A Point Mutation in \textit{atpC1} Raises the Redox Potential of the \textit{Arabidopsis} Chloroplast ATP Synthase \textgreek{\gamma}-Subunit Regulatory Disulfide above the Range of Thioredoxin Modulation\textsuperscript{*5,\textsection}\

Received for publication, August 21, 2007, and in revised form, October 19, 2007 Published, JBC Papers in Press, October 24, 2007, DOI 10.1074/jbc.M707007200

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The light-dependent regulation of chloroplast ATP synthase activity depends on an intricate but ill defined interplay between the proton electrochemical potential across the thylakoid membrane and thioredoxin-mediated redox modulation of a cysteine bridge located on the ATP synthase \textgreek{\gamma}-subunit. The abnormal light-dependent regulation of the chloroplast ATP synthase in the \textit{Arabidopsis thaliana} \textit{cfq} (coupling factor quick recovery) mutant was caused by a point mutation (G to \textdelta) in the \textit{atpC1} gene, which caused an amino acid substitution (E244K) in the vicinity of the redox modulation domain in the \textgreek{\gamma}-subunit of ATP synthase. Equilibrium redox titration revealed that this mutation made the regulatory sulfhydryl group energetically much more difficult to reduce relative to the wild type (i.e. raised the \( E_{m,7/3} \) by 39 mV). Enzymatic studies using isolated chloroplasts showed significantly lower light-induced ATPase and ATP synthase activity in the mutant compared with the wild type. The lower ATP synthesis capacity in turn restricted overall rates of leaf photosynthesis in the \textit{cfq} mutant under low light. This work provides \textit{in situ} validation of the concept that thioredoxin-dependent reduction of the \textgreek{\gamma}-subunit regulatory disulfide modulates the proton electrochemical potential energy requirement for activation of the chloroplast ATP synthase and that the activation state of the ATP synthase can limit leaf level photosynthesis.

The chloroplast ATP synthase complex catalyzes the synthesis of ATP from ADP and free phosphate by coupling anhydride bond formation with the proton transmembrane electrochemical potential (\( \Delta \mu_{H^+} \)) across the thylakoid membrane and is one of the light-regulated enzymes of chloroplasts. In the light, the enzyme complex can be fully activated to achieve high capacity of ATP synthetic activity, whereas in the dark, it is converted to a catalytically inactive state, thereby preventing the dissipative cleavage of stromal ATP that would otherwise occur. The regulatory mechanisms governing the activity of this enzyme have been intensely investigated over the past several decades, revealing that regulation of the chloroplast ATP synthase catalytic activity is more intricate than that of its counterparts in mitochondria and bacteria.

There are at least three components involved in the regulation of chloroplast ATP synthase: \( \Delta \mu_{H^+} \) formation across the thylakoid membrane, nucleotide binding and release to the catalytic and regulatory sites, and thiol modulation of disulfide bridge-forming cysteine residues on the \textgreek{\gamma}-subunit (1). \( \Delta \mu_{H^+} \) plays a central role in the regulation of chloroplast ATP synthase. In the light, the generation of \( \Delta \mu_{H^+} \) induces conformational changes within the ATP synthase complex causing the release of bound ADP, activating the ATP synthase. As \( \Delta \mu_{H^+} \) dissipates in the dark, these conformational changes and ADP release are reversed, inactivating the enzyme (2). These simple but subtle control devices avoid energy losses in the dark (3).

Redox modulation of the \textgreek{\gamma}-subunit also plays an important role in regulating chloroplast ATP synthase activity and represents a level of regulation that is absent in the bacterial and mitochondrial homologs. In plants and green algae, the \textgreek{\gamma}-subunit contains an extra domain of \textasciitilde40 amino acids (3) in which there is nested a unique sequence motif of nine amino acid residues, including two cysteines (4). Reversible disulfide bridge formation between the two cysteines enables regulation of the enzyme via redox thiol modulation. \textit{In vitro} reduction can be achieved by dithiothreitol (DTT)\textsuperscript{5} addition, whereas \textit{in vivo} reduction is through thioredoxin that is reduced by photosystem I via ferredoxin-thioredoxin reductase (5). Unsurprisingly, deletion of the nine-amino acid motif containing the disulfide bridge-forming cysteine or replacement of the cysteines with alanine makes the enzyme insensitive to thiol modulation (6, 7). Redox modulation of the \textgreek{\gamma}-subunit likely affects binding of the \epsilon-subunit, which acts with the \textgreek{\gamma}-subunit to repress activity (6, 8, 9).

