The unicellular green alga *Chlamydomonas reinhardtii* is capable of photosynthetic H₂ production. H₂ evolution occurs under anaerobic conditions and is difficult to sustain due to 1) competition between [FeFe]-hydrogenase (H₂ase), the key enzyme responsible for H₂ metabolism in algae, and the Calvin-Benson–Bassham (CBB) cycle for photosynthetic reductants and 2) inactivation of H₂ase by O₂ coevolved in photosynthesis. Recently, we achieved sustainable H₂ photoproduction by shifting algae from continuous illumination to a train of short (1 s) light pulses, interrupted by longer (9 s) dark periods. This illumination regime prevents activation of the CBB cycle and redirects photosynthetic electrons to H₂ase. Employing membrane-inlet mass spectrometry and H₂/O₂, we now present clear evidence that efficient H₂ photoproduction in pulse-illuminated algae depends primarily on direct water biophotolysis, where water oxidation at the donor side of photosystem II (PSII) provides electrons for the reduction of protons by H₂ase downstream of photosystem I. This occurs exclusively in the absence of CO₂ fixation, while with the activation of the CBB cycle by longer (8 s) light pulses the H₂ photoproduction ceases and instead a slow overall H₂ uptake is observed. We also demonstrate that the loss of PSII activity in DCMU-treated algae or in PSII-deficient mutant cells can be partly compensated for by the indirect (PSII-independent) H₂ photoproduction pathway, but only for a short (<1 h) period. Thus, PSII activity is indispensable for a sustained process, where it is responsible for more than 92% of the final H₂ yield.

**Significance**

*Chlamydomonas reinhardtii* is the most studied alga. This alga possesses two [FeFe]-H₂ases in the chloroplast, HYDA1 and HYDA2 (3, 4). In the light, they accept electrons from photosynthetically reduced ferredoxin (FDX1) (5), while in the dark electrons come from the activity of pyruvate ferredoxin oxidoreductase (PFR1) (6). PFRI catalyzes the oxidation of pyruvate to acetyl-CoA, and its activity is linked to H₂ase via FDX1 (7). Since [FeFe]-H₂ases interact with the photosynthetic electron transport chain at the level of ferredoxin, they may accept electrons originating both from water oxidation via the photosystem II (PSII)-dependent pathway (“direct water biophotolysis”) and from the degradation of organic substrates via a PSII-independent mechanism (“indirect water biophotolysis” or “indirect pathway”) (8). In the latter case, the reductants are supplied to the plastoquinone (PQ) pool by the type II NADPH dehydrogenase (NDA2), thus bypassing PSII (9, 10).

The release of H₂ leads to a loss of metabolic energy. In healthy, actively growing *C. reinhardtii* cultures, H₂ production is therefore only a temporal phenomenon observed during dark anaoxia and upon subsequent onset of illumination (11). In contrast to dark fermentation, H₂ photoproduction is a very efficient process that proceeds for only a short period of time (from a few seconds to a few minutes). Two theories have been developed to explain the short duration. The first is based on the oxygen sensitivity of H₂ases (12, 13). In the light, algae accumulate O₂ that is produced by water oxidation at PSII (14). As a result, H₂ photoproduction may cease over time (14, 15), and the duration of this process is reported to shorten with increased light intensity (16). Because of the negative correlation between the rates of H₂ photoproduction and O₂ evolution, the inhibition of H₂ases by O₂ is frequently quoted as the primary reason for the rapid loss in H₂ photoproduction after the onset of illumination (17).

Alternatively, the loss in the H₂ photoproduction efficiency during illumination could be explained by the light-induced induction of competitive pathways, which may drain reducing equivalents away from the [FeFe]-H₂ase enzyme (18, 19). Candidates for this role are the Mehler-like reaction driven by flavodiron proteins (FDPs) (15, 20, 21) and the Calvin–Benson–Bassham (CBB) cycle (22). Compelling evidence for the competition between these two pathways is catalyzed by O₂-sensitive [FeFe]-hydrogenases, which accept electrons from photosynthetically reduced ferredoxin and reduce protons to H₂. Since the process occurs downstream of the Calvin–Benson–Bassham (CBB) cycle, the electrons are delivered from the type II NADPH dehydrogenase (NDA2) and bypass the direct pathway of O₂ evolution. The authors declare no competing interest.
pathways and H₂ production has been accumulated in recent studies (23–25). As CO₂ fixation provides the strongest sink for photosynthetic reductants, it should play a major role in the cessation of H₂ photoproduction in algae when the CBB cycle is active (19, 22).

