

Light- and Metabolism-related Regulation of the Chloroplast ATP Synthase Has Distinct Mechanisms and Functions*

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Background: Chloroplast ATP synthase activity is regulated by both light and metabolic factors, but the relationship between these regulatory modes is not established.

Results: Mutating three highly conserved acidic amino acid residues in the γ subunit alters light- but not metabolism-induced regulation.

Conclusion: Metabolism and light regulation operates via distinct mechanisms.

Significance: The chloroplast ATP synthase is a key control point for the light and dark reactions of photosynthesis.

The chloroplast CF₀-CF₁-ATP synthase (ATP synthase) is activated in the light and inactivated in the dark by thioredoxin-mediated redox modulation of a disulfide bridge on its γ subunit. The activity of the ATP synthase is also fine-tuned during steady-state photosynthesis in response to metabolic changes, e.g. altering CO₂ levels to adjust the thylakoid proton gradient and thus the regulation of light harvesting and electron transfer. The mechanism of this fine-tuning is unknown. We test here the possibility that it also involves redox modulation. We found that modifying the *Arabidopsis thaliana* γ subunit by mutating three highly conserved acidic amino acids, D211V, E212L, and E226L, resulted in a mutant, termed *mothra*, in which ATP synthase which lacked light-dark regulation had relatively small effects on maximal activity *in vivo*. *In situ* equilibrium redox titrations and thiol redox-sensitive labeling studies showed that the γ subunit disulfide/sulfhydryl couple in the modified ATP synthase has a more reducing redox potential and thus remains predominantly oxidized under physiological conditions, implying that the highly conserved acidic residues in the γ subunit influence thiol redox potential. In contrast to its altered light-dark regulation, *mothra* retained wild-type fine-tuning of ATP synthase activity in response to changes in ambient CO₂ concentrations, indicating that the light-dark- and metabolic-related regulation occur through different mechanisms, possibly via small molecule allosteric effectors or covalent modification.

The chloroplast CF₀-CF₁-ATP synthase (ATP synthase)² drives the reversible synthesis of ATP from ADP and P_i using

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² The abbreviations used are: ATP synthase, chloroplast CF₀-CF₁-ATP synthase; AMS, 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonate; ECS,

energy from the light-driven proton electrochemical gradient, or proton motive force (pmf) (1, 2). This multisubunit complex is composed of two subcomplexes. The membrane-embedded CF₀ subcomplex converts energy from proton flux into rotational motion. The water-soluble CF₁ subcomplex couples this rotational motion to the synthesis of ATP. The architecture of the chloroplast complex is similar to that of bacterial and mitochondrial orthologues (3, 4). The F₁ subcomplex, composed of five subunits designated (in common nomenclature) α , β , γ , δ , and ϵ , with stoichiometry of 3:3:1:1:1, contains three catalytic nucleotide binding sites in the β subunits and three noncatalytic nucleotide-binding sites at the water-soluble thylakoid membrane surface (5, 6). The α and β subunits form a hexagonal $\alpha_3\beta_3$ ring around the γ subunit as a central stalk, whereas δ and ϵ subunits have a function in stabilizing the structure and inhibiting ATP hydrolysis activity, respectively (7). The integral membrane CF₀ subcomplex contains four subunits (I, II, III₁₄, and IV, also called b, b', c, and a) and couples transmembrane proton movement with rotational torque generation (8, 9).

The chloroplast ATP synthase is regulated at multiple levels. As with other ATP synthases, it is activated by imposition of pmf. An additional level of regulation occurs by redox modulation of a disulfide/sulfhydryl pair on the γ subunit via thioredoxin (1, 9–13). This redox regulation modulates the amplitude of pmf required to activate the ATP synthase and has been proposed to prevent “wasteful” ATP hydrolysis in the dark by reversal of the ATP synthase reaction (14). Thiol modulation of the chloroplast ATP synthase is structurally assigned to a chloroplast-specific 9-amino acid “loop” inserted in the γ subunit containing a pair of redox-active cysteine residues (Cys¹⁹⁹ and Cys²⁰⁵ in *Arabidopsis thaliana*) (Fig. 1A).

Thiol activation occurs at very low irradiances and thus has been proposed to act as an “on-off” switch (15), probably

electrochromic shift; g_{H+}, conductivity of thylakoid membrane to protons; LEF, linear electron flow; *mothra*, modified thioredoxin-regulated ATP synthase; pmf, proton motive force; q_E, energy-dependent exciton quenching; 35S, cauliflower mosaic virus 35S promoter; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