Although the redox regulation of chloroplast ATPase and ATP synthase activity has been extensively studied, the \textit{in planta} physiological function remains unclear in that redox modulation is not a prerequisite for either activation or deactivation.
vation of the chloroplast ATP synthase. Activation of the ATP synthase is a rapid process that normally precedes the thioredoxin-dependent reduction of γ-subunits following a dark-to-light transition (10). It has been shown that reduction of the γ-subunit lowers the ΔµH+ threshold for activation of the chloroplast ATP synthase (11–13) and increases the efficiency of ATP formation as a function of ΔµH+ (14, 15), likely by stabilizing the activated conformational state of the enzyme complex (16, 17). This makes it possible to sustain activated ATP synthase in low light. Additionally, the physiological significance of the oxidation, which results in disulfide bridge formation within the γ-subunit, is also uncertain because deactivation of the ATP synthase following a light-to-dark transition is normally more rapid than γ-subunit oxidation (10, 18, 19).

To investigate the physiological significance of the thiol modulation, we adopted a genetic approach to study the in situ regulation of ATP synthase in Arabidopsis thaliana. We previously developed a two-step screening strategy for the selection of ATP synthase activation mutants in Arabidopsis and chose several candidates in which redox responses of chloroplast ATP synthase differed compared with the wild type (20, 21). In one mutant, cfq, the energetic threshold for activation of the ATP synthase was insensitive to pre-illumination, behaving as though the γ-subunit regulatory disulfide was not reduced in the light. In the work reported here, we investigated the activity of the ATP synthase in the cfq mutant and discovered a point mutation in the cfq mutant atpC1 gene that resulted in an amino acid substitution (E244K) in the vicinity of the redox modulation domain in the γ-subunit of ATP synthase. Redox titration revealed that the midpoint potential of the regulatory sulfhydryl group on the cfq γ-subunit was nearly 40 mV more reducing than that of the wild type. The more negative midpoint potential resulted in a higher energetic threshold (i.e. higher ΔµH+) for sustaining the activation of ATP synthase and thus lowered ATP synthase activity in the mutant under limiting light conditions. Photosynthetic rates of the mutant were lower than those of the wild type under the low light, consistent with the higher energy requirement for sustained activation of the ATP synthase and a lower capacity of ATP synthase in the mutant.

EXPERIMENTAL PROCEDURES

Plant Materials and Growth Conditions—The M2 generation of the cfq mutant (20) was back-crossed three times with wild-type Col-0, and a homozygous line was chosen from the F3 generation. Wild-type and mutant plants were grown at 20 °C on soil at 70–80% humidity under a 12/12-h light/dark photoperiod with a photon flux density of 150 μmol m−2 s−1.

Measurement of ΔA518 Relaxation Kinetics—Plants ~45 days old were initially dark-adapted for 12 h to ensure that γ-subunits of ATP synthase within the leaves were fully oxidized. A kinetic spectrophotometer was used to monitor the flash-induced absorbance change at 518 nm with detached leaves as described previously (20) with the exception that 7 s of continuous red light (65 μmol m−2 s−1) was used to induce γ-subunit reduction. ΔA518 relaxation kinetics were analyzed, and the relaxation time constants were calculated by fitting the initial 600 ms of the decay to a first-order exponential using an iterative nonlinear least-squares program (OriginLab Corp., Northampton, MA). The r² values of the fits were all ≥0.93.

