Mitochondrial respiration is essential for photosynthesis-dependent ATP supply of the plant cytosol

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Summary

- Plants rely on solar energy to synthesize ATP and NADPH for photosynthetic carbon fixation and all cellular need. Mitochondrial respiration is essential in plants, but this may be due to heterotrophic bottlenecks during plant development or because it is also necessary in photosynthetically active cells.
- In this study, we examined *in vivo* changes of cytosolic ATP concentration in response to light, employing a biosensing strategy in the moss *Physcomitrium patens* and revealing increased cytosolic ATP concentration caused by photosynthetic activity.
- Plants depleted of respiratory Complex I showed decreased cytosolic ATP accumulation, highlighting a critical role of mitochondrial respiration in light-dependent ATP supply of the cytosol. Consistently, targeting mitochondrial ATP production directly, through the construction of mutants deficient in mitochondrial ATPase (complex V), led to drastic growth reduction, despite only minor alterations in photosynthetic electron transport activity.
- Since *P. patens* is photoautotrophic throughout its development, we conclude that heterotrophic bottlenecks cannot account for the indispensable role of mitochondrial respiration in plants. Instead, our results support that mitochondrial respiration is essential for ATP provision to the cytosol in photosynthesizing cells. Mitochondrial respiration provides metabolic integration, ensuring supply of cytosolic ATP essential for supporting plant growth and development.

Introduction

Photosynthetic organisms are the main primary producers on our planet, fixing *c.* 110 Gt Carbon per year and providing the chemical energy supporting most lifeforms (Ringsmuth *et al.*, 2016). Sunlight powers the photosynthetic electron flow catalysed by photosystem (PS) I and II, cytochrome b_6f and the ATP synthase that together mediate the synthesis of NADPH and ATP to drive cellular metabolism. All eukaryotic photosynthetic organisms require mitochondrial respiration in addition to photosynthesis. Respiration allows for the transfer of electrons from metabolic intermediates to oxygen through the activity of the respiratory complexes localized in the inner mitochondrial membrane, Complex I, II, III and IV, as well as alternative electron transport proteins, such as NAD(P)H dehydrogenases and alternative oxidases. Electron transport via complexes I, III and IV is coupled to proton transport and the generation of an electrochemical gradient across the inner mitochondrial membrane that drives the synthesis of ATP through the F_1F_O ATP synthase, also called complex V. The NADH:ubiquinone oxidoreductase complex (Complex I, CI) is under most conditions a major site of electron entry into the mitochondrial electron transport chain (mETC) and it can provide up to 40% of the protons exploited for mitochondrial ATP synthesis (Watt *et al.*, 2010; Braun *et al.*, 2014), even though this varies widely depending on metabolic status and the contribution of alternative electron transport components.

Respiration in plants is essential for survival. Functional knockouts of respiratory complexes II, III, IV and V lead to lethality (León *et al.*, 2007; Robison *et al.*, 2009; Radin *et al.*, 2015; Kolli *et al.*, 2020). One evident explanation that applies for many plants is the fact that mitochondria cover cellular energy demand during the night and in nonphotosynthetic tissues, such as roots. Importantly, seed plants typically go through developmental stages where photosynthesis is not active, such as embryogenesis and seed germination. While the need for energy supply in the absence of photosynthetic activity provides a potential explanation for why respiration is essential in plants, a tight functional link between chloroplast and mitochondrial energy metabolism has been described in multiple photosynthetic organisms. Mitochondria participates to photorespiration, recycling the glycolate produced because of oxygenase activity of RuBisCO. In the diatom *Phaeodactylum tricornutum* the metabolite exchange between chloroplast and mitochondria was demonstrated to be essential for carbon fixation (Bailleul *et al.*, 2015). Experiments on the green alga *Chlamydomonas reinhardtii* remarked the relevance of metabolite exchange between chloroplasts and mitochondria for

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photosynthesis optimization (Cardol *et al.*, 2003; Schönfeld *et al.*, 2004; Burlacot & Peltier, 2023). Studies using used barley leaf protoplasts showed that glycine of photorespiratory origin has a key role in the energization of cytosol and chloroplasts, and in the maintenance of the redox balance in illuminated protoplast (Gardeström & Wigge, 1988; Igamberdiev *et al.*, 1998, 2001). Additional evidence of mitochondrial modulation of photosynthetic performance is available also from studies conducted using green tissues of plants, where excess reducing power produced via photosynthesis can be routed to mitochondrial respiration, preventing over-reduction of photosynthetic electron transport components and generation of reactive oxygen species (ROS) in the plastid (Dutilleul *et al.*, 2003; Noguchi & Yoshida, 2008; Zhang *et al.*, 2012; Mellon *et al.*, 2021).

The assessment of impact of mitochondrial respiration in photosynthetic cell metabolism, however, has been in part limited by the lack of genetic resources. While only knock-down plants have been isolated and studied for complexes II, III, IV and V so far, plants completely lacking mitochondrial Complex I activity have been described in *Arabidopsis thaliana* as well as in *Nicotiana sylvestris* and the moss *Physcomitrium patens* where they showed a severe growth phenotype and, in the formers, alterations in germination, fertilization, and pollen development (Gutierres *et al.*, 1997; Fromm *et al.*, 2016; Mellon *et al.*, 2021). Interestingly, European mistletoe *Viscum album* was shown to be able to live without a functional CI and most of the relevant corresponding genes have been lost (Maclean *et al.*, 2018; Senkler *et al.*, 2018; Schröder *et al.*, 2022). While this is an extreme example of evolutionary rearrangement of central energy metabolism, probably linked to the specific lifestyle as an obligate semi-parasite (although currently unique to mistletoes and not found in other parasites), also *Viscum* shows respiratory activity, likely based on alternative NADH dehydrogenases.

In contrast to plants, a range of respiratory mutants depleted in all respiratory complexes has been isolated in the green alga *Chlamydomonas reinhardtii*. Those mutants show strong phenotypes under heterotrophic conditions while their growth is generally unaltered or just moderately reduced under photoautotrophic conditions (Duby & Matagne, 1999; Cardol *et al.*, 2009; Salinas *et al.*, 2014; Larosa *et al.*, 2018). Those observations may be taken as evidence that mitochondrial respiration is required to support phases of heterotrophic lifestyles and is not essential when photosynthesis is active. Alternatively, there may be a fundamental difference in the role that respiration plays in plants and algae that may have evolved to making plant, but not algal, photosynthesis strictly dependent on mitochondrial respiration (Mellon *et al.*, 2021).

In photosynthetically active cells, both the chloroplasts and the mitochondria are able to synthesize ATP. ATP is, however, also essential in other subcellular compartments. In the cytosol major ATP consumption occurs to support protein synthesis, membrane transport or primary metabolic pathways. It was proposed that when chloroplasts actively reduce CO_2, cytosolic ATP originates mainly from the mitochondria as exported directly via the ATP-ADP carriers (AACs) (Voon *et al.*, 2018). By contrast, ATP export from chloroplast was suggested to be enhanced under stress conditions that slow CO_2 fixation down and thus lower the ATP consumption rate in the chloroplast (Gardeström & Igamberdiev, 2016). Even in that case ATP export would be indirect via the triose-phosphate shuttle, since there is currently no evidence for any direct and substantial ATP export from the stroma to the cytosol. These conclusions derive from observations made from experiments using biochemical fractionation and inhibitors (Gardeström & Wigge, 1988; Krömer *et al.*, 1993; Wigge *et al.*, 1993) and were more recently supported also by metabolic modelling approaches (Shameer *et al.*, 2019). Yet, information from intact systems, that enable the exploration of dynamic transitions and responses to variable environmental conditions are largely missing, which represents a remarkable shortcoming in our current understanding of energy metabolism considering the central role of ATP for (plant) life.

In this work we set out to address the question why mitochondrial respiration is essential in plants. Specifically, we aimed at distinguishing between the two hypotheses that either (i) mitochondrial respiration is essential due to the need to sustain heterotrophic cells and heterotrophic phases of development where photosynthesis is absent, or (ii) mitochondrial respiration is critical for cellular ATP supply in the presence of photosynthesis. We chose the moss *P. patens* as a model that is able to complete its life cycle entirely without any heterotrophic developmental stage, enabling isolation of mutants that are not viable in other plants. Further, we addressed the methodological limitation of insufficient subcellular resolution in ATP measurements by exploiting a genetically encoded FRET sensor (ATeam1.03-nD/nA) that allows monitoring of rapid ATP changes as recently demonstrated in Arabidopsis (De Col *et al.*, 2017; Voon *et al.*, 2018; Lim *et al.*, 2022), an approach that enables to study metabolite dynamics in intact cells (Lim *et al.*, 2020). To measure the impact of photosynthetic activity on ATP *in situ* we further adapted an on-stage illumination approach (Elsässer *et al.*, 2020). Exploiting cytosol-specific ATP monitoring in *P. patens* enabled us to explore the role of the mitochondria in cytosolic ATP supply. Genetic inactivation of respiratory complexes I and V allowed us to gather compelling evidence that mitochondrial respiration is essential for cytosolic ATP supply also while photosynthesis is active.

