Short Title:

Regulation of photosynthetic control

Title:

The algal PETC-Pro171-Leu suppresses electron transfer in the cytochrome *b₆f* under acidic lumenal condition

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One sentence summary:

Change of PETC to P171L via site directed mutagenesis alters the pH dependency of the photosynthetic control mechanism

Author Contributions:

S.O., F.B., M.H., and Y.T. conceived the original screening and research plans; S.O., F.B., M.H., and Y.T. supervised the experiments; S.O., F.B., and R.R. designed and performed the experiments; S.O., F.B., and R.R. analyzed the data; S.O., F.B., M.H., and Y.T. conceived the project and wrote the article with contributions of all the authors; S.O., F.B., M.H., and Y.T. supervised and completed the writing. S.O. agrees to serve as the author responsible for contact and ensures communication.

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Abstract

Linear photosynthetic electron flow (LEF) produces NADPH and generates a proton electrochemical potential gradient across the thylakoid membrane used to synthesize ATP, both of which are required for CO₂ fixation. As cellular demand for ATP and NADPH are variable, cyclic electron flow (CEF) between PSI and cytochrome *b₆f* complex (*b₆f*) produces extra ATP. The *b₆f* regulates LEF and CEF via photosynthetic control, which is a pH-dependent *b₆f* slowdown of plastoquinol oxidation at the lumenal site. This protection mechanism is triggered at more alkaline lumen pH in the *pgr1* mutant of the vascular plant *Arabidopsis thaliana*, carrying Pro194Leu in the *b₆f* Rieske Iron-sulfur protein. In this work, we introduced *pgr1* mutation in the green alga *Chlamydomonas reinhardtii* (*PETC-PI71L*). Consistent with *pgr1* phenotype, *PETC-PI71L* displayed a limited photosynthesis along with slower photoautotrophic growth under high light conditions. Our data under low oxygen revealed that the ΔpH component in algae was already sufficient to trigger the effect in *PETC-PI71L* in sub-saturating light conditions where the mutant *b₆f* was more restricted to oxidize the PQ pool and revealed a diminished electron flow.

Introduction

Oxygenic photosynthesis fixes carbon dioxide and generates oxygen as a result of intra- and intermolecular photochemical electron transfer reactions. The electron transfer pathways in oxygenic photosynthesis either follows Linear Electron Flow (LEF) or, as an example of alternative passages, Cyclic Electron Flow (CEF). Electrons stemming from water oxidation reduce NADP^+ during LEF, thus providing the reducing power for CO_2 fixation in the Calvin Benson cycle. In the CEF, electrons from reduced ferredoxin (Fd) are differently partitioned and redirected into the plastoquinone (PQ) pool. A possible CEF route reinjects electrons into the cytochrome b_6f complex (b_6f) from the stromal side. In LEF and CEF, the b_6f contributes to proton gradient formation across thylakoid membrane by translocating protons from chloroplast stroma to thylakoid lumen. Light-driven charge separation, water splitting as well as b_6f activity generate a membrane potential ($\Delta\Psi$) and a proton gradient (ΔpH), respectively, and this proton motive force (pmf) drives ATP synthesis. During prolonged electron transfer, the lumen will get further acidified, which in turn will slow down plastoquinol (PQH_2) oxidation at the Q_o -site of b_6f , and thereby establish photosynthetic control to protect photosystem I (PSI) from acceptor side limitation (Rumberg and Siggel, 1969; Tikhonov et al., 1981; Nishio and Whitmarsh, 1993). Lumen acidification is also important to induce Non-Photochemical Quenching (NPQ) (Kanazawa and Kramer, 2002), which is the most rapid photoprotective response to excess light. The fastest constituent of NPQ is q_E , which operates at a time scale of seconds to minutes and regulates the thermal dissipation of excess absorbed light energy, thereby providing effective photo-protection of photosystem II (PSII). In vascular plants, the PSII protein PSBS is essential for q_E (Li et al, 2000), whereas q_E induction in the green alga *Chlamydomonas reinhardtii* is mediated by LHCSR3, an ancient light-harvesting protein that is missing in vascular plants (Peers et al., 2009).

Electron transfer through the b_6f is governed by the light-induced introduction of an electron hole (positive charge) into the high-potential chain. The latter involves redox centers heme f and the $[\text{2Fe-2S}]$ type Rieske Iron Sulfur Cluster (Rieske ISC). The electron hole depends on photo-oxidized plastocyanin and is successively passed from heme f via Rieske ISC to PQH_2 . The Q-cycle within the b_6f is responsible to generate a pmf across the thylakoid membrane, owing to a sophisticated electron bifurcation. Two electrons are pulled from PQH_2 at the Q_o site, forming PQ within the membrane

while simultaneously releasing two H^+ into the lumen. During the PQH_2 oxidation at the Q_o site, the first electron is transferred into the high-potential chain (Rieske ISC reducing heme f), and the second electron into the low-potential chain (b_L heme reducing b_H heme, terminal acceptor is c_i heme). A second PQH_2 oxidation at the Q_o site forms a reduced form of b_H / c_i heme pair which reduces a PQ bound at Q_i site to complete the Q-cycle (note that alternative annotations for Q_o , Q_i , b_H and c_i can be found in literatures). It was suggested that under LEF and CEF conditions two types of Q-cycle mechanism operate in the b_6f with specific lumen acidification efficiencies per electron transferred via the high-potential chain (Buchert et al., 2020).

The molecular structure of b_6f complex was first resolved in the green alga *Chlamydomonas reinhardtii* and the cyanobacteria *Mastigocladus laminosus* independently (Kurisu et al., 2003; Stroebel et al., 2003). The b_6f functions as a homodimer, and each monomer consists of four core subunits (PetA, PetB, PETC, and PetD) and four stably associated small subunits (PetG, PetL, PETM, and PETN) to stabilize the complex (Figure 1A). The cofactors and quinone binding pockets involved in electron transfer are found in the b_6f core subunits which resemble the cytochrome bc_1 complex of the respiratory chain. The structure-function similarities suggest that the hydrophilic domain of PETC subunit would move toward PetA subunit in transferring the electrons from Rieske ISC to heme f .

