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ATPG is required for the accumulation and function of chloroplast ATP synthase in *Arabidopsis*

KONG MengMeng[1], WANG FenFei[1], YANG ZhongNan[2] & MI HuaLing[1*]

[1] *National Key Laboratory of Plant Molecular Genetics, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200032, China;*
[2] *College of Life and Environmental Sciences, Shanghai Normal University, Shanghai 200234, China*

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The subunit II of chloroplast ATP synthase is one of the two peripheral stalks, which associates the catalytic CF_1 with membrane-spanning CF_o. Although the structural and functional roles of chloroplast ATP synthase have been extensively examined, the physiological significance of subunit II *in vivo* is still unclear. In this work, we identified one *Arabidopsis* T-DNA insertion mutant of *atpG* gene encoding the subunit II of chloroplast ATP synthase. The *atpg* null mutant displayed an albino lethal phenotype, as it could not grow photoautotrophically. Transmission electron microscopy analysis showed that chloroplasts of *atpg* lacked the organized thylakoid membranes. Loss of subunit II affected the accumulation of CF_1-CF_o complex, however, it did not seem to have an effect on the CF_1 assembly. The light induced ATP formation of *atpg* was significantly reduced compared with the wild type. Based on these results, we suggested that ATPG was essential for the accumulation and function of chloroplast ATP synthase.

ATPG, subunit II of chloroplast ATP synthase, albino, *Arabidopsis*

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The chloroplast ATP synthase (CF_1-CF_o complex, H^+-ATPase) is a multisubunit complex that catalyzes ATP synthesis from ADP and orthophosphate coupling with the proton electrochemical potential across thylakoid membranes. It is mainly located in the unstacked regions of thylakoid membranes. The chloroplast ATP synthase consists of two parts, the hydrophilic CF_1 (subunit α_3, β_3, γ, δ and ε) and the membrane integrated CF_o (subunits I, II, III_{14} and IV). The CF_1 contains the nucleotide binding, catalytic and regulatory sites of ATP complex. The CF_o is involved in the proton transport through thylakoids [1]. These two parts are connected by a central stalk consisting of subunits γ, ε, III_{14} and a peripheral stalk composed of subunits I, II and δ [2]. The proton movement through subunit III complex drives the rotation of subunits γ and ε in catalytic core of $\alpha_3\beta_3$ hexamer to catalyze ATP synthesis [3,4]. Many researches have been carried out to explore the construction and function of chloroplast ATP synthase by biochemical technologies *in vitro* [5,6].

The peripheral stalk, which locates at the edge of chloroplast ATP synthase, is described as a highly flexible and elastic structure to stabilize the rotating machinery [7,8]. Two stalks were observed directly by electron microscopy of negatively stained CF_1-CF_o particles in 1998 [9]. Different species exhibit different subunit compositions of the peripheral stalk. In *E. coli*, it is composed of two copies of subunit b to form a homodimer, which has the left-handed coiled-coil interactions [10]. However, b′b heterodimer in photosynthetic bacteria constitutes the peripheral stalk corresponding to subunits II and I of chloroplast ATP synthase [11]. Different subunit compositions of the peripheral stalk in photosynthetic organisms appear to be a better adaptation to combine with different sites of CF_1 [12]. Although the construction and function of subunit b in *E. coli* have been

*Corresponding author (email: mihl@sippe.ac.cn)

studied in great detail [13,14], the knowledge about subunits II and I of chloroplast ATP synthase is limited. The nuclear *atpG* gene encoding the subunit II of chloroplast ATP synthase has been thought to be a duplication of *atpF* encoding the subunit I [15], but the secondary structures of two subunits are different significantly. In *Spinach*, the subunit II is shorter than subunit I and it has been predicted to contain less α-helix [12]. The subunit II has a membrane-anchored part at N-terminus and a hydrophilic part at C-terminus interacting with CF_1 subunits [16].

The chloroplast ATP synthase contains nine subunits and six of them are encoded by the chloroplast genes locating in two gene clusters [17]. Only subunits, γ, δ and II are encoded by the nuclear genes *atpC*, *atpD* and *atpG*, respectively. Two *Arabidopsis* seedling-lethal mutants (*dpa1* and *atpd*) for the genes encoding γ and δ subunits of ATP synthase have already been characterized [18,19]. The subunit γ contains the redox regulatory domain of ATP synthase [20]. Recently, evidence showed that subunit γ catalyzes ATP hydrolysis-driven proton translocation in non-photosynthetic plastids, suggesting its roles in maintaining sufficient transthylakoid proton gradient to drive protein translocation or other processes [21]. Deficiency of subunits, γ (ATPC) or δ (ATPD) affects the composition and function of chloroplast ATP synthase. As a consequence of destabilized ATP complex, accumulation of increased proton gradient through the thylakoid membranes induces high non-photochemical quenching in both mutants. However, the genetic and physiological studies on ATPG are still lacking. In this study, we have identified the *atpg* mutant from *Arabidopsis* T-DNA insertion lines. We investigated the effect of ATPG on the function and constitution of chloroplast ATP synthase. We also discuss the possible role of ATPG in chloroplast development.