Materials and Methods

Plant materials and growth conditions

The wild-type (WT) strain used in this study was the Gransden WT strain of *Physcomitrium patens* subsp. *patens*. Two different media were used for growing protonemal tissue, the minimal $PpNO_3$ medium and the enriched version $PpNH_4$ (Ashton *et al.*, 1979).

For propagation, protonemal tissue was grown on cellophane-layered $PpNH_4$ medium at 24°C under moderate light intensity (50–80 μmol photons $m^{-2}\,s^{-1}$) and long day conditions (16 h : 8 h, light : dark) in growth chambers. WT and WT-ATeam plants were harvested after 5 or 6 d of growth, and

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ndufa5 and *ndufa5*-ATeam plants were harvested after 7 to 10 d. *$f_A d$* plants were grown for months. Growth of *$f_A d$* KO lines was also tested under continuous illumination. For confocal imaging experiments, protonemal tissue was grown on cellophane-layered minimal $PpNO_3$ medium under the same conditions and used after 10 d of growth.

Moss transformation and transgenic plant selection

For generation of ATP reporter lines, the *P. patens* cytATeam_pT1OG expression vector was generated, using the coding sequence of ATeam1.03-nD/nA isolated from the Arabidopsis Gateway expression vector pH2GW7_cytATeam1.03.nDnA (De Col *et al*., 2017).

Transformation of *P. patens* was carried out by the polyethylene glycol (PEG)-mediated method (Nishiyama *et al*., 2000), using either protoplasts of WT or *ndufa5* lines (the latter published previously; Mellon *et al*., 2021) to generate reporter lines in both WT and *ndufa5* backgrounds. Screening of stably resistant lines was done through quantification of mVenus fluorescence, as described in the main results section.

For generation of plants missing a functional mitochondrial ATP synthase, we generated the Fad_KO_BHRf construct by inserting the PCR-amplified flanking regions of the locus encoding F_Ad (Pp3c9_7910) (Fig. S4A) in the BHRf vector (kindly provided by F. Nogue, INRA Versailles, France). Transformation of *P. patens* was carried out as described for ATP reporter lines. In this case screening of stable resistant lines was done through PCR verification of disruption of the genomic F_Ad locus (Fig. S4B). Genomic DNA extraction protocol was adapted from a previously published protocol (Edwards *et al*., 1991). PCR amplifications of the recombination cassette were performed on extracted gDNA. Primers used for construct design and line validation are the following (5′ to 3′ sequence). Fad-p1: GTGGAATGGCATGATTTTAT; Fad-p2: ACTAAACAACCAGACCAGGA; Fad-p3: CTTGGGATTGATGATGCTAT; Fad-p4: TGGGTATTATCGGAGTCAAC; Fad-p5: CATCTACCATTTTGGGTTTC; Fad-p6: ACTTCGAACCAGTTCCAGTA; HNZ Up Rev: TGCGCAACTGTTGGGAAG; 35S terminator: CGCTGAAATCACCAGTCTCTCT.

Confocal imaging

Plants were grown for 10 d on solid $PpNO_3$ medium and dark adapted for 40 min. A piece of protonema was extended on a slide with a drop of liquid $PpNO_3$ medium (without agar) and covered with a coverslip. The coverslip was then fixed with duct tape to avoid desiccation of the sample during the measurement. This process was done in a dark room to keep the samples dark-adapted.

Samples were illuminated with 50 µmol photons $m^{-2}\,s^{-1}$ using a red-filtered (580–630 nm) light (halogen lamp connected to an optic fibre). Light intensity was set to 50 µmol photons $m^{-2}\,s^{-1}$ using a light meter (LI-250A; Li-Cor, Lincoln, NE, USA). The spectrum of the filtered light is included in the Fig. S1(D) and was measured using a LI-COR LI-180 Spectrometer.

Imaging was performed using a 40× oil immersion lens using a Leica SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany). An Argon laser (488 nm) was used to excite both CFP and chlorophylls, with laser power set at 12.5%. A scan was performed every 10 s. The first scan was done at darkness to quantify the basal ATP levels. Light was then turned on and off according to the described protocols. Three emission channels were set up: CFP (465–500 nm), mVenus (525–561 nm) and auto-fluorescence of Chls (670–690 nm) (see Fig. S1).

The acquired fluorescence datasets were analysed using the MATLAB-based Redox Ratio Analysis software (RRA) (Fricker, 2016). For each dataset, five square regions of interest (ROIs) were analysed. Each ROI was analysed as a single replicate. First, background signal was removed using the integrated functionality of the RRA software. The intensities of the single mVenus and CFP channels were then exported and used for further analysis and plotting with OriginPro. We calculated the mVenus/CFP intensity ratio, which was then log-transformed to approach the normal distribution. The log-normalized values were then normalized to the value at time 0, that is to the steady state in the dark.

The slope of the increase in the ATeam signal during dark-to-light transitions was calculated by fitting the datapoints (norm. log(mVenus/CFP)) to the formula $y = a + bx$ using the Linear Fitting tool of OriginPro, where b is the reported slope.

Statistical significance between two samples was analysed by two-tailed Student's *t*-test (alpha = 0.05) using OriginPro. The number of replicates and meaning of asterisks are indicated in the figure legends.

Chlorophyll fluorescence experiments

Photosynthetic parameters were retrieved from Chl fluorescence to analyse photosystem II (Y_{II}, qL) and a dual wavelength absorbance spectrometry to analyse photosystem I (Y_I), as described previously (Gerotto *et al*., 2016). Chlorophyll fluorescence and near-infrared (NIR) absorption analyses were performed at room temperature using a Dual-PAM 100 system (Walz) on protonema grown for 10 d in $PpNO_3$ in WT and for *c*. 4 months for the fad mutant. Before the analysis, plants were adapted in the dark for 40 min. Induction curves were obtained by setting actinic red light at (*c*.) 50 or 330 µmol photons $m^{-2}\,s^{-1}$, and photosynthetic parameters were recorded every 30 s. At each step, the photosynthetic parameters were calculated as follows: Y_{II} as $(F'_m - F_o)/F'_m$; qL as $(F'_m - F)/(F'_m - F'_o) \times F'_o/F$; Y_I as $1 - Y_{ND} - Y_{NA}$, with Y_{NA} as $(P_m - P'_m)/P_m$ and Y_{ND} as $(P - P_o/P_m)$ (Klughammer & Schreiber, 1994).

Results

Generation of *P. patens* plants accumulating ATP probe ATeam in the cytosol

Stable *P. patens* WT lines constitutively accumulating ATeam1.03-nD/nA in the cytosol, referred to as WT-ATeam, were generated by protoplast transformation. After transformation, lines stably resistant to zeocin were screened through the fluorescence of the mVenus channel to select the ones with

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detectable accumulation of the probe. At least three independent lines were selected among the ones showing mVenus fluorescence signal (Fig. S1A,B). The ATeam-expressing lines did not show any visible growth defect and showed normal development (Fig. S1A,B) with the ability to grow leaflets, rhizoids and sporophytes and produce viable spores as well as the parental line, suggesting that the probe accumulation did not cause major alterations in metabolism and development.

The localization of the mVenus fluorescent signal in the lines was verified by confocal microscopy, with Chl fluorescence marking chloroplast localization. The mVenus signal showed no overlap with Chl fluorescence and was localized in large areas inside the cell, consistent with a cytosolic expression, confirming the expected localization (Fig. 1a).

In the long term (> 1 yr, corresponding to > 5 tissue regeneration) we observed a decrease in the fluorescence signal, most likely due to silencing effects, as similarly observed for different biosensor proteins in Arabidopsis (Schwarzländer *et al.*, 2016; De Col *et al.*, 2017; Sadoine *et al.*, 2021) and for other overexpressed proteins in *P. patens* (Kubo *et al.*, 2017). In case of any evidence of silencing appeared, new lines were generated.

All imaging experiments were performed on protonema, a young tissue regenerated vegetatively, and we did not observe any mosaic silencing of the probe, meaning that the silencing, if present, was homogenous in all the cells. Because the probe is ratiometric, a moderate silencing would not cause any drift in FRET, as the value is self-normalized. In any case, if silencing effects appeared, new WT-ATeam plants were isolated to ensure maintenance of a good signal-to-noise ratio.

Photosynthesis drives the increase of ATP concentration in the cytosol during dark-to-light transitions

In each round of transformation, three independent lines generated were used to observe the cytosolic $MgATP^{2-}$ dynamics in the cytosol during dark-to-light transitions and assess changes in $MgATP^{2-}$ levels associated with the activation of photosynthesis. To this end, photosynthesis activity was induced using 50 µmol photons $m^{-2} s^{-1}$ of red ($\lambda > 630$ nm) actinic light. Red light is absorbed well by chlorophylls, but it does not interfere with the acquisition parameters of mVenus or CFP channels (465–561 nm). This choice of wavelengths thus enabled to keep the actinic light on during the acquisition of fluorescence without adding signal to the detector (Fig. S1C,D). Taking advantage of the ratiometric nature of the probe and the natural distribution of protonema in single-cell layers, we quantified the CFP and mVenus signals and calculated the corresponding FRET ratio (mVenus/CFP) in whole focal planes that contained dozens of different cells (Figs 1a, S1C).