In *Arabidopsis thaliana*, various photosynthetic mutants have been isolated and characterized by forward genetics. One study employed ethyl methanesulfonate as mutagen to isolate the CE10-10-1 mutant which exhibited reduced NPQ with normal PSI/PSII quantum yields (Shikanai et al., 1999). A follow-up study pinpointed the mutation as a proline-to-leucine substitution at position 194 of Arabidopsis PETC, linking the NPQ defect to insufficient ΔpH formation in the light (Munekage et al., 2001). The Arabidopsis PETC-P194L mutation was dubbed *pgr1* (*proton gradient regulation 1*).

It is known that green algal photosystems are protected from excess light illumination differently than plants. For instance, algal state transition redistributes excitation energy more effectively than plant (Delepelaire and Wollman, 1985). Moreover, flavodiiron protein was found in algae to protect PSI acceptor side limitation induced by fluctuating light illumination (Jokel et al., 2018). Induction of qE relies on LHCSR3, which requires a preceding stress period to trigger light to heat dissipation upon lumen acidification (Peers et al., 2009; Bonente et al., 2011). Moreover, the algal PETO protein

interacts with *b₆f* and functions as a CEF effectors under anoxic condition, which might point to a different CEF regulation (Takahashi et al., 2016; Buchert et al., 2018).

Herein, we introduced the Arabidopsis *pgr1* mutation into *Chlamydomonas reinhardtii* (*PETC-PI71L*) to analyze the effects of the mutation on Photosynthetic Control in the green alga. The *PETC-PI71L* cytochrome *b₆f* showed a normal accumulation and electron flow activity in cells grown under oxic conditions. However, unlike Arabidopsis *pgr1* mutant, the *PETC-PI71L* displayed an impaired photoautotrophic growth because the electron transfer activity of the mutated *b₆f* was significantly decreased in cells grown under anoxic conditions. Our experiments also revealed that the Δ pH component in *PETC-PI71L* is dependent on oxygen availability under light conditions that are permissive under other circumstances. Under low oxygen condition the mutant *b₆f* was more restricted to oxidize the PQ pool and limited electron flow.

Materials and methods

Strains and growth condition

The cells of *Chlamydomonas reinhardtii* were grown in Tris-acetate phosphate (TAP), Tris-Phosphate (TP) medium, or minimum medium (MM) (Harris, 1988) under 50-100 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ or 600 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The previously isolated PETC gene disrupted strain *petC-Δ1* (mating type minus) described in (de Vitry et al., 1999) was crossed with cc-125 (mating type plus) and the resultant PETC deficient progeny was used as a recipient strain for transformation. Doubling time of the cell growth is calculated by dividing 0.30 by specific growth rate obtained as the slope which is computed by linear approximation for logarithmic growth curve in common logarithmic axis. Linear fitting was performed at the highest coefficient of determination (R-square) with no restrictions by using built-in linear fitting program of the Origin Pro 2021b version 9.8.5.212 (OriginLab corporation, USA). Refer the Appendix 1 for derivation of the formula to calculate the doubling time with specific growth rate.

Generation of transformation vectors

The BamHI fragment containing PETC gene (Cre11.g467689.t1.1, *Chlamydomonas* version 5.5) in the pACR4.5 (de Vitry et al., 1999) was digested with BamHI and subsequently the resulting 4153 bp fragment containing the *PETC* gene and 528 bp upstream of 5' -UTR of PETC was recloned into pBSSK⁺ vector at BamHI site to yield pgcC plasmid. The mutation Pro171Leu in PETC was introduced by In-Fusion^R (Clontech, TAKARA, Japan) with the DNA fragment between BbvCI and SnaBI sites which was generated by PCR with If-BbvCI_P171L_gcC_F and If-SnaBI_gcC_R primers. The sequence of this study's oligos is shown in Table 1. The DNA sequence of the inserted PCR product was validated by DNA sequencing with the primers used to generate the PCR product and we obtained pgcC-P171L plasmid.

We also prepared the DNA construct in which the *PETC* gene expression is controlled by *Chlamydomonas PSAD* promoter and UTRs. We amplified spliced *PETC* coding DNA sequence fragment by nested PCR with cDNA library as a template DNA. The 1st round of PCR from cDNA library with primers HK#1483 and HK#1484 generated a 731 bp DNA fragment. The following 2nd

round of PCR from the 1000 times diluted 1st round of PCR product with primers HK#1485 and HK#1486. The resulting DNA fragment of the PCR was ligated into the pSL18 vector (Depège et al., 2003) digested with NdeI and XbaI. The cloned DNA sequence of spliced *PETC* coding DNA sequence was validated by DNA sequencing with HK#1491 and HK#1492 primers. To introduce Pro171Leu in *PETC*, the *PETC* coding DNA sequence was replaced by In-Fusion HD cloning kit at NdeI and XbaI site with the PCR product consisting of two overlapping DNA fragments generated by the following primers; one fragment is generated with If-paDcC-NdeI_F and ccC_mP171L_R while the other fragment is generated with ccC_mP171L_F and If-paDcC-XbaI_R. The replaced DNA sequence was validated by DNA sequencing with the primers used to generate PCR product and cC_cdn131-137_F and cC_cdn137-131_R, and we obtained pccC-P171L plasmid.

Nuclear gene transformation

Nuclear transformation was performed with NEPA21 (Nepagene, Japan) according to the previous research (Yamano et al., 2013). The plasmid DNA was linearized by digesting with ScaI and the digested DNA was delivered into the Δ *PETC*. Selection of transformants was performed on MM solid medium under continuous illumination ($50\text{-}100\text{ }\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$).

SDS-PAGE

The whole cellular proteins were denatured by heating at 100°C for 2 minutes in the presence of SDS and DTT and the polypeptides were separated by SDS-PAGE (Figures 2, 3, 7 and 8). Subsequent solubilization of dried pellets and SDS-PAGE conditions were as above. The polypeptides were transferred on nitrocellulose membrane for immunodetection. The signals were visualized with a CCD camera equipped imagers (Amersham Imager, LAS-4000, GE healthcare, or Chemidoc, Biorad) and the signal intensities were quantified with Multigauge ver. 3.0 (FujiFilm, Japan)

Low temperature fluorescence emission spectra

Cells were resuspended in TP medium at $2\text{ }\mu\text{g Chl}\cdot\text{mL}^{-1}$ and incubated for overnight under illumination at $300\text{ }\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ prior to measurements. After the incubation, culture was

directly, state 1 inducible treatment, or state 2 inducible treatment at 25°C. For the case of state 1 inducible treatment, cells were incubated under 300 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ in the presence of 10 μM of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). For the case of state 2 inducible treatment, cells were incubated in the darkness in the presence of 100 mM glucose and 50 $\text{U}\cdot\text{mL}^{-1}$ of glucose oxidase. A sample in a NMR tube was in liquid nitrogen, excited with 430 nm illumination, and emission spectrum was recorded from 400 to 800 nm with FL-7000 (Hitachi, Japan).