For preventing competition between the [FeFe]-H₂ases and the CBB cycle, we recently devised a pulse-illumination protocol that allows H₂ production in nutrient-replete algal cultures for up to 3 d (23). To achieve this, we specifically selected the duration of light pulses in the light/dark sequence to avoid activation of the CBB cycle, thus allowing for the redirection of photosynthetic electrons toward the [FeFe]-H₂ases. Typically, a train of 1- to 6-s light pulses interrupted by 9-s dark periods is sufficient for sustained H₂ photoproduction in C. reinhardtii cultures (23, 25). Our protocol thus differs from earlier pulse-illumination approaches that aimed at preventing the accumulation of O₂ in the cultures (26).

While we could demonstrate competition of [FeFe]-H₂ase with FDPs (25), the origin of reductants for H₂ photoproduction in the pulse-illuminated algae remained unclear. The relatively high efficiency of the process suggests the involvement of water oxidation by PSII, and consequently the simultaneous production of H₂ and O₂. Although widely proposed in the current literature (8, 24), the presence of the direct water biophotolysis in H₂-producing green algae has not yet been proven by direct experimental data.

In the present study, we provide clear evidence for the presence of PSII-dependent oxidation of ¹⁸O-labeled water (H₂¹⁸O) with concomitant evolution of ¹⁶O₂ and ¹⁶,¹⁸O₂ during H₂ photoproduction in the pulse-illuminated green alga C. reinhardtii under anoxic conditions. O₂ evolution is balanced by light-dependent and light-independent respiration that sustains the anoxic condition. We also demonstrate that the loss of PSII activity in algae can be partly compensated by the PSII-independent H₂ photoproduction pathway. Nevertheless, the activity of PSII is indispensable for the sustained process, where it contributes to more than 92% of the final H₂ yield.

**Results**

**Net H₂ Photoproduction, Water Oxidation, and O₂ Exchange.** Employing membrane-inlet mass spectrometry (MIMS), we confirmed the induction of sustained H₂ photoproduction in anoxic C. reinhardtii cultures by a train of 1-s light pulses interrupted by 9-s dark periods (Fig. 1A). The signal exhibited a typical sawtooth wave with the H₂ production transients during pulse illumination and a strong H₂ uptake on the shift to darkness, which is in line with previous data (23). The H₂ uptake is the result of H₂ consumption by the MIMS setup and the reversible action of the H₂ase (as demonstrated in SI Appendix, Fig. S1). The response of anoxic algae to the train of light pulses was strain-specific. While in the cell-deficient mutant the first H₂ peak appeared already on the first 1-s flash (Fig. 1C, CC-4533), the wild-type CC-124 strain required some time before demonstrating the pronounced H₂ photoproduction yield (Fig. 1A).

The application of ¹⁸O-labeled water (H₂¹⁸O) to the algae allowed simultaneous monitoring of PSII activity via both nonlabeled and ¹⁸O-labeled O₂ isotopologues (27). Thus, as shown in Fig. 1B, the injection of H₂¹⁸O into the MIMS cell filled with the algal suspension led to the detection of O₂ evolution at m/z 32 (for ambient ¹⁸O₂) and m/z 34 (for singly labeled ¹⁶,¹⁸O₂) signals as a result of the water-oxidizing activity of PSII centers during pulse illumination:

\[ 2\text{H}_2\text{O} \rightarrow \text{O}_2 + 4\text{H}^+ + 4\text{e}^- \]  
\[ \text{H}_2\text{O} + \text{H}_2\text{O} \rightarrow \text{O}_2^{16,18} + 4\text{H}^+ \]  
\[ \text{m/z } 34 \]  

The m/z 34 signal showed O₂ evolution at the first 1-s light pulse, and algal cultures reached maximum O₂ production yields after 10 to 60 pulses (Fig. 1B). Then, the signal slowly declined but kept the sawtooth shape until the end of the pulse-illumination period. The decline in the net O₂ evolution coincided well with the simultaneous increase in the H₂ photoproduction yield (Fig. 1A). Surprisingly, ¹⁶O₂ production (m/z 32) of midlog-phase C. reinhardtii cultures was significantly delayed by strong light-dependent uptake at the beginning of pulse illumination (Fig. 1B). Since ambient ¹⁶O₂ dominates in the MIMS chamber and the level of ¹⁶,¹⁸O₂ is negligible in the beginning of pulse illumination, the pronounced uptake of ¹⁶O₂ in the first few cycles of pulse illumination indicates the domination of light-dependent respiration over water oxidation during this period. On the contrary, the mature (close to the stationary phase) algae did not show any signs of the light-dependent ¹⁶O₂ uptake (SI Appendix, Fig. S2). In the latter case, the m/z 32 signal simply repeated the shape of the m/z 34 curve. Thus, light-dependent O₂ uptake is not caused by the self-shading effect and activation of respiration in dense cultures. This phenomenon is most likely linked to O₂ photoreduction by FDPs that operate during the dark-to-light (or low-light to high-light) transients under oxic and microoxic conditions (25, 26).

