PGR5 is required for efficient Q cycle in the cytochrome b₆f complex during cyclic electron flow

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Abstract

Proton Gradient Regulation 5 (PGR5) is involved in the control of photosynthetic electron transfer, but its mechanistic role is not yet clear. Several models have been proposed to explain phenotypes such as a diminished steady state proton motive force (pmf) and increased photodamage of photosystem I (PSI). Playing a regulatory role in cyclic electron flow (CEF) around PSI, PGR5 contributes indirectly to PSI protection by enhancing photosynthetic control, which is a pH-dependent downregulation of electron transfer at the cytochrome b₆f complex (b₆f). Here, we re-evaluated the role of PGR5 in the green alga Chlamydomonas reinhardtii and conclude that pgr5 possesses a dysfunctional b₆f. Our data indicate that the b₆f low-potential chain redox activity likely operated in two distinct modes – via the canonical Q cycle during linear electron flow and via an alternative Q cycle during CEF, which allowed efficient oxidation of the low-potential chain in the WT b₆f. A switch between the two Q cycle modes was dependent on PGR5 and relied on unknown stromal electron carrier(s), which were a general requirement for b₆f activity. In CEF-favoring conditions, the electron transfer bottleneck in pgr5 was the b₆f, in which insufficient low-potential chain redox tuning might account for the mutant pmf phenotype. By attributing a ferredoxin-plastoquinone reductase activity to the b₆f and investigating a PGR5 cysteine mutant, a current model of CEF is challenged.
Introduction

In linear electron flow (LEF), the two photosystems (PSII and PSI) act in series to ultimately reduce NADP⁺ via the enzyme ferredoxin (Fd)-NADP(H) oxidoreductase (FNR). The cytochrome \textit{b}6\textit{f} complex (\textit{b}6\textit{f}) functionally interconnects the two photosystems [reviewed in 1], accepting electrons from plastoquinol (PQH₂) and donating electrons to plastocyanin (PC). Functional \textit{b}6\textit{f} occurs as a homodimer, each monomer consisting of four major subunits (cytochrome \textit{b}₆, subunit-IV, cytochrome \textit{f} (cyt.\textit{f}), and the Rieske iron sulfur protein (ISP)), as well as four minor subunits (PetG, L, M and N). In addition, each monomer includes six cofactors: two \textit{b}-hemes (\textit{b}l and \textit{b}h), two \textit{c}-hemes (cyt.\textit{f} and \textit{c}i), one chlorophyll \textit{a} and one \textit{β}-carotene. Light indirectly induces \textit{b}6\textit{f} turnover: Upon oxidation by the primary PSI electron donor P700, PC extracts one electron from cyt.\textit{f}, which is re-reduced by the Rieske ISP. The positively charged Rieske FeS domain moves towards the lumenal Qo-site, where an electron flow bifurcation occurs: PQH₂ donates one electron to the Rieske ISP (part of the high-potential chain with a midpoint potential \(E_m = 300-350\) mV) and a second electron to \textit{b}l (low-potential chain; \(E_m = -35\) mV) and/or \textit{c}i (\(E_m = 100\) mV, flexible as described below). Via the canonical Q cycle, the production of one PQH₂ at Qi requires the oxidation of two PQH₂ at Qo. The spatial proximity between \textit{b}h and \textit{c}i suggests electron sharing between the two and the presence of a membrane potential (\(\Delta\Psi\)) promotes the shared electron to rest on \(b_{h\text{red}}/c_{i\text{ox}}\) [2]. Furthermore, presence of a \(\Delta\Psi\) is a general prerequisite for efficient \textit{b}-heme oxidation [3] but it remains enigmatic by which mechanism the \textit{b}6\textit{f} senses the \(\Delta\Psi\). It is of note that heme-\textit{c}i is unique since it lacks an amino acid axial ligand and thus might ligate with the semiquinone analogue NQNO [4, 5], which downshifted the heme-\textit{c}i midpoint potential from 100 mV to approximately \(-150\) mV [6]. Furthermore, heme-\textit{c}i was proposed to engage in a Qi-site gating function [7] by either ligating tightly with the phenyl group of F40 in subunit-IV in the oxidized state (closed Qi-site), or, after transient heme-\textit{c}i reduction, with (semi-)PQ. A recent cryo-EM structure of the spinach \textit{b}6\textit{f} complex contained the native PQ in proximity to heme-\textit{c}i [8]. Following the ligation-associated midpoint potential downshift of heme-\textit{c}i [6], it is not clarified yet whether heme-\textit{c}i reduces heme-\textit{b}h or the quinone. Since not more than half of the \textit{b}-heme population is reduced per Qo-site turnover in uninhibited complexes [9, 10, 11 and references therein], occurrence of \(b_{h\text{red}}/b_{h\text{red}}\) is unlikely. By approaching \(b_{h\text{red}}/b_{h\text{red}}\), i.e. during a Qo-site turnover in the presence of \(b_{h\text{red}}\), the strongly reducing redox potential in the low-potential chain injects the first electron into the quinone-\textit{c}i\text{ox} ensemble [7]. Thereby, heme-\textit{c}i could force a quasi-concerted PQ reduction [5, 12]. The deprotonation of PQH₂ at Qo and the protonation of PQ at Qi couple electron transfer to proton translocation into the thylakoid lumen. The resulting transmembrane electrochemical proton gradient (\(pmf\)) fuels ATP synthesis via the chloroplast ATP-synthase. Besides LEF, which produces both NADPH₂ and ATP, diverse auxiliary electron flow pathways, including cyclic electron flow (CEF) around PSI, contribute to the \(pmf\) and thereby equilibrate the NADPH₂ to ATP output ratio of the light reactions [reviewed in 13]. In addition, the \(pmf\) plays an integral photoprotective role, since the chemical component (\(\Delta\text{H}\)) induces energy-dependent quenching (qE) and modulates the rate-limiting, pH-dependent oxidation of PQH₂ at the Qo-site, which is termed photosynthetic control [14, reviewed in 15]. Hence, CEF creates a regulatory feedback loop, linking the stromal redox poise to the efficiency of light harvesting and the rate of electron transfer.
PGR5 (proton gradient regulation 5) has been first identified in Arabidopsis thaliana as component being involved in the regulation of the pmf via CEF [16]. The corresponding knockout mutant in C. reinhardtii features multi-faceted phenotypes resembling its vascular plant counterpart [17, 18]: The algal pgr5 fails to induce qE-dependent NPQ and is extremely susceptible to PSI photodamage in response to high light [19, 20] as well as fluctuating illumination [21]. These defects have been attributed to an impaired acidification of the thylakoid lumen due to compromised Fd-PQ reductase-dependent CEF and a resulting lack of photosynthetic control in response to enhanced stromal redox pressure [19, 22]. However, the detailed mechanism of this CEF route is still elusive, as is the molecular role of PGR5. In the past, the association of FNR with the b6f [23-25] has been proposed to induce a switch from LEF to CEF: According to this model, FNR would tether reduced Fd in the vicinity of b$_{h}$ and c$_{i}$, ultimately facilitating PQ reduction via a modified Q cycle that combines luminal and stromal electrons [26-28]. Our previous work showed less stable binding of algal FNR to the thylakoid membrane in the absence of PGR5 [20], suggesting a structural or regulatory contribution of PGR5 to this CEF pathway by influencing the localization of FNR. By contributing to photosynthetic control and potentially providing the Fd-PQ reductase activity required for CEF, the b$_{6f}$ seems to be at the core of the phenotypes the absence of PGR5 produces. Therefore, we spectroscopically reinvestigated the impact of PGR5 on photosynthetic electron transfer in C. reinhardtii, with a focus on b$_{6f}$ functionality by measuring the behavior of the high- and low-potential chain as well as the electrogenic efficiency of the photosynthetic machinery. We provide evidence that during CEF, a Fd-assisted Q cycle is active which requires PGR5 for sustained b$_{6f}$ function in the light.
Materials and methods

Strains and cell cultures

As described previously [19], the *Chlamydomonas reinhardtii* WT strain t222+, *pgr5* and a complemented line, termed C1, were used. The complemented C1 strain accumulated ~75% of WT PGR5 levels [19]. Cells were cultivated at 20 μmol photons m⁻² s⁻¹ on agar-supplemented Tris-acetate-phosphate (TAP) plates [29]. When growing cells for experiments, liquid Tris-phosphate medium was devoid of acetate (TP). Stirred cultures were grown at 10 μmol photons m⁻² s⁻¹ (16 h light/8 h dark) and were bubbled with sterile air at 25 °C. Grown cultures were diluted ~6-fold at least once after inoculation and grown to a density of ~2 × 10⁵ cells mL⁻¹ before harvesting (5000 rpm, 5 min, 25°C). For experiments with PGR5-complemented lines that feature zeocin resistance, WT, *pgr5* and complemented lines were grown in TAP in the same conditions as TP cells, but without air bubbling. One day before the experiments, cells were diluted in fresh TAP and 5 μg mL⁻¹ zeocin was added to resistant cultures to drive PGR5 expression. Before the measurements, cells were resuspended at 20 μg chlorophyll mL⁻¹ (determined as in [30]) in TP supplemented with 20% (w/v) Ficoll. Before transferring the samples to an open cuvette, cells were shaken vigorously in dim light. Figure 1 pictures the routine of sample handling and dark adaptation in open cuvettes involved regular mixing of the 2-mL oxic sample. For oxygen-deprived conditions in the dark, cells were supplemented with 50 mM glucose, 10 U glucose oxidase and 30 U catalase in the cuvette, and then overlaid with mineral oil for at least 30 min. Inhibition of mitochondrial respiration by oxygen deprivation results in a strongly reduced chloroplast stroma [reviewed in 31]. This redox poise is known to promote PGR5-dependent CEF in algae [22]. Independent of PSII photochemistry, these illuminated cells will be referred to as anoxic. In some experiments throughout this study, 1mM hydroxylamine (HA, from 1M aqueous stock) and 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU, from 10 mM ethanolic stock) were used as PSII inhibitors. Where indicated, oxic control samples were poised for at least 20 min with 10 mM methyl viologen (MV, added from 1M aqueous stock) to abolish PSI acceptor side limitation and inhibit CEF.

Generation of PGR5 complemented lines using a bicistronic system.

The PGR5 gene (Cre05.g242400) was amplified from genomic DNA extracts using forward (5'-GCCCGGAATTCTGCTGCTCCAAGCCGTTTG) and reverse primer (5'-CTAGTCTAGATTAAGCCAGGAAGCCCG), harboring the underlined EcoRI/XbaI restriction sites. The reverse primer was used in combination with a second forward primer (5'-GGACTTCTCCAGCTGCTTTGC, underlined base alters codon 113 from Cys to Ser) to generate a 107 bp mega primer [32]. The latter served to generate the PGR5C113S template, amplified with the EcoRI/XbaI primer pair above. The digested PGR5 fragments were introduced into a bicistronic expression vector [33, 34] under the control of the PSAD promoter. In addition, the construct conferred zeocin resistance, since PGR5 expression was linked to ble via a skipping peptide FMDV2A. The DNA was introduced to the pgr5 nuclear genome by electroporation (25 μF, 1kV). Transformants were pre-selected on TAP agar plates supplemented with 10 μg mL⁻¹ zeocin. Picked colonies, putatively carrying the bicistronic PGR5 construct, were grown at 10 μmol photons m⁻² s⁻¹ and submitted to spot tests performed on TP agar plates.
Chlorophyll fluorescence analysis