To follow the dynamic response of ATP, plants were first incubated in the dark for 40 min to relax all photosynthesis-related processes and enable all samples to start from a homogeneous, dark-adapted state. When actinic illumination was switched on the mVenus signal increased immediately with a corresponding decrease in the CFP channel with the same kinetics (Fig. 1b) a behaviour that indicates a bona fide increase in FRET efficiency between probes. mVenus : CFP ratio indeed increased within seconds of illumination, reaching a plateau after 1.5 min (Fig. 1c). After light was turned off, FRET signal remained steady for *c.*

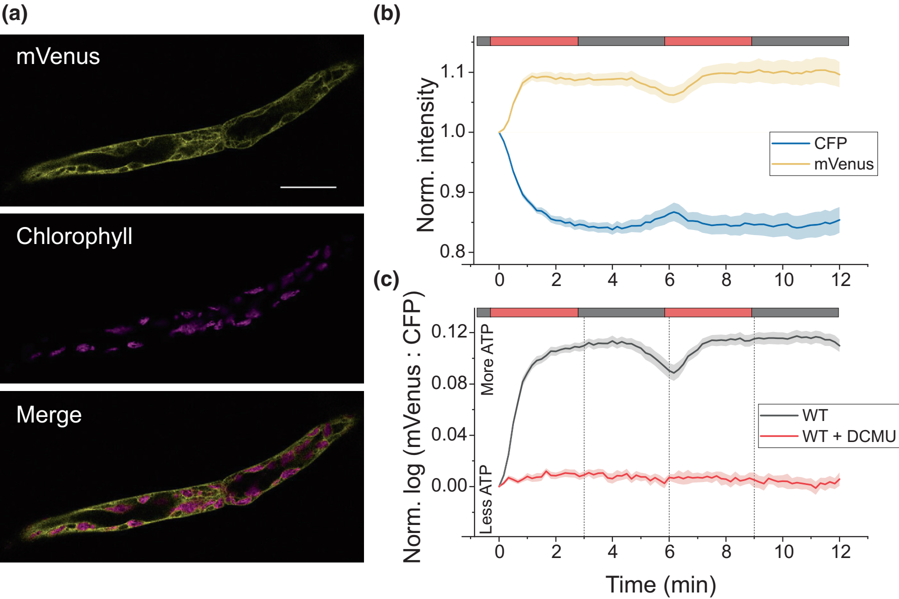

Fig. 1 Light-induced $MgATP^{2-}$ accumulation in the cytosol of *Physcomitrium patens*. (a) Confocal images of two cells of protonema of one of the WT-ATeam lines showing mVenus channel (yellow), Chl auto-fluorescence (magenta) and the merged image. Bar, 50 µm. (b) Normalized intensities of the single channels of WT-ATeam lines showing their changes upon dark-to-light transitions. Plants were dark-adapted for 30 min before the measurements, and the first datapoint was measured before switching light on. Light periods are marked with a red bar and correspond to 50 µmol photons $m^{-2} s^{-1}$ of red light. Error bands represent the SE of mean ($n = 40$) (c) Normalized log-transformed FRET ratio during dark-to-light transitions of control and DCMU-treated WT-ATeam plants. Light periods are marked with a red bar and correspond to 50 µmol photons $m^{-2} s^{-1}$ of red light. Error bands represent the SE of mean. Sample sizes are $n = 40$ wild-type (WT) and $n = 13$ (WT + DCMU). A live experiment is included as Supporting Information Video S1.

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1.5 min and then started to decrease, with a kinetic less steep than the light-driven increase (Fig. 1c). When the light was switched on a second time, the signal increased again, confirming that $MgATP^{2-}$ concentration responded to the light presence. In the second exposure to dark we observed a longer period of stability (*c.* 2 min), before it started to decrease again.

The same measurements were repeated in the presence of the PSII inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) that blocks all photosynthetic electron transport, that completely abolished all the above-mentioned dynamics (Figs 1c, S1E), demonstrating that light alone did not affect the FRET signal and that the observed dynamics were fully dependent on photosynthetic electron transport fuelled by the actinic light.

To assess the eventual effect of light quality on the cytosolic $MgATP^{2-}$ dynamics, the same experiment was repeated using a different setup already used for similar experiments in Arabidopsis, where photosynthesis is induced with white light that is switched off during the confocal measurement, creating a pseudo-continuous light effect (Elsässer *et al.*, 2020). These experiments showed very similar results, confirming that the dynamics observed were indeed the result of $MgATP^{2-}$ accumulation in cytosol induced by light, independently from the setup and light wavelength (Fig. S2A–D). The comparison further validates that both on-stage live illumination approaches as usable.

To test the effect of light intensity on the increase of $MgATP^{2-}$ during dark-to-light transitions, we compared the kinetics of FRET upon illumination with different intensities, namely 5, 50 or 200 μmol photons $m^{-2}\,s^{-1}$ of red light (Fig. 2).

An intensity of 5 μmol photons $m^{-2}\,s^{-1}$ of red light, which is low and limiting for *P. patens* growth but high enough to initiate photosynthetic activity, was sufficient to trigger an increase in cytosolic $MgATP^{2-}$ (Fig. 2a), that was, however, slower than in 50 μmol photons $m^{-2}\,s^{-1}$ (Fig. 2b). This is in line with our finding that the increase in cytosolic ATP is fully dependent on the activation of electron transport in the thylakoids. On the other hand, using a higher light intensity (200 μmol photons $m^{-2}\,s^{-1}$) did not alter the shape or magnitude of the increase of cytosolic ATP, even before reaching the plateau (Fig. 2a,b), meaning that 50 μmol photons $m^{-2}\,s^{-1}$ of red light are sufficient to saturate ATP biosynthesis capacity in these conditions. This was also the case using the white light setup (Fig. S2A).

Mitochondrial respiration has seminal role in ATP supply in the cytosol

Mitochondrial activity has been suggested to contribute to cytosolic ATP biosynthesis in photosynthetic organisms and inhibitors of the mitochondrial respiratory chain have been shown to alter cytosolic ATP in Arabidopsis seedlings (De Col *et al.*, 2017).

We monitored cytosolic ATP levels after blocking both the cyanide-sensitive and cyanide-insensitive electron transfer pathways, by treating plants with both KCN and salicylhydroxamic acid (SHAM), inhibitors of the Complex IV and the alternative oxidase (AOX), respectively. After this treatment, the basal ATP levels were drastically reduced (Fig. S3C). Exposure to actinic light also triggered an increase of cytosolic ATP in KCN/SHAM-treated samples but with slower kinetics compared to control plants (Fig. S2E). This observation suggests that mitochondrial respiration provides a relevant contribution to cytosolic ATP biosynthesis. To assess impact of mitochondrial respiration on ATP biosynthesis using an orthogonal genetic approach and exclude any secondary effects due to inhibitor treatments, the ATeam1.03–nD/nA probe was introduced in *P. patens* lines lacking the Complex I structural subunit NDUFA5 (*ndufa5* KO) previously shown to completely lack mitochondrial NADH dehydrogenase activity (Mellon *et al.*, 2021). Three independent

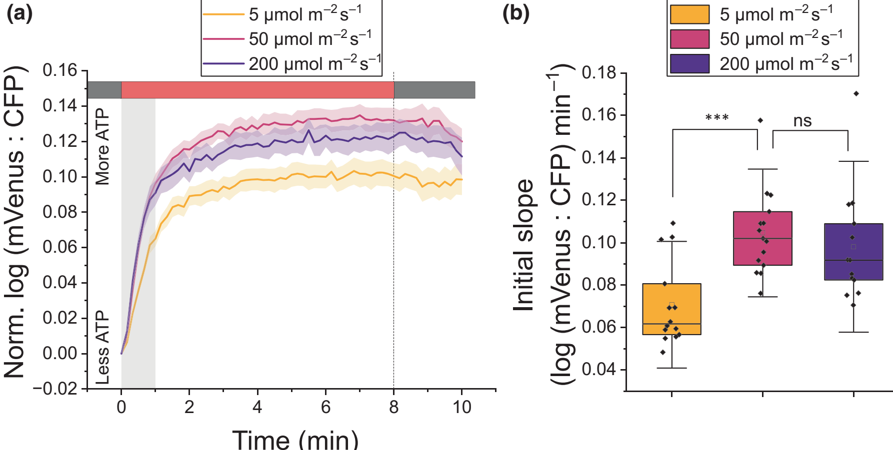

Fig. 2 Effect of light intensity on the cytosolic increase of ATP during dark-to-light transitions in *P. patens*. (a) Dark-adapted WT-ATeam plants were illuminated for 8 min with red light of either 5 (yellow), 50 (pink) or 200 (purple) μmol photons $m^{-2}\,s^{-1}$, followed by 2 min of darkness. Dark and light periods are marked with grey or red bars, respectively. The shadowed region marks the timelapse used for the calculation of slopes shown in (b). The vertical dashed bar corresponds to the light-to-dark transition. Error bands represent the SE of mean. Sample sizes are $n = 14$ (5 μmol photons $m^{-2}\,s^{-1}$), $n = 15$ (50 μmol photons $m^{-2}\,s^{-1}$) and $n = 13$ (200 μmol photons $m^{-2}\,s^{-1}$). (b) Slope during the first minute of illumination. Error bars represent 1.5 times the SD. Statistics: two-sample *t*-test, ***, $P < 0.001$; ns, $P > 0.05$. The box size indicates the interquartile range. Mean of data is represented as an empty square, median of data is represented by a horizontal line.