The rise of the H₂ (m/z 2) signal occurred almost immediately upon the firing of each light pulse. This was followed by a rise in the O₂ (m/z 34) signal with ~3-s delay (Fig. 1C). As a result, the H₂ production wave showed maxima at almost the minimum of the m/z 34 signal. Since the diffusivity of H₂ in the polydimethylsiloxane membrane used in the MIMS cell (applied to separate the liquid phase of the sample from the high vacuum of the mass spectrometer) is approximately four times faster than for O₂ (29), this...
The addition of 30 μM of DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea], a specific inhibitor that binds to the QB-pocket of PSII and blocks electron transport from PSII to the PQ pool, did not affect H2 photoproduction much during the first 20 min of pulse illumination (Fig. 2A). The m/z 2 signal of the DCMU-treated algae (CC-124 + DCMU) was only slightly reduced as compared to the control (CC-124). This response of pulse-illuminated algae to DCMU was confirmed using an H2 electrode (SI Appendix, Fig. S3). In contrast, the presence of DCMU significantly reduced the 16,18O2-evolving activity in algae at the start of pulse illumination (Fig. 2B), but inhibition was not complete. Thus, as seen in Fig. 2B, Inset a residual water-oxidizing activity (<1.5% of the control) was observed in the DCMU-treated samples. At the same time, DCMU-treated algae demonstrated no light-induced evolution of nonlabeled 18O2 (m/z 32) but a strong light-dependent 16O2 uptake (Fig. 2C).

Since the m/z 34 signal represents the net 16,18O2 exchange (as seen from the m/z 32 signal in Fig. 2C), the contribution of the residual PSII activity in the H2 photoproduction yield of the DCMU-treated samples remained unclear. Therefore, in the next experiment, a train of light pulses was applied to the PSII-deficient (FuD7) mutant. Due the absence of fully assembled PSII centers in the thylakoid membrane (30), this strain completely lacks water-oxidizing activity (SI Appendix, Fig. S4A). Nevertheless, FuD7 still produced H2, albeit at the decreased rate (Fig. 2A and SI Appendix, Fig. S3). Similar to DCMU-treated algae, the PSII-deficient mutant showed a strong light-dependent 16O2 uptake (SI Appendix, Fig. S4B).

The short-term experiments undertaken with the DCMU-treated wild-type strain and the FuD7 mutant revealed functioning of the PSII-independent H2 photoproduction pathway when PSII was not available. Therefore, it was worth checking whether this pathway could sustain H2 photoproduction in the long-term process. It was subsequently observed that the PSII-independent pathway could not substitute the PSII-dependent pathway in the long-term process (Fig. 3A). The H2 photoproduction yields both in the FuD7 mutant and in the DCMU-treated wild-type strain began decreasing during the first hour (Fig. 3A, Inset). This result is in good agreement with those of the short-term experiments (SI Appendix, Fig. S3). In the CC-124 and CC-5325 strains (Fig. 3A and B), which possess full PSII activity, DCMU addition stopped H2 photoproduction after 6 h, while H2 production of the FuD7 mutant continued, but at a greatly reduced rate. In all cases, final H2 yields were only slightly higher than in the dark samples.

In contrast to PSII deficiency, the absence of NDA2 (responsible for nonphotochemical reduction of the PQ pool) (9, 10) in C. reinhardtii cells was almost completely compensated for by the PSII-dependent pathway. This was particularly evident in the first 4 h of the experiment (Fig. 3B, Inset). Here, the input of the PSII-independent pathway was noticeable only by the end of the

Fig. 2. The effect of DCMU and the psbA deletion (FuD7) on H2 (A), 16,18O2 (B), and 16O2 (C) yields in pulse-illuminated algae. The experimental conditions were the same as in Fig. 1, except that the total Chl content was increased to ~20 mg L⁻¹ (to distinguish the difference between O2 production and O2 consumption in the control and DCMU-treated samples). DCMU was introduced to aerobic algae at a final concentration of 30 μM. Curves are the mean of two to four independent experiments. The H2 photoproduction of the FuD7 mutant is a result of one measurement with MIMS but was repeated nine times with a H2 sensor, producing similar results (SI Appendix, Fig. S3). Additional experimental data can also be found in SI Appendix, Fig. S10.

