Enhanced abundance and activity of the chloroplast ATP synthase in rice through the overexpression of the AtpD subunit

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Received 18 April 2022; Editorial decision 15 July 2022; Accepted 21 July 2022

Editor: Elizabete Carmo-Silva, Lancaster University, UK

Abstract

ATP, produced by the light reactions of photosynthesis, acts as the universal cellular energy cofactor fuelling all life processes. Chloroplast ATP synthase produces ATP using the proton motive force created by solar energy-driven thylakoid electron transport reactions. Here we investigate how increasing abundance of ATP synthase affects leaf photosynthesis and growth of rice, Oryza sativa variety Kitaake. We show that overexpression of AtpD, the nuclear-encoded subunit of the chloroplast ATP synthase, stimulates both abundance of the complex, confirmed by immunodetection of thylakoid complexes separated by Blue Native-PAGE, and ATP synthase activity, detected as higher proton conductivity of the thylakoid membrane. Plants with increased AtpD content had higher CO₂ assimilation rates when a stepwise increase in CO₂ partial pressure was imposed on leaves at high irradiance. Fitting of the CO₂ response curves of assimilation revealed that plants overexpressing AtpD had a higher electron transport rate (J) at high CO₂, despite having wild-type-like abundance of the cytochrome b₆f complex. A higher maximum carboxylation rate (V_{cmax}) and lower cyclic electron flow detected in transgenic plants both pointed to an increased ATP production compared with wild-type plants. Our results present evidence that the activity of ATP synthase modulates the rate of electron transport at high CO₂ and high irradiance.

Keywords: ATP synthase, CO₂ assimilation, electron transport, photosynthesis, proton motive force, thylakoid membrane.

Introduction

The efficiency of light interception is one of the major factors affecting crop yield (Zhu et al., 2010), and its improvement presents a promising route for increasing plant productivity (Evans, 2013; Long et al., 2015; Walter and Kromdijk, 2022). Absorption of light and conversion of light into chemical energy are performed by the light reactions of photosynthesis which are localized to the thylakoid membranes of chloroplasts. Using the energy of light, electrons originating from...
water split by PSII are transferred by the chain of cofactors to PSI to reduce NADP⁺. This process is known as linear electron flow (LEF). Between the two photosystems, the cytochrome b₆f complex (Cytb₆f) links electron transport with the translocation of protons across the thylakoid membrane, from stroma to lumen, which establishes the electrochemical proton gradient termed the proton motive force (pmf). The latter is used by the ATP synthase complex to produce ATP from ADP and Pᵢ. During the so-called dark reactions of photosynthesis, CO₂ is fixed into sugars by enzymes of the photosynthetic carbon reduction cycle which requires a minimum of 3 ATP and 2 NADPH to fix 1 CO₂. Since the chloroplast ATP synthase needs 4.7 protons to produce 1 ATP (von Ballmoos et al., 2008), LEF only results in production of 2.6 ATP molecules per 2 NADPH. To achieve a ratio of 3 ATP to 2 NADPH and to supply ATP for other metabolic processes, plants run cyclic electron flow (CEF) around PSI that establishes additional pmf and thus increases ATP production (Joliot and Joliot, 2005).

The capacity of the photosynthetic light reactions must be closely adjusted to their metabolic consumption by the photosynthetic carbon reduction cycle and other anabolic pathways (Cruz et al., 2005; Walker et al., 2020). Therefore, ATP synthase activity is tightly controlled by multilevel regulatory systems providing feedback from both light and dark reactions (Kanazawa et al., 2017). Redox modulation of ATP synthase through the thioredoxin system stimulates its activity under low light and in response to dark–light transition (Carrillo et al., 2016; Kohzuma et al., 2017). At low CO₂, the activity of ATP synthase is rapidly inhibited (Kanazawa and Kramer, 2002), which allows a build-up of the transmembrane proton gradient, a major component of pmf, that triggers non-photochemical quenching (NPQ) (Takizawa et al., 2007). The latter is a suite of photoprotective reactions aimed at reducing the excitation energy reaching the reaction centres of PSII by dissipating a part of absorbed light as heat in the PSII antennae (Malmøe, 2018). PROTON GRADIENT REGULATION5 (PGR5), mediating one of the CEF routes, cooperates with ATP synthase in building up the proton gradient to up-regulate NPQ (Ruban, 2016; Yamori and Shikanai, 2016).

