobiological H<sub>2</sub> production by green algae and cyanobacteria is an area of biotechnology that could become important to fill this gap.<sup>2,3</sup> The H<sub>2</sub> producing ability of some unicellular green algae has been known for more than seven decades.<sup>6</sup> However, there are some shortfalls that prevent its use on the industrial scale. Green alga *C. reinhardtii* possess both a highly active water oxidation photocatalyst, photosystem II (PSII),<sup>7</sup> and a productive H<sub>2</sub> producing catalyst, Fe–Fe hydrogenase (HydA).<sup>8</sup> This seems to be an ideal system, but in nature, the photoproduction of H<sub>2</sub> by microalgae is limited to short bursts that occur only when dark adapted cells in pre-established anaerobic conditions are exposed to light.<sup>9</sup> Long-term H<sub>2</sub> production is not possible due to the high O<sub>2</sub> sensitivity of the HydA enzyme activity and maturation, and the fact that O<sub>2</sub> is a by-product of the water oxidation reaction of PSII during its normal turnover.<sup>10</sup>

Molecular Biomimetics, Department of Chemistry – Ångström Laboratory, Uppsala University, 751 20 Uppsala, Sweden. E-mail: fikret.mamedov@kemi.uu.se;  
Tel: +46 18 471 65 81

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‡Present address: Department of Molecular Microbiology, John Innes Centre, Norwich, NR4 7UH, United Kingdom.



This problem can be partially solved by the temporal separation of the PSII activity and H<sub>2</sub> production in the two-stage process that occurs during sulfur (S) deprivation in *C. reinhardtii*.<sup>11</sup> Such separation generally leads to several (5–7) days of H<sub>2</sub> production in the second stage and has been widely used to study the biochemistry and physiology of this phenomenon.<sup>12–15</sup> S-deprivation (the most studied) and other similar two-stage processes (P, N, and Mg-deprivation) commonly lead to an inhibition of the *de novo* PSII synthesis.<sup>16–18</sup> A significant decrease in the PSII amount in the thylakoid membrane eventually makes the cellular O<sub>2</sub> consumption due to various respiratory processes overcome O<sub>2</sub> evolution in PSII. This situation creates local anaerobiosis in the cell which is a suitable condition for HydA activation and H<sub>2</sub> production. It takes 24–48 h of S-deprivation before HydA is activated. During this time, starch is accumulated in the cell.<sup>11,19</sup> Both starch and the remaining PSII are electron sources for H<sup>+</sup> reduction by HydA.<sup>20–22</sup> The reduction of HydA is light-driven by photosystem I (PSI) and wires the plastoquinone (PQ) pool with a stroma soluble ferredoxin (Fd) protein that is the terminal electron carrier to HydA.<sup>4</sup> Anaerobiosis is an immediate precondition for HydA expression and function,<sup>10,23</sup> although the remaining PSII centers are still active and can supply as much as 80% of electrons that finally reach HydA.<sup>12</sup>

Significant efforts have been made to increase the efficiency and durability of the H<sub>2</sub> production by S-deprived cells of *C. reinhardtii*. Two major strategies were employed to achieve this goal. The first one employs changing the culture conditions such as light intensity,<sup>24</sup> cell immobilization<sup>25</sup> and addition of fresh cells growing in the S-deprived medium or dosed sulfate addition.<sup>26–28</sup> The second strategy uses a wide range of mutant strains which have limited antenna properties,<sup>29–31</sup> decreased PSII activity,<sup>32,33</sup> increased respiration,<sup>12,34</sup> inhibited cyclic electron flow<sup>14,15</sup> or controlled CO<sub>2</sub> fixation.<sup>35</sup>

Several studies have reported a higher H<sub>2</sub> yield using S-deprivation than in the original study.<sup>11</sup> Scoma *et al.*<sup>32</sup> reported a mutant with double substitution in the PSII D1 protein (L159I-N230Y) with the light to H<sub>2</sub> conversion efficiency reaching 3.3%, which is nearly 2 times higher than that in the corresponding WT cells. The *stm6* mutant with a high respiration rate showed a 4 times higher final H<sub>2</sub> formation than in the WT,<sup>12,34</sup> with the total yield reaching more than 13 mmol L<sup>−1</sup> after 130 h of S-deprivation. Similarly, in a proton gradient regulation *pgr5* mutant, a 10-fold higher H<sub>2</sub> yield was shown,<sup>15</sup> while in another proton gradient regulation mutant *hpm91*, a 30-fold increase was obtained.<sup>14</sup>

The long-term durability and stability of the H<sub>2</sub> production is crucial for any technical application. Again, several strategies have been employed. Immobilization of S-deprived cells of *C. reinhardtii* increased continuous H<sub>2</sub> photoproduction for at least 90 days under a constant flow of the Tris Acetate Phosphate (TAP) medium containing μM sulfate concentrations. The longest reported period of H<sub>2</sub> production so far was achieved by using a two-stage sulfate limited chemostat

system with the inflow of fresh culture for 167 days;<sup>28</sup> see also ref. 26 and 27. However, all these approaches still have significant limitations such as application of a two-stage process, complex reactor designs and high operation costs.

