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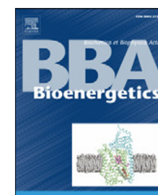

Loss of mitochondrial ATP synthase subunit beta (Atp2) alters mitochondrial and chloroplastic function and morphology in Chlamydomonas

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ABSTRACT

Mitochondrial F_1F_O ATP synthase (Complex V) catalyses ATP synthesis from ADP and inorganic phosphate using the proton-motive force generated by the substrate-driven electron transfer chain. In this work, we investigated the impact of the loss of activity of the mitochondrial enzyme in a photosynthetic organism. In this purpose, we inactivated by RNA interference the expression of the *ATP2* gene, coding for the catalytic subunit β, in the green alga *Chlamydomonas reinhardtii*. We demonstrate that in the absence of β subunit, complex V is not assembled, respiratory rate is decreased by half and ATP synthesis coupled to the respiratory activity is fully impaired. Lack of ATP synthase also affects the morphology of mitochondria which are deprived of cristae. We also show that mutants are obligate phototrophs and that rearrangements of the photosynthetic apparatus occur in the chloroplast as a response to ATP synthase deficiency in mitochondria. Altogether, our results contribute to the understanding of the yet poorly studied bioenergetic interactions between organelles in photosynthetic organisms.

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1. Introduction

F-ATP synthases are molecular rotary motors that catalyze ATP synthesis from ADP and inorganic phosphate using the proton-motive force generated by substrate-driven electron transfer chains. The enzyme can be found in the plasma membrane of eubacteria, in thylakoids of chloroplast and in the inner mitochondrial membrane of eukaryotic cells [1]. In bacteria, it is constituted of two multisubunit domains called F_1 and F_O. Composed of 3 subunits in a ratio ab_2c_{10-14}, the membrane-bound sector F_O forms a proton channel. The catalytic soluble fraction F_1 contains 5 subunits in a $\alpha_3\beta_3\gamma\delta\varepsilon$ stoichiometry and is connected to F_O by two stalks: a peripheral (stator) stalk ($b_2\delta$), and a central (rotary) stalk (γε) that couples proton translocation with the catalytic activity [2].

The function and the structure of the mitochondrial F_1F_O ATP synthase have been extensively studied in yeast and mammals. The eukaryotic enzyme shows striking similitudes to the bacterial counterpart in the overall topology, structure and subunit composition. However, the mitochondrial enzyme comprises at least eight auxiliary subunits that play a regulatory role or are implicated in the dimerization of the enzyme [3–5]. Genetic studies in yeast suggested that in the absence of each constitutive subunit of the rotor (ε, δ, γ), an enzyme complex can still be assembled but proton translocation through F_O is no longer coupled to ATP synthesis (reviewed in Ref. [6]). In contrast, in the absence of either α or β, or when these subunits are not incorporated into a functional F_1 sector, the enzyme cannot be properly assembled and it appears also that the F_O channel is not functional, thereby preventing proton leakage [7–9]. Unlike yeast and mammalian mitochondrial ATP synthase, the plant enzyme has been poorly characterized. Pioneer works have identified mitochondrial and nuclear genes coding for α and β subunits respectively in *Nicotiana plumbaginifolia* [10,11] while both subunits are nucleus-encoded in green algae like *Chlamydomonas reinhardtii* and *Polytomella* sp. [12–14]. A proteomic analysis of the mitochondrial ATP synthase in *Arabidopsis thaliana* later revealed that the majority of mammalian and yeast subunits were conserved in plants [15]. By contrast, the enzyme from three chlorophycean algae (*C. reinhardtii, Polytomella sp.* and *Volvox carteri*) lacks eight subunits conserved in other eukaryotes (*b*, *d*, *e*, *f*, *g*, *h*, F6, IF_1); instead, it contains nine subunits of unknown evolutionary origin, which were named Asa1 to 9 for "ATP Synthase Associated" proteins [16–18]. As the missing subunits are involved in building the peripheral stalk and in the dimerization of the enzyme, it was hypothesized that Asa subunits build a novel peripheral stator and dimerization module architecture. This hypothesis was supported by the construction of a *C. reinhardtii ASA7*-silenced strain, in which the enzyme dimeric form is unstable *in vitro* [19]. In *Brassica napus*, alteration of mitochondrial *atp6* gene transcription was shown to be responsible for cytoplasmic male-

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sterility [20]. Recently, antisense expression of subunits OSCP (ATP5) and γ (ATP3) were shown to affect cellular ATP levels in the dark and to alter fertility and leaf morphology [21]. Besides these data, no information is yet available about the consequences of ATP synthase activity loss on cell bioenergetics and structures in photosynthetic organisms. In this work, we investigated the consequences of the loss of the mitochondrial ATP synthase in *C. reinhardtii* by inactivating the expression of the ATP2 gene (coding for the β subunit) by RNA interference. In the absence of β subunit, the ATP synthase cannot be assembled into an enzyme complex, mitochondrial respiration is decreased, and cells growth must rely on photosynthesis. In addition, mutant cells showed altered mitochondrial and chloroplastic ultrastructure, which can be ascribed to mitochondrial ATP synthase defects and to rearrangements in the photosynthetic apparatus, respectively.