Pre-selection of pgr5::PGR5 and pgr5::PGR5 C113S was done with colonies grown on TP plates after 24-h exposure to 200 μmol photons m⁻² s⁻¹ (supplementary Figure S1), using a Maxi-Imaging PAM chlorophyll fluorometer (Walz, Germany). All other experiments were carried out in an LED-based spectrophotometer (JTS-10, BioLogic, France). The device was equipped with a Fluo_59 accessory in fluorescence mode with separate light sources to induce photosynthetic processes and measure the fluorescence yield [35]. Regarding the latter, weak 10-μs pulses (LED435-03, Roithner, Austria) were used for excitation and were either placed in the dark and 100 μs after cessation of the actinic light, respectively. The light-detecting photodiodes were protected from scattered actinic light and the measuring pulses by using appropriate 3-mm filters (reference diode: BG39; measuring diode: LPF650+RG665, Schott, Mainz, Germany). Note that the experimental routines in Figure 1 are not in the same order they are referenced in the following text. After 30-min dark-adaptation (Figure 1 item a), Fo was measured in the dark and Fm was obtained by a 250-ms saturating pulse (520-nm LED, 5000 μmol photons m⁻² s⁻¹) to give Fv/Fm ((Fm–Fo)/Fm). The photochemical quantum yield of PSII, ΦPSII, was calculated as (Fm′–Fo′)/Fm′ by measuring maximal (Fm′) and steady state (Fs) fluorescence in the light. Actinic light exposure (630-nm LEDs emitting ~150 μmol photons m⁻² s⁻¹) was either a brief 10-s (Figure 1 item b) or 30-min to establish a steady state regime (Figure 1 item d). For the latter, oxic samples were regularly resuspended in an open cuvette and oxygen-deprived cells were not mixed. PSII operation efficiency depends, in part, on the number of open centers. Two different parameters were calculated, aiming to describe the pool of oxidized QA [reviewed in 36]. The PSII efficiency factor qP was derived from (Fm′–Fo’)/(Fm–Fo) in light-adapted samples [37]. Fo’ was estimated from Fo/(Fs/Fm′+Fo/Fm′) [38]. qP is based on a puddle model, but when a lake model for light harvesting is assumed, the redox state of the QA pool in the light can be more accurately assessed by qL, which was derived from qP×Fo’/Fs [39]. Therefore, qL is preferred over qP [reviewed in 36], although controversy exists [reviewed in 40]. We included both parameters and high values of (1–qL), or (1–qP), approximate a high proportion of reduced QA⁻ over total QA [41, 42].

Time-resolved absorption spectroscopy

All time-resolved absorption measurements are expressed as ΔI/I and were carried out in the JTS-10 with light-adapted cells (630-nm LEDs emitting ~150 μmol photons m⁻² s⁻¹, Figure 1 item d). Using white pulsed LED detection light, the electrochromic shift (ECS) signals were measured as the difference of the absorbance changes at 520 and 546 nm (ΔI/I₂0nm–546nm, respective interference filters FWHM: 20 nm). The light-detecting diodes were protected from scattered actinic light by 3-mm BG39 filters (Schott, Mainz, Germany). Illumination of the samples was interrupted by short dark intervals (250 μs) during which 10-μs detecting pulses were placed after 200 μs. The light intensity used to establish a steady state regime was further described by the initial ECS slope during 2-ms when transitioning from darkness to ~150 μmol photons m⁻² s⁻¹ red actinic light (Figure 1 item c). Assuming that the light excited both photosystems equally, the initial photochemical rates (kPSII ~ kPSI) are expressed in charge separations PS⁻¹ s⁻¹ [43]. The unit of the slope arises from signal normalization (see next paragraph). A saturating pulse (22-ms of 630-nm LED, ~3000 μmol photons m⁻² s⁻¹...
resulting in \( k_{\text{PSII}} \sim k_{\text{PSI}} \sim 1700 \) charge separations \( \text{s}^{-1} \) was used to follow the kinetics of the ECS and the \( b_{\text{OF}} \) redox reactions (Figure 1 item f). The pulse duration was timed to generate a stable ECS level in WT. Using appropriate interference filters (FWHM: 10 nm), the \( b_{\text{OF}} \) redox reactions were monitored on the level of cyt. \( f \) (554 nm) and cytochrome \( b \) (563 nm) with a baseline drawn between 546 and 573 nm [44]. At variance with the previous deconvolution [44], 554-nm signals were corrected with \( 0.23 \times (563 \text{ nm} - 546 \text{ nm}) \) for a spectral overlap with reduced cytochrome \( b \), estimated from extinction coefficients [6]. Only this correction abolished cyt. \( f \) signal offsets in reducing conditions, thus approximating the dark reference several tens of ms after a flash (supplementary Figure S2). The kinetics of 520-nm to 573-nm signals were obtained from back and forth recordings. Four and nine technical replicates were averaged for each wavelength in the multiple and single turnover setups, respectively (Figure 1).

The functional PSI:PSII ratios under the growth conditions were obtained by comparing the ECS amplitudes that were produced after a saturating \( \sim 6-\text{ns} \) laser flash (\( a \)-phase). The flashes were provided by a dye laser (DCM, exciton laser dye) pumped by a frequency doubled Nd:YAG laser (Minilite II, Continuum). The ECS signals developed in less than 1 \( \mu \text{sec} \), i.e. before the first detection point in the dark at 300 \( \mu \text{sec} \). The \( a \)-phase amplitude was measured in oxic samples before and after the addition of PSII inhibitors (Figure 1 items c and h). In the presence of HA and DCMU, PSII fails to promote flash-induced charge separations. Accordingly, since the \( a \)-phase corresponded to 1 charge separation PSI\(^{-1} \), it revealed the concentration of active PSI in the sample. At 20 \( \mu \text{g} \) chlorophyll \( \text{mL}^{-1} \) in the cuvette, \( \Delta I/I_{520\text{nm}-546\text{nm}} \) \( a \)-phase amplitudes of \( \sim 900 \) to \( \sim 1100 \) were typically observed (used to normalize all \( \Delta I/I \) signals). In the absence of PSII inhibitors, \( \Delta I/I_{520\text{nm}-546\text{nm}} \) \( a \)-phase amplitudes of \( \sim 2000 \) were produced in oxic samples.

The apparent \( b \)-phase of single turnover kinetics (\( \sim 10-\text{ms} \) of electrogenic \( b_{\text{OF}} \) contribution to the ECS signal; Figure 1 item i) was deconvoluted to obtain \( k_{\text{AV}} \) by subtracting the \( c \)-phase (ATP-synthase activity that results in ECS decay), which followed a first-order exponential decay [45, 46]. To avoid double turnover, subsaturating laser flashes were used in this type of experiments, which generated an \( a \)-phase of \( \sim 0.35 \) – 0.45 charge separations PSI\(^{-1} \). Like the \( b \)- and \( c \)-phase, the dark relaxation kinetics of the \( b_{\text{OF}} \) were fitted with the mono-exponential decay function ExpDec1 of the OriginPro software. For \( k_{f_{\text{red}}} \) and \( k_{b_{\text{ox}}} \), the 600-\( \text{ms} \) phase was used for fitting. However, in multiple turnover experiments (Figure 1 item f), a shorter time window for anoxic \( k_{b_{\text{ox}}} \) calculation was necessary for a seamless transition to the fit of \( k_{b_{\text{ox}}} \) up to 9-\( \text{s} \) dark.

The overall membrane potential formation rates in saturating light (Figure 1 item f) were determined via a dark pulse-based protocol [47, reviewed in 48], which yielded a similar parameter as \( \psi_{\text{H+}} \) developed by Kramer and colleagues [49]. Accordingly, the ECS signals were continuously recorded when changing the light intensities (from weak steady state light to saturating light, and from there to darkness). Therefore, the light-dependent photochemical rates were instantaneously altered, whereas the turnover rates of the ATP-synthase and the \( b_{\text{OF}} \) stayed initially unchanged. Linear slopes were calculated during the initial 2-\( \text{ms} \) of transitioning from steady state to saturating light (\( S_{\text{ini}} \)), which reflected the initial electrogenic efficiency in light-saturating conditions (\( k_{\text{ini}} \), obtained in light-adapted samples at variance with \( k_{\text{PSII}} \sim k_{\text{PSI}} \) above). In the light-adapted state, the initial electrogenic efficiency depended on the number of electrons in the high-potential chain that was immediately available [50].
These electron pools were exhausted during the 22-ms saturating light pulse, i.e. the absence of acceptors (oxidized PQ or oxidized Fd) or donors (reduced P700) was promoted. The linear slopes at the end of the pulse (Sf; final 6-ms) and in subsequent darkness (Sd) were calculated and yielded the effective electron transfer after several turnovers in saturating light (keff = Sf - Sd). The initial dark signals were disregarded since they may include electrogenic PSI charge recombinations [51], and linear Sd was obtained from 2-ms to 16-ms darkness.

As described previously [52], P700 redox changes (Figure 1 item e) were measured with short-pulsed detection LEDs, which peaked at 700 nm and were used in combination with interference filters at 705 and 740 nm (FWHM: 10 nm and 15 nm, respectively). The light-detecting photodiodes were protected from scattered actinic light by a 3-mm RG695 filter (Schott, Mainz, Germany). Illumination of the samples was interrupted by short dark intervals (250 μs) during which 10-μs detecting pulses were placed after 200 μs. The presented P700 kinetics originated from light-adapted cells which had a functional PSII. The steady state kinetics were recorded in the light (Ps) and during a saturating 12-ms pulse, using a 630-nm LED ring (same as above, kPSII ~ kPSI ~ 1700 charge separations PS−1 s−1). The pulse served to drive a multiple turnover process which increased the efficiency of photosynthetic control and emptied the immediate donor pools of reduced plastocyanin and cytochrome f [50]. Thus, the strong pulse yielded in each condition the maximal P700 oxidation state (Pm), producing a lower ΔI/I at 705 nm—740 nm than Ps. By fitting the mono-exponential decay kinetics during the 12-ms pulse (ExpDec1 function of the OriginPro software), the P700 oxidation rate kP-ox was obtained. Prolonging the pulse did not change Pm (not shown). As a reference (Figure 1 item h), PSII inhibitors HA and DCMU were added in light-adapted samples to obtain the maximal P700 oxidation during the saturating pulse (P′m, kinetics not shown). After the 12-ms pulse, dark recovery of P700 was recorded for several seconds and the dark kinetics were corrected for a linear drift that developed in steady state light-adapted cells, especially in anoxic conditions (supplementary Figure S3). For each kinetics, the ΔI/I of the P700 redox state in darkness served as zero baseline (P0, only detecting light present). P0 was calculated from the offset of a two-component exponential decay function (OriginPro software, using dark kinetics from 4-ms to 2550-ms). The first decay rate of this function, k1P-red, describes the fast component of the P700 dark relaxation. Using the P0-corrected values, the yield of PSI (ΦPSI) is defined as (Pm−Ps)/P′m [50]. The PSI acceptor side limitation (YNA, non-photo-oxidizable P700) is defined as (P′m−Ps)/P′m and the PSI donor side limitation (YND, photo-oxidized P700 in steady state light) is defined as Ps/P′m.
Results

When assessing the function of PGR5 in photosynthetic electron transfer, PSI photodamage has to be anticipated in pgr5 under high to moderate, as well as fluctuating light conditions [19-21]. To avoid such alterations of functional PSI, we conducted experiments at low light conditions under a photo-autotrophic growth regime. In fact, the weak irradiance during growth and measurements provided permissive conditions for the original pgr5 phenotype by avoiding photodamage [19-21]. To re-evaluate the role of PGR5 in electron transfer regulation, we combined several in vivo measurement protocols to assess PGR5-dependent electron transfer under these conditions in C. reinhardtii. For this dataset, we compared the pgr5 strain with WT, and furthermore investigated a partially PGR5-rescued strain C1 [19].

To determine the functional PSI:PSII ratio, the amplitudes of the electrochromic shift (ECS) signals were calculated after a laser flash. Thereby, we obtained a PSI:PSII ratio of 1.13 ± 0.13, 1.18 ± 0.13 and 1.24 ± 0.18 in WT, pgr5 and complemented C1, respectively (N = 3 ± SD). Another indication that permissive conditions were found was provided by the initial photochemical rates (kPSII ~ kPSI) of 216 ± 23, 193 ± 11 and 185 ± 12 charge separations PSI$^{-1}$ s$^{-1}$ (N = 3 ± SD) in WT, pgr5 and C1, respectively. In the following sections, we will present redox and electrogenic parameters obtained with cultures that were comparable on the levels of the PSI:PSII ratio and kPSII ~ kPSI.