Equilibrium Redox Titrations—Fully expanded detached leaves were infiltrated with varying ratios of oxidized and reduced 20 mM DTT solutions in 10 mM Tricine/NaOH (pH 7.9) containing 1% (v/v) Tween 20 (22). After 4 h of incubation in the dark, ΔA518 kinetic measurements were carried out. The equilibrium redox potential for DTT at pH 7.9 was calculated according to the following equation: E mΔH+ = E mΔH+ (59/0.059(9/7.9) in the dark, ΔA518 kinetic measurements were carried out. The equilibrium redox potential for DTT at pH 7.9 was calculated according to the following equation: E mΔH+ = E mΔH+ (59/0.059(9/7.9) was equal to −380 mV when using the E mΔH+ value of −327 mV at 25 °C (23). The redox potentials (Eh) were calculated according to the Nernst equation: Eh = EmH+ + (59/n)log([DTT+]/[DTT−]), where n is equal to 2 for the two electrons involved in the thioredoxin-mediated thiol/disulfide exchange. The pH value of 7.9 mimics the stromal pH in the light (24).

DNA Sequencing of atpC1 of the cfq Mutant—Genomic DNA from cfq leaves was isolated; the entire atpC1 coding region for the γ-subunit of chloroplast ATP synthase was amplified by PCR using primers gama-1 (5′- TGTTCACC-ACTCCAGGGTCTC-3′) and gama-2A (5′- TCAAAGAG-GGTCTAAACAAAATCAAAC-3′); and the product was sequenced (25).

Complementation Analysis—λC31 (26) containing the Arabidopsis Col-0 atpC1 gene was used as the PCR template, and a 1456-bp insert spanning the coding region was subcloned into the multicloning XbaI site of the pBI-121 binary vector (Clontech, Mountain View, CA). The insert was positioned downstream of the cauliflower mosaic virus 35S promoter and used for transformation of cfq mutant plants using the infiltration method (27). Transformants (T1 generation) were selected on Murashige-Skoog medium containing 0.8% agar and 50 mg/liter kanamycin. Resistant plants were transferred to soil. The homozygous lines (T2′) were further selected on the same medium.

DNA Gel Blot Analysis—The genomic DNA was extracted from 3-week-old plants. Approximately 8 μg of DNA/lane was digested with EcoRV overnight, resolved on 0.7% (w/v) agarose gel, and transferred onto a Hybond-N nylon membrane (Amerham Biosciences). The blots were probed with 32P-dCTP-labeled XbaI fragment (Megaprime DNA labeling system, Amerham Biosciences) from λC31 containing the whole atpC1 coding region. The blots were hybridized at 60 °C in hybridization solution (6X SSC (3 M NaCl and 3 M sodium citrate), 0.01 M EDTA (pH 8.0), 5X Denhardt’s solution, 0.5% (w/v) SDS, and 100 mg/ml sheared denatured salmon sperm DNA) overnight; washed repeatedly for 15 min in a solution containing 30 mM NaCl, 3 mM Na2HPO4, 0.1% (w/v) SDS; and then exposed to x-ray film (Kodak X-Omat AR) for 1 day.

RNA Gel Blot Analysis—Total RNA was isolated from fully expanded leaves using TRizol reagent (Invitrogen). 10 μg of RNA was resolved on formaldehyde-containing 1% (w/v) agarose gel and transferred to a Hybond-N nylon membrane. Preparation of the atpC1 probe, hybridization, and washing were carried out essentially as described above for DNA gel blotting.

Immunoblot Analysis—Chloroplast thylakoid membranes were isolated following a published protocol (28). The membrane pellet was washed three times with ice-cold 10 mM NaCl and centrifuged at 7000 × g. The resulting thylakoid pellet was
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FIGURE 1. $\Delta A_{518}$ relaxation kinetics in wild-type and cfq Arabidopsis leaves. $\Delta A_{518}$ relaxation kinetics were measured in >20 leaves using a flash kinetic spectrophotometer, and some representative responses are shown. Wild-type (A) and cfq (B) plants were dark-adapted for 12 h ($D_{ad}$ traces) and then illuminated with 65 $\mu$mol m$^{-2}$ s$^{-1}$ red light for 7 s and dark-adapted for 4 min ($L_{ad}$ traces). Wild-type (C) and cfq (D) plants were dark-adapted for 12 h ($D_{ad}$ traces), and leaves were then infiltrated with 1 mM DTT for 20 min ($DTT$ traces).