2. Materials and methods

2.1. Strain and growth conditions

The *C. reinhardtii* strain used in this study is the *cw15 arg7-8 mt*$^+$ mutant. This strain lacks a cell wall and is auxotroph for arginine because of a mutation in the *ARG7* gene coding for argininosuccinate lyase [22]. Cells were routinely grown under moderate light (50 µmol photon m^{-2} s^{-1}) at 25 °C in liquid or solid agar medium (Tris-acetate phosphate (TAP) medium or minimal medium (MIN)) [23].

2.2. Construction of plasmid ATP2-RNAi (3772 kbp)

Escherichia coli DH5α was used for cloning gene and cDNA sequences, and *E. coli* transformants were grown in Luria medium in the presence of ampicillin (50 µg/ml) at 37 °C. The pNB1 plasmid (2895 bp) was used to express double-stranded RNA (dsRNA) [65]. An *ATP2* cDNA (347 bp) and the corresponding genomic (518 bp) fragment were amplified by PCR using forward (5'-GTGGATGTGCGTTTCG-3') and reverse (5'-CCGGTCACCAGGATCT-3') primers containing *Cla*I/*Hind*III (forward) or *HindIII*/*SpeI* (reverse) restriction sites at their 5' ends for further constructions. These PCR fragments were cloned into pGEM-T Easy Vector (Promega) to obtain pATP2-1 and pATP2-8, respectively. The excised *Hin*dIII fragment of pATP2-1 was inserted into the pNB1 plasmid and the construct with inverse orientation of cDNA fragment was selected by a PCR analysis to obtain pATP2-68. The *Cla*I–*Spe*I fragment of pATP2-8 was then inserted into the *Cla*I–*Spe*I sites of pATP2-68, giving the plasmid pATP2-RNAi, used for RNA inactivation of *ATP2*.

2.3. Transformation of C. reinhardtii

Transformation of the *C. reinhardtii cw15 arg7-8 mt*$^+$ strain was carried out using the glass bead method [24] with 4 µg of plasmid pATP2-RNAi (linearized with *Sac*I) and 1 µg of pASL, linearized with *Bam*HI. This pASL plasmid bears the *Chlamydomonas ARG7* gene encoding for the enzyme argininosuccinate lyase [22] and is used as a selectable marker. Prototroph transformants were selected on TAP agar plates.

2.4. RNA analyses

Total RNA (15 µg) prepared according to Ref. [25] was separated on 0.8% agarose-formaldehyde gels and transferred onto Hybond-N membrane (Amersham Pharmacia Biotech). Digoxigenin-labeled PCR products of cDNA fragments were used as gene probes and detected with anti-digoxigenin-AP conjugates and CDP-Star as substrate (Roche, Basel, Switzerland.). Hybridization and washing steps were performed according to standard protocols. The oligonucleotides listed above and the pair of 5'-AACACCTTCTTCTCGGAGAC-3'/5'-GAGCTGAGCATGAAGTGGAT-3' primers were used to synthesize the probe for detection of *ATP2* and α-tubulin transcripts, respectively.

2.5. Protein analyses

C. reinhardtii crude total membrane fractions were obtained according to Ref. [26]. The protein content was determined by the Bradford method [27]. To conduct Blue native polyacrylamide gel electrophoresis (BN-PAGE) analyses [28], protein complexes were first solubilized in the presence of n-dodecyl-β-D-maltoside 2% (w/v), 375 mM 6-aminohexanoic acid, 250 mM EDTA, and 25 mM Bis-Tris, pH 7.0, and centrifuged for 20 min at 15,000*g* at 4 °C to remove insoluble matters. 0.4% (w/v) sodium taurodeoxycholate was then added to the supernatant prior to separation by electrophoresis on a 4% to 12% polyacrylamide gradient BN gel. ATP synthase activity was detected by incubating the gel in 50 mM HEPES, pH 8.0, containing 10 mM ATP and 30 mM $CaCl_2$. Coomassie blue staining was performed as described in Ref. [29]. For Western blot analysis, protein extracts were loaded onto 10% SDS-gels and electroblotted according to standard protocols onto PVDF membranes (Amersham GE Healthcare, Buckinghamshire, England). Detection was performed using a BM Chemiluminescence Western blotting kit (Roche, Basel, Switzerland) with anti-rabbit peroxidase conjugated antibodies. We used rabbit sera obtained against *Polytomella* sp. Atp2 (1:200,000).