From our previous studies it became clear that independent of the method used to initiate the H<sub>2</sub> production in *C. reinhardtii*, the starting conditions of this process are (i) decrease in the PSII amount and/or activity that lead to anaerobic conditions in the cell (shown in ref. 12 and 36) and (ii) reduction of the PQ-pool. It is our hypothesis that both these conditions could be achieved by simply altering the balance between two photosystems, PSII and PSI which both consecutively drive the electron transfer in the thylakoid membrane. We tested this hypothesis and now report that, in the C3 mutant strain of *C. reinhardtii*,<sup>37</sup> with an abnormally low PSI to PSII ratio, we indeed observed H<sub>2</sub> production for a record period of 42 days in the standard growth media without any modifications.

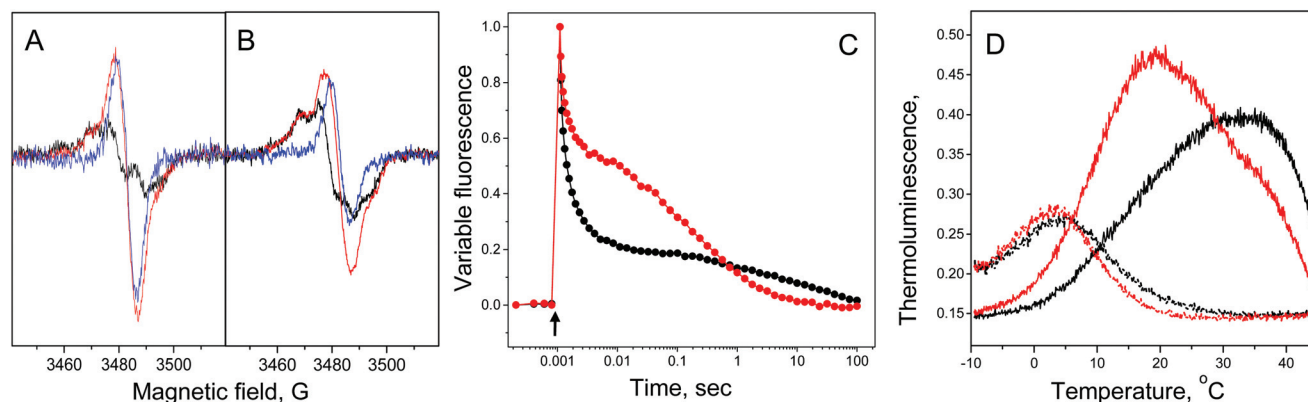
## 2. Materials and methods

The C3 mutant strain was obtained from the *Chlamydomonas* Resource Center (<http://www.chlamycollection.org>). The C3 mutant and the corresponding WT-137C strain were grown photoheterotrophically in 1 L Erlenmeyer flasks containing 300 mL TAP medium at pH 7.0, 25 °C (ref. 49) under continuous white light illumination of 30 μmol photons per m<sup>2</sup> per s and constant shaking at 100 rpm for 6 days. After the culture reached a concentration of 12 μg Chl per mL, the cells were collected by centrifugation at 3000g for 2 min. The cell pellet was gently re-suspended in fresh TAP and placed in sealed bioreactors (air tight flasks, 300 ml culture + 20 ml air headspace) at a concentration of 6.0 μg Chl per mL for the long-term incubation experiments that were performed at the same light and temperature conditions. Samples were anaerobically withdrawn for analysis from the sealed bioreactors at the indicated time points. H<sub>2</sub> and O<sub>2</sub> gas quantifications from the head space were done as described previously.<sup>12</sup>

After every measurement from 24 h and beyond, the H<sub>2</sub> amount in the air space of the bioreactor was made zero by flushing with argon. For the experiments in the presence of 20 μM DCMU, 25 mL aliquots were sealed anaerobically in incubation tubes and the H<sub>2</sub> yield was determined after 24 h of light incubation. Chl content, flash-induced fluorescence and thermoluminescence measurements were performed according to the published methods.<sup>12,36</sup> EPR quantifications of Y<sub>D</sub><sup>•</sup> and P<sub>700</sub><sup>+</sup> to determine the amounts of PSII and PSI, respectively, were performed with a Bruker BioSpin EMX-micro spectrometer equipped with an EMX-Premium bridge and an ER4119HS resonator using a quartz flat cell as described previously.<sup>12,38</sup>

All data shown are average of at least triplicate data sets. For EPR, fluorescence and thermoluminescence results, typical spectra or traces are shown.