1. Absence of PGR5 facilitates PSI oxidation in Chlamydomonas. The P700 redox state was analyzed in steady state light-adapted cells. The resulting kinetics are shown in Figures 2A and 2B for WT and pgr5, respectively. The C1 line is shown in supplementary Figure S4A. At a first glance, the kinetics were very similar, with exception of a strong pulse-induced P700 oxidation signal in anoxic pgr5. To further deconvolute the signals, the corresponding P700 redox states are shown in Figure 2C (see supplementary Figure S4B for C1). It became evident that the yield of PSI (ΦPSI) was comparable in oxic conditions, also in the presence of methyl viologen (MV). Due to the artificial electron acceptor, the MV treated samples were not limited on the PSI acceptor side (YNA) and showed larger ΦPSI as well as larger donor side limitation (YNDD). The anoxic WT exhibited an increased YNA, unlike pgr5. Largely owing to YNA, the anoxic WT showed a significant decrease in YND compared to oxic samples (Student’s t-test, P = 0.03). On the contrary, YND increased in anoxic pgr5 (P = 0.02).

When comparing the black traces in panels A and B of Figure 2, P700 was oxidized faster during the saturating light pulse in oxic pgr5, since $k_{P-ox}$ was significantly larger than in WT (Figure 2D, see Materials and Methods). The oxic C1 sample was intermediary and $k_{P-ox}$ remained similar in the presence of MV, like in pgr5. In WT, $k_{P-ox}$ was faster after MV addition. As expected, $k_{P-ox}$ was increased in all strains due to an increased PSI antenna size in anoxic conditions. After increasing photosynthetic control efficiency during the pulse and thus emptying PSI electron donors, the P700 dark relaxation rate $k_{1P-red}$ was slower in anoxic conditions (Figure 2E, see Materials and Methods). An insignificant slowdown was seen in anoxic pgr5 and MV addition had an inhibiting effect on $k_{1P-red}$ in all strains.

2. The functional $b_{6}f$ is subjected to enhanced oxidation by PSI in oxic pgr5 and becomes strongly impaired in anoxic pgr5. The $b_{6}f$ redox behavior of the samples in Figure 2 is
presented in Figure 3. The kinetics in the high- and low-potential chain will be described first in WT and pgr5 (for C1 see supplementary Figure S5), followed by calculations of the apparent dark relaxation rates. At variance with the representations in Figure 2, the steady state signals before the strong pulse were the reference levels. It is of note that the bsf signals, although small in amplitude, displayed specific redox information, since the signals were absent in bsf-lacking mutants (supplementary Figure S6). When oxic WT in the steady state light was exposed to the saturating ms-pulse, a net cyt.f oxidation of about −0.4 a.u. was observed (hatched box in Figure 3A). In the dark, after the pulse, the fast cyt.f net reduction phase had an amplitude of about +0.65 a.u. and finished in ~50 ms. Both amplitudes of cyt.f net oxidation (~0.2 a.u.) and reduction phase (+0.4 a.u.) were smaller in anoxic samples, compared to oxic conditions. The time during the pulse to reach maximal cyt.f oxidation was shorter compared to oxic cells, which was expected after showing lower ΦPSI (Figure 2C) and thus less injection of positive charges into the high-potential chain. The cyt.f re-reduction kinetics in darkness were faster in these samples as well (Figure 3A). When MV was present, there was almost no cyt.f net oxidation during the pulse. No distinct fast cyt.f reduction phase was observed during 100-ms of darkness. When monitoring redox changes of the hemes b/bs in the saturating ms-pulse in oxic WT samples (Figure 3B), a net reduction of about +0.1 a.u. was observed. The hemes b/bs net oxidation in the dark was finished within ~50 ms after the saturating pulse in oxic WT samples. The redox state of hemes b/bs was slightly more oxidized for several seconds darkness compared to the steady state before the pulse. The net reduction amplitude of the hemes b/bs during the ms-pulse was slightly more pronounced in the presence of MV. After the pulse, however, there was a significantly larger amplitude of hemes b/bs net oxidation in the MV treated samples, which transiently reached −0.2 a.u. compared to the steady state reference. The oxidation was finished after ~300-ms of darkness. In anoxic samples during the ms-pulse, the hemes b/bs net reduction amplitude reached a slightly lower plateau earlier. In darkness, the hemes b/bs net oxidation amplitude was small and the phase finished within ~25 ms. In contrast to oxic samples, a unique hemes b/bs redox feature was a net reduction phase, that had started by 100-ms of darkness in anoxic WT.

The net cyt.f oxidation amplitude during the pulse was smaller in oxic pgr5 (~0.2 a.u., Figure 3C), and thus half as large as in WT (cf. Figure 3A). In this sample, the cyt.f reduction phase amplitude was slightly larger (+0.8 a.u.) but the decay kinetics resembled WT. Whereas MV treated samples were indistinguishable from WT, cyt.f redox changes in anoxic pgr5 differed in several aspects. Unlike anoxic WT, fast cyt.f reduction kinetics were absent after the pulse in anoxic pgr5. The net oxidation amplitude was almost non-existent in these cells. As expected from the elevated ΦPSI and YND, which indicated the amount of P700⁺ before darkness in Figure 2C, the cyt.f reduction amplitude in anoxic pgr5 was indistinguishable from oxic cells (Figure 3C) and contrasted with the difference observed in WT (cf. Figure 3A). The hemes b/bs redox signals during and after the saturating ms-pulse were like WT with two exceptions in anoxic pgr5 (Figure 3D): The hemes b/bs oxidation phase after the pulse finished after ~25-ms, i.e., later than in WT (cf. Figures 3A and 3D). Moreover, the onset of re-reduction was significantly delayed.

To quantitate the observations after the pulse, the apparent net reduction rate of cyt.f was calculated (k_f-red, Figure 3E). With k_f-red between ~40 and ~45 s⁻¹, oxic samples were comparable and significantly slowed down by ~75% in the presence of MV. Compared to oxic samples, k_f-red was increased by a factor of ~2 in anoxic WT cells. On the other hand, k_f-red was lowered by a factor of ~2 and ~8 in anoxic C1 and pgr5, respectively. After the pulse,
the apparent net oxidation kinetics in the low-potential chain are expressed as $k_{b-ox}$ (Figure 3F). In WT and C1, $k_{b-ox}$ was ~50 s$^{-1}$ and was significantly lowered upon MV addition. Oxic pgr5 controls trended towards lower $k_{b-ox}$ compared to WT and C1, and the inhibitory effect of MV was statistically not significant. The anoxic WT displayed higher $k_{b-ox}$, whereas the pgr5 mutant and the partially complemented C1 strain did not show this effect. Yet, the C1 line was less severely affected than pgr5. Figure 3G shows the slow dark-reduction rates of hemes $b_l/b_h$ in anoxia, $k_{b-red}$. The WT rate was significantly faster than C1 and pgr5.

Using a multiple turnover protocol, it is reasonable to assume that the presented rates also strongly depended on the oxidation level of the PC pool as well as the PQ pool redox state. Regarding the former, the P700 parameters in Figure 2 served as a satisfactory proxy, since PC equilibrates with P700. The next section will present various chlorophyll fluorescence parameters that reflect PSII photochemistry, and to some extent the PQ pool redox state.

3. The enhanced PSII photochemistry in oxic pgr5 stands in stark contrast to the anoxic mutant phenotype. During the workflow presented in Figure 1, samples have also been examined for their chlorophyll fluorescence yields. After 30-min dark adaptation, the maximum quantum efficiency, $F_v/F_m$, was comparable in all strains (supplementary Table S1). Figure 4A shows the photochemical quantum yield of PSII ($\Phi_{PSII}$) in WT and pgr5, which was significantly lower in anoxic samples as expected. $\Phi_{PSII}$ was determined after a short 10-s period of light acclimation as well as in the steady state after 30 min (the parameters for C1 are summarized in supplementary Figure S7). Only oxic pgr5 showed a significant increase of $\Phi_{PSII}$ in the steady state, which was lowered to WT-levels upon MV addition. Figure 4B shows a similar picture of the PSII efficiency factor ($qP$), yielding higher values in oxic steady state pgr5. Moreover, $qP$ inhibition in the presence of MV was observed in oxic algae, which has been reported for vascular plants previously [53]. Figure 4B also shows that, in contrast to oxic conditions, the steady state $qP$ in anoxic WT was higher than in pgr5. Like the closely related $qP$, the fraction of open PSII centers ($qL$) in Figure 4C revealed the same phenotypes of 10-s vs. 30-min light adaptation. Furthermore, $qL$ increased significantly only in fully light-acclimated anoxic WT cells.

4. The initial electrogenic efficiency of the light-adapted photosynthetic chain in Chlamydomonas depends on PGR5. The data in Figures 2, 3 and 4 display alterations in the mutant electron transfer chain. In a similar fashion to the P700 measurements, in which a superimposed saturating light pulse emptied the immediate donor pool of PSI during several turnovers, we analyzed the charge separation efficiency of the photosynthetic apparatus by recording the electrochromic shift (ECS). The results are shown in Figure 5 (see supplementary Figure S8 for C1). The ECS, which serves as intrinsic voltmeter, was recorded in the background light (as reference), during and after the saturating pulse. In the steady state, before the short pulse, the slope of the signal was zero. The additional membrane potential ($\Delta\Psi$) that was built up at the very onset of the 22-ms pulse depended on the immediate availability of electrons in the photosynthetic chain, and thus the photochemical yield of both photosystems (which we determined above). The initial rate of this process, $k_{ini}$ (see Materials and Methods), was calculated from the data shown in green symbols for WT (inset of Figure 5A) and pgr5 (inset of Figure 5B). Oxic samples produced ~4 additional,
stable charge separations during the pulse, also in the presence of MV. Anoxic cells generated less additional $\Delta \Psi$ during the pulse in general, and WT generated more extra $\Delta \Psi$ than $pgr5$. During several seconds of darkness while ATP-synthase was active, the $\Delta \Psi$ collapsed to $\sim$4 units in oxic and anoxic samples, and to $\sim$8 units in MV treated samples. At the end of the pulse, a new steady state (zero slope) was established in oxic and anoxic WT only. The progressed $\Delta \Psi$ production rate at the end of the pulse, $k_{\text{end}}$ (see Materials and Methods), gave an indication of how electron transfer in the chain was diminished upon exhaustion of electron donors (reduced P700) or acceptors (oxidized PQ or oxidized Fd).

The $k_{\text{ini}}$ values are shown in Figure 5C and indicate that oxic controls produced similar rates between $\sim$630 and $\sim$730 charge separations PSI$^{-1}$ s$^{-1}$. In the presence of MV, with exception of $pgr5$, $k_{\text{ini}}$ was less efficient compared to the controls. Nonetheless, the MV trend existed in $pgr5$ as well, which showed a relatively low $k_{\text{ini}}$ in oxic controls already. In anoxic samples, $k_{\text{ini}}$ was compromised in all strains compared to oxic conditions. WT $k_{\text{ini}}$ in anoxia showed the smallest decrease, followed by C1 and $pgr5$. As shown in Figure 5D, $k_{\text{end}}$ was diminished to a similar extent in oxic samples, and anoxic conditions lowered $k_{\text{end}}$ further in all strains.