RESULTS

Light-dependent Reduction of the Chloroplast ATP Synthase $\gamma$-Subunit Cysteine Disulfide Bond Is Impaired in the Arabidopsis cfq Mutant—Chloroplast ATP synthase activity can be monitored in intact leaves by taking advantage of the fact that the amplitude of light-induced electrochromic absorbance change with a peak at 518 nm ($\Delta A_{518}$) is closely linear to the amplitude of the electrical component of $\Delta \mu_{H}^{+}$ that is formed across the thylakoid membrane in the light (e.g. Refs. 10, 19, and 20). Fig. 1A shows that a single flash of light, strong and brief enough to excite all of the photosystem reaction centers in unison, generated a large $\Delta \mu_{H}^{+}$ across the thylakoid membrane. The slow first-order decay of $\Delta A_{518}$ (time constant of $\Delta A_{518}$ decay (τ) = 257 ms) in dark-adapted wild-type leaves (Fig. 1A, $D_{ad}$ trace) indicated that the catalytically inactive form of the ATP synthase predominated (20) and that the decay was due mainly to slow depolarization by ion movement across the thylakoid membrane rather than proton efflux through the ATP synthase. In wild-type leaves, 7 s of pre-illumination with 65 $\mu$mol m$^{-2}$ s$^{-1}$ red light was sufficient to fully activate the ATP synthase and to bring about the thioredoxin-dependent reduc-
tion of the γ-subunit. As we (20) and others (32) have shown, a subsequent 4-min dark adaptation results in deactivation of the ATP synthase but not oxidation of the γ-subunit. Under this condition, a single flash was sufficient to activate the ATP synthase, and the decay of ΔA_{518} was accordingly accelerated (τ = 73 ms) because of the increased conductance of protons through the ATP synthase associated with ATP formation (Fig. 1A, L_{ad} trace). In cfq, the dark-adapted leaves exhibited the same slow decay of ΔA_{518} (τ = 248 ms) (Fig. 1B, D_{ad} trace) seen in the wild type. However, in the mutant, the pre-illumination and subsequent 4-min dark adaptation caused only modest acceleration of the decay of ΔA_{518} (τ = 161 ms) (Fig. 1B, L_{ad} trace), indicating substantially less activation of the ATP synthase.

To determine whether the lower activation of the ATP synthase in cfq was caused by impaired reduction of the γ-subunit, we pretreated leaves for 20 min with 1 mM DTT, which normally has the ability to at least partially reduce CF₁ γ-subunits (14). Fig. 1C illustrates that although 1 mM DTT pretreatment was not as efficient as pre-illumination, the reductant pretreatment substantially accelerated the decay of ΔA_{518} (τ = 120 ms) in wild-type leaves. In contrast, there was no effect of 1 mM DTT pretreatment on the decay of ΔA_{518} in cfq (Fig. 1D), indicating that the γ-subunit of ATP synthase in cfq was still in the oxidized form after DTT pretreatment.

**FIGURE 3.** Alignment of amino acid sequences of chloroplast γ-subunits. The amino acid sequence of the γ-subunits of ATP synthase encoded by *A. thaliana* atpC1 (At4G04640) is aligned with those of *Spinacia oleracea* (CA68727) and *Chlamydomonas reinhardtii* (180328A) as described previously (26). Shaded boxes indicate sequence identity. The E244K substitution in atpC1 of the cfq mutant is shown above the alignment. The two regulatory cysteine residues are shown at positions 199 and 205.

**FIGURE 2.** Equilibrium redox titrations of thiol/disulfide regulatory groups in the γ-subunit of the chloroplast ATP synthase. Leaves of the wild type (WT), the cfq mutant, and cfq mutants transformed with wild-type atpC1 (T₁-2, T₂-3, and T₄-4) were infiltrated with 20 mM DTT at various reduced/oxidized ratios for 4 h at pH 7.9. The ΔA_{518} relaxation kinetics were then measured and used to calculate the equilibrium midpoint potential of ATP synthase γ-subunit regulatory cysteines. The 95% confidence limits were calculated and are presented rounded to the nearest whole number.