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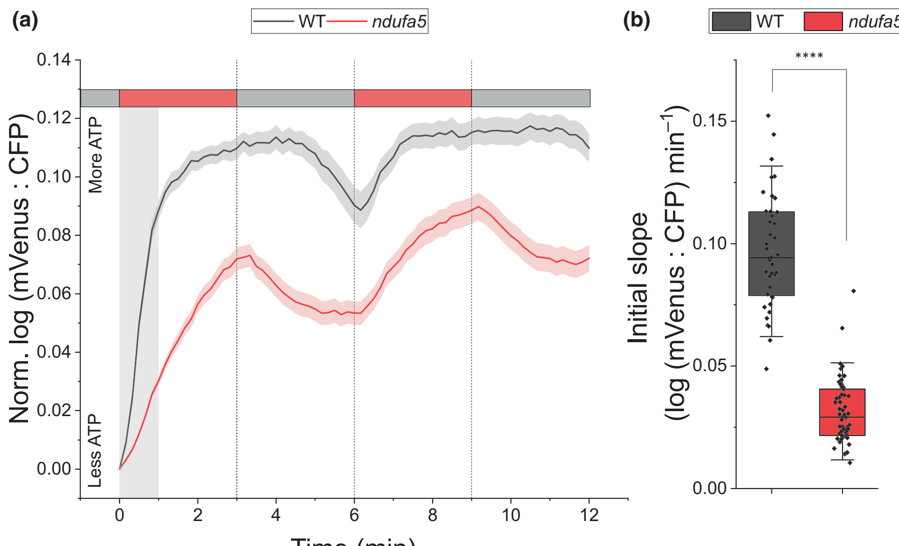

Fig. 3 Cytosolic ATP dynamics in the *P. patens* Complex I deficient plant *ndufa5*-ATeam. (a) Normalized FRET ratio during dark-to-light transitions in wild-type (WT) and *ndufa5* KO. Dark and light periods are marked with grey or red bars, respectively. The shadowed region marks the timelapse used for the calculation of slopes shown in (b). The vertical dashed bars correspond to light–dark transitions. Error bands represent the SE of mean. Sample sizes are $n = 40$ (WT) and $n = 55$ (*ndufa5*). (b) Slope of the normalized FRET ratio during the first minute of illumination. The box size indicates the interquartile range. Mean of data is represented as an empty square, median of data is represented by a horizontal line. Error bars represent 1.5 times the SD. Statistics: two-sample *t*-test, ****, $P < 0.0001$.

lines stably expressing the cytosolic probe in *ndufa5* background, that we will refer to as *ndufa5*-ATeam, were isolated.

Complex I mutants showed impaired cell and tissue morphology: cells are smaller and protonema filaments more condensed than in WT (Mellon *et al.*, 2021) (Fig. S3A,B). The probe expression showed no additional impact on growth beyond the defects associated with *ndufa5* depletion (Fig. S3A,B). The fluorescence intensity of the single channels was in all cases in a range close to the WT-ATeam lines and thus the same confocal settings were used for both lines to enable a reliable comparison of FRET signals between WT and *ndufa5* KO. There were no significant differences in the FRET ratio between WT-ATeam and *ndufa5*-ATeam dark-acclimated plants, meaning that the basal cytosolic ATP levels did not show significant differences between genotypes (Fig. S3C).

ndufa5-ATeam plants were exposed to the same light treatment shown in Fig. 1, observing an increase in ATP during dark-to-light transitions also in these mutants (Fig. 3). However, the kinetics of FRET signal in *ndufa5*-ATeam plants was clearly affected and the initial slope 2.7 times slower than in WT-ATeam lines (Fig. 3b). Differently to WT-ATeam, the signal also rapidly decreased when the light was turned off after the first 3 min of illumination. A second light treatment caused a further increase in cytosolic ATP, as in WT-ATeam, without reaching a plateau as observed in WT plants. These results suggest that mitochondrial respiration is an important contributor to the synthesis of cytosolic ATP and, remarkably, that Complex I is strictly required for efficient cytosolic ATP kinetics during dark-to-light transitions.

To further investigate the ATP dynamics, WT-ATeam and *ndufa5*-ATeam plants were exposed to light treatments of different duration. The extension of the illumination phase up to 8 min (Fig. 4a) showed that even though the rate of ATP biosynthesis was slower in the CI mutant, it almost reached the same normalized value of FRET as WT.

On the other hand, when the illumination was reduced to 1 min (Fig. 4b), this time was sufficient for WT-ATeam to show a sustained increase in cytosolic ATP while 1 min of darkness was instead not enough to observe a signal decrease. A following longer darkness period indeed showed that at least one and a half minutes of darkness were needed to observe the signal decrease. In *ndufa5*-ATeam, instead, FRET signal increase was slower during illumination, and it decreased immediately in the dark, confirming that rate of ATP biosynthesis was reduced in the mutant.

Impairment of mitochondrial ATP biosynthesis drastically decrease growth without impacting photosynthesis

To further verify the biological relevance of mitochondrial ATP synthesis for plant metabolism we aimed to generate knockout mutants of the mitochondrial F_1F_O-ATP synthase (complex V), the complex responsible of exploiting the electrochemical gradient generated by the mETC to synthesize ATP.

The F_1F_O-ATP synthase is largely conserved among eukaryotes with homologs of yeast and mammals subunits found conserved in green algae and plants and those could be identified in *P. patens* genome as well (Table S1). Proteomics approaches further identified two additional subunits in plants associated with the F_O domain, referred as F_Ad and 6 kDa, that present no counterparts in mammals or yeast (Senkler *et al.*, 2017). Both subunits are also conserved in *P. patens* genome (Table S1). In particular, the subunit F_Ad has been linked with development and fertility in wheat where its repression leads to sterile plants (Li *et al.*, 2010). In *Arabidopsis*, the gene is highly expressed in pollen during late developmental stages and the homozygous mutants are not viable. The hemizygous mutant shows altered mitochondrial morphology during the dehydration phase of pollen, causing their degeneration (Li *et al.*, 2010).

Considering its functional impact and the fact that a single nuclear gene encodes for F_Ad, this subunit was chosen as target for the inactivation of complex V in *P. patens*. Two independent *fAd* lines, depleted of the gene Pp3c9_7910, were isolated and verified to have an insertion of the resistance cassette in the locus

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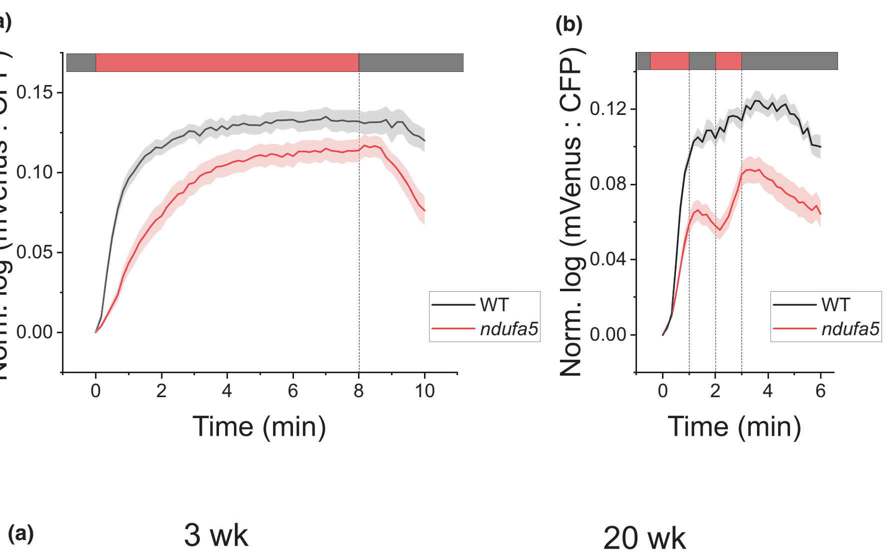
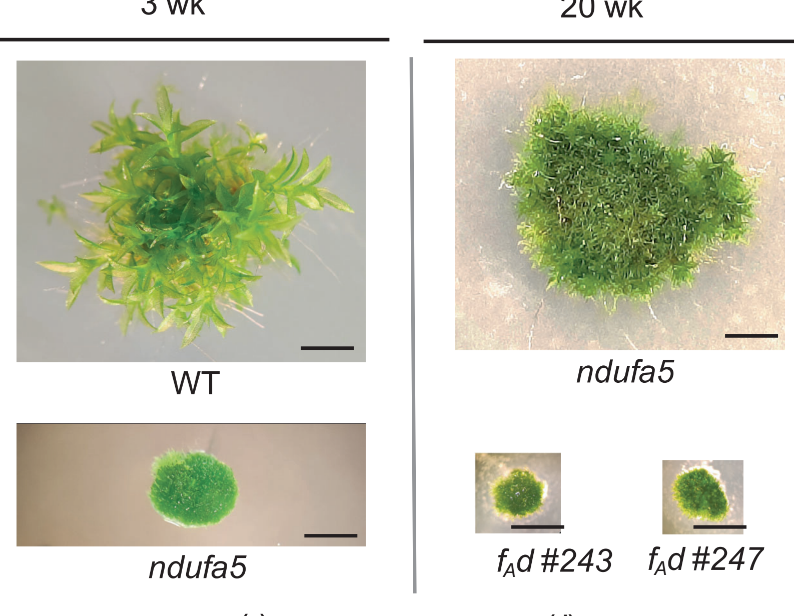


