The nuclear atpC1 gene encoding the γ subunit of the plastid ATP synthase has been inactivated by T-DNA insertion mutagenesis in Arabidopsis thaliana. In the seedling-lethal dpa1 (deficiency of plastid ATP synthase 1) mutant, the absence of detectable amounts of the γ subunit destabilizes the entire ATP synthase complex. The expression of a second gene copy, atpC2, is unaltered in dpa1 and is not sufficient to compensate for the lack of atpC1 expression. However, in vivo protein labeling analysis suggests that assembly of the ATP synthase α and β subunits into the thylakoid membrane still occurs in dpa1. As a consequence of the destabilized ATP synthase complex, photophosphorylation is abolished even under reducing conditions. Further effects of the mutation include an increased light sensitivity of the plant and an altered photosystem II activity. At low light intensity, chlorophyll fluorescence induction kinetics is close to those found in wild type, but non-photothermal quenching strongly increases with increasing actinic light intensity resulting in steady state fluorescence levels of about 60% of the minimal dark fluorescence. Most fluorescence quenching relaxed within 3 min after dark incubation. Spectroscopic and biochemical studies have shown that a high proton gradient is responsible for most quenching. Thylakoids of illuminated dpa1 plants were swollen due to an increased proton accumulation in the lumen. Expression profiling of 3292 nuclear genes encoding mainly chloroplast proteins demonstrates that most organelle functions are down-regulated. On the contrary, the mRNA expression of some photosynthesis genes is significantly up-regulated, probably to compensate for the defect in dpa1.

In oxygenic photosynthesis light-dependent electron transport from water to NADP⁺ is coupled to synthesis of ATP (photophosphorylation) and energetically mediated by a transmembrane electrochemical proton gradient. Photophosphorylation, which essentially resembles oxidative phosphorylation, is carried out at the thylakoid membrane of photosynthetic eubacteria and chloroplasts and is catalyzed by a proton-translocating reversible ATPase (“ATP synthase”). The basic organization, structure, and composition of this protein complex have been extensively investigated on the eu-bacterial level as well as in mitochondria and plas-tids and were found to be vastly conserved (1, 2).

The plastid ATP synthase complex consists of nine different subunits, four of them are localized in the membrane integral CF₀ subcomplex (α, b’, b’, and c₁γ) which is responsible for proton translocation, and five subunits constitute the extrinsic CF₁ subcomplex (a₂, β₂, γ, δ, and e) which forms the catalytic entity (3, 4). Three independent studies proved the rotation of the γ subunit relative to (αβ)₅ during ATP hydrolysis in the isolated F₁ (5–7) and recently in isolated F₀F₁ complexes (8). The γ subunit is responsible for the so-called “thiol modulation” or “redox modulation” due to two Cys residues able to form an intrapeptide disulfide bond (9). Upon reduction, the activation profile of ATP synthase is shifted toward lower proton gradients (10, 11). The seven amino acids Cys₁⁹⁹–Cys₂⁰⁵ (numbering in Arabidopsis) are present only in land plants and green algae as in Chlamydomonas reinhardtii (10, 12). Nevertheless, differences in the redox regulation mechanism have been pointed out between algae and higher plants (13). Wherein in higher plants the re-oxidation of CF₁, following a light-dark transition, takes over 1 h, in the alga Dunaliella salina CF₁ is several times faster re-oxidized by a specific endogenous oxidant (14). Only recently redox modulation of the γ rotation has also been reported (15).

In bacteria the genes for the two subcomplexes are organized in separate operons suggesting that the enzyme evolutionarily derived from a proton channel (F₀) and an ATPase (F₁) (16). The organization of the genes reflects also a possible mechanism of assembly. The bacterial F₀ and F₁ accumulate independently and associate then to the membrane to assemble a functional complex (17). However, only little is known about the assembly of the chloroplast enzyme (1). In Chlamydomonas neither CF₀ nor CF₁ is assembled, although the unimpaired polypeptides are synthesized, if one of the nine subunits is missing. Several mutants in Chlamydomonas affected in different chloroplast-encoded subunits of the ATP synthase have been used to characterize the assembly process (18).

In Arabidopsis two genes, atpC1 and atpC2 (accession numbers M61741 and J05761, respectively), located on chromosomes 4 and 1, respectively, encode for the plastid γ subunit (19, 20). In plants grown under continuous illumination, atpC1

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is much higher expressed than atpC2 (19). The two AtpC proteins in Arabidopsis share 73% sequence homology, whereas the homology of the AtpC subunits between other plants and AtpC1 in Arabidopsis is about 88 ± 4%. This raises the intriguing question about the role(s) of the two AtpC gene copies in Arabidopsis, and whether the two genes possess distinct functions and under which physiological conditions they become operative.

Proton efflux through the plastid ATP synthase, associated with ATP synthesis, results in an accelerated relaxation of the electric field-associated absorption change at 518 nm (21). This property has been used to identify coupling factor reduction mutants in Arabidopsis which grow poorly in dim light and showed slowed Δψ518 decay after dark-light transition (22). One coupling factor quick recovery mutant, cfq, contains an E244D point mutation in the γ subunit leading to a decreased acidification of the lumen compared with wild type in the initial few minutes of the induction period under subsaturating light conditions. This mutant has been used to correlate non-photochemical chlorophyll fluorescence quenching (NPQ)1 with Δψ (23).