1 Materials and methods

1.1 Plant materials and growth conditions

The *Arabidopsis* wild-type plants used in this study were Columbia-0 (Col). The *atpg* mutants were screened from pSKI15 activation-tagging T-DNA mutant pools [22]. Surface-sterilized seeds were sown on Murashige and Skoog (MS) medium supplemented with 2% sucrose at 22°C under 12 h light/12 h dark conditions at 15 μmol photons m^{-2} s^{-1}.

1.2 Molecular cloning of *atpG* gene and complementation

The Tail-PCR and arbitrary degenerate primers used in this work were as described [23]. T-DNA insertion site was identified by PCR amplified with primers of T-DNA left border (AtLB1, 5′-ATACGACGGATCGTAATTTGTC-3′; AtLB2, 5′-TAATAACGCTGCGGACATCTAC-3′; AtLB3 5′-TTGACCATCATACTCATTGCTG-3′) and gene specific primers (LP, 5′-CGACAAGGTCTATTACTCTCCG-3′; RP, 5′-CTGGAGTGTTTGGCTCTGAA-3′). For mutants, PCR with AtLB3 and RP primers amplified a DNA fragment about 450 bp. For wild-type plants, PCR with LP and RP primers amplified a DNA fragment about 780 bp. For heterozygous plants, PCR showed both fragments. For complementation analyses, full-length cDNA fragment of *atpG* was cloned into binary vector pMON530 under the control of CaMV 35S promoter. Specific primers 5′-ATGGCTGCTAATTCGATAATG-3′ and 5′-GACGAACAATCCAAGAAACAG-3′ were chosen for PCR amplification. A high-fidelity phusion DNA polymerase (BioLabs) was used and the construct was verified by sequencing. Complementation construct was transformed into heterozygous plants using floral dip transformation as described [24]. The transformants were selected on MS medium with 40 mg L^{-1} kanamysin.

1.3 Chlorophyll analysis

Total chlorophylls were determined according to Lichtenthaler [25]. Fresh leaves from two-week-old seedlings were homogenized in 80% acetone for 12 h at 4°C. Spectrophotometric quantification was carried out in a HITACHI U-1900 spectrophotometer. Pigment measurements were repeated in three independent experiments.

1.4 Transmission electron microscopy

Leaf segments were fixed with 2.5% glutaraldehyde in phosphate buffer (pH 7.2) for 24 h at 4°C and then washed three times with the same buffer. The tissues were post-fixed overnight in 1% OsO_4 at 4°C. The fixed samples were dehydrated through a series of ethanol solutions, infiltrated with a series of epoxy resin in epoxy propane and embedded in Epon 812 resin. Ultrathin sections were cut with a diamond knife and mounted onto copper grids. Then the samples were stained with 2% uranyl acetate for 10 min followed by lead citrate for 2 min and observed with a transmission electron microscope (Phillips CM120).

1.5 RT-PCR and northern analysis

Total RNA was isolated from three-week-old plants with RNAgents Denaturing Solution (Promega). RNA samples (2–5 μg) were used as templates for cDNA synthesis using SuperScript III First-Strand synthesis system (Invitrogen) according to the manufacturer's instructions. RT-PCR reactions were performed with specific primers for *at4g32260* (5′-CTAATCTCCCTGTCATCCAACC-3′ and 5′-GAGCAGCAATCTGAGAATCCA-3′) its neighbor genes *at4g32250* (5′-CAAAGAAGGGTTGTGGTAGA-3′ and 5′-TCAGAACTTGGTAAAGGGATA-3′), and *at4g32270* (5′-TTTCTCACTTGCCCATCTC-3′ and 5′-CATCCTTCTCGGAATTACAC-3′). *Actin2* (*at3g18780*) was used as an internal control with its primers (5′-TCTTCTTCCGCTCTTTCTTTCC-3′

and 5′-TCTTACAATTTCCCGCTCTGC-3′). Northern analysis was performed as described [26]. Primer pairs are listed in Table S2.

1.6 Preparation of thylakoids

Fresh leaves of three-week-old plants were grinded with ice-cold STN solution containing 50 mmol L^{-1} Tris-HCl (pH 7.4), 400 mmol L^{-1} sucrose, 10 mmol L^{-1} NaCl and 2 mmol L^{-1} $MgCl_2$. The extract was filtered through a nylon membrane and centrifuged at 200×*g* for 3 min at 4°C. The supernatant was centrifuged at 6000×*g* for 10 min, washed twice with STN solution and resuspended in STN solution.

1.7 Immunoblot analysis

Thylakoid membrane proteins containing 2 μg of chlorophyll were denatured with SDS sample buffer in a boiling water bath for 5 min and separated by SDS-PAGE in a 12% polyacrylamide gel with 4 mol L^{-1} urea. Then the proteins were transferred electrophoretically onto a PVDF membrane (Millipore) and incubated with different antibodies against chloroplast proteins (Agrisera). Signals were identified by an ECL plus Western blotting detection system (GE) as manufacturer's instructions.