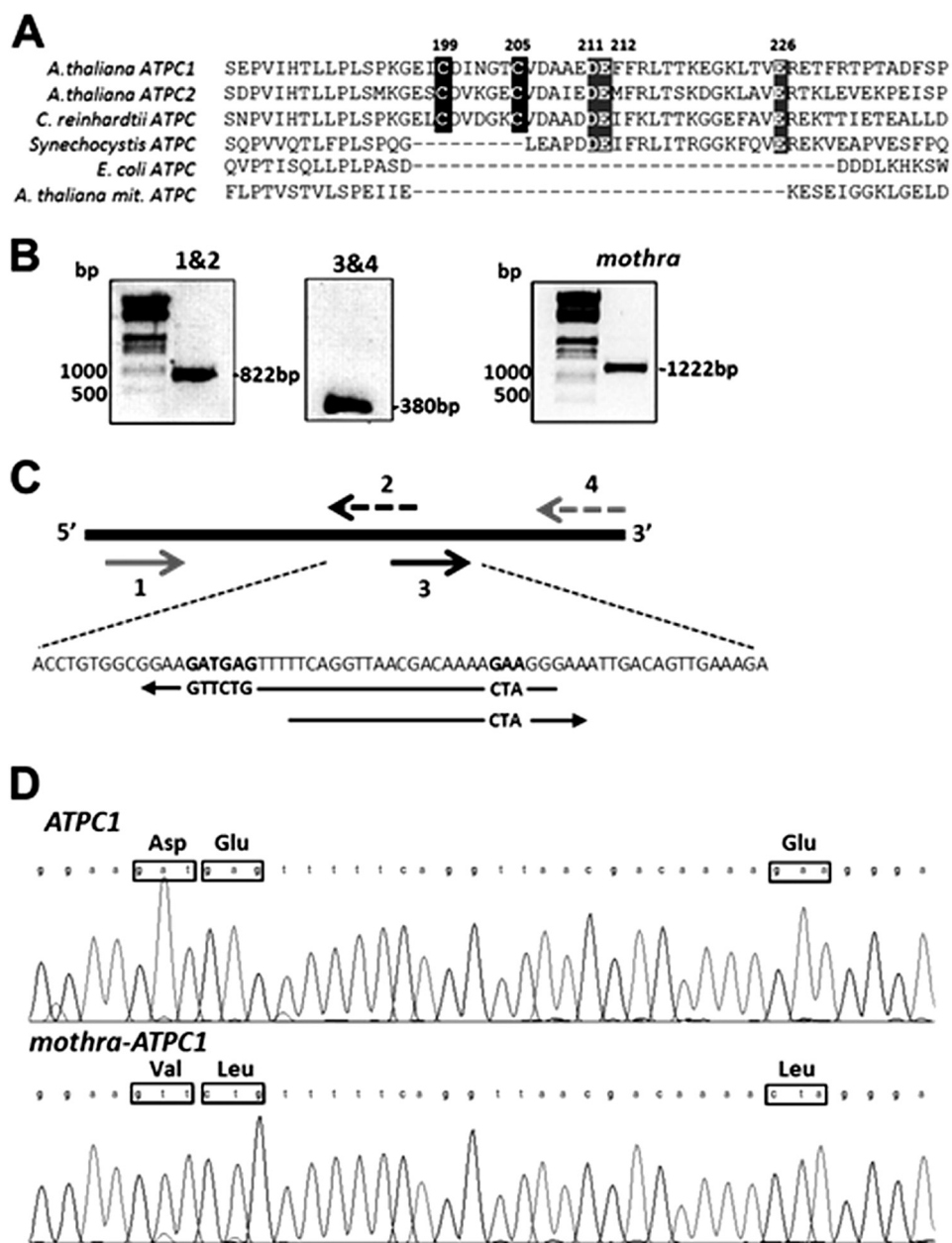


FIGURE 1. *A*, alignment of partial protein sequences of γ subunits from different sources, chloroplast ATPC1 and ATPC2 from *A. thaliana*, ATPC of *Chlamydomonas reinhardtii*, *Synechocystis*, *Escherichia coli*, and mitochondrial ATPC subunit of *A. thaliana*. The cysteine residues (black background) involved the redox modulation of plant and algal plastid ATP synthase, and the highly conserved acidic residues are indicated by a gray background. *B*, oligonucleotide-directed mutagenesis of the ATPC1 gene. Small restriction-sited forward primer and modified ATPC1 reverse primer (1 and 2) amplified a fragment of 822 bp; modified ATPC1 forward primer and XbaI restriction-sited reverse primer (3 and 4) amplified a fragment of 380 bp. *C*, details of the mutagenesis strategy. *D*, sequence analysis of wild-type and mutant γ subunit.

because of the relatively high redox midpoint potential of the regulatory thiol/disulfide couple. The determinants of this redox potential are not understood, but the effects of mutagenesis suggest the involvement of charged amino acids in a region (Glu²¹⁰-Glu²¹²) (16–19).

It has also been shown that ATP synthase activity is modulated during steady-state photosynthesis *in vivo* upon altering metabolic or physiological conditions, e.g. by decreasing atmospheric CO₂ or O₂ levels (20), imposing environmental stress conditions (e.g. drought) (21), or altering the capacity of the Calvin-Benson cycle and starch synthesis (22–24). It has thus been proposed that ATP synthase modulation represents an

important feedback regulatory mechanism, sensing the metabolic status of the stroma and, in response, adjusting the efflux of protons from the lumen to modulate the lumen pH-dependent down-regulation of light capture and electron transfer. Several mechanisms for this “metabolism-related” regulation of the ATP synthase have been proposed: thiol modulation, depletion of substrate P_i, or binding of small allosteric effectors or phosphorylation; but these have not yet been directly tested. In this work, we describe a site-directed γ subunit mutant of *Arabidopsis*, which alters the redox modulation of the ATP synthase without decreasing its maximal activity, allowing us to test one of these models.