In the present study we have identified the Arabidopsis thaliana mutant dpa1 in which the atpC1 gene has been inactivated by T-DNA insertion. The mutant has been characterized on the level of accumulation, activity, and assembly of ATP synthase. Deficiency of the γ subunit leads to loss of ATP synthesis and unusual NPQ which is much below the dark Δψ, level due to proton accumulation in the lumen. The pattern of mRNA expression of nuclear genes coding for chloroplast proteins in dpa1 is interpreted as a compensatory effect.

EXPERIMENTAL PROCEDURES
Plant Material, Growth Conditions, and Mutant Selection—Seed sterilization and growth conditions of dpa1 and wild type were as described (24) with the exception that only 1.4% (w/v) sucrose has been supplemented to the medium. Seedlings were grown under continuous light at a photon flux density of 20 μmol photons m⁻² s⁻¹ and at a constant temperature of 21 °C if not otherwise indicated. Prior to illumination, plates were placed for 2 days at 4 °C to synchronize germination. Propagation of the dpa1 mutant occurred via heterozygous offsprings. All comparisons between mutant and wild type were carried out with leaf material of the same developmental stage. Individual mutant plants of segregants grown in Petri dishes have been identified by chlorophyll (chl) fluorescence video imaging (FluorCam 690M; Photon Systems Instruments, Brno, Czech Republic). The FluorCam software package (FluorCam) has been used for chl fluorescence induction and to determine fluorescence parameters and PSI yield. The dpa1 mutant could easily be distinguished from wild type plants due to a strong NPQ (see below).

Molecular Mapping and Complementation Studies—F₁ populations were produced by pollinating emasculated flowers of the accession Landsberg erecta with Wassilewskija plants heterozygous for the dpa1 mutation. F₂ families selected for the mutant offsprings were grown on medium, and individual mutant plants were chosen for genetic mapping with the molecular markers. The oligonucleotides 5′-CAGTCAGAAGGCATCTTAATAGATG-3′ and 5′-GGTCGCGTCGCTCT-3′ were used as a simple sequence length polymorphism marker Cer52226 (25) located 850 kb upstream of the atpC1 gene on the bacterial artificial chromosome F42C1. This marker is polymorphic between Landsberg and Wassilewskija producing 203- and 285-bp PCR products, respectively.

The full-length coding sequence of the intron-less atpC1 gene was amplified by PCR with genomic DNA from wild type and the reverse primer 5′-AACAAAAAAATGGCTTGCTCTAATCTAACA-3′ and the reverse primer 5′-AAGAGGGTTCTAGACAAATCAAACCTG-3′, which inserts an XbaI restriction site at the 3′-end of the gene. The XbaI-digested and purified PCR product was ligated into the Smal/XbaI sites of the binary vector pS001-VS under control of the cauliflower mosaic virus 35S rRNA promoter (26). Successful cloning was verified by sequencing. The construct was introduced into progeny of plants that segregate the mutation via Agrobacterium using the floral dip method (27). Transformant plants were efficiently selected on non-nutrient medium (Grodan, Hobro, Denmark) immersed in 10 mg/liter sulfadiazine prior to spreading the seeds (28).

For PCR analysis of transformed lines (see Fig. 1) the following primers have been used: Atact(5′-GCTCATTTTGGCTGGGCTTACG-3′) and Atact(5′-TCTGATTATTTGGTGTGGTTCCTCG-3′) of the actin3 gene as a control; primer 1 (5′-GACCGACCTACATATAAATTCCCAAA-3′) and primer 2 (5′-GCTCATTTTGGCTGGGCTTACG-3′) of the 5′-end atpC1 coding region; primer 3 (5′-CATAGATGCACTCGAAATCAGCC-3′) of the 5′-end of the 5′-end atpC1 coding region; and primer Pv (5′-GCCATGTTGAAAGTGCCTGTC-3′) of the 35S promoter.

Northern and Southern Analyses—Total RNA and DNA were isolated and subjected to Northern and Southern analysis, respectively, as described (24, 29–31). Hybridizations with 32P-labeled probes were carried out for 1 h at 68 °C in Rapid Hyridization Buffer (Amersham Biosciences). The signals were analyzed on autoradiographs using Fuji Bio Imaging plates type BASII, a Fuji Bio Imaging analyzer, and the Aida software package (Raytest, Sprockhövel, Germany). Probes for atpHIII, atpF, atpA, and atpC1 used for Northern analysis were as described (32). To confirm the array expression data, probes for petC, psbS, lbc6, and psaD1 genes were used in Northern analysis. The full-length cDNAs obtained from the ABRC (Arabidopsis Biological Resource Center at Ohio State) were amplified using the vectorial (pZL1, Invitrogen) forward primer 5′-TATAGCAGTCTAGAAGCCCG-3′ and the reverse primer 5′-ATTAGGTCACATATAAGG-3′. Primer combinations specific for the 5′-ends of atpC1 (primer 2 and 5′-GGTTAGGGAACACGACAATCATCGT-3′), atpC2 (5′-GCTCGAGTCGAGTCTACTCTT-3′ and 5′-CAGTACACAACTCAACCTGGCC-3′), and the 18S rRNA (5′-GCTAAGGAGAGCTTACGAG-3′) were used in Northern analysis. The membranes were incubated with antisera against thylakoid membrane proteins, and signals were identified by chemiluminescence or by detection with the X-ray film (33).

Photophosphorylation—Chloroplast thylakoids from Arabidopsis leaves were prepared as described for spinach (34). The reactions were conducted in a Δψ clamp instrument as described (35). The reaction cell of 2.5-ml volume contained a medium consisting of 25 mM Tricine buffer, pH 8.0, 5 mM dithiothreitol, 5 mM MgCl₂, 5 mM 32P-labeled Na₂HPO₄, 50 mM KCl, 50 μM phenazine methosulfate, and thylakoids corresponding to a chl concentration of 25 μg/ml. The experiments were conducted at pre-chosen Δψ values (35, 36) which were kept constant throughout the experiment by the employed clamp device. Δψ was continuously controlled by the fluorescence quenching of 9-aminoacridine. The fluorescence signal was calibrated as described (37).