**Fig. 1** Photosynthetic properties of the WT and C3 mutant of *C. reinhardtii*. EPR based quantification of PSI and PSII in WT (A) and C3 mutant (B) cells. The spectra shown are from dark adapted cells ( $Y_D^\bullet$ , black), the same cells in the presence of 10 mM  $K_4[Fe(CN)_6]$  and following oxidation for 5 min (red) and difference spectra (red spectrum minus black spectrum) respectively ( $P_{700}^+$ , blue). The EPR quantification of respective photosystems was done according to ref. 38. EPR conditions: microwave frequency 9.76 GHz, microwave power 8 mW, modulation amplitude 5 G, and room temperature. Normalized flash-induced fluorescence decay kinetics (C) and thermoluminescence curves (D) from the control WT (black) and C3 mutant (red) cells. Thermoluminescence was measured in the absence (solid) and presence of 20  $\mu$ M DCMU (dotted lines).

### 3. Results

#### The C3 mutant has a lower PSI content and an over-reduced PQ-pool

The C3 mutant cells grow at a similar rate as the WT cells in our standard conditions. The relative amounts of PSI and PSII, present in the thylakoid membrane were determined by EPR spectroscopy.<sup>38</sup> Two characteristic EPR signals are observed from the photosystems in photosynthetically active cells at room temperature: the stable  $Y_D^\bullet$  radical from PSII and the light or chemically oxidizable  $P_{700}^+$  chlorophyll (Chl) radical from the PSI reaction center. Both radicals, when present, correspond to 1 spin per respective photosystem and serve as a robust quantitative probe.<sup>12,38</sup> Fig. 1A and B show the EPR spectra from fully oxidized  $Y_D^\bullet$ , present in the dark adapted cells (black spectra) and spectra from the same cells in the presence of 10 mM  $K_4[Fe(CN)_6]$ , which in addition to  $Y_D^\bullet$ , already present in the dark, also contain fully oxidized  $P_{700}^+$  (red spectra). The difference spectra show the deconvoluted, pure EPR spectra from the maximally achievable  $P_{700}^+$  (blue spectra). Quantification of  $Y_D^\bullet$  and  $P_{700}^+$  in the WT cells showed a PSI/PSII ratio of 0.85, which is normal for *C. reinhardtii* cells (Fig. 1A). In the C3 mutant cells, this ratio was much lower and found to be 0.33. This is almost 3 times lower than that in the WT (Fig. 1B). Thus, there is only one PSI center for every 3 PSII centers present in the C3 mutant.

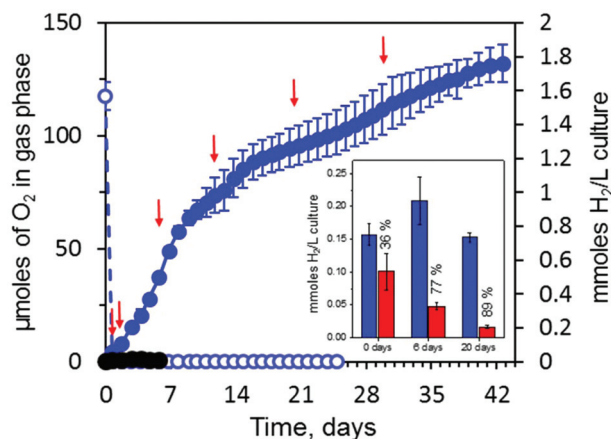
We also studied the reduction level of the PQ pool in the WT and C3 mutant by flash-induced variable fluorescence decay and thermoluminescence measurements. Fluorescence decay kinetics report on the electron transfer from the primary quinone electron acceptor in PSII ( $Q_A^-$ ) after a single flash in PSII.<sup>39–41</sup> Fig. 1C shows the fluorescence decay traces in the control WT (black) and the C3 mutant cells (red). The WT cells show a fast fluorescence decay of more than 80% of the total variable fluorescence. This is normal, and the fast decay

reflects efficient forward electron transfer from  $Q_A^-$  to  $Q_B$  in PSII centers with an occupied  $Q_B$ -site with  $Q_B$  bound to its pocket in PSII ( $t_{1/2} = 232 \mu$ s, 58% of the total amplitude) or an empty  $Q_B$ -site (which first requires  $Q_B$  binding,  $t_{1/2} = 3.5$  ms, 25%).<sup>39–41</sup> In the C3 mutant cells, the fluorescence decay was very different: the  $\mu$ sec phase was smaller (38%) and 2 times slower ( $t_{1/2} = 462 \mu$ sec) indicating that less  $Q_B$  is present in its site. The rest of the decay is dominating by a decay phase ( $t_{1/2} = 119$  ms, 44%) representing a very slow  $Q_B$  binding. A severe delay of the msec decay phase indicates that the  $Q_B$  binding was significantly perturbed in the C3 mutant. Together, these data show that forward electron transport from  $Q_A^-$  is severely slowed down or even inhibited in the C3 mutant.