1.8 Two-dimensional blue native/SDS-PAGE

One-dimensional blue native-polyacrylamide gel electrophoresis (BN-PAGE) was carried out as described [27,28]. Thylakoid membrane proteins isolated from three-week-old plants were solubilized in 25 mmol L^{-1} BisTris-HCl (pH 7.0), 20% glycerol and 2% dodecyl-β-D-maltopyranoside (DM). After incubation on ice for 15 min, samples were centrifuged at 16000×*g* for 20 min at 4°C. The supernatant was thoroughly mixed with loading buffer (10∶1, v/v) containing 100 mmol L^{-1} BisTris-HCl (pH 7.0), 500 mmol L^{-1} 6-aminocaproic acid, 30% sucrose and 5% Coomassie Blue G250. Then each sample equivalent to 5 μg of chlorophyll was loaded on a 4.5%–12% polyacrylamide gradient gel. For two-dimensional gel analysis, the gel strips obtained by BN-PAGE were stained with Coomassie brilliant blue and incubated in SDS sample buffer containing 5% β-mercaptoethanol for 15 min. Then gel strips were put on the top of SDS-PAGE in a 12% polyacrylamide gel with 4 mol L^{-1} urea. After electrophoresis, the proteins were transferred onto a PVDF membrane for immunoblot analysis.

1.9 Measurement of light induced ATP production

The prepared thylakoids were added to 1 mL reaction solution containing 50 mmol L^{-1} Tris-HCl (pH 7.6), 5 mmol L^{-1} NaCl, 5 mmol L^{-1} $MgCl_2$, 10 mmol L^{-1} Na_2HPO_4, 1 mmol L^{-1} FeCY and 1 mmol L^{-1} ADP. The reaction mixture was incubated for 1 min under illumination of 85 μmol photons m^{-2} s^{-1} at 25°C and inactivated by boiling water bath for 5 min. The ATP synthesis was measured by a luciferase luminescence assay [29].

1.10 Accession numbers

Sequence data from this article can be found in the GenBank data libraries under accession number: *Volvox carteri* (AAD55575), *Chlamydomonas reinhardtii* (EDP00272), *Oryza sativa* (NP_001049730), *Sorghum bicolor* (EER92459), *Spinacia* (CAA50520), *Vitis vinifera* (XP_002285183), *Populus trichocarpa* (ABK94675), *Physcomitrella patens* (XP_001754735.1), *Cyanophora paradoxa* (AAA81256) and *Arabidopsis* ATPG (AT4G32260).

2 Results

2.1 The nuclear *atpG* gene encodes the subunit II of chloroplast ATP synthase

The subunit II of chloroplast ATP synthase (ATPG) is a polypeptide of 219 amino acids with a molecular weight of 23 kD. Structural analysis revealed that ATPG contains a highly conserved domain belonging to ATP-synt_B family at C-terminal. The TargerP [30] and Predotar [31] programs predicted that ATPG protein is targeted to chloroplast. Sequence alignment showed that the ATPG homologs existed in various plants, including *Spinacia* [15], *Vitis vinifera*, *Oryza sativa*, *Populus trichocarpa*, *Sorghum bicolor* and *Physcomitrella patens*. In addition, the homologs were also found in *Volvox carteri*, *Chlamydomonas reinhardtii* and *Cyanophora paradoxa* (Figure 1(a)). We further performed a phylogenetic analysis of ATPG and its homologs to investigate the evolutionary relationship among these proteins. As shown in Figure 1(b), the proteins from angiosperms formed one clade. The proteins from monocots and dicots were divided into two separated groups within the angiosperm clade. While the proteins from alga were distant from angiosperms and bryophyte. Our data indicated that the monocot-dicot divergence was well reflected from the phylogenetic tree. Therefore, ATPG is a highly conserved ancient protein, which exists widely in photosynthetic organisms.

To analyze the *atpG* expression profile, we isolated total RNA from various tissues of three-week-old wild type. RT-PCR analysis showed that *atpG* mRNA was detectable in all tissues with high expression in rosette and cauline leaves, but it was weakly expressed in roots and siliques (Figure 1(d)). As an important signal, light plays a vital role during the process of proplastids differentiating into chloroplasts. Many genes involved in photosynthsis are expressed after exposure to 6–24 h illumination [33,34]. To examine the effect of light on *atpG* mRNA expression, we extracted total RNA from five-day-old etiolated seedlings exposed to 0, 2, 6, 9, 12 and 24 h of continuous light, respectively. RT-