ATP synthase is comprised of nine subunits organized in two major subcomplexes, CF₀ and CF₁. The membrane-integral CF₀ subcomplex consists of four subunits (a, b, b’, and c) and the extrinsic CF₁ subcomplex is made up of five subunits (α, β, γ, δ, and ε) (Capaldi and Aggeler, 2002; Hahn et al., 2018). ATP synthesis in CF₁ is powered by the CF₀ rotary motor in the membrane. In Arabidopsis thaliana (Arabidopsis), the b’, δ, and γ subunits are encoded by the nuclear genes atpG, atpD, and atpC1/C2, respectively. The δ subunit (hereafter AtpD) plays an important role in stabilizing the structure of the complex and regulating ATP synthesis because it forms a peripheral stalk together with subunits b and b’ that acts as a stator to prevent unproductive rotation of CF₁ with CF₀ (Maiwald et al., 2003; Hahn et al., 2018). Plants with reduced AtpD abundance show decreased LEF and increased NPQ (Price et al., 1995; Yamori et al., 2011).

Rice (Oryza sativa) is one of the world’s staple crops, and substantial efforts are being made towards improving rice productivity. The Kitaake variety is often used as a model crop because it is smaller and has a shorter life cycle compared with indica varieties, and transformation techniques have been established. Here we test whether overexpression of the AtpD subunit of ATP synthase affects the complex formation and photosynthesis in rice. We show that overexpressing AtpD results in enhanced abundance and activity of ATP synthase and has the potential to be used for photosynthesis improvement.

**Materials and methods**

**Construct assembly and transformation**

The coding sequence of O. sativa atpD (OsKitaake02g334900.1, Phytozome, https://phytozome.jgi.doe.gov/) was codon optimized for rice and the Golden Gate cloning system (Engler et al., 2014) using the IDT online tool (https://sg.idtdna.com/CodonOpt) and translationally fused at the 3’ end with the glycine linker and the Myc-tag-coding sequence (EQKLISEEDL). The resulting sequence was assembled with the Zea mays UbiquitinI promoter and the bacterial Nos terminator into the second expression module of the pAGM4723 binary vector. The first module of the binary vector contained the coding sequence of the hygromycin phosphotransferase gene (hpt) combined with the O. sativa ActinI promoter and Nos terminator. The construct was transformed into O. sativa ssp. japonica variety Kitaake using Agrobacterium tumefaciens strain AGL1 following the procedure described in Ermakova et al. (2021). T₀ plants resistant to hygromycin were transferred to soil and analysed for the presence of AtpD-Myc by immunoblotting with Myc antibodies and for the hpt copy number by digital PCR (idDNA Genetics, Norwich, UK). Lines 2, 9, and 15 were selected for further analysis based on a stronger AtpD-Myc signal from immunoblots per transgene insertion number and the availability of seeds. Homozygous T₂ seeds were obtained by selfing T₀ and T₁ plants and then used in all experiments unless stated otherwise. Wild-type (WT) plants were used as control in all experiments.

**Plant growth conditions**

Plants were grown in a controlled-environment chamber (Model PGC Flex, Conviron, Winnipeg, Canada) under ambient CO₂ partial pressure (pCO₂), 16 h photoperiod, 28 °C day, 22 °C night, and 60% humidity. Irradiance at 400 μmol photons m⁻² s⁻¹ was supplied by a mixture of Pentron Hg 4 ft fluorescent tubes (54 W 4100 K cool white, Sylvia, Wilmington, MA, USA) and halogen incandescent globes (42 W 2800 K warm white clear glass 630 lumens, CLA, Brookvale, Australia). Plants were grown in 1.2 litre pots in a potting mix composed of 80% peat/10% perlite/10% vermiculite (pH 5.6–5.8) mixed with 5 g l⁻¹ of slow-release fertilizer (Osmocote, Evergreen Garden Care, Australia). All pots were kept at field water capacity. All measurements were performed on the mid-distal leaf blade portions of the youngest fully expanded leaves from the central stem of 4-week-old plants.