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EXPERIMENTAL PROCEDURES

Construction of Modified Thioredoxin-regulated ATP Synthase (*Mothra*)—The full-length coding sequence of the intronless *ATPC1* nuclear gene was amplified by *Pyrococcus furiosus* polymerase from wild-type genomic DNA using the *Sma*I restriction site forward primer in the 5' end of the gene 5'-AACAA-AAAAATGGCTTGCTCTAATCTAACA-3' and the *Xba*I restriction-sited reverse primer in the 5' end of the gene 5'-AAGAGGGTTCTAGACAAATCAAACCTGTGC-3'.

The PCR fragment was inserted in the cloning vector PCR-Script Amp SK(+) (Stratagene). Three conserved acidic residues in the regulatory loop region (Fig. 1, B and C), namely Asp²¹¹, Glu²¹², and Glu²²⁶, were modified by oligonucleotide-directed mutagenesis using the cloned *ATPC1* gene as template DNA. The *ATPC1* gene was amplified in two-step amplification with *Sma*I restriction sites forward primer and modified *ATPC1* reverse primer 5'-CCCTAGTTTTGTCGTTAACCT-GAAAAACAGAACTTCCGC-3' and then, as second step, modified *ATPC1* forward primer 5'-GTTTTTCAGGTTAAC-GACAAAAGTAGGGAAATT-3' and *Xba*I restriction-sited reverse primer on the PCR-Script Amp SK(+). The mutated DNA was excised with *Sma*I and *Xba*I and inserted into binary vector pSEX001-VS under control of the cauliflower mosaic virus 35S promoter (25). Successful cloning was confirmed by sequencing (Fig. 1D). The modified *ATPC1* on the binary vector was transformed into *Agrobacterium* GV3101 strain and infected into plants heterozygous for the lethal *dpa1* mutation (26).

Transformants carrying *mothra* were segregated on solid Murashige-Skoog medium with 10 mg/liter sulfadiazine. A *dpa1* line complemented with *ATPC1* also was constructed using the same strategy, yielding *dpa1* lines carrying 35S::*ATPC1*, which we term the "complemented lines."

Wild-type *A. thaliana* (ecotype Wassileskija), complemented lines, and *mothra* (containing neutral residues Val²¹¹, Leu²¹², and Leu²²⁶ in place of highly conserved acidic amino acids Asp²¹¹, Glu²¹², and Glu²²⁶) were grown on soil under continuous light period at 30–50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at 22 °C for 4 weeks as described (17).

Protein Extraction and Western Blot Analyses—Plant leaves were frozen and ground in liquid nitrogen, and proteins were extracted in 50 mM Tris-HCl (pH 8.0), 2 mM EGTA, 10 mM EDTA, and 10 mM DTT. The extracts were fractionated by centrifugation at 10,000 $\times g$ for 10 min at 4 °C. The separated insoluble fractions, including thylakoid membrane proteins, were washed twice with extraction buffer and once with 80% acetone to remove pigments. The insoluble proteins were separated by SDS-PAGE after dissolving in 100 mM Na₂CO₃, 10% (w/v) sucrose, 50 mM DTT, and 1.5% (w/v) SDS. The relative contents of γ subunit in wild type and *mothra* were estimated by Western blotting, as described previously (17). The γ subunit protein was detected by specific antibodies raised against chloroplast γ_1 subunit.

In Vivo Spectroscopic Assays—Chlorophyll *a* fluorescence and flash-induced electrochromic shift (ECS) parameters were measured, as described previously (27, 28). Linear electron flow (LEF) and energy-dependent exciton quenching (q_E) were esti-

mated from saturation-pulse fluorescence yield under steady-state actinic lights and light-induced *pmf* (ECSt). The conductivity of thylakoid membrane to protons (g_{H^+}), attributable to activity of the ATP synthase, was estimated from the first-order exponential decay of the dark interval relaxation kinetic changes in absorbance associated with the ECS at 520 nm, with reference wavelengths taken at 505 and 535 nm (21, 23, 27, 28). The relative extent of steady-state proton flux across the thylakoid membrane (v_{H^+}) was estimated from the initial slope of the ECS decay (27, 29). To account for variations in leaf thickness and pigmentation, ECS measurements were normalized to the extent of the rapid rise in ECS induced by a saturating, single-turnover flash (15). The correction was small, with rapid ECS phases *mothra* and complemented lines that were 1.00 ± 0.004 and 1.17 ± 0.01 times than wild type. Steady-state fluorescence and ECS assays also were repeated under a range of atmospheric CO₂ concentrations using a gas exchange system (LI-COR) to control CO₂ levels.

Non saturating flash-induced relaxation kinetics analysis of the ECS signal was performed to determine the activation state of the ATP synthase in the dark. Flash-induced relaxation kinetics experiments were performed 1 and 60 min after preillumination for 2 min with 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ as described previously (17, 21).

Equilibrium Redox Titrations and Redox State of ATP Synthase γ -Subunit—*In situ* redox titrations of ATP synthase were performed using the approach of Wu and co-workers (1, 18), detecting the ATP synthase activity by analyzing the kinetics of flash-induced ECS signals as described in Ref. 17. Relative ATP synthase activity was estimated from the reciprocal ECS decay lifetime, assuming pseudo-first-order behavior (27).