***In vivo* Spectroscopy**

For *in vivo* spectrophotometric analysis, cells were maintained at 20 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ on agar-supplemented TAP plates (Sueoka, 1960). When growing cells for experiments, liquid TP medium was used. The cultures were grown at 40 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (16 h light/8 h dark) and were bubbled with sterile air at 25°C while shaking at 120 rpm. Cells were harvested after ~2-h in the light cycle. Grown cultures were diluted ~6-fold at least once after inoculation and grown to a density of $\sim 1 \times 10^6$ $\text{cells}\cdot\text{mL}^{-1}$ before harvesting (5000 rpm, 5 min, 25°C). Cells were resuspended at 20 $\mu\text{g Chlorophyll}\cdot\text{mL}^{-1}$ in TP supplemented with 20% (w/v) Ficoll. Oxic samples were resuspended throughout the measurements in 2 mL open cuvettes. For oxygen-deprived conditions, cells were supplemented with 50 mM glucose, 10 U glucose oxidase (Carl Roth, Germany) and 30 U catalase (Sigma-Aldrich, USA) in the cuvette, and then overlaid with mineral oil for 20 min in the dark. The enzyme stock solutions to obtain anoxic samples were freshly prepared. Cell incubation in the presence of 10 μM nigericin (from 10 mM ethanolic stock) was carried out for 20 min in the light before the measurements. All samples were light-adapted in the cuvette for 20 min before the measurements using LEDs emitting ~ 150 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ of 630 nm actinic light (AL). Measurements of the electrochromic shift (ECS) and P700 were obtained using a Joliot-type spectrophotometer (JTS-10, Biologic France in Figure 4; JTS-150, Spectrologix USA in Figure 5) and are described in detail elsewhere (Buchert et al., 2020). In brief, all absorption changes were normalized to the ECS $\Delta I/I$ signals (520 nm – 546 nm) produced after a saturating 6-ns laser flash in the presence of 1 mM hydroxylamine (HA) and 10 μM DCMU, which relate to the density of active PSI centers in the sample. Thus, the unit of 1 charge separation $\cdot\text{PSI}^{-1}$ is produced when measuring the

flash-induced ECS amplitude (rapidly established before the first detection at ~300-ms). This optical ruler was determined in separate measurements to compare sample activities regardless of the presence of HA and DCMU. The shown flash-induced ECS kinetics were obtained after lowering the flash intensity to 30-45% saturation to avoid double turnovers of the cytochrome *b₆f* complex. The ECS was also deconvoluted in the dark pulse method (Joliot and Joliot, 2002; reviewed in Bailleul et al., 2010; Nawrocki et al., 2019). Accordingly, AL-adapted cells experienced 30 s darkness and were re-illuminated with AL (Supplemental Figure 1). During this 10 s AL induction phase, activities of stable charge separations $\cdot s^{-1} \cdot PSI^{-1}$ were measured by brief light shuttering at discrete time points (four technical replicates with 1 min AL in between; data assembly from two independent measurement sequences). For AL interruptions at $t = 0$ ms, the linear ECS slopes before (S_L from -4 ms to -1 ms) and after darkness (S_D from +1 ms to +5 ms) gave electron transfer rates before darkness ($S_L - S_D$). Rates at 1 ms illumination of light-adapted cells were calculated from separate slopes obtained during 2 ms AL after 30 s dark (15 replicates spaced by 5 s). P700 measurements (705 nm – 740 nm) are based on the method by (Klughammer and Schreiber, 1994), measuring AL-adapted cells. As shown in Supplemental Figure 2, the fractions of photooxidizable (by a 22 ms saturating pulse), pre-oxidized in AL and non-photooxidizable P700 were determined by referring to the fully oxidized P700 signals in PSII-inhibited HA/DCMU samples (Buchert et al., 2020). Since the above-mentioned ECS slopes obtained during 2 ms AL are a function of the P700 oxidation rate in PSII-inhibited HA/DCMU samples, the obtained YI parameter in those samples served to calculate CEF (by multiplication with the P700 oxidation rate) as it a function of the concentration of reduced P700 in AL (Takahashi et al., 2013).

Chlorophyll fluorescence measurement

Cells were grown in liquid TP medium to $\sim 1 \times 10^6$ cells $\cdot mL^{-1}$ with gentle shaking at 25°C under continuous light (50 μmol photons $\cdot m^{-2} \cdot s^{-1}$). The cells were harvested by centrifugation at 4000 rpm, suspended in fresh liquid TP medium at 5 μg Chl $\cdot mL^{-1}$, and placed in darkness for 30 min (growth light control samples). Photosynthesis driven by 450 nm LEDs was measured with a Maxi-Imaging PAM chlorophyll fluorometer (Heinz Walz, Germany). The effective photosynthetic active radiation

1 in the measuring chamber was determined with a Quantitherm PAR/Temp Sensor (Hansatech,
2 England). Cell aliquots were also incubated shaking for 4 h under high light illumination ($500 \mu\text{mol}$
3 $\text{photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$). The maximal PSII quantum yield in dark-adapted samples and the PS II quantum
4 yield during illumination $Y(\text{II})$ were determined as $(F_m - F_o)/F_m$ and $(F_m' - F)/F_m'$, respectively. F_o
5 and F_m of dark-adapted samples are respective minimal (in the presence of detection light) and
6 maximal fluorescence yields (due to closed PSII centers after saturating pulse). F and F_m' are the
7 fluorescence yields in light-adapted cells obtained before and after a saturating pulse, respectively.
8 The nonphotochemical quenching $\text{NPQ} = (F_m - F_m')/F_m'$ was also determined, as well as estimation
9 of the plastoquinone pool redox state using the $(1 - q_L)$ parameter (Kramer et al., 2004). The latter
10 required F_o' measurements (minimal fluorescence in illuminated samples) and q_L was calculated as
11 $((F_m' - F) \times F_o') / ((F_m' - F_o') \times F)$. These parameters were either measured after 30 s during light curve
12 experiments or at distinct time points when a 10 min continuous illumination at $95 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$
13 was applied.