Fig. 3. Long-term H2 photoproduction by pulse-illuminated algae. The cultures of the wild-type (CC-124) strain and the PSII-deficient (FuD7) mutant (A), and NDA2-deficient (NDA2) mutant and its wild-type progenitor (CC-5325) (B) were exposed to a train of 1-s light/9-s dark pulses in the absence and in the presence of 30 μM DCMU under an Ar atmosphere. The H2 production activity of the same strains was also checked under complete darkness. ( Insets) The same samples in the first 4 h of pulse illumination. Values are mean of 6 to 13 independent replicates (± SD).
\textbf{CO}_2 \text{ Exchange of Algal Cultures Correlates with O}_2 \text{ Evolution and H}_2 \text{ Photoproduction}.\text{ Previously, we demonstrated that } \textit{C. reinhardtii} cells exposed to a train of 1-s light pulses interrupted by 9-s dark periods do not fix CO}_2 \text{ and as a result do not accumulate biomass (23, 25). These previous experiments were performed at low cell densities (<10 mg total Chl per L) to ensure optimal photosynthetic performance. In the current work, we employed sensitive MIMS methodology at a higher cell density of around 20 mg Chl L\textsuperscript{-1} to improve the resolution of CO}_2 \text{ analysis. Thus, the culture was observed to release CO}_2 \text{ during the 1-s light/9-s dark pulse-illumination train (Fig. 4). The ambient } (m/z \ 44) \text{ CO}_2 \text{ signal rose within 5 min of engaging the pulse-illumination train and then slowly declined until train cycling was concluded. At this point, the } m/z \ 44 \text{ signal decayed exponentially to reach the initial state. Along with ambient } CO}_2 \text{ release, } \textit{C. reinhardtii} \text{ cells also produced } ^{18}\text{O-labeled CO}_2 \text{ isotopologues: } \textit{C}^{16,18}\text{O}_2 (m/z \ 46) \text{ and } \textit{C}^{18,18}\text{O}_2 (m/z \ 48) \text{ (Fig. 4). Observe, the release of all CO}_2 \text{ isotopologues was light-dependent, but the signals did not fluctuate during pulse illumination (Fig. 4). The typical sawtooth shape appeared only when the duration of the light pulse in the light/dark sequence was extended to 8 s. In this regime, a clear sign of CO}_2 \text{ fixation appeared 5 min after the start of pulse illumination (Fig. 5A). All three } CO}_2 \text{ signals, } m/z \ 44, m/z \ 46, \text{ and } m/z \ 48, \text{ were affected (Fig. 5A and SI Appendix, Fig. S5). A similar trend in behavior of } m/z \ 44, m/z \ 46, \text{ and } m/z \ 48 \text{ on activation of the CBB cycle indicates that these three signals primarily represent } \textit{C}^{16}\text{O}_2, \textit{C}^{16,18}\text{O}_2, \text{ and } \textit{C}^{18,18}\text{O}_2, \text{ but not other possible gases with a similar molecular weight like N}_2\textsuperscript{16,18}\text{O} \text{ and N}_2\textsuperscript{18,18}\text{O}. \text{ For example, the latter two may appear in } \textit{C. reinhardtii} \text{ as a result of NO detoxification (31).}

\text{As expected, prolonging the light pulse in the light/dark sequence led to enhanced } O}_2 \text{ release, but the burst amounts of } O}_2 \text{ were produced only in the 8-s/9-s regime where the CBB cycle was activated (Fig. 5B; notice the different scale for the 8-s/9-s pulse protocol). By contrast, } \textit{C. reinhardtii} \text{ cells exposed to the 5-s light/9-s dark protocol yielded almost the same amount of } O}_2 \text{ as the algae exposed to 3-s/9-s pulse illumination, presumably due to a more pronounced } O}_2 \text{ consumption during the dark phases of the same duration. The enhanced } O}_2 \text{ consumption, however, did not correlate with } CO}_2 \text{ release (Fig. 5A). Interestingly, 3-s/9-s and 5-s/9-s pulse-illumination protocols yielded almost the same amounts of } H}_2 \text{ (Fig. 5C). By contrast, the 8-s/9-s protocol released } H}_2 \text{ only in the beginning of pulse illumination. Thus, the activation of the CBB cycle immediately led to } H}_2 \text{ uptake (Fig. 5C). As seen in Fig. S5A and C, the } H}_2 \text{ trace } (m/z \ 2) \text{ starts to decline at exactly the time when } CO}_2 \text{ signals establish a pronounced sawtooth wave.}