The redox state of the γ subunit of chloroplast ATP synthase was probed using the binding of 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonate (AMS) followed by nonreducing SDS-PAGE (17, 30). Oxidizing and reducing conditions were achieved by infiltration into 1-cm leaf discs of 0.1% (v/v) Tween 20 and 20 mM Tricine containing 100 μM methyl viologen for oxidizing conditions or 20 mM reduced dithiothreitol (DTT) for reducing conditions. Following these treatments, discs were incubated for 30 min in darkness at room temperature, followed by freezing and grinding in liquid nitrogen. Insoluble proteins were isolated by centrifugation and washing with 80% acetone. The protein precipitates were dissolved in freshly prepared solution containing 1% SDS, 50 mM Tris-HCl (pH 8.0), and 15 mM AMS and then immunoblotted by SDS-PAGE using running buffer lacking reducing agent as described in Ref. 16. Redox states of the γ subunits in wild type and the *mothra* were visually detected using an antibody against the γ_1 subunit using Western blotting.

RESULTS

Effects of Altering Three Highly Conserved Acidic Residues in the Regulatory γ Subunit—As a part of a wider effort to determine the functions of key γ subunit residues in ATP synthase catalysis and regulation, three conserved acidic residues in the vicinity of the γ subunit regulatory cysteine residues, Asp²¹¹, Glu²¹², and Glu²²⁶ (Fig. 1A), were mutated by oligonucleotide-directed mutagenesis to generate a mutant, *mothra*, in which

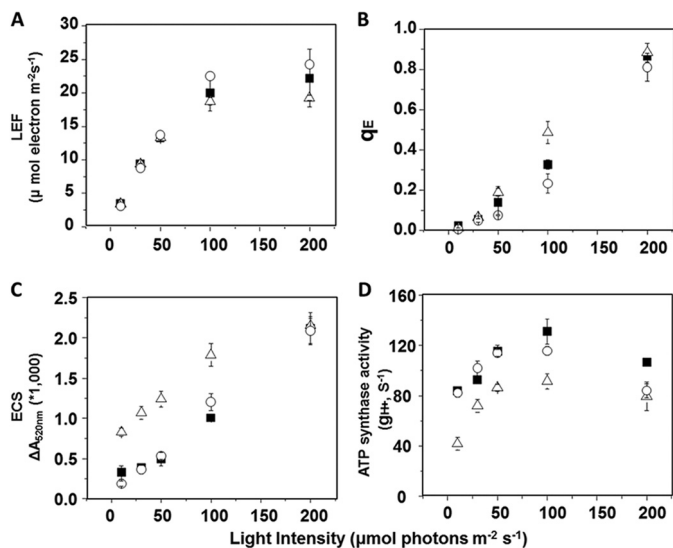


FIGURE 2. Wild type (filled squares), a representative complemented line (35S::ATPC1 expressed in *dpa1*) (open circles), and *mothra* (open triangles) were compared for differences in light intensity-dependence of LEF (A), q_E (B), light-induced pmf estimated by analysis of the electrochromic shift (ECS) (C), and g_{H^+} (D), based on first exponential ECS decay kinetics. Data are for attached leaves ($n = 3$).

these were modified to neutral residues Val²¹¹, Leu²¹², and Leu²²⁶, respectively, as described in Fig. 1, B–D. These residues are in the loop containing the regulatory cysteine residues, a region that is apparently quite flexible and can accommodate large changes (point mutations and deletions) albeit with altered regulatory and catalytic behaviors (18, 19, 31).

Effects of *Mothra* on Photosynthesis under Ambient CO₂ and O₂—Wild-type, *mothra*, and complemented lines had similar maximal photosystem II quantum yields (0.80 ± 0.0028 , 0.79 ± 0.005 , and 0.79 ± 0.002 , respectively, $n = 3$), as measured by saturation pulse fluorescence yield changes (F_V/F_M), likely indicating no effects on the stability of photosystem II. LEF was nearly identical in wild type, complemented lines, and *mothra* at low to 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ irradiance and ambient CO₂ and 21% O₂ (Fig. 2A). LEF saturated more readily at irradiances higher than 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, LEF in *mothra*, and complemented lines, indicating the imposition of a rate limitation at high fluxes. The extents of photoprotective q_E response in *mothra* were 20–50% higher in *mothra* than wild-type or the complemented line at irradiance above approximately 50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, possibly indicating increased light-induced acidification of the lumen (Fig. 2B).

The total amplitude of ECS signal in steady-state was used to assess the light-driven pmf across thylakoid membranes. Fig. 2C shows light-induced pmf responses under increasing actinic light. Wild-type and complemented lines showed similar pmf extents and light responses. The *mothra* mutant showed higher pmf responses at lower actinic light.

The relative activities of the ATP synthase, as estimated by the initial first-order exponential decay for the ECS signal (g_{H^+}) (for review, see Refs. 27, 32), were smaller in *mothra* than wild-type and complemented lines (Fig. 2D). Because lower ATP synthase activity results in slowing of proton efflux from the lumen, this effect can explain the higher pmf observed in *mothra*.