The thylakoids were pre-illuminated for 2 min to obtain the pre-chosen proton gradient. Then 0.5 mM ADP was added in the light. After 10, 20, and 30 s, 0.2-ml samples were taken and deproteinized with HClO₄ (final concentration 0.6 M). The formed 32P-labeled organic phosphate was determined as described for spinach (33).

Immunological and Translation Analyses—Thylakoid membrane proteins of 3-week-old plants were isolated as described (39). Proteins were quantified (40), and samples were heated for 5 min at 80 °C in 2% SDS mixed with 1/10 volume of glycerol/dye solution and immediately applied onto SDS-PAGE at 30 mA for 12–15 h at room temperature. The protein pattern was visualized by silver-staining analysis. For primary electron detection, proteins were transferred to PVDF membranes (Pall Bio- dyne, Dreieich, Germany) by semi-dry electroblotting (Pebqlab, Erlangen, Germany). The membranes were incubated with antisera raised against thylakoid membrane proteins, and signals were identi-
ified by the enhanced chemiluminescence technique (Amersham Biosciences). Most of the antibodies used in this study were raised in rabbits against Chlamydomonas or spinach polypeptides (24). For in vivo labeling analysis intact leaves of 3-week-old plants were immersed in a 1/50 MS solution containing 50 μCi of [35S]methionine for 20 min (42). Subsequently, thylakoid membrane proteins were isolated, subjected to SDS gel electrophoreses, and transferred to PVDF membranes. Inactivation was detected by fluorography (43).

Electron Microscopy—Sample preparations for ultrastructural analysis and electron microscopy were performed as described (44).

Fluorometric and Absorption Studies—chla fluorescence measurements were performed with 3-week-old plants using a commercial pulse amplitude modulated fluorometer PAM 101 interfaced with the PAM data acquisition system FDA-100 (Walz, Effeltrich, Germany). The fiber optic probe was held 2 mm distant from the upper side of plants grown under sterile conditions in Petri dishes. Leaves were dark-adapted for 5 min prior to the induction fluorescence measurements. The minimal (F₁), steady state (Fₛ), and maximal (Fₘ) fluorescence yield, and the variable 

Expression Profiling Using Nuclear Arrays—The 3292 gene sequence tags array representing genes known or predicted to code for proteins having a chloroplast transit peptide has been described previously (48, 49). At least three experiments with different filters and independent cDNA probes derived from plant material corresponding to pools of at least 50 individuals were performed for each condition or genotype tested, thus minimizing variations between individual plants, filters, or experiments.

Miscellaneous—Basic molecular biology methods were performed as described (31). Nucleotide sequences were determined by the dideoxy chain termination method (50). Energy transfer was determined by fluorometry using a mixture of oligonucleotides matching the 3292 genes in antisense orientation as primer, and hybridized to the gene sequence tags array as described previously (48, 49).

RESULTS

Identification and Phenotype of the Dpa1 Mutant—The F2 progeny of 1100 EMS-treated seeds and 75 preselected pale mutants from T-DNA collections (53) obtained from the Arabidopsis Biological Resource Centre (Ohio State University, Columbus, OH) have been used for screening. 87 mutant plants were selected by their non-photoautotrophic growth on soil. They developed pale green cotyledons but no primary leaves. Cultivation on sucrose-supplemented MS medium (52) often rescued the mutant seedlings, leading to a nearly normal pigmentation and development. Under these conditions the mutants could often hardly be distinguished from wild type plants, although growth rates were slightly retarded. Seven plants showing a high dark level of fluorescence (F₀) but a lower level during induction were selected from the collection by imaging analysis (Fig. 1A). In six plants the lowest fluorescence level during induction was 4000 Fm/F₀, and the maximal fluorescence during induction, Fₘ', was 4000 μmol photons m⁻² s⁻¹. For nigericin studies, leaves of 3-week-old plants were harvested and cut into small pieces with a sharp razor blade at 4 °C in 50 mM HEPES (pH 7.6), 330 mM sorbitol, 1 mM MgCl₂, 1 mM MnCl₂, 2 mM EDTA, and 0.2% (w/v) defatted bovine serum albumin. The resuspension buffer was supplied with 0.1 mM methylviologen as an electron acceptor in all samples and, when indicated, with 2 μM nigericin as a ionophore (47).

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This increased NPQ was indicative of photosynthetic electron transport activity in dpa1 in contrast to a high chlorophyll fluorescence phenotype of photosystem I mutants such as hcf101 (Fig. 1A) (24). The screened seedling lethal dpa1 mutant of A. thaliana, accession Wassilewskija, originated from the T-DNA insertion collection.