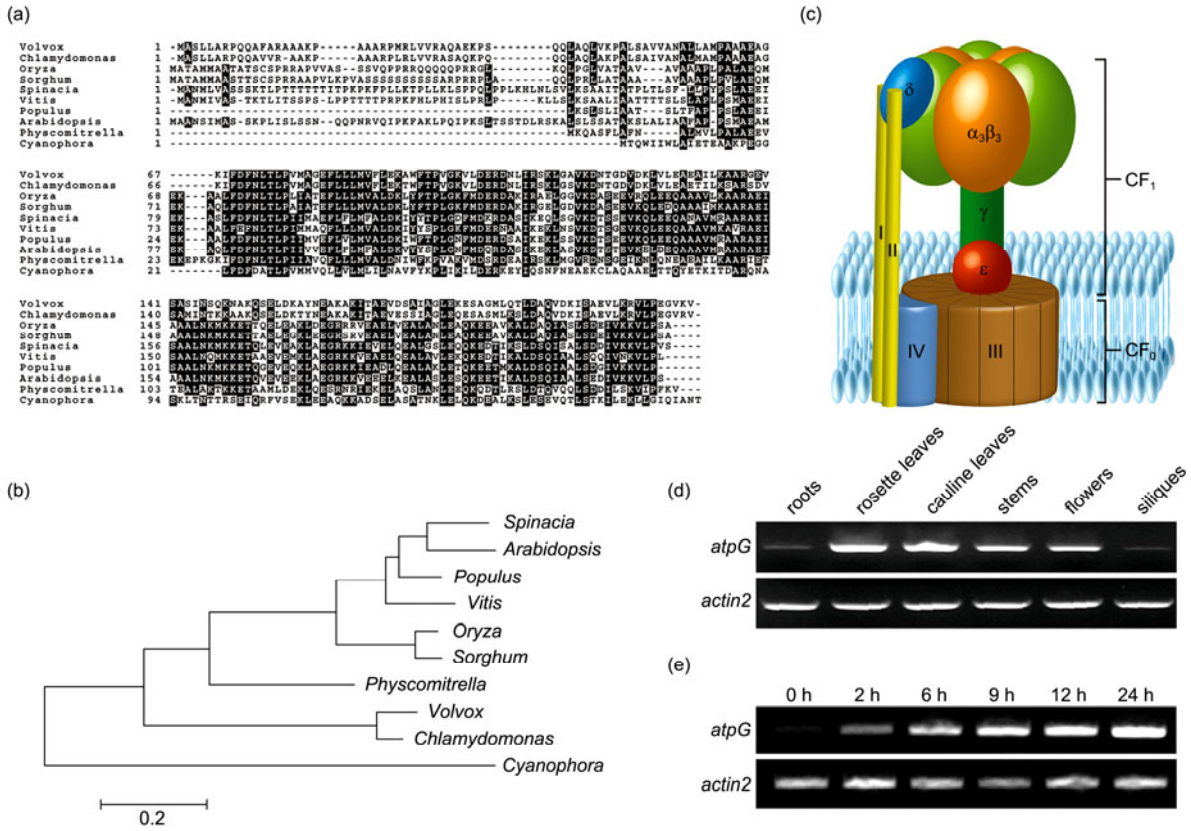

Figure 1 Structural organization of chloroplast ATP synthase and sequence analysis of the ATPG protein. (a) Alignment analysis between ATPG and its homologs. The sequences were aligned using Clustal W [32] and displayed using BOXSHADE (www.ch.embnet.org/software/BOX_form.html); (b) unrooted phylogenetic tree of ATPG and its homologs. Amino acid sequences were analyzed by MEGA 5.0; (c) the structure of chloroplast ATP synthase is based on the model of Richter et al. [4]; (d) tissue-specific expression of *atpG* gene. Total RNA was extracted from various tissues of wild-type plants; (e) light induction of *atpG* gene. Total RNA was extracted from five-day-old etiolated wild-type seedlings treated with illumination for 0, 2, 6, 9, 12 and 24 h, *actin2* gene was used as an internal control.

PCR results showed that *atpG* was slightly expressed in the dark, but after transfer of the etiolated plants to light, *atpG* mRNA was gradually increased (Figure 1(e)). This result suggests that the expression of *atpG* mRNA is induced by light.

2.2 Knockout of *atpG* leads to the seedling-lethal phenotype

We screened the *atpg* mutant from *Arabidopsis* pSKI15 activation-tagging T-DNA mutant pools [22]. The mutant was albino and seedling lethal on soil. When cultivated on sucrose-supplemented medium, the mutant survived for three weeks with 6–8 leaves (Figure 2(a)). Thermal asymmetric interlaced (TAIL) PCR analysis revealed that the T-DNA was inserted into the second exon of *atpG*, 649 bp downstream from the ATG start codon (Figure 2(b)). No chlorophyll fluorescence signal was detected in the mutant, which might have been a result of low chlorophyll content of *atpg* (Table S1). The microscopy analysis showed that the mutant had less mesophyll cells, which were loosely arranged around the vascular with larger intercellular spaces (Figure S1).

When we backcrossed the heterozygous plants with wild type, all the F_1 plants behaved like wild type. F_2 progenies segregated at a 3∶1 ratio (green∶albino=537∶169; χ^2=0.37, P<0.05), indicating that *atpg* was caused by a single recessive mutation. RT-PCR results showed that no expression of *atpG* was detected in mutant, whereas the transcripts of its neighbor genes were at similar levels as those in wild type (Figure 2(c)). In accordance with RT-PCR results, ATPG protein was undetectable in the mutant by immunodetection (Figure 2(d)). To further confirm the albino phenotype was caused by a T-DNA insertion into *atpG* locus, we carried out the complementation experiment. Full-length *atpG* cDNA under the control of CaMV 35S promoter was transformed into heterozygous plants. We obtained 16 independent transformants with the mutant background, and all these lines had restored the wild-type phenotype (Figure S2). These results demonstrated that the mutation was due to a lack of *atpG* function.