Fig. 4 Cytosolic ATP dynamics in the *P. patens* Complex I deficient plant *ndufa5* under alternative light–dark cycles. Dark-adapted plants were exposed (a) to 8 min of light followed by 2 min of darkness or (b) to a light fluctuation that consisted in 1 min of light, 1 min of darkness, 1 min of light and 4 min of darkness. In both cases light used was 50 μmol photons $m^{-2} s^{-1}$ of red light. Light and dark periods are marked with red and grey bars, respectively. Error bands represent the SE of mean. Sample sizes are $n = 15$ (a, wild-type (WT)), $n = 15$ (a, *ndufa5*), $n = 5$ (b, WT) and $n = 25$ (b, *ndufa5*).



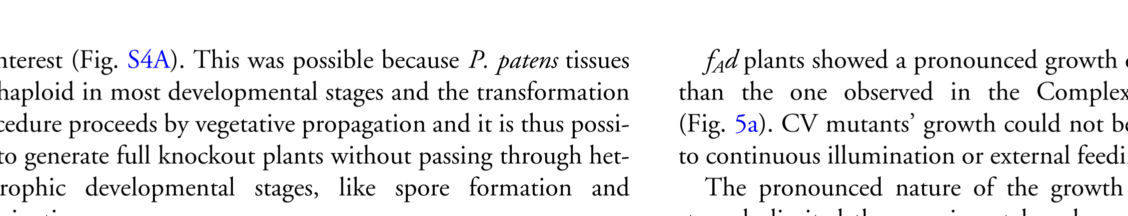


Fig. 5 Growth phenotype and photosynthetic properties of *P. patens* $f_A d$ plants. (a) Comparison of wild-type (WT), *ndufa5* and *fAd* colony size after 3 or 20 wk of growth. WT plants cannot be cultivated for months because of depletion of nutrients in the medium. Bars, 2 mm. (b, c) Quantum yield of Photosystem I (Y_I, b) and Photosystem II (Y_{II}, c) monitored with PAM during exposition to 8 min of actinic light at 50 μmol photons $m^{-2} s^{-1}$ followed by 8 min of dark. (d) Redox state of plastoquinone assessed by the fluorescence parameter 1-qL. All kinetics were measured after 40 min of dark adaptation. WT and $f_A d$ KO plants were grown photoautotrophically for, respectively, 10 d and 4 months. Data are expressed as the means ±SD, $n = 4$.

of interest (Fig. S4A). This was possible because *P. patens* tissues are haploid in most developmental stages and the transformation procedure proceeds by vegetative propagation and it is thus possible to generate full knockout plants without passing through heterotrophic developmental stages, like spore formation and germination.

$f_A d$ plants showed a pronounced growth defect, much stronger than the one observed in the Complex I deficient *ndufa5* (Fig. 5a). CV mutants' growth could not be rescued by exposure to continuous illumination or external feeding with glucose.

The pronounced nature of the growth defect unfortunately strongly limited the experimental analyses that were feasible for

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these plants. Yet, after *c.* 4 months it was possible to obtain enough tissue to measure photosynthetic performance using Chl fluorescence (Fig. 5b). Upon exposition to mild illumination (50 μmol photons m^{-2} s^{-1}), efficiency of photosystem (PS)I (Y_I) was indistinguishable between WT and f_Ad plants. The efficiency of PSII (Y_{II}) instead in the same conditions showed larger saturation and correspondingly the mutant showed a stronger reduction of plastoquinone, as estimated from 1-qL, suggesting that PSII electron transport capacity was reduced in the mutant with respect to the WT. The same measurements repeated with a stronger, saturating, light intensity (330 μmol photons m^{-2} s^{-1}), however, showed instead no difference between WT and the Complex V mutant (Fig. S4C), suggesting the photosynthetic light conversion is functional in the mutant. The mild alterations in photosynthetic activity observed could also be due to the different age and growth rate of plants but in any case, they cannot explain the major growth phenotype of the mutants.

Discussion

Light-driven ATP accumulation in the cytosol of *P. patens*

Dark-adapted cotyledons of Arabidopsis seedlings exhibited an illumination-driven increase in ATP levels (Voon *et al.*, 2018). However, due to the high and saturating steady-state levels of cytosolic ATP in Arabidopsis cotyledons, light-induced differences were only observable when ATP levels were reduced before illumination through pretreatment with the Complex I inhibitor rotenone, ensuring observations within the dynamic range of the probe (Voon *et al.*, 2018). *Physcomitrium patens* lines expressing the FRET-based sensor ATeam1.03-nD/nA did not require pretreatment with inhibitors. Their analysis revealed that plant illumination triggers a rise in cytosolic ATP in the absence of pharmacological inhibition of mitochondrial electron transport. This increase is entirely dependent on photosynthetic activity, as it is fully abolished by the PSII inhibitor DCMU. Using different light intensities, it was determined that 50 μmol photons m^{-2} s^{-1} of red light, a wavelength efficiently absorbed by chlorophylls, was already saturating for the ATP biosynthesis in the experimental system employed here, consistent with *P. patens* growth saturating at a light intensity between 50 and 150 μmol photons m^{-2} s^{-1} (Storti *et al.*, 2020).

The $MgATP^{2-}$, as the physiologically most relevant form of ATP, is stable in the slightly alkaline pH milieu of the cytosol leading the stable sensor readings between pH 7.5 and 8.5 (De Col *et al.*, 2017). While illumination induces alkalinization in various compartments in Arabidopsis it was verified that it did not cause an increase in ATeam FRET (Elsässer *et al.*, 2020). Hence, the most plausible explanation for the ATeam response in *P. patens* is a bona fide rise in ATP concentration. Under the assumption of no major changes in total adenylate pool size in the observed time frame, this indicates an increase in adenylate charge.

The differences in cytosolic ATP basal levels and dynamics between Arabidopsis and *P. patens* could be associated to the evolutionary distance between the species or to a different developmental stage, Arabidopsis seedlings vs *P. patens* protonema cells in minimal medium and fully photosynthetically active. Remarkably, *P. patens* can have a life cycle completely photoautotroph, which is not the case for Arabidopsis.

In *P. patens* WT plants, the light-induced increase in FRET signal reaches a plateau *c.* 1 min after illumination. Conversely, it takes *c.* 1.5 min of darkness for the FRET signal to decrease (Fig. 1). This dynamic behaviour was highly reproducible. A potential technical explanation for the presence of a plateau may be probe saturation with $MgATP^{2-}$, where the probe cannot resolve any further increases in $MgATP^{2-}$, as previously observed in Arabidopsis (Voon *et al.*, 2018). In that case $MgATP^{2-}$ concentration surpasses the probe's binding maximum of *c.* 3 mM (at 25°C) (De Col *et al.*, 2017) after 1 min of illumination, and it takes 1.5 min in the dark to consume enough ATP to re-enter the probe's dynamic range. If this was the case, however, in experiments with shorter illumination (1 min) FRET signal would be expected to decrease immediately after the light is switched off. The ATeam signal remains stable after the end of the illumination phase for *c.* 1.5 min, independent of the duration of the light treatment (1, 3, or 8 min). An alternative biological explanation consistent with all observations is instead that $MgATP^{2-}$ concentration reaches a steady state as a result of ATP buffering by a characteristic feature of the metabolic system and/or its regulation.

This ATP buffering activity can be attributed to mitochondrial respiration that utilizes reducing equivalents produced upstream by photosynthesis and transported to the mitochondria through redox shuttles (Igamberdiev & Bykova, 2023), thus maintaining a supply of ATP in the cytosol. Following this hypothesis the decrease of reducing equivalent export from the chloroplast would thus only start impacting cytosolic ATP levels if the dark period exceeded 1.5 min. The concept of 'metabolic lag' between chloroplasts and mitochondria is not new, as it is the most accepted hypothesis to explain the phenomenon referred to as light-enhanced dark respiration (LEDR) (Lehmann *et al.*, 2016). This phenomenon describes the large value of respiration that can be measured, either as O_2 consumption or CO_2 evolution, in plants immediately after exposure to dark. Values of respiration will then approach a lower steady value in circa 30 min (Azcón-Bieto & Osmond, 1983). Behind LEDR it is believed to be the accumulation of reduced substrates, exported by chloroplasts, that are still available for mitochondria for some minutes even though they are no longer produced after a recent light exposure (Gessler *et al.*, 2017). These can drive respiration, which could as well be responsible for the 'residual' ATP production that we describe here as ATP buffering.