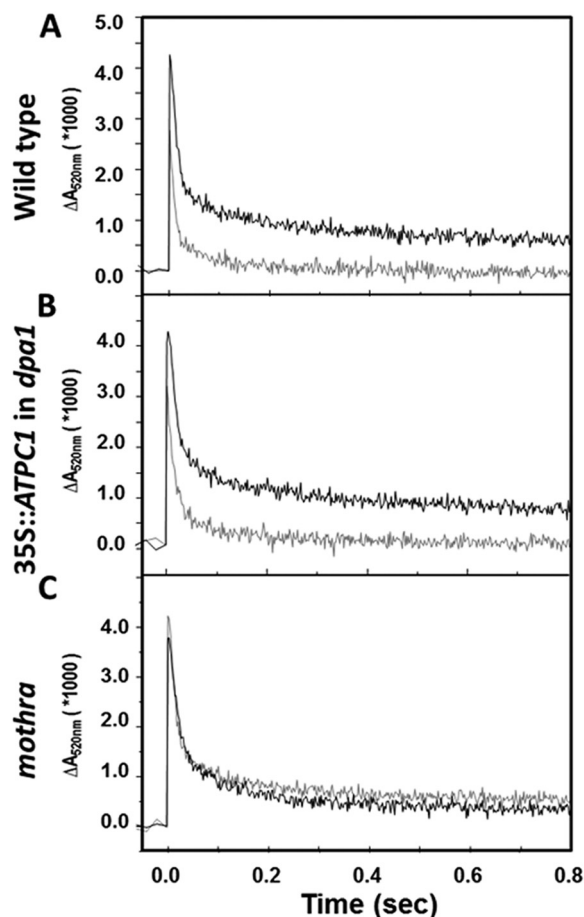


FIGURE 3. Activity of the ATP synthase probed by the decay of the ECS signal. Flash-induced relaxation kinetics of ECS (15, 33) were measured for wild-type (A), complemented line (35S::ATPC1 expressed in *dpa1*) (B), and *mothra* (C) at 1 min (gray curves) and 60 min (black curves) after 2-min preillumination at 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ actinic light.

ATP Synthase in *Mothra* Is Equally Active in the Light- and Dark-adapted Leaves—We analyzed ATP synthase activation state in attached leaves by probing the decay of the thylakoid pmf via the ECS signal after excitation with short (100 μs), nonsaturating light-emitting diode pulses. As discussed previously (15, 33), the decay of the ECS under these conditions is a good measure of the redox state of the γ subunit, *i.e.* the extent of the rapid phase is large when the γ subunit is reduced and slow when oxidized. Fig. 3A shows that preillumination of wild type for 2 min at 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ resulted in a large rapid ECS decay phase, indicating reduction of the γ subunit. Dark adaptation for 60 min led to reoxidation. The same response was observed in the complemented lines (Fig. 3B). In contrast, the modified γ -ATP synthase in *mothra* showed similar ECS decay kinetics in both light- and dark-adapted leaves, implying a lack of redox regulation. The extents of the fast and slow phases and thus the ATP synthase activity in *mothra* were intermediate between those seen in light- and dark-adapted wild type.

ATP Synthase in *Mothra* Shows a Shifted γ Subunit Redox Midpoint Potential—The γ subunit protein expression levels in two independent lines each of *mothra*, and the complemented lines were investigated by immunoblotting analyses against an

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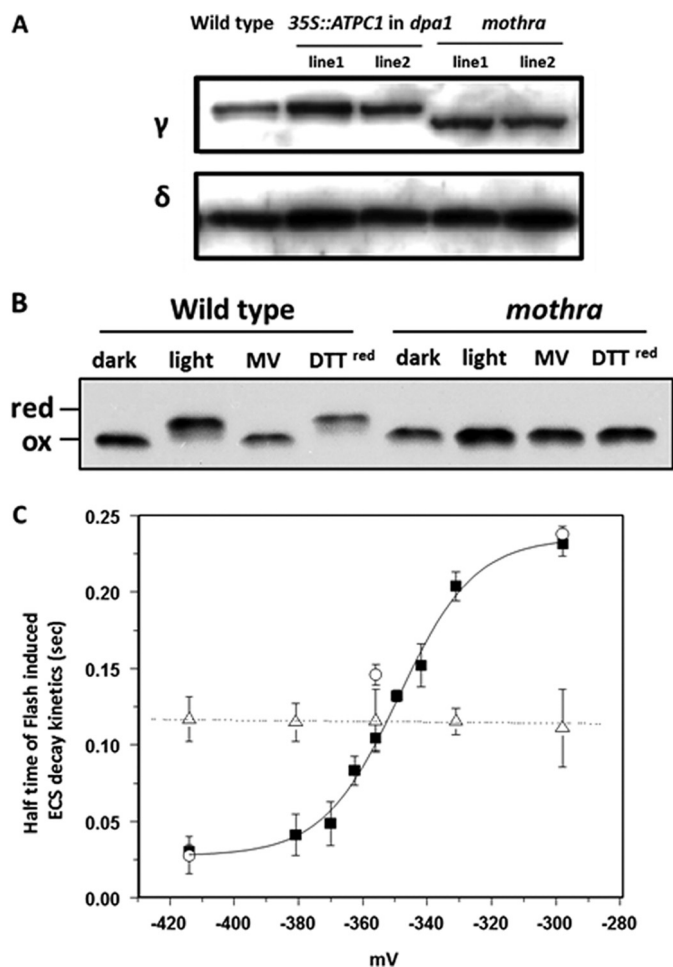