5. The phenotype in Chlamydomonas $pgr5$ is rescued by overexpressing PGR5 and PGR5Cys$^{113}$Ser, respectively. It is of note that throughout the study, the partially complemented C1 line, which accumulates $\sim$75% of WT PGR5 levels under the control of its native promoter [19], resembled WT in oxic conditions, whereas it tended to partially perform like $pgr5$ in anoxia. Although P700 of WT and C1 behaved similarly in anoxic conditions (cf. Figure 2C and supplementary Figure S4B), the $pgr5$ resemblance was most apparent on the levels of $b$-hemes oxidation ($k_{b-\text{ox}}$ in Figure 3F). For cyt.f reduction ($k_{f-\text{red}}$ in Figure 3E) and electrogenticity ($k_{\text{ini}}$ in Figure 5C), both rates were significantly faster under anoxia as compared to $pgr5$, but still slower than WT. To eliminate PGR5 titration effects in anoxic C1, we generated independent PGR5-complemented lines which, besides the P700 redox behavior (Figure 6A), also produced WT-like cyt.f reduction rates after the pulse ($k_{f-\text{red}}$ in Figure 6B), as well as $k_{b-\text{ox}}$ (Figure 6C) and $k_{\text{ini}}$ (Figure 6D). Figure 6 also includes a mutated version of PGR5, in which the sole Cys at position 113 was replaced with Ser. Interestingly, this mutation did not interfere with restoration of a WT-like phenotype when overexpressing PGR5Cys$^{113}$Ser. The only exception was an intermediate $k_{b-\text{ox}}$ (Figure 6C) which was significantly faster than $pgr5$, but not as fast as WT and the $pgr5$::PGR5 strain, respectively. We noted that hetero-phototrophic cells varied slightly in their rates, compared to photoautotrophic cells (cf. Figures 4 to 6). Although the $pgr5$ phenotype was more effectively recovered in the overexpression lines compared to C1, slow photoautotrophic growth at low irradiance was not feasible to maintain controlled zeocin levels. Therefore, we introduced the C1 strain with the native PGR5 promotor in the first part of the manuscript. In the following part, we will focus on single turnover $b_6f$ kinetics in WT and $pgr5$ since the complex appeared to underperform under anoxic conditions in the mutant.

6. Redox finetuning of the $b_6f$ low-potential chain is PGR5-dependent. In order to rule out possible dark redox equilibration artefacts (owing to different pre-oxidation levels in the light-adapted steady state), this section introduces single turnover measurements. Here, the light-adapted cells have been investigated in the absence of PSII photochemistry and upon a 30-s
dark period (Figure 1 item i). This dark period ensured the reduction of primary and secondary PSI donors as well as pmf consumption, especially since the ΔpH governs photosynthetic control [14, reviewed in 15]. Furthermore, as seen from the varying number of open PSII centers in Figure 4C, we intended to mitigate differences in the PQ pool redox state by inhibiting PSII activity and exerting a more homogeneous reducing pressure on the b$_{6f}$ samples. During the single b$_{6f}$ turnover, an electron hole is passed from the oxidized c-heme in cyt.f to the Rieske ISP which, after swapping back the FeS domain closer to the cytochrome b$_{6}$ subunit, is reduced by PQH$_{2}$ at the Qo-site. The latter is a bifurcated process that also reduces hemes b$_i$/b$_h$. When heme-b$_h$ receives an electron from the Qo-site, a ΔΨ is generated, which we monitored via ECS signals. Redox changes in the b$_{6f}$ (Figure 7A) and the corresponding ECS changes (Figure 7B) were assayed in oxic samples. The ECS kinetics in Figure 7 are relative and are composed of three phases [reviewed in 48, 54]. The deconvolution is explained in Materials and Methods. We observed no significant differences in the two strains regarding the decay rate of the phases, related to ATP synthesis (supplementary Figure S10). We also measured the b$_{6f}$ redox kinetics and ECS in the presence of MV (Figures 7C and 7D) as well as in anoxic conditions (Figures 7E and 7F). On a time scale after injecting an electron hole into the b$_{6f}$, cyt.f reduction ($k_{f_{\text{red}}}$) preceded the electrogenic b-phase ($k_{\text{AΨ}}$). The last phase was the relatively slow oxidation of the hemes b$_i$/b$_h$ ($k_{b_{\text{ox}}}$). A statistical evaluation of $k_{f_{\text{red}}}$ (Figure 7G), $k_{\text{AΨ}}$ (Figure 7H), and $k_{b_{\text{ox}}}$ (Figure 7I) is shown, as well as the amplitude of the b-phase relative to one charge separations per PSI (Figure 7J).

In oxic samples, whether MV was present or not, cyt.f oxidation was finished before the first record at 300 μs after the flash and resulted in an amplitude of ~0.1 units compared to the reference signal before the flash (circle symbols in the b$_{6f}$ redox kinetics panels of Figure 7A, 7C and 7E). In anoxic samples, oxidation of cyt.f was slowed down slightly, finishing between ~1-ms and ~2-ms after the flash. With exception of the MV treated samples, cyt.f reduction by the FeS domain was initiated at ~1-ms, yielding similar $k_{f_{\text{red}}}$ values in oxic and anoxic conditions. When MV was added, $k_{f_{\text{red}}}$ was lowered significantly (5% and 9% residual rates of oxic WT and pgr5, respectively) and a delayed onset of reduction became apparent between ~5-ms and ~10-ms.

Before cyt.f was getting reduced in oxic and anoxic samples (during the first ms), net redox changes of the hemes b$_i$/b$_h$ were very small (square symbols in b$_{6f}$ redox kinetics panels of Figure 7A, 7C and 7E). Only after the onset of cyt.f reduction, a net reduction of the hemes b$_i$/b$_h$ became varyingly apparent. In the same timescale, the different hemes b$_i$/b$_h$ net reduction amplitudes coincided with the electrogenic b-phase (square symbols in ECS kinetics panels of, Figure 7B, 7D and 7F). The sequential reduction of cyt.f and hemes b$_i$/b$_h$ was expected and was also observed in MV treated samples but there was a significant slowdown of the low-potential chain turnover. The signal amplitude of the hemes b$_i$/b$_h$ net reduction was a function of Qo- ($k_{f_{\text{red}}}$ or $k_{\text{AΨ}}$ as proxy) and Qi-site activity ($k_{b_{\text{ox}}}$ as proxy). Accordingly, the amplitude appeared larger in the presence of MV since Qo-site turnover was less slowed down than Qi-site activity. For instance, 23% and 26% residual $k_{\text{AΨ}}$ were measured in oxic WT and pgr5 after adding MV (Figure 7H), compared to 10% and 28% residual $k_{b_{\text{ox}}}$ (Figure 7I). Since the inhibitory MV effect on $k_{b_{\text{ox}}}$ was less pronounced in pgr5, the hemes b$_i$/b$_h$ net reduction amplitude was smaller during the first 10-ms after the flash in the mutant (Figure 7C).

When comparing the respective strains under oxic and anoxic conditions, the net reduction amplitudes in the hemes b$_i$/b$_h$ redox kinetics differed during the first 10-ms (Figure 7A and
Both anoxic strains showed a relatively small net reduction of the hemes $b_l/b_h$. As mentioned above, this amplitude was a function of Qo- and Qi-site activity. Since the Qo-site, i.e., $k_{\text{red}}$ (Figure 7G) and $k_{\Delta\Psi}$ (Figure 7H), was not significantly different from oxic conditions in the respective strains, the small amplitude in anoxia was related to Qi-site events, where electrons exited the low-potential chain during the 10-ms phase.

On the other hand, $k_{\Delta\Psi}$ differed between WT and the less efficient $pgr5$, although the hemes $b_l/b_h$ net reduction amplitude was comparable. After injection of Qo-site electrons into the low-potential chain finished, $k_{b-ox}$ was faster in anoxic WT only (Figure 7I). The relative amplitude of the $b$-phase was between 50-85% of the $a$-phase and tended to be slightly higher in $pgr5$ (Figure 7J). The $\Delta\Psi$ generated by one $b$ turnover in our conditions was close to the values in earlier reports [55, 56], which attributed similar fractions of one charge separation when measuring electron transfer ‘within’ the membrane bilayer from hemes $b_l$ to $b_h$ in the $bc_1$ complex, as opposed to across the whole membrane.
Discussion

PGR5 is an important regulator of photosynthetic electron transfer, however, its function has not been linked to the operation of the \( b_{6f} \). Our data indicate a dysfunctional \( b_{6f} \) in the absence of PGR5 which is manifested in an impaired redox cross talk between the \( b_{6f} \) complex and PSI. The Q cycle of the \( b_{6f} \) complex is modified in strongly reducing as compared to oxic conditions. We conclude that the \( b_{6f} \) is inhibited when being disconnected from signals downstream of PSI in the absence of PGR5 or in the presence of artificial electron acceptors. Moreover, PGR5 is functionally involved in a modified Q cycle which has access to stromal electrons and operates in WT but less efficiently in pgr5. To facilitate interpretation of pgr5 performance in the steady state measurements, we will first discuss the single turnover experiments. For simplicity of redox signal discussions, the \( b_{6f} \) will be treated as a homogeneous population, although distinct subsets may exist in the sample, e.g. in close vicinity to PSI in CEF-supercomplexes [57-60]. As summarized below, six previous findings are the conceptual framework for the interpretation of the hemes \( b_{i}/b_{i} \) signal evolution shown in Figure 7. Moreover, Table 1 is a guide for Figure 8:

(i) The light-induced \( \Delta \Psi \) produces \( b_{h}^{\text{red}}/c_{i}^{\text{ox}} \), which converts to \( b_{h}^{\text{ox}}/c_{i}^{\text{red}} \) in the dark [2].

(ii) Presence of a \( \Delta \Psi \) is crucial for Qi-site activity, i.e., oxidation of the \( b_{i} \)-hemes after a Qo-site turnover [3]. How exactly \( \Delta \Psi \) is sensed in the \( b_{6f} \) is not yet understood.

(iii) As reviewed elsewhere for the cytochrome \( bc_{1} \) complex [12, 61], an intrinsic short-circuit-preventing process influences the cyt.\( f \) reduction rate by governing the interaction between the Rieske ISP FeS domain and cyt.\( f \). Only after the \( b_{i} \)-hemes become oxidized, the reduced FeS domain will swap closer to cyt.\( f \) to release the “trapped” high-potential chain electron.

(iv) In order to fill the Qi-site with substrate (PQ and/or \( \text{H}^{+} \)), \( c_{i}^{\text{red}} \) is transiently required. Besides having an effect on other side chains in the cavity [8], \( c_{i}^{\text{red}} \) likely weakens the interaction with the moiety of F40 in subunit-IV [7]. In fact, slight changes of the F40 aromatic ring position relative to the heme-\( c_{i} \) plane were observed depending on the Qi-site occupation in spinach [8] and cyanobacterial \( b_{6f} \) [62]. Considering (i) and the possible gating mechanism, the Qi-site pocket may remain free after a flash and fill with substrate in the dark.

(v) \( b_{i}^{\text{red}}/b_{i}^{\text{red}} \) has not been observed in single turnover measurements [9, 10, 11 and references therein]. Accordingly, when semi-PQ reduces \( b_{h}^{\text{ox}} \) in the presence of \( b_{h}^{\text{red}} \), simultaneous transitions of \( b_{i}^{\text{ox}} \rightarrow b_{i}^{\text{red}} \) and \( b_{h}^{\text{red}} \rightarrow b_{h}^{\text{ox}} \) occur, followed by electrogenic \( b_{i}^{\text{ox}}/b_{h}^{\text{red}} \)-equilibration. The inability to accumulate electrons in the low-potential chain can be linked to the terminal electron acceptor heme-\( c_{i} \). The tight ligation of the heme-\( c_{i} \) with PQ (or quinone analogue inhibitors) is favored by \( c_{i}^{\text{ox}} \) [6], and thus by \( \Delta \Psi \) (i). A reducing pressure on the \( b_{6f} \) is required for PQH\(_{2} \) formation since, compared to the second step, tight \( c_{i}^{\text{ox}}/\text{PQ} \) ligation requires more energy for the first reduction step and prevents semi-PQ accumulation [5, 7].

