Central in respiration or photosynthesis, the cytochrome bc₁ and b₆f complexes are regarded as functionally similar quinol oxidoreductases. They both catalyse a redox loop, the Q-cycle, which couples electron and proton transfer. This loop involves a bifurcated electron transfer step considered as being mechanistically mandatory, making the Q-cycle indispensable for growth. Attempts to falsify this paradigm in the case of cytochrome bc₁ have failed. The rapid proteolytic degradation of b₆f complexes bearing mutations aimed at hindering the Q-cycle has precluded so far the experimental assessment of this model in the photosynthetic chain. Here we combine mutations in Chlamydomonas that inactivate the redox loop but preserve high accumulation levels of b₆f complexes. The oxidoreductase activity of these crippled complexes is sufficient to sustain photosynthetic growth, which demonstrates that the Q-cycle is dispensable for oxygenic photosynthesis.
Cytochrome b₆f and bc₁ are homologous protein complexes having a major role in photosynthetic and respiratory electron transport chains. They contribute to building up the proton motive force via the Q-cycle¹⁻³ depicted in Figure 1 and Supplementary Figure S1. This redox loop couples the consecutive oxidation of two quinolines at the Qᵣ site to the reduction of one quinone at the Qₛ site through the low-potential chain and of two plasto-cyanins along the high-potential chain. It increases the ratio of H⁺ pumped per electron transferred and thus the overall energetic efficiency of the complex. In cytochrome b₆f, the low-potential chain involves two b haems, b₈ and b₆ (the subscripts l and h stand for low and high midpoint potential), and a single covalently bound c-type haem, c₁⁻⁴, in close vicinity with the b₈ haem as depicted in Figure 1. In the conditions tested so far, the inactivation of the Qₛ site of the cytochrome bc₁ of purple photosynthetic bacteria forbids photosynthetic growth⁴⁻¹¹. In the oxygenic photosynthetic chain, attempts to inactivate the Q-cycle by knocking out the b₈ haem with mutation of its histidine axial ligand have failed until now because, at variance with the bc₁ case⁶, mutation of His202 dramatically decreases the accumulation of the b₆f complex¹¹.

Here we engineered in the green alga *Chlamydomonas reinhardtii* a strain restoring the accumulation of b₆f complexes although lacking haems b₈ and c. We show that it sustains photosynthetic growth and propose a mechanism accounting for this growth despite a broken Q-cycle.

**Results**

The Q₆KO strain has b₆f complexes but lacks haems b₈ and c. To overcome the accumulation defect resulting from the H₂O₂Q mutation in the petB gene encoding cytochrome b₈, we genetically combined it with the R420C mutation¹² in the chloroplast protease FtsH1. The double mutant, referred to as Q₆KO, contains a wild-type level of cytochrome b₆f complex (Fig. 2a,b), which is in marked contrast with the parental single mutant petB-H₂O₂Q. We purified by affinity chromatography the Q₆KO b₆f complex, which contains, expectedly, a decreased amount of b₈ haem (30%) as shown by the UV-visible spectra in Figure 2c. This lower-than-expected content (30 versus 50%) stems from the instability of the solubilized complex. Indeed, we assessed the amount of remaining b₈ haem *in vivo* and found that it matched the amount of b₈ haem from the control strain (Fig. 3a, black filled symbols). Importantly, in addition to lacking the b₈ haem, the rescued b₆f complex also lacked the c haem as evidenced by Figure 2b,c, as suggested for petB-H₂O₂Q.⁶ We thus successfully recovered, in the Q₆KO strain, a b₆f complex with a fully inactivated Qₛ site without affecting the other cofactors.

Q₆KO has a disabled Qₛ site but retains a wild-type Qᵣ site. This was further demonstrated by the functional characterization of the cytochrome b₆f variant *in vivo*. The oxidation kinetics of cytochrome f was identical to the wild-type one (Supplementary Fig. S2). Figure 3a shows the transient absorbance changes associated with the redox changes of the b haem. Under mildly reducing conditions (Fig. 3a, black trace and inset), the reduction of a b haem is similar to that of the wild type, with a half-time of ~2 ms (ref. 15).