2.6. ATP determination

ATP was extracted according to Ref. [30]. 15 µl perchloric acid was added to 300 µl of cell culture in exponential growth (5×10^5 to 5×10^6 cells/ml). When indicated, samples were incubated for 4 h in the dark prior to ATP extraction. The samples were homogenized and 485 µl of a saturated $NaHCO_3$ solution were added. After a brief centrifugation, samples were diluted in a 50 mM Tris-acetate buffer (pH 7.75) and ATP content was determined using the Enliten luciferase/luciferin kit (Promega) with a Lumat LB9501 apparatus (Berthold). Values were calculated on a chlorophyll basis.

2.7. Oxygen evolution

The cells were harvested during exponential phase (5×10^5 to 5×10^6 cells/ml). Dark respiration rates were measured using a Clark Electrode (Hansatech Instruments, King's Lynn, England) as previously described [31]. The cytochrome pathway and the alternative pathway of respiration were inhibited by addition of 1 mM potassium cyanide (KCN) in aqueous solution and 1 mM salicylhydroxamic acid (SHAM) in DMSO (final concentration 1%), respectively. The possible inhibitory effect of DMSO alone was subtracted from the measurements. The apparent capacity of each pathway corresponds to the following respiratory rates: for the cytochrome pathway, the oxygen consumption inhibited by KCN after addition of SHAM; for the alternative pathway, the oxygen consumption inhibited by SHAM after addition of KCN.

2.8. Enzyme activity analyses

Enzyme activity analyses were performed on total membrane fractions prepared as described by Remacle et al. [26]. Complex I (rotenone-sensitive NADH:duroquinone oxidoreductase), complex II (succinate:2,6-dichlorophenol-indophenol (DCIP) oxidoreductase) and complex III (decylbenzoquinone:cytochrome *c* oxidoreductase) were measured following published procedures [26,32].

2.9. Fluorescence emission spectra

Fluorescence emission spectra at 77 K were recorded using a LS 50B spectrofluorometer (Perkin Elmer). The excitation wavelength

was 440 nm and excitation and emission slits were 10 and 5 nm, respectively. A broad blue filter (CS-4-96, Corning, Corning, NY) was placed between the excitation window and the sample to minimize stray light. Cells were treated to induce state transitions before freezing in liquid nitrogen. Chlorophyll concentration was lower than 2 µg mL^{-1}, and it was verified that no changes in the intensity ratio of the 685- and 715-nm emission bands arose from reabsorption artifacts. Spectra were corrected for the wavelength-dependent photomultiplier response.

2.10. Transmission electron microscopy

Cell pellets were fixed for 60 min at 4 °C in 2.5% glutaraldehyde in 0.1M Sörensen's buffer (0.2M Na_2HP0_4, 0.2M NaH_2PO_4; pH 7.4). After several washes in the same buffer, the samples were postfixed for 30 min in 2% aqueous osmium tetroxide, dehydrated in a graded series of ethanol (70%, 95%, 100%), and embedded in Epon. Ultrathin sections were contrasted with uranyl acetate and lead citrate before being examined under a Jeol CX 100 II transmission electron microscope at 60 kV.

3. Results

To suppress the *ATP2* gene expression, a cell wall-less *Chlamydomonas* strain auxotrophic for arginine was cotransformed with the plasmid pASL (bearing the *ARG7* gene) and the plasmid designed for RNAi (pATP2- RNAi) (see Sections 2.2 and 2.3). Since we assumed that, as in yeast, a defect in ATP synthase activity should impair cell growth, we analyzed the growth of two hundred arg^+ colonies in heterotrophic conditions (dark + acetate). Thirty transformants showed a reduced growth in the dark. The amount of β subunit in these strains was estimated by immunological reactions with crude membrane protein extracts by using polyclonal antibodies raised against β subunit from *Polytomella* sp. (a *C. reinhardtii* close relative). In wild type, the immunological reaction gave rise to two signals at 67 and 51 kDa (Fig. 1A). To determine which of the 2 bands corresponded to the β subunit from mitochondrial complex V, we solubilized membrane proteins from a wild-type mitochondrial preparation by addition of n-dodecyl-β-D-maltoside (2% w/v), and high molecular mass complexes were separated by Blue Native PolyAcrylamide Gel Electrophoresis (BN-PAGE). The band corresponding to the mitochondrial ATP synthase was cut and loaded on a second dimensional SDS PAGE which was subjected to immunodetection. Antibodies directed against β only reacted with the upper one of the two bands in the complex V lane (Fig. 1B). Among the clones analyzed, four (hereafter called ATP2-1 to ATP2-4) showed only a faint signal for the upper band, indicative of a decreased β amount (Fig. 1A) while several clones showed reduced amount of β subunit (data not shown). One clone bearing no defect in *ATP2* expression was also conserved as a control for further experiments (referred as C-8). To confirm that loss of the β subunit in deficient strains was mediated by an inactivation of its gene expression by RNAi, RNA blots from control strains and ATP2-deficient strains were hybridized with *ATP2* and α-tubulin cDNA probes. The signal obtained with the *ATP2* probe was strongly attenuated in the *ATP2*-deficient strains compared to the controls (Fig. 1C).