**Gas exchange**

Gas exchange and fluorescence analyses were performed using a LI-6800 (LI-COR Biosciences, Lincoln, NE, USA) equipped with a fluorometer head 6800-01A (LI-COR Biosciences). Leaves were first equilibrated at 381 μbar pCO₂ in the reference side, leaf temperature 25
Thylakoid isolation and Blue Native-PAGE

Thylakoid membranes from the mid portions of the three youngest fully expanded leaves collected from one plant were ground in 100 ml of ice-cold grinding buffer (50 mM HEPES-NaOH, pH 7.5, 330 mM sorbitol, 10 mM MgCl₂) and centrifuged to a nitrocellulose membrane. Western blotting was then performed using Image Lab software (Biorad, Hercules, CA, USA).

Electron transport and electrochromic shift analyses

To obtain PSI parameters (PhiPSI, the effective quantum yield; PhiNPQ, the yield of non-photochemical quenching; PhiNO, the yield of non-regulated non-photochemical quenching) and PSI parameters (PhiPSI, the effective quantum yield; PhiNPQ, the non-photochemical yield caused by donor side limitation; PhiNA, the non-photochemical yield caused by the acceptor side limitation), fluorescence analysis was performed simultaneously with the spectroscopic measurements at 820 nm using the Dual-PAM-100 (Heinz Walz, Effeltrich, Germany). Measurements were done using red actinic light and 300 ms saturating pulses of 10 000 µmol m⁻² s⁻¹. Leaves were dark-adapted for 30 min to record the minimal and maximal levels of fluorescence in the dark. Then a saturating pulse was given after pre-illumination with far-red light to record the maximal and minimal oxidation levels of P700 (the reaction centre of PSI). To allow for a brief photoactivation, the leaves were next illuminated for 8 min with actinic light of 378 µmol m⁻² s⁻¹ and briefly dark-adapted again for 2 min. After that, photosynthetic parameters were assessed over a range of irradiances from 0 to 2043 µmol m⁻² s⁻¹ at 2 min intervals by applying a saturating pulse at the end of each illumination period. The parameters were calculated according to Kramer et al. (2004) and Klughammer and Schreiber (2008). The kinetics of P700 oxidation upon the change of light intensity presented in Fig. 5 were extracted from these measurements.

The electrochromic shift (ECS) signal was monitored as the absorbance change at 515–550 nm using the Dual-PAM-100 equipped with the PS15/535 emitter–detector module (Heinz Walz). Leaves were first dark-adapted for 40 min, and the absorbance change induced by a single turnover flash was measured. Dark interval relaxation of the ECS signal was recalculated after 3 min of illumination with red actinic light of increasing irradiance. Proton conductivity of the thylakoid membrane through the ATP synthase was calculated as an inverse of the time constant obtained by fitting the first-order ECS relaxation (Sacksteder and Kramer, 2000). Total pnfs was estimated from the amplitude of the rapid decay of the ECS signal normalized for ECS signal change induced by the single turnover flash.

Chlorophyll, protein, and Rubisco active sites

Frozen leaf discs were ground using the Qiagen TissueLyser II (Qiagen, Venlo, The Netherlands) and total Chl (a+b) was extracted in 80% acetone buffered with 25 mM HEPES-KOH and measured according to Porra et al. (1989). The amount of Rubisco sites was assayed by [¹⁴C]carboxyarabinitol bisphosphate binding as described in Ruuska et al. (2000). Total protein content was measured from the same samples by Coomassie Plus protein assay reagent (Thermo Fisher Scientific).

Statistical analysis

For all measurements, the relationship between mean values of transgenic and WT plants was tested using heteroscedastic Student’s t-tests.

Results

Overexpression of AtpD increases abundance of ATP synthase

The gene construct for AtpD overexpression (AtpD-OE hereafter) was transformed into rice calli, and T₀ plants resistant to hygromycin were selected and transferred to soil. T₀ plants were analysed for the presence of AtpD-Myc protein and for gene insertions based on the hpt copy number (Fig. 1). Out of 26 T₀ plants, 19 plants showed detectable levels of AtpD-Myc. Lines 2, 9, and 15 were selected for further analysis, and homozygous T₂ plants were studied in all experiments.