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**Figure 1 | The cytochrome b₆f dimer operates through a modified Q-cycle.** Left, box schematic: the b₆f complex transfers two protons (green arrows) per electron transferred (blue arrows) along high (Fe₅S₆ cluster, cytochrome f) and low potential chains (b₈, b₆, c haems). Quinol (QH₂) oxidation at Qₛ site, Quinone (Q) reduction at Qᵣ site. Right, structure (redrawn from ref. 35) depicting haems b (purple), c and f (red), Fe₅S₆ cluster (yellow and orange ball-and-stick model), cytochrome b₈ (cyan), subunit IV (blue), Rieske subunit (yellow), cytochrome f (red), PetG, L, M and N subunits (green). Magnification of Qₛ site comprising b₈ and c haems.

**Figure 2 | Characterization of cytochrome b₆f complex in the Q₆KO strain.** (a) Immunoblot chemiluminescence analysis of the major subunits of cytochrome b₆f. Subunit β of chloroplast ATPase as a loading control. Q₆KO shows wild-type level of all cytochrome b₆f subunits with the doublet signature for cytochrome b₈ missing the c haem after SDS-urea PAGE. (b) Covalent haem peroxidatic activity confirms the absence of the c haem in Q₆KO strain. (c) Dithionite minus ascorbate spectra from purified b₆f complexes. Black, WT; red, Q₆KO. Haems b₈ (peaks at 434 and 564 nm) and c (broad band at 425 nm) components in Q₆KO the decreased amplitude at 434 and 564 nm demonstrates the absence of b₈ and the trough at 420 nm the absence of c. The spectra have been normalized to the cytochrome f content (inset).
Thus, the Q site is not impaired. However, contrary to the wild-type case (Fig. 4, green trace), this reduction is not electrogenic (Fig. 4, black trace), showing that the reduced b haem is on the luminal side of the membrane and that, as a corollary, the b, haem and the Q site are indeed knocked out. The QKO bL complex is thus genuinely the long-sought variant, inactivated in its Q site yet retaining a wild type like Q site, required to assess the dispensable character of the Q-cycle in the photosynthetic chain.

**QiKO sustains phototrophic growth.** *In vitro* assay (reduction of plastocyanin in the presence of excess plastoquinol) showed that the QKO bL complex sustains a notable electron-transfer flux. The turnover rate is, taking into account that only 30% of b haem is present, 20 ± 4 s⁻¹, 5% that of wild type (WT). Although faint, this flux proved to be vital in essence. Indeed, it sustained photosynthetic growth (Fig. 5 and Supplementary Fig. S3). Figure 5 shows growth efficiencies under moderate illumination, in the presence (a) and absence (b) of oxygen. As expected from the block in the photosynthetic electron-transfer chain, a bL-lacking mutant showed no phototrophic growth under either condition. This was in contrast to the QKO strain, which grew moderately but markedly under phototrophic conditions (Fig. 5). Although this phototrophic growth is oxygen sensitive, the bL-lacking and QKO strains grew at a rate similar to that of the wild type in the presence of oxygen, which allows mitochondrial respiration, under heterotrophic conditions (acetate) (Fig. 5e). Oxygen per se is thus not detrimental, but the combination of light and oxygen is (Fig. 5c), suggesting that photosynthetic activity over-produces reactive oxygen species in the QKO strain, as found in the H212N bL case.