Results

The PETC protein consists of one transmembrane helix and a luminal hydrophilic domain in which Rieske iron sulfur cluster (ISC) is present, and both domains are connected through a flexible hinge. Based on the structural similarities with the Rieske subunit in *bc₁* complex of respiratory chain, photosynthetic electron transfer between Rieske ISC and heme *f* in PetA involves reversible movements of the luminal hydrophilic domain of PETC. The PETC amino acid sequence alignment shows that chloroplast targeting transit peptide of *A. thaliana* is 23 amino acids longer than that of *C. reinhardtii* while the mature protein sequence is highly conserved (Supplemental Figure 3). The four amino acids for ligation of two iron atoms and a flexible hinge region are conserved. Moreover, the mutated proline residue in Arabidopsis *pgrl* (Pro194) is conserved as Pro171 in *C. reinhardtii*. The location of this proline is close to Rieske ISC and is on the surface of hydrophilic domain (Figure 1B, C, and D), and its proximity to the Rieske ISC is conserved in the *b₆f* structures (Supplemental Figure 4).

Generation of *PETC-P171L* mutant in the green alga *Chlamydomonas reinhardtii*

The Arabidopsis *pgrl* mutant carrying single amino acid mutation in PETC (P194L) exhibits a hypersensitive photosynthetic control and downregulate the electron transfer flow under strong light illumination more strongly than wild type (Munekage et al., 2001). To characterize the corresponding PETC mutation in the green alga *C. reinhardtii*, we generated a *PETC-P171L* strain in which proline 171 is substituted by leucine. In our complementation-based screening approach, *petC-ΔI* was selected as a recipient strain since it has a frameshift upon TG base deletions within the 36th codon and does not accumulate *PETC* transcripts, thus showing no spontaneous reversion (de Vitry et al., 1999). Since the *petC-ΔI* exhibited a reduced accumulation of PSI protein, we backcrossed the *petC-ΔI* to wild type strain (cc-125) to assess whether the mutation of PETC gene affects the accumulation of PSI proteins. We obtained several tetrads and analyzed the accumulation of PsaA and PETC by immunoblotting (Figure 2). The T₁₅₋₃ progeny accumulates no PETC but PsaA normally and is referred to as the ΔPETC for the subsequent transformation since the two phenotypes were segregated. As described in Materials and Methods, we generated two types of vectors for transforming *PETC* gene; one consists

of genomic DNA fragment around PETC gene and the other contains PETC cDNA fused with *PSAD* promoter and UTRs. We delivered each transforming vector into the Δ PETC cells by electroporation and obtained numerous colonies after screening under photoautotrophic condition (on minimum solid medium in the light of $50 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). The resulting transformants recovered the accumulation of PETC to wild-type level, indicating that both vectors efficiently complemented the Δ PETC phenotype (Figure 3). Subsequently, we transformed Δ PETC with vectors containing the mutation PETC-P171L and obtained complemented strains expressing the mutated PETC. Immunoblot shows that the complemented PETC-P171L cells accumulated PETC as well as PetA at wild-type levels, suggesting that the mutation does not affect the stability of *b₆f*.

The *PETC-P171L* accumulates more oxidizable P700 and displays slower onset of electron transfer in anaerobic algae which relied on the lumen pH under these conditions.

Previous studies showed that high light treatment slows down ATP synthase activity in *Chlamydomonas* (Buchert et al., 2017), which eventually facilitates the induction of photosynthetic control. Since this type of H^+ conductivity regulation involves reactive oxygen species formation during photosynthetic electron transfer, which may differ between the strains, we chose a permissive light intensity for the following experiments ($\sim 160 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ actinic red light) where this ATP synthase regulation is not expected. Instead, *pmf* partitioning was modulated on a metabolic level in light-adapted cells by lowering oxygen availability. A similar situation may occur in the natural habitat of *Chlamydomonas* which shares it with respiring microbes. Accordingly, the alga faces microoxic environments in which photosynthesis needs to be fine-tuned. Figure 4 shows a spectroscopic *in vivo* analysis of control and the *PETC-P171L* in oxic conditions and upon anaerobic treatment. Mitochondrial congestion in anoxic samples coincides with a redox poise shown to trigger CEF (Alric, 2014) and a *pmf* re-partitioning towards a more pronounced Δ pH (Finazzi and Rappaport, 1998).

Figures 4A and 4B display the redox state of P700, the primary electron donor of PSI (see Materials and Methods and Supplemental Figure 2 for representative kinetics). Under oxic conditions when PSII was active, the P700 redox states were indistinguishable between control and *PETC-P171L* (Figure

4A). About 20% of P700 were oxidized during actinic background light which is ascribed to similar donor side limitation (YND) in both strains. P700 was able to be further photooxidized by a short saturating light pulse, as shown by the yield of PSI (YI). A similar fraction (about 40%) of P700 was not oxidizable due to acceptor side limitation (YNA). On the contrary, YND was remarkably large when PSII was inhibited (HA+DCMU, Figure 4A) because electrons from PSII were suppressed. This coincided with YI of ~ 0.2 in control, i.e., 20% of P700 remained reduced in the light-adapted state in absence of PSII activity and were only oxidized by saturating pulses. This finding suggests that this 20% of P700 is reduced by electrons through CEF. YI in controls was roughly twice as large as in *PETC-PI7IL*, which translates in lower CEF rates in the mutant (indicated as $12 \text{ electrons} \cdot \text{s}^{-1} \cdot \text{PSI}^{-1}$ in parenthesis of Figure 4A).

Under anoxic conditions, control showed no significant difference in redox states of P700 (Figure 4B). However, the *PETC-PI7IL* established a larger YND and a lower YNA compared to control when PSII was active. In the absence of PSII activity, control and *PI7IL* showed similar P700 parameters. This finding shows that CEF rates in the steady state were unaffected in both strains (shown as $\text{electrons} \cdot \text{s}^{-1} \cdot \text{PSI}^{-1}$ in parenthesis of Figure 4B). Overall, we observed subtle differences on the level of light-adapted steady-state of P700. The differences were produced in the absence of PSII activity under oxic conditions and in the presence of PSII activity under anoxic conditions.