Since *atpg* mutant exhibited an albino phenotype, we further compared the ultrastructure of chloroplasts in both ecotypes grown under different light conditions by transmission electron microscopy. As shown in Figure 2(e), the wild-type chloroplasts contained well-structured thylakoid

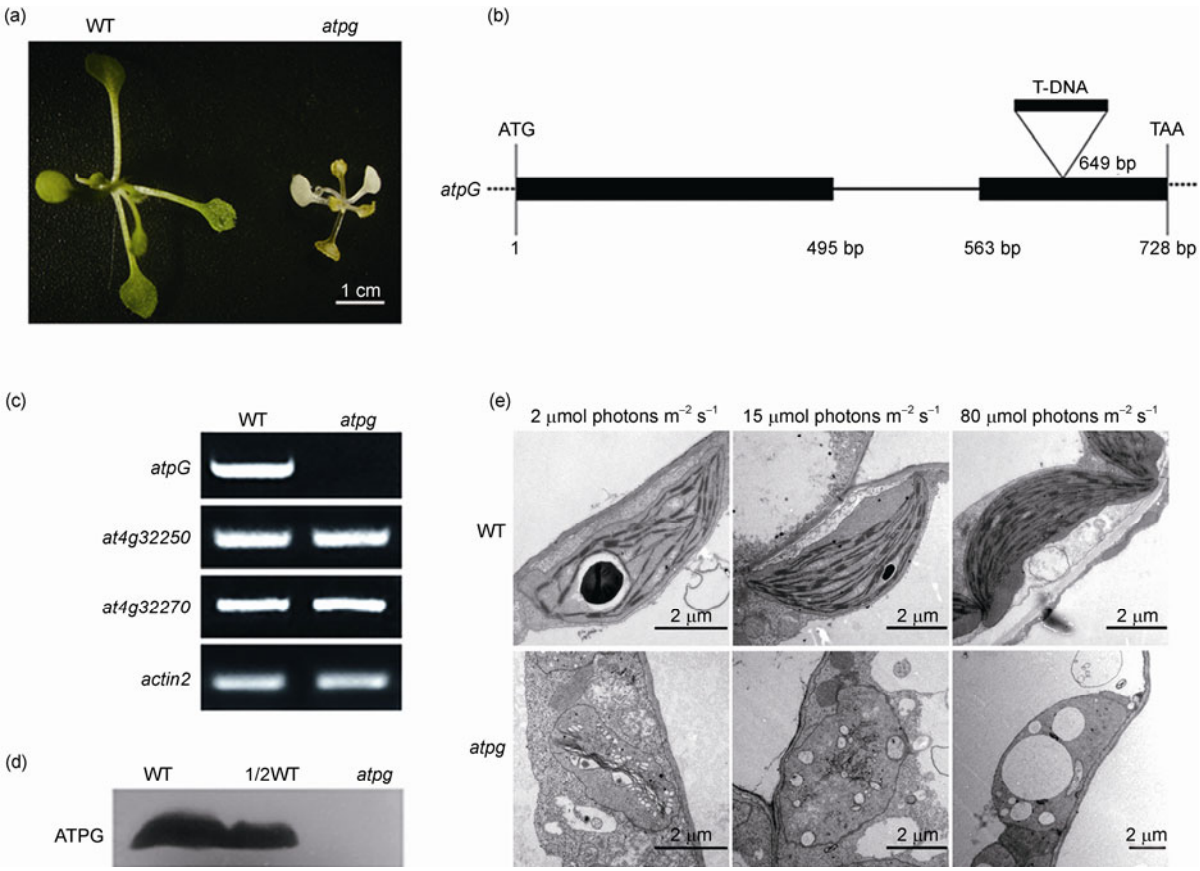

Figure 2 Visible phenotype of wild type (WT) and *atpg*. (a) Photograph of seedlings growth on MS medium supplemented with 2% sucrose at 15 μmol photons m^{-2} s^{-1}; (b) schematic representation of the T-DNA insertion into *atpG* gene. The boxes and bar represent exons and intron, respectively. The numbers below show the position of exon and T-DNA insertion; (c) RT-PCR analysis of *atpG* and its neighbor genes; (d) immunoblot analysis of ATPG protein; (e) transmission electron microscope of chloroplasts from wild type and *atpg*. The second leaves of two-week-old plants grown under different light intensity were fixed for transmission electron microscope analysis, scale bars are indicated.

membranes composed of stroma thylakoids and granna thylakoids. While in the *atpg* mutant, some stacked but disorganized thylakoid membranes with swollen lumen space were observed when grown under low intensity light of 2 μmol photons m^{-2} s^{-1}. When light intensity increased to 15 μmol photons m^{-2} s^{-1}, only few short appressed membranes and vesicle-like structures were observed. The chloroplasts were completely vacuolated lacking the internal membrane when grown under normal light of 80 μmol photons m^{-2} s^{-1}. These results indicated that the chloroplast development in *atpg* was arrested at an early stage, especially during thylakoid membrane formation and this might have been aggravated by photo-oxidative stress.