The three selected AtpD-OE lines demonstrated increased abundance of the whole ATP synthase complex in their thylakoid membranes, compared with WT plants, when the samples were normalized on a Chl (a+b) basis (Fig. 2A). When total leaf protein extracts were loaded on a leaf area basis, lines 2 and 9 had more AtpD-Myc than line 15 and also increased abundance of...
AtpH, the ε subunit of the $F_0$ complex, compared with WT plants (Fig. 2B). Abundances of D1 (PSII core subunit), Rieske (Cyt$b_6$ core subunit), and PGR5 were unaltered in transgenic lines 2 and 9, whilst line 15 had significantly less AtpH, Rieske, and PGR5, compared with the WT (Fig. 2C). Line 9 had increased leaf Chl content compared with the WT (Table 1). Despite having an increased abundance of ATP synthase on a chlorophyll basis, line 15 contained significantly less chlorophyll per leaf area (Table 1) and was excluded from further physiological analysis.

**Increased ATP synthase activity in AtpD-OE plants**

Proton conductivity of the thylakoid membrane and pmf in leaves of WT and AtpD-OE plants were estimated from the speed and amplitude of ECS relaxation upon the light–dark transition. Lines 2 and 9 showed significantly increased thylakoid proton conductivity at 400 $\mu$mol m$^{-2}$ s$^{-1}$, indicating a higher activity of ATP synthase compared with the WT (Fig. 3). When measured at 1600 $\mu$mol m$^{-2}$ s$^{-1}$, no significant differences in proton conductivity were detected between the genotypes. In line with that, the amplitude of the fast ECS decay, representing a balance between the build-up and dissipation of pmf, was significantly decreased in both AtpD-OE lines at 400 $\mu$mol m$^{-2}$ s$^{-1}$ but not at 1600 $\mu$mol m$^{-2}$ s$^{-1}$, compared with WT plants (Fig. 3).

**Electron transport properties of AtpD-OE plants**

Fluorescence analysis of WT and AtpD-OE plants demonstrated that the maximum quantum efficiency of PSII ($F_V/F_M$) did not differ between genotypes (Table 1), and neither did the PSII electron transport parameters PhiPSII, PhiNPQ, and PhiNO when measured at different irradiances and ambient $p$CO$_2$ (Fig. 4, left panels). Spectroscopic analysis of the redox state of P700, the reaction centre of PSI, at different irradiances (Fig. 4, right panels) showed that the quantum yield of PSI (PhiPSI) was increased in AtpD-OE lines (significant for line 9 at 548 $\mu$mol m$^{-2}$ s$^{-1}$ and for line 2 at 417 and 548 $\mu$mol m$^{-2}$ s$^{-1}$). The detected increase in PhiPSI in AtpD-OE plants could be attributed.
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...which was significant in line 2 between 218 µmol m\(^{-2}\) s\(^{-1}\) and 548 µmol m\(^{-2}\) s\(^{-1}\). The donor side limitation of PSI (PhiND) did not differ between the WT and AtpD-OE plants at any irradiance (Fig. 4, right panels). Further comparison of the kinetics of P700 oxidation during the increase of irradiance from 218 µmol m\(^{-2}\) s\(^{-1}\) to 417 µmol m\(^{-2}\) s\(^{-1}\) revealed faster oxidation in AtpD-OE lines, compared with the WT (Fig. 5). This result suggested that PhiNA in AtpD-OE plants was probably reduced due to an increased electron sink capacity downstream of PSI and not due to up-regulated CEF (Joliot and Johnson, 2011).

Gas exchange analysis of plants with increased ATP synthase abundance

CO\(_2\) assimilation rates and PhiPSII were assayed in WT and AtpD-OE plants at different irradiances and pCO\(_2\) (Fig. 6). When the light response of photosynthesis was analysed at 381 µbar pCO\(_2\) and high light, line 9 showed significantly increased assimilation rates between 1000 µmol m\(^{-2}\) s\(^{-1}\) and 1500 µmol m\(^{-2}\) s\(^{-1}\), compared with the WT (Fig. 6, left panels). The response of CO\(_2\) assimilation to intercellular pCO\(_2\) (AC\(_i\) curves) was measured at a constant irradiance of 1500 µmol m\(^{-2}\) s\(^{-1}\) (Fig. 6, right panels). Line 9 had significantly increased assimilation rates at all intercellular pCO\(_2\) except the lowest one, compared with the WT. Line 2 also had significantly increased assimilation rates at ambient and high intercellular pCO\(_2\). Both AtpD-OE lines showed increased PhiPSII, compared with WT plants (significant for line 2 at 133–650 µbar and for line 9 at 74–257 µbar).