The light-induced oxidation of pre-reduced b, What mechanism underlies the unexpected finding that, despite its inactivated Q-cycle, the QKO bL complex sustains a flux compatible with photosynthetic growth? In the mechanistic framework of the Q-cycle, the oxidation of a quinol at the Q site relies on the bifurcated electron

![Figure 3 | Probing electron transfer in vivo. (a) Light-induced redox changes of cytochrome b in QKO. Black, filled symbols, mildly reducing conditions; open symbols, after pre-illumination to get similar contents of pre-reduced and pre-oxidized haem b; red, strongly reducing conditions. Inset: reduction component on a smaller time scale. (b) Light-induced redox changes of cytochrome f in strongly reducing conditions. Black, WT; blue, QKO, normalized on the photosystem I amount. Re-reduction of cytochrome f in QKO is rate-limited by the re-oxidation of the b, haem. The reduction of cytochrome f in QKO is biphasic, with the fast component being similar to the WT one (t_{1/2}, ~3 ms) (see inset), and the slow component being concomitant with the oxidation of b, (t_{1/2}, ~250 ms).

![Figure 4 | Light-induced electrogenicity in QKO and WT (520–546 nm). QKO (squares) and WT (circles), black and green, mildly reducing conditions; red and blue, strongly reducing conditions.

![Figure 5 | Remaining electron flow in QKO devoid of Q-cycle sustains phototrophic growth with light enhanced oxygen sensitivity. Cells were plated on minimal medium and grown for 10 days under 15 (a, b) or 45 (c, d) μE m⁻² s⁻¹ of light and a controlled atmosphere combining 2% CO₂ and 98% air (a, c) and or 98% N₂ (b, d) to test phototrophic growth. (e) Cells were plated on acetate medium as heterotrophic growth control at very low light fluxes (1 μE m⁻² s⁻¹).
transfer to the oxidized FeS₄ cluster of the Rieske subunit and to the oxidized b₃ haem. In the wild type, b₃ is quickly reoxidized by the b₃ haem, and thus made available as an electron acceptor for the next quinol oxidation. Consequently, in QKO, the long-lived reduction of the b₃ haem (Fig. 3a, black trace) should only allow a single turnover of the Qₐ site and not a steady flux as observed here.

We thus studied in vivo the function of the Qₐ site in conditions where a significant fraction of the b₃ haem was reduced (~60%, Fig. 3a, red trace) prior to the light activation of the complex. As a fraction of b₃ haem was pre-reduced, the relative amplitude of the flash-induced reduction phase was smaller than that under oxidizing conditions. Saliently, we observed a net oxidation of a b haem that developed with a ~250 ms half-time. The Qₐ site being knocked out, this net oxidation of a b haem was not electrongenic (Fig. 4, red trace). It must thus reflect the electron transfer from the reduced b haem to an electron acceptor produced by the light-induced injection of an oxidizing equivalent, or hole, in the high-potential chain. In principle, this hole may be borne by cytochrome f, the FeS₄ cluster or the semiquinone produced at the Qₐ site. The oxidized cytochrome f can be excluded as it is separated from b by too large a distance (more than 30 Å) to allow electron transfer in a few hundred ms (ref. 17). The edge-to-edge distance between b and the FeS₄ cluster (~23 Å) is compatible with such an electron-transfer rate. However, 2-iodo-2',4',4'-trinitro-3-methyl-6-isopropyl diphenyl ether (DNP-INT), specifically inhibiting quinol access to the Qₐ site while permitting FeS₄-neryl ether (DNP-INT), specifically inhibiting quinol access to the Qₐ site, should only allow a single turnover of the Qₐ site in conditions (Supplementary Fig. S4), prevented b₃ oxidation (Supplementary Fig. S5). The semiquinone thus stands as the most likely candidate (Fig. 6).