Inactivation of Chloroplast ATP Synthase γ Subunit

FIG. 1. Mutant screen, T-DNA insertion, and complementation studies. A, chla fluorescence of seedling lethal mutants was initially recorded by fluorescence imaging. The dpa1 mutant has been selected due to its high dark fluorescence level, F₀ (D), and its normal steady state fluorescence level (Fₛ) during illumination (L). Photosynthetic electron transport mutants (hcf101) showed a high fluorescence level under both conditions, whereas complemented homozygous mutant plants (dpa1c) behaved like wild type. B, schematic presentation of the insertion of the truncated T-DNA into the atpC1 gene and of the primers used for the PCR analysis. Primer 1 was chosen from the atpC1 promoter, primers 2 and 4 from the atpC1 coding region, and primer 3 from the left border of the T-DNA. C, PCR analysis of wild type (WT), heterozygous lines (Het), and mutant plants. The data demonstrate homozygosity of dpa1 because primers 2 and 4 did not amplify, whereas the internal control reaction using primers of the actin3 gene was positive in dpa1. The PCR product using primers 3 and 4 demonstrates the insertion and the orientation of the T-DNA. D, analysis of complemented dpa1c plants. Primers 1 and 4 did not amplify in the complemented line due to the insertion of the T-DNA into the coding region of the endogenous gene. Successful complementation is proven by amplification with primers 2 and 4 of atpC1 as well as primers 4 and Pv (chosen from the 35S RNA promoter of the binary vector).
Inactivation of the atpC1 Gene—Initially, we have tried to isolate flanking genomic regions of the right border of the T-DNA by inverse PCR. Each attempt to get a specific product failed. We backcrossed the mutation in order to confirm the Mendelian segregation of dpa1, to remove possible background mutations and to generate a mapping population. The dpa1 mutation was mapped to the upper part of chromosome 4 by the use of the molecular Cer452226 marker closely located to atpC1 (see “Experimental Procedures”). Forty-eight meiotic chromosomes of individual F2 mutant plants derived from backcrosses to the accession Landsberg have been used in this study. No recombinations have been identified, indicating that the dpa1 mutation is closely linked to the atpC1 gene.

Southern analyses with genomic DNA from dpa1, wild type, and heterozygous plants using probes of the T-DNA left border and the atpC1 gene resulted in restriction fragment length polymorphisms showing that there is a T-DNA insertion in the region of atpC1 (data not shown).

Due to the close location of the mutation to atpC1 and the observed restriction fragment length polymorphism, primers of the atpC1 gene and of the T-DNA borders were chosen in order to identify the site of T-DNA insertion. Only primers of the left border together with gene-specific primers were able to amplify indicating that the right border was truncated (Fig. 1, B and C). By sequencing PCR products obtained with primers 3 and 4, the exact location of the T-DNA at position +603 bp relative to the start codon of the atpC1 gene could be defined (Fig. 1, B and C). With primers 2 and 4, atpC1 could not be amplified in the mutant, but the expected product of 578 bp was generated in wild type and heterozygous plants (Fig. 1C). The PCRs also included the two primers of the actin3 gene as an internal control that amplified in both wild type and mutant plants (Fig. 1C).

Northern analyses of transcripts of plastid genes encoding subunits of the chloroplast ATP synthase, i.e. atpA, atpHII, and atpF, showed that their expression was not altered in the mutant (Fig. 2A). On the contrary, atpC1 was not expressed in dpa1 (Fig. 2B). Real time reverse transcriptase-PCR analysis has shown that atpC2 is 209 ± 35 times lower expressed than atpC1 in wild type leaves and that the expression of atpC2 is unaltered in dpa1 as compared with the wild type.

The atpC1 Gene Complements the dpa1 Mutation—In order to confirm that the phenotype of the dpa1 mutant is indeed caused solely by the insertional inactivation of the atpC1 gene, complementation studies were performed. Mutant plants expressing the wild type atpC1 gene under control of the 35S RNA cauliflower mosaic virus promoter were selected in the progeny of transformed plants heterozygous for the mutation (Fig. 1D). Northern analysis of complemented lines confirmed that atpC1 is significantly overexpressed as compared with wild type plants (Fig. 2B).

Homozygous dpa1 transformants were able to grow photoautotrophically, exhibited normal growth rates, and were green in appearance. The chl fluorescence parameters \( F_v/F_m \) as well as photochemical quenching (0.85) and NPQ (0.18) of complemented lines were within the standard deviations of the wild type (see below).

Accumulation of the Plastid ATP Synthase Is Abolished in dpa1—The accumulation of the ATP synthase and other complexes of thylakoid membranes of dpa1 and wild type was compared by using a collection of antisera raised against eight individual ATP synthase polypeptides and, as a control, repre-
sentative polypeptides of PSI, PSII, and the cytochrome b$_{6}$f complex (24). All nuclear and plastid-encoded subunits of the ATP synthase analyzed failed to accumulate in significant amounts in *dpa1* (Fig. 3A). The dilution series of wild type membranes demonstrated that levels of ATP synthase subunits were reduced to less than 5%. Amounts of several subunits such as AtpA, AtpC, AtpH, AtpD, and AtpG were below the limit of detection. Only little differences in steady state levels were observed for other nuclear and plastome-encoded proteins of PSII, e.g. LHClII, PsbO, PsbD (D2), PSI, and PsaC, analyzed in *dpa1* (Fig. 3B). In contrast, levels of proteins of the cytochrome b$_{6}$f complex, e.g. PetA and PetB, were increased in *dpa1*.

Some ATP synthase complexes might assemble in the mutant, which contain the *atpc2* gene product. However, expression of AtpC2 is not sufficient to allow photoautotrophic growth, detection of the γ subunit, and significant accumulation of any other subunit that belongs to the ATP synthase. Taken together, immunoblot analyses indicate that loss of the γ subunit nor the δ subunit.