The situation is further clarified by using thermoluminescence measurements which are a useful complement to the analysis of the fluorescence kinetics.<sup>36,42,43</sup> Fig. 1D shows the thermoluminescence glow curves from the WT (black) and the C3 mutant cells (red). The WT cells exhibited the characteristic so called the  $B_1$ -band with the dominating peak at 38 °C. This peak reflects the recombination between  $Q_B^-$  and the  $S_2$  state of the oxygen evolving complex (OEC) (Fig. 1D, solid black curve, see also Fig. 2S, B†). In contrast, in the C3 mutant cells, the main fraction of the thermoluminescence band, measured under the same conditions, was shifted to 19 °C (Fig. 1D, solid red curve). This clearly indicates that a large proportion of the charge recombination in PSII instead occurs between  $Q_A^-$  and the  $S_2$  state (the so-called Q-band). The presence of a significant amount of  $Q_A^-$  is additional evidence of the mostly empty  $Q_B$ -site in the C3 mutant, again pointing to the reduced state of the PQ-pool favoring  $Q_A^- S_2$  recombination instead of  $Q_B^- S_2$  recombination.

Upon addition of 20  $\mu$ M 3-(3,4-dichlorophenyl)-L,L-dimethylurea (DCMU), which completely blocks the  $Q_B$ -site, the clean Q-band could be observed at 3 °C in both WT and C3 mutant (Fig. 1D, dotted curves, WT (black) and C3 (red)).





**Fig. 2** Long-term  $\text{H}_2$  production in the C3 mutant (filled blue circles) and its absence in the WT (filled black circles) cells of *C. reinhardtii* in sealed bioreactors under illumination of  $30 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  in the complete TAP medium. The arrows show the time points for the fluorescence and EPR measurements (Fig. 3). The inset shows the effect of DCMU addition on the  $\text{H}_2$  production in the C3 mutant. Blue bars –  $\text{H}_2$  production in the absence and red bars in the presence of  $20 \mu\text{M}$  DCMU at different time points. The numbers at the top indicate the percentage of inhibition. Decrease in oxygen evolution at the beginning of incubation is shown by open circles for the WT (black) and in the C3 mutant (blue).

### Long-term $\text{H}_2$ production by the C3 mutant

The reduced state of the PQ pool, the low PSI content and the correspondingly low PSI/PSII ratio in the C3 mutant are indicative of severe limitations in the photosynthetic electron transport and will result in a slow PSII turnover and low  $\text{O}_2$  formation. This could lead to a situation where local anaerobic conditions are established in the cell that could then activate HydA expression.

Indeed, when the active C3 mutant culture was placed in gas-tight bioreactors, the  $\text{O}_2$  concentration quickly decreased and after 17 h, no  $\text{O}_2$  was detected in the bioreactor's air space. It is important that this occurred without any further modifications of the growth medium or nutrient limitations (Fig. 2, open blue circles). In addition, immediately after the disappearance of  $\text{O}_2$ ,  $\text{H}_2$  production commenced in the C3 mutant culture (Fig. 2, closed blue circles).

The  $\text{H}_2$  production for the first 42 days is shown in Fig. 2. The amount of  $\text{H}_2$  released from the C3 cells was increasing during the initial 9 days to more than  $3 \text{ mL}$  of  $\text{H}_2$  per  $\text{L}$  of culture per day. It then decreased somewhat for the next 6 days (15 days in total) to  $1 \text{ mL}$  of  $\text{H}_2$  per  $\text{L}$  of culture per day, and from there on, the cells produced  $\text{H}_2$  at a relatively stable rate of  $0.5\text{--}0.7 \text{ mL}$  of  $\text{H}_2$  per  $\text{L}$  of culture per day for the rest of the incubation (Fig. 2, Table 1). Remarkably, the  $\text{H}_2$  production by the C3 cells continued for at least 6 weeks (42 days) without any modification of the culture or change in the incubation conditions.

It should be mentioned, that when the WT cells were placed in the bioreactors under similar conditions, no  $\text{H}_2$  production was detected during 30 days of incubation (the first 7 days are shown in Fig. 2, black circles).

We also estimated the contribution of the PSII produced electrons to the  $\text{H}_2$  production in the C3 mutant. Aliquots of  $25 \text{ mL}$  of the C3 mutant culture were taken after 1, 6 and 20 days of  $\text{H}_2$  production and incubated for 24 h in the absence or presence of  $20 \mu\text{M}$  DCMU, a well-known inhibitor of PSII activity. The results are shown in the inset to Fig. 2. After 1 day, only 36% of  $\text{H}_2$  production was inhibited by the DCMU addition. After 6 days, the inhibition was more than 2 times stronger – 77% and after 20 days, it reached almost 90% (Fig. 2, inset). These results indicate that although PSII is an important supplier of electrons to HydA in the beginning of the  $\text{H}_2$  production, it becomes indispensable in this capacity during the long-term  $\text{H}_2$  production in the C3 mutant.