2.3 The impaired accumulation of other subunits of ATP synthase in *atpg*

To investigate the level of other subunits of ATP synthase in the absence of subunit II, chloroplast proteins from three-week-old wild type and *atpg* leaves were isolated for immunoblot analysis. We examined all five subunits of CF_1 and detected four of them (subunits α, β, γ and ε) in *atpg*. The amount of subunit γ was significantly reduced compared to the wild type. However, the level of subunit ε was increased, which might be due to the fact that the protein concentration in *atpg* was more than ten times of those in the wild type, when equal amounts of chlorophyll were loaded. No accumulation of subunit I was detected in *atpg*, indicating that the mutation probably caused the destabilization of membrane integrated CF_o (Figure 3(a)). We further examined the transcript levels of genes encoding ATP synthase subunits. Northern analysis demonstrated that the plastid-encoded genes *atpA*, *atpB*, *atpF* and nuclear-encoded genes *atpC* and *atpD* did not show major changes in *atpg* (Figure 3(c)). This result suggested that a severe defect in the accumulation of other subunits of ATP synthase in *atpg* was not due to the defective transcriptional activity.

In addition, we also checked the changes of other photosynthetic proteins in *atpg*. Immunoblot analysis showed that the photosystem II reaction center D1 (PsbA) and D2 (PsbD) proteins, cytochrome *f* (Cytf) were undetected in *atpg*. Levels of CP47 protein of photosystem II (PsbB), the D subunit of photosystem I (PsaD), the chlorophyll-binding protein Lhca1 and Lhcb1 were slightly decreased compared to thoses

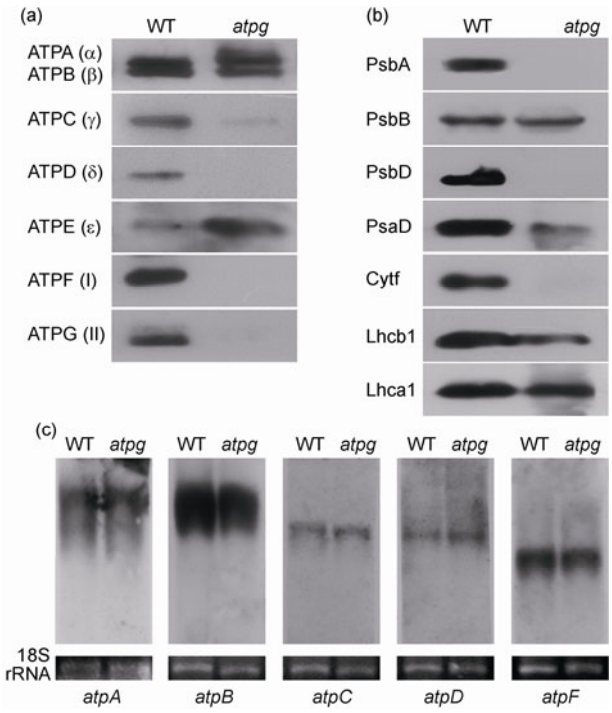

Figure 3 Immunoblot analysis of chloroplast proteins and northern analysis of nuclear- and plastid-encoded genes in wild type and *atpg*. (a) Immunoblot analysis of chloroplast ATP synthase subunits; (b) immunoblot analysis of other chloroplast proteins. Thylakoid membranes containing 2 μg of chlorophyll were separated by SDS-PAGE, transferred to a PVDF membrane and immunoblotted with antibodies against photosynthetic proteins; (c) Northern analysis of nuclear- and plastid-encoded genes. About 10 μg total RNA was transferred to a nylonmembrane after electrophoresis and further probed with DIG-labeled probes for *atpA*, *atpB*, *atpC*, *atpD* and *atpF*.

in the wild type (Figure 3(b)).

2.4 The defect in the CF_1-CF_o complex in *atpg*

The subunit II together with subunit I act as a peripheral stalk to connect the CF_1 and CF_o [2]. To explore the function of ATPG on the enzyme accumulation, chloroplast proteins were separated by two-dimensional blue native/SDS-PAGE and subsequently immunoblotted with specific antibodies raised against subunits β (ATPB) and γ (ATPC). In wild-type plants, the chloroplast ATP synthase can be separated into three distinguishable subcomplexes as previously reported [35]. The first protein spot corresponding to a molecular mass of 550 kD represents the monomeric form of CF_1-CF_o complex, 440 kD protein spot represents the assembled CF_1 particles and 140 kD protein spot represents free α and β subunits (Figure 4). In *atpg* mutant, only the second and third spots were detected when probed with an antibody against subunit β, whereas the 550 kD protein spot was missed. Moreover, the level of free subunit β was shown to be increased relative to the wild type. The subunit γ was also found in the assembled CF_1 spot corresponding to a molecular mass of 440 kD in *atpg*. These results suggested