Next, we focused on the transition from dark to light, beginning with electrochromic shift measurements after 30-s darkness (Figure 4C). As mentioned above, a significant ΔpH is generated when algae are kept in the dark under anoxic conditions (Finazzi and Rappaport, 1998), which supposedly slows down the Qo-site activity in *PETC-PI7IL*. The electrochromic shift (ECS) of photosynthetic pigments allows to measure the membrane potential as the ECS signals linearly follow the electric *pmf* component (Junge and Witt, 1968; Witt, 1979). Figure 4C resolves the averaged *b*- and *c*-phases of the ECS kinetics produced by a sub-saturating laser flash (reviewed in Bailleul et al., 2010). The non-resolved *a*-phase with an amplitude of ~ 0.6 charge separations $\cdot \text{PSI}^{-1}$ relied on the charge separation activity of both photosystems. The *b*-phase, which is a ~ 10 -ms ECS rise, involves charge separation activity via the *b_{6f}* and the *c*-phase depends on ATP synthase activity. The latter phase overlapped the *b*-phase since ATP synthase activity was driven by the *pmf* provided from

photosystem activity. Under oxic conditions, the fast *c*-phase prevented the appearance of a *b*-phase (Supplemental Figure 5). Therefore, the detection of an apparent *b*-phase requires a slow *c*-phase in anoxic algae (Velthuys, 1978). The ECS kinetics in anoxic *PETC-PI7IL* displayed a flat *b*-phase and comparably slow *c*-phase kinetics (Figure 4C), suggesting a compromised *b₆f* charge separation activity in *PETC-PI7IL*. To explore whether the *b₆f* performance during single turnover measurements affects the photosynthetic induction phase, we re-illuminated cells after a 30-s dark period. More specifically (see Materials and Methods), we measured electron transfer transients during the first 10-s of light in anoxic (Figure 4D) and oxic conditions (Figure 4E). The developing induction kinetics under anoxic conditions showed that the sum of LEF and CEF activities was transiently less efficient in *PETC-PI7IL*, producing the largest differences within the first 25 ms of light (cf. filled symbols and asterisk in Figure 4D). When PSII was inhibited (open symbols Figure 4D), no differences were observed between both strains. On the total electron transfer level, the induction kinetics were similar in both strains under oxic conditions (Figure 4E). The sum of LEF and CEF activities in the control strain was slightly higher at 4-s and 10-s of light (filled asterisks, Figure 4E). The kinetics were almost identical when PSII was inhibited, except for 100-ms of light where *PETC-PI7IL* produced slightly higher rates (open asterisk, Figure 4E).

To examine phenotype of anoxic *PETC-PI7IL* cells further, we repeated the electron transfer and P700 experiments in the presence and absence of nigericin (Figure 5). The H^+/K^+ ionophore exchanges the osmotic *pmf* component ΔpH for the electric one, $\Delta \Psi$. Thus, the drug was suitable to test the contribution of the previously reported ΔpH buildup (Finazzi and Rappaport, 1998), and its impact during the transition to sub-saturating light in dark-adapted anoxic *PETC-PI7IL*. Indeed, Figure 5A shows that nigericin action in the preceding 30-s of darkness abolished the *PETC-PI7IL* electron transfer bottleneck in the first ~50-ms of light. Moreover, the steady state rates were elevated in both strains when nigericin was present. In these cells, P700 redox states were changed upon nigericin addition (Figure 5B). In the absence of the drug, the control strain established a larger YNA and thus a smaller YI than *PETC-PI7IL*. The YND parameter was lowered to similar levels after adding nigericin. Upon nigericin addition, *PETC-PI7IL* cells established YNA and YI like control. Moreover, samples were indistinguishable in the absence of PSII activity (HA+DCMU, Figure 5B).

Taken together, these spectroscopic findings reveal that the P700 redox states under the actinic light illumination are differently distributed in *PETC-PI7IL* cells when oxygen levels were low. The different P700 redox state in *PETC-PI7IL* relied on upstream events on the level of the cytochrome *b₆f* and the Δ pH component of the *pmf* could be identified as being one of the limiting factors in oxygen-deprived algae. In most experiments at $\sim 160 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ actinic red light, *PETC-PI7IL* performed like control strains when cells were well aerated, suggesting that the acidification of the lumen pH in the permissive light was insufficient to trigger the cytochrome *b₆f* phenotype under these conditions. In the last two results sections, we will explore the *PETC-PI7IL* performance in the presence of oxygen at various light intensities.

The *PETC-PI7IL* mutant showed an impaired photoautotrophic growth rate under high light

We evaluated whether the *PETC-PI7IL* mutation has an impact on cellular growth over the course of days. The control and *PETC-PI7IL* cells grown in liquid medium were spotted on solid medium and the cell growth was observed (Figure 6A). The cells showed a similar photoheterotrophic growth under moderate light illumination on solid TAP medium. On the other hand, when cells were grown photoautotrophically on solid TP medium, *PETC-PI7IL* cells grew more slowly than control cells under illumination of $100 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and $600 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Although the *PETC-PI7IL* cells were able to grow photoautotrophically, the relative growth rate of the mutant cells was apparently was slower in the stronger light (Figure 6A). We also analyzed the photoautotrophic growth of control and *PETC-PI7IL* strains in liquid medium under illumination of $200 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. We monitored the chlorophyll concentration of liquid culture as a measure of cell growth (Figure 6B). The doubling times of control and *PETC-PI7IL* were estimated to be 9.4 and 14 hours, respectively, indicating that *PETC-PI7IL* cells grow 1.5 times slowly under the high light condition.

The *PETC-PI7IL* shows an impaired NPQ induction under strong light illumination

The slower photoautotrophic growth of *PETC-PI7IL* cells may be ascribed to the lower activity of the mutated *b₆f* and/or to their stronger photosensitivity under strong light illumination. Then, we analyzed

the expression of LHCSR3 by immunoblotting since the protein is crucial for induction of algal NPQ. It was revealed that LHCSR3 was similarly induced after 4 h of strong light illumination in control and *PETC-PI71L* cells (Figure 7A). Subsequently the induction of state transitions was also assessed by measuring fluorescence emission spectra at 77K. It was shown that control and the *PETC-PI71L* cells exhibited higher intensity of PSII core fluorescence emission (peaking at 688 nm) in state 1 while the PSI core fluorescence emission (peaking at 716 nm) was higher in state 2, indicating both state 1 and state 2 were fully induced (Figure 7B). Thus, the *PETC-PI71L* mutation showed normal expression of LHCSR3 and induction of state transitions.