Another important conclusion that can be drawn from the observation of rapid cytosolic ATP accumulation after onset of illumination and the delayed decrease after the onset of darkness is an ability of the plant cells to mitigate the metabolic impact of changes in illumination intensity, maintaining relatively steady ATP levels even in the presence of abrupt light fluctuations. In a natural and dynamic environment, light changes are common and significantly impact photosynthetic productivity and yield in crops (Wang *et al.*, 2020; De Souza *et al.*, 2022). The observed kinetics imply that while photosynthetic activity responds rapidly to changes in light availability, plant cells can stabilize their

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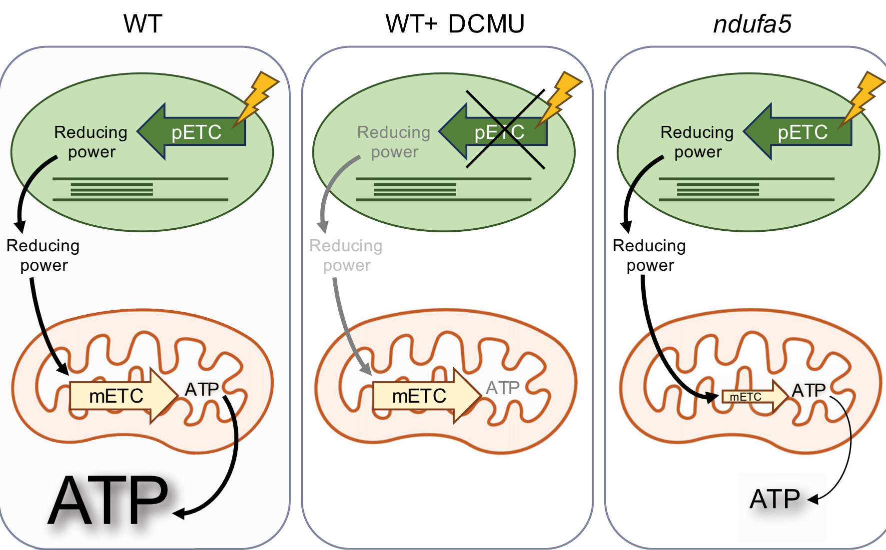

Fig. 6 Scheme depicting the proposed hypothesis for differential ATP dynamics during dark-to-light transitions in *P. patens*. In untreated wild-type (WT) plants (left), exposure to light triggers the transfer of electrons through the photosynthetic electron transport chain (pETC) which increases the abundance of reducing species, which are exported to the cytosol and used in mitochondria to sustain a transfer of electrons through the mitochondrial electron transport chain (mETC), coupled to ATP production. In presence of the PSII inhibitor DCMU (centre), transfer of electrons through the pETC is blocked, and therefore, an increased activity of mETC is not induced. In *ndufa5* plants (right), the capacity of mETC is reduced, and therefore, the ATP production rate is slower.

cytosolic ATP levels to uncouple cytosolic energy physiology from abrupt light fluctuations, while maintaining a link to long-lived changes in illumination. Using mitochondrial respiration as an integrated strategy to supply the cytosol with photosynthesis-derived ATP, rather than taking a more direct strategy such as the triose-phosphate shuttle, may provide a decisive advantage under natural highly dynamic circumstances. As such the ability to maintain photosynthesis-derived ATP supply of the cytosol stable by taking the 'detour' via mitochondrial respiration may represent a central element of the strategy of plants to cope with dynamic light conditions and to thrive under natural growth conditions (Long *et al.*, 2022).

Mitochondrial respiration is essential for cytosolic ATP accumulation

About 1 min of illumination is sufficient to elevate cytosolic $MgATP^{2-}$ levels to a steady state in WT. That steady state is maintained for 1.5 min after onset of darkness (Fig. 4b). Contrarily, in *ndufa5* plants lacking functional mitochondrial Complex I, $MgATP^{2-}$ accumulation in the cytosol occurs at an approximately three times slower rate. As a result of slower accumulation, a steady state in ATP levels is reached only after prolonged light exposure of up to 8 min. Then similar $MgATP^{2-}$ levels are reached as in WT.

The impact of respiration in cytosolic ATP supply is confirmed by the dynamics immediately after light cessation. In the *ndufa5* background, the sensor FRET signal decreases promptly in the dark, whereas in WT background, it remains stable for *c.* 1.5 min. This response behaviour supports the hypothesis that mitochondrial respiration contributes to ATP supply to the cytosol not only during illumination but also during dark-to-light transitions. *ndufa5* plants, deficient in Complex I, which contributes to 40% of proton translocation for mitochondrial ATP biosynthesis (Braun *et al.*, 2014), exhibit a significant reduction in ATP accumulation in the cytosol. The degree by which *ndufa5* plants are affected, even though the lines retain proton translocation capacity from respiratory complexes III and IV, emphasizes the importance of functional mitochondrial respiration to act as major contributor to cytosolic ATP supply in plants.

The essential role of mitochondrial ATP biosynthesis is further underscored by the phenotype of mutants (F_Ad) with impaired mitochondrial ATP biosynthesis, showing massive growth reduction even under continuous illumination. The observation that those mutants exhibit only mild alterations in photosynthetic activity, further emphasize that it is mitochondrial ATP biosynthesis and ATP supply to the cytosol that make respiration indispensable in photosynthetically active cells.

While the mutant data support that the mitochondrial contribution is crucial, all observed dynamics strictly depend on photosynthetic activity, as evidenced by their dependence from light and the abolition of ATP accumulation by the PSII inhibitor DCMU. This aligns with the hypothesis that chloroplasts predominantly export reducing equivalents, readily imported and utilized by mitochondria for ATP synthesis, which is promptly exported to the cytosol (Fig. 6).

These findings provide critical and hitherto lacking *in vivo* validation of biochemical studies on plant cell ATP compartmentation, highlighting limited capacity of chloroplasts to export ATP in mature leaves. Our findings are consistent with the idea that mitochondria play a quantitatively major role in ATP supply to the cytosol, as proposed by previous work based on biochemical considerations (Millar *et al.*, 2011; Gardeström & Igamberdiev, 2016). Metabolic flux balance analysis also recently showed that cytosolic ATP supply by the mitochondria is an efficient solution to the problem that arises when the capacity to export ATP directly from the chloroplast is limited (Shameer *et al.*, 2019). It is interesting to underline the model-based quantitative predictions on the importance of mitochondrial ATP synthesis for the cytosol under various conditions presented in the latter are fully consistent with the results presented here showing the role of mitochondrial respiration in determining the cytosolic ATP level in illuminated cells. Chloroplast capacity for ATP biosynthesis pathways that is impacted by alterations in

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mitochondrial respiration. *Chlamydomonas reinhardtii* with altered respiration increase their chloroplast ATP synthesis through activation of Cyclic Electron Transport (Cardol *et al.*, 2003). We also previously showed in *P. patens* that chloroplast ATPase activity was induced by CI inactivation in *ndufa5* (Mellon *et al.*, 2021).

While chloroplast pathways are surely contributing to ATP supply in the cell, the f_Ad mutants phenotype as well as the *in vivo* ATP dynamics in *ndufa5* suggest that they are unable to quantitatively compensate for mitochondrial inactivation, emphasizing the pivotal and dominant role of mitochondria in cytosol ATP supply.

The use of *P. patens*, which is a bryophyte, allows us to elaborate further conclusions. Opposite to Arabidopsis, this model is photoautotrophic throughout its development. Therefore, the impact of depleting respiration is not due to presence of heterotrophic tissues that rely exclusively on respiration for obtaining energy and thus enable to conclude instead that the respiration is essential in plants for the supply of ATP in the cytosol in active photosynthetic tissues.

Evolutionary perspectives on photosynthesis-dependent ATP biosynthesis in the mitochondria

Recent studies have shed light on the role of respiration in various photosynthetic organisms, including diatoms, green algae, and plants. While these investigations share a common recognition of the biological relevance of respiration, mechanistic differences have been observed. In the green alga *Chlamydomonas reinhardtii* photosynthesis and growth can proceed in photoautotrophic conditions even if respiratory Complexes III and IV are inactive (Salinas *et al.*, 2014). This contrasts with plants, where an impairment of Complexes III and IV has a major impact on growth and their complete inactivation is lethal. This divergence can be attributed to the fact that *Chlamydomonas* in its natural environment may also encounter anoxic conditions, resulting in respiratory inhibition. By contrast, plants are mostly obligatory autotrophs and have adapted to the constant presence of oxygen, rendering them hypoxia sensitive (Loreti & Perata, 2020). Since plants are normally exposed to oxygen, respiration can be continually active in plant cells without running any major risk of dysfunction. That may be sufficient to account for the evolutionary adaptation of their photosynthetic energy metabolism to strictly work in concert with respiration.