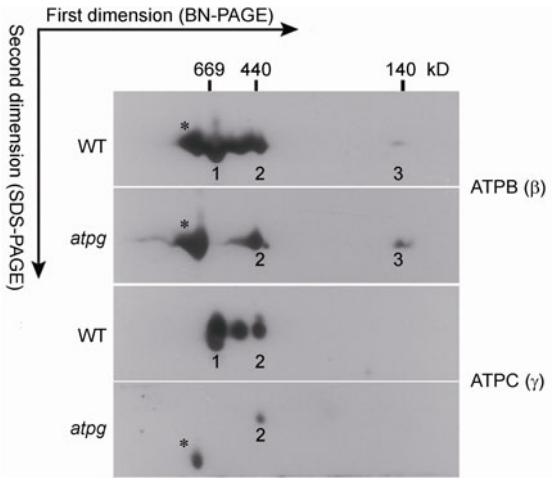

Figure 4 Immunodetection analysis of chloroplast ATP synthase subunits separated by two-dimensional blue native/SDS-PAGE in wild type and *atpg*. Chloroplasts from three-week-old plants containing 5 μg of chlorophyll were separated by one-dimensional blue native-PAGE. Then the gel strips were subjected to SDS-PAGE in the second dimension and immunoblotted with antibodies raised against subunits β and γ. The numbers below indicate different subcomplexes of chloroplast ATP synthase. 1 represents the monomeric form of ATP complex, 2 represents the assembled CF_1 part, 3 represents free α and β subunits, respectively. The asterisks (*) indicate the non-specific binding of antibodies with unknown proteins.

that subunits β and γ were probably assembled into CF_1 without the subunit II, but the accumulation of integral CF_1-CF_o complex was disturbed.

2.5 The suppression of the activity of light induced ATP production in *atpg*

Adenosine triphosphate (ATP) has long been recognized as a universal energy currency of living cells. In chloroplast, ATP is required for the photosynthetic assimilation of carbon dioxide into carbohydrates. To study whether the chloroplast ATP synthase activity is affected in the absence of subunit II, we measured the light induced ATP production in both ecotypes using potassium ferricyanide (FeCY) as an electron acceptor. As shown in Figure 5, the content of light induced ATP synthesis in the wild type was about 39.26± 9.45 μmol mg^{-1} chl h^{-1}. In contrast, it was 62.8% lower in *atpg* than that of wild type (14.61±7.23 μmol mg^{-1}chl h^{-1}). The result indicates that deletion of subunit II of chloroplast ATP synthesis causes the severe suppression of photophosphorylation activity.

3 Discussion

3.1 ATPG is essential for the CF_1-CF_o complex accumulation in chloroplast

It is well known that chloroplast ATP synthase catalyzes the light-dependent synthesis of ATP coupling with transmembrane proton transport. In higher plants, isolated chloroplasts

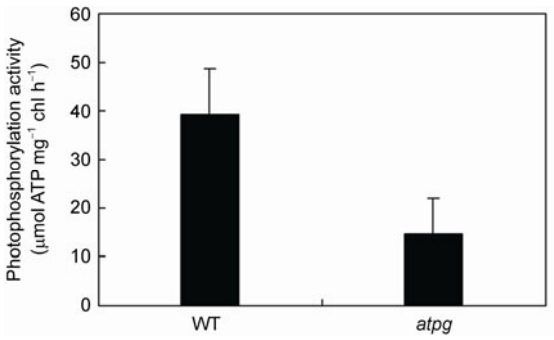

Figure 5 The light induced ATP production of chloroplasts in wild type and *atpg*. The reactions were carried out at room temperature and potassium ferricyanide (FeCY) was used as an electron acceptor. The ATP content was measured by a luciferase luminescence assay.

synthesize most ATP synthase subunits that are chloroplast-encoded. These chloroplast-made components can form a subcomplex at the earlier stage of ATP synthase assembly [36]. Only subunits γ, δ and II are synthesized outside the chloroplast and then imported. Previous studies have shown that the chloroplast ATP synthase was destabilized without subunits γ or δ, suggesting that both were essential for the enzyme assembly [18,19].

The subunit II of CF_o functions as a peripheral stalk to link the hydrophilic CF_1 with membrane-spanning CF_o. Complete deletion of this subunit in cyanobacterium is lethal, suggesting the subunit b′ is essential for cell viability [37]. In this study, we reported the isolation and characterization of a null mutant of *atpG* encoding the subunit II of ATP synthase in *Arabidopsis*, which might shed light on the assembly mechanism of chloroplast ATP enzyme. Choquet and Vallon [38] have pointed out that the assembly of ATP synthase is an intricate process involving various inter-subunit interactions within CF_1 and CF_o or between these subcomplexes. Some protein factors have been proven to interact with certain ATP synthase subunits to stabilize or promote assembly of the enzyme, such as Alb4 [35]. By two-dimensional blue native/SDS-PAGE analysis, we found that lack of subunit II affected the CF_1-CF_o complex accumulation (Figure 4). This result suggests that the subunit II might be involved in the stabilization and/or assembly of CF_1-CF_o complex. However, it is not clear if this is the secondary effect of other ATP synthase subunits deficiency. Together with *dpa1* and *atpd* mutants [18,19], we conclude that the chloroplast ATP synthase is a highly organized complex, one subunit deletion often affects the accumulation of whole complex.