We first compared the growth of *ATP2-silenced* and control strains on agar plates in low light (~5 µmol photons m^{-2} s^{-1}). In phototrophic conditions, the mutant and control strains grew exactly the same way. In contrast, the presence of acetate (5 mM) did not stimulate the growth of the mutant strains while it boosted the growth of wild-type control cells. These observations are exemplified in Fig. 1D for the ATP2-1 mutant and C-8 control strains.

A few months after their isolation, the strains were tested again for their growth and for their β subunit content. It turned that they grew like the wild-type strain and that this reversion was due to the loss of the inactivation of *ATP2* gene expression, as judged by immunological

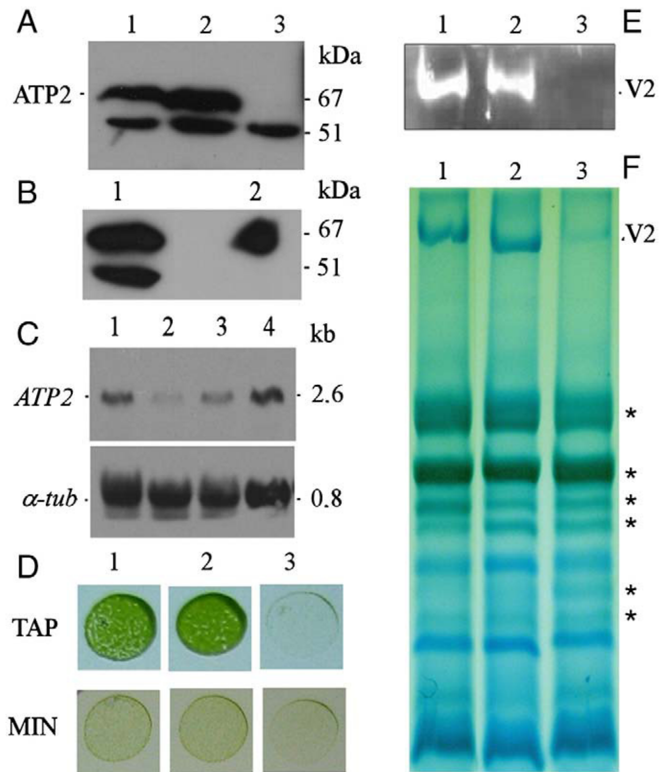

Fig. 1. Analysis of β protein amounts, *ATP2* gene transcript levels, growth phenotypes, and Complex V assembly in wild type and *ATP2*-silenced transformants. (A, B) Western blot analysis against β subunit. Proteins were resolved on a 10% acrylamide SDS-PAGE, transferred onto PVDF membranes and immunodetected with the anti-Atp2 antibody from *Polytomella sp.* (A) 15 µg proteins of the crude membrane fractions from wild-type (1), C-8 (2) and ATP2-1 (3) strains (B) 15 µg proteins of purified wild-type mitochondria (1) prepared as described in Ref. [38], purified Complex V from *Chlamydomonas* extracted from a BN-PAGE (2) loaded with 100 µg mitochondrial extract (see Fig. 1E, F for details on the procedure). (C) RNA blot analysis of the ATP2 gene transcript. Hybridization patterns were obtained with *ATP2* and α-*tubulin* probes on RNA (15 µg) blots from wild-type (1), ATP2-1 (2), ATP2-2 (3) and C-8 (4) strains. (D) Growth phenotype. Wild-type (1), C-8 control and ATP2-1 mutant cells were cultivated in low light (~5 µE m^{-2} s^{-1}). MIN, minimal medium; TAP, medium supplemented with 5 mM acetate. Cell suspensions ($A_{750\ nm}$ = 0.05) were spotted on solid agar plates and growth was evaluated after 5 days. (E, F) Analysis of ATP synthase activity and assembly by BN-PAGE. Crude membrane fractions (150 µg of protein) of *C. reinhardtii* wild-type (1), C-8 (2) and *ATP2-1*(3) strains were loaded onto a BN gel after solubilisation with n-dodecyl-β-D-maltoside (2% w/v). After electrophoresis, the gel was stained for ATPase activity (E), or with Coomassie blue (F). Asterisks indicate the position of chlorophyll-associated protein complexes.

detections against β subunit. The four original mutant strains were then subcloned and we recovered some clones that behaved as the original mutants (no growth in the dark, absence of β subunit). In less than a month, the ATP2-deficient phenotype of the recovered subclones again disappeared. The instability of the RNAi phenotype of our ATP2-silenced strains prevented us from conducting long-term time-course experiments, such as those that required large amounts of biological material (e.g. purification of mitochondria). In the next part of the manuscript, for each experiment conducted with subclones of the four original ATP2-silenced mutants, a sample was analyzed to check that the amount of β subunit was null.