The dual role of the semiquinone. As depicted in Figure 6, the oxidation of the quinol by the FeS₄ cluster is thought to be endergonic. Recently, Zhang et al. located the midpoint potential of the quinol/semiquinone couple 200 mV above that of the FeS₄ (at pH 9.0). This makes the equilibrium constant of the forward electron transfer from the quinol to the FeS₄ cluster much lower than 1. Consequently, the concentration of the reactive semiquinone is kept extremely low (see ref. 20) and has remained undetectable under functional conditions or barely detectable (0.1–10%) under extreme ones. Notably, in a cytochrome bc₃ mutant lacking the b₃ haem, that is, under even harsher conditions than those described here, the semiquinone has kept elusive. In most of the currently considered scenarios, even though they may cover different mechanistic details, the formation of the semiquinone resulting from the oxidation of the quinol by the FeS₄ is strongly uphill in energy and is thus pulled forward by the depletion of its semiquinone product through the subsequent downhill electron transfer to the b₃ haem. As a strong support to this mechanistic scenario, changing the driving force for quinol oxidation by changing the redox properties of the FeS₄ cluster results in linear changes in the activation energy of the Q-cycle with a slope near unity. In this sequential scenario (see ref. 22), the injection of an electron into the high-potential chain is effectively driven by the second step, that is, the consumption of the semiquinone, and both reactions are concurrent. Importantly, this behaviour is not restricted to the regular function of the Qₐ site but it also holds when, as we propose, the consumption of the semiquinone involves its reduction by the pre-reduced b₃ haem. We found accordingly that, the injection of an electron into the high-potential chain, as probed by the redox changes of cytochrome f, paralleled in time the redox changes of the b₃ haem irrespective of the redox poise, or, in other words, was concomitant with the redox changes of the b₃ haem (Fig. 3b).

Altogether these findings show that a pre-reduced haem factors, but does not preclude, the injection of an electron in the high-potential chain, that is, the quinol-plastoquinone oxidoreductase activity. A parsimonious mechanistic model accounting for these observations is a ping-pong play in which the semiquinone and the b₃ haem act, one after the other, as the electron donor and electron acceptor (Fig. 6). This mechanism relies on the dual properties of the semiquinone species, which can act either as an electron-acceptor-yielding quinol or as an electron-donor-yielding quinone. In the currently accepted energy landscape of the bc₃ and bc₃f complexes, the semiquinone is a much stronger electron acceptor than the quinone and can thus, on thermodynamic grounds, readily oxidize the b₃ haem to form the quinol species (Fig. 6b). Notably, it is yet a sluggish process with an overall rate (4 s⁻¹) being several orders of magnitude slower than the theoretically predicted rate (10¹² s⁻¹). It is thus kinetically limited, bringing experimental support to a hypothesized gating mechanism (see refs 19, 20, 25–27). Interestingly, the overall electron-transfer flux sustained by the QKO bc₃f is similar to that found with the antimycin-inhibited cytochrome bc₃ and the homodimer H212N bc₃. This suggests that the mechanism proposed here may also apply to the bc₃ complex, as considered in refs 28, 29 as one among other possible scenarios.

Discussion
As any energy-converting enzymes, cytochrome bc₃ and bc₃f are prone to undergo short circuits in their reaction pathways. Although expected on thermodynamic grounds, appropriate mechanistic control can regulate these to extremely slow processes and thus make them negligible with regard to their yield. In keeping with this framework, we propose that a short-circuit reaction between the reduced b₃ and a semiquinone may occur, but that its rate would...
make it a poor competitor with the forward-productive electron transfer reactions. Yet, under conditions inactivating the redox loop, the occurrence of such short circuit would provide an ‘emergency exit’ pathway bypassing the Q-cycle and making it dispensable. It would thereby rescue its quinol-plastoquinone oxidoreductase activity and thus the function of the entire photosynthetic chain.