\textbf{Discussion}

\textbf{Clear Evidence for Simultaneous Water Oxidation and } H}_2 \text{ Photoproduction in Green Algae}. \text{ The role of PSII in } H}_2 \text{ photoproduction in green algae has long been a subject of debate (32). The PSII-dependent net } O}_2 \text{ release of dark-adapted anoxic algae, when exposed to light, appears only after a period of efficient } H}_2 \text{ production (14). Therefore, direct involvement of PSII in } H}_2 \text{ photoproduction is not obvious, especially because DCMU does not completely inhibit } H}_2 \text{ production (33, 34). In sulfur (S)-deprived } \textit{C. reinhardtii} \text{ cells, where the recovery of PSII reaction centers is affected by stress (35), the involvement of residual PSII activity in the } H}_2 \text{ photoproduction yield has been proposed based on inhibitory analysis, electron paramagnetic resonance spectroscopy, and fluorescence data (36–38). Nevertheless, Clark-type } E}_2 \text{ electrodes used directly in algal suspensions have not indicated water-splitting activity in S-deprived cells (36), either due to low sensitivity of the technique (39) or due to the absence of net } O}_2 \text{ release in the actively respiring cells (40). The situation improved with application of a high-sensitive MIMS approach (15, 22). The publications showed that the release of } O}_2 \text{ in dark-adapted algae occurs a few seconds after appearance of } H}_2 \text{ in the system, suggesting direct involvement of PSII in } H}_2 \text{ photoproduction.}

\text{As shown in Fig. 1, the application of the 1-s light/9-s dark protocol to anoxic algae results in sustained } H}_2 \text{ photoproduction and also leads to the simultaneous evolution of } O}_2 \text{ (detected at } m/z \ 32 \text{ and } m/z \ 34). \text{ The appearance of both nonlabeled and } ^{18}\text{O-labeled isotopologues of } O}_2 \text{ upon enrichment with } H}_2\textsuperscript{18}O \text{ is well known for monitoring PSII-driven water oxidation reaction (41). The appearance of photosynthetically produced } O}_2 \text{ in algal cultures should indicate a shift in the environment from anoxic to
microoxic. However, the situation is not so simple. This is for two reasons: First, light activates strong O2 uptake, which is observed in midlog-phase cultures (Fig. 1B, m/z 32 signal) in the FuD7 mutant (SI Appendix, Fig. S4B) and in the DCMU-treated algae (Fig. 2C, +DCMU trace); second, the release of photosynthetically produced O2 occurs simultaneously with mitochondrial respiration (which was subtracted from all signals during data processing; SI Appendix, Fig. S6 A and B). These respiratory processes under normal atmospheric pressure balance photosynthetically produced O2 to a level undetectable by polarographic techniques (Clark-type O2 electrode) (23). Nevertheless, the competition of H2 photoproduction with FDP-driven O2 photoreduction under such low O2 levels is still highly possible (25), especially in the beginning of the experiment where extracellular O2 concentrations are at around 0.1 to 0.5 μM. In this context, the gradually increasing H2 photoproduction rate (Fig. 1A) may reflect a release of FDPs of such competition. Indeed, the level of extracellular O2 does decrease in the course of the experiment due to its consumption by the instrument and the culture itself (SI Appendix, Fig. S6A).

Surprisingly, the cell wall-deficient CC-4533 strain showed the production of H2 on the first light pulse (Fig. 1C, CC-4533). The rapid response of cells to the establishment of anoxic conditions (~3 min from fully aerobic environment) suggests the expression of the O2-sensitive [FeFe]-Hase in C. reinhardtii cells at the time of aerobic cultivation (43, 44). Since the HYDA1/HYDA2 proteins are also already available in aerobic C. reinhardtii (23), the delay in net H2 evolution of the CC-124 strain (Fig. 1A) is most likely caused by the limitation of H2 diffusion through the cell wall. Assuming the reversible nature of the H2ase-driven reaction (45, 46), enhanced intracellular levels of H2 may increase H2 consumption during dark periods and decrease the final H2 yield. In accordance with this suggestion, prolongation of the light pulse duration to above 3 s in the pulse-illumination sequence demonstrates the early appearance of the H2 signal in the same strain (Fig. 5C). Similar to the CC-4533 strain, the periods of H2 photoproduction in the CC-124 algae coincide well with O2 evolution (Fig. 1C, CC-124). Simultaneous production of H2 and O2 in the light indicates the direct involvement of electrons from water oxidation by PSI in H2 photoproduction.