Fitting of the AC\(_i\) curves revealed significantly increased J, the rate of photosynthetic electron transport, and TPU, the triose phosphate use, in AtpD-OE lines 2 and 9, compared with the WT (Table 1). Line 9 also had significantly increased V\(_{\text{cmax}}\), the maximum carboxylation rate allowed by Rubisco (P=0.18 for line 2). The J/V\(_{\text{cmax}}\) ratio and the rate of respiration in the dark (R\(_d\)) did not differ between WT and AtpD-OE plants, whilst the R\(_d)/V_{\text{cmax}}\) ratio was significantly lower than in the WT in lines 2 and 9. In line

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**Table 1.** Gas exchange and fluorescence parameters of wild-type (WT) and AtpD-OE rice plants

| Parameter | WT | Line 2 | Line 9 | Line 15 |
|-----------|----|--------|--------|---------|
| Chl (a+b), mmol m\(^{-2}\) | 0.66 ± 0.06 | 0.72 ± 0.03 | 0.86 ± 0.02* | 0.40 ± 0.03* |
| Chl a/b | 4.44 ± 0.04 | 4.40 ± 0.06 | 4.50 ± 0.06 | 4.59 ± 0.05* |
| Soluble protein, g m\(^{-2}\) | 8.49 ± 0.78 | 10.23 ± 0.37 | 12.45 ± 0.35* | 5.84 ± 0.58* |
| F\(_{v}/F\(_{m}\) | 0.806 ± 0.003 | 0.799 ± 0.005 | 0.804 ± 0.003 | 0.791 ± 0.009 |
| V\(_{\text{cmax}}\) µmol CO\(_2\) m\(^{-2}\) s\(^{-1}\) | 106.8 ± 8.3 | 133.8 ± 14.5 | 157.4 ± 12.6* | N/A |
| J (LEF), µmol s\(^{-2}\) m\(^{-2}\) s\(^{-1}\) | 125.1 ± 8.0 | 153.0 ± 1.7* | 166.8 ± 5.1* | N/A |
| J/V\(_{\text{cmax}}\) | 1.17 ± 0.03 | 1.17 ± 0.13 | 1.09 ± 0.11 | N/A |
| TPU, µmol CO\(_2\) m\(^{-2}\) s\(^{-1}\) | 9.02 ± 0.39 | 10.65 ± 0.08* | 11.24 ± 0.40* | N/A |
| R\(_d\), µmol CO\(_2\) m\(^{-2}\) s\(^{-1}\) | 1.52 ± 0.12 | 1.32 ± 0.07 | 1.40 ± 0.13 | N/A |
| R\(_d)/V_{\text{cmax}}\) | 0.0142 ± 0.0005 | 0.0100 ± 0.0010* | 0.0090 ± 0.0006* | N/A |
| Rubisco sites, µmol m\(^{-2}\) | 34.1 ± 1.7 | 35.0 ± 1.9 | 36.9 ± 1.4 | N/A |
| Rubisco sites/soluble protein | 4.02 | 3.42 | 2.97 | N/A |
| LMA, g(DW) m\(^{-2}\) | 54.2 ± 2.6 | 50.5 ± 3.6 | 53.2 ± 1.4 | 51 ± 2.2 |

F\(_v)/F\(_m\), the maximum quantum efficiency of PSII; V\(_{\text{cmax}}\), maximum carboxylation rate allowed by Rubisco; J, the rate of photosynthetic electron transport based on NADPH requirement; TPU, triose phosphate use; R\(_d\), dark respiration rate. Mean ±SE, n=4 biological replicates. Asterisks indicate statistically significant differences between the WT and transgenic plants (t-test, P<0.05). N/A, not assessed.
with the observed decrease in $R_d/V_{\text{max}}$, AtpD-OE plants also showed a lower CO$_2$ compensation point, significant for line 9 at ambient and high pO$_2$ and for line 2 at high pO$_2$ (Fig. 7). Overall, a positive correlation was observed between both $V_{\text{max}}$ and $J$ values and ATP synthase abundance (estimated as the relative abundance of the AtpH subunit from immunoblots on Fig. 2) for all three genotypes (Fig. 8). The total abundance of Rubisco active centres measured in the leaves subjected to gas exchange analysis did not differ between WT and AtpD-OE plants, but the total soluble protein content was significantly increased in line 9, compared with the WT (Table 1).
Biomass and grain yield of AtpD-OE plants