The Midpoint Redox Potential of the Regulatory Sulphydryl Groups on γ-Subunits of ATP Synthase Is Shifted to a More Reducing Potential by the cfq Mutation—To further investigate the alteration of redox properties in the mutant, we performed an equilibrium redox titration by infiltrating leaves with different proportions of oxidized and reduced DTT at a combined total concentration of 20 mM. This was done to poise the chloroplast stroma at different redox potentials, allowing the accurate estimation of the equilibrium midpoint potential of the ATP synthase activity (22). In this experiment, the ΔA_{518} relaxation time constants were used to calculate the equilibrium midpoint potential of the γ-subunit regulatory cysteines. We set 100% activity of ATP synthase based on the decay of ΔA_{518} when the leaves were treated with 20 mM reduced DTT. In the wild type, the midpoint potential of ATP synthase was estimated at −337 ± 4 mV (E_m(7.9)), whereas in cfq, the midpoint potential of ATP synthase had been strongly shifted to the more reducing midpoint potential of −376 ± 5 mV (Fig. 2). Increasing the combined concentration of oxidized plus reduced DTT from 20 to 50 mM did not alter the measured midpoint potentials. The results indicated that the thermodynamics of the thiol/disulfide exchange between thioredoxin f (E_m(7.9) = −325 ± 10 mV) (33) and the regulatory sulphydryl groups on γ-subunits of ATP synthase were much less favorable in cfq than in the wild type. This strong effect of the mutation on the redox properties of the ATP synthase suggested that the mutation was in the γ-subunit.

Identification of a Point Mutation in the γ-Subunit Encoding the atpC1 Gene of cfq—In *A. thaliana*, there are two nuclear genes encoding the γ-subunit of the chloroplast ATP synthase, *atpC1* (At4G04640) and *atpC2* (At1G15700). *atpC1* is a light-regulated gene with a GT-1-binding site upstream of the coding region (26). The expression of *atpC2* is negligible (26) and was found not to be sufficient to compensate for the lack of *atpC1* in the *dpa1* null mutant (34), implying the γ-subunit of the *Arabidopsis* chloroplast ATP synthase to be exclusively the product of the *atpC1* gene. The entire coding region of the *cfq* mutant *atpC1* gene was amplified by PCR and sequenced. Comparison of the mutant sequence with the wild-type sequence identified a point mutation (G to A) located at position 2447 in the coding region of the gene. The substitution resulted in a change in the amino acid sequence from negatively charged Glu to positively charged Lys at position 244 within a region of the protein sequence that is highly conserved among photosynthetic eukaryotes (Fig. 3). This conserved region is located close to the C-terminal end of the protein and 38 amino acids from the second cysteine residue in the redox regulatory domain, strongly suggesting that this substitution caused the specific alteration in the redox properties of γ-subunits present in cfq.

To obtain conclusive evidence that alteration of the *atpC1* gene led to the abnormal ΔA_{518} relaxation kinetic phenotype and midpoint
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The redox potential of cfq, a Pro35S::atpC1 construct was introduced into cfq plants. Several kanamycin-resistant transformants were isolated, and the presence of the transgene construct was confirmed by DNA gel blot analysis (supplemental Fig. 1A). RNA blot analysis demonstrated that atpC1 was highly expressed in the transgenic lines compared (supplemental Fig. 1B).

Flash-induced absorbance changes were measured in detached leaves of the T3 generation transgenic plants and compared with those in the wild type and the cfq mutant (Fig. 4). As was seen previously (Fig. 1), pre-illumination accelerated the decay of $\Delta A_{518}$ ($\tau$ from 257 to 73 ms) in the wild type, whereas the decay in cfq was less enhanced ($\tau$ from 248 to 160 ms). In T3 generation transgenic plants, the relaxation time constant after pre-illumination averaged 98 ms, close to what was observed in wild-type plants. We also performed equilibrium redox titrations on the complemented transformants (T3-2, T3-3, and T3-4) (Fig. 2) and found that the midpoint potential was shifted an average of +30 mV, close to restoring the wild-type value. Thus, introduction of wild-type atpC1 into the cfq mutant largely restored the wild-type phenotype, confirming that the observed mutant phenotype was caused by a point mutation detected in the atpC1 gene. That the wild-type phenotype was not fully restored indicated that despite the very high expression level of the introduced wild-type gene, there remained some incorporation of the $\gamma$-subunit.