Next, we measured light-dependent steady state chlorophyll fluorescence parameters under high light conditions and, like in Figure 7A, the analyzed *PETC-PI71L* cells showed control-like LHCSR3 accumulation upon high light treatment (Figure 8A). The latter observation ensured that NPQ could be measured *per se*. As expected, PAM fluorimetry light curve experiments revealed low NPQ in both strains which were not exposed to 4 h high light (cf. filled symbols in Figure 8B). Interestingly, only control cells showed increased NPQ when pre-treated in high light (cf. open symbols in Figure 8B). The PSII quantum yield $Y(II)$ was very similar between the strains at irradiances below $30 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ of actinic blue light, except for the F_v/F_m in high light treated cells which was lower in *PETC-PI71L* (cf. $Y(II)$ at lowest irradiance, inset in Figure 8C). At higher irradiances, $Y(II)$ in controls was above that in *PETC-PI71L* irrespective of high light pre-treatment (Figure 8C). The NPQ induction over a 10 min light period indicated that a lower capacity in *PETC-PI71L*, and that high light exposure stimulated NPQ only in the control strain (Figure 8D). After the 10 min light, NPQ collapsed rapidly within the first 30 s of darkness which was followed by a transient NPQ increase. The $Y(II)$ was lowest in the mutant during 10 min of illumination, but the values recovered similarly in all strains during subsequent darkness (Figure 8E). The related $(1-qL)$, indicative for the PQ pool reduction state, was highest in illuminated *PETC-PI71L* and the NPQ-displaying control strains maintained a more oxidized PQ pool during 10 min of light (Figure 8F). Taken together, the chlorophyll fluorescence parameters in *PETC-PI71L* suggested a limitation of photosynthesis at elevated irradiances on the level of cytochrome *b₆f* complex.

Discussion

In the current work we successfully generated a *PETC-P171L* strain in *C. reinhardtii*, harboring a genetic site-directed alteration corresponding to the mutation in *pgr1* of *A. thaliana* (Shikanai et al., 1999; Munekage et al., 2001; Jahns et al., 2002; Okegawa et al., 2005). In *C. reinhardtii*, this mutation did not affect *b₆f* accumulation but diminished the electron transfer rate due to lumenal acidification. In Arabidopsis, the *pgr1* mutant showed reduced NPQ owing to cessation of total electron transfer at the level of *b₆f* at elevated light intensities above $\sim 200 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (Munekage et al., 2001). It was further found that the pH-dependent inactivation of electron transfer via the *b₆f* was shifted by about 1 pH unit towards more alkaline pH values using *pgr1* thylakoids from Arabidopsis (Jahns et al., 2002). Our data indicated similar effects that *PETC-P171L* is sensitive to high light due to an inefficient photosynthesis. The algal mutant was capable of state transitions but grew slower, and anoxic treatment revealed that the ΔpH -dependent slow-down of electron transfer through the *PETC-P171L b₆f* complex was already induced at lower light intensities.

The *PETC-P171L* mutation slows down the electron transfer through *b₆f* in lumenal pH acidification.

The previous studies observed strong NPQ reduction in *pgr1* after high light illumination, linked to suppression of electron transfer in the mutant plants (Munekage et al., 2001; Okegawa et al., 2005; Yamamoto and Shikanai, 2019). Our investigation of factors triggering photosynthetic control in algae revealed similarities to vascular plants, i.e., the bottleneck of electron transfer in *PETC-P171L* was detected in high light conditions (Figure 8). We ruled out that state transitions were affected by the Qo-site mutation (Figure 7) and also evaluated the photosynthetic machinery of *PETC-P171L* during permissive actinic illumination (Figures 4 and 5). The light intensity was kept low to avoid a potential source of variability by a downregulation of ATP synthase as a side effect of photosynthesis in high light (Buchert et al. 2017). Here, we identified a significant *b₆f* defect in *PETC-P171L* under anoxia which had an impact on faster electron flow cessation during photosynthetic induction and facilitated P700 oxidation in the light. The addition of nigericin, exchanging ΔpH for $\Delta\Psi$, abolished the impact of the mutation. Anaerobiosis increases the ΔpH contribution to the *pmf* in darkness, which depends

on functional ATP synthase (Finazzi and Rappaport, 1998; Rappaport et al., 1999). Oxygen deprivation caused a charge separation deficiency of the b_6f in *PETC-PI71L*, which is a consequence of delayed mutant Qo-site turnover. In fact, a cooperativity of high- and low-potential chain reactions has been documented in previous Rieske iron sulfur protein (ISP) mutants of the cytochrome bc_1 complex from yeast (Snyder et al., 2000). Still, this coupling mechanism operating between Qo- and Qi-site remains elusive. The authors mutated homologous Rieske ISP residues at position 157 and 159 (orange-colored rectangle in Supplemental Figure 3), not too far from proline 171 of *C. reinhardtii* PETC, which eventually result in a more reducing E_m and a slowdown of b -heme reduction (Snyder et al., 2000). Thus, similar effects could be anticipated in *PETC-PI71L* provided that the mutation lowers the Rieske ISP E_m b_6f as originally proposed (Munekage et al., 2001; Jahns et al., 2002). However, the Rieske ISP E_m potential has not been determined in *pgr1*. Previous data indicated that the E_m potential of reconstituted recombinant *C. reinhardtii* Rieske ISP is pH-dependent with a pK_{ox} of 6.2 and a pK_{red} of 8.2 (Soriano et al., 2002). The ISP E_m potential changes in the pH-dependent region with -40mV/pH. Thus, lowering the Rieske ISP E_m via the PETC-PI71L mutation could impact pK_{ox} and pK_{red} and might alter inter b_6f electron transfer as shown in yeast (Snyder et al., 2000). In an alternative scenario, the PETC-PI71L mutation might alter binding of PQH₂ to the Qo-site and consecutively change the pH-dependent redox properties and oxidation of PQH₂. The Rieske ISC is found in the hydrophilic domain of PETC (Figure 1B) and, based on structural similarities with bc_1 complex (Darrouzet, Valkova-Valchanova, Moser, Dutton, & Daldal, 2000), it is supposed to be a flexible hydrophilic domain to realize electron transfer between redox centers Rieske ISC and heme f . Additionally, proline 171 mutated in this study is the second proline of the “Proline-X-Proline” motif, which is a common structure at the beginning of turn structure (MacArthur & Thornton, 1991). This motif is exclusively found in PETC protein and conserved among Rieske ISP (Supplemental Figure 3). Accordingly, this motif forms a typical hairpin structure and the substitution of the second proline to leucine may disturb conformation of PETC hydrophilic domain structure, causing the functional impact in the redox chemistry of b_6f .