### Photosynthetic changes during $\text{H}_2$ production in the C3 mutant: Chl content

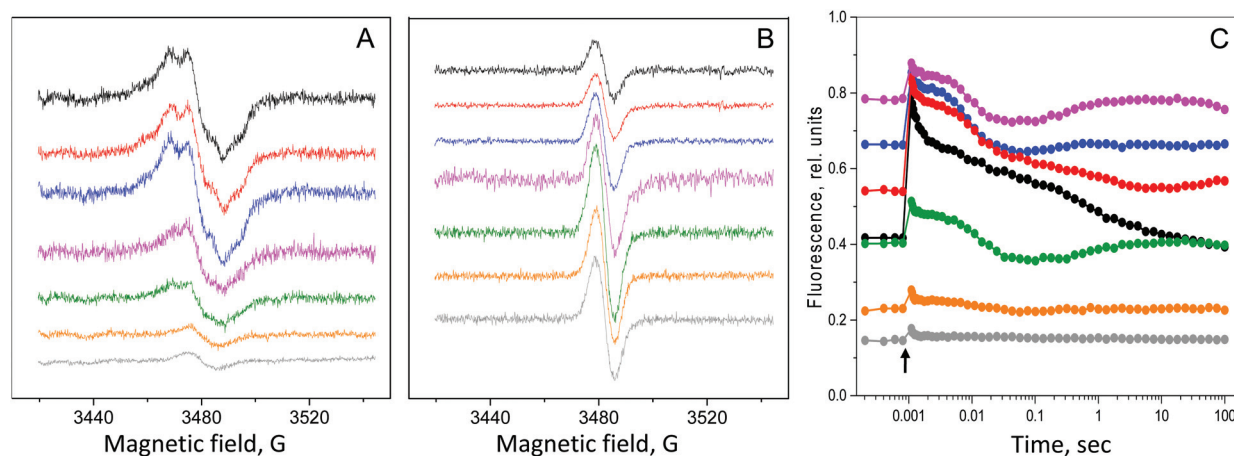
During the long-term  $\text{H}_2$  production, the cells of the C3 mutant of *C. reinhardtii* underwent changes in the Chl content, antenna composition and photosynthetic electron transfer properties. The total amount of Chl in the culture increased during the first 6 days of  $\text{H}_2$  production from  $6$  to  $8 \mu\text{g mL}^{-1}$  and then decreased to  $3.5 \mu\text{g mL}^{-1}$  during the next 6 days (12 days in total). After this, the total Chl concentration decreased very slowly (Table 1). The Chl composition also changed: the Chl  $a/b$  ratio steadily increased for the first 20 days from  $2.00$  to  $3.47$  and then remained on the same level over the next 10 days (Table 1). The total number of cells did

**Table 1** Changes in the photosynthetic properties of the C3 mutant cells of *C. reinhardtii* during long-term  $\text{H}_2$  production in bioreactors. The total Chl ( $a + b$ ) is given in  $\mu\text{g mL}^{-1}$ .  $F_V/F_M$  was determined from the flash-induced fluorescence traces shown in Fig. 3C. The amounts of PSII and PSI were determined after double integration of the EPR spectra shown in Fig. 3A and B. The amount of PSII and PSI in the control sample was defined as 100%. The standard error in the EPR measurements is less than 5%

Culture	Chl ( $a + b$ )	Chl $a/b$	$F_V/F_M$	PSII (%)	PSI (%)	PSI/PSII	$\text{H}_2 \text{ mL L}^{-1} \text{ d}^{-1}$
0 h (control)	$5.8 \pm 0.10$	$2.00 \pm 0.06$	$0.47 \pm 0.02$	100	100	0.33	0
1 day	$6.4 \pm 0.07$	$2.56 \pm 0.05$	$0.36 \pm 0.03$	95	112	0.39	$1.10 \pm 0.12$
2 days	$7.0 \pm 0.53$	$2.44 \pm 0.33$	$0.22 \pm 0.05$	103	166	0.53	$1.25 \pm 0.33$
6 days	$8.0 \pm 0.11$	$2.65 \pm 0.11$	$0.23 \pm 0.02$	58	233	1.31	$3.11 \pm 0.54$
12 days	$3.5 \pm 0.06$	$3.16 \pm 0.18$	$0.11 \pm 0.06$	38	287	2.51	$1.05 \pm 0.47$
20 days	$3.4 \pm 0.08$	$3.47 \pm 0.27$	$0.20 \pm 0.06$	18	236	4.36	$0.51 \pm 0.14$
30 days	$3.2 \pm 0.06$	$3.35 \pm 0.21$	$0.19 \pm 0.04$	14	218	5.15	$0.76 \pm 0.13$







**Fig. 3** Changes in photosynthetic activity in the cells of the C3 mutant of *C. reinhardtii* during long-term  $H_2$  production in sealed bioreactors. The EPR signal from  $Y_D^\bullet$  in PSII (A) and the EPR signal from  $P_{700}^{\bullet+}$  in PSI (B) measured by EPR spectroscopy. The amount of respective radicals in the control sample was taken as 100%. EPR conditions as in Fig. 1A and B. The spectra in A and B are normalized to the same Chl concentration. (C) Flash-induced fluorescence decay kinetics of the C3 mutant during the long-term  $H_2$  production in sealed bioreactors. The samples from cells of 0 days (control, black), 1 day (red), 2 days (blue), 6 days (pink), 12 days (green), 20 days (orange) and 30 days (grey) of incubation are shown.

not change significantly during these 30 days of  $H_2$  production. Therefore, these changes are most likely associated with a decrease of Chl *b* containing the light harvesting complex II (LHCII) antenna associated with both PSII and PSI centers. This conclusion is corroborated by the 77 K fluorescence emission spectra obtained during the course of  $H_2$  production (see the ESI and Fig. 1S†).