To investigate the assembly and activity of complex V in ATP2-silenced strains, high-molecular-mass complexes were solubilized from crude membrane extracts with n-dodecyl-β-D-maltoside (2% w/v) and were separated by BN-PAGE. After migration, the gel was either stained with Coomassie blue or tested for ATPase activity. By both techniques, the complex V was detected in control lanes as a band of about 1700 kDa (Fig. 1E, F), which corresponds to the dimeric enzyme as previously reported [18,33]. In *ATP2*-silenced strains, only a very faint blue-band with no activity could be observed, indicating that in *Chlamydomonas* as

in yeast [7], the ATP synthase cannot assemble in the absence of the β subunit.

The steady-state levels of intracellular ATP were also measured (Fig. 2). For the wild-type strain, the content of ATP was slightly lower after 4 h of incubation in the dark compared to the steady-state level in the light. In the dark, the addition of myxothiazol (cytochrome bc_1 inhibitor) led to a 50% decrease in the intracellular ATP content. The results obtained in the light for the silenced strains showed a 40% average decrease of the ATP level compared to the wild type. In the dark, the ATP content of the mutants dropped to a level close to the ATP content achieved by the wild-type strain in presence of myxothiazol, and subsequent addition of the inhibitor to the mutants cells had no further significant effect. This set of data thus confirms that the β-deficient mutants lack mitochondrial ATP synthase activity.

In a next step, we investigated the impact of the loss of ATP synthase activity on mitochondrial respiration of mixotrophically grown cells by comparing oxygen consumption rates in the dark. Total respiratory rate was in average 35% lower in *ATP2*-silenced cells (Table 1). Apparent cytochrome and alternative oxidase (AOX) pathways capacities were then evaluated using respiratory inhibitors (potassium cyanide (KCN) for complex IV of the cytochrome pathway and salicylhydroxamic acid (SHAM) for the alternative oxidase pathway). Respiratory capacities were calculated as described in Section 2.7. Alternative pathway capacity did not significantly differ between wild-type and mutant strains. In contrast, the apparent capacity of cytochrome pathway in *ATP2-silenced* mutants represented 30% of the wild-type value. The presence of an uncoupler (10 μM CCCP) did not modify the respiratory rate or the cytochrome pathway capacity of control cells. The addition of CCCP to *ATP2-silenced* cells almost doubled the cytochrome pathway capacity. On the one hand, these results indicated that the activity of the cytochrome pathway is partly limited in *ATP2*-silenced cells, probably because the proton-motive force generated by the respiratory chain is no longer consumed to synthesize ATP. On the other hand, the absence of respiratory control (RC) in wild-type cells indicated that the respiratory chain operates at an apparent maximal rate. In contrast, phototrophically grown wild-type cells (Cardol P., unpublished data) or purified mitochondria from mixotrophically-grown cells (Mathy G., Sluse F, personal communication) have a RC value of *ca.* 2 to 2.5.

In the yeast *S. cerevisiae*, mutants affected in the assembly of complex V exhibit abnormal mitochondrial structures (e.g. [34]). We thus investigated the impact of the loss of mitochondrial ATP synthase in the *ATP2*-silenced strain on intracellular morphology by electron microscopy. The size, shape and cristae of wild-type control mitochondria (Fig. 3C) were consistent with previous analysis [35]. In contrast, while the outer mitochondrial membrane of *ATP2*-silenced mitochondria appeared normal, typical cristae were absent and the matrix stained rather uniformly (Fig. 3D). The ultrastructure

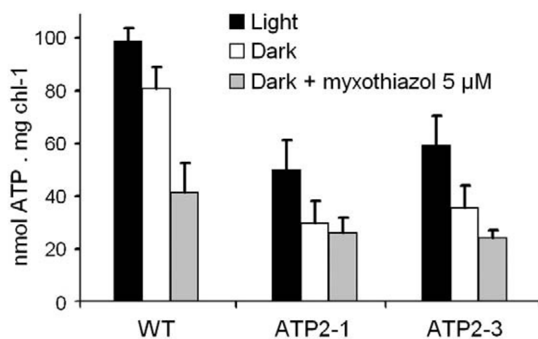

Fig. 2. Steady-state ATP levels in wild-type and *ATP2-silenced* mutant cells. Cells were fixed after continuous illumination with low light (50 μE m^{-2} s^{-1}) or after 3 h in the dark. Values are expressed in nmol ATP mg chl^{-1}. Error bars indicate standard deviations relative to 3 replicates.

Table 1
Dark respiratory rate and respiratory-chain specific activities of wild-type and *ATP2-silenced* mutant cells. The whole-cell respiratory rate and the capacity of the cytochrome and alternative pathways were measured in the absence or in the presence of an uncoupler (10 μM CCCP). Values expressed in nmol of O_2 min^{-1} 10^{-7} cells correspond to the means of three experiments (± standard deviations). Specific activities (± SD from three to six experiments) were measured in crude membrane fractions: Complex I or rotenone-sensitive NADH:duroquinone oxidoreductase (nmoles of NADH oxidized min^{-1} mg $protein^{-1}$), Complex II or succinate: DCIP oxidoreductase (nmoles DCIP reduced min^{-1} mg $protein^{-1}$), Complex III or decyl-benzoquinone: cytochrome *c* oxidoreductase (nmoles Cyt *c* reduced min^{-1} mg $protein^{-1}$), nd = not determined.