FIGURE 4. Accumulation and redox properties of chloroplast ATP synthase in wild type and *mothra*. *A*, accumulation of ATP synthase subunit γ and δ using Western blot analysis of thylakoid membrane proteins from wild type, representative complemented line (35S::ATPC1 expressed in *dpa1*), and *dpa1* complemented with modified ATPC1 (*mothra*). *B*, separation of reduced and oxidized γ subunits using AMS gel shift assay. To obtain reducing conditions (red), leaf discs were infiltrated with reagents and illuminated with $\sim 50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. Wild-type and *mothra* were treated with buffer as a control, methyl viologen (MV), and reduced DTT under light or dark condition. *C*, equilibrium redox titrations (18) of thiol/disulfide regulatory groups in the γ subunit of the chloroplast ATP synthase in wild type (filled squares), complemented line (35S::ATPC1 expressed in *dpa1*) (open circles), and *mothra* (open triangles). The data for the wild-type titration were well fit with an $n = 2$ Nernst curve as expected for a disulfide/sulfhydryl transition. The ΔA_{520} relaxation kinetics were measured and used to calculate the half-time of the kinetics. Data represent the average \pm S.D. (error bars) of $n = 4-5$.

antibody of the γ_1 subunit (Fig. 4A). The γ_1 subunit was highly expressed in the complemented line and *mothra* compared with wild type. Expression of ATP synthase δ subunit was constant. The apparent molecular weight of the *mothra* γ subunit was decreased compared with that of wild-type and the complemented lines, possibly due to effects of mutations on protein charge or secondary structure, e.g. the ability to form the regulatory disulfide bond.

The redox state of wild-type and mutant γ subunits was probed by modification of free sulfhydryl groups with AMS followed by separation on SDS-PAGE (16, 17). In wild type, the ATP synthase γ subunit showed the expected redox dependence upon exposure of dark-adapted leaves for 60 min to light, with the apparent molecular weight being completely shifted

from low to high molecular weight, indicating transition from oxidized to reduced forms (Fig. 4B). Infiltration with methyl viologen blocked the transition to the reduced form, consistent with its ability to divert electrons from photosystem I electron acceptors to O_2 , thus preventing reduction of the γ subunit (15). Addition of reduced DTT mimicked the effects of light, consistent with its expected effects on thiol redox state. In contrast, the *mothra* γ subunit showed no response to light-induced reduction, as shown by the maintenance of a lower molecular weight band under light of DTT exposure (Fig. 4B), indicating that the *mothra* γ subunit is deficient in redox regulation *in vivo*.

We performed *in situ* equilibrium redox titrations using ratios of oxidized and reduced DTT and detected changes in activity by flash-induced ECS measurements (17, 18). The ATP synthase in wild type showed a transition from inactive to active at calculated ambient redox potential of about -350 mV , similar to results from previous work (17). In contrast, ATP synthase in *mothra* showed no substantial changes in activity over the entire range of redox potentials obtained by DTT, indicating that its redox potential is out of the range accessible by this method.

The lack of effect of redox potential on *mothra* ATP synthase could be caused by the γ subunit remaining in either the oxidized or reduced forms. Infiltration with strong oxidants H_2O_2 or diamide readily oxidized wild-type ATP synthase, as shown by ECS decay kinetics (Fig. 5, A and B) and AMS gel shift (Fig. 5, C and D) assays. In contrast, the ECS decay kinetics in *mothra* were insensitive to H_2O_2 and diamide and showed the same behavior as wild type in the presence of the two oxidants (Fig. 5, A and B). In addition, AMS had no significant effect on the apparent molecular weight of *mothra* γ subunit, likely indicating a lack of free sulfhydryl groups under any redox conditions tested, indicating a substantially higher redox potential, so that it remains in its oxidized form under physiological conditions.

Metabolism-related Regulation of ATP Synthase in Wild Type and *Mothra*—To test whether thiol modulation is responsible for metabolism-related ATP synthase regulation, we compared the responses of ATP synthase activity (measured as g_{H^+}) to CO_2 levels in wild-type, *mothra*, and complemented lines with constant photosynthetically active radiation of $200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Fig. 6). The value of g_{H^+} at ambient CO_2 was approximately 25–30% higher in wild-type than either *mothra* or the complemented lines under all conditions, possibly reflecting slight differences in overall activity. However, decreasing CO_2 levels from ambient to 50 or near 0 ppm resulted in similar, strong decreases in g_{H^+} in all lines, quantitatively similar to the effects reported earlier for *Arabidopsis* (34), tobacco (20), or a range of C_4 plants (22). Increasing CO_2 to 2000 ppm also resulted in similar decreases in g_{H^+} in wild-type, *mothra*, and complemented lines. This high CO_2 effect was reported earlier and ascribed to feedback limitations related to depletion of stromal inorganic phosphate (22, 29). Overall, these results indicate that, whereas the ATP synthase in *mothra* is deficient in redox regulation, its responses to metabolic changes were essentially unaffected.