#### Photosynthetic changes during $H_2$ production in the C3 mutant: relative content of the photosystems

The relative amounts of PSII and PSI during the long-term  $H_2$  production in the C3 mutant were determined by electron paramagnetic resonance (EPR) spectroscopy (Fig. 3A and B). In the control sample, there was an imbalance in the photosystems ratio, where 3 PSII centers were found for each PSI center (see above, Fig. 1A and B). During the first 2 days of incubation in the closed bioreactors, the amount of PSII did not change, while the amount of PSI increased by 40% (Fig. 3A, B, 4 and Table 1). Correspondingly, the PSI/PSII ratio had increased to 0.5 (2 PSII centers per 1 PSI center (Fig. 4 and Table 1)).

During the next 4 days (6 days in total), the amount of PSII decreased to 58% of the amount in the control. Thereafter, the PSII concentration continued to decrease to 14% of the control amount after 30 days (Fig. 3A, 4 and Table 1). The fate of PSI was very different – during the next 10 days (12 days in total), the PSI amount increased almost 3 times to 287% (reaching the amount of PSII in the control sample), and then during the next 18 days (30 days in total), decreased to 218% (Fig. 3B, 4 and Table 1).

As a result, the PSI/PSII ratio underwent significant changes associated with the  $H_2$  production in the C3 mutant. It steadily increased from a ratio of 0.33 and reached a ratio of more than 5 (Fig. 4 and Table 1). Thus, while at the beginning of  $H_2$  production there were *ca.* 3 PSII centers per 1 PSI center, this

imbalance between the two photosystems was completely reversed during the next 30 days and resulted in a ratio of 5 : 1 PSI to PSII centers in the thylakoid membrane of the C3 mutant.

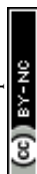
#### Photosynthetic changes during $H_2$ production in the C3 mutant: PSII and the PQ pool

The changes in the PSI/PSII ratio that occurred during the  $H_2$  production stage in the C3 mutant had a marked effect on the electron transfer between PSII and PSI. This effect was studied by flash-induced variable fluorescence decay kinetics and thermoluminescence measurements and the results are shown in Table 1, Fig. 3C and ESI Fig. S2.†

The  $F_0$  level of the fluorescence increased almost two times during the first 6 days of  $H_2$  production from *ca.* 0.4 to 0.8, Fig. 3C, and then started to decrease to reach 0.15 which is a very low level during the next 24 days (30 days in total, Fig. 3C). The decrease in the  $F_0$  level occurred concomitantly with a decrease in the  $F_M$  level which decreased *ca.* 5-fold during the same time (Fig. 3C).

The increase in the  $F_0$  level reflects the rise in the reduced  $Q_A^-$  in PSII which is a consequence of the over-reduced PQ-pool and increasingly reduced conditions in the thylakoid membrane.<sup>12,36</sup> While the membrane stays over-reduced during  $H_2$  production, the overall decrease in the PSII amount was reflected in the decrease in both  $F_0$  and  $F_M$  and correspondingly in the  $F_V/F_M$  ratio (from 0.47 in the control cells to 0.19 after 30 days of  $H_2$  production, see Table 1). It follows the decrease in the amount of PSII centers which was directly quantified by EPR spectroscopy (Table 1 and Fig. 3A), although these two parameters are not directly comparable, since the  $F_V/F_M$  ratio also represents the photochemical efficiency of PSII.<sup>44,45</sup>

Very informative results were also derived from the analysis of the fluorescence decay kinetics. In the control C3 mutant



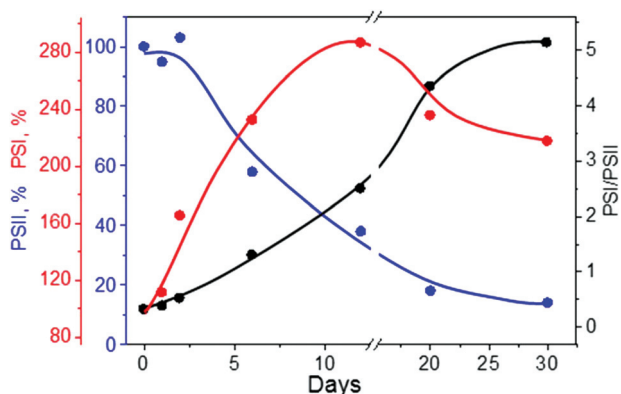


Fig. 4 Changes in the amount of PSII (blue), PSI (red) and the PSI/PSII ratio (black) during the first 30 days of  $H_2$  photoproduction in the C3 mutant of *C. reinhardtii*.

cells, the flash induced fluorescence decay was slower than in the WT cells as was discussed above (Fig. 1C; also shown in Fig. 3C, black trace). The kinetics were then slowed down further with the establishment of anaerobic conditions and beginning of the  $H_2$  production after 1 day in the sealed bioreactors (Fig. 3C, red trace). The  $\mu$ sec decay phase (reflecting forward electron transfer from  $Q_A^-$  to bound  $Q_B$ ), completely disappeared and was replaced by the slower decay phases in the msec time regime. These slow decay kinetics became even slower after 2 days of incubation (Fig. 3C, blue trace).