	WT	C-8	ATP2-1	ATP2-2
Respiratory rates				
Total	30.8 ± 8.5	34.6 ± 8.8	25.1 ± 4.6	18.2 ± 1.9
Capacity of the cytochrome pathway	23 ± 6.6	28 ± 5	9.5 ± 2.2	8.6 ± 5.6
Capacity of the alternative pathway	8.8 ± 2.4	6.9 ± 1	11.1 ± 3	4.7 ± 4.7
Respiratory rates in presence of CCCP 10 μM				
Total	31.9 ± 9.9	34.7 ± 13.6	29 ± 4.6	23.4 ± 2.4
Capacity of the cytochrome pathway	26.2 ± 6.4	24 ± 9.6	16.1 ± 4.8	14.2 ± 5.6
Specific activities on membrane fractions				
Complex I	18.2 ± 3.4	nd	nd	15.9 ± 4
Complex II	14.4 ± 2.7	nd	nd	11.3 ± 3.3
Complex III	161.5 ± 26	nd	nd	166.3 ± 6.6

of the remainder of the cell seemed normal at the exception of another notable difference at the level of thylakoid organization. The thylakoids from the *ATP2*-silenced cells formed thinner lamellae stacks than in the wild-type chloroplast (Fig. 3E, F). No stacks consisting of more than two layers of thylakoids could be observed in the mutant whereas stacks with up to 5 layers of thylakoids were present in the wild type.

Previous reports have indicated that the chloroplast responds to the decrease of intracellular ATP concentration by displacing Light Harvesting complexes of PSII (LHCII) on PSI (a transition called State II) (reviewed in Ref. [36]). To gain insight into the organization of the photosynthetic apparatus, we analyzed the distribution of excitation energy between PSII and PSI by determining fluorescence emission spectra of intact dark-adapted cells at low temperature (77 °K). Control cells gave rise to a spectra in which PSI (715 nm) and PSII (685 nm) contributed roughly equally to the emitted fluorescence ($F_{715}/F_{685} = 0.96 \pm 0.06$) while the ratio was increased (1.55 ± 0.09) for *ATP2*-silenced cells (Fig. 4). This result indicated a shift toward state II in the absence of mitochondrial synthase activity.

4. Discussion

In this work, we succeeded in transiently inactivating the expression of the *ATP2* gene coding for the mitochondrial ATP synthase β subunit in the green alga *Chlamydomonas*. We used an RNA interference strategy similar to the one that was successfully employed in many other studies on this organism (reviewed in Ref. [37]). Phenotypically, the *ATP2*-silenced mutant is an obligate phototroph. It thus behaves like previously characterized *Chlamydomonas* mitochondrial mutants defective in the activity of complexes I + III or I + IV, which are unable to generate a proton-motive force across the inner mitochondrial membrane [26,31,38,39]. The phenotype of the *ATP2*-silenced mutants was however unstable. As it was reported for some unstable RNAi phenotypes in *Chlamydomonas* (reviewed by Ref. [37]), we cannot decide whether the loss of the silenced phenotype is due to a strong selection against the detrimental underexpression of the essential β subunit involved in F_1-ATPase activity or due to other reasons such as the inactivation of the expression of the RNAi construct. To address this issue, attempts to inactivate the expression of the *ATP2* by means of other constructs or the expression of *ATP1 encoding the* α *subunit of F1*