It was a possibility that the $\gamma$-subunit mutation resulted in assembly or stability problems and that the mutant phenotype was the result of a decrease in the ATP synthase content in the thylakoid membrane of the mutant. Additionally, the overexpression of wild-type atpC1 in the transgenic plants may have driven an increase in the content of the ATP synthase in the thylakoid membrane, accounting for the observed acceleration of the decay of $\Delta A_{518}$. To investigate this possibility, immunoblotting was performed to determine the ATP synthase $\gamma$-subunit content. Each ATP synthase contains three $\beta$-subunits, and quantifying the $\beta$-subunits enables the determination of the total chloroplast ATP synthase content. The immunoblot of the $\beta$-subunits revealed that the chloroplast ATP synthase in cfq and overexpression lines accumulated normally and to the same level as in the wild type (supplemental Fig. 2), indicating that the mutation in the $\gamma$-subunit of the cfq mutant and overexpression of the wild-type atpC1 gene in the cfq background had no significant influence on the content of ATP synthase. An increased expression of several or all genes of the ATP synthase appears to be necessary to regulate the abundance of the complex. Thus, the cfq and transgenic phenotypes were caused by the differences in the function of the $\gamma$-subunit of ATP synthase rather than a change in the ATP synthase content in the thylakoid membrane.

**ATPase and ATP Synthase Activity**—The amino acid substitution of Glu to Lys at position 244 in cfq makes the $\gamma$-subunit energetically more difficult to reduce especially under low light conditions. To study the impact of the substitution on the energy requirement for the activation of ATP synthase in cfq, $\Delta A_{518}$ relaxation kinetics were measured after pre-illumination of the leaf with 65 $\mu$mol m$^{-2}$ s$^{-1}$ red light for a range of times, followed by a 4-min dark interval (Fig. 5A). Pre-illumination times of >4 s decreased the relaxation time constant in the wild type, whereas >6 s of pre-illumination was required for cfq plants. In wild-type controls, ~8 s of pre-illumination was
were treated with 20 mM reduced DTT, and the ATP synthase activity increased during the first 10 min. As a control, wild-type leaves pre-illumination with 65 \textmu M DTT were dark adapted of the leaves for varying times following a 7-s illumination. The deactivation of ATP synthase was also investigated by pre-illumination required to fully activated the ATP synthase in cfq mutant, indicating a greater energy requirement (i.e., $\mu_{1H}$) for the activation of the mutant ATP synthase.

The deactivation of ATP synthase was also investigated by dark adaptation of the leaves for varying times following a 7-s pre-illumination with 65 \textmu M DTT. Fig. 5B illustrates that the ATP synthase activity in the wild type gradually decreased as the time of dark adaptation increased, whereas the ATP synthase activity in cfq was lower and decreased more rapidly during the first 10 min. As a control, wild-type leaves were treated with 20 \textmu M reduced DTT, and the ATP synthase activity remained unchanged.

The time course for the light-dependent activation of ATPase activity was determined in chloroplasts isolated from wild-type and cfq leaves (Fig. 6A). Illumination with low light increased the enzyme activity by 2-fold in the wild type, whereas the ATPase activity responded only slightly to the illumination in the cfq mutant. The ATP synthase in isolated wild-type chloroplasts increased as light intensity was increased, responding to the rise of the proton gradient across the thylakoid membranes, and saturated at 600 \textmu M m$^{-2}$ s$^{-1}$ (Fig. 6B). Although the cfq mutant showed a similar trend compared with the wild type, the ATP synthase activities were lower at all intensities (Fig. 6B).

**FIGURE 6.** Dependence of ATP synthase/hydrolyase activity on illumination intensity. A, ATP hydrolyase activity was measured as inorganic phosphate released by thylakoid membranes exposed to various light intensities for 2 min. Each data point represents the mean ± S.E. of four individual samples. B, ATP synthase activity was measured as inorganic phosphate depletion by thylakoid membranes as a function of light intensity. Each data point represents the mean ± S.E. of four individual samples. chl, chlorophyll.