**PSI Plays a Major Role in Sustained H2 Photoproduction.** Reductants for the H2 photoproduction of C. reinhardtii are suggested to be supplied not only by PSI but also by the degradation of stored organic substrates, primarily starch (47). In a similar manner to the PSI-dependent process, the indirect pathway requires active PSI for the donation of electrons to H2ase. However, by contrast, the indirect process results in CO2 release with a maximum molar stoichiometry of 1 CO2 per 2 H2. It has previously been shown that the contribution of the indirect pathway to the H2 photoproduction yield might be significant in S-deprived cells, where the PSI activity is substantially affected by the stress (48).

The results obtained with DCMU-treated algae and the PSI-deficient mutant (Fig. 24 and SI Appendix, Fig. S3) clearly show that the PSI-independent H2 production pathway can partly compensate for the loss of the PSI-dependent H2 production pathway toward H2 photoproduction of pulse-illuminated cells. The indirect process, however, could not sustain H2 production for longer than 6 h (Fig. 3A), and the specific rate of H2 production had already started to decline during the first hour (Fig. 3 A, Inset). On the contrary, the elimination of NDA2 in C. reinhardtii, which is the main player of the PSI-dependent pathway (10), shows almost no effect on H2 photoproduction under pulse-illumination conditions (Fig. 3B). It has been previously demonstrated that NDA2 deficiency decreases the H2 photoproduction activity of S-deprived algae (9, 10). However, S deprivation leads to a significant accumulation of starch reserves in cells during the photosynthetic stage (36). Therefore, it is not surprising that S deprivation increases the contribution of the NDA2-dependent pathway in the H2 photoproduction yield, especially when the activity of PSI is limited or fully absent (49). Thus, S-deprivation data do not contradict our conclusions, since in actively growing algae, which are used in this study, the starch reserves are limited. In the presence of DCMU, which blocks the electron flow from PSI, the nda2 deletion mutant produces almost the same amount of H2 as algae placed in complete darkness. The slightly higher H2 yield of the DCMU-treated nda2 deletion mutant might be attributed to 1) the light activation of fermentation, 2) incomplete inhibition of electron flow by DCMU (Fig. 2B), or 3) the presence of other player(s) in the PSI-independent pathway. If any, their input in the total H2 yield is negligible and limited to the beginning of pulse-illumination (Fig. 3 B, Inset). A full compensation of algal NDA2 elimination by PSI confirms that H2 photoproduction during 1-s illumination periods proceeds via the most efficient mechanism of direct water biophotolysis. This conclusion is in agreement with fluorescence data showing that PSI activity in dark-adapted C. reinhardtii cells is linearly related to the hydrogenase capacity observed during the first seconds of illumination (50).

In the 1-s/o-s pulse-illumination regime, algae spend most of the time in darkness, where fermentation plays a major role. According to the data presented in Fig. 3A, up to 4% of the H2 yield in the CC-124 strain is supported by dark fermentation, while 96% is supported by PSI. In the presence of DCMU, the PSI-independent pathway doubles the H2 yield in pulse-illuminated algae as compared to the dark samples (Fig. 3A). Thus, even if both pathways (PSI-independent and dark) operate in the CC-124 cells during pulse illumination, PSI is still responsible for about 92% of the final H2 yield. Very similar results (93% of the PSI input) were obtained for the nda2 deletion mutant, where the contribution of fermentation was close to 7% (Fig. 3B). However, the NDA2 deficiency may also enhance fermentative H2 production in the mutant. Taking into account all data, we conclude that H2 production during 1-s light pulses is driven primarily or even exclusively by the PSI-dependent pathway.

**Efficient H2 Photoproduction in C. reinhardtii Occurs before Activation of the CBB Cycle.** The light-dependent water oxidation performed by PSI results in O2 evolution with a simultaneous release of protons (H+) into the thylakoid lumen. In addition, protons are pumped into the lumen by the PQ/plastohydroquinone cycle driven by PSI and the cytochrome b/f complex. The accumulation of H+ in the lumen builds up the ΔpH across the thylakoid membrane and ensures adenosine 5′-triphosphate (ATP) biosynthesis. Simultaneously, a relatively small release of CO2, which was first detected by MIMS in PSI membranes isolated from spinach (51), could be observed. This CO2 is known to be released by PSI and is the result of HCO3− reaction with H+ (produced during water splitting) followed by the subsequent bicarbonate dehydration:

\[
\text{H}^+ + \text{HCO}_3^- \rightarrow \text{H}_2\text{O} + \text{CO}_2
\]