During prolonged incubation, 6 to 30 days, concomitantly with a sharp decrease in the  $F_M$  and  $F_0$  parameters (Table 1), the single kinetics with half times of 200–300 ms dominated the fluorescence decay (Fig. 3C, rest of the traces). This phase represents a mixture of very slow  $Q_B$  binding and recombination to the donor side of PSII. In addition, a small rise of variable fluorescence developed after the initial decay which is similar to the kinetics observed in the WT control. This decay pattern indicates that the electron transfer from PSII, although very slow and inefficient, still exist in a strongly reduced environment in the C3 mutant during the whole 30 days of  $H_2$  production in the C3 mutant (Fig. 3C). These conclusions from the fluorescence decay data were also supported by the thermoluminescence measurements obtained during  $H_2$  production (see the ESI and Fig. S2†).

To conclude, our analysis reveals that PSII in the C3 mutant cells is functional and provides significant electron pressure at the level of the PQ pool even at the over-reduced conditions in the thylakoid membrane. This electron pressure exists if cells are producing  $H_2$ .

## 4. Discussion

### PSII is both good and bad for long-term $H_2$ production

Long-term direct and sustainable  $H_2$  production by photosynthetic microorganisms is an ideal approach to produce renewable fuel from solar energy. There are scientific and technological challenges in the implementation of this approach, the

most crucial of which is the  $O_2$  sensitivity of HydA. Since photosynthesis is an ultimate electron and proton source for HydA,  $O_2$  released by PSII is an unavoidable companion of any long-term  $H_2$  production.

Two main strategies were undertaken to eliminate this problem. The first one was to increase the  $O_2$  tolerance of HydA by genetic manipulation. Although this partially worked for the Ni-Fe type hydrogenases, the results for the algal Fe-Fe HydA have not been encouraging so far.<sup>46,47</sup> The second strategy is to separate  $O_2$  evolution and  $H_2$  production in time by the so-called two-stage processes namely S-deprivation.<sup>11,16,17</sup> However, this approach is not suitable for sustainable long-term  $H_2$  production.

In this study, we suggest a new strategy to circumvent the  $O_2$  sensitivity of HydA without implementation of any two-stage processes, which we see as very promising for long-term  $H_2$  production. The strategy is to suppress the  $O_2$  concentration in the algal cell to the level where it does not inhibit the HydA activity. This should be achieved under the standard growth conditions, *i.e.* without application of any two-stage processes. The way to achieve this is to create a specific balance between PSII and HydA activity in such a way that will allow continuous supply of electrons from PSII and at the same time to have a low intracellular (stromal)  $O_2$  concentration which will not inhibit the HydA activity. This means that the PSII activity must be confined to the certain level. The PSII activity is indispensable for long-term cell survival, but it is also important for  $H_2$  production since large amounts of electrons for  $H_2$  production will be provided by the water splitting activity of PSII (Fig. 2, inset).<sup>12</sup> Therefore, we can postulate that PSII is necessary but harmful, *i.e.* both good and bad for  $H_2$  production in green algae. We should also mention that starch reserves as an alternative electron source for HydA will be obsolete in the absence of the two-stage process.

Thus, a novel strategy for long-term  $H_2$  production in *C. reinhardtii* should be to find a right balance between PSII and HydA activities under normal growth conditions. The PSII activity must be high enough to sustain algal cells and, at the same time, low enough in order not to inhibit the HydA activity. Seemingly, the most optimal way to approach this is to limit the PSII activity, for example by mutagenesis.

There are many mutants in PSII (including *C. reinhardtii*) with a wide range of inhibition of  $O_2$  evolution to an almost desirable extent. However, the diminished PSII activity in these mutants will most likely result in the oxidized state of the PQ-pool, and due to the large prevalence of the PSI mediated electron transfer, increase the PSI/PSII ratio. From previous studies, we know that one of the important pre-conditions for the initiation of  $H_2$  production in *C. reinhardtii* is the over-reduced state of the PQ pool which is prerequisite to cellular anaerobiosis.<sup>12,16</sup> While inhibition of the PSII activity by mutagenesis could lead to the anaerobic conditions in stroma where HydA is located, it is not the optimal way to achieve the high reduction state of the thylakoid membrane. Under illumination, unaffected PSI activity will mainly keep the PQ pool in the oxidized state.



Alternatively, imposing a limitation on the PSI activity is a more promising approach and we believe it could lead to conditions where the PQ-pool will stay in the reduced state all the time. It will also limit the PSII activity since the electron transfer chain beyond PSII will be log-jammed. In this case, HydA activation and sustainable  $H_2$  production could be achieved under the standard growth conditions. This hypothesis was successfully tested in the C3 mutant of *C. reinhardtii*.