As introduced and in agreement with the general view [1, 63], the \( b_{6f} \) displayed a canonical Q cycle in oxic conditions (Figure 8A and 8B, referring to Figure 7). Thus, the \( b_{6f} \) low-potential chain harbors \( b_{i}^{\text{ox}}/b_{i}^{\text{ox}}/c_{i}^{\text{red}} \) after the first (Figure 8A), and \( b_{i}^{\text{ox}}/b_{h}^{\text{ox}}/c_{i}^{\text{ox}} \) after the second Qo-site turnover that is associated with PQH\(_{2} \) formation at Qi (Figure 8B). The (semi-)PQ in the Qi-site receives the electrons from \( c_{i}^{\text{red}} \) and/or \( b_{h}^{\text{red}} \) in the presence of \( \Delta \Psi \), probably in a concerted and closely spaced process. Since the redox signals re-equilibrated near the zero baseline in oxic samples, an electron that entered the low-potential chain at the Qo-site did not reside on either \( b_{i} \)-heme after the reactions ceased. Note that in the absence of PSII activity in the light the PQ pool is oxidized, so that equilibration of \( b_{h}^{\text{red}} \) during 30-s dark was unlikely.
The b-heme re-equilibration near the zero baseline was also true for oxic samples in the presence of MV (not shown in Figure 8). However, according to (iii), the bO inhibition by MV can be attributed to altered k_box. Before the laser flash was applied, the FeS domain of the Rieske ISP might rest in an unusual position more distant to cyt.f, thus producing slow k_f_is. The observed bO redox kinetics in the presence of MV showed attenuated resemblance to bO samples treated with the Qi-site inhibitors MOA-stilbene [10] and NQNO [64]. This observation links the Qi-site functionality to a stromal redox poise, which we also examined in the anoxic samples by creating a reduced stroma. Here, the major finding concerns the k_box tuning (Figure 7E and 7I) which was missing in anoxic pgr5 (Figure 8C) and produced high rates in WT (Figure 8D). As expected, the b-heme redox re-equilibration below the zero reference in the anoxic samples can be attributed to the accumulation of electrons in the PQ pool [reviewed in 31] and the generation of DΨ precluded a Qi-site turnover. The establishment of k_red, which requires several minutes darkness [65], depended on a flash-induced Qo-site turnover after the 30-s of darkness. According to (v), an electrogenic oxidation/reduction of heme-bO occurred within 10-ms after the flash. Meanwhile ((i) and (ii)), bO/b_red converted to bO/b_red/k_is and yielded one PQH2 at the Qi-site in both strains. The 10-ms phase entails a difference between WT and pgr5 (not shown in Figure 8) that arises from panels H (kox) and J (b-α-phase ratio) of Figure 7. Further experiments need to clarify whether less efficient PQH2 formation at the pgr5 Qi-site was responsible for the slightly slower charge separation and higher b-phase amplitudes in the mutant. It could be that the distance between stromal H+ and the heme-cI is larger in pgr5 due to a modified protein interaction in the stromal bO domain. This may slow down the b-heme redox rates (v), while increasing relative electrogenicity. Involvement of H+ movements in bO electrogenicity has been discussed recently in the context of heme-cI protonation upon reduction [6]. The b-heme redox re-equilibration after the flash below the zero reference, i.e., formation of k_box/k_red in the ~100-ms range, was slow in pgr5 (right part in Figure 8C). According to (i), the consumption of DΨ was required which yielded a similar k_box as oxic samples (cf. right part in Figure 8D). On the other hand, the WT (right part in Figure 8D) showed a faster k_box in the same time range which strongly suggests a modified Qi-site turnover as a response to the stromal redox poise. Considering that thylakoid membranes isolated from WT retain more bound FNR than those from pgr5 [20] and that FNR copurifies with the bO [23-25], it could be an allosteric regulator of the bO. Thus, in the presence of ΔΨ, bO-associated FNR overcomes the energetic barrier (i) by using stromal electrons from Fd to produce c_red (Em of Fd in Fd:FNR = ~ -500 mV [67]). Importantly, this modified Q-cycle yields a second PQH2 at the WT Qi-site (right part in Figure 8D), when inducing a single turnover of a strongly pre-reduced WT complex. In turn, such a modified Q-cycle would facilitate more efficient proton pumping into the lumen as compared to the canonical Q-cycle, which would be hampered in pgr5 especially under conditions where the modified Q-cycle is favored (see also below). Switching to the Fd-assisted Q cycle might (in)directly rely on reduced Fd since FNR membrane recruitment is stimulated in anoxic conditions and requires functional bO and PSI [68]. Moreover, MV-treated thylakoid membranes do not retain bound FNR in the light [69]. Considering the above-mentioned consequences of MV on the Qi-site functionality upon illumination, the stromal interface of the bO might be modulated due to events downstream of PSI.

As evidenced by the large b-heme oxidation amplitudes after the pulse (Figures 3B and 3C), MV addition in the steady state experiments also led to an electron backup in the bO low-
potential chain due to an underperforming Qi-site (Figure 9A). According to (iii) and in agreement with the low $k_{f\text{-red}}$ (Figure 3E), the elevated YND (Figure 2C) and lower $k_{1P\text{-red}}$ (Figure 2E), this eventually imposed a bottleneck on LEF. If one target of the MV effect is localized at the $b_{6f}$, an altered pmf composition with a lower $\Delta p$H component should be expected. At least the larger ECS decay amplitude in Figure 5 indicates a larger $\Delta \Psi$ component that drives ATP synthesis after the pulse. Moreover, future pmf parsing studies might correlate $\Delta \Psi$-induced charge recombination in PSII [70] with altered chlorophyll fluorescence that we (Figure 4) and others [53] observed.

In agreement with previous studies [71, 72], the chlorophyll fluorescence pattern in oxic pgr5 suggests that the mutant displayed an altered electron flow downstream of PSI (Figure 9B). Accordingly, the higher $\Phi_{PSI}$ in light-adapted pgr5, which we (Figure 4A) and others [19] observed in oxic conditions, indicate that PSI oxidized the high-potential chain more efficiently between PQH₂ and PC. This resulted in a stronger pre-oxidation of cyt.f in the steady state (Figure 3C) and a faster PSI oxidation during saturating light pulses (Figure 2D). Accordingly, higher LEF rates via an unthrottled PSI may be expected in the mutant, but not in the WT (Figure 9C). The higher LEF rates in oxic pgr5 are associated with a drain of electron carriers downstream of PSI, at the expense of a PQ pool reduction via CEF. This shortage of immediate electron donors in the photosynthetic chain might account for a lowered initial electrogenic efficiency in light-saturating conditions (Figure 5C). When the photon flux density is higher than in our permissive growth conditions [19, 20], it is reasonable to link the PSI photodamage phenotype in pgr5 to a deregulation of events downstream of PSI. As suggested previously, the resulting lower PSI:$b_{6f}$ ratio creates a situation where more electrons are funneled through the remaining PSI centers, which lowers $\Phi_{PSI}$ [19]. We prevented this scenario in low light growth since algal mutants of the PGR5-dependent CEF pathway cope with the drained stromal electrons, up to a certain light intensity and time period, by increasing metabolic cooperation with mitochondria [21, 73]. Algal photosynthesis features additional electron sinks like flavodiiron proteins, which are absent in angiosperms. Thus, in addition to the weak growth light, flavodiiron proteins might prevent PSI overreduction in oxic pgr5 as opposed to Arabidopsis [16]. Accordingly, introducing flavodiiron proteins in the Arabidopsis pgr5 background alleviates the PSI phenotype [74].

When depriving oxygen, the substrate for mitochondrial respiration and flavodiiron proteins, the remaining acceptor for the drained stromal electrons is hydrogenase. In fact, the hydrogenase successfully competes for reduced Fd in anoxic pgr5 [75, 76]. On the contrary, the ATP-depleted WT is engaged in CEF and thereby generates the pmf, in part, via the Fd-assisted Q cycle (Figure 9D). Thus, YNA is maintained in anoxic WT and the electrons are evenly distributed in the photosynthetic chain. The pgr5 fails to switch to a Fd-assisted Q cycle in anoxic conditions (Figure 9E). Since we did not observe a $b_{6f}$ phenotype in oxic pgr5 during steady state experiments, the intrinsic Q cycle switch in anoxia could involve posttranslational modifications that impede a $b_{6f}$ operation as under oxic conditions. By failing to efficiently generate a $\Delta p$H upon Qi-site tuning, the $b_{6f}$ in pgr5 did not underlie photosynthetic control imposed by a strong light pulse in the WT. This resulted in an insignificant slowdown of $k_{1P\text{-red}}$ in anoxia (Figure 2E). Moreover, probably as response to the reduced PQ pool, more PSII centers were closed (Figure 4C) and the electron donors downstream of the $b_{6f}$ were strongly oxidized (Figures 2C and 3C). According to (iii), the underperforming $b_{6f}$ was responsible for the redox pool imbalance between PQ and PC/P700. This strongly impaired the initial electrogenic efficiency in light-saturating conditions (Figure
Moreover, the slow and delayed $b$-heme reduction phase in $pgr5$ (Figures 3G) might be indicative of the underperformance since it could be the sum of a coincident $b$-heme oxidation phase when the $b_6f$ relaxes in the dark (i). The small number of electrons that flow downstream of the $b_6f$ over time might promote the unbinding of FNR from PSI and/or $b_6f$. As elaborated above, FNR binding to the membrane might rely on a critical Fd photoreduction rate and was affected in $pgr5$, thus favoring hydrogen production. An interesting side note of our in vivo results in algae is that they challenge the suggested role of the PGRL1-PGR5 complex, which is believed to be the Fd-PQ reductase in Arabidopsis, where PGR5 supports PGRL1 reduction [77]. The authors demonstrated in vitro that PGR5 forms heterodimers with PGRL1. According to their non-reducing SDS-PAGE, several Cys in PGRL1 were crucial for the dimerization interface. Our results rule out that mixed disulfides play a role in the corresponding algal model. Moreover, they exclude that the only cysteine in PGR5 is involved in PGRL1 reduction or that PGRL1 redox state is relevant for the effects we observed.

Taken together, we propose that the $b_6f$ receives a stromal redox feedback from PSI and represents the Fd-PQ reductase that is switched on in ATP-depleted conditions. PGR5 is required for the activation and/or tuning of the Fd-assisted Q cycle.
Abbreviations

bsf, cytochrome bsf complex; CEF, cyclic electron flow; cyt.f, cytochrome f; ECS, electrochromic shift; Fd, ferredoxin; FNR, Fd-NADP(H) oxidoreductase; ISP, iron sulfur protein; LEF, linear electron flow; ΔΨ, membrane potential; MV, methyl viologen; PSI / PSII, photosystem I / II; PC, plastocyanin; PQ, plastoquinone; PQH₂, plastoquinol; PGR5, proton gradient regulation 5; pmf, proton motive force.

Author contribution

F.B. Conceptualization, Investigation, Formal analysis, Visualization, Writing – original draft; L.M. Formal analysis, Writing – review & editing; P.G. Resources, Formal analysis; M.H. Funding acquisition, Conceptualization, Formal analysis, Writing – review & editing.
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References

1. Cramer, W. A. and Hasan, S. S. (2016) Structure-function of the cytochrome b_{6f} lipoprotein complex. In Cytochrome complexes: Evolution, structures, energy transduction, and signaling (Cramer, W. A. and Kallas, T., eds.). pp. 177-207, Springer Netherlands, Dordrecht.

2. Lavergne, J. (1983) Membrane potential-dependent reduction of cytochrome b_{6} in an algal mutant lacking photosystem I centers. Biochim. Biophys. Acta. 725, 25-33.

3. Barbagallo, R. P., Breyton, C. and Finazzi, G. (2000) Kinetic effects of the electrochemical proton gradient on plastoquinone reduction at the Qi site of the cytochrome b_{6f} complex. J. Biol. Chem. 275, 26121-26127.

4. Zatsman, A. I., Zhang, H., Gunderson, W. A., Cramer, W. A. and Hendrich, M. P. (2006) Heme-heme interactions in the cytochrome b_{6f} complex: EPR spectroscopy and correlation with structure. J. Am. Chem. Soc. 128, 14246-14247.

5. Baymann, F., Giusti, F., Picot, D. and Nitschke, W. (2007) The c_{i}/b_{11} moiety in the b_{6f} complex studied by EPR: A pair of strongly interacting hemes. Proc Natl Acad Sci U S A. 104, 519-524.

6. Alric, J., Pierre, Y., Picot, D., Lavergne, J. and Rappaport, F. (2005) Spectral and redox characterization of the heme c_{i} of the cytochrome b_{6f} complex. Proc Natl Acad Sci U S A. 102, 15860-15865.