This reaction facilitates the removal of H+ from PSI. Moreover, the electron-acceptor side of PSI is known to be another source for CO2 evolution under intensive light illumination (52, 53). In C. reinhardtii, the bicarbonate dehydration reaction (and in general interconversions of inorganic carbon) can be accelerated by a luminal carbonic anhydrase, CrCAH3 (54–56). However, in whole cells under normal conditions, the light-dependent CO2 release is barely detectable due to the presence of two processes: 1) the consumption of CO2 by Rubisco (i.e., by CBB cycle) and 2) mitochondrial respiration. Therefore, it is not surprising that we observed CO2 release in the pulse-illuminated algae, when the CBB cycle is not active (Fig. 4) and mitochondrial respiration...
is restricted by an extremely low level of O2 in the microoxic environment (because of the efficient O2 consumption by the MIMS setup; SI Appendix, Fig. S8A). As shown in Fig. 4, the light-induced rise of m/z 44, m/z 46, and m/z 48 signals occurs simultaneously with O2 evolution and H2 photoproduction (Fig. 1). The appearance of m/z 46 and m/z 48 signals together with ambient CO2 (m/z 44) indicate a CO2–water interexchange:

\[
H_2O + C^{16}O = H_2C^{16}O + C^{16}O_2 \quad [5]
\]

\[
H_2O + C^{16,18}O = H_2C^{16,18}O + C^{16,18}O_2. \quad [6]
\]

Alternatively, the inclusion of labeled O2, which appears as a product of water oxidation (reaction 3), in the organic substrate and its immediate degradation should be expected:

\[
nC^{16}O_2 + \left[CH_{2}O\right]_n \rightarrow nH^{16}O + n C^{16}O_2. \quad [7]
\]

\[
nC^{16,18}O_2 + \left[CH_2O\right]_n \rightarrow nH^{16}O + n C^{16,18}O_2. \quad [8]
\]

The probability of reaction 7, and especially reaction 8, is much lower than reactions 5 and 6, unless they proceed at the same site as reaction 3. Since the reactions 7 and 8 are expected to occur in the stroma of chloroplasts, they are unlikely to satisfy kinetics of light-dependent release of CO2 isotopologues during pulse illumination (Fig. 4).

Although the observed CO2 evolution could be potentially caused by activation of substrate degradation in the light (photofermentation), the positive correlation between O2 evolution (SI Appendix, Fig. S2) and CO2 release (Fig. 4), and relatively fast recovery of all three CO2 signals after conclusion of pulse illumination (Fig. 4) strongly suggest direct involvement of PSII in CO2 evolution. Since the equilibration rate between labeled water and CO2 occurs with half-times of t1/2 ≈ 30 s or less (55, 57), any donor-side CO2 formation or other acidification of the lumen would produce CO2 isotopologues reflecting the H18O enrichment of the water (given that measurements were initiated about 5 min after H218O addition). By contrast, CO2 release at the acceptor side of PSII, where bicarbonate is bound to the non-heme iron and equilibrates slowly with bulk water, would be predominantly C16O2. Thus, the different time dependence of C16O2 versus C16,18O2 and C16O2 (Fig. 4, m/z 46 and m/z 48 signals) suggests that light-induced CO2 release may originate from both the acceptor and donor sides of PSII, as well as the acidification of the lumen. This is further supported by estimating the total amount of released CO2. If just the acceptor side would contribute, we should expect around 2 nmol of CO2 released per mg Chl [assuming 1.83 nmol of O2 per mol of total Chl in photomixotrophic C. reinhardtii cells (58)]. Instead, formation of around 4 to 8 nmol CO2 per mg Chl is typically observed in algae cultures (Figs. 4 and 5 and SI Appendix, Fig. S11). These data show PSII-associated CO2 formation in intact cells, which supports the idea that it has an important regulatory and protective function for PSII in vivo.

The prolongation of light pulses to 8 s in the pulse-illumination sequence gradually activates the CBB cycle, leading to a pronounced O2 uptake after 5 min of pulse illumination (Fig. 5A and SI Appendix, Fig. S5). As shown in Fig. 5, the activation of the CBB cycle immediately results in the burst release of O2 (Fig. 5B) and inhibition of H2 evolution followed by H2 uptake (Fig. 5C). These data support the previously proposed hypothesis that the primary loss of H2 evolution activity in C. reinhardtii cells is caused by competition between ferrodoxin–NADP+ oxidoreductase and H2ases for reduced ferrodoxin, rather than by the sensitivity of hydrogenase to oxygen (19, 22). Besides the activation of the CBB cycle, increasing O2 levels inside the chloroplast can also enhance the flow of photosynthetic electrons toward FDPs. Such competition indeed becomes more pronounced with prolongation of the light pulse in the sequence (25). In long-term experiments, prolongation of the light pulse results in biomass accumulation (25). These experiments show that the pulse-illumination approach allows a fine tuning of algal metabolism between microoxic H2 photoproduction and aerobic CO2 fixation, thus enabling PSII-dependent water-oxidation activity to be maintained at the desired level.