As mentioned above, the finding that the Q-cycle is dispensable from a mechanistic point of view also applies to the bc₁ complex, as such complex with an inhibited Q site can sustain an overall electron-transfer flux.13,30–33. However, it is also dispensable from an energetic standpoint in the oxygenic photosynthetic chain, whereas similar mutants of the bc₁ complex from photosynthetic purple bacteria forbid photosynthetic growth. A rationale behind this physiological difference may lie in the relative contribution of the two complexes to their respective energy-converting chain. With a fully active Q-cycle, the ratio of H⁺ transferred across the membrane per electron transferred through the high potential chain increases from 1 to 2. The total H⁺/e⁻ ratio being 2 in the photosynthetic chain of purple bacteria and 3 in the oxygenic photosynthetic chain (see Supplementary Fig. S1), inactivating the low-potential chain and its associated H⁺ transfer impacts only a third of the H⁺/e⁻ in the latter case and up to 50% in the former. In addition, whereas the photosynthetic chain of purple bacteria promotes a cyclic electron transfer, the oxygenic photosynthetic chain allows the linear electron transfer from water to NADPH. As the mechanism we propose preserves linear electron transfer, at least partially, the impaired photosynthetic chain still yields oxygen and reducing power that can fuel the respiratory chain and thereby compensate the decreased production of ATP resulting from the inactivation of the Q-cycle and meet the requirement of the Benson-Calvin cycle in terms of ATP and reduced nicotinamide adenine dinucleotide phosphate (NADPH).

To conclude, the present data show that, as in the bc₁ complex case, a disabled Q site does not completely inhibit the function of the Q site, which can still sustain an electron-transfer flux. In addition, we show that, at odds with the bc₁ complex case, this flux is large enough to allow photosynthetic growth, thus demonstrating that, in the oxygenic photosynthetic chain, the Q-cycle is dispensable from an energetic standpoint.

Methods

Strains and growth conditions. The following C. reinhardtii strains were grown heteroestratically in continuous white light (35µE m⁻² s⁻¹) in Tris–acetate–phosphate medium, pH 7.2 at 25 °C: wild type, deletion of chloroplast petB gene encoding cytochrome b₃, ΔpetB, substitution of b₃ heme ligand petB-H202Q,Δ-S tag addition in chloroplast petA gene encoding cytochrome f (petA-CterH, ref. 5), substitution of ATP-dependent FtsH protease arginine finger that is essential for ATPase and protease activity in nuclear-encoded FtsH1 gene (ftsH1-R420C)Δ4, and combinations isolated by sexual crosses ftsH1-R420C{petA-CterH,Δ-Q} and ftsH1-R420C{petA-CterH,Δ-Q}Δ4. Growth tests were initiated by spotting 10⁵ cells of log-phase cultures onto agar plates. Plates were placed in tight-sealed chambers applying 2% glucose oxidase to achieve anoxia, and mediators and inhibitors were added as described above. Cells were kept in the sample cell in the dark for a time ranging from 1 to 3 min depending on the strain used and their respiration rate. Strongly reducing conditions were obtained by adding 20 mM glucose, 2 mg ml⁻¹ glucose oxidase to achieve anoxia, and mediators antifuraginone (−100 µM) and antifuraginone–2-sulfonate (−225 µM) at 1 µM to promote redox poising of the cells and thus b₃ heme reduction.6 The sample was kept in the dark for 25 min under complete anoxia. We have checked (not shown) that the first quinone acceptor of photosystem II was fully reduced under such conditions. The time between two consecutive actinic flashes was set at 5 min, to allow the redox equilibration of the samples between flashes. To obtain a similar content of pre-reduced and pre-oxidized b₃ heme in the QKO strain, the cells were first dark adapted for 25 min under complete anoxia, yielding the strongly reducing content described above, and then submitted to a series of 5 pre-illuminating flashes at 0.2 Hz.

Inhibitors. Two distinct Q site inhibitors were used: tridecyl-stigmatellin and 2-sodio-2′,4′-trinitro-3-methyl-6-isopropyl diphenyl ether. PSII was inhibited by 1 mM hydroxyamine and 10 µM 3-(3,4-dichlorophenyl)-1,1-dimethylethylene (DCMU) to determine the PSI:PSII ratio for normalization.

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Author contributions

A.M. and C.V. isolated *Chlamydomonas reinhardtii* strains and performed genetic, molecular, biochemical and physiological analyses; A.M. and F.R. performed spectroscopic analyses; A.M., F.A.W. and F.R. designed the study, analysed the data and wrote the paper.

Additional information

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