Leaf mass per area in AtpD-OE plants was similar to that of the WT (Table 1). During the mid-tillering stage, AtpD-OE plants of lines 2 and 9 were slightly larger than WT plants: $P=0.097$ for line 2 and $P=0.111$ for line 9 (Fig. 9). The total weight of seeds produced by AtpD-OE lines 2 and 9 was similar to that of the WT, whilst plants of line 15 produced significantly fewer seeds (Fig. 9C).

Discussion

Increasing the rate of electron transfer during the light reactions of photosynthesis is predicted to be of benefit for enhancing crop yield (Simkin et al., 2019). Promising strategies for accelerating electron transport include up-regulating the abundance of Cyt$b_f$ by overexpressing the Rieske subunit to alleviate rate limitation at the step of plastoquinol oxidation (Simkin et al., 2017; Ermakova et al., 2019), speeding up the delivery of electrons to PSI by engineering plants with algal cytochrome $c_6$ (Chida et al., 2007; López-Calcagno et al., 2020), and facilitating faster relaxation of NPQ (Kromdijk et al., 2016). Although the low productivity of the chloroplast ATP synthase with the high $H^+/ATP$ ratio of 4.67 was suggested to be favourable for avoiding photodamage (Davis and Kramer, 2020), we were interested to test the effects of increased ATP synthase activity on electron transport. Although the activity of ATP synthase correlates with transcript and protein levels of its subunits (Kohzuma et al., 2009), multiple additional levels of regulation make this complex a regulatory hub collecting signals from light-harvesting reactions, the photosynthetic carbon reduction cycle, and central metabolism (Schöttler and Tóth, 2014).
Rice plants overexpressing the AtpD subunit of ATP synthase demonstrated increased abundance of the whole complex, and two AtpD-OE lines had increased ATP synthase activity detected as higher proton conductivity of the thylakoid membrane (Figs 2, 3). Moreover, two AtpD-OE lines showed a proportional increase in whole-chain electron transport and CO₂ assimilation rates at high pCO₂ (Table 1; Figs 6, 8). This is in line with the C₃ photosynthesis model which predicts electron transport limitations at high irradiance and high pCO₂ as well as at lower irradiance (Farquhar and von Caemmerer, 1981; von Caemmerer and Farquhar, 1981). Studies on tobacco plants with reduced atpD transcript levels have previously shown a close correlation between CO₂ assimilation rate and AtpD abundance (Price et al., 1995; Yamori et al., 2011). Given that there are equally close correlations between Rieske content and chloroplast electron transport and CO₂ assimilation rates, previous works suggested that Cytb₆f and ATP synthase co-limit electron transport (Price et al., 1995; Yamori et al., 2011, 2016). Our results indicate that, at high irradiance and high CO₂, electron transport is primarily limited by ATP synthase, and the limitation at Cytb₆f could be overcome by increasing AtpD content. At ambient pCO₂, a significant increase of thylakoid proton conductivity was only detected in AtpD-OE plants at growth irradiance but not at high irradiance (Fig. 3), supporting a down-regulation of ATP synthase in conditions when the light reactions are limited by Rubisco activity and metabolic regeneration of NADP⁺, ADP, and Pi (Kohzuma et al., 2017).