**Summary**

The illumination of anoxic C. reinhardtii cultures with a train of short light pulses interrupted by longer dark periods demonstrates that efficient H2 photoproduction in algal cells occurs exclusively in the absence of photosynthetic CO2 fixation, thus when the CBB cycle is not active. The cells produce H2 via the most efficient mechanism of direct water biophotolysis, where water oxidation at the donor side of PSII provides the electrons for reduction of protons by the [FeFe]-H2ase enzyme(s) downstream of PSI. Thus, the two reactions occur simultaneously. However, under normal conditions O2 is not released by the cells due to its consumption by respiration. During short periods of light illumination, the H2ase activity supports a linear photosynthetic electron flow from PSI to PSII, promoting proton translocation across the thylakoid membrane, and ensuring efficient water oxidation in the CBB cycle. Thus, [FeFe]-H2ase in concert with PSI, creates favorable conditions for O2 accumulation and ATP biosynthesis to levels sufficient for the activation of mitochondrial respiration and the CBB cycle. If the duration of light pulses is not sufficient to fulfill the above condition, the algae continue to produce H2. The long-term production of H2 in the absence of CO2 fixation suggests the central role of [FeFe]-H2ase in supporting algal photosynthesis and cell fitness under anoxic conditions.

**Materials and Methods**

The wild-type C. reinhardtii strain CC-124, cell wall-deficient strains CC-4533 and CC-5325, psa4 deletion (Fud7) mutant CC-4147, and the nda2 deletion mutant (LMJ.RY0402.257129) were obtained from the Chlamydomonas Resource Center at the University of Minnesota, St. Paul, MN. The nda2 deletion is characterized as described in SI Appendix, Fig. S7. All cultures were maintained, grown, and checked for H2 photoproduction activity under pulse illumination with H2 and O2 microsensors (H2-NO and OX-NO; Unisense A/S) as described in detail by Kosourova et al. (23). Conditions for the long-term experiments were reported in the same publication. All experiments were conducted in TAP medium under anaerobic conditions with or without photomixotrophic algae, which were pipetted into the MIMS chamber or vials just before measurements.

The gas exchange in the suspension of algal cells was studied by time-resolved MIMS setup (SI Appendix, Fig. S8) as described previously (59, 60). Briefly, the setup consisted of an isotope ratio mass spectrometer (Delta V Plus; Thermo Fisher Scientific), a cooling trap (~78 °C; dry ice + ETOH), and an in-house-built gas-tight membrane-inlet chamber with 200-μL working volume. Before the measurements, H18O (97%; Larodan Fine Chemicals AB) was added to the MIMS chamber to a final enrichment of 4%. Analysis of O2 reactions (evolution/consumption) was based on the m/z 32 (18O2) and m/z 34 (16,18O2) signals, with Faraday cup amplification of 3 × 1010 and 1 × 1011, respectively. The signal m/z 36 (18O18O2) was not considered due to its low evolution at quite low H18O-enrichment and contamination of this signal by the presence of 18Ar. Analysis of CO2 reactions was based on simultaneous monitoring of m/z 44 (18O16O), m/z 46 (16,18O2), and m/z 48 (12C18O2) signals with cup amplification of 1 × 1010, 1 × 1011, and 1 × 1012, accordingly. The argon signal (m/z 40) with cup amplification of 1 × 1011 was used as a control. H2 photoproduction activity was studied in separate runs by monitoring the m/z 2 (H2) signal with an amplification of 1 × 1012. No H18O2 was added for H2 assays. The white light-emitting diode light pulses (~1,000 μmol photons m−2 s−1) were applied using the STM32F103 microcontroller board controlled by the OxygenHydrogen software. Before each measurement, microoxic environment inside the MIMS chamber was achieved within ~2 min after sealing by degassing the sample with the vacuum pump of the mass spectrometer. The initial levels of oxygen slightly varied from experiment to experiment from 0.1 to 0.5 μM. The final gas exchange
Data Availability. All data needed to evaluate conclusions of this paper are present in the paper and in SI Appendix.

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