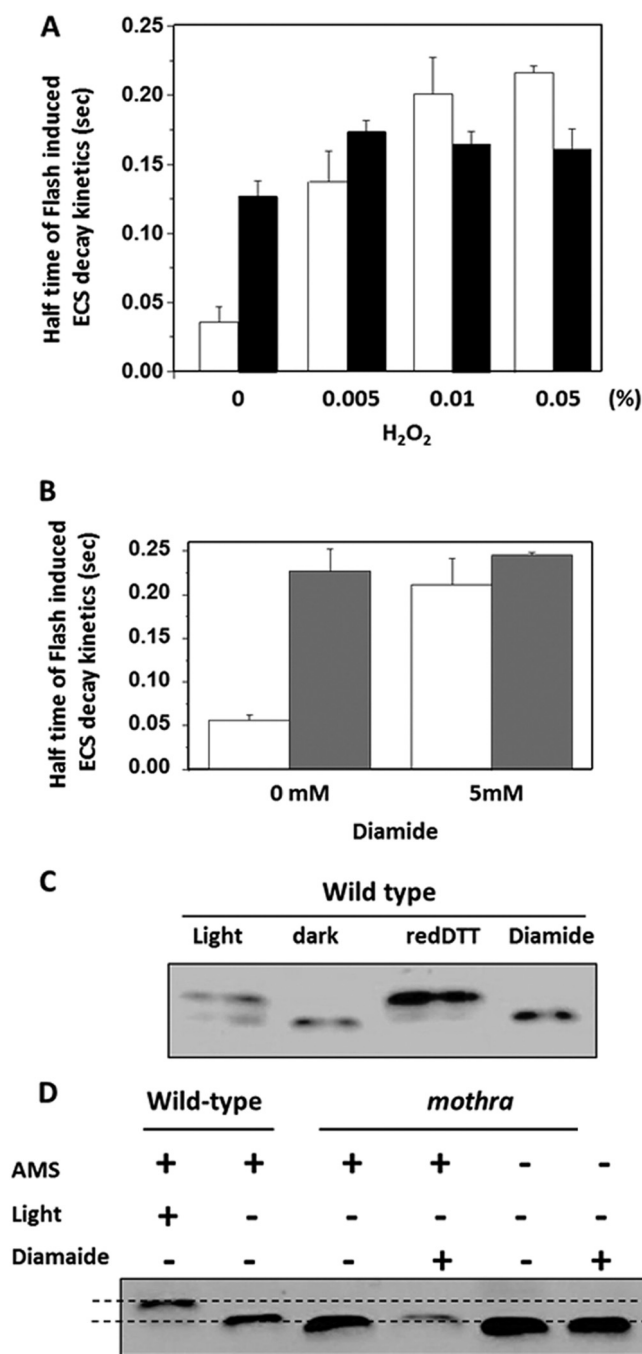


FIGURE 5. Modulation of ATP synthase activities and γ subunit structural properties by infiltration with strong oxidants. *A*, decay half-times of flash-induced ECS kinetics after preillumination, performed as described under "Experimental Procedures," in leaf discs of wild-type (white bars) and *mothra* (black bars) infiltrated with buffer (20 mM Tricine-KOH, pH 7.5, 1% Tween 20) or a range of hydrogen peroxide (H_2O_2) concentrations. *B*, leaf discs of wild type (white bars) and *mothra* (gray bars) infiltrated as in *A*, but with 5 mM strong oxidant diamide. *C*, control demonstrating the use of AMS gel shift assay for assessing modulation of γ subunit redox state in wild type under conditions described in Ref. 17. As indicated in the figure, wild-type leaf discs were vacuum-infiltrated with buffer as in *A*, during illumination with $100 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ or in darkness, reduced DTT, 5 mM diamide. Insoluble proteins were solubilized in sample buffer without addition of reducing reagents, incubated in the thiol binding probe AMS, and separated by SDS-PAGE. Reduced proteins bands migrate less and appear in the upper part of the gel, whereas oxidized proteins migrate further (16, 17). *D*, AMS gel shift assay for redox state of wild-type and *mothra* γ subunit by light and diamide using the methods described for *C*. As a control, wild-type and leaf discs were

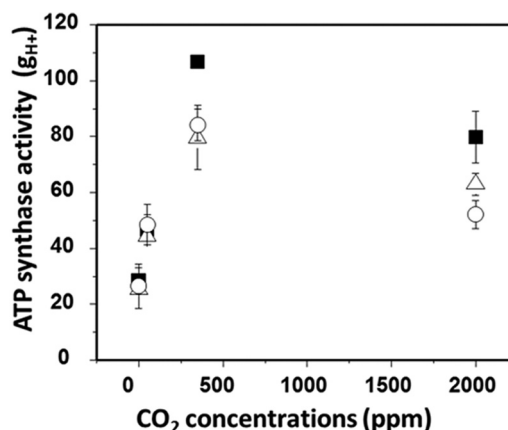


FIGURE 6. Change in ATP synthase activity (g_{H^+}) from steady-state photosynthetic parameters in wild-type (filled squares), complemented line (35S::ATPC1 expressed in *dpa1*) (open circles), and *mothra* (open triangles) under 0, 50, 350, and 2000 ppm CO_2 under $200 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Data are for attached leaves ($n = 3-4$).