Curiously, we also observed an increase in in vivo Rubisco activity (Vₐmax) in two AtpD-OE lines, despite similar amounts of Rubisco in the leaves (Table 1), which matched similar abundances of Rieske and other electron transport components (Fig. 2; Table 1). The lower CO₂ compensation point detected in two AtpD-OE lines was also in line with the increased Vₐmax and lower Rₐ/Vₐmax (Table 1; Fig. 7) (Azcon-Bieto and Osmond, 1983; von Caemmerer, 2000). The higher Vₐmax was probably due to the higher activation state of Rubisco in plants overexpressing AtpD. The active sites of Rubisco become inactivated by binding sugar phosphates and require Rubisco activase to restore their activity (Salvucci et al., 1985). The activase is strongly dependent on ATP as it is both regulated by the
ATP/ADP ratio and uses ATP for the reaction \( \text{Streusand and Portis, 1987; Robinson and Portis, 1988} \). Higher thylakoid proton conductivity in AtpD-OE plants could provide more ATP for Rubisco activate and sustain Rubisco carboxylation activity in conditions promoting the deactivation, for instance upon exposure to low CO$_2$ \( \text{von Caemmerer and Edmondson, 1986} \). Since CEF is strongly inhibited by ATP \( \text{Fisher et al., 2019} \), a lower CEF, seen as a lower PhiNA and faster P700 oxidation kinetics \( \text{Figs 4, 5} \), indicated an increased ATP production in AtpD-OE plants. Because activation of Rubisco is one of the promising traits for improving crop photosynthesis \( \text{Parry et al., 2013; Carmo-Silva et al., 2015; Taylor et al., 2022} \), exploring the relationship between the thylakoid proton conductivity and Rubisco activation could be of great interest for future research.

Importantly, we have developed a method for increasing the abundance and activity of ATP synthase by overexpressing one subunit of the complex. It was previously shown that AtpD is critical for stabilizing the complex and that its abundance correlates with the electron transport rate \( \text{Engelbrecht et al., 1989; Price et al., 1995} \). AtpD is one of the two nuclear gene-encoded subunits of chloroplast ATP synthase. Interestingly, despite the transfer of organellar genes to the nucleus being favourable for the cell energy budget, only a limited number of genes encoding subunits of the thylakoid complexes were translocated to the nucleus; proteins with the highest abundance are more likely to be retained in an organellar genome \( \text{Kelly, 2021} \). This suggests that nuclear-encoded subunits limit the assembly of thylakoid complexes and are involved in retrograde–anterograde signalling pathways integrating chloroplast light reactions with cellular metabolism \( \text{Lyska et al., 2013; Pribil et al., 2014} \). Taking advantage of these signalling pathways provides an opportunity for increasing the abundance of complexes by overexpressing single subunits, as previously reported for Cyt$b$ \( \text{Simkin et al., 2017; Ermakova et al., 2019} \) and as shown here for ATP synthase. However, whilst overexpression of Rieske in Arabidopsis allowed higher electron transport and CO$_2$ assimilation rates, this was accompanied by the increased abundance of not only Cyt$b$f but also ATP synthase subunits, as well as PSII and PSI \( \text{Simkin et al., 2017} \). In contrast, two rice AtpD-OE lines did not display increases in thylakoid membrane complexes other than the ATP synthase and showed a lower \( \text{pmf} \), suggesting that ATP synthase activity exceeded Cyt$b$f activity. Therefore, increasing Cyt$b$f abundance could be complementary to AtpD overexpression to further boost the electron transport rate and stimulate photosynthesis.

**Conclusion**

Photosynthetic light reactions are tightly regulated to prevent photodamage, but in some conditions overcoming or reducing the regulation of photosynthesis could be of benefit for plant productivity \( \text{Kromdijk et al., 2016} \). Here we show that overexpressing the AtpD subunit of the chloroplast ATP synthase was sufficient to increase abundance of the whole complex. Moreover, two AtpD-OE lines showed increased ATP synthase activity, resulting in higher electron transport rates at high $p$CO$_2$ and high irradiance, as well as higher assimilation rates. The gas exchange properties of AtpD-OE plants suggested that increased biomass and seed yield could be expected when plants are grown at high light and high $p$CO$_2$. Further experiments including additional lines and targeted overexpression in leaves will clarify whether increasing the AtpD content presents a novel route for enhancing crop yield.

**Acknowledgements**

We thank Aleu Mani George for [14C]carboyarabinitol bisphosphate binding assays, and the Australian Plant Phenomics Facility supported under the National Collaborative Research Infrastructure Strategy of the Australian Government for plant growth facilities.

**Author contributions**

SvC, HB, and ME: design; ME, EH, RW, BM, and HB: performing the research and data analysis; ME and SvC: writing.

**Conflict of interest**

The authors declare no conflict of interest.

**Funding**

This research was supported by the Australian Research Council Centre of Excellence for Translational Photosynthesis (CE140100015).

**Data availability**

Raw data and materials are available from the corresponding author upon request.

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