Delegating cytosolic ATP supply to mitochondrial respiration in plant cells generates carbon and O_2 cycling between the chloroplast and the mitochondria, implying that O_2 and reducing power produced by photosynthesis are consumed by mitochondrial respiration. This allocation of metabolic roles between mitochondria and chloroplasts also has an additional cost posed by the requirement of several enzymes and transporters to sustain major carbon flux rates–from carbon fixation by highly abundant Rubisco and the enzymes of the Calvin-Benson-Bassham cycle, to mitochondrial respiration by the enzymes of the Krebs cycle and the OXPHOS protein complexes – as opposed to a seemingly simple solution by using a chloroplast ATP exporter. For instance, a recent modelling approach suggests that higher capacity for mitochondrial ATP synthase flux is required in the light than in the dark in source leaves (Shameer *et al.*, 2019), underpinning the possibility that additional investment into protein biosynthesis is needed specifically for mitochondrial ATP supply of the cytosol in the light, and beyond of what is required anyway under other conditions. Assuming that these costs apply, relying on mitochondrial respiration for cytosolic ATP supply is expected to provide an evolutionary advantage that outweighs these costs.

One possible advantage that is suggested by our observations is the ability to maintain a steady ATP supply to the cytosol. Buffering photosynthesis-derived ATP supply to the cytosol under changeable light conditions may help mitigating changes in photosynthetic activity induced by rapid light fluctuations while maintaining a steady supply of ATP to maintain critical housekeeping functions. By contrast, if ATP supply were more directly linked to photosynthetic activity, cytosolic ATP availability would closely follow illumination dynamics, resulting in rapid dynamics in concentration (and possibly energy charge) following any alteration in light availability.

Another potential advantage may lie in the regulation of photosynthesis. The proton gradient (ΔpH) across the thylakoid membrane serves as the energy source for ATP biosynthesis in the chloroplast but also is a key signal for the regulation of photosynthesis, controlling the modulation of multiple mechanisms such as nonphotochemical quenching (NPQ), xanthophyll cycle and photosynthetic control (Eberhard *et al.*, 2008). If mitochondria are the primary contributors to ATP supply for the cytosol, this will allow the metabolic demand for ATP supply to be decoupled from photosynthetic regulation. In an alternative scenario where ATP synthesis is localized in the chloroplast, limiting ATP synthase activity to increase ΔpH and induce photosynthesis regulation would also restrict ATP supply to the cell. Under stress conditions, active regulation of photosynthesis via regulating the activity state of ATP synthase may thus impede ATP supply to other parts of the cell, potentially impairing essential functions of housekeeping and the ability to mount efficient stress responses, such as membrane energization, active transport, cytoskeletal remodelling and gene expression.

In both scenarios, photosynthesis-derived ATP supply via mitochondrial respiration enables plants to respond effectively to variable environmental conditions. This adaptability is a decisive evolutionary driving force for plants and may even outweigh potential disadvantages in simple energy efficiency. Hence, the data we present here offer *in vivo* evidence that mitochondrial respiration is strictly required and maintained in plants to ensure cellular supply with photosynthesis-derived ATP.

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Competing interests

None declared.

Author contributions

AA and TM contributed to the conceptualization. AMV-V, PN, KZ and S-LT contributed to the investigation. MS contributed to the resources. MS, AA and TM contributed to the supervision. AMV-V and TM contributed to the writing – original draft. AA, KZ and MS contributed to the writing – review and editing.

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Data availability

The data supporting the findings of this study are available within the article and its Supporting Information.

References

Ashton NW, Grimsley NH, Cove DJ. 1979. Analysis of gametophytic development in the moss, *Physcomitrella patens*, using auxin and cytokinin resistant mutants. *Planta* **144**: 427–435.

Azcón-Bieto J, Osmond CB. 1983. Relationship between photosynthesis and respiration: the effect of carbohydrate status on the rate of CO_2 production by respiration in darkened and illuminated wheat leaves. *Plant Physiology* **71**: 574–581.

Bailleul B, Berne N, Murik O, Petroutsos D, Prihoda J, Tanaka A, Villanova V, Bligny R, Flori S, Falconet D *et al.* 2015. Energetic coupling between plastids and mitochondria drives CO_2 assimilation in diatoms. *Nature* **524**: 366–369.

Braun HP, Binder S, Brennicke A, Eubel H, Fernie AR, Finkemeier I, Klodmann J, König AC, Kühn K, Meyer E *et al.* 2014. The life of plant mitochondrial Complex I. *Mitochondrion* **19**: 295–313.

Burlacot A, Peltier G. 2023. Energy crosstalk between photosynthesis and the algal CO_2-concentrating mechanisms. *Trends in Plant Science* **28**: 795–807.

Cardol P, Alric J, Girard-Bascou J, Franck F, Wollman FA, Finazzi G. 2009. Impaired respiration discloses the physiological significance of state transitions in Chlamydomonas. *Proceedings of the National Academy of Sciences, USA* **106**: 15979–15984.

Cardol P, Gloire G, Havaux M, Remacle C, Matagne Ŕ, Franck F. 2003. Photosynthesis and state transitions in mitochondrial mutants of *Chlamydomonas reinhardtii* affected in respiration. *Plant Physiology* **133**: 2010–2020.

De Col V, Alric J, Girard-Bascou J, Franck F, Wollman F-A, Finazzi G. 2017. ATP sensing in living plant cells reveals tissue gradients and stress dynamics of energy physiology. *eLife* **6**: 1–29.

De Souza AP, Burgess SJ, Doran L, Hansen J, Manukyan L, Maryn N, Gotarkar D. 2022. Soybean photosynthesis and crop yield are improved by accelerating recovery from photoprotection. *Science* **377**: 851–854.

Duby F, Matagne RF. 1999. Alteration of dark respiration and reduction of phototrophic growth in a mitochondrial DNA deletion mutant of Chlamydomonas lacking cob, nd4, and the 3′ end of nd5. *Plant Cell* **11**: 115–125.

Dutilleul C, Driscoll S, Cornic G, de Paepe R, Foyer CH, Noctor G. 2003. Functional mitochondrial Complex I is required by tobacco leaves for optimal photosynthetic performance in photorespiratory conditions and during transients. *Plant Physiology* **131**: 264–275.

Eberhard S, Finazzi G, Wollman F-A. 2008. The dynamics of photosynthesis. *Annual Review of Genetics* **42**: 463–515.

Edwards K, Johnstone C, Thompson C. 1991. A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Research* **19**: 1349.

Elsässer M, Feitosa-Araujo E, Lichtenauer S, Wagner S, Fuchs P, Giese J, Kotnik F, Hippler M, Meyer AJ, Maurino VG *et al.* 2020. Photosynthetic activity triggers pH and NAD redox signatures across different plant cell compartments. *bioRxiv*. doi: 10.1101/2020.10.31.363051.

Fricker MD. 2016. Quantitative Redox Imaging software. *Antioxidants & Redox Signaling* **24**: 752–762.

Fromm S, Braun HP, Peterhansel C. 2016. Mitochondrial gamma carbonic anhydrases are required for Complex I assembly and plant reproductive development. *New Phytologist* **211**: 194–207.

Gardeström P, Igamberdiev AU. 2016. The origin of cytosolic ATP in photosynthetic cells. *Physiologia Plantarum* **157**: 367–379.

Gardeström P, Wigge B. 1988. Influence of photorespiration on ATP/ADP ratios in the chloroplasts, mitochondria, and cytosol, studied by rapid fractionation of Barley (*Hordeum vulgare*) protoplasts. *Plant Physiology* **88**: 69–76.

Gerotto C, Alboresi A, Meneghesso A, Jokel M, Suorsa M, Aro EM, Morosinotto T. 2016. Flavodiiron proteins act as safety valve for electrons in *Physcomitrella patens*. *Proceedings of the National Academy of Sciences, USA* **113**: 12322–12327.

Gessler A, Roy J, Kayler Z, Ferrio JP, Alday JG, Bahn M, del Castillo J, Devidal S, García-Muñoz S, Landais D *et al.* 2017. Night and day – circadian regulation of night-time dark respiration and light-enhanced dark respiration in plant leaves and canopies. *Environmental and Experimental Botany* **137**: 14–25.

Gutierres S, Sabar M, Lelandais C, Chetrit P, Diolez P, Degand H, Boutry M, Vedel F, de Kouchkovsky Y, de Paepe R. 1997. Lack of mitochondrial and nuclear-encoded subunits of Complex I and alteration of the respiratory chain in Nicotiana sylvestris mitochondrial deletion mutants. *Proceedings of the National Academy of Sciences, USA* **94**: 3436–3441.

Igamberdiev AU, Bykova NV. 2023. Mitochondria in photosynthetic cells: coordinating redox control and energy balance. *Plant Physiology* **191**: 2104–2119.