DISCUSSION

Highly Conserved Acidic Amino Acid Residues Modulate the Redox Potential of ATP Synthase Regulatory Thiols—The γ subunit of the $\text{F}_0\text{-F}_1$ -ATP synthase forms the central rotor of the ATP synthase (5, 7). The chloroplast homologue contains a novel regulatory domain, in which two redox-active cysteine residues are sandwiched between the long α helices (7). It is now well established that these cysteine residues are reduced to free thiols by thioredoxin in the light and reoxidized in the dark, regulating the activity of the ATP synthase (for review, see Refs 19, 31). We found that mutating residues, Asp²¹¹, Glu²¹², and Glu²²⁶ to Val, Leu, and Leu, respectively, eliminated the classical light-dark regulation of the ATP synthase (Fig. 3), accompanied by a shift in the redox potential of the thiol/disulfide transition (Fig. 4), similar to the elimination of the regulatory cysteine residues (19). We conclude that these acidic residues are important for adjusting thiol redox properties. In another *Arabidopsis* mutant, *cfq* (18, 35), a single substitution of another highly conserved glutamate group (E244K) in the γ subunit resulted in more negative redox potential, rendering the complex more prone to oxidation in the dark. Deletion or mutation of the negatively charged residues Glu²¹⁰-Asp²¹¹-Glu²¹² in this region in the thermophilic bacterium *Bacillus* PS3 (30, 31, 36) resulted in reverse redox sensitivity, *i.e.* becoming more active in the oxidized form and less in the reduced. We thus conclude that redox potential and its effects on activation of the ATP synthase are highly dependent on the charges and overall conformation of this regulatory loop. These suggest that the thiol modulation in chloroplast ATP synthase is highly dependent upon the structure of the γ subunit, particularly because it revealed that acidic amino acids in the regulation domain induce smooth transition to reduced state.

Physiological Consequences of Altered Thiol Modulation in Mothra—The chloroplast ATP synthase is controlled by the interplay of *pmf* and redox modulation (15, 37). In the light, the

treated with buffer in light ($100 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) or darkness, as described in Ref. 17. Leaf discs from *mothra* were treated with buffer and 5 mM diamide in the presence and absence of AMS labeling.

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γ subunit is reduced by thioredoxin, causing the ATP synthase to be activated when pmf reaches a relatively low threshold level. Because this low pmf is close to that sustained by ATP hydrolysis in the dark, the ATP synthase remains activated in the dark (15). Oxidation of the γ subunit in the dark imposes a higher activation threshold for ATP synthase leading to inactivation, presumably to prevent wasteful hydrolysis of ATP in the dark. These regulatory behaviors are clearly seen in Fig. 3, where the decay of the ECS signals in light-adapted wild type (with reduced ATP synthase) was monotonic, reflecting a continuously active ATP synthase. After dark adaptation, ECS decay underwent a sharp transition from fast to slow, when pmf decreased below the activation threshold. In *mothra*, ECS decay kinetics remained biphasic in both light- and dark-adapted leaves, implying that the ATP synthase activity was redox-insensitive. Moreover, ECS decay kinetics in *mothra* appeared intermediate between those of light- and dark-adapted wild type, suggesting that the mutant enzyme was locked in a partially activated state.

The differences in ATP synthase regulation and activities have consequences for steady-state photosynthesis. The inability to fully activate the ATP synthase in *mothra* results in lower steady-state proton conductivity (g_{H^+} , Fig. 2D). The effects were especially pronounced at low irradiance where g_{H^+} was 50% of that of wild type, as expected from the requirement for additional pmf to fully activate ATP synthase in *mothra*. The lower g_{H^+} slows the release of protons from the thylakoid lumen, increasing light-driven pmf (Fig. 2C). Above 50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, where feedback regulation of the light reactions is engaged, the increased pmf in *mothra* results in stronger activation of the q_E response (Fig. 2B) and slowing of LEF (Fig. 2B).

We did not observe substantial differences in cyclic electron flow around photosystem I in wild type and *mothra* as estimated by two methods: redox state of photosystem I by 820 nm absorbance changes of P700 and proton flux by ECS (data not shown), indicating that the differences in the relationship between pmf and LEF can be attributed to altered activity of the chloroplast ATP synthase (see discussion in Refs. 27, 28). Thus, whereas regulation of light capture was altered by the mutations, the overall ATP/NADPH output ratio was largely unaffected.

Thiol and Metabolism Modulation of the ATP Synthase Activity Is Mechanistically and Functionally Distinct—ATP synthase is regulated under steady-state illumination in response to metabolic status, e.g. in response to changing CO_2 , O_2 , or mutations that affect assimilation (20, 22, 27). This metabolism-related regulation modulates the thylakoid pmf (27, 29), thus affecting the regulation of light capture and subsequent electron transfer. The mechanism of the metabolism-related regulation is not known, but one possible model would involve thiol modulation. To test this possibility, we used *mothra*, which shows redox-insensitive ATP synthase activity, with only small effects on sustained photosynthesis at growth light irradiance (Fig. 2). Although the basal rate of ATP synthase in *mothra* was somewhat lower than in wild type, altering CO_2 levels had nearly identical effects on ATP synthase activity *in vivo* in wild type and *mothra* (Fig. 6), indicating that,

although *mothra*-ATP synthase was defective in light-dark regulation, it retained its capacity for metabolic fine-tuning. We thus conclude that metabolism-related regulation of ATP synthase does not involve thiol modulation of the γ subunit. This result is consistent with previous observations that thiol activation of the ATP synthase occurs at very low irradiances and thus likely acts as an on-off switch to activate the complex at very early times during induction of photosynthesis (15, 33). The binding of substrates (ADP or P_i) (27, 29, 38, 39) or allosteric effectors or covalent modification (e.g. phosphorylation) (40–42) are most likely fine-tuning effectors.

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