7. de Lavalette, A. D., Barucq, L., Alric, J., Rappaport, F. and Zito, F. (2009) Is the redox state of the c_{i} heme of the cytochrome b_{6f} complex dependent on the occupation and structure of the Qi site and vice versa? J. Biol. Chem. 284, 20822-20829.

8. Malone, L. A., Qian, P., Mayneord, G. E., Hitchcock, A., Farmer, D. A., Thompson, R. F., Swainsbury, D. J. K., Ranson, N. A., Hunter, C. N. and Johnson, M. P. (2019) Cryo-EM structure of the spinach cytochrome b_{6f} complex at 3.6 A resolution. Nature.

9. Furbacher, P. N., Girvin, M. E. and Cramer, W. A. (1989) On the question of interheme electron transfer in the chloroplast cytochrome b_{6} in situ. Biochemistry. 28, 8990-8998.

10. Rich, P. R., Madgwick, S. A., Brown, S., von Jagow, G. and Brandt, U. (1992) MOA-stilbene: A new tool for investigation of the reactions of the chloroplast cytochrome b_{6f} complex. Photosynth Res. 34, 465-477.

11. Mulkidjianian, A. Y. (2010) Activated Q-cycle as a common mechanism for cytochrome b_{1} and cytochrome b_{6f} complexes. Biochim. Biophys. Acta. 1797, 1858-1868.

12. Allen, J. F. (2004) Cytochrome b_{6f}: structure for signalling and vectorial metabolism. Trends Plant Sci. 9, 130-137.

13. Nawrocki, W. J., Bailleul, B., Picot, D., Cardol, P., Rappaport, F., Wollman, F. A. and Joliot, P. (2019) The mechanism of cyclic electron flow. Biochim. Biophys. Acta. 1860, 433-438.

14. Steihl, H. H. and Witt, H. T. (1969) Quantitative treatment of function of plastoquinone in photosynthesis. Z Naturforsch. B 24, 1588-1598.

15. Kramer, D. M., Sacksteder, C. A. and Cruz, J. A. (1999) How acidic is the lumen? Photosynth Res. 60, 151-163.

16. Muñekage, Y., Hojo, M., Meurer, J., Endo, T., Tasaka, M. and Shikanai, T. (2002) PGR5 is involved in cyclic electron flow around photosystem I and is essential for photoprotection in Arabidopsis. Cell. 110, 361-371.

17. Nandha, B., Finazzi, G., Joliot, P., Hald, S. and Johnson, G. N. (2007) The role of PGR5 in the redox poising of photosynthetic electron transport. Biochim. Biophys. Acta. 1767, 1252-1259.

18. Suorsa, M., Jarvi, S., Grieco, M., Nurmi, M., Pietrzykowska, M., Rantalä, M., Kangasjarvi, S., Paakkarinen, V., Tikkanen, M., Jansson, S. and Aro, E. M. (2012) PROTON
GRADIENT REGULATION5 is essential for proper acclimation of Arabidopsis photosystem I to naturally and artificially fluctuating light conditions. Plant Cell. 24, 2934-2948
19 Johnson, X., Steinbeck, J., Dent, R. M., Takahashi, H., Richaud, P., Ozawa, S., Houille-Vernes, L., Petroutsos, D., Rappaport, F., Grossman, A. R., Niyogi, K. K., Hippler, M. and Alric, J. (2014) Proton gradient regulation 5-mediated cyclic electron flow under ATP- or redox-limited conditions: a study of ΔATPase pgr5 and ΔrbcL pgr5 mutants in the green alga Chlamydomonas reinhardtii. Plant Physiol. 165, 438-452
20 Mosebach, L., Heilmann, C., Mutoh, R., Gabelein, P., Steinbeck, J., Happe, T., Ikegami, T., Hanke, G., Kurisu, G. and Hippler, M. (2017) Association of Ferredoxin:NADP(+) oxidoreductase with the photosynthetic apparatus modulates electron transfer in Chlamydomonas reinhardtii. Photosynth Res. 134, 291-306
21 Jokel, M., Johnson, X., Peltier, G., Aro, E. M. and Allahverdiyeva, Y. (2018) Hunting the main player enabling Chlamydomonas reinhardtii growth under fluctuating light. Plant J. 94, 822-835
22 Alric, J. (2014) Redox and ATP control of photosynthetic cyclic electron flow in Chlamydomonas reinhardtii: (II) involvement of the PGR5-PGRL1 pathway under anaerobic conditions. Biochim. Biophys. Acta. 1837, 825-834
23 Clark, R. D., Hawkesford, M. J., Coughlan, S. J., Bennett, J. and Hind, G. (1984) Association of ferredoxin-NADP+ oxidoreductase with the chloroplast cytochrome b-f complex. FEBS Lett. 174, 137-142
24 Zhang, H., Whitelegge, J. P. and Cramer, W. A. (2001) Ferredoxin:NADP+ oxidoreductase is a subunit of the chloroplast cytochrome b-f complex. J. Biol. Chem. 276, 38159-38165
25 Okutani, S., Hanke, G. T., Satomi, Y., Takao, T., Kurisu, G., Suzuki, A. and Hase, T. (2005) Three maize leaf ferredoxin:NADPH oxidoreductases vary in subchloroplast location, expression, and interaction with ferredoxin. Plant Physiol. 139, 1451-1459
26 Joliot, P., Béal, D. and Joliot, A. (2004) Cyclic electron flow under saturating excitation of dark-adapted Arabidopsis leaves. Biochim. Biophys. Acta. 1656, 166-176
27 Joliot, P. and Joliot, A. (2006) Cyclic electron flow in C3 plants. Biochim. Biophys. Acta. 1757, 362-368
28 Joliot, P. and Johnson, G. N. (2011) Regulation of cyclic and linear electron flow in higher plants. Proc Natl Acad Sci U S A. 108, 13317-13322
29 Sueoka, N. (1960) Mitotic replication of deoxyribonucleic acid in Chlamydomonas reinhardi. Proc Natl Acad Sci U S A. 46, 83-91
30 Porra, R. J., Thompson, W. A. and Kriedemann, P. E. (1989) Determination of accurate extinction coefficients and simultaneous-equations for assaying chlorophyll-a and chlorophyll-b extracted with 4 different solvents - Verification of the concentration of chlorophyll standards by atomic-absorption spectroscopy. Biochim. Biophys. Acta. 975, 384-394
31 Johnson, X. and Alric, J. (2013) Central carbon metabolism and electron transport in Chlamydomonas reinhardtii: metabolic constraints for carbon partitioning between oil and starch. Eukaryot. Cell. 12, 776-793
32 Landt, O., Grunert, H. P. and Hahn, U. (1990) A general method for rapid site-directed mutagenesis using the polymerase chain reaction. Gene. 96, 125-128
33 Scholz, M., Gabelein, P., Xue, H., Mosebach, L., Bergner, S. V. and Hippler, M. (2019) Light-dependent N-terminal phosphorylation of LHCSR3 and LHCb4 are interlinked in Chlamydomonas reinhardtii. Plant J. 99, 877-894
34 Rasala, B. A., Lee, P. A., Shen, Z., Briggs, S. P., Mendez, M. and Mayfield, S. P. (2012) Robust expression and secretion of Xylanase1 in Chlamydomonas reinhardtii by fusion to a selection gene and processing with the FMDV 2A peptide. PLoS One. 7, e43349
Rappaport, F., Beal, D., Joliot, A. and Joliot, P. (2007) On the advantages of using green light to study fluorescence yield changes in leaves. Biochim. Biophys. Acta. 1767, 56-65

Baker, N. R. (2008) Chlorophyll fluorescence: a probe of photosynthesis in vivo. Annu. Rev. Plant Biol. 59, 89-113

Genty, B., Briantais, J.-M. and Baker, N. R. (1989) The relationship between the yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochim. Biophys. Acta. 990, 87-92

Oxborough, K. and Baker, N. R. (1997) Resolving chlorophyll a fluorescence images of photosynthetic efficiency into photochemical and non-photochemical components - calculation of qP and Fv'/Fm' without measuring Fv'. Photosynth Res. 54, 135-142

Kramer, D. M., Johnson, G., Kiirats, O. and Edwards, G. E. (2004) New fluorescence parameters for the determination of QA redox state and excitation energy fluxes. Photosynth Res. 79, 209

Kalaji, H. M., Schansker, G., Brestic, M., Bussotti, F., Calatayud, A., Ferroni, L., Goltsev, V., Guidi, L., Jajoo, A., Li, P., Losciaie, P., Mishra, V. K., Misra, A. N., Nebauer, S. G., Pancaldi, S., Penella, C., Pollastrini, M., Suresh, K., Tambussi, E., Yannicciari, M., Zivcek, M., Cetner, M. D., Samborska, I. A., Stirbet, A., Olsovskia, K., Kunderlikova, K., Sholenzek, H., Rusinowski, S. and Baba, W. (2017) Frequently asked questions about chlorophyll fluorescence, the sequel. Photosynth Res. 132, 13-66

Schreiber, U. and Bilger, W. (1987) Rapid assessment of stress effects on plant leaves by chlorophyll fluorescence measurements. ed.)^eds.). pp. 27-53, Springer Berlin Heidelberg, Berlin, Heidelberg

Weis, E. and Berry, J. A. (1987) Quantum efficiency of photosystem II in relation to 'energy'-dependent quenching of chlorophyll fluorescence. Biochim. Biophys. Acta. 894, 198-208

Joliot, P. and Joliot, A. (2008) Quantification of the electrochemical proton gradient and activation of ATP synthase in leaves. Biochim. Biophys. Acta. 1777, 676-683

Malnoe, A., Wollman, F. A., de Vitry, C. and Rappaport, F. (2011) Photosynthetic growth despite a broken Q-cycle. Nat Commun. 2, 301

Joliot, P. and Joliot, A. (2001) Electrogenic events associated with electron and proton transfers within the cytochrome b6/f complex. Biochim. Biophys. Acta. 1503, 369-376

Zito, F., Finazzi, G., Joliot, P. and Wollman, F. A. (1998) Glu78, from the conserved PEWY sequence of subunit IV, has a key function in cytochrome b6f turnover. Biochemistry. 37, 10395-10403

Joliot, P. and Joliot, A. (2002) Cyclic electron transfer in plant leaf. Proc Natl Acad Sci USA. 99, 10209-10214

Bailleul, B., Cardol, P., Breyton, C. and Finazzi, G. (2010) Electrochromism: a useful probe to study algal photosynthesis. Photosynthesis Res. 106, 179-189

Avenson, T. J., Cruz, J. A. and Kramer, D. M. (2004) Modulation of energy-dependent quenching of excitons in antennae of higher plants. Proc Natl Acad Sci USA. 101, 5530-5535

Klughammer, C. and Schreiber, U. (1994) An improved method, using saturating light-pulses, for the determination of photosystem-I quantum yield via P700' -absorbance changes at 830nm. Planta. 192, 261-268

Brettel, K. (1997) Electron transfer and arrangement of the redox cofactors in photosystem I. Biochim. Biophys. Acta. 1318, 322-373

Alric, J., Lavergne, J. and Rappaport, F. (2010) Redox and ATP control of photosynthetic cyclic electron flow in Chlamydomonas reinhardtii (I) aerobic conditions. Biochim. Biophys. Acta. 1797, 44-51