Igamberdiev AU, Hurry V, Krömer S, Gardeström P. 1998. The role of mitochondrial electron transport during photosynthetic induction. A study with barley (*Hordeum vulgare*) protoplasts incubated with rotenone and oligomycin. *Physiologia Plantarum* **104**: 431–439.

Igamberdiev AU, Romanowska E, Gardeström P. 2001. Photorespiratory flux and mitochondrial contribution to energy and redox balance of barley leaf protoplasts in the light and during light-dark transitions. *Journal of Plant Physiology* **158**: 1325–1332.

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

Kolli R, Engstler C, Akbaş Ş, Mower JP, Soll J, Carrie C. 2020. The OXA2a insertase of arabidopsis is required for cytochrome c maturation. *Plant Physiology* **184**: 1042–1055.

Krömer S, Malmberg G, Gardeström P. 1993. Mitochondrial contribution to photosynthetic metabolism (A study with barley (*Hordeum vulgare* L.) leaf protoplasts at different light intensities and CO_2 concentrations). *Plant Physiology* **102**: 947–955.

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Kubo M, Fujita T, Hasebe M. 2017. PHYSCOmanual, v.2.0, Chapter 11.6, Overexpression. [WWW document] URL https://www.nibb.ac.jp/evodevo/PHYSCOmanual/11.6_revised_170727.htm [accessed 25 January 2024].

Larosa V, Meneghesso A, la Rocca N, Steinbeck J, Hippler M, Szabò I, Morosinotto T. 2018. Mitochondria affect photosynthetic electron transport and photosensitivity in a green alga. *Plant Physiology* 176: 2305–2314.

Lehmann MM, Wegener F, Werner RA, Werner C. 2016. Diel variations in carbon isotopic composition and concentration of organic acids and their impact on plant dark respiration in different species. *Plant Biology* 18: 776–784.

León G, Holuigue L, Jordana X. 2007. Mitochondrial Complex II is essential for gametophyte development in Arabidopsis. *Plant Physiology* 143: 1534–1546.

Li WQ, Zhang XQ, Xia C, Deng Y, Ye D. 2010. MALE GAMETOPHYTE DEFECTIVE 1, encoding the FAd subunit of mitochondrial F1F0-ATP synthase, is essential for pollen formation in *Arabidopsis thaliana*. *Plant and Cell Physiology* 51: 923–935.

Lim S-L, Flütsch S, Liu J, Distefano L, Santelia D, Lim BL. 2022. Arabidopsis guard cell chloroplasts import cytosolic ATP for starch turnover and stomatal opening. *Nature Communications* 13: 652.

Lim SL, Voon CP, Guan X, Yang Y, Gardeström P, Lim BL. 2020. In planta study of photosynthesis and photorespiration using NADPH and NADH/NAD^+ fluorescent protein sensors. *Nature Communications* 11: 17056.

Long SP, Taylor SH, Burgess SJ, Carmo-Silva E, Lawson T, De Souza AP, Leonelli L, Wang Y. 2022. Into the shadows and back into sunlight: photosynthesis in fluctuating light. *Annual Review of Plant Biology* 73: 617–648.

Loreti E, Perata P. 2020. The many facets of hypoxia in plants. *Plants* 9: 745.

Maclean AE, Hertle AP, Ligas J, Bock R, Balk J, Meyer EH. 2018. Absence of Complex I is associated with diminished respiratory chain function in European mistletoe. *Current Biology* 28: 1614–1619.

Mellon M, Storti M, Vera-Vives AM, Kramer DM, Alboresi A, Morosinotto T. 2021. Inactivation of mitochondrial Complex I stimulates chloroplast ATPase in *Physcomitrium patens*. *Plant Physiology* 187: 931–946.

Millar AH, Whelan J, Soole KL, Day DA. 2011. Organization and regulation of mitochondrial respiration in plants. *Annual Review of Plant Biology* 62: 79–104.

Nishiyama T, Hiwatashi Y, Sakakibara I, Kato M, Hasebe M. 2000. Tagged mutagenesis and gene-trap in the Moss, *Physcomitrella patens* by shuttle mutagenesis. *DNA Research* 7: 9–17.

Noguchi K, Yoshida K. 2008. Interaction between photosynthesis and respiration in illuminated leaves. *Mitochondrion* 8: 87–99.

Radin I, Mansilla N, Rödel G, Steinebrunner I. 2015. The Arabidopsis COX11 homolog is essential for cytochrome c oxidase activity. *Frontiers in Plant Science* 6: 1–17.

Ringsmuth AK, Landsberg MJ, Hankamer B. 2016. Can photosynthesis enable a global transition from fossil fuels to solar fuels, to mitigate climate change and fuel-supply limitations? *Renewable and Sustainable Energy Reviews* 62: 134–163.

Robison MM, Ling X, Smid MPL, Zarei A, Wolyn DJ. 2009. Antisense expression of mitochondrial ATP synthase subunits OSCP (ATP5) and gamma (ATP3) alters leaf morphology, metabolism and gene expression in Arabidopsis. *Plant & Cell Physiology* 50: 1840–1850.

Sadoine M, Ishikawa Y, Kleist TJ, Wudick MM, Nakamura M, Grossmann G, Frommer WB, Ho CH. 2021. Designs, applications, and limitations of genetically encoded fluorescent sensors to explore plant biology. *Plant Physiology* 187: 485–503.

Salinas T, Larosa V, Cardol P, Maréchal-Drouard L, Remacle C. 2014. Respiratory-deficient mutants of the unicellular green alga *Chlamydomonas*: a review. *Biochimie* 100: 207–218.

Schönfeld C, Wobbe L, Borgstädt R, Kienast A, Nixon PJ, Kruse O. 2004. The nucleus-encoded protein MOC1 is essential for mitochondrial light acclimation in *Chlamydomonas reinhardtii*. *Journal of Biological Chemistry* 279: 50366–50374.

Schröder L, Hegermann J, Pille P, Braun HP. 2022. The photosynthesis apparatus of European mistletoe (*Viscum album*). *Plant Physiology* 190: 1896–1914.

Schwarzländer M, Dick TP, Meyer AJ, Morgan B. 2016. Dissecting redox biology using fluorescent protein sensors. *Antioxidants & Redox Signaling* 24: 680–712.

Senkler J, Rugen N, Eubel H, Hegermann J, Braun HP. 2018. Absence of Complex I implicates rearrangement of the respiratory chain in European mistletoe. *Current Biology* 28: 1606–1613.

Senkler J, Senkler M, Eubel H, Hildebrandt T, Lengwenus C, Schertl P, Schwarzländer M, Wagner S, Wittig I, Braun HP. 2017. The mitochondrial complexome of *Arabidopsis thaliana*. *The Plant Journal* 89: 1079–1092.

Shameer S, Ratcliffe RG, Sweetlove LJ. 2019. Leaf energy balance requires mitochondrial respiration and export of chloroplast NADPH in the light. *Plant Physiology* 180: 1947–1961.

Storti M, Segalla A, Mellon M, Alboresi A, Morosinotto T. 2020. Regulation of electron transport is essential for photosystem I stability and plant growth. *New Phytologist* 228: 1316–1326.

Voon CP, Guan X, Sun Y, Sahu A, Chan MN, Gardeström P, Wagner S, Fuchs P, Nietzel T, Versaw WK *et al.* 2018. ATP compartmentation in plastids and cytosol of *Arabidopsis thaliana* revealed by fluorescent protein sensing. *Proceedings of the National Academy of Sciences, USA* 115: E10778–E10787.

Wang Y, Burgess SJ, de Becker EM, Long SP. 2020. Photosynthesis in the fleeting shadows: an overlooked opportunity for increasing crop productivity? *The Plant Journal* 101: 874–884.

Watt IN, Montgomery MG, Runswick MJ, Leslie AGW, Walker JE. 2010. Bioenergetic cost of making an adenosine triphosphate molecule in animal mitochondria. *Proceedings of the National Academy of Sciences, USA* 107: 16823–16827.

Wigge B, Krömer S, Gardeström P. 1993. The redox levels and subcellular distribution of pyridine nucleotides in illuminated barley leaf protoplasts studied by rapid fractionation. *Physiologia Plantarum* 88: 10–18.

Zhang LT, Zhang ZS, Gao HY, Meng XL, Yang C, Liu JG, Meng QW. 2012. The mitochondrial alternative oxidase pathway protects the photosynthetic apparatus against photodamage in Rumex K-1 leaves. *BMC Plant Biology* 12: 1–18.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Isolation of WT-ATeam *Physcomitrium patens* plants and verification of impact of actinic light on probes fluorescence.

Fig. S2 Cytosolic ATP dynamics during dark-to-light transitions using a pseudo-continuous light system.

Fig. S3 Isolation of ndufa5-Ateam plants and basal levels of cytosolic ATP.

Fig. S4 Generation of *Physcomitrium patens* mutant lines lacking ATP synthase subunit FAd and photosynthetic properties.

Table S1 Identification of homologous sequences for respiratory Complex V in eukaryotic organisms.

Video S1 Increase in FRET signal on a WT-ATeam plant during dark-to-light transitions.

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