Our findings revealed that the generated ΔpH in anaerobic *Chlamydomonas* during 30-s of darkness was sufficient to trigger severe photosynthetic control in *PETC-PI71L*, preventing efficient b -hemes

reduction. Moreover, the nigericin data also revealed an important contribution of the ΔpH in anaerobic cells. Accordingly, b_6f regulation is important for PSI photoprotection in a reducing cellular environment such as anaerobiosis. The differential regulation of pH dependent inactivation of electron transfer via the b_6f in oxic and anoxic conditions in *PETC-PI71L* is surprising. This might suggest that depending on oxygen availability (or metabolic congestion), the regulation of photosynthetic control is distinct in *C. reinhardtii*. However, revealing a low-light *pgr1* phenotype may also occur in plants when metabolism becomes restricted, e.g. under drought stress conditions where a downregulated ATP synthase activity favors large ΔpH amplitudes (Kohzuma et al., 2009). How can this be rationalized? Three non-exclusive scenarios may occur: First, metabolic control of ATP synthase downregulates H^+ conductivity which theoretically facilitates a light-dependent ΔpH build up in anaerobic conditions. Second, sustaining a ΔpH in an ATP synthase-dependent manner is favored in anoxic algae even in darkness (Finazzi and Rappaport, 1998; Rappaport et al., 1999). Third, the regulation of b_6f electron transfer via the Q-cycle is distinct under oxic and anoxic conditions (Buchert et al., 2020). Under anoxic conditions stromal electron intake into b_6f changes the Q-cycle from a canonical cycle during LEF to an alternative cycle during CEF, which would facilitate more efficient proton translocating into the lumen as compared with the canonical Q-cycle (Buchert et al., 2020). In line, photosynthetic control would be faster activated under anoxia and revealed the *PETC-PI71L* phenotype even in low-light conditions.

Taken together, *PETC-PI71L* may be a useful tool for future investigations under various metabolic and environmental conditions to uncover details of the cooperative mechanism between Qo - and Qi -site. Moreover, *PETC-PI71L* may help to elucidate the interplay of various *pmf*-shaping ion channels/antiporters involved to fine-tune *pmf* partitioning in algae in response to metabolic constraints.

PETC-PI71L mutation retards photoautotrophic cell growth.

The growth phenotype of *pgr1* plants under different light intensities has not been addressed in literature (Shikanai et al., 1999; Munekage et al., 2001; Yamamoto and Shikanai, 2019). We found

that *PETC-PI7IL* grew less efficiently under photoautotrophic condition at growth rates 1.5 times slower in the logarithmic phase than control (Figure 6 and Supplemental Figure 6). Despite similar amounts of LHCSR3 in the control and *PETC-PI7IL* cells, NPQ could not be fully established in *PETC-PI7IL* and PSII performance was low due to a more reduced PQ pool in the mutant (Figures 7 and 8). This analysis shows the *PETC-PI7IL* mutation impacts cellular growth and underlines the importance of a balanced photosynthetic control in wild type cells (control strain), avoiding premature cessation of photochemistry as a function of light intensity.

Taken together, *PETC-PI7IL* exhibits a reduced photosynthetic electron transfer rate because of its hypersensitive photosynthetic control mechanism. The light intensity threshold to trigger the hypersensitive effect in the mutant *b₆f* can be modulated by the metabolic state of the cell. The molecular machinery to realize energy quenching and balancing of light harvesting is established as in wild type *b₆f* strains. These results are consistent with Arabidopsis studies, indicating that the effect of the proline substitution in PETC on electron transfer is *per se* conserved between the two organisms.

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1 **Figures**

2 **Figure 1**

3 **The position of Rieske ISC and PETC-Pro171 in the *Chlamydomonas b₆f* complex**

4 Structures of the dimeric *b₆f* from the *C. reinhardtii* (PDB; 1Q90). The flexible hinge of PETC is not
5 modeled. The Rieske ISCs are shown in space filling representation and of which ligand amino acids
6 (Cys134, His136, Cys152, and His155 of PETC) and Pro171 of PETC (colored in magenta) and four
7 hemes (heme *c_i* is yellow, heme *b_H* and heme *b_L* are blue, and heme *f* is orange) are shown in ball-stick
8 model. The PetA (orange), PetB (blue), PETC (gray), PetB (green), PetG (magenta), PetL (yellow),
9 PETM (cyan), and PETN (red) subunits are shown. (A) and (C). Overall structure with the luminal
10 side at bottom and the stromal side at the top. The position of one of the Rieske ISC is indicated with
11 arrow. (B) and (D). The enlarged view around the Rieske iron sulfur cluster. (C) and (D). Protein
12 surface is modeled. The subunits and cofactors except for PETC subunit are transparent in panel A and
13 B.

Figure 2

Isolation of PSI suppression free PETC deleted strain

The *petC-Δ1* was backcrossed with cc-125 and the resultant progenies were analyzed by immunoblotting using the antibodies against PsaA or PETC. Whole cellular proteins (0.5 μg Chl per lane) were denatured, separated by SDS-PAGE, and analyzed by immunoblot. The polypeptides transferred on nitrocellulose membrane were stained with Ponceau (the migration positions of protein standard are shown). The proteins of progenies (1 to 4) from four independent perfect tetrads (T₉, T₁₀, T₁₅, and T₂₁) and their parent strains (cc-125 and *petC-Δ1*) are loaded.

Figure 3

Generation of *PETC-P171L* mutant

The accumulation of PSI (PsaA) and *b₆f* (PETC and PetA) proteins was analyzed by immunoblotting. The cells were grown in TAP liquid medium in the light of 50 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The Pro171Leu of PETC (*PETC-P171L*) and wild type sequence of PETC (control), the PETC gene expressing with own promoter and UTRs (genomic) or the spliced PETC gene expressing with PSAD promoter UTRs (cDNA). Four independent clones from each transformant, a dilution series for the wild type (wild type (%)), and Δ PETC (T_{15-3} in Figure 2) were also analyzed.