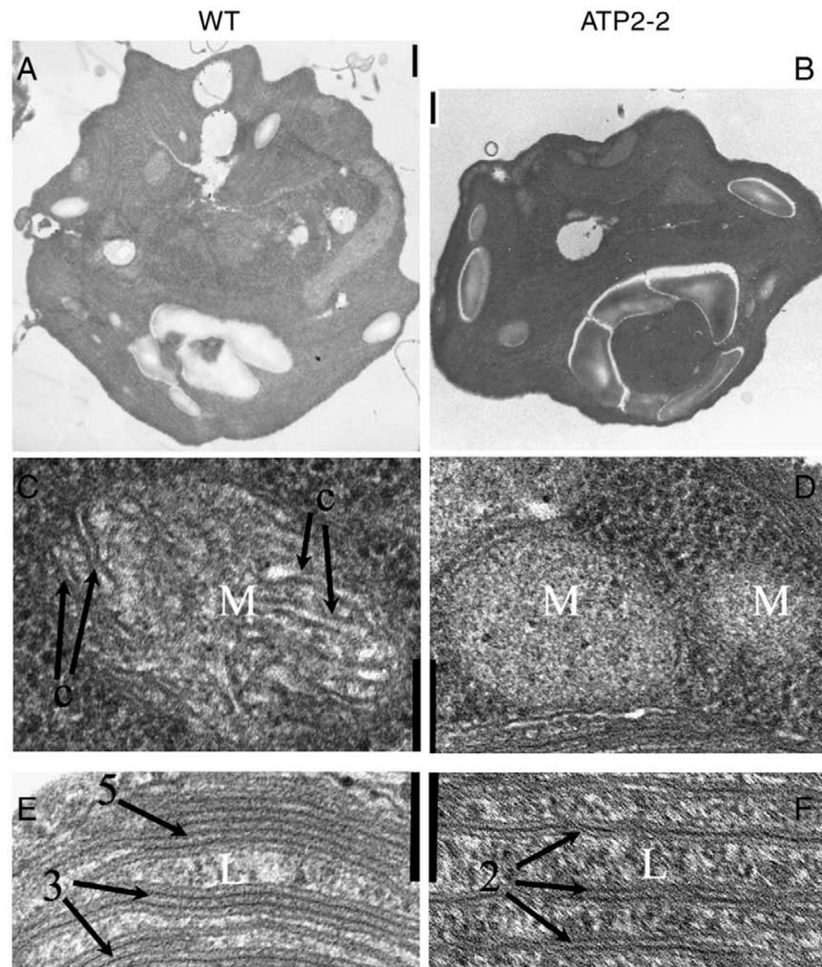

Fig. 3. Ultrastructure of wild-type and ATP2-2 mutant cells. Magnification showing the ultrastructure from cells grown in the light in the presence of 5 mM acetate. A, C and E: wild type; B, D and F: ATP2-2. M, mitochondria; C, cristae; L, lumen; numbers indicate the number of thylakoid lamellae in stacks. Black bars = 0.5 μm (A), 0.68 μm (B) or 0.2 μm (C, D, E, F).

and present in two copies in the Chlamydomonas nuclear genome [16], will be envisaged in a near future.

The silencing of *ATP2* gene expression resulted in the loss of ATP synthase assembly, and thus in the lack of ATP synthesis in mitochondria. The absence of α or β subunits constitutive of the $\alpha_3\beta_3$ catalytic hexamer also led to a dramatic loss of assembled active enzyme in the yeasts *S. cerevisiae* [40] and *S. pombe* [41], or in *Trypanosoma brucei* [42]. In *Chlamydomonas ATP2*-silenced mutants, the total oxygen consumption *in vivo* is diminished by less than a half and is thus higher

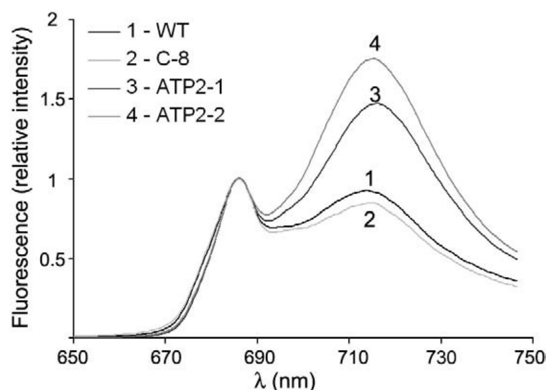

Fig. 4. Analysis of state transitions by 77 K fluorescence spectroscopy. Dark-adapted cells from wild-type control and ATP2-silenced mutant strains were frozen in liquid nitrogen. Fluorescence emission spectra (excitation wavelength = 440 nm) were recorded, normalized at 685 nm and. F_{715} to F_{685} ratios were determined (see text).

to the respiratory rate of mutant cells lacking complex I + III or I + IV activities (about 25% of wild-type value) [26,31]. In these mutants, the remaining respiration can be achieved in a non coupling way, via complex II and additional monomeric type-II NADH dehydrogenases and alternative oxidases (AOX). These alternative enzymes encountered in mitochondria from plants, several fungi, and several protists, drive the electrons from NAD(P)H to molecular oxygen in a non phosphorylating way [43,44]. In the *ATP2*-silenced cells, the cytochrome *c* pathway activity also contribute to respiration (about 35% of the total oxygen consumption) however being restricted by a lower dissipation of the proton gradient, as judged by the stimulation of the cytochrome pathway in presence of a protonophore (CCCP). Although a large stimulation by CCCP of the succinate-driven respiration is also observed on isolated mitochondria of the yeast *ATP2*-mutant [40], the relatively high respiration values of *Chlamydomonas ATP2*-silenced mutants contrasts with the low respiratory rate (<5%) and with the particularly diminished cytochrome *c* activity typically measured in yeast lacking ATP synthase (e.g. [45–47]). The respiratory chain is the main site where electron leakage to oxygen occurs, resulting in the production of the toxic reactive oxygen species (ROS). In *Chlamydomonas*, the sustained respiration in the absence of ATP synthase activity probably alleviates the redox pressure on the respiratory-chain and prevents ROS generation. In the *ATP2*-silenced mutant, the still partly functional cytochrome c pathway however raises the question of how the mitochondrial proton gradient is dissipated in the absence of coupling with ATP synthesis. In yeast, it was demonstrated that the loss of subunits α or β results in an impaired assembly of the proton channel, at variance with the loss of rotor subunits γ, δ and ε that leads to proton