**Decreased Stability of the Plastid ATP Synthase Subunits**—In silver-stained gels it was already visible that the two large α and β subunits of the ATP synthase and the γ subunit were depleted in *dpa1* (Fig. 3C), although amounts and patterns of all the other thylakoid membrane proteins were comparable with those of the wild type. A diffuse band, the identity of which is presently unknown, comigrates with the AtpB protein in the gels. The rate of synthesis of plastid-encoded ATP synthase proteins was investigated by pulse-labeling experiments. Labeled proteins were purified and analyzed by SDS-gel electrophoreses. The appearing signals in the gels could be assigned to individual subunits of the thylakoid membrane, i.e. Psaa/B, Atpa/B, PsbB/C, and PsbA/D, due to their abundance and known electrophoretic mobilities and by mutant analysis (32). The protein labeling patterns in the mutant and wild type were identical with respect to the size and the numbers of all detectable polypeptides and their intensity of labeling. The PSII subunits Psaa/B, PsbB/C, and PsbA/D, and the ATP synthase α (Atpa) and β (Atpb) subunits were synthesized at about normal rates in the mutant, whereas steady state levels of the α and β subunits were reduced by more than 95% as compared with the wild type (Fig. 3A).

The Activity of the Plastid ATP Synthase Is Abolished in *dpa1*—The immunological data have been supported by analysis of ATP production with isolated thylakoids (Table I). The rate of photophosphorylation depended on the magnitude of ΔpH applied and attained 112.1 μmol of ATP mg of chl$^{-1}$ h$^{-1}$ at ΔpH 3.0 in wild type thylakoids. As reported for spinach chloroplasts, the ATP synthesis rate was increased in an intermediate ΔpH range from 2.5 to 3.0 under reducing conditions, indicating a lower ΔpH profile for activation of the ATP synthase (12). In mutant thylakoids, on the other hand, phosphorylation activity was low and ΔpH-independent both under oxidized and reduced conditions, i.e. probably unrelated to photophosphorylation.

### Table I

| pH Difference | WT Oxidized | Reduced | dpa1 Oxidized | Reduced |
|--------------|-------------|---------|--------------|---------|
| 2.4          | 15          | 15      | 2.7          | 6.6     |
| 2.5          | 31.7        | 64      | 1.9          | 6.2     |
| 2.8          | 73.1        | 116.5   | 1.9          | 5.4     |
| 2.9          | 89.3        | 140     | 2.6          | 6.8     |

The inhibition of chloroplast ATP synthase activity by light in *dpa1* mutants was investigated by pulse-labeling experiments with $^{35}$S-methionine (Fig. 3D). To reduce the complexity of labeling, synthesis of the nuclear encoded chloroplast proteins was blocked with cycloheximide so that only plastome-encoded proteins appeared. Labeled proteins were purified and subjected to SDS-gel electrophoreses. The appearing signals in the gels could be assigned to individual subunits of the thylakoid membrane, i.e. Psaa/B, Atpa/B, PsbB/C, and PsbA/D, due to their abundance and known electrophoretic mobilities and by mutant analysis (32). The protein labeling patterns in the mutant and wild type were identical with respect to the size and the numbers of all detectable polypeptides and their intensity of labeling. The PSII subunits Psaa/B, PsbB/C, and PsbA/D, and the ATP synthase α (Atpa) and β (Atpb) subunits were synthesized at about normal rates in the mutant, whereas steady state levels of the α and β subunits were reduced by more than 95% as compared with the wild type (Fig. 3A).

Therefore, the functional state of PSII has been monitored by non-invasive chl fluorescence measurements (45). Analysis of *dpa1* mutant seedlings revealed a decrease in F$_{v}$/F$_{m}$ (0.60 ± 0.05 versus 0.80 ± 0.02 in the wild type) which is indicative of a partially impaired PSII. Light intensities of 2 μmol photons m$^{-2}$ s$^{-1}$ for fluorescence induction did not significantly increase the F$_{v}$ above the F$_{m}$ level in wild type plants, and saturating light pulses were able to completely induce the F$_{m}$ level during induction (Fig. 4). In *dpa1* low light significantly increased the F$_{v}$ to 20% of F$_{m}$ indicating that electrons are not efficiently released from PSII and probably keep the primary quinone acceptor of PSII, QA, partially reduced. F$_{m}$ could be reached during induction indicating that no significant proton accumulation or NPQ takes place at this low light intensity in *dpa1*. Light-induced quenching of chl fluorescence severely depended on the chosen light intensity. Under low light of 2 μmol of photons m$^{-2}$ s$^{-1}$ NPQ was close to zero in both wild type and *dpa1*; under still moderate light intensities of 30 μmol of photons m$^{-2}$ s$^{-1}$ a very strong NPQ was observed in *dpa1* reaching values of 1 after 2 min of induction, while in wild type NPQ was below 0.2 (Fig. 4). Depending on the light intensity the fluorescence dropped far below the dark F$_{m}$ level which could be quenched up to 40%. This high non-photochemical quenching mechanism did not allow light pulse-dependent increase of the fluorescence above the F$_{m}$ level after 2 min of induction (Fig. 4).
The quenching below $F_o$ was reversible and relaxed to about 90% within 3 min dark incubation; complete relaxation took about 10 min (Fig. 5). It was investigated whether the post-illumination increase of the fluorescence in $dpa1$ was mainly due to high energy quenching ($qE$), which is known to depend on the luminal proton concentration (55). Far-red light, which selectively excites PSI, has been applied after induction but was unable to change the relaxation rate, independently of which intensity has been used (Fig. 5A). Therefore, the fluorescence increase in the dark after switching off actinic light could not be caused by reduction of quinones in $dpa1$.