**FIGURE 7.** Effect of the cfq mutation on photosynthetic and growth parameters. A, CO$_2$ assimilation rates of cfq and the wild type (WT) were compared at illumination intensities of 50, 100, and 200 \textmu M m$^{-2}$ s$^{-1}$. Error bars represent the mean ± S.E. of five or six individual plants. B, wild-type and cfq plants with two true leaves were moved to growth light intensities of 50, 100, and 150 \textmu M m$^{-2}$ s$^{-1}$. After 30 days, shoot fresh weights were measured. Error bars represent the mean fresh weight ± S.E. of five plants from three individual experiments. PFD, photon flux density.

**Photosynthesis and Growth Are Impaired in the cfq Mutant**—Because the change in the redox properties of the cfq mutant had an impact on the activity of the ATP synthase at low light, the possible effects of the mutation on photosynthesis and plant growth were investigated (Fig. 7). The cfq mutant exhibited a CO$_2$ assimilation rate indistinguishable from that of wild-type plants under a growth light intensity of 200 \textmu M m$^{-2}$ s$^{-1}$. At irradiance levels lower than growth light intensity (50 and 100 \textmu M m$^{-2}$ s$^{-1}$), the cfq mutant had an ~10% lower assimilatory capacity with 95% statistical confidence (Fig. 7A). However, small differences in CO$_2$ assimilation rate can sum over time to large differences in growth, as evident here in the comparison of wild-type and cfq plants grown under low light. At 60 \textmu M m$^{-2}$ s$^{-1}$ growth intensity, wild-type shoot fresh weight was ~2-fold more than cfq shoot fresh weight (Fig. 7B). Chlorophyll content was also analyzed in plants grown at the three light levels (Table 1). Although there was no change in the chlorophyll a/b ratios, the total chlorophyll content in cfq plants grown at 50 \textmu M m$^{-2}$ s$^{-1}$ was lower than that in wild-type plants.

**DISCUSSION**

In contrast to other thioredoxin-regulated photosynthetic enzymes, reduction of the ATP synthase disulfide bond is not...
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TABLE 1
Chlorophyll content and chlorophyll a/b ratio

| Growth light intensity | Wild type | cfq |
|------------------------|-----------|-----|
| 50 μmol m⁻² s⁻¹        |           |     |
| 100 μmol m⁻² s⁻¹       |           |     |
| 150 μmol m⁻² s⁻¹       |           |     |

| Wild type Chlorophyll a/b (μmol/g leaf) | 2.61 ± 0.05 | 2.56 ± 0.05 | 2.61 ± 0.06 |
| Total chlorophyll (μmol/g leaf)       | 1.00 ± 0.05 | 1.10 ± 0.10 | 1.18 ± 0.02 |

| cfq Chlorophyll a/b (μmol/g leaf) | 2.58 ± 0.02 | 2.62 ± 0.07 | 2.62 ± 0.03 |
| Total chlorophyll (μmol/g leaf)   | 0.71 ± 0.02 | 0.94 ± 0.04 | 1.09 ± 0.06 |

required for activation. Instead, the reduction occurs following the ΔµH⁺-dependent activation of the enzyme, leaving the significance of this thiol modulation unclear. A previous study showed that trypsin treatment resulted in cleavage of a small peptide containing a cysteine residue of the reduced disulfide bridge of the γ-subunit (35). The resulting CF₁ retained full ATPase activity (36). However, concurrent cleavage of α-, δ-, and ε-subunits made it unclear whether or to what extent the dithiol bridge in the γ-subunit was involved (37, 38). The cfq mutant, which is distinguished by an ATP synthase γ-subunit dithiul with a much more reducing midpoint potential, allowed us to further study the role of thiol modulation in the γ-subunit and its physiological significance. Compared with the wild type, the slower decay of ΔΔΨ in cfq after pre-illumination (7 s at 65 μmol m⁻² s⁻¹ red light) indicated that the ATP synthase activity in cfq was restricted because the low light pre-illumination was not able to generate sufficient reducing power in the form of the ratio of reduced to oxidized thioredoxin to reverse the oxidation of the γ-subunit (Fig. 1A). The oxidized form of the ATP synthase in the mutant required 3 s more illumination compared with the wild type for activation due to the requirement of a higher ΔµH⁺-threshold (Fig. 5).