Figure 4

The *PETC-P171L* mutation restricts photosynthetic performance of light- and dark-adapted cells predominantly in anaerobic conditions.

P700 redox states were measured in (A) oxic and (B) anoxic light-adapted cells. The fractional yields of P700 in the photo-oxidizable state (YI), the non-oxidizable form due to acceptor side limitation (YNA), and the pre-oxidized P700 due to donor side limitation (YND) are shown (parameter-specific one-way ANOVA, $n = 3$, $P < 0.05$). The oxic controls in absence of PSII activity (HA+DCMU) retained a higher YI due to enhanced steady state CEF (expressed charge separations $\cdot \text{s}^{-1} \cdot \text{PSI}^{-1} \pm \text{SD}$). In anoxic conditions, *PETC-P171L* failed to establish the YNA due to larger YND. (C) The electrochromic shift ~ 10 -ms rising phase, associated with charge separation activity upon a single b_6f turnover, was less pronounced in anoxic light-adapted *PETC-P171L*. Sub-saturating flashes were fired on light-adapted cells after a 30-s dark period (averaged kinetics $\pm \text{SD}$, $n = 3$). (D) After a 30-s dark period, electron transfer rates over a 10-s photosynthetic induction phase were measured in anoxic

1 light-adapted cells. Total electron transfer in *PETC-P171L* was significantly less efficient at discrete
 2 time points, especially in the initial ~25-ms (filled asterisk, Student's t-test $P < 0.05$). A significant
 3 difference during induction required PSII activity. **(E)** The induction phase was indistinguishable in
 4 oxic conditions. Total electron transfer rates in the controls were enhanced at a later stage of
 5 illumination (filled asterisks), and *PETC-P171L* showed transiently higher rates at 100-ms in absence
 6 of PSII activity (open asterisk).

Figure 5

The electron transfer restrictions in *PETC-P171L* cells under anaerobic conditions is overcome by diminishing the ΔpH via nigericin.

(A) After a 30-s dark period, electron transfer rates over a 10-s photosynthetic induction phase were measured in anoxic light-adapted cells (averaged kinetics $\pm$ SD, $n = 3$). Total electron transfer in *PETC-P171L* was significantly less efficient within the first ~50-ms (filled asterisk, Student's t-test $P < 0.05$). The different induction kinetics between the strains relied on the ΔpH and became similar after addition of the H^+/K^+ ionophore nigericin (Nig., 10 μM), although the control showed higher rates at discrete sampling intervals (open asterisk). In both strains the steady state rates were higher when Nig. was present. (B) On the level of co-dependent PSI parameters, the fractional yields of P700 in the photo-oxidizable state (YI), the non-oxidizable form due to acceptor side limitation (YNA), and the pre-oxidized P700 due to donor side limitation (YND) are shown (parameter-specific one-way ANOVA, $n = 3$, $P < 0.05$). The YI was larger in *PETC-P171L* owing to a diminished YNA. YND was lowered to similar levels after adding Nig. and diminishing ΔpH -dependent photosynthetic control. Moreover, further PSII inhibition (HA+DCMU) produced similar P700 yields.

Figure 6

The photoautotrophic growth rate is slowed down at low cell density in *PETC-P171L*.

(A) Cells were grown photoheterotrophically on solid TAP medium ($50 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) or photoautotrophically on solid TP medium (100 or $600 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$). Three independent clones of the control and *PETC-P171L* strain (*P171L*) were analyzed. (B) Photoautotrophic cell growth is monitored by measuring Chlorophyll concentration of the TP liquid culture medium grown under continuous light ($200 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$). Black line represents control and red line represents *PETC-P171L* (*P171L*). Three biological replicates were averaged ($\pm$ standard errors).

Figure 7

The *PETC-P171L* mutation does neither affect LHCSR3 expression induction and/or stability, nor induction of state-transitions.

(A) The cells grown in TP medium were shifted from low light ($15 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) to stronger light ($200 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and immune-chase experiment with the antibody against LHCSR3 protein was performed for cellular proteins. Cells were harvested before shifting to higher light (0), after 4, 8, 12, and 24 hours shifting to high light. Ponceau staining panel served as loading control. (B) The TP-grown *Chlamydomonas* cells acclimated under high light ($300 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) were resuspended with TP at $2 \mu\text{g Chlorophyll}\cdot\text{mL}^{-1}$ and incubated in state 1 or state 2 inducible condition. In the case of state 1 inducible treatment, cells were incubated under $200 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ in the presence of DCMU for 10 minutes. In the case of state 2 inducible treatment, cells were incubated in the darkness for 10 minutes in the presence of 100 mM glucose and $50 \text{ U}\cdot\text{mL}^{-1}$ glucose oxidase. Samples were excited with 430 nm and the emitted fluorescence were recorded from 600 to 800 nm in liquid nitrogen. The fluorescence spectra were normalized at 688 nm .

Figure 8

The chlorophyll fluorescence parameters reveal a limited photosynthetic capacity in *PETC-P171L*.

(A) Immunodetection of LHCSR3 protein was obtained from growth light samples (50 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and after 4 h high light treatment (500 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Based on chlorophyll loading (100% = 3 μg) and the AtpB reference, LHCSR3 accumulation and induction was comparable in the control and *PETC-P171L* cells. A representative Western Blot is shown and the chlorophyll fluorescence parameters in panels B to F are means three biological replicates ($\pm$ standard deviations).

(B) The control strains showed more NPQ during light curve experiments which was further increased upon high light treatment, unlike in *PETC-P171L*. (C) The PSII quantum yield, $Y(II)$, was comparable in growth light samples up to 30 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ of actinic light (inset), and $Y(II)$ remained higher in the control strains when light was further increased. The strain-specific $Y(II)$ developments showed a similar trend in high light samples. (D) The NPQ induction over 10 min of light was more efficient in control strains irrespectively of high light pre-treatment, although it enhanced NPQ capacity. (E) The corresponding $Y(II)$ was also lower in *PETC-P171L* during the light period. The $Y(II)$ in high light treated mutants increased rapidly upon transition to darkness and (F) the increased

1 (1-qL) in those samples suggested a strongly reduced plastoquinone pool during the preceding
2 illumination.
3

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