In order to exclude the possibility that the weak measuring beam (setting of the PAM beam intensity was 3 and 4), which is permanently on during measurements in the dark, was responsible for the dark increase of the fluorescence in $dpa1$, both the measuring beam and the actinic light were switched off for 10 min after induction (Fig. 5B). At this time the dark level could be reached again within a few seconds when applying the measuring beam confirming that the fluorescence increase is independent of reduction of $Q_A$ by the weak measuring beam.

Therefore, we suppose that the fluorescence increase in the dark is reflected by relaxation of the high energization of the thylakoid membrane. This assumption is consistent with the finding that the fluorescence increase was severely slowed down at a lower temperature when the thylakoid membrane is less leaky for protons (Fig. 5C). Lowering the temperature to 0°C decreased the relaxation rate considerably, and increasing the temperature to 35°C increased the relaxation rate. In addition, the kinetics of fluorescence quenching is also temperature-sensitive (Fig. 5C). Increasing the temperature to 35°C accelerated the quenching process, and decreasing the temperature to 0°C slowed down the kinetics. However, the steady state fluorescence level below $F_o$ was nearly identical at 0, 20, and 35°C after 15 min. This might well be explained by the temperature dependence of the xanthophyll cycle.

To investigate further the quenching mechanism, fluorescence induction curves were analyzed in the presence of the ionophore nigericin (Fig. 6). If quenching in $dpa1$ was due to accumulating protons in the lumen, the action of the uncoupler should prevent this tendency. Indeed, when nigericin was applied during induction, quenching of $F_o$ started to relax in $dpa1$ and fluorescence again reached levels above $F_o$ (Fig. 6B). When nigericin was supplied 10 min before starting the fluorescence measurement, $dpa1$ failed to perform strong NPQ, and the fluorescence traces were close to that found in wild type. Quenching of $F_o$ in the darkness immediately after switching off actinic light was almost negligible (Fig. 6C).

Ultrastructure of $dpa1$ Chloroplasts—Electron micrographs of leaves from illuminated 3-week-old plants revealed an impaired structure of the chloroplast (Fig. 7). The mutant formed grana stacks which were disordered and not strictly oriented in parallel to each other probably because they were not always interconnected by stroma thylakoid membranes. The luminal space was swollen indicative of a light-dependent high proton gradient across the thylakoid membrane which leads to influx of osmotically active ions (54). The thylakoid membrane system was not reduced in $dpa1$, but mutant chloroplasts were deficient of any starch grains.

Expression of mRNAs for Nucleus-encoded Chloroplast Proteins in $dpa1$—To investigate further effects of the $atpC1$ mutation, the expression levels of nuclear genes that contribute to chloroplast functions were determined by DNA array analysis. This was carried out on a set of 3292 gene sequence tags, about
81% of which code for chloroplast-targeted proteins, and their expression patterns in *dpa1* mutants were compared with those in wild type (48, 49). Differential gene expression values (*dpa1 versus* wild type) were determined by comparing hybridization signals. Statistical analysis of the expression data revealed that 1930 genes showed significant differential expression in *dpa1*. An unbalanced response of the nuclear chloroplast transcriptome was observed in the mutant, with the vast majority of differentially expressed genes being down-regulated (1765 genes down- and 165 genes up-regulated).

The differentially expressed genes in *dpa1* were grouped into 13 major functional categories, including photosynthesis (dark or light reaction), primary and secondary metabolism, transcription, protein synthesis, transport, and others (Fig. 8) (Supplemental Material). Most of the different functional gene classes followed the general trend of down-regulation. However, genes for the light and dark phase of photosynthesis were up-regulated more than others. Closer inspection of photosynthetic gene expression uncovered that in particular genes coding for proteins of the ATP synthase, the cytochrome *bbf* complex, PSI, or the Calvin cycle were up-regulated in *dpa1* (Table II), whereas differentially expressed genes coding for PSII tended to be up- and down-regulated. Because the analysis of the expression of photosynthetic proteins (Fig. 3) and of chlorophyll fluorescence characteristics implied that photosynthetic electron flow still occurs in the *dpa1* mutant, the mRNA expression changes might indicate that the plant is able to monitor the altered photosynthetic state of the chloroplast (e.g. the increased lumen acidification) and react by regulating appropriate photosynthetic genes. For example, from the analysis of steady state levels of photosynthetic proteins, it appears that the level of PSI-C is decreased in the mutant (Fig. 3B) and the up-regulation of other PSI genes could be a compensatory reaction of the plant to prevent an even higher reduction of this photosystem. In the same way expression of the two remaining nuclear genes of the ATP synthase, *atpD* and *atpG*, is increased in *dpa1* (Table II).

**DISCUSSION**

*The γ Subunit Is Essential for Assembly of the Plastid ATP Synthase*—In the present study we characterized the *dpa1* mutation in *Arabidopsis* which has been induced by T-DNA insertion into the *atpC1* gene encoding the γ subunit of the chloroplast ATP synthase. The low endogenous expression of *atpC2* is not altered in *dpa1* and does not result in detectable amounts of the γ subunit in the mutant (Fig. 2B). *AtpC2* contains the unique domain, not present in mitochondrial and bacterial γ subunits, which is known to be responsible for redox regulation of the enzyme (56, 57). Due to the low homology between *AtpC1* and *AtpC2* it is conceivable that the two subunits have been maintained for adjustment under changing environmental or tissue-specific conditions or in order to keep the plastid ATP synthase under proper photosynthetic control. The two *atpC* genes are not conserved in the amino-terminal part of the transit peptide and are not found in duplicated genomic blocks assuming two independent gene transfers to the nucleus (58, 59).