### C3 mutant has an optimal ratio between two photosystems for long-term $H_2$ production

The C3 mutant cells have a low PSI/PSII ratio and possesses only 1 PSI center for 3 PSII centers as was shown by our EPR measurements (Fig. 1B). However, most of the antenna was associated with the PSI reaction centers (Fig. 1S, A†) to rebalance the excitation energy distribution towards PSI. Still, the electron transfer on the acceptor side of PSII in the C3 mutant was significantly slowed down as was shown by our flash-induced fluorescence and thermoluminescence measurements (Fig. 1C and D). The slow electron transfer from  $Q_A^-$  to  $Q_B$  and the dominating  $Q_A^- S_2$  state recombination band are both indicative of the limited electron transfer beyond the PSII complex in the thylakoid membrane of the C3 mutant. Thus, a low PSI content resulted in the reduced state of the PQ-pool at our light growth conditions (30  $\mu\text{mol}$  photons per  $\text{m}^2$  per  $\text{s}^{-1}$ ) in the C3 mutant.

When the culture of the C3 mutant in the standard TAP medium was placed in the sealed bioreactors, anaerobic conditions were quickly established. The lower PSI content limited the PSII activity allowing respiration to overtake  $O_2$  evolution within 17 h (Fig. 2, open circles). This altered ratio of the two photosystems to 1 : 3 for PSI : PSII, providing favorable initial conditions for  $H_2$  production by the C3 mutant.

### Tuning of photosynthesis during long-term $H_2$ production

After anaerobiosis was established in the C3 mutant culture,  $H_2$  evolution commenced after 17 h (Fig. 2). Due to the specific starting conditions in the thylakoid membrane of the C3 mutant, this process took place in the complete TAP media without any deprivation procedure in a one-stage direct process. The remarkable result was that this  $H_2$  production continued for a long period of time (Fig. 2). Our experiments routinely showed that the  $H_2$  production continues for more than 40 days (6 weeks). To our knowledge, this is the longest direct  $H_2$  production by green algae under the standard cultivation conditions (light, growth medium, and absence of any nutrient manipulations) known so far.

The rate of  $H_2$  production in the C3 mutant was not constant during these 40 days. During the first 6 days of  $H_2$  production, it increased 3-fold, and then during the next 6 days (12 days in total), it decreased to the initial value and stayed constant for the rest of the experiment (Table 1). These unique  $H_2$  production properties are based on changes in the amount and activity of both photosystems and the retuning of the overall electron transport from PSII to HydA.

Although the amount of PSII did not change during the first 2 days of  $H_2$  production, these PSII centers become more active as was shown by the complete reduction of the PQ pool (Fig. 3C and Fig. 2S, C†). In addition, more LHCII was re-associated with these PSII centers during this time in order to help its activation (Fig. 1S, B†). This boost in the PSII activity was, most probably, initiated by an increase in the amount of PSI which was occurring steadily during the first 12 days of  $H_2$  production (Table 1 and Fig. 3B, 4). This allowed relief of the reductive electron pressure produced by PSII and opened the electron flow from PSII to HydA as was observed by fluorescence and thermoluminescence measurements (Fig. 3C and Fig. 2S, D–F†). Thus, the initial increase in the PSI amount is responsible for the high rates of  $H_2$  production during the first 12 days (Fig. 2).

In the next 18 days (30 days in total), the burden of the long-term anaerobic incubation started to be seen as the internal cell supplies became exhausted. The amount of PSI started to decrease consequently with the amount of PSII which also had significantly diminished to less than 15% (Table 1 and Fig. 3A). It was accompanied by the decrease in the associated antenna and loss of the total Chl (Table 1 and Fig. 1S†). Taken together, these resulted in the decrease in the  $H_2$  production rates (Table 1 and Fig. 2).

Our data show that fine tuning of the PSI/PSII ratio is needed not only for initiation but also for prolongation of the direct  $H_2$  production in one-stage process. While in the beginning of  $H_2$  production, there was an excess of PSII over PSI, after 2–6 days, the amount of PSI had equalized with PSII. After 6 days, it had exceeded the amount of PSII, reaching a PSI/PSII ratio of more than 5 (Fig. 4 and Table 1). Thus, a small amount of PSI is required to quickly initiate  $H_2$  production in the C3 mutant and then an excess of PSI over PSII is needed at the later stages for long-term production.

Interestingly, the amount of PSI correlated with the rate of  $H_2$  production in the C3 mutant (Table 1). This is not surprising since PSI is indispensable for light-induced  $H_2$  production in *C. reinhardtii*.<sup>48</sup> In addition to the important role of PSII in  $H_2$  production as a primary electron source, our experiments also show that by changing the PSI properties,  $H_2$  production could be initiated and supported for at least 42 days. This introduces a new approach to achieve sustainable, long-term  $H_2$  fuel production from solar energy in green algae.

## Conflicts of interest

There are no conflicts to declare.

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