leakage through Fo [6,48,49]. In *Chlamydomonas*, several alternative pathways that compete with the ATP synthase for the H^+ gradient might be considered such as uncoupling proteins (UCPs) and the ADP/ATP carrier. These proteins are anchored in the mitochondrial inner membrane and belong to the anion carrier protein family. The function of UCPs might be the maintenance of energy balance during phosphorylating conditions (reviewed in Ref. [50,51]). In yeast and mammals, ADP/ATP exchange between the cytosol and the mitochondrial matrix catalyzed by the ADP/ATP-antiporter has been shown to be also involved in the uncoupling of oxidative phosphorylation by free fatty acids [52,53]. Although genes coding for these proteins have been found in the Chlamydomonas nuclear genome [54,55], nothing is yet known about their activity and regulation in this organism. Nonetheless, maintaining a sufficient electrical potential across mitochondrial membrane is also required for the import of many proteins from the cytosol to the matrix (e.g. [56]). The way by which *Chlamydomonas* mitochondrial mutants, which lack mitochondrial respiratory activity coupled to proton gradient generation [31,57], maintain an electrical field across the membrane also remains to be elucidated. In this respect, it was shown in yeast that ATP hydrolysis by F_1 in the absence of respiration would maintain an electrochemical potential across the mitochondrial inner membrane [58]. In photosynthetic organisms, as stated here above, stable ATP synthase mutants will be necessary to study these regulatory aspects of the proton gradient generation/dissipation.

In mitochondria, the enzymes of the electron transport chain and the ATP synthase reside within the inner membrane, the surface of which is increased by invaginations into the matrix (cristae). Several studies suggest a link between the presence of complex V and the cristae morphology in yeast. When the catalytic hexamer $\alpha_3\beta_3$ is missing, mutant mitochondria are characterized by the absence of cristae and by the intramitochondrial accumulation of α and β subunit in large aggregates [7,9]. In contrast, in the absence of either subunit e or g, required for the dimerisation and oligomerisation of the enzyme, mitochondria appear to have numerous digitations and an onion-like structure (reviewed in Ref. [59]). Loss of ATP synthase assembly in *Chlamydomonas* mutant defective in β subunit also results in mitochondria deprived of cristae structures. The same observation made in yeast, mammals and plants thus reinforces the idea that the ATP synthase is a key component in the biogenesis of the inner membrane.

From a structural point of view, *ATP2*-silenced *Chlamydomonas* cells also show a peculiar organization of thylakoid stacks in the chloroplast with a reduced number of lamellae, as compared to the wild-type. In first approach, this effect cannot be ascribed to an unbalanced stoichiometry of the photosynthetic complexes (including a defect in chloroplastic ATP synthase) as indicated by the absence of phenotype of the mutant cultivated in phototrophic conditions or by the similar patterns of chlorophyll-associated complexes resolved in BN-PAGE experiments. This structural change is however accompanied by a transition to State II, as judged by 77K fluorescence emission spectra. State transitions are short-term adaptations to light quality that allow plants and algae to redistribute the light excitation energy between the two reaction centers of the photosynthetic apparatus, which operate in series and have different absorption properties. In response to persistent reduction of the plastoquinone pool (PQ), i.e. when the activity of photosystem II (PSII) exceeds the one of photosystem I (PSI), a mobile pool of PSII light harvesting complexes II (LHCII) migrates from PSII to PSI (transition from state I to state II) (reviewed in Ref. [36]). Previous studies also showed that in the absence of mitochondrial oxidative phosphorylation in *Chlamydomonas*, either by the use of inhibitors, uncouplers, in anaerobiosis [60] or in mutants affected in complexes I, III, and IV of the respiratory chain [39,61], the chloroplast reacts to the decrease of intracellular ATP by switching to state II, which promotes cyclic electron flow over linear in chloroplast (reviewed in Ref. [36]). In the cyclic electron flow, PSI operates independently of PSII and recycles electrons to the intersystem carriers instead of reducing $NADP^+$. This process might favor ATP synthesis at the expense of NAD(P)H. In *A. thaliana,* massive structural rearrangements in the chloroplast, including a decrease in the diameter of the grana stacks, were also observed under transition to state II [62]. According to the authors, LHCII migration from PSII-rich grana to PSI-rich stroma lamellae upon transition to state II destabilizes membrane bridges that interconnect lamellar sheets. In *Chlamydomonas*, thylakoids do not form grana stacks but are arranged in a complex network where thylakoids come irregularly in contact with each other (e.g. [63]). It is also known that cytochrome b_6/f complex migrates from stacked to unstacked regions of thylakoids in plant and algae upon transition to State II [64]. The fact that *Chlamydomonas* cells deficient for mitochondrial ATP synthase activity are in State II and show thylakoid rearrangement reinforces the proposal that thylakoid remodeling upon state transition is critical for cell bioenergetics upon ATP deficiency [39].

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