22
Fan, D. Y., Jia, H., Barber, J. and Chow, W. S. (2009) Novel effects of methyl viologen on photosystem II function in spinach leaves. Eur. Biophys. J. 39, 191-199
54 Joliot, P. and Delosme, R. (1974) Flash-induced 519 nm absorption change in green algae. Biochim. Biophys. Acta. 357, 267-284
55 Robertson, D. E. and Dutton, P. L. (1988) The nature and magnitude of the charge-separation reactions of ubiquinol cytochrome c2 oxidoreductase. Biochim. Biophys. Acta. 935, 273-291
56 Glaser, E. G. and Crofts, A. R. (1984) A new electrogenic step in the ubiquinol:cytochrome c2 oxidoreductase complex of Rhodopseudomonas sphaeroides. Biochim. Biophys. Acta. 766, 322-333
57 Iwai, M., Takizawa, K., Tokutsu, R., Okamuro, A., Takahashi, Y. and Minagawa, J. (2010) Isolation of the elusive supercomplex that drives cyclic electron flow in photosynthesis. Nature. 464, 1210-1213
58 Terashima, M., Petroutos, D., Hudig, M., Tolstygina, I., Trompelt, K., Gäbelein, P., Fufezan, C., Kudla, J., Weinl, S., Finazzi, G. and Hippler, M. (2012) Calcium-dependent regulation of cyclic photosynthetic electron transfer by a CAS, ANR1, and PGRL1 complex. Proc Natl Acad Sci U S A. 109, 17717-17722
59 Takahashi, H., Clowez, S., Wollman, F. A., Vallon, O. and Rappaport, F. (2013) Cyclic electron flow is redox-controlled but independent of state transition. Nat Commun. 4, 1954
60 Steinbeck, J., Ross, I. L., Rothnagel, R., Gäbelein, P., Schulze, S., Giles, N., Ali, R., Drysdale, R., Sierecki, E., Gambin, Y., Stahlberg, H., Takahashi, Y., Hippler, M. and Hankamer, B. (2018) Structure of a PSI–LHCl–cyt b6f supercomplex in Chlamydomonas reinhardtii promoting cyclic electron flow under anaerobic conditions. Proc Natl Acad Sci U S A. 115, 10517-10522
61 Mukidjanian, A. Y. (2005) Ubiquinol oxidation in the cytochrome bc1 complex: Reaction mechanism and prevention of short-circuiting. Biochim. Biophys. Acta. 1709, 5-34
62 Yamashita, E., Zhang, H. and Cramer, W. A. (2007) Structure of the cytochrome b6f complex: quinone analogue inhibitors as ligands of heme c6. J. Mol. Biol. 370, 39-52
63 Mitchell, P. (1975) The protonmotive Q cycle: A general formulation. FEBS Lett. 59, 137-139
64 Jones, R. W. and Whitmarsh, J. (1988) Inhibition of electron transfer and the electrogenic reaction in the cytochrome b6f complex by 2-n-nonyl-4-hydroxyquinoline N-oxide (NQNO) and 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB). Biochim. Biophys. Acta. 933, 258-268
65 Joliot, P. and Joliot, A. (1988) The low-potential electron-transfer chain in the cytochrome b6f complex. Biochim. Biophys. Acta. 933, 319-333
66 Joliot, P. and Joliot, A. (1986) Mechanism of proton-pumping in the cytochrome b6f complex. Photosynth Res. 9, 113-124
67 Batie, C. J. and Kamin, H. (1981) The relation of pH and oxidation-reduction potential to the association state of the ferredoxin · ferredoxin:NADP+ reductase complex. J. Biol. Chem. 256, 7756-7763
68 Takahashi, H., Okamuro, A., Minagawa, J. and Takahashi, Y. (2014) Biochemical characterization of photosystem I-associated light-harvesting complexes I and II isolated from state 2 cells of Chlamydomonas reinhardtii. Plant Cell Physiol. 55, 1437-1449
69 Palatnik, J. F., Valle, E. M. and Carrillo, N. (1997) Oxidative stress causes ferredoxin-NADP+ reductase solubilization from the thylakoid membranes in methyl viologen-treated plants. Plant Physiol. 115, 1721-1727
70 Davis, G. A., Kanazawa, A., Schottler, M. A., Kohzuma, K., Froehlich, J. E., Rutherford, A. W., Satoh-Cruz, M., Minhas, D., Tietz, S., Dhingra, A. and Kramer, D. M.
(2016) Limitations to photosynthesis by proton motive force-induced photosystem II photodamage. Elife. 5

71 DalCorso, G., Pesaresi, P., Masiero, S., Aseeva, E., Schunemann, D., Finazzi, G., Joliot, P., Barbato, R. and Leister, D. (2008) A complex containing PGRL1 and PGR5 is involved in the switch between linear and cyclic electron flow in Arabidopsis. Cell. 132, 273-285

72 Takagi, D. and Miyake, C. (2018) PROTON GRADIENT REGULATION 5 supports linear electron flow to oxidize photosystem I. Physiol. Plant. 164, 337-348

73 Dang, K. V., Plet, J., Tolleter, D., Jokel, M., Cuine, S., Carrier, P., Aubry, P., Richaud, P., Johnson, X., Alric, J., Allahverdiyeva, Y. and Peltier, G. (2014) Combined increases in mitochondrial cooperation and oxygen photoreduction compensate for deficiency in cyclic electron flow in Chlamydomonas reinhardtii. Plant Cell. 26, 337-348

74 Yamamoto, H., Takahashi, S., Badger, M. R. and Shikanai, T. (2016) Artificial remodelling of alternative electron flow by flavodiiron proteins in Arabidopsis. Nat Plants. 2, 16012

75 Steinbeck, J., Nikolova, D., Weingarten, R., Johnson, X., Richaud, P., Peltier, G., Hermann, M., Magneschi, L. and Hippler, M. (2015) Deletion of Proton Gradient Regulation 5 (PGR5) and PGR5-Like 1 (PGRL1) proteins promote sustainable light-driven hydrogen production in Chlamydomonas reinhardtii due to increased PSII activity under sulfur deprivation. Front Plant Sci. 6, 892

76 Chen, M., Zhang, J., Zhao, L., Xing, J. L., Peng, L. W., Kuang, T. Y., Rochaix, J. D. and Huang, F. (2016) Loss of algal Proton Gradient Regulation 5 increases reactive oxygen species scavenging and H2 evolution. J Integr Plant Biol. 58, 943-946

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

78 Kramer, D. M. and Crofts, A. R. (1989) Activation of the chloroplast ATPase measured by the electrochromic change in leaves of intact plants. Biochimica et Biophysica Acta. 976, 28-41
Figure 1. Experimental workflow of the steady state protocol in items a to h is shown, as well as the single turnover experiments in i. The references are in parenthesis. AL: actinic light (~150 μmol photons m⁻² s⁻¹); ECS: electrochromic shift; MV: methyl viologen; HA: hydroxylamine; DCMU: 3-(3,4-dichlorophenyl)-1,1-dimethylurea

Figure 2. The electron transfer via P700 is changed in pgr5 under different cellular redox states, pointing to a more photo-oxidizable PSI. For (A) WT and (B) pgr5, saturating pulse-induced P700 redox changes are shown in the absence and presence of 10 mM methyl viologen (MV), as well as in anoxic cells. The 12-ms pulse (hatched red box) was applied on light-adapted cells in the steady state (red box), followed by several seconds dark measurements (black box). (C) As described in Materials and Methods, the different P700 populations were deconvoluted as photo-oxidizable fraction (ΦPSI, yield of PSI), non-oxidizable P700 owing to acceptor side limitation (YN), and pre-oxidized P700 due to donor side limitation (YND). The electron acceptor MV abolished YN and increased YND despite PSII activity. In WT, anoxic conditions lowered ΦPSI and increased YN. The latter effect was absent in anoxic pgr5. (D) As described in Materials and Methods, the rate of P700 oxidation during the pulse, kPox, and (E) the fast P700 dark relaxation rate after the pulse, k1Pred, were calculated from the kinetics in panels A and B for WT and pgr5, respectively (N = 3 ± SD; Student’s t-test *P < 0.05 and **P < 0.005). The parameters for the partially complemented line C1 are shown as well (see supplementary Figure S4 for P700 kinetics and redox states). kPox was faster in oxic pgr5. Only MV samples in the WT showed faster kPox. Anoxic cells also produced faster kPox than oxic controls. k1Pred was slowed down in MV samples. The anoxic WT and C1 also showed slower k1Pred.

Figure 3. The electron transfer via b6f is changed under different cellular redox states, pointing to a more oxidized high-potential chain in pgr5, especially in anoxic cells, and to a b6f inhibition in the low-potential chain in the absence of cyclic electron flow. (A) Cyt.f redox changes in a steady state light/pulse/dark regime showed different pulse-induced oxidation magnitudes in WT cells with oxic > anoxic > oxic+MV amplitudes, compared to the steady state reference. Addition of the cyclic electron flow inhibitor methyl viologen (MV, 10 mM) caused no significant cyt.f net oxidation during the pulse. Most of the dark relaxation finished in ~50-ms, except for the slow re-reduction in MV samples. (B) Redox changes of b-hemes in WT were comparable during the pulse and most of the oxidation was finished in ~50-ms, ~300-ms, ~25-ms in oxic, oxic +MV and anoxic samples, respectively. The latter samples showed a slow re-reduction phase with an onset at less than 100-ms darkness. (C) In pgr5, cyt.f showed different pulse-induced oxidation magnitudes with oxic > anoxic = oxic+MV amplitudes, compared to the steady state reference. Oxidation amplitudes in oxic controls were smaller than in WT (cf. panel A). Mutant kinetics in the presence of MV and in anoxia were similarly slowed down. (D) Redox changes of b-hemes in pgr5 were comparable during the pulse and showed a similar trend as WT, except for a slightly longer oxidation phase after the pulse in anoxia (cf. panel B). Anoxic pgr5 also showed a re-reduction phase which started later and was slower than in WT. (E) After the pulse in panels A and C, fitting the cyt.f
reduction phase yielded a $k_{f\text{-red}}$ which was slowed down by MV ($N = 3 \pm SD$; Student’s t-test $^*P < 0.05$ and $^{**}P < 0.005$, see Materials and Methods). Anoxic WT showed a faster $k_{f\text{-red}}$, whereas it was much slower in anoxic $pgr5$, followed by C1 (for intermediary C1, see supplementary Figure S5). (F) Following the pulse, $b$-heme oxidation rates were calculated as $k_{b\text{-ox}}$ which was slowed down by MV in WT and C1. In anoxic WT, $k_{b\text{-ox}}$ was faster compared to the oxic control and the other anoxic strains. (G) The slow $b$-heme re-reduction rate in anoxia, $k_{b\text{-red}}$, was fastest in WT.

Figure 4. The electron transfer via PSII is changed under different cellular redox states, pointing to higher PSII efficiency in oxic $pgr5$ and to a lower efficiency in reducing conditions ($N = 3 \pm SD$; Student’s t-test $^*P < 0.05$ and $^{**}P < 0.005$). Panels (A), (B) and (C) show the chlorophyll fluorescence-derived quantum yield of PSII ($\Phi_{PSII}$), the PSII efficiency factor ($qP$), and the fraction of open PSII centers ($qL$) for WT and $pgr5$. During light adaptation in oxic conditions, all parameters were unaltered in WT after 10-s illumination and in the steady state after 30-min light. In oxic $pgr5$, all parameters were like in WT after 10-s light but increased significantly after 30-min light. In the presence of 10 mM methyl viologen (MV), all parameters in $pgr5$ were lowered significantly to levels as in the WT, in which MV lowered $qP$ only. During light adaptation in anoxic conditions, all parameters were at the same low level after 10-s light. They remained low in anoxic $pgr5$ after 30-min illumination and $qL$ was significantly changed in anoxic WT only. The light-adapted anoxic WT showed a significantly higher $qP$ and $qL$ than $pgr5$.

Figure 5. The electrogenic efficiency of the photosynthetic electron transfer chain is compromised in $pgr5$ under anoxic conditions. The ability to generate and dissipate an electric field is assessed by measuring the electrochromic shift, ECS. Signals were recorded in steady state light, during a saturating pulse and in darkness. (A) The ECS kinetics of the WT and (B) $pgr5$ indicated that the 22-ms pulse led to equilibration of a new membrane potential $\Delta \Psi$ (for ECS kinetics of C1 refer to supplementary Figure S8). The initial efficiency to generate a higher $\Delta \Psi$ level at the onset of the pulse was calculated with data from the green symbols, yielding $k_{\text{ini}}$ from the linear slope during the first 2-ms of the pulse (inset, see Materials and Methods). The apparent $\Delta \Psi$ generation efficiency at the end of the pulse ($k_{\text{end}}$) was corrected with the $\Delta \Psi$-consuming activity of the ATP-synthase. (C) $k_{\text{ini}}$ was similar in oxic strains and, except for $pgr5$, slower upon addition of 10 mM methyl viologen (MV; $N = 3 \pm SD$; Student’s t-test $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$ and $^{****}P < 0.00005$). In anoxic cells, $k_{\text{ini}}$ was diminished in all strains but highest in WT, followed by C1 and $pgr5$. (D) $k_{\text{end}}$ was low in the strains due to exhaustion of electron acceptors or donors. Several small differences among treatments and strains were observed.