Overexpression of *atpC1* is not sufficient to increase amounts of the ATP synthase complex in complemented lines (Fig. 3C). Thus, an increased expression of several or all genes of the ATP synthase might be necessary to regulate the abundance of the complex or, more likely, that regulation of complex abundance takes place on a translational or post-translational level (18). Biogenesis of ATP synthases in bacteria and mitochondria proceeds through assembly of the soluble F1 followed by association to the transmembrane F0 subcomplex (17, 60). The independence of assembly of the two subcomplexes is abolished in plastids of the green algae *Chlamydomonas* where a concerted assembly of CF1 and CF0 subunits has been reported for mutants affected in different subunits (61–63). The same holds true for *dpa1*, where lack of the nuclear encoded γ subunit affects the accumulation of all other nuclear and plastid-encoded subunits that constitute the ATP synthase. A model for the assembly pathway that resulted from different mutant analyses and reconstitution experiments predicts an early stage formation of αβ heterodimers followed by trimerization upon interaction with the γ subunit (18). *In vivo* labeling studies have shown that the plastid-encoded α and β proteins of the CF0 subcomplex of 55.4 and 53.8 kDa, respectively, are translated at normal rates (Fig. 3D). Surprisingly, *de novo* synthesized α and β subunits are associated with the membrane even in the absence of the γ subunit in *dpa1*. This provides new insights into the assembly pathway of the plastid ATP synthase and may imply that either γ is not necessary for the formation of the αβ hexamers prior to assembling into the membrane or that the αβ dimer can already associate to the membrane mediated by yet unidentified chaperones in the chloroplast. In yeast mitochondria at least two chaperones play an important role in the assembly of the α and β subunits into the F0 oligomer (64). Nevertheless, binding of α and β to CF0 mediated by δ and b' cannot be excluded. The *dpa1* mutant now provides a starting material to investigate the function of *atpC2*, recombinant forms of the γ subunit, and the assembly mechanism of the ATP synthase in *Arabidopsis*.

**Loss of the Chloroplast ATP Synthase Causes High Non-photochemical Quenching**—Several ATP synthase mutants in *Chlamydomonas* have been shown to be light-sensitive (54) and the same is true for *dpa1*. As a consequence of the ATP synthase disruption, accumulation of a large electrochemical gradient through the thylakoid membrane that induces qE, the energetic component of NPQ, might be expected (55). Therefore, quenching of the fluorescence was in fact also expected to occur in *Chlamydomonas* ATP synthase mutants. Surprisingly, the major effect in the *Chlamydomonas* mutants is a light intensity-dependent rise of the steady state fluorescence level (F<sub>0</sub>) up to a level very close to F<sub>m</sub> (54). It has been suggested that Δψ<sub>hi</sub>-dependent NPQ was not induced under the experimental conditions. In contrast, in *dpa1*, increasing light intensity results in increasing NPQ and a drop of F<sub>0</sub> even below the initial dark F<sub>0</sub> level. This difference shows that fluorescence signals and quenching mechanisms differ significantly between the green algae and higher plants. A different extent in the dissipation of excess absorbed light energy has been described.
in respect to the xanthophyll cycle for the npq mutants of Chlamydomonas and Arabidopsis (65, 66). Inactivation of the violaxanthin de-epoxidase results in an impaired NPQ of 25% in Chlamydomonas and of 85% in Arabidopsis, suggesting a larger contribution of xanthophyll cycle-dependent NPQ in Arabidopsis compared with the green alga (66).

Post-illumination Fluorescence Increase Is Due to Relaxation of Thylakoid Proton Gradient—We further characterized the fluorescence quenching mechanism and the unusual relaxation that occurs in darkness. Analysis of the relaxation kinetics in the dark allowed us to investigate different factors separately (Fig. 5). Both far-red light and the measuring beam did not influence the relaxation kinetics of the quenching in the dark. Therefore, the post-illumination fluorescence increase in the dark is not due to reduction of plastquinone or Q$_A$.

In the presence of the uncoupler nigericin strong quenching was abolished in dpa1 and exciton quenching relaxed when nigericin was applied during fluorescence induction (Fig. 6). These results demonstrate that a low luminal pH causes strong NPQ below the $F_p$ level in dpa1. This is in contrast to the cfq mutant that is affected by a decreased ΔpH across the thylakoid membrane due to a higher proton conductivity of the ATP synthase.

**Fig. 8.** mRNA expression profiling of 3292 nuclear genes in dpa1. Total RNA isolated from 3-week-old plants grown under continuous light at a photon flux density of 20 μmol photons m$^{-2}$ s$^{-1}$ was used. Up- and down-regulated genes were classified in 13 major functional categories. Except for photosynthetic genes the expression of all other classes was strongly down-regulated. The array data were confirmed by Northern analysis with randomly chosen gene probes (see “Experimental Procedures”).

**Table II**

Representative expression levels of up- and down-regulated nuclear photosynthetic genes in dpa1