The CF_1-CF_o complex is able to catalyze a reversible reaction: ATP synthesis and hydrolysis. When treated with EDTA or other chelating agents, the pure CF_1 could be isolated from thylakoid membranes. The isolated CF_1 is a latent ATPase [39]. Despite the fact that *atpg* lacked the organized thylakoid membranes, four subunits (subunits α, β, γ and ε) appeared to assemble into an incomplete CF_1 complex which was not associated with the thylakoid membranes. This result further proved that the subunit II is involved in CF_1 integrating into the proton channel CF_o. Our result was consistent with some ATP synthase mutants of *Chlamydomonas reinhardtii*, in which the mutants defective in CF_o subunits allowed the accumulation of some CF_1 components, but the mutant lacking a CF_1 subunit appeared to lose all the CF_o subunits [40]. In summary, loss of the subunit II causes the catalytic CF_1 detached from transmembrane CF_o, but the assembly of CF_1 seems to be less affected. Furthermore, we could infer that assembly of catalytic CF_1 head is independent of the membrane-bound CF_o.

3.2 ATPG is required for the photophosphorylation activity

In chloroplast, the light induced ATP production through the use of a proton gradient is referred to as photophosphorylation. There are three modes of photophosphorylation *in vivo* including cyclic, non-cyclic and pseudocyclic photophosphorylation. Cyclic photophosphorylation involves photosystem I and synthesizes ATP only. Non-cyclic photophosphorylation produces both NADPH and ATP, which could be used in the Calvin cycle [41]. In our study, the light induced ATP production of *atpg* was decreased to 37.2% of the wild type level (Figure 5), indicating that inactivation of subunit II inhibited the photophosphorylation activity significantly.

ATP synthesis involves the transfer of electrons from the intermembrane space, through inner membrane, back to the matrix. We noticed that although the electron transfer chain was damaged due to the photosystems deficiency in the mutant, a small amount of light induced ATP formation was still detected when substrates were supplied to the reaction. The reasonable explanation is that extensive early studies have confirmed that the main catalytic site of ATP synthase is subunit β with some contributions from side chains in the subunit α [42,43]. So we hypothesized that the incomplete CF_1 head composing of subunits β and α in the mutant maintained partial photophosphorylation activity *in vivo*. And the residual thylakoid membranes in *atpg* might provide a prerequisite for the catalysis of ATP formation.

3.3 ATPG is required for the chloroplast development

Mutations of many nuclear genes that are involved in chloroplast development would cause albino phenotype. The chloroplasts of these mutants often lack internal membrane or contain few appressed membranes, such as *apg3*, *dxr* and *agy1* [44–46]. Electron microscopy analysis showed that the chloroplast development in *atpg* was blocked even grown under low light conditions (Figure 2(e)). These findings suggest that ATPG is required for chloroplast development, especially during the thylakoid formation. In higher plants, chloroplast development is an extremely complex procedure,

which involves many energy-dependent processes. Generally, at the onset of illumination, Pchlide is converted to Chlide and the prolamellar body breaks up into some small vesicles, the photosystem I and II reaction centers start to form in these vesicles and the photosystem I-mediated cyclic photophosphorylation appears within the first few minutes [47,48]. Baker and Butler [49] inferred that these vesicles contain an active proton pump, implying that photophosphorylation has an important role at the early stage of chloroplast development. In this study, the loss of the subunit II affected the CF_1-CF_o complex accumulation, further impaired the photophosphorylation activity. Therefore, it is reasonable to assume that the defective chloroplast development in *atpg* results from the lack of ATP supply. As previously reported, knockout of subunit γ affected the chloroplast development [18], we conclude that inactivation of single subunit of chloroplast ATP synthase would lead to the blockage of chloroplast development.

On the other hand, chloroplast ATP synthase is one of the four photosynthetic complexes localized in thylakoid membranes. Failure in the assembly of ATP synthase would also affect the elongation of thylakoids and the secondary effect of impaired thylakoids is degradation of most thylakoid proteins. As shown in Figure 3(b), some core proteins of photosystem were absent in *atpg*, including CF_o subunits. Incomplete photosystems would cause the photo-damage of photosynthetic organization so that the mutant could hardly survive even under weak light conditions.

4 Conclusions

In this study, we isolated one *Arabidopsis* T-DNA insertion mutant of *atpG* gene encoding the subunit II of chloroplast ATP synthase. We demonstrated that loss of subunit II affects the accumulation of CF_1-CF_o complex, resulting in impairment of the function of chloroplast ATP synthase and chloroplast development. We suggest that the subunit II is of structural and functional importance for the chloroplast ATP synthase.

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Supporting Information

Table S1 Chlorophyll content of wild-type plants and *atpg* mutants
Table S2 Primers used in northern analysis
Figure S1 Microscopy images of leaves from two-week-old wild type and *atpg* mutant.
Figure S2 Phenotype of wild type (WT) and complementary plant (*atpgc*).

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