Figure 6. Measurements of various redox parameters under anoxic conditions are shown, which demonstrate the restoration of the $pgr5$ phenotype by overexpression of PGR5 and a Cys113Ser variant of the polypeptide. (A) The P700 redox state in TAP-grown WT and $pgr5$ follows a similar trend as in anoxic samples of Figure 2C. Both overexpression lines had a WT-like P700 redox state. (B) After the pulse (for kinetics, see supplementary Figure S9A), the reduction rates of cytochrome $f$ ($k_{f \text{-red}}$) were significantly slower in the mutant and were
recovered to WT-level in both overexpressors \( (N = 3 \pm SD; \text{Student’s } t\text{-test } *P < 0.05 \text{ and } ***P < 0.0005) \). (C) Simultaneously, the oxidation rates of the b-hemes after the pulse \( (k_{b-ox}) \) were significantly slowed down in pgr5 and, to a lesser extent, in pgr5::PGR5 C113S (for kinetics, see supplementary Figure S9B). (D) During the saturating pulse, the high initial electrogenic charge separation rates \( (k_{\text{ini}}) \) in steady state cells were PGR5-dependent, and were also recovered in the C113S variant.

Figure 7. Redox kinetics and electrogenic signals reveal a PGR5-dependent low-potential chain tuning in anoxia as well as an inhibitory effect of methyl viologen on the single bof turnover. (A) Cyt.f and b-heme signals are shown for oxic WT and pgr5. Cyt.f is rapidly (<300 μs) oxidized after the flash and re-reduced within 100-ms darkness (fitted curves). The b-heme net reduction lasted between ~1-ms and 10-ms darkness, followed by a slower oxidation phase (fitted curves) for several tens of ms. (B) The corresponding oxic ECS kinetics were normalized to the signal produced by a flash hitting ~40% of PSI centers \( \Delta I/I_{270-546} < 300 \mu s, a\text{-phase} \). The following b-phase (fitted curve of squares) resulted from bof-dependent charge separation activity in the ~10-ms range. As described in Materials and Methods, the b-phase was deconvoluted from raw ECS kinetics by subtracting the c-phase (fitted curve of circles). In this sample, the b-phase developed slower in pgr5 and showed a slightly larger relative amplitude. (C) Addition of 10 mM methyl viologen (MV) slowed down cyt.f reduction and b-heme oxidation in both strains, and the mutant was slightly less affected. The inhibition of b-heme oxidation allowed larger reduction amplitudes compared to panel A. (D) Evolution of the b-phase was slowed down by MV but the amplitude was not altered. MV also slowed down the c-phase upon disulfide promotion in the ATP-synthase γ-subunit [78]. (E) The bof redox kinetics in anoxia showed slightly slower cyt.f oxidation whereas reduction was like in oxic cells. Net reduction of b-hemes in the first 10-ms was of negligible amplitude and a large oxidation phase followed. (F) ECS and b-phase kinetics in anoxia resembled oxia (B). (G) Cyt.f reduction rates \( k_{\text{red}} \) were calculated and showed significant slowdown in the presence of MV \( (N = 3 \pm SD; \text{Student’s } t\text{-test } *P < 0.05, **P < 0.005 \text{ and } ***P < 0.0005) \). (H) The electrogenic b-phase also evolved at slower rates \( (k_{\Delta\psi}) \) in MV samples. Compared to WT, the pgr5 mutant showed slower k\_\(\Delta\psi\) in oxic and anoxic conditions. (I) After the varying apparent b-heme reduction phase, the slow oxidation rates were expressed as \( k_{b-ox} \). MV slowed down \( k_{b-ox} \) and the WT showed faster \( k_{b-ox} \) in anoxia, whereas the anoxic mutant \( k_{b-ox} \) remained unchanged. (J) Compared to the amplitude of the a-phase, the mutant had a slight tendency to produce a larger b-phase, which was significant for pgr5 MV samples.

Figure 8. Different operation modes of the bof are shown which highlight Qi-site alterations between (A–B) the canonical Q cycle in oxic conditions and (C–D) an alternative Fd-assisted Q cycle in anoxic conditions. Referring to Figure 7, the panels display schematics of the developing electrochromic shift (ECS) signals and b-heme redox changes in the 10-ms range after a subsaturating single turnover flash and, separated by the dashed line, beyond the initial phase. Each turnover is induced by a laser flash, which besides an electron hole also generates a membrane potential \( (\Delta\Psi) \) via PSI charge separation. This further influences bof properties and the figure focuses on events after the reduction of cyt.f and the swapping of the elliptic FeS domain of the Rieske ISP to a cytochrome bo-proximal position. (A) Per PQH\_2 formed by
Qi-site turnover, the Qo-site turns over twice in a canonical Q cycle. After the first full oxidation of PQH$_2$, the $b_6f$ produces a $\Delta\Psi$ by converting the redox couple $b_{h6}^{\text{red}}/b_{h6}^{\text{ox}}$ to $b_{h6}^{\text{red}}/b_{h6}^{\text{red}}$. With the decay of $\Delta\Psi$ upon ATP synthesis, the prevalent $b_{h6}^{\text{red}}/c_{i6}^{\text{ox}}$ converts to $b_{h6}^{\text{red}}/c_{i6}^{\text{red}}$. Reduction of heme-c$_i$ opens the Qi-site for the PQ substrate, which ligates with heme-c$_i$. Swapping of the FeS domain closer to cyt.f is linked to $b$-heme oxidation. (B) The flash-induced $\Delta\Psi$ converts $b_{h6}^{\text{ox}}/c_{i6}^{\text{red}}$ to $b_{h6}^{\text{red}}/c_{i6}^{\text{ox}}$, which tightly ligates heme-c$_i$ with substrate. To accomplish the $c_i$/semi-PQ reduction, reducing pressure is accumulated on the low-potential chain upon a second Qo-site turnover by reduction of $b_{h6}^{\text{ox}}$. In the presence of $\Delta\Psi$, this drives the quasi-simultaneous $b_{h6}^{\text{red}}$ oxidation since $b_{h6}^{\text{red}}/b_{h6}^{\text{red}}$ is unlikely during single turnover measurements. The (semi-)PQ in the Qi-site receives the electrons from $c_i^{\text{red}}$ and/or $b_{h6}^{\text{red}}$ in a concerted and closely spaced process. (C–D) In anoxic samples, the low-potential chain was partially pre-reduced before the flash since, with the consumption of $\Delta\Psi$ during 30-s darkness, equilibration of $b_{h6}^{\text{red}}$ follows $c_i^{\text{red}}$. The initial reaction steps left from the dashed line were very similar in both (C) $pgr5$ and (D) WT. The pre-flash $b_{h6}^{\text{ox}}/b_{h6}^{\text{red}}$ re-equilibrated, again, to $b_{h6}^{\text{ox}}/b_{h6}^{\text{red}}$ after the electrogenic charge transfer associated with a Qi-site turnover. During this initial phase, one PQH$_2$ is formed at the Qi-site by utilizing the pre-reduced $b_{h6}^{\text{red}}/c_{i6}^{\text{red}}$ redox couple. (C) The following $b$-heme oxidation phase is slower in the mutant and may be attributed to poor Qi-site substrate availability, which depends on $c_i^{\text{red}}$. In the presence of $\Delta\Psi$, $b_{h6}^{\text{red}}/c_{i6}^{\text{ox}}$ is prevalent and a slower transition to $b_{h6}^{\text{ox}}/c_{i6}^{\text{red}}$ follows the $\Delta\Psi$ decay in $pgr5$ (gray and violet curves). (D) Unlike $pgr5$, the WT retains more FNR at the thylakoid membrane, which might be an allosteric modulator by interacting with the $b_{h6}$. Thus, FNR-bound Fd may drive the transient generation of $c_i^{\text{red}}$ in the presence of $\Delta\Psi$. This creates the ‘PQ-accessible’ Qi-site specifically in WT and, due to the $b_{h6}^{\text{red}}/c_{i6}^{\text{red}}$ redox couple, produces faster $b$-heme oxidation and a second PQH$_2$ molecule. Compared to $pgr5$ and possibly important during multiple turnover, the faster $b$-heme oxidation also accelerates the Rieske FeS domain to swap closer to cyt.f.

Figure 9. Model summarizing the multiple turnover measurements. Except for PC and NADPH$_2$ pools, the redox levels were measured (blue and red stand for oxidized and reduced, respectively). PSI acceptor side limitation (YNA) and photosynthetic control (ΔpH) were established between weakly (transparent) and strongly contributing levels (bold). Modified forward reaction efficiencies are highlighted (Qo: PQH$_2$ oxidation, Rieske ISP/cyt.f interaction; Qi: PQ reduction, $b_{h6}/c_i$ electron sharing, Fd-dependent $c_i$ reduction; NADPH$_2$ formation). (A) Qi-site inhibition by methyl viologen (MV) disturbs the low-potential chain oxidation and thus ISP/cyt.f interaction. MV likely interferes with the redox signal that mediates FNR tethering to the membrane. (B) The $pgr5$ mutant shows unregulated electron utilisation downstream of PSI, (C) unlike WT. Faster electron flow via PSI was sustained without increasing YNA in algal $pgr5$ since alternative electron acceptors, such as flavodiiron proteins, prevented reduction of the Fd pool and PSI photodamage. The mutant showed impaired FNR membrane recruitment and the PQ pool and cyt.f were slightly more oxidised. (D) When anoxic WT $b_{h6}$ operates in the Fd-assisted Q cycle mode, $b_{h6}$-electrogenicity is maintained. Thus ΔpH protects PSI, which also throttles electron flow via YNA. (E) Inefficient utilization of excess stromal electrons at the Qi-site stalls the $pgr5$ $b_{h6}$ in anoxic conditions, thus weakening YNA. The backup of low-potential chain electrons could be
linked to a disturbed ISP/cyt.f interaction and loss of efficient pmf generation of the photosynthetic chain.
### Table 1. Key events of Figure 8.

| sample      | $b_l/b_h$ after 30-s D $(c_i$ at low $\Delta\Psi$) | $b_l/b_h$ after 10-ms $b$-phase $(\text{PQH}_2$ formed at Qi) | $b_l/b_h$ after ~100-ms $(\text{PQH}_2$ formed at Qi) |
|-------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| oxic        | ox/ox (red)                                    | ox/red (0 or 0.5)                                | ox/ox (0.5 or 1)                                 |
| anoxic WT   | ox/red (red)                                   | ox/red (1)                                      | ox/ox (2, Fd-assisted $c_i$ red)                  |
| anoxic $pgr5$ | ox/red (red)                                   | ox/red (1)                                      | ox/ox (1, $\Delta\Psi$-assisted $c_i$ red)       |
| Sample         | $b_i/b_h$ after 30-s D ($c_1$ at low $\Delta\Psi$) | $b_i/b_h$ after 10-ms $b$-phase (PQH$_2$ formed at Qi) | $b_i/b_h$ after ~100-ms (PQH$_2$ formed at Qi) |
|---------------|-----------------------------------------------|-------------------------------------------------|-----------------------------------------------|
| oxic          | ox/ox (red)                                   | ox/red (0 or 0.5)                               | ox/ox (0.5 or 1)                              |
| anoxic WT     | ox/red (red)                                  | ox/red (1)                                      | ox/ox (2, Fd-assisted $c_1$ red)              |
| anoxic pgr5   | ox/red (red)                                  | ox/red (1)                                      | ox/ox (1, $\Delta\Psi$-assisted $c_1$ red)   |