| Accession no. | Annotation | Ratio dpa1/WT | Accession no. | Annotation | Ratio dpa1/WT |
|---------------|------------|---------------|---------------|------------|---------------|
| Electron carrier | At1g20340 | PetE2 (plastocyanin) | 3.49 | At4g14890 | PetF5 (ferredoxin) | 0.40 |
| PSI | At1g155670 | PSI-A | 2.63 | At1g503600 | PSI-A | 0.36 |
| PSII | At1g79040 | PSII-R | 2.96 | At2g30790 | PSII-P2 | 0.34 |
| Cytb6/f | At1g44570 | PsbS | 2.41 | At4g15510 | PSII-P related protein | 0.32 |
| ATP synthase | At1g67740 | PsbX | 1.73 | At3g01440 | PSII-Q related protein | 0.19 |
| Dark reaction | At2g06520 | PetM | 1.52 | At2g2650 | PetM | 1.91 |
| | At4g09650 | AtpD | 1.37 | At4g15530 | Pyruvate, orthophosphate dikinase | 0.23 |
| | At4g32260 | AtpG | 1.37 | At4g15530 | Pyruvate, orthophosphate dikinase | 0.23 |
| | At1g58420 | Rubisco small subunit 2b | 4.92 | At1g73110 | Putative Rubisco activase | 0.36 |
| | At5g38430 | Rubisco large subunit | 4.65 | At4g35580 | Sedoheptulose-bisphosphatase | 0.34 |
| | At1g67090 | Putative Rubisco small subunit | 4.43 | At4g15530 | Pyruvate, orthophosphate dikinase | 0.23 |
| | At1g58190 | Putative phosphoglycerate kinase | 3.73 | | | |
| | At1g32060 | Phosphoribulokinase | 2.39 | | | |

Inactivation of Chloroplast ATP Synthase γ Subunit

FIG. 8.
Inactivation of Chloroplast ATP Synthase γ Subunit

Involvement of the ATP synthase in the regulation of photoinhibition was suggested by Ketcham et al. (23), who found that the level of NPQ was reduced in mutant plants with decreased ATP synthase activity. This observation was supported by the fact that the ATP synthase is a key component of the thylakoid membrane, where it is involved in the proton gradient that drives the synthesis of ATP. The ATP synthase is also responsible for the accumulation of photodamage to the thylakoid membranes, with the accumulation of photodamage being inversely proportional to the level of ATP synthase activity. In addition, the ATP synthase is involved in the regulation of the Calvin cycle, which is the primary source of ATP in plants. Therefore, the involvement of the ATP synthase in the regulation of photoinhibition is not surprising, as it is a key component of the thylakoid membrane and is involved in the regulation of the Calvin cycle.

The important role of NPQ in photoprotection implies that NPQ itself has to be modulated in response to rapid changes of physiological conditions (77, 78). Recently, it has been shown that the chloroplast ATP synthase is involved in modulation of NPQ (79). The proton conductivity of the enzyme, as estimated by measurement of the electrochromic shift at 520 nm, is altered at different CO2 concentrations. This allows the modulation of the relationship between photosynthetic electron transport and NPQ. The dpa1 mutant represents an extreme case with maximal proton accumulation which only relaxes through leakage of the membrane.

Up-regulation of Nuclear Photosynthetic Gene Expression in dpa1—In the dpa1 mutant a preferential down-regulation of the differentially expressed genes is observed. A different, and more balanced, response of transcriptional regulation was observed for photosynthetic genes, for which about an equal number of genes were up- or down-regulated. The changes in the accumulation of transcripts for photosynthetic genes could be interpreted as a reaction of the plant to the altered physiological states of thylakoids due to the loss of ATP synthase function. It appears possible that under the conditions induced by the dpa1 mutation particular components of the photosynthetic apparatus are more rapidly degraded and that as a compensatory response corresponding nuclear genes are up-regulated, e.g. genes of the ATP synthase. On the other hand, up-regulation of genes encoding the cytochrome b,f complex, e.g. petM, might result in higher levels of this complex (Fig. 3B and Table II).

Another possibility is that certain subunits of the photosynthetic apparatus are down-regulated to decrease the photosynthetic activity of corresponding complexes that could be harmful in the absence of ATP synthase activity. However, a previous comparison of the differential expression of photosynthetic proteins and their transcripts in the prpl11-I mutant (48) has shown that complex interdependencies of mRNA and protein levels can occur in photosynthetic mutants. This includes situations where nuclear genes are up-regulated but the levels of corresponding proteins are decreased, due to the interaction of plastome- and nuclear encoded proteins in photosynthetic multiprotein complexes (48). However, taken together, the transcriptional regulation of photosynthetic genes differs from the general trend (Fig. 8), clearly indicating that the plant might be able to maintain the altered photosynthetic state due to loss of ATP synthase activity and to respond by regulating the expression of appropriate photosynthetic genes (see Supplemental Material).

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REFERENCES

1. Strotmann, H., Shavit, N., and Leo, S. (1998) The Molecular Biology of Chloroplast and Mitochondria in Chlamydomonas, pp. 477–500, Klauer Academic Publishers, Norwell, MA.

2. Groth, G., and Pohl, E. (1999) Physiol. Plant. 106, 145–148.

3. Groth, G., and Strotmann, H. (1999) J. Biol. Chem. 274, 13521–13527.

4. Nishio, K., Iwamoto-Kihara, A., Yamamoto, A., Wada, Y., and Futai, M. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 10964–10968.

5. Noji, H., Yasuda, R., Yoshida, M., and Kinosita, K. (1997) Nature 386, 299–302.

6. Sabbert, D., Engelbrecht, S., and Junge, W. (1996) Nature 381, 623–625.

7. Noji, H., Yasuda, R., Yoshida, M., and Kinosita, K. (1997) Nature 386, 299–302.

8. Nishio, K., Iwamoto-Kihara, A., Yamamoto, A., Wada, Y., and Futai, M. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 13448–13452.

9. Nalin, C. M., and McCarty, R. E. (1984) J. Biol. Chem. 259, 7275–7280.

10. Ross, S. A., Davenport, J. W., Warncke, K., and McCarty, R. E. (1984) J. Biol. Chem. 259, 7286–7293.

11. Rust, B. W., and Oxborough, K. (1992) Annu. Rev. Plant Physiol. Plant Mol. Biol. 43, 269–291.