In an atpC₁ null mutant missing the γ-subunit of chloroplast ATP synthase, the entire ATP synthase complex is destabilized, leading to a lethal phenotype (34) and highlighting the importance of this gene. The atpC₁ gene in cfq was determined to carry a single base pair mutation changing the amino acid sequence (4, 39) of the chloroplast γ-subunit that placed the point mutation of cfq close to the thiol-modulated cysteine motif and within a conserved domain of the γ-subunit (Fig. 3), we hypothesized that the point mutation might change the redox properties of the two cysteines. The midpoint redox potential (Eₘₚₗₚ) for the wild-type ATP synthase was −337 ± 4 mV, whereas that for the cfq mutant markedly shifted to a more reducing potential of −376 ± 5 mV (Fig. 2). The shift of the γ-subunit redox potential in cfq would make the ATP synthase more difficult to reduce by the natural reductant thioredoxin f, which has an equilibrium midpoint potential near −325 ± 10 mV at pH 7.9 (33). Indeed, under conditions in which the wild-type γ-subunit would be 75% reduced (i.e. when thioredoxin f is 90% reduced), the cfq γ-subunit would be only 10% reduced. The substitution of negatively charged glutamic acid (Glu) with positively charged lysine represents a significant change in the local electrostatic environment of the protein. The electrostatic change alone or coupled with a charge-driven conformational change in the γ-subunit is a likely candidate for making the disulfide bond between the two cysteines more stable and difficult to reduce. Our results demonstrate that the conserved amino acid residue segment proximal to the regulatory dithiol bridge is necessary for maintaining the physiologically appropriate redox range for the cysteine thiol/disulfide equilibrium.

It is thought that four protons pass through the ATP synthase from the thylakoid lumen to the stroma to generate one ATP from ADP and phosphate. To investigate the possibility of impaired proton utilization efficiency in cfq, we also measured ATP hydrolysis and synthesis activity. The ATP synthase activity of cfq (monitored as the dissipation of ΔµH⁺ following a light flash) could reach the same magnitude as that of the wild type after a longer pre-illumination period (Fig. 5), but the ATP hydrolysis and synthesis activity were lower than those of the wild type at all times and all intensities under continuous light (Fig. 6). This difference in behavior is explained by the fact that ATP synthesis following a flash of light is less limited by the amount of active ATP synthase than is ATP synthesis in continuous light (10, 11). Together, these results show that the γ-subunit point mutation lowered the efficiency of ATP formation.

That lower photosynthetic rates in cfq were observed only under low light conditions (Fig. 7A) is in agreement with predictions of the well validated biochemical model of leaf photosynthesis (40) that, in limiting light, photosynthesis is primarily limited by the rate of ATP formation and NADPH production. As light intensities approach saturation, photosynthesis is increasingly ribulose-bisphosphate carboxylase/oxygenase-limited, and thus, the difference in net CO₂ reduction between wild-type and cfq leaves would be expected to disappear. The cfq plants grown under low light intensity (50 μmol m⁻² s⁻¹) showed substantially slower shoot growth rates compared with the wild-type plants, almost certainly due to the lower photosynthetic rate of cfq at low light (Fig. 7B), suggesting that plants may benefit from the thiol modulation system under low light conditions. That cfq but not wild-type plants growing under
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low light accumulated less chlorophyll than those growing under normal light conditions (Table 1) is likely another manifestation of lower rates of ATP formation due to the cfq mutation in the γ-subunit.

Acknowledgments—We are most grateful to M. L. Richter for providing antiserum against the spinach chloroplast ATP synthase β-subunits and M. Futai for providing the αC31 construct. We acknowledge Dr. Aleel Grennan for expert assistance with the manuscript and Dr. Carl Bernacchi for assistance